

CARESS FLOW

OXYGEN FOR VULVO - VAGINAL TREATMENTS

SCIENTIFIC PUBLICATIONS

A decorative background consisting of a dense field of small dots in various shades of blue and green, arranged in a pattern that suggests a scientific or medical theme.

SCIENTIFIC PUBLICATIONS

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OXYGEN FOR VULVO - VAGINAL TREATMENTS

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Effectiveness of concomitant administration of hyaluronic acid and topical hyperbaric oxygen therapy via a vaginal probe in improving symptoms of vulvovaginal atrophy

BostonUroGyn, Wellesley, MA
[Dr. Neeraj Kohli, BA, MD, MBA](#)

Impact of Caress Flow treatment on vaginal microbiota

S. Orsola Hospital – Bologna
[Prof. Maria Cristina Meriggiola](#)

Concomitant treatment with topical hyaluronic acid and topical oxygenotherapy in the improvement of symptoms related to genito-urinary syndrome in women in physiological and iatrogenic menopause

Fondazione IRCCS Policlinico
San Matteo di Pavia
[Prof. Rossella Nappi](#)

A prospective study to assess the performance and safety of vaginal molecular oxygen (Caress Flow®) plus a systemic symbiotic compound (Vibioma®) in the prevention of recurrent vaginitis: a randomized controlled clinical trial.

Lower Genital Tract Unit-Obst. and Gyn, Dept. V. Buzzi Hospital – University of the Study of Milan
[Prof. Filippo Murina](#)

Vaginal microbiota changes following Caress Flow therapy. Prof. Fabio Facchinetti -

University of Modena and Reggio Emilia.
[Prof. Fabio Facchinetti](#)



1. SCIENTIFIC RATIONALE

INTRODUCTION: OXYGEN AND HYALURONIC ACID IN THE TREATMENT OF GYNAECOLOGICAL DISEASES

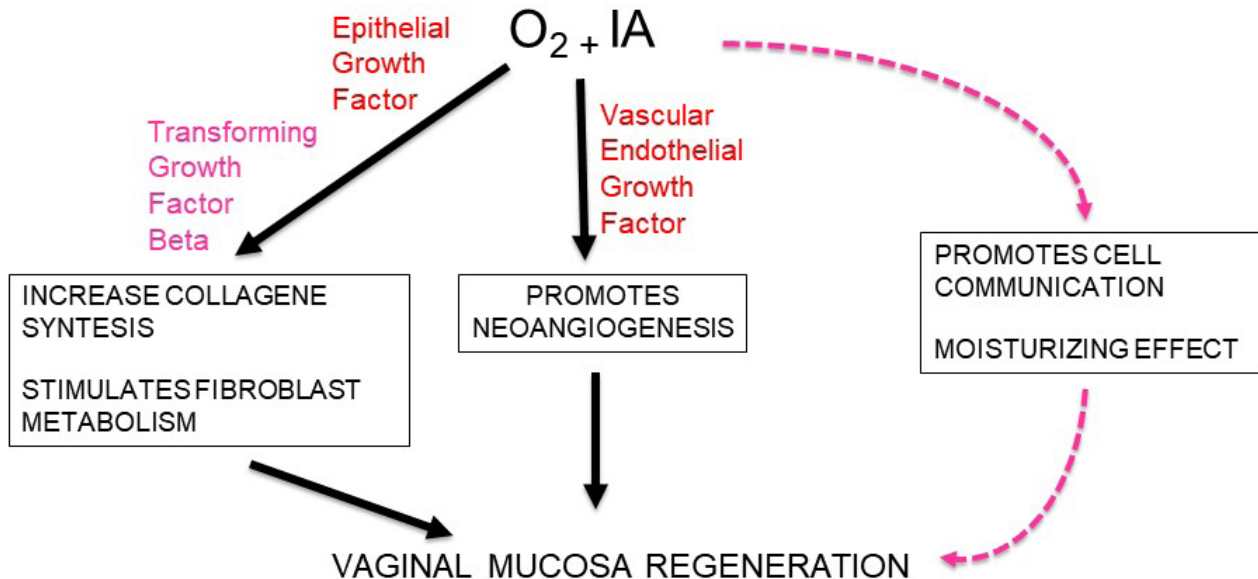
Topical oxygen therapy is a cosmetic procedure that is becoming popular in skin treatment and other medical applications. Oxygen therapy is not a recent innovation in the medical field, other techniques of oxygen therapy, such as hyperbaric oxygen treatments, are widely used in the treatment of other skin diseases, including wound healing, burns, skin grafts, and others. Hyperbaric oxygen therapy was also proposed as a skin rejuvenation and antiaging treatment.

Oxygen therapy has a powerful regenerative, antibacterial and biostimulating effect, increases the availability of oxygen to the tissues, promotes the increase in tissue repair processes and increases the synthesis of collagen allowing normal hydroxylation of this protein. In fact, at tissue oxygen tensions lower than normal, collagen is not synthesized correctly, slowing the healing of ulcers and wounds. In addition, oxygen induces a neo-angiogenic stimulus by releasing factors such as the Vascular Endothelial Growth Factor (VEGF). This function is essential for the restoration of the microcirculation in compromised vascular situations, re-establishing a vascular flow in the hypoxic areas that guarantees correct tissue regeneration.

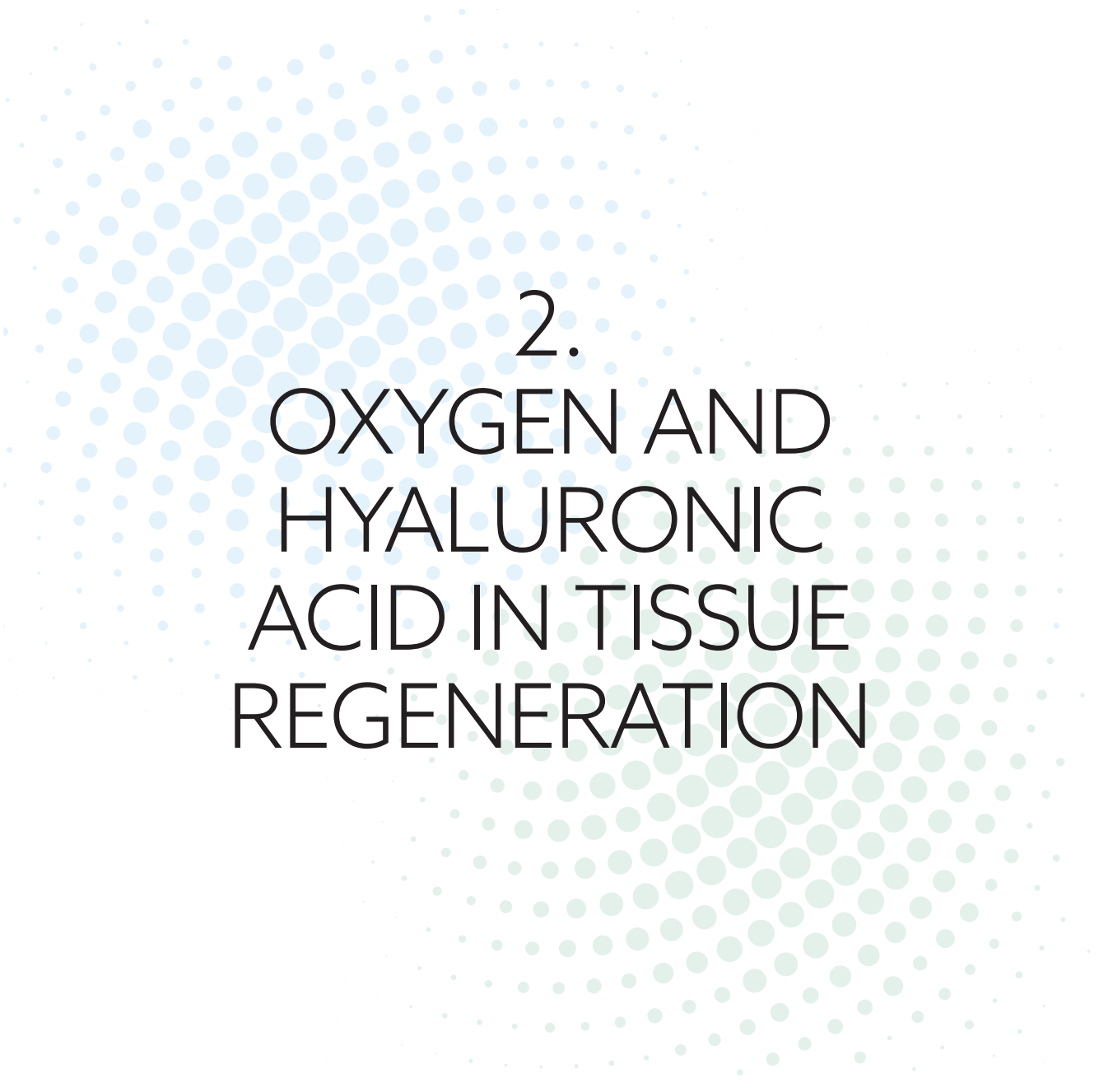
Hyaluronic acid is a natural polysaccharide that forms a fundamental part of the extracellular matrix of the skin and cartilage. Hyaluronic acid: has remarkable adhesive, moisturizing and reparative properties of the vaginal mucosa.

Because of these properties, the synergistic association of high concentration oxygen and hyaluronic acid has been shown to have therapeutic effects in diseases involving tissue regeneration problems, including many gynaecological diseases.

OXYGEN AND HYALURONIC ACID IN THE TREATMENT OF GYNECOLOGICAL DISEASES : THE SINERGY



Topical oxygen therapy should be proposed as an adjuvant therapy in the treatment of these conditions. The aim of this issue is to review the role of oxygen and hyaluronic acid in tissue pathophysiology and to analyse the available methods that have been proposed to improve tissue regeneration, with a particular focus on the potential usefulness of oxygen and Hyaluronic acid treatment for gynaecological diseases.



2. OXYGEN AND HYALURONIC ACID IN TISSUE REGENERATION

Postmenopausal vulvovaginal atrophy (VVA) is positively improved by topical hyaluronic acid application. A prospective, observational study

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Abstract. – **OBJECTIVE:** To evaluate the effectiveness of a topical vaginal preparation containing hyaluronic acid in controlling signs and symptoms correlated with postmenopausal vulvovaginal atrophy (VVA).

PATIENTS AND METHODS: A prospective, observational study has been performed at the Obstetrics and Gynecology Department of the Vita Salute San Raffaele University of Milan, Italy. Forty-six (46) consecutive postmenopausal women complaining of genital discomfort due to postmenopausal estrogen lack have been enrolled. All patients have been investigated by the use of the Vaginal Health Index (VHI) and of a Visual Analogic Scale (VAS) of symptoms at baseline and one month after the end of the study. The treatment protocol consisted of the administration of a hyaluronic acid-based liquid preparation for vaginal use (Justgin®, Just Pharma, Rome, Italy) three times a week, for a total of 8 weeks. Statistical analysis of VHI and VAS scores has been performed by the use of the Wilcoxon signed-rank test for repeated values, assuming a p-value < 0.05 as significant.

RESULTS: Both Vaginal Health Index (VHI) and Visual Analogic Scale (VAS) of genital symptoms showed statistically significant ($p < 0.0001$) improvements at the end of the study protocol. Patients' degree of satisfaction at the end of treatment was reported as high.

CONCLUSIONS: Conventional treatment of the postmenopausal syndrome, either in terms of systemic and genital symptoms, is based on hormonal replacement therapy (HRT). The limitations to this approach are represented by the need to discontinue the treatment after some years and the contraindications that some women present about the estrogens. For these reasons, alternative approaches have been recently investigated and indicate promising perspectives. Hyaluronic acid topical approach with a liquid preparation for vaginal use (Justgin®, Just Pharma, Roma, Italy) to control signs and symptoms of vulvovaginal atrophy (VVA) in

postmenopausal women demonstrated significant effectiveness both in terms of objective and subjective improvement.

Key Words:

Menopause, VVA, Vulvovaginal atrophy, Vaginal Dryness, Dyspareunia, Hyaluronic acid.

Introduction

During women reproductive ages, the vaginal epithelium undergoes changes in response to the level of circulating estrogens. When menopause occurs, circulating estrogens levels show a dramatic reduction. Parallel with the onset of systematic negative effects, the estrogens lack also determines several modifications of the genital tissues; significant cytological transformations follow estrogen reduction, including the proliferation of connective tissue, fragmentation of elastin and collagen hyalinization. These changes may result in granulation, fissures, ecchymosis, telangiectasia and ulcerations, resulting in a condition described as vulvovaginal atrophy (VVA)¹. The major symptoms of VVA are: decreased vaginal lubrication, leading to vaginal dryness, followed by other vaginal and urinary symptoms, such as burning, itching, bleeding, leucorrhea, dyspareunia and dysuria. These symptoms usually appear some 2-4 years after the onset of menopause. VVA is particularly relevant regarding the prevalence, affecting 20-45% of women. In contrast to postmenopausal vasomotor symptoms, VVA demonstrates a progressive and worsening feature over time and a less likely attitude to solve without targeted interventions. Several surveys have reported that VVA

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symptoms significantly contribute to determine an adverse emotional and physical impact on patients and their partners through unsatisfactory sexual relationships^{2,3}. The main therapeutic objective in managing VVA is to relieve genital symptoms as well as trying to restore the vaginal environment to a healthy condition⁴. Historically, vaginal estrogens have been considered the gold standard of treatment for the relief of VVA symptoms; topical estrogen administration has been widely preferred compared to the systemic modality when genital symptoms are the only complaint and, moreover, low-dose vaginal estrogen administration has been proven to reach optimal effectiveness with minimal side effects and systemic absorption⁵. On the other side, the estrogen-based approach represents, in a significant percentage of postmenopausal women, a kind of limiting factor for several reasons: fear of cancer risk increase, personal and/or cultural reasons, or even identified contraindications. In fact, no conclusive data are available about the long-term safety of vaginal administration of estrogens in particular subgroups of patients⁶. In this view, the state-of-the-art position statements about the optimal approach for the relief of VVA symptoms take into great consideration the patients' preferences and willing, the personal needs and the cultural positions. A quite extensive number of non-hormonal vaginal preparations has been suggested in recent years and proposed as an alternative approach, lubricants and vaginal moisturizers being the most frequently prescribed in clinical practice. For this, it is now a good practice to give patients a comprehensive and detailed information about the available options. In the group of non-hormonal treatments, hyaluronic acid owns many of the characteristics that represent a consistent background for the treatment of VVA symptoms⁷.

Background

The vaginal epithelium is particularly sensitive to the effects of circulating estrogens in women; at menopause, when a dramatic reduction of estrogens occurs, this epithelium strongly modify its structure, with progressive and worsening atrophic changes. Hyaluronic acid is a natural polysaccharide that represents an important part of the extra-cellular matrix of the skin and cartilage. This substance is able to storage large amounts of water molecules and has a key role due to the properties of formation and conservation of extra-cellular inflation, skin moistening in

the case of inflammation and preservation of water equilibrium. Also, it is widely effective in the treatment of skin diseases due to the preservation of tissue consistency, facilitating the cellular migration in cases of inflammation and also the process of improvement and regeneration of damaged tissues⁸.

Patients and Methods

The study has been performed between 2015 and 2016 at the Department of Obstetrics and Gynecology of Vita Salute San Raffaele University School of Medicine, Milan, Italy. In this period, 46 consecutive postmenopausal women have been identified, recruited and enrolled in the trial. All patients participated to the trial on a volunteer basis after having received the proposal in the office visits of the Gynecological Department consultations. The selection criteria of cases were based on the presence of patients' complaint of vulvovaginal signs and symptoms correlated with postmenopausal lack of estrogens: vulvovaginal atrophy, genital dryness, vaginal burning, itching and painful intercours-es were the major discomforts reported. Exclusion criteria were: previous use of systemic or topical estrogen-based preparations. All patients were extensively informed of the study design and hypothesis and a signed consent was obtained. According to the study design and the preparation administered, Institutional Review Board (IRB) gave exempt to ethical approval. All patients were prospectively enrolled in the trial after having been comprehensively investigated on the basis of: duration of menopause, gynecological visit, Vaginal Health Index (VHI) score, Visual Analogic Score (VAS) of signs and symptoms. VHI is an objective, score-based investigation tool, firstly elaborated and published by Gloria Bachmann⁹ and comprising of five vaginal parameters evaluated by clinical inspection: elasticity, fluid volume, pH, epithelial integrity and moisture. Each parameter is graded from 1 (worst condition) to 5 (best condition). The VAS of signs and symptoms was subjectively recorded by patients on a personal diary; the parameters evaluated were: vaginal dryness, burning, itching and painful intercours-es. Patients were asked to score their symptoms from 1 (best condition) to 10 (worst condition). The study protocol of treatment was based on the application of a hyaluronic acid-based liquid

preparation for vaginal use (Justgin®, Just Pharma, Rome, Italy) three times a week for a total of 8 weeks. All cases have been again investigated with the use both VHI and VAS of symptoms one month after the end of treatment. At the same time, patients were asked to report their degree of satisfaction, according to a 5-point Likert scale (very satisfied, satisfied, uncertain, dissatisfied and very dissatisfied). Treatment was considered satisfactory when patients were very satisfied or satisfied.

Statistical Analysis

Statistical analysis of VHI and VAS scores has been performed by the use of the Wilcoxon signed-rank test for repeated values, assuming a p -value < 0.05 as statistically significant.

Results

The study group consisted of 46 consecutive postmenopausal women; mean age of patients was 57.8 years (min. 49 – max. 67 yrs, 95% CI 8.9 to 10.4) and mean duration of menopause was 6.5 years from onset. All 46 patients completed the trial since neither adverse effects nor spontaneous dropouts were observed. As far as it concerned the Vaginal Health Index (VHI), mean VHI at the enrollment in the trial was 9.65 (min. 5 – max. 16; 95% CI 8.90 to 10.40) underlying that vulvovaginal atrophy objective signs were particularly relevant. When the patients underwent final VHI evaluation one month after the end of the hyaluronic acid-based protocol, mean VHI showed a statistically significant improvement to the value of 19.96 (min. 15 – max. 23; 95% CI 19.38 to 20.54) ($p < 0.0001$) (Figure 1).

With regard to patients' subjective Visual Analogic Scale (VAS) of symptoms – dryness, burning, itching and dyspareunia – a mean value of 7.65, 5.15, 2.21, and 5.63 was reported for the four parameters respectively; it is noteworthy that vaginal dryness (mean VAS 7.65) and

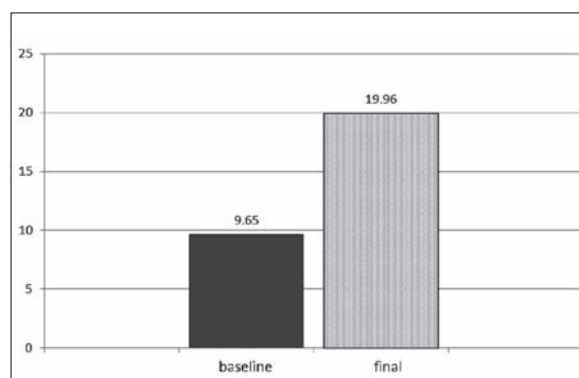


Figure 1. Vaginal Health Index (VHI) mean scores at baseline and after treatment (Wilcoxon signed-rank test: $p < 0.0001$).

painful intercours (mean VAS 5.63) emerged as the most relevant negative symptoms in this group of patients. On the other end, vaginal itching (mean VAS 2.21) was reported as less invalidating. At the end of treatment, all the baseline VAS scores showed a statistically significant ($p < 0.0001$) improvement as high as 3.95, 2.93, 1.13 and 3.54 respectively; these improvements are summarized in Table I and Figure 2. Concerning the personal satisfaction, according to the 5-point Likert scale, a 95% degree of patients' satisfaction was obtained.

Discussion

VVA is a chronic, progressive and worsening condition depending on the estrogen dramatic decrease that occurs at menopause. According to several surveys published in recent years, the prevalence of VVA among postmenopausal women is particularly relevant, accounting for almost 50% of women. These findings can reasonably underestimate the real dimension of the condition, as these patients are frequently reluctant to complain and report their symptoms,

Table I. Visual Analogic Scale (VAS) mean scores of symptoms.

	Baseline	Final	p -value
Vaginal dryness	7.65	3.95	< 0.0001
Vaginal burning	5.15	2.93	< 0.0001
Genital itching	2.21	1.13	< 0.0001
Painful intercours	5.63	3.54	< 0.0001

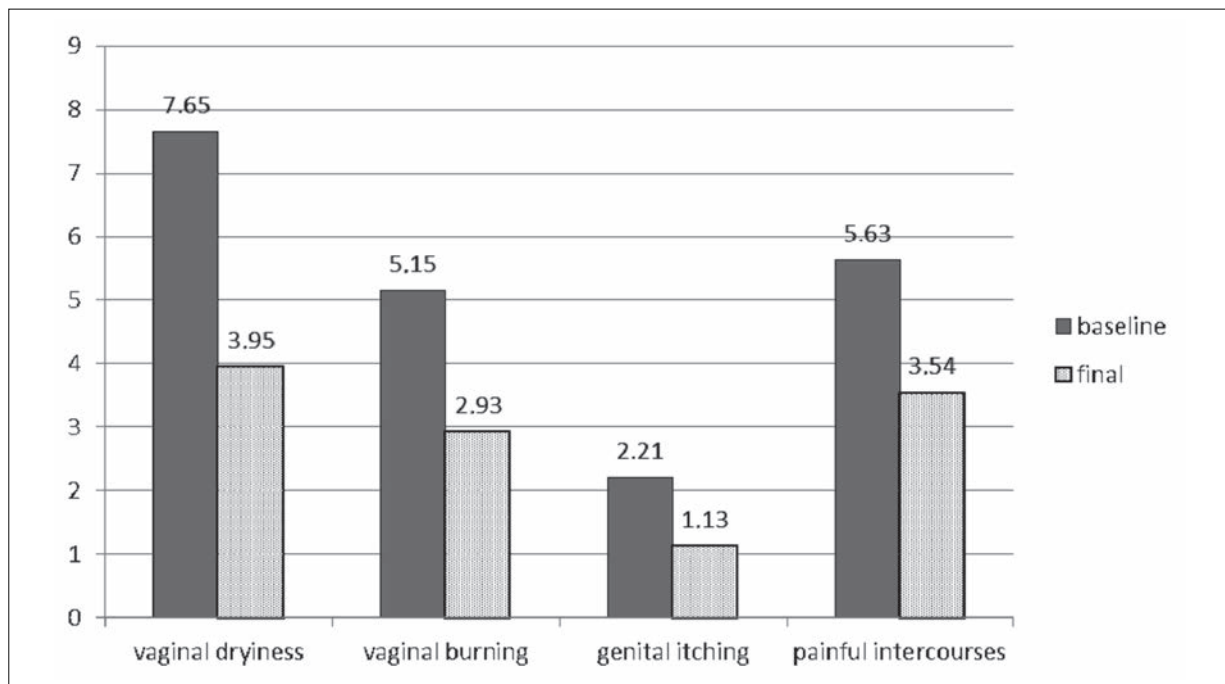


Figure 2. Visual Analogic Scale (VAS) mean scores of symptoms at baseline and after treatment (Wilcoxon signed-rank test: $p < 0.0001$).

GPs are often poorly educated to investigate properly their patients and gynecologists should be encouraged to be proactive. For these reasons, VVA despite its high prevalence remains poorly recognized and undertreated¹⁰. Based on the available data, systemic estrogen replacement therapy is not recommended for the sole purpose of VVA symptoms treatment. Symptomatic postmenopausal women might derive benefit from systemic hormones, which could improve some aspects of sexual function by addressing bothersome estrogen deficiency symptoms. However, the decision to start a systemic estrogen therapy must be personalized and patients must be adequately informed about the risks and benefits, with particular attention to cardiovascular, thrombotic, and breast cancer risks. There is clear and consistent evidence for the benefits of vaginal estrogen therapy in women who have vaginal symptoms and dyspareunia. Vaginal estrogen therapy and ospemifene are effective and indicated for the treatment of VVA^{11,12}. In spite of available reassurances to the contrary, many women still fear estrogen therapy and feel very uncomfortable or present some kind of limitations, according to personal history and/or risk factors. Moreover, no data are available regarding the long-term

safety of vaginal estrogen therapy. Alternative options to obtain relief of VVA symptoms include non-hormonal vaginal lubricants and moisturizers, as well as regular sexual activity. Regular use of non-hormonal vaginal lubricants and moisturizers may reduce friction-related irritation of atrophic tissue during vaginal intercourse, thus providing a transient benefit. Recently, very interesting evidence of safety, effectiveness and acceptability of fractional CO₂ laser treatment of VVA symptoms has been reported in the literature¹³⁻¹⁶. As far as it concerns hyaluronic acid, this compound has been largely studied and investigated in many dermatologic, ocular and osteoarticular applications requiring tissue remodeling; vaginal atrophy has also been investigated with promising results¹⁷⁻¹⁹. The results of the present investigation with a hyaluronic acid-based vaginal liquid preparation commercially available in Italy (Justgin®, Just Pharma, Rome, Italy), strongly supports the indication of such a preparation for the relief of genital symptoms due to VVA. In terms of mechanisms of action details, the high molecular weight hyaluronic acid acts as a protective macromolecule for the vaginal mucosa and also favours the penetration of the low molecular weight molecule into the deeper vaginal layers.

Our results indicate a robust statistical significance of the improvements of symptoms, both objectively by the use of the Vaginal health Index (VHI) and subjectively with a Visual Analogic Scale (VAS) of symptoms, after a 2-month treatment with the hyaluronic acid liquid preparation for vaginal use. In particular, the most significant improvements have been reported as far as it concerned vaginal dryness and painful intercourses, therefore strongly improving the Quality of Life (QoL) of these women; in this view, the patients' degree of satisfaction at the end of the trial was reported as high as 95%. Previous positive results of the use of vaginal hyaluronic acid preparations in symptomatic postmenopausal women²⁰⁻²² are available in literature and, even when compared with vaginal estrogen, hyaluronic acid demonstrated similar effectiveness¹⁹. The results of the present work differ from the previous experiences concerning the hyaluronic acid way of vaginal administration; in fact, all the previously published results refer to vaginal creams and/or suppositories that, as underlined by several surveys, often determine a low degree of satisfaction and compliance, leading to a significant percentage of treatment discontinuation¹⁰. The liquid preparation, due to its intrinsic characteristics, appears to be very well tolerated and accepted by patients, determining the absence of dropout cases from the study group and contributing to the final degree of satisfaction.

Conclusions

In accordance with the actual statement that postmenopausal women complaining of VVA discomforts should always comprehensively be informed of all the therapeutic available alternatives, and in accordance with experts' opinions that sexual satisfaction and QoL after menopause should be of primary importance for care providers, the results of the present study support the effectiveness of hyaluronic acid in obtaining significant improvements of VVA related symptoms and the inclusion of hyaluronic acid in the pool of effective non-hormonal treatments.

Conflict of Interest

All the authors of the submitted manuscript declare under their responsibility that no financial interests related to the article have to be disclosed.

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Comparison of the Hyaluronic Acid Vaginal Cream and Conjugated Estrogen Used in Treatment of Vaginal Atrophy of Menopause Women: A Randomized Controlled Clinical Trial

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ABSTRACT

Background: Vaginal atrophy is a common complication in menopause which does not improve with time and, if untreated, can affect the quality of life for women. The aim of this study was to compare the effectiveness of the vaginal cream of hyaluronic acid and conjugated estrogen (Premarin) in treatment of vaginal atrophy.

Methods: This study was a randomized controlled clinical trial on 56 menopausal women with symptoms of vaginal atrophy; they were randomly allocated to two groups (recipient conjugated estrogen and hyaluronic acid). The severity of each sign of atrophy was evaluated by visual analog signals (VAS) and on the basis of a four point scale. Also to recognize the cellular maturation with pap smear and the maturation degree were calculated according to the formula and scores 0-100. As to the vaginal PH, we used PH marker band, the rate of which was divided into 4 degrees. Data were analyzed using SPSS, version 20, and $P \leq 0.05$ was considered as significant.

Results: The results of this study showed that the symptoms of vaginal atrophy compared with the baseline level were relieved significantly in both groups. Dryness, itching, maturation index, PH and composite score of the vaginal symptoms were relieved significantly in both groups ($P < 0.001$). Dyspareunia in Premarin ($P < 0.05$) and hyaluronic acid ($P < 0.001$) decreased compared with pre-treatment. Urinary incontinence only showed improvement in the hyaluronic acid group ($P < 0.05$). Improvement in urinary incontinence, dryness, maturation index ($P < 0.05$) and composite score of vaginal symptoms ($P < 0.001$) in the hyaluronic acid group was better than those in the Premarin group.

Conclusion: According to the results of the present study, hyaluronic acid and conjugated estrogen improved the symptoms of vaginal atrophy. But hyaluronic acid was more effective and this drug is suggested for those who do not want to or cannot take local hormone treatment.

Trial Registration Number: IRCT2013022712644N1

KEYWORDS: Atrophic vaginitis; Estrogen; Hyaluronic acid; Menopause

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INTRODUCTION

Menopause is defined as the permanent experience of long-lasting endocrinal, somatic and psychological changes.¹ During these periods, women experience some symptoms which begin with vasomotor signs (like flushing, night sweat, etc.), changes in menstruation cycle, vaginal dryness, Itching and dyspareunia and continue with temper changes, memory reduction, disorders of sexual arousal reduction, stress urinary incontinence and complaint from musculo-eskeletal pains.

Even though some of the complications subside during the time, the symptoms of vasomotor, vaginal dryness and dyspareunia which are connected to disorder in sexual function related to lack of sexual hormones (especially Estrogen) irrespective of treatment will progress markedly and unfortunately will not be solved without treatment.^{2,3}

Following the subsidence or discontinuity of this hormone, women are affected by symptomatic vaginal atrophy and basic changes will occur in their genitor-urinary mucous.⁴ These changes include vaginal dryness, irritation, itching, post-coital bleeding, vaginal discharge and dyspareunia and in the urinary system, urine frequency and urinary incontinence appear.^{3,5}

As a whole, it is estimated that 10.0-40.0% of women experience the symptoms connected with atrophy and on the other hand about 16 million women (500 thousand new cases) show such symptoms every year.⁴ In confirmation to the prevalence of this problem, Crandall C et-al. (2004) and Mac Bride et-al. (2010) considered this matter and reported that the vaginal dryness was observed from 23.4% pre-menopause to 61.5% post-menopause among the women under the study.^{3,6} The results of the researches conducted by Kingenberg et-al. (2009) and Mehta and Bachman also showed that 10.0-40.0% of women at the post-menopause stage face inconvenience and problems related to vulva and vaginal atrophy that requires treatment but only 25.0% of them refer for treatment.^{7,8}

Two hormonal and non-hormonal methods are usually used in treatment of such problems. In the studies which applied non-hormonal method, materials like lubricants and vaginal moistures,^{4,9,10} vitamin E oil and improving lifestyle like stopping cigarette smoking have been mentioned.⁵ For hormonal methods also the conjugated Estrogen in two forms of systemic (oral and parenteral) and topical are prescribed.^{11,12} The systemic method is useful for those women who are suffering from flushing and sleep disorder related to vaginal atrophy.^{13,14}

On the other hand, the contraindication of this method for tumors sensitive to Estrogen, liver failure and having thromboembolization history related to Estrogen should also be considered. Also, attention should be paid to their side effects like breast sensitivity, nausea and vomiting, vaginal bleeding, mild increase in the risk of affecting the neoplasms dependent on Estrogen and in lesser amount the pain in the perineal area.^{13,15,16}

Topical treatment in the form of cream, tablet and ring (conjugated Estrogen 0.625) which has been confirmed by FDA (Food and Drug Association) with the objective of preparing sufficient Estrogen for reducing the symptoms of atrophy and relief of its resultant complications is applied.^{11,17} In this regard, researches show that topical drugs have similar effect^{18,19} and even though the probability of general absorption is there, as to the influence and improvement of the symptoms to the rate of 80.0-90.0%, which is expected, they are similar.¹²

Topical hormones are also not without complication; the results of a study (2004) showed that they have similar effects in the incidence rate of hyperplasia and endometrial thickening.²⁰ Creams are probably accompanied with more side-effects compared with ring or tablet which may be due to the application of a dose more than recommended.⁴

Considering the aforesaid points, for those who do not select Estrogen-therapy due to the medical prohibitions or having side-effects,¹

the non-hormonal interventions which are mostly neglected for the sexual problems are propounded. In these methods, applying lubricants, moisture creams and using dilators are recommended. It was reported in a study in the year 2010 that their use will reduce the complications of vaginal atrophy.²¹ Also, in this connection, materials which could be applied as gel in the form of the extract of some plants like Vitex Agnus-Castus and compounds like Hyaluronic Acid alone or in combination with Vitamin E for the treatment of sexual disorders and vaginal atrophy are mentioned.^{22,23}

Regarding Hyaluronic Acid which is a natural polysaccharide, it can be mentioned that, it forms an important part of extra-cellular matrix of the skin and cartilage. This substance is able to conserve a large amount of water molecules and due to the properties like formation and conservation of extra-cellular inflation, skin moistening in the case of inflammation and preservation of water equilibrium has a key role. Also, it is effective widely in treatment of skin diseases due to preservation of tissue consistency, facilitating the cellular emigration in the cases of inflammation and also the process of improvement and regeneration of the tissues.²⁴

Various studies carried out regarding Hyaluronic Acid have shown that this compound has been tolerated well without side-effects among patients and the complications have been observed only when applied in the form of parenteral jelly by creating susceptibility at injection sites as mild inconveniences, redness, edema and cyanosis.^{25,26} It should be notified that this medicine in the form of suppositories or tablets has rarely been used in Iran for treatment of atrophy of genitor-urinary system.

The present study aimed at achieving an appropriate and uncomplicated treatment, which is accepted by those who have contraindication for hormonal drug and or desire to use non-hormonal methods. Therefore, we tried to compare the effects of conjugated Estrogen cream 0.625 mg

(hormonal) and hyaluronic acid cream (non-hormonal) for the treatment of atrophy and its complications with the aim to promote the health of menopause women. In spite of the importance of the matter and effect of vaginal atrophy symptoms on women's life, they are mostly not reported and do not go under treatment subsequently. Therefore, to remove such problems, beginning of treatment and taking care of them by physical evaluation, talking about their sexual problems and the qualitative problems of their life seem necessary.²⁷

MATERIALS AND METHODS

This study was approved by ethics committee under number: CT-92-6681 on Oct 2013 and was carried out in multi-stages as a simple randomized controlled clinical trial on 56 menopause women referred to Shahid Motahari Clinic during 6 months (September to March 2013-2014). They were selected on the basis of the aim and by sample size determination with error of 5% , confidence of 95%, a power of 80%, an effect size of 6.0 (mean=4 and standard deviation=6), and correlation of 70% using the formula:

$$n = \frac{2(z_{1-\alpha/2} + z_{1-\beta})\delta^2}{d^2}$$

To consider the possibility of loss of 15% and the longitudinal nature of the study and sizes repeated, we used the formula:

$$n' = n \times \frac{1}{1 - p}$$

Finally, the sample size in each group was determined 28.

Inclusion Criteria

Married and menopause women, existence of moderate to severe dryness at the vaginal region, endometrial thickening with vaginal sonography maximum 5mm were among the inclusion criteria.

Exclusion Criteria

Smoking cigarette, and using anti-coagulate

drugs (Heparin), topical hormonal and nonhormonal drugs one month before the study, existence of vaginal infection requiring treatment in the primary examination for Pup Smear, sensitivity (such as Rash, Erythema, Inflammation) to drug or its compounds, existence of doubtful or known history of hormone relative diseases (such as breast cancer, unknown cases of vaginal bleeding, severe Thrombophlebitis or Thromboebolism disorders related to Estrogen), existence of chronic diseases (such as cardiac diseases, hypertension, diabetes) .

At first, multi-stage sampling was done with no blinding eligible individuals. In this way, the researcher selected the existing centers in proportion to the number of referrals from 30 to 50 percent through simple sampling method. The design and protocol of the study is shown in Figure 1.

Then, the numbers from 1 to 80 were written on the same card and then put in a bag covered; then we assigned the odd numbers to one group (group A) and even numbers

to the other group (group B). Groups of A and B (both groups had inclusion criteria) filled in the informed consent form. Group A received conjugated Estrogen 0.625 mg cream (production by Aborayhan Pharmaceutical Company) and group B received Hyaluronic Acid vaginal cream (containing 5 mg sodium salt), which had been prepared from Shiraz Pharmacy College.

Group A applied one applicator of drug (0.058 mg) every night before sleep for a period of two weeks and two times a week for the next six weeks and group B used one applicator 5(mg) every night before sleep for a period of 8 weeks. The manner of putting cream inside vagina, proper place and using at a specified time were explained for both groups and follow-up through telephone calls.

The study of the rate of vaginal atrophy symptoms at zero week (before treatment) and eighth week (after treatment) was carried out for both groups with a compound scale including vaginal dryness and itching, dyspareunia and urinary incontinence. Stress

CONSORT Flow Diagram

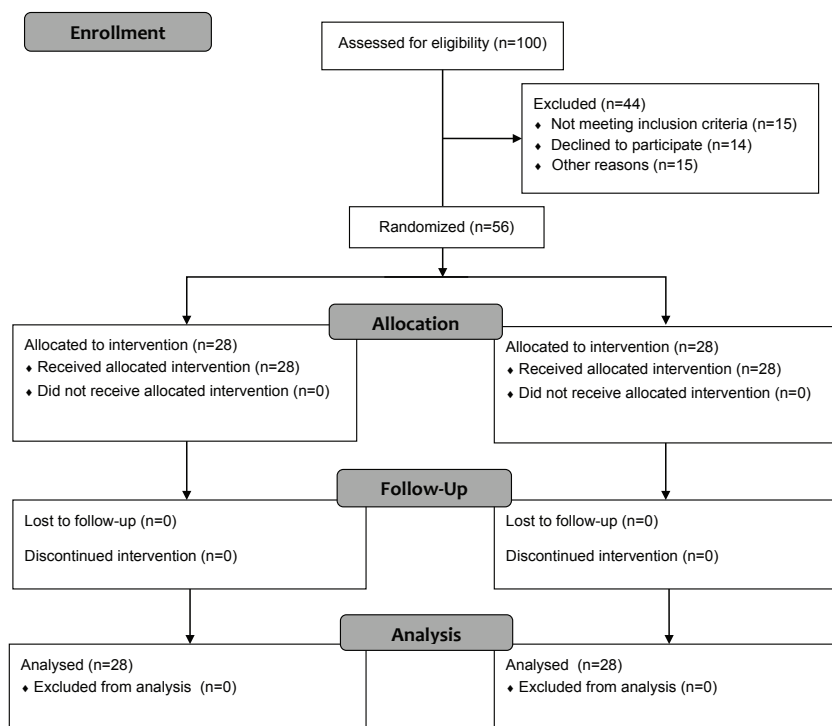


Figure 1: Consort flow diagram of participants

urinary incontinence (the urinary incontinence followed by increasing the intra-abdominal pressure at the time of sneezing, coughing, etc.) and urgency (sudden and severe feeling in urinary urgency) were considered in this study.

The severity of each sign of atrophy was evaluated by VAS (Visual Analog Signals) before and after the intervention and on the basis of four points scale in which zero=asymptomatic, one=mild (score 1 to 3), two=moderate (score 4 to 6) and three=severe (score 7 to 10) were propounded, respectively. The signs were evaluated by the researcher with attending the interview sessions.

To recognize the cellular maturation (before and after intervention) also by carrying out vaginal and cervical Pap Smear, the available rate and type of cells (para-basal, medial and surface) were determined. The Smear samples were colored with Ethyl alcohol 90.0%, studied as uni-blind by a cytologist (unaware of the type of treatment) and the maturation degree was calculated according to the formula [(percentage of surface cells x1)+(percentage of medial cells x0.5)+(percentage of Para-basal cellsx0)=maturation degree] with index: Lack 0-25, low estrogenic effect=26-49, moderate estrogenic effect=50-75, severe estrogenic effect=76-100.⁹

The vaginal PH was studied before and after the intervention using PH indicator strip inserted into the vagina; its rate was divided into 4 degrees as zero (PH<5.0), one (PH=5.0-5.49), two (PH=5.5-6.49) and three (PH>6.49) (9). It is necessary to mention that the cytologist, sample taker, the type of PH marker band and the laboratory were fixed throughout the study. Finally, the collected data were analyzed through SPSS

20 software, using descriptive statistics, Chi-square, paired and independent t-tests, and retest by Wilcoxon and Mann-Whitney tests at the confidence interval of 95.0%.

RESULTS

The results showed that from the view point of age, menarche, age of menopause beginning, number of pregnancies, occupation, education, disease history and drug consumption both therapeutic groups were similar and the Chi-square test did not show any statistical significant difference ($P>0.05$) (Table 1).

As to the atrophy symptoms before and after the intervention, it was specified that vaginal dryness and itching and also dyspareunia were significantly improved after the intervention among the groups ($P<0.001$). The relief of vaginal dryness in the hyaluronic acid group was observed more in the intra-group comparison ($P<0.05$) and urinary incontinence was also improved in this group only ($P<0.05$).

On the other hand, studying the compound mean score from vaginal atrophy symptoms (itching, dryness, dyspareunia and urinary incontinence) after treatment showed a reduction in group A at a rate of 1.7 ± 0.62 and in group B at a rate of 3.32 ± 0.76 (the mean after-the mean before the intervention) and a significant result was obtained ($P<0.001$). This reduction was more in the hyaluronic acid group; comparison of the results of both groups showed that the drug has had a better effect on group B ($P<0.001$) (Table 2).

As to the vaginal cellular maturation, the results indicated the maturity of cells; both groups had a significant difference

Table 1: Demographic characteristics of the samples in the study groups

Variables	Hyaluronic acid group (n=28)	Premarin group (n=28)	P value
	Mean±SD	Mean±SD	
Age (year)	56.4±5.47	51.92±4.31	0.44
Age menarche (year)	13±1.26	12.5±1.40	0.139
Age menopause (year)	47.71±5.26	46.2±4.16	0.823
Parity	4.92±2.32	5±2	0.551

*Frequency Mean±SD Chi-square

Table 2: Comparison of the mean±SD within and between groups of the composite score of vaginal atrophy symptoms (vaginal dryness and itching, dyspareunia and urinary incontinence) before and after the treatment

Composite score of vaginal atrophy symptoms	Premarin group (n=28)	P-value within groups	Hyaluronic acid group (n=28)	P-value within groups	P-value between groups
	Mean±SD		Mean±SD		
Before treatment	5.8±2.28	*P<0.001	5.92±2.15	*P<0.001	0.904
After treatment	4.10±1.66		2.60±1.39		*P<0.001

* Mean±SD paired and independent t retest by Wilcoxon and Mann-Whitney tests

compared with the results before treatment ($P<0.05$) (Table 3).

Also, studies showed that comparing the results of intra-group (before and after treatment), the amount of PH of both groups reduced, moving towards acidity ($P<0.001$) and no significant difference was obtained when the results of both groups were compared ($P>0.05$) (Table 4).

DISCUSSION

The results showed that the women in both

groups did not have any difference and were similar with respect to some of the demographic characteristics ($P>0.05$). In a study by Ziagham et-al. (2012) which compared the effect of hyaluronic acid vaginal suppository with vitamin E in the treatment of vaginal atrophy among menopause women, both groups were similar as to the age, menopause duration, occupation, educational level and economical status and had no statistical difference.²⁸

In a study, the effect of Hyaluronic Acid vaginal tablet and Estradiol was compared on the vaginal atrophy for a period of 8 weeks; no

Table 3: Comparison of the frequency of vaginal cell maturation index in the groups treated before and after the intervention

VCM ^a		Premarin group N (%)	P value within groups	Hyaluronic acid group N (%)	P value within groups	P value between groups
Before	None (0-25)	10 (35.7)	*P<0.001	3 (10.7)	*P<0.001	0.593
	Mild (26-49)	14 (50)		25 (89.3)		
	Moderate (50-75)	4 (14.3)		0 (0)		
	Severe (76-100)	0 (0)		0 (0)		
After	None (0-25)	0 (0)		0 (0)		*0.018
	Mild (26-49)	0 (0)		1 (3.6)		
	Moderate (50-75)	25 (89.3)		25 (89.3)		
	Severe (76-100)	3 (10.7)		2 (7.1)		

^aVaginal Cell Maturation Index; *Paired and independent t test retest by Wilcoxon and Mann-Whitney tests

Table 4: Comparison of the frequency of vaginal pH in the groups treated before and after the intervention

Vaginal pH		Premarin group N (%)	P value within groups	Hyaluronic acid group N (%)	P value within groups	P value between groups
Before intervention	<5.0	11 (39.3)	*P<0.001	11 (39.3)	*P<0.001	0.507
	5-5.49	1 (3.6)		4 (14.3)		
	5.5-6.49	3 (10.7)		3 (10.7)		
	>6.49	13 (46.4)		10 (35.7)		
After intervention	<5	12 (42.9)		17 (60.7)		0.463
	5-5.49	6 (21.4)		7 (25.1)		
	5.5-6.49	6 (21.4)		2 (7.1)		
	>6.49	4 (14.3)		2 (7.1)		

*Paired and independent t-test retest by Wilcoxon and Mann-Whitney tests

significant difference was observed between the mean age and the age of menopause beginning among both groups.²⁹ In another study, the researchers compared the effect of Genestine with Hyaluronic Acid on vaginal Atrophy also no significant difference was observed between age, menopause age and their effects on vaginal symptoms.³⁰

The results of the research also indicated that both Hyaluronic Acid and Permarine improved the vaginal atrophy symptoms, cellular maturation increase and reduced PH; this improvement was sometimes more among the Hyaluronic Acid group.

We did not have access to the same study. But many researchers studied separately the effect of two aforesaid drugs on atrophy and their results were similar to those of the present study in selection of the objective group and effect of drug but there were some differences in terms of drug form and duration of the intervention.

In this respect, we reviewed some studies as Castelo-Branco C et al (2005) to study the management of post-menopausal vaginal atrophy and atrophic vaginitis with focuses on the changes involved in vaginal aging. It was shown that estrogen increased the content of the skin collagen, and hyaluronic acid to improve the skin moisture and genitourinary symptoms.³¹

Another researcher studied the effect of conjugated Estrogen cream in treatment of atrophy which was consumed daily or two times a week for a period of 12 weeks. The results indicated that applying both methods of using the drug caused more improvement in symptoms of atrophy, maturation index and vaginal PH significantly compared with the placebo group.³²

In another study, the conjugated Estrogen vaginal cream was used twice a week for a period of 12 weeks for the treatment of vaginal atrophy. The results showed that Estrogen cream caused a reduction in the vaginal dryness, dyspareunia and PH and an increase in the vaginal cells maturation index.³³ The findings of our study is similar

to the results of the above studies in terms of improving atrophy symptoms after taking estrogen, but does not agree in the amount of consumption, the duration of intervention and use of a single drug without comparison with other drugs.

The effect of hyaluronic acid on treatment of vaginal atrophy was studied in other research-, the results of which were similar to those of the present study as to improving the symptoms of atrophy in the genito-urinary system. Especially, the results of evaluating the effect of hyaluronic acid suppository of another study in Iran on the severity of the symptoms of vaginal Atrophy 2, 4 and 8 weeks after treatment became significant and more effect was observed in improvement of symptoms compared with the group receiving vitamin E.²⁸ Our findings are similar to the mentioned study in terms of reduction of symptoms and duration of the use of drugs but doesn't match in terms of drug form and comparison with vitamin E.

Another researcher studied the effectiveness of three drugs, i.e. hyaluronic acid, vitamin E and A in the form of suppository for a period of one month firstly in continuous days and then every other day on menopausal women and the results showed that using hyaluronic acid caused a significant reduction in signs and symptoms of vaginal atrophy.²⁵

The results of the present study showed a significant reduction in symptoms with using hyaluronic acid that is similar to the results obtained by Castantino and Guaraldi's study. But there was a difference in terms of comparison of vitamin A and E and drug form.

It is also reported that the effect of the gel of hyaluronic acid on the symptoms of vaginal atrophy was due to the chemo-therapy started from the first week.²⁸ Our results are in the same line with Tea et al.'s study in terms of improving the symptoms using hyaluronic acid and is different in terms of drug form, target - group and comparison with other drugs.

Another study on the effect of prescribing genestine vaginal suppository compared with hyaluronic acid suppository on the

epithelium atrophy showed that using drugs for 15 continuous days in each month for a period of 3 months improved the symptoms of vaginal dryness and itching, dyspareunia, colposcopy specialties and the rate of vaginal cellular maturation.³⁰ The results of our study were similar to those of Le Donne et-al.'s survey in term of drug effects on atrophy and drug type, but they were different as to the drug form and duration of the intervention.

To the best of our knowledge, no study was previously done in Iran that used hyaluronic acid cream for vaginal atrophy treatment. The variety of admitted patients to the clinic and finding the menopause women from different department were the limitations of this study.

CONCLUSION

This study showed the better and more relief of the symptoms of urinary incontinence, cellular maturation and vaginal dryness in menopause women in the hyaluronic acid cream therapeutic group compared with Estrogen-therapy group. Therefore, the hyaluronic acid could be a suitable alternative for those women who suffer from the complications of atrophy of the genital system and those with medical contraindications or negative experience in using hormonal drugs.

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Conflict of Interest: None declared.

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Effectiveness and safety of vaginal suppositories for the treatment of the vaginal atrophy in postmenopausal women: an open, non-controlled clinical trial

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Abstract. – Menopause, due to the physiological decrease in the estrogens levels, is often associated with many symptoms related to vaginal atrophy such vaginal dryness, dyspareunia, burning, itching, decreasing in libido and therefore a worsening of the quality of life and in particular of the sexual activity.

There are many pharmacological remedies to solve these events, first of all hormone replacement therapy (HRT) that up to the 90s was the therapy of choice for the care of the menopause symptoms. This hormonal therapy, however, has been re-considered due to its side effects.

As alternative, a clinical trial has been performed to investigate the efficacy and safety, in postmenopausal women with urogenital atrophy, of the use of suppositories for vaginal use, containing hyaluronic acid, vitamin E and vitamin A. The trial, according to a open, non-controlled design, was performed on 150 postmenopausal women, 1 vaginal suppository per day, for the first 14 days and then a vaginal suppository, day in and day out, for other 14 days.

The primary endpoint was the evaluation of vaginal dryness assessed by a Visual Analogue Scale (VAS) both by the investigator and the patient. The secondary endpoints were the evaluation of all the other symptoms and signs associated with the vaginal atrophy (itching, burning, dyspareunia, vaginal inflammation or swelling, irritation, assessed by a 4-point scale, presence of vaginal abrasions and irritation), and the recording of the adverse events occurring during the trial.

The patients have not reported adverse effects during the treatment, and the results in terms of effectiveness on the vaginal atrophy symptoms were markedly positive. A high level of compliance was registered.

The product tested can therefore be considered a safe and effective alternative for the treatment of vaginal atrophy symptoms in postmenopausal women, especially when HRT is not recommended.

Key Words:

Medical device, Menopause, Vaginal atrophy, Vaginal dryness, Hyaluronic acid.

Introduction

Menopause is a physiological step in a woman's life, which is associated with a decrease in the estrogens levels, due to a reduction in their endogenous production. Low estrogens circulating levels lead to some negative effects on all those organs whose function and health is linked to their presence. The lower genitor-urinary tract is negatively affected by a decrease in endogenous estrogens production^{1,2}. The vaginal epithelium becomes atrophic and its physiological lubrication is consistently reduced. This modifications lead to many physical discomforts as local itching and dyspareunia. In addition, the thinning of endometrium tissues and the enhancing of the vaginal pH due to estrogens deficiency, leads to increased incidence of vaginal infections and to a marked structural weakness). The vaginal dryness is also responsible for sexual dysfunctions and for libido reduction^{4,5}. The condition of vaginal atrophy is associated with a reduction of estrogens and thus it can be associated to all those women who are in premenopause but that have lower levels of endogenous estrogens for medical (anti-estrogenic drugs administration) or surgical reasons. The aging of female reproductive tract, due to reduced estrogens levels have serious influences on the quality of women's life, especially when its expectation is going to increase⁵.

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Hormonal replacement therapy, per systemic or topical way, have been often used, up to the 90s, for the treatment of menopausal effects on the urogenital tract, including those related to vaginal atrophy⁶. The WHI study (Women's Health Initiative) in 2002 and other published studies from that date onwards have changed radically the knowledge about the relationship between the risks and the benefits of the HRT^{7,8}. In particular, with regard to the risks of developing endometrial and breast cancer, the study published in 2005⁹, performed on approximately one million of postmenopausal women treated with HRT, reported a significant increase of the incidence of such cancer forms in women treated with estrogens alone or with a combined therapy (estrogens plus progestin compounds)¹⁰.

An alternative to the HRT was the use of estrogenic products for topical use^{11,12}. However, they are still considered at risk in case of prolonged use.

Considering these last results, there is an increasing need for new safe and effective therapies for the treatment of vaginal atrophy in postmenopausal women, which can be a valid alternative to hormonal therapy¹³.

To reduce the disorders associated with vaginal dryness, in postmenopausal women vaginal moisturizing or lubricants can be effective. The first can have short or long term effects, by improving the balance of intracellular fluids in the vaginal epithelium. The tissues seem to be more trophic and the physical disorders seem to get better.

The vaginal lubricants rather have a short term action as mechanical barrier between the vaginal epithelium and the external environment, and are mainly used to improve the dryness related to sexual activity.

The results of the clinical trial in open, non-controlled design are reported. It investigated the use of vaginal suppositories contain hyaluronic acid, vitamin E and vitamin A, intravaginal administered in women suffering from vaginal atrophy. The trial doesn't include the placebo group, due to the fact that the vaginal administration of a placebo can cause local alterations of the clinical results (e.g. vaginal pH and local epithelial alterations)¹⁴.

Hyaluronic acid sodium salt is a high weight molecule belonging to the class of glycosaminoglycans and consists of repeated disaccharides units (glycuronic acid and N-acetylglucosamine). Hyaluronic acid is able to retain very high amount of water molecules, forming a moisturizing, not greasy and permeable to water and light

film on skin. Hyaluronic acid is the main component of the derma's fundamental substance, and it is widely used in dermatology because it helps to:

- Form extracellular water film;
- Maintain the extracellular swelling;
- Moisturize the skin in case of inflammation.

It then helps to maintain water balance and thus makes the skin smooth and elastic¹⁵. Also plays a key role to maintain the tissues integrity and to ease the cells migration in case of inflammation. Hyaluronic acid inhibits the pericellular migration of viruses and bacteria and sets on its structure the free radicals (antioxidant action), facilitating the healing process and the tissue regeneration^{16,17}. Vitamin E, a fat-soluble vitamin with great antioxidant properties, takes part to the metabolism of all cells. It prevents the degradation of the tissue due to oxidant agents. However, vitamin E, despite the lack of literature, has a rational in this context: it can be used on the skin, because of its properties of antioxidant, anti-inflammatory and healing active agent. Even though few studies have been performed on the effects of vitamin E on vaginal mucosa, the Authors recommend it (taken orally, topically, or vaginally) for certain types of vaginosis¹⁸. In fact, Vitamin E as vaginal suppositories or oil can be used once or twice per day for 3 to 14 days to soothe the vaginal and vulvar mucosa. The use of vitamin E suppositories dates back to 1954 to treat yeast vulvovaginitis¹⁹. A very high soothing effect has been found when they are used once or twice daily for 7 or more days to reduce the symptoms associated with vaginal infections: vaginal irritation, swelling, local redness, burning and itching. The tissue becomes less irritated with a decrease in redness, swelling, and congestion. Vitamin A is a fat-soluble vitamin that has shown to have properties to increase the function of the immune local cells as well as ensure the integrity of morphological and functional vaginal epithelium^{20,21}. The lack of vitamin A also leads to growth inhibition and bones deformation, to serious changes in epithelial structures and reproductive organs^{22,23}.

Materials and Methods

One-hundred and fifty women, aged between 44 to 64, were admitted to participate to the trial,

all in surgical or physiological menopause from even 1 year, presenting vaginal dryness and correlated symptoms. Exclusions criteria were: no compliance to the treatment, genital abnormalities, positive Pap-test within the last three months, vaginal infections (confirmed by microbiological analysis), contact allergy in vulvo-vaginal zone, the use of drugs for vaginal administration in the last 15 days before the beginning of the study, alcohol or drugs abuse, taking part to other studies in the month before the recruitment.

All patients gave a written informed consent to the procedure. The trial was approved by the local Ethics Committee. The study followed the good clinical practice (GCP), and was performed according to an open, non-controlled design to evaluate local and systemic effects of the administration of a preparation of hyaluronic acid, vitamin E and vitamin A (Santes® ovuli, LO.LI. Pharma Srl, Italy) to treat post-menopausal vaginal atrophy.

All the patients were treated with daily application of one suppository, deeply in vagina (in the evening, before going to bed), for 14 days continuously. Then, for other 14 days, the administration of one suppository per day, one day in one day out.

Within the period of the study (4 weeks), four visits were performed: at baseline, after 7 days from the beginning of the treatment (visit 1), after 14 days (visit 2) and at the end of the treatment (day 28) (visit 3).

At baseline visit the exclusion/inclusion criteria were evaluated. Furthermore, demographic, medical and gynecological data were collected. A Pap-test was performed when not available in the last 3 months, urine and vaginal tampons were obtained.

During each visit a gynaecological inspection was done including vulvoscopy, vaginoscopy, cervix, ovarian, uterus and tubal analysis. Registration of objective vaginal symptoms (inflammation, edema, vulvo-vaginal abrasions) and of subjective vaginal symptoms (dryness, itching, burning, dyspareunia) were made.

The evaluation of the vaginal dryness (primary endpoint) was performed according to an analogical scale between 0 (no vaginal dryness feeling) to 10 (unbearable vaginal dryness feeling), by reporting data in the patient's diary.

Other symptoms and signs were evaluated by the investigator using a 4-point scale (1 = absent,

2 = mild, 3 = moderate, 4 = severe). Vaginal abrasions were only assessed as present or absent.

During all the treatment each patient noted daily the symptoms (itching, burning, dyspareunia), evaluating by a 4 points scale (1 = absent, 2 = mild, 3 = moderate, 4 = severe).

Statistical Analysis

Statistical analysis have been performed with SAS (version 8). Data belonging to all the patients that followed the protocol were included in the statistical analysis (PP population). Some results are expressed as means $\pm$ SD. The primary endpoint (vaginal dryness) differences were compared using the two-tailed Student's *t* test for independent data and *chi*² test.

A *P* value < .05 was considered statistically significant.

Analysis of secondary variables has performed by using Friedman test.

Results

Among 165 women recruited for the study, 150 were enrolled. 15 women do not copy with the inclusion criteria. 150 women enrolled, aged between 44 and 64 (mean $\pm$ SD, 51.6 $\pm$ 7.6), with a BMI between 18.1 and 37.47 (mean $\pm$ SD; 24 $\pm$ 10.6) were assigned to the treatment according to the study protocol; demographic characteristics of the patients are reported in Table I.

Twenty patients did not complete the study: four spontaneously abandoned the study, seven did not have compliance to the treatment, nine had a negative follow-up. A total of 130 patients completed the study (PP population).

The vaginal dryness (primary endpoint), measured with VAS scale, decreased in PP population from 7.92 at baseline to 4.22 at visit 1 (7 days after the beginning of the treatment), to 0.84

Table I. Demographic characteristics of the women taking part to the trial.

Patients	Values
Included in the trial	150
Age	51.6 $\pm$ 7.6 (min 44 max 64)
BMI (Body Mass Index)	24 $\pm$ 10.6 (min 18.1 max 37.47)

at visit 2 (14 days), to 0.0 at visit 3 (28 days, end of the treatment). Differences revealed by ANOVA analysis was highly significant ($F = 1029,2$; $P < 0.001$). Multiple comparison confirmed a significant decrease of the symptom, even at the first time point of the study (visit 1, after 7 days), in comparison with the baseline value.

During the baseline visit, the hitching symptom was severe in 106 women and moderate in 24 women; it disappeared progressively during the treatment period and only 4 women still reported the symptom at the end of the treatment (visit 3). The results are reported in Table II. The mean values of hitching, measured with a 4-point scale decreased from 3.82 at baseline, to 2.89 at visit 1, to 1.35 at visit 2 and to 1.03 at visit 3 ($P < 0.001$).

During the baseline visit, burning was present as severe symptom in 93 women, moderate in 26 women and mild in 11. The symptom is progressively disappeared during the treatment. At the end of the treatment only 4 women complained about a mild burning symptom. Data are reported in Table II. The mean values of burning, measured with a 4-point scale decreased from 3.63 at baseline, to 2.45 at visit 1, to 1.31 at visit 2 and to 1.03 at visit 3 ($P < 0.001$).

Dyspareunia, at the baseline visit, was registered as severe symptom in 18 women, as moderate in 78 women and as mild in 34. The symptom decrease progressively and only 5 women reported mild dyspareunia at the end of the treatment. Data are reported in Table II. The

mean values of dyspareunia, measured with a 4-point scale decreased from 2.88 at baseline, to 2.19 at visit 1, to 1.43 at visit 2 and to 1.04 at visit 3 ($P < 0.001$).

During the baseline visit, the inflammation/edema of vaginal mucosa was registered severe in 30 women, moderate in 52 women, mild in 28 and absent in 20; it disappeared progressively during the treatment, and only 5 women still reported the mild symptom at the end of the treatment (visit 3). The mean values of the symptom, measured with a 4-point scale decreased from 2.71 at baseline, to 1.93 at visit 1, to 1.65 at visit 2 and to 1.04 at visit 3 ($P < 0.001$).

During the baseline visit, the vaginal mucosa irritation was severe in 25 women, moderate in 32, mild in 50 and absent in 23 women; it disappeared progressively during the treatment in all the patients. The mean values of the irritation, measured with a 4-point scale decreased from 2.45 at baseline, to 1.78 at visit 1, to 1.34 at visit 2 and to 1.00 at visit 3 ($P < 0.001$).

The compliance to the treatment was complete for 126 patients and partial for the other 4 patients. Data are reported in Figure 1A.

The overall judgement of the product's effectiveness reported by the investigator was optimal in 108 patients, good in 20 patients and moderate in 2 patients. Data are reported in Figure 1B.

The overall judgement of the product's safety was reported by the investigator optimal in 124 patients, good in 4 and sufficient in 2 patients. Data are reported in Figure 1C.

Table II. Subjective symptoms in post-menopausal women treated with the product for 28 days (visit 1 = 7 days; visit 2 = 14 days; visit 3 = 28 days).

		Hitching	Burning	Dyspareunia
Baseline	Absent	0	0	0
	Mild	0	11	34
	Moderate	24	26	78
	Severe	106	93	18
VISIT 1	Absent	0	15	27
	Mild	20	45	53
	Moderate	104	67	48
	Severe	6	3	2
VISIT 2	Absent	95	100	78
	Mild	25	20	48
	Moderate	10	10	4
	Severe	0	0	0
VISIT 3	Absent	126	126	125
	Mild	4	4	5
	Moderate	0	0	0
	Severe	0	0	0

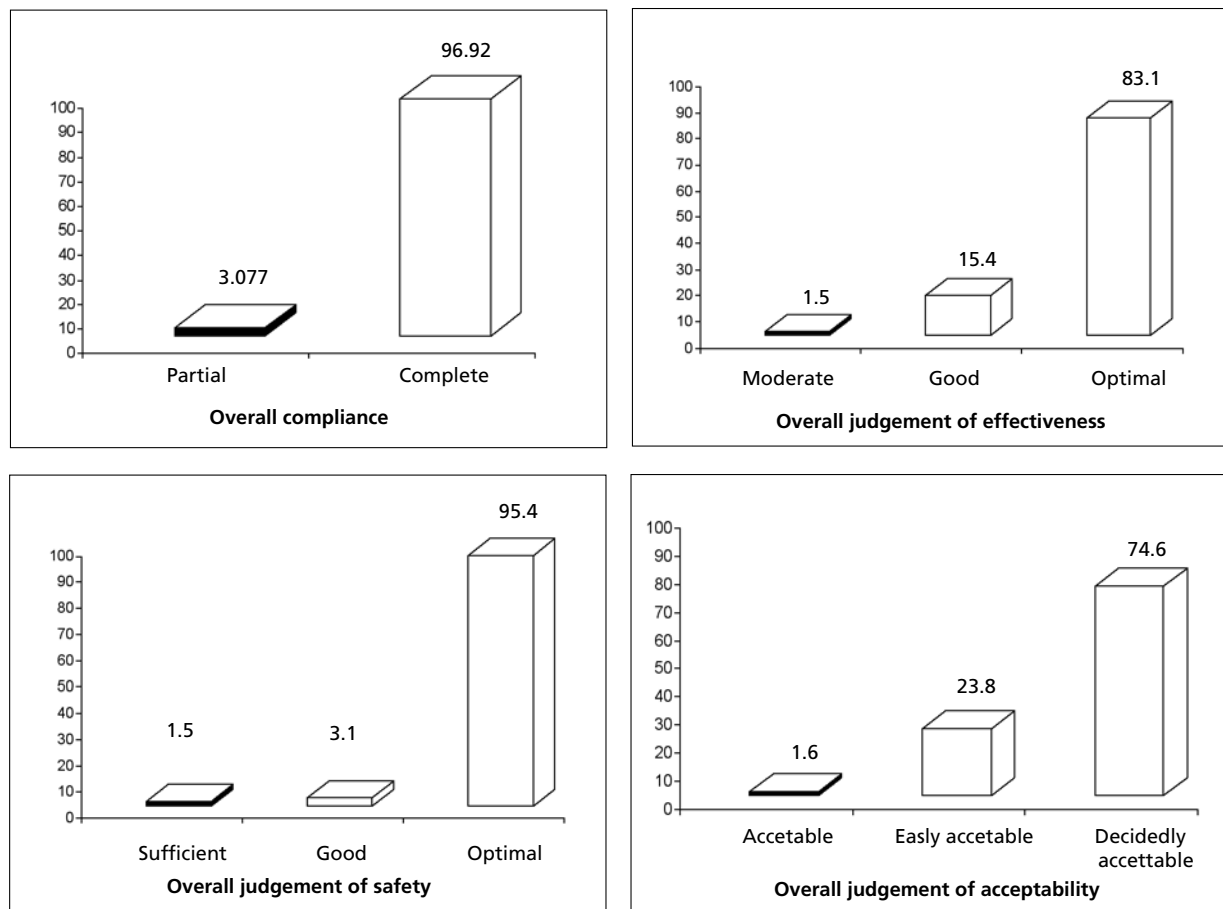


Figure 2. A-D, Overall judgement and compliance in post-menopausal women after 28 days of treatment (data expressed in percentage).

Acceptability of the medical device by the patients was evaluated as decidedly acceptable by 97 patients, easily acceptable by 31 and acceptable by 2 patients. Data are reported in Figure 1D.

Discussion

In the last years is great the need of new therapeutic forms, safe and effective, to restore normal condition in post-menopausal women affected from vaginal atrophy. This new product in suppositories form has been studied in order to be a new alternative form of treatment of the symptoms correlated to genital atrophy.

The trial was assessed on post-menopausal women, in order to evaluate the safety and the effectiveness of this product on the clinical and subjective symptoms correlated to vaginal atrophy.

The results confirmed a favourable safety profile of the product even after a longer period of treatment. The effectiveness on vaginal dryness and on correlated symptoms was good right from the first week of treatment. The overall judgement on effectiveness and safety was optimal for the main part of the patients. The compliance was favourable in almost the whole group of women treated.

The only guidelines available for the study were those correlated to the treatment of vaginal condition: “Guidance for industry. Bacterial vaginosis – Developing antimicrobial drugs for treatment” of FDA (Food and Drugs Administration) that suggest do not use placebo to avoid any alteration of the clinical results¹⁴.

The components of the suppositories are responsible for slowing down the natural process of aging of the vaginal and vulvar tissues and of increasing their natural hydration. Effectiveness of the single components of the product should be investigate separately in further trials.

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Topical Oxygen Therapy Induces VEGF Expression and Improves Closure of Clinically Presented Chronic Wounds

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Abstract

1. Chronic wounds, especially in diabetics, represent a serious threat to human health.
2. Correcting a compromised state of tissue oxygenation by the administration of supplemental O₂ is known to benefit wound healing. Beyond its role as a nutrient and antibiotic, O₂ supports wound healing by driving redox-signaling.
3. HBO (hyperbaric oxygen) therapy is widely used and approved by CMS to treat specific ulcerations. The current literature supports that approaches to topically oxygenate wounds may be productive.
4. Here, we present the results of two simultaneous studies testing the effects of HBO and portable topical oxygen (TO) therapies. These two therapeutic approaches have several contrasting features.
5. A total of 1854 patients were screened in outpatient wound clinics for non-randomized enrollments into the HBO (n=32, 31% diabetic) and TO (n=25, 52% diabetic) studies.
6. Under the conditions of the current study, HBO treatment seemed to benefit some wounds while not benefiting the others. Overall, HBO did not result in statistically significant improvements in wound size in the given population over the time monitored in this study.
7. TO significantly improved wound size. Among the three (VEGF, TGFβ1 and COL1A1) O₂-sensitive genes studied in wound-edge tissue biopsies, TO treatment was associated with higher VEGF165 expression in healing wounds. Expression of the other genes mentioned was not affected by TO. All of the genes studied did not significantly change in expression in patients of the HBO study. This work establishes a link between VEGF gene expression and healing outcome for TO therapy.
8. Taken together, this report presents evidence demonstrating that TO treatment benefits wound healing in patients suffering from chronic wounds. TO treatment is associated with induction in VEGF expression in the wound edge tissue and improvement in wound size.

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Keywords

redox; wound therapy; wound care; tissue repair; wound patient; topical therapeutics; skin; translational research

Introduction

Hypoxia, caused by disrupted vasculature as well as complicating vasculopathies and other systemic limitations, limits wound healing. Correcting a compromised state of tissue oxygenation by the administration of supplemental O₂ benefits wound healing in the peri-operative and outpatient settings¹. Clinical trials have shown that keeping patients warm and administering supplemental O₂, both of which enhance wound oxygenation, decreases the rate of wound infection in surgical patients and shortens the average length of hospitalization^{2,3}. Beyond its role as a nutrient and antibiotic, O₂ supports wound healing by driving redox-sensitive gene expression and signal transduction which influences a wide array of healing responses^{4–6}.

Clinical use of O₂ to promote wound healing began in the 1960's with administration of systemic HBO to treat wounds⁷. Today, HBO therapy is approved by the Center for Medicare and Medicaid Services in the United States to treat specific ulcerations. Our own laboratory has noted beneficial effects of HBO therapy in restoring wound healing that was impaired by psychological stress⁸. Encouragingly, recent reports have demonstrated that HBO therapy may mobilize bone marrow-derived endothelial progenitor cells which could benefit the healing of chronic wounds affected by diabetes and peripheral arterial disease⁹. On the down side, HBO poses the threat of oxygen toxicity in specific cases^{10–12}. This risk may be managed by adopting a personalized approach for HBO therapy where the treatment specifically aims at addressing wound hypoxia on a case by case basis. Although HBO is a clearly promising mode of wound therapy, it requires extensive facilities which may not be available to all patients. Furthermore, a population of wound patients may simply not qualify or consent to receive HBO therapy. The approach to topically oxygenate wounds using a variety of approaches is distinct from the conventional HBO therapy in many ways. For example, topical approaches do not involve high pressure, are not systemic in nature and therefore do not pose the uncommon risk of systemic oxygen toxicity¹³. The hypothesis that wounds may benefit when oxygenated topically is supported by the current literature^{13–16}.

Among many known growth factors, VEGF is believed to be the most prevalent, efficacious and long-term signal that is known to stimulate angiogenesis in wounds¹⁷. This work represents the side-by-side presentation from the result of two simultaneous studies testing the effects of HBO therapy and topical oxygen (TO) therapy, respectively. The goals were to examine changes in wound closure outcomes and in the expression of oxygen-sensitive genes including VEGF in biopsies collected from the wound-edge tissue.

Materials and Methods

Study Design

This study was approved by the Institutional Review Board of The Ohio State University. Subjects were duly consented and enrolled at Ohio State University's Comprehensive Wound Center (CWC) outpatient sites. A total of 1854 patients that visited our wound clinics were screened to successfully enroll 57 patients in the HBO and TO studies. The inclusion criteria were: (a) age: 30–70 years; (b) the wound had been present for at least 4 weeks; (c) patients were not immunosuppressed or therapeutically anticoagulated; (d) patients were able to give their own consent. The selected age group represents >90% of the patients seen in our wound

clinics. Patients were excluded if they had previously received oxygen therapy for the wound being studied. The type of oxygen therapy administered was based upon the clinical decision of the physician managing the wound. Patients that did not qualify for HBO were asked if they wanted to enroll in the TO study. In order to qualify for HBO therapy, patients were required to meet CMS criteria which include standards that have been established for fourteen clinical diagnoses that are currently approved for HBO.

Wound-edge tissues were rapidly freed from blood by rinsing in saline and snap frozen in liquid nitrogen. For those subjects who consented, wound-edge biopsies were obtained three time points (T_0 , T_1 and T_2) during the 14 week study period. Three mm punch biopsy was performed exactly on the perimeter of the wound which represented the wound edge. The tissue harvested was immediately freed of blood using ice-cold saline and placed in OCT and snap-frozen in liquid nitrogen. Wound dimension recording was performed at all of these time-points on all patients enrolled. The three time points were defined as follows:

β -actin	GTACCACTGGCATCGTGATGGACT
	CCGCTCATTGCCAATGGTGAT
VEGF-A ₁₆₅	TGCCCACTGAGGAGTCCAACAT
	CACGTCTGCGGATCTTGTAACAAACA

T_0 , just before the first round of oxygen therapy

T_1 , seven weeks into the study or upon meeting specific criteria set for the HBO and TO studies whichever came first. For the HBO study, completion of 50% of therapy was set as the criterion. This would refer to say completion of five out of a total of ten dives prescribed. For the TO study, 50% wound closure was set as the criterion.

T_2 , fourteen weeks into the study or wound closure whichever came first.

Supplemental Oxygen Therapy

HBO was administered to patients that met specified CMS criteria for receiving HBO therapy. It was administered at the CWC outpatient clinics under the supervision of physicians according to the Undersea and Hyperbaric Medicine Society (UHMS) guidelines¹⁸. Sechrist model 3200 chambers (Sechrist Industries, Anaheim, CA) were used for the HBO treatments.

The first TO treatment for all patients was performed in outpatient sites of CWC so that pre-treatment wound-edge tissue biopsies could be obtained and patients could be instructed on the use of the device. Treatments, other than the T_0 , T_1 and T_2 time-points during which biopsies were collected, were performed in the patients' homes. Treatments which followed the collection of wound-edge biopsies were performed in the clinic. The TO device (GWR Medical Inc., Chadds Ford, PA) is a single use disposable device that connects to a portable oxygen source as described previously¹⁹. The device inflates to approximately 1 atmosphere of pressure and has a release valve in the event of excess pressure build up. Representatives from the manufacturer of the TO devices provided patients with instruction on device usage. TO was administered to the wound for 90 minutes per day for 4 consecutive days in a week followed by three days of no oxygen supplementation. This cycle was repeated each week. TO therapy was discontinued at the discretion of the managing physician.

RNA Isolation

Total RNA was isolated from wound biopsy tissue material stored in liquid nitrogen using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Samples were placed in a 2 ml microcentrifuge tube with RLT buffer from the RNeasy Fibrous Tissue Mini Kit and 5 mm stainless steel beads. Tissue samples were disrupted and homogenized using a TissueLyser

equipment (Qiagen, Valencia, CA). Tissue disruption was carried out twice for 3 minutes each at 20–30 Hz.

mRNA Quantification

mRNA were quantified by real-time PCR assay using double-stranded DNA binding dye SYBR green-I as described previously^{6,20,21}. Gene expression results were standardized relative to β -actin. The primer-set used for the individual genes were as follows:

Primer sets for TGF β -1 (PPH00508A) and collagen1A1 (PPH01299E) were obtained from SuperArray Bioscience Corporation (Frederick, MD).

Statistical Analyses

Patient demographics were compared across the two studies (HBO and TO) in order to present a comparative account of the two study populations. Differences in normally distributed continuous variables were tested using the two-sample *t*-test while continuous variables that were not normally distributed were tested using the Wilcoxon rank-sum test. Differences in categorical variables were tested using Fisher's exact test. Wound volume, in cm³, was calculated from the wound surface area and depth as recording during standard clinical practice. The wound volume data was transformed using a cube root for variance stabilization and normality assumptions^{22–26}. The difference in the cubed root of the final minus the cubed root of the initial wound volume was regressed on oxygen treatment (HBO or TO) and adjusted for the cubed root of the initial wound volume. Covariate interactions with the treatment variable were considered significant if the *p*-value ≤ 0.05 and the scale of the cubed root of the initial volume was tested using fractional polynomials²⁷. Gene expression data was collected from only those patients who consented to provide biopsies. Some patients provided biopsies for one time point but not the other. Patients from whom paired gene expression data was available for the two time points being compared statistically were included in the relevant analyses. For presentation of gene expression data, wound closure was dichotomized *a priori* as healing if the final wound volume was less than or equal to the initial wound volume and not healing if the final wound volume was greater than the initial wound volume. Wound size that got bigger therefore fell into the non-healing category. This categorization was solely for the purposes of grouping and had nothing to do with the testing of efficacy (as in Fig. 3) where a ratio of initial:final wound volume of 1 was interpreted as no effect. Data from VEGF, TGF β 1, and collagen 1A1 gene expression measurements were natural log transformed and regressed on dichotomized wound healing and adjusted for other covariates. This linear regression was run separately for both studies *i.e.* HBO and TO. One-sample *t*-tests were used to compare at various time points the VEGF ratio (one time point : another time point as indicated in the respective illustrations) to 1.0 (reflecting no change) for only those subjects that were in the healing group. All analyses were conducted using Stata 10.0, Stata Corporation, College Station, Texas.

Results

A total of 1854 patients were screened in the outpatient clinics of CWC for enrollments into the HBO and TO studies. The demographics of the subject populations of the HBO and TO studies were comparable as illustrated in Table 1. A total of fifty-seven patients with chronic wounds were enrolled. Based on the assessment of the respective physicians, thirty-two patients qualified for HBO therapy. Twenty-five patients consented to receive TO therapy. The mean age of the subjects in the HBO and TO studies was 52.3 and 54.7 years, respectively. The subject population of the HBO study primarily consisted of men who represented 90.6% of the total population. The subject population in the TO study was more even balanced for gender-distribution. Fifty-two percent of the TO subject population were men while the balance of

48% were women. Wound site was confined to either the trunk or lower extremity and there were no significant differences between the subject populations of the HBO and TO studies. The fractions of known diabetics in the HBO and TO studies were 31% and 52%, respectively. The wound etiology and location for each study are illustrated in Figure 1. While enrollment into the two treatment modalities was not randomized thus making direct comparison of the findings between the two studies not possible, the study design enabled the determination of whether the two modalities share a common mechanism of action.

The wound volume data was transformed using a cube root for variance stabilization and normality assumptions^{22–26}. The difference in the cubed root of the final minus the cubed root of the initial wound volume was regressed on oxygen treatment (HBO or TO) and adjusted for the cubed root of the initial wound volume. Analysis of the effects of HBO on wound closure was based on a model which included the cubed root of the initial volume as a covariate and the best fit was determined to be linear using fractional polynomials. The untransformed results are shown in Figure 2. The diagonally dashed line of reference represents no change in wound volume in response to treatment. Observations (black dots) on or above the dashed line represents no benefit in wound size in response to treatment. Observations plotted below the dashed line of reference represent that the treatment improved wound closure outcome. The solid line represents the linear regression model based on wound closure data as collected from the HBO study (Fig. 2). Covariate interactions with the treatment variable were considered significant if the p -value ≤ 0.05 and the scale of the cubed root of the initial volume was tested using fractional polynomials²⁷. In the case of the HBO study, the p -value was observed to be 0.150 ($R^2=0.068$). Thus, under the conditions of the current study, HBO treatment did not result in significant improvements in wound closure (Fig. 2). The statistical approach to determine the efficacy of TO treatment on wound closure outcomes was exactly identical to the approach described above for the HBO study. The untransformed results related to changes in wound volume in response to TO treatment are illustrated in Figure 3. In the present study, TO treatment significantly improved wound closure by decreasing wound volume. For the TO study, the regression line shown in solid was significantly (p -value, 0.001, $R^2=0.414$) different from the reference dashed line (Fig. 3).

Next, we turned towards the examination of O_2 -sensitive genes in the wound-edge tissue biopsies collected from consenting patients. Three genes, VEGF, TGF β 1 and collagen 1A1 (COL1A1) were selected based on their known sensitivity to oxygen and functional relevance of the gene products to wound healing. Each subject had a baseline level of gene expression determined by using the T_0 biopsy for real-time PCR measurements, since they were obtained prior to any exposure to oxygen therapy. All measured mRNA expression levels were standardized against β -actin mRNA expression. The effect of supplemental oxygen therapy was analyzed by measuring the relative change in target gene expression for each individual compared to their baseline (T_0). This allowed each patient to serve as their own control for these analyses. Relative change in gene expression was calculated dividing the rate of gene expression at a selected time-point by the observed baseline. All data were log transformed to perform statistical comparisons between ratios.

At the intermediate time-point T_1 , results adjusted for age of each subject were analyzed. Both TGF β 1 as well as COL1A1 did not exhibit any statistically meaningful responsiveness to TO treatment (Table 2). In contrast, VEGF expression was significantly higher in TO treated healing wounds (Table 2). This finding led to our emphasis on the study of VEGF. The expression of VEGF was analyzed also for the final time-point T_2 . Analysis of the VEGF expression data from the three time-points in TO treated healing wounds are illustrated in Table 3. Interestingly, when the initial time-point (T_0) was compared with the intermediate time-point (T_1), a trend in favor of TO-induced VEGF expression in the wound-edge tissue was noted (Table 3). However, the observation was not statistically significant (p -value = 0.07).

This effect became statistically significant if the initial time-point (T_0) was compared with the final time-point (T_2). Plotting of individual VEGF response data points against log-transformed wound closure ($T_2:T_0$) demonstrated the results presented in Table 3 on an individual basis (Fig. 4). Data points to the left of the vertical line (wound volume ratio < 1) indicate that the wound was smaller than the original size while observations to the right indicate the wound got bigger with time as represented in the Y-axis. Data points below the horizontal line (VEGF ratio < 1) indicate lowering of VEGF gene expression over the specified time period. Data points above the horizontal line (VEGF ratio > 1) indicate induction of VEGF gene expression. Of note, all the healing wounds in the TO treatment study showed VEGF induction (Fig. 4c), an effect that is listed to be statistically significant in Table 3.

The difference in gene expression over dichotomized (healing vs. non-healing) wound outcomes for the HBO study was not statistically significant for VEGF, TGF β 1, or collagen 1A1 for any of the time intervals compared (Table 4). Analysis of the VEGF expression data from the three time-points in HBO treated healing wounds are illustrated in Table 5. HBO did not cause a significant increase in VEGF expression at any interval ($T_1:T_0$ or $T_2:T_1$) or cumulatively over the course of treatment ($T_2:T_0$). In the contrast to the findings noted in the TO study, changes in VEGF expression in the wound-edge tissue of patients enrolled in this study were not statistically significant. However, a closer look at the scatter plot data reveals that in numerous subjects HBO treatment did markedly induce VEGF. However, there were some subjects did not respond. Therefore, taken together, the effect was not statistically significant (Fig. 5). It would be of interest to identify the conditions under which HBO is effective in inducing VEGF in the wound tissue of patients.

Discussion

Achieving closure in a chronic wound requires provision of adequate oxygen delivery to the tissue, adequate protein and other nutritional factors, a moist environment, an appropriate inflammatory *milieu*, debridement, appropriate management of infection, and correction of contributing medical diagnoses. Hypoxia is a limitation that is commonly noted in problem wounds. Achieving appropriate levels of tissue oxygenation to support healing represents a major requirement in the treatment of chronic wounds^{28–30}. Systemic HBO represents a therapeutic modality that is widely utilized as a standard of care in numerous wound clinics. Adjunctive HBO therapy has been demonstrated to be clinically effective in several studies^{31–34}. In the treatment of hypoxic and ischemic wounds, the most important effects of hyperbaric oxygenation are the stimulation of fibroblast proliferation and differentiation, increased collagen formation and cross-linking, augmented neovascularization, and the stimulation of leukocyte microbial killing. Ischemic soft tissues also benefit from hyperoxygenation through improved preservation of energy metabolism and reduction of edema³⁵. In addition, HBO therapy may have important effects on the biology of cytokines and other mediators of inflammation³⁶. In patients whose wounds were favorably affected by HBO therapy, increased levels of nitric oxide were observed in response to HBO treatment³⁷. Therapeutic HBO can increase the mobilization of endothelial progenitor cells from the bone marrow into peripheral blood which has the clear potential of benefiting wound healing in patients affected by diabetes and peripheral arterial disease⁹. A randomized blind study examining the effects of HBO therapy on experimental wounds in humans noted that the HBO group significantly benefited from a 42% reduction in wound hyperemia, a 35% reduction in the size of the lesion, and a 22% reduction in wound exudation³⁸. The favorable effects of HBO in clinical studies are supported by numerous experimental studies demonstrating that HBO therapy improves tissue oxygenation^{39–44}. Our own studies in mice have demonstrated that impairments in wound closure caused by psychological stress can be corrected by HBO therapy⁸.

Factors that have limited a wider acceptance of HBO therapy in mainstream wound care include inconsistent results in a clinical setting^{45,46}, arguable flaws in some study designs⁴⁷ and insufficient number of clinical trials^{48,49}. The high cost of providing HBO has also raised concerns⁴⁷. In that vein, the cost of not accepting a potentially productive treatment modality may be also counter-argued⁵⁰. Our observation that HBO therapy did not favorably impact wound closure outcome in the total population studies is on one hand consistent with the previous literature reporting lack of efficacy of HBO under specific conditions. On the other hand, however, examination of individual outcomes revealed that the lack of efficacy of HBO on wound closure was not uniformly noted over the entire population of subjects studied. One may rationally argue that the HBO study included responders as well as non-responders. The mixed findings resulted in a lack of statistical significance under the conditions specified. It becomes increasingly important to identify the specific conditions under which HBO becomes effective in favorably affecting wound outcomes. Of note in that context is the fact that HBO superoxygenates tissues to levels multi-fold higher than their baseline pO_2 ^{51–53}. From a mechanistic standpoint, it is important to recognize that while correction of wound hypoxia is desirable, excessive oxygenation may pose the risk of oxygen toxicity and cell cycle arrest^{11,54–56}. This notion is supported by findings of a mathematical model developed to assess the effect of wound tissue pO_2 on healing outcomes⁵⁷. We posit that a personalized approach to utilize HBO therapy that is based on achieving a prescribed wound tissue oxygen tension as opposed to utilizing the same regimen for all patients will provide more consistent favorable beneficial effects of HBO therapy.

Topically applied oxygen gas is able to modestly raise the pO_2 of the superficial wound tissue¹⁵. Of note, a series of recent observations demonstrate that the topical route of wound oxygenation may be effective in benefiting wound healing^{13–16}. Both HBO as well as TO devices are FDA approved. Recently, FDA has proposed to reclassify TO devices from the most stringent class III (pre-market approval) to a safer class II (<http://www.fda.gov/cdrh/ode/guidance/1582.html>). Encouraging results obtained from the use of TO in both clinical¹³ as well as pre-clinical¹⁵ settings warrant further interest addressing the significance of TO in treating problem wounds in a clinical setting. If proven to be effective, portable TO therapy has the added advantage of benefiting a much larger potential patient population especially under conditions of public disaster and in a field-setting where HBO therapy may not be applicable. In this context it is important to recognize that although HBO and TO both seek to oxygenate wounds, they are quite different treatment approaches. The current study examined the effects of TO in patients that were not selected for HBO therapy. For the first time, the effects of TO has been studied not only to examine closure outcomes in a clinical setting but to also gain mechanistic insight on how TO therapy may influence wound healing.

The state of tissue oxygenation is a key determinant of inducible VEGF expression and angiogenesis. While hypoxia can initiate neovascularization by inducing angiogenic factor expression, it cannot sustain it. A threshold level of oxygenation is required to support the metabolic needs of tissue remodeling. Acute hypoxia facilitates the angiogenic process⁵⁹ while chronic hypoxia impairs wound angiogenesis⁶⁰. Sustained hypoxia causes death and dysfunction of tissue. VEGF is a major long-term angiogenic stimulus at the wound site. On one hand, hypoxia is a potent trigger of inducible VEGF expression⁶¹. On the other hand, hyperoxia induces VEGF as well^{54,62–65}. This study provides the first evidence that supplemental TO treatment significantly induces VEGF expression in the wound-edge tissue of patients suffering from chronic wounds. This work is consistent with previous findings suggesting that TO treatment may induce wound angiogenesis⁶⁶.

Given the essential role of angiogenesis in wound healing, it is not surprising that there was a statistically significant correlation between VEGF and healing outcomes, as shown with TO

therapy. A similar effect was expected with HBO, but was not observed. Healing responses induced by HBO in this patient population occurred by means that were independent of VEGF expression. This hypothesis is consistent with the current literature^{9,37}. One potential reason for this is the differences in the levels of wound tissue oxygenation achieved with each of these modalities. The levels of oxygenation achieved with 2 atmospheres of pressure in HBO therapy can range from 300–1200 mmHg, which far exceeds the pO_2 of healthy skin or that achieved in the wound tissue with TO^{15,19}. This work establishes a link between VEGF gene expression and healing outcome for TO therapy. If validated in a larger population, this finding could help identify a biomarker to gauge response to TO treatment. Taken together, this study presents evidence supporting that TO treatment may benefit wound healing in patients suffering from chronic wounds. TO treatment is associated with induction in VEGF expression in the wound edge tissue and improvement in wound closure outcome. Approaches to topically oxygenate exposed dermal wound tissue warrant serious interest.

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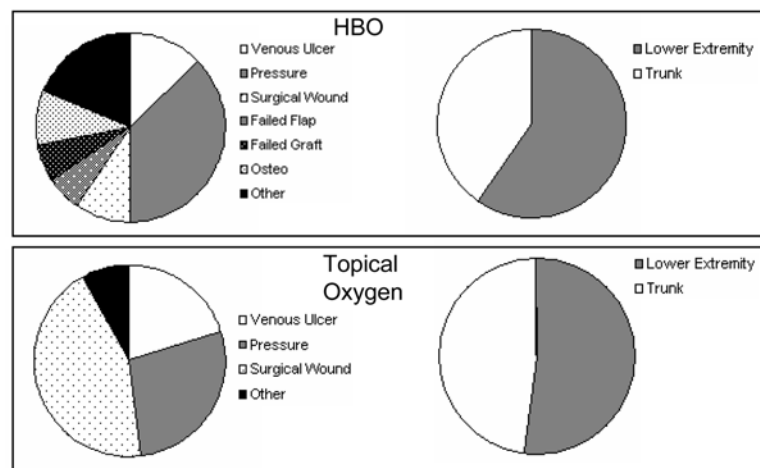


Figure 1.
Wound etiology and locations for patients enrolled in the HBO and topical oxygen studies.

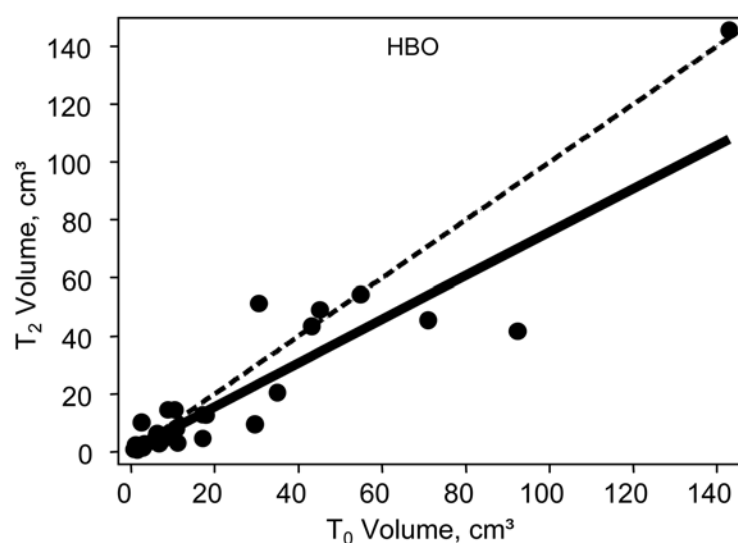


Figure 2. Wound closure in response to HBO treatment

Analysis of the effects of HBO on wound closure was based on a model which included the cubed root of the initial volume as a covariate and the best fit was determined to be linear using fractional polynomials. The untransformed results are shown here. The diagonally dashed line of reference represents no change in wound volume in response to treatment. Observations (black dots) on or above the dashed line represents no benefit in wound size in response to treatment. Observations plotted below the dashed line of reference represent that the treatment improved wound closure outcome. The solid line represents the linear regression model based on wound closure data. The solid line was tested to be statistically not significantly different from the reference dashed line (p -value = 0.150, R^2 = 0.068)

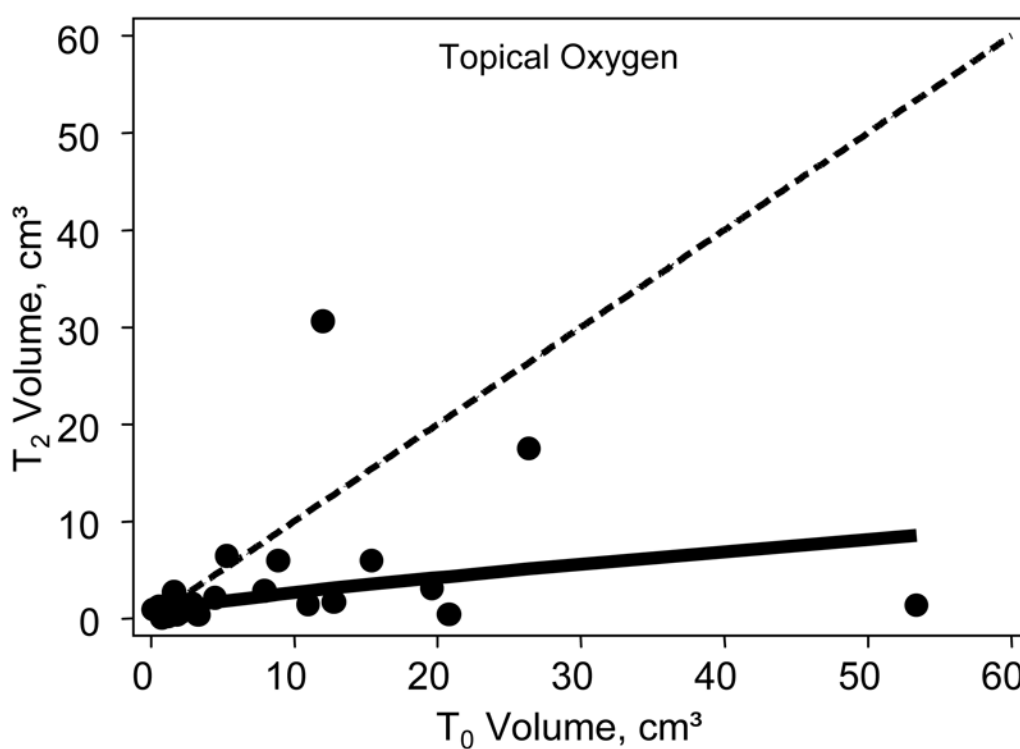


Figure 3. Wound closure in response to topical oxygen treatment

Analysis of the effects of topical oxygen on wound closure was conducted as described in the legend of Figure 2. The solid line represents the linear regression model based on wound closure data from the topical oxygen study. The solid line was tested to be significantly different from the reference dashed line (p -value < 0.001, $R^2 = 0.414$) indicating that topical oxygen treatment improved wound closure.

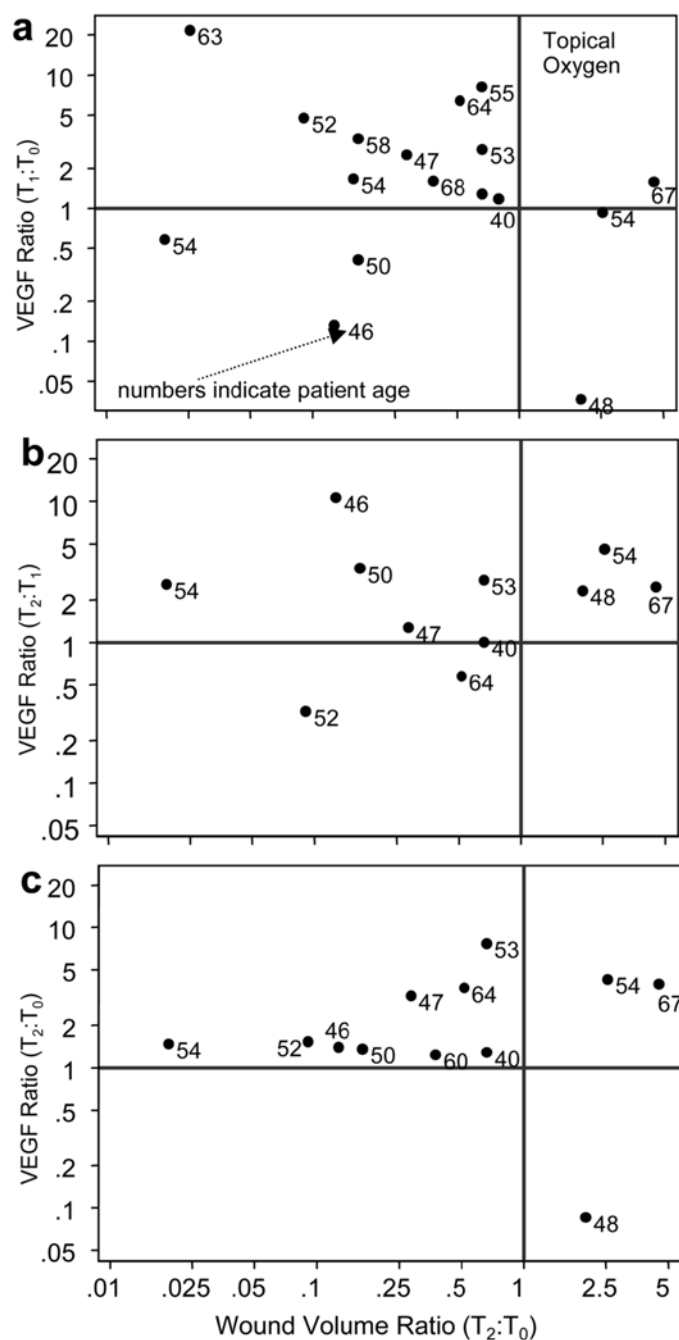


Figure 4. Scatter plot illustrating individual data points plotting topical oxygen induced changes in VEGF gene expression in the time period specified on the y-axis against changes in wound volume over the entire study period

Both the VEGF ratio and the wound volume ratio were log transformed. The number against each data point represent the age of the respective patient in years. a, VEGF changes during the time period T_0 (initial) to T_1 (interim); b, VEGF changes during the time period T_1 (interim) to T_2 (final); and c, VEGF changes during the time period T_0 (initial) to T_2 (final) *i.e* the entire study duration.

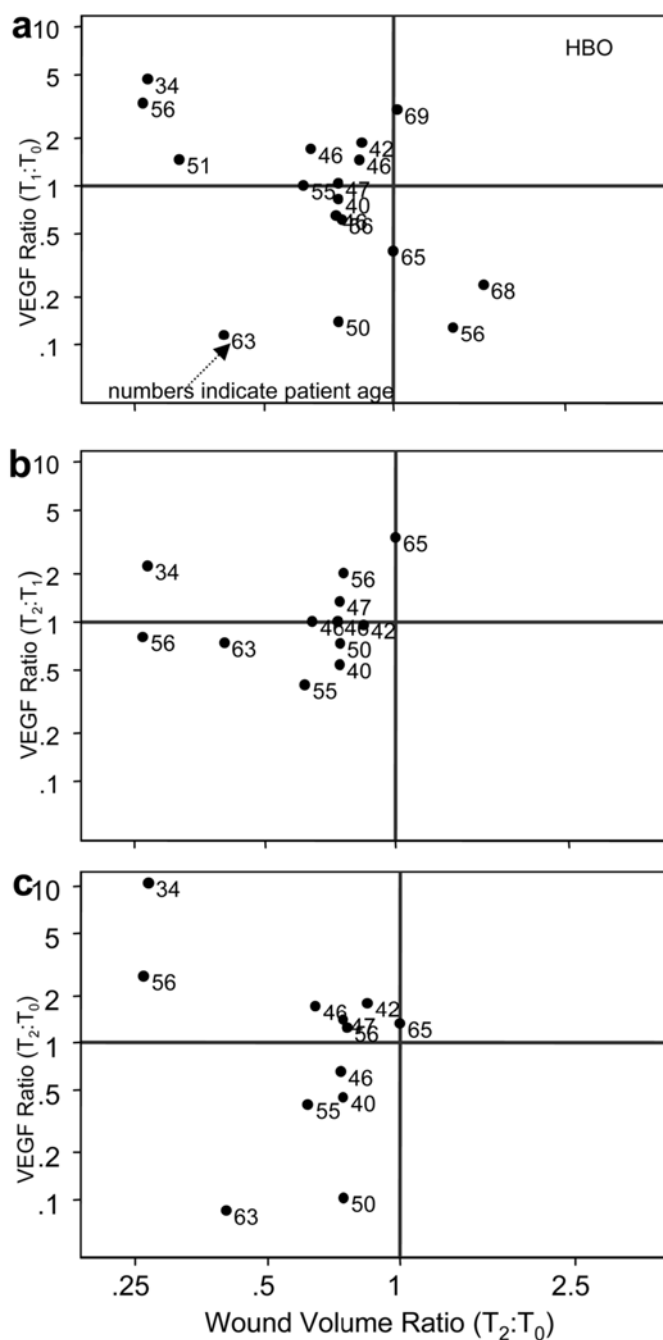


Figure 5. Scatter plot illustrating individual data points plotting changes in HBO-induced VEGF gene expression in the time period specified on the y-axis against changes in wound volume over the entire study period

Both the VEGF ratio and the wound volume ratio were log transformed. The number against each data point represent the age of the respective patient in years. a, VEGF changes during the time period T_0 (initial) to T_1 (interim); b, VEGF changes during the time period T_1 (interim) to T_2 (final); and c, VEGF changes during the time period T_0 (initial) to T_2 (final) *i.e* the entire study duration.

Table 1

Patient Demographics by Treatment Type

Attribute	HBO	Topical Oxygen
Count (% diabetic)	32 (31)	25 (52)
Age, mean (standard deviation)	52.3 (11.0)	54.7 (8.9)
Male, percent	90.6	52.0
Initial volume (cm ³), median (IQR)	9.4 (2.8 – 30.1)	3.3 (1.2 – 12.0)
Final volume (cm ³), median (IQR)	6.1 (2.5 – 17.5)	1.4 (0.6 – 2.8)

HBO: Hyperbaric Oxygen, IQR: Interquartile range

Table 2
Changes in Oxygen-Sensitive Genes in Wound-Edge of Patients Treated with TO

Gene	Oxygen Treatment	Count Healing : Not Healing	Expression, mean		Wounds Healing to not healing Ratio	p-value ¹
			Wounds Not Healing	Wounds Healing		
VEGF	Topical Oxygen	14 : 3	0.85	4.02	4.73	0.031
TGFβ1	Topical Oxygen	15 : 3	0.62	2.52	4.06	0.347
COL1A1	Topical Oxygen	15 : 3	2.22	2.92	1.32	0.520

Results from T0 versus T1 shown.

¹ p-value is testing if the healing to not-healing wound volume is significantly greater than 1.0 as determined from linear regression of natural log transformed gene expression values from healing (yes/no) and is adjusted for the patient's age

Table 3
VEGF Expression for Healing Wounds Treated with HBO

Time Interval ¹	Oxygen Treatment	Number healing wounds	VEGF Ratio ²	p-value ³
T ₂ to T ₀	HBO	12	1.86	0.741
T ₂ to T ₁	HBO	12	1.27	0.767
T ₁ to T ₀	HBO	14	1.38	0.716

¹ T₀= baseline measurement; T₁ = midpoint of therapy; T₂ = 14 weeks or imminent closure

² VEGF ratio = log transformed ratio of VEGF/ β -actin (T_x): VEGF/ β -actin (T_y) where T_x = first T in time interval and T_y = second T in time interval

³ p-value is testing if the healing VEGF ratio is significantly greater than 1.0 and is generated from a one-sample t-test that uses the natural logarithm of the VEGF ratio.

Table 4
Changes in Oxygen-Sensitive Genes in Wound-Edge of Patients Treated with HBO

Gene	Oxygen Treatment	Count Healing : Not Healing	Expression, mean		Wounds Healing to not healing Ratio	p- value ¹
			Wounds Not Healing	Wounds Healing		
VEGF	HBO	14 : 3	1.12	1.38	1.23	0.995
TGFβ1	HBO	14 : 3	1.67	2.29	1.37	0.190
COL1A1	HBO	14 : 3	0.87	2.49	2.86	0.415

Results from T0 versus T1 shown.

¹ p-value is testing if the healing to not-healing wound volume is significantly greater than 1.0 as determined from linear regression of natural log transformed gene expression values from healing (yes/no) and is adjusted for the patient's age

Table 5
VEGF Expression for Healing Wounds Treated with TO

Time Interval ¹	Oxygen Treatment	Number healing wounds	VEGF Ratio ²	p-value ³
T ₂ to T ₀	Topical Oxygen	9	2.54	0.010
T ₂ to T ₁	Topical Oxygen	8	2.81	0.232
T ₁ to T ₀	Topical Oxygen	14	4.02	0.070

¹ T₀= baseline measurement; T₁ = midpoint of therapy; T₂ = 14 weeks or imminent closure

² VEGF ratio = log transformed ratio of VEGF/ β -actin (T_x): VEGF/ β -actin (T_y) where T_x = first T in time interval and T_y = second T in time interval

³ p-value is testing if the healing VEGF ratio is significantly greater than 1.0 and is generated from a one-sample t-test that uses the natural logarithm of the VEGF ratio.

Topically delivered dissolved oxygen reduces inflammation and positively influences structural proteins in healthy intact human skin

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Summary

Background As oxygen is essential for wound healing and there is limited diffusion across the stratum corneum into the epidermis, we wanted to evaluate whether the topical delivery of a total dissolved oxygen in dressing form on intact human subject skin would improve clinical and histologic skin functioning.

Aims Fifty normal, healthy subjects completed a pilot clinical evaluation to assess the efficacy and tolerability of a dissolved oxygen dressing (OxygeneSys™-Continuous) to improve the health and appearance of intact skin.

Methods Clinical analysis was performed on 50 subjects; histological and gene expression analysis was performed on 12 of the 50 subjects to assess the effect of the dissolved oxygen dressing.

Results Clinical data demonstrate that the dressing is well tolerated, and several measures of skin health and integrity showed improvements compared with a control dressing site. Skin hydration measurements showed a statistically significant increase in skin hydration at 0–4, 4–8, and 0–8 weeks ($P < 0.05$ at each time point). The blinded clinical investigator's grading of desquamation, roughness, and skin texture show significant decreases from baseline to the 8-week time point ($P < 0.05$). The dressings were removed prior to the blinded clinical investigator's grading. These data were supported by the histological and gene expression studies, which showed a general reduction in inflammatory response markers and transcription products (IL-6, IL-8, TNF- α , MMP-1, and MMP-12), while facilitating a general increase in structural skin proteins (collagen I, elastin, and filaggrin). Additionally, p53 signals from biopsy samples support the clinical investigator's observations of no safety concerns.

Conclusion The data from this study demonstrate that the dressing has no deleterious effects and stimulates beneficial effects on intact, nonwounded skin.

Keywords: skin, topically dissolved oxygen, inflammation, structural proteins, filaggrin, aquaporin, aquaglyceroporin channel, AQP3

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Introduction

Skin, the largest organ serves many functions, including protection from external environmental insults such

as pathogenic organisms, UV radiation, regulation of water and temperature and participating in the immune system.¹ Skin health is dependent on a number of fluctuating physiologic mechanisms. Many of these physiologic processes are compromised with age. For example, wound healing is significantly compromised in older subjects.¹ If left untreated, these wounds can become chronic in nature and present serious clinical sequelae for patients.² Nonhealing chronic wounds, pressure ulcers, and bed sores can be especially debilitating in the geriatric population.

Our aging population is not the only subject group with issues or concerns about skin health. As the largest and most esthetically important organ in the body, the skin is a growing area of focus for individuals from all age groups. Geriatric people are interested in curbing the effects of age, while younger people are interested in maintaining a youthful, healthy skin condition that will last as long as possible. Rapid and complete healing from all kinds of skin wounds is an essential component of skin health. Therefore, therapies and conventions that target not only healthy but also wounded skin have appealing clinical and quality-of-life benefits.

A central dogma in skin care states that the nutritional supply of oxygen to the skin is delivered through the internal circulation. However, recent data have shown that significant amounts of oxygen may enter via diffusion from the external overlying surface.³ There is a close dependency between tissue oxygenation and wound healing. Specifically, it has been shown that wounds with <30 mmHg are considered to be hypoxic and have more clinical issues such as being slow to heal, having little or no granulation tissue, and having accumulations of necrotic deposits.⁴ In contrast, those wounds with oxygen levels >30 mmHg usually have few longer-term clinical issues and follow a normal course of wound healing.⁴

Maintaining physiologic oxygen levels is critical for normal homeostasis in all tissues. In the skin, white blood cells require oxygen for the respiratory burst mechanism that is necessary for killing ingested bacteria.^{5–7} Skin fibroblasts normally secrete a variety of extracellular matrix molecules; this process is critically dependent on physiologic oxygen levels.^{8,9} The events of angiogenesis and granulation tissue formation are dependent upon oxygenation.^{5,10} Wounds deprived of oxygen deposit collagen poorly and are easily infected. Epithelialization represents a final resolution of the wound, and its mechanisms are optimized at high oxygen levels.¹¹ As oxygen is essential for wound healing

and there is limited diffusion across the stratum corneum into the epidermis, we wanted to evaluate whether the topical delivery of a total dissolved oxygen in dressing form on intact human subject skin would improve clinical and histologic skin functioning. Biopsy samples were taken from subjects at active and control sites following 8 weeks of treatment. Biopsy samples were coronally sectioned, with one half processed for histopathology to assess impact on hydration, oxidative stress, and structural proteins, and the second half processed for real-time RT-PCR analysis to assess impact on inflammatory markers. Results from these evaluations suggest active mechanisms are in play with the use of topical oxygen therapy to intact, healthy skin. No safety issues were seen in the current study, and structurally significant and biologically relevant differences were detected as a result of 8 weeks of active treatment.

Materials and methods

Human subjects

A total of 50 healthy subjects (men and women ages 50–69 years; mean age 58.4) completed a single site, randomized controlled, 8-week study. Subjects had age-appropriate photoaging and stable concomitant medications. Informed consent was obtained from all subjects in the study, which was approved by the Concordia Clinical Research Institutional Review Board, New Jersey.

The semi-occlusive, absorbent, oxygen-enriched dressing (Active Group, OxygeneSys™-Continuous, AcryMed, Inc., Beaverton, OR, USA) was affixed to the skin covering the anterior tibia on one limb, and the contralateral limb was covered with a Kling® bandage (Johnson & Johnson Consumer Products Company, Skillman, NJ, USA) to function as the control. A computer-generated randomization scheme determined which limb (left or right) would receive the experimental dressing. The dressing was wet with an ampule of eye moisturizer and affixed to the shin with a Kling® dressing held together with paper tape. The dressing was applied daily by the subject following bathing and worn for 24 h continuously. The location of the dressing placement was noted by the investigator with black indelible ink. Subjects were permitted to continue using their own skin care, cleansing, and makeup products, but were not allowed to begin any new products for the 8-week duration of the study. No skin care products of any kind were used on the shins where the dressing was applied.

Clinical measurements

Subjects were evaluated in a blinded fashion by collecting a variety of observations at the designated time points. All study subjects were evaluated by a single investigator. The dressings were removed prior to the blinded clinical investigator's grading. A 5-point ordinal scale was used for all investigator and subject-assessed parameters at baseline, 1 week (compliance check visit), 4 weeks, and 8 weeks, ranging from 0 = no signs or symptoms to 4 = very dramatic signs and symptoms resulting in discomfort, representing an adverse reaction. Investigator-assessed efficacy parameters were as follows: desquamation, roughness, erythema, skin texture, and tolerability parameters were as follows: itching, stinging, and burning. Subject-assessed parameters included: flakiness, roughness, redness, and overall problems. Digital photography of each shin was taken at baseline, 4 and 8 weeks (Fig. 1).

Skin hydration (μS , micro siemens) was measured with the Dermalab pin probe corneometer (Cortex Technology, Hadsund, Denmark) that used low grade current and conductivity to indirectly measure water content. Transepidermal water loss (TEWL; $\text{g}/\text{m}^2 \text{ h}$) was measured with the Dermalab TransEpidermal Water Loss module (Cortex Technology). Elasticity was measured with the Dermalab skin elasticity module (Cortex Technology, Denmark), and skin coloration was measured with the Dermalab DSM II Colormeter (Cortex Technology). Sensory monofilament test was performed by drawing a cotton fiber over the skin.

Biopsy

Biopsies were performed in a controlled office environment, 25% humidity, 22°C, during the summer season. Full-thickness biopsies were collected from 12 randomly selected subjects. One 3 mm biopsy was taken from each shin (randomized active and control) at week 8. Biopsies were immediately cut in coronal halves (superficial to deep). One half was immediately placed in ice-cold 2% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA, USA) in PBS for histological and immunohistologic analysis and the other half in ice-cold RNAlater (Sigma Chemical Company, St. Louis, MO, USA) for real-time RT-PCR analysis. Histology samples were stored in 2% PFA at 4 °C for 48 h then transferred to 70% ethanol and stored at 4 °C until processed and paraffin embedded. RNAlater samples were stored overnight

at 4 °C then stored at −80 °C until processed for RNA.

Histology

All histologic analyses were performed at the 8-week time point, comparing active site to control. Histopathologic evaluations were assessed from paraffin embedded tissues, serially sectioned at 5 μm and stained with hematoxylin and eosin. Immunohistochemistry evaluations were performed by reacting 5 μm sections with the following primary antibodies: oxidative stress DNA adduct 8-hydroxy 2-deoxyguanosine (8-OHdG; Abcam, Cambridge, MA, USA), water-glycerol channel aquaporin-3 (AQP3; Santa Cruz Biotechnology, Santa Cruz, CA, USA), structural proteins filaggrin (Vector Laboratories, Burlingame, CA, USA), collagen I (Abcam), and elastin (Abcam), and processed using standard immunohistochemistry methods. Digital, whole-slide scans (Aperio ScanScope CS; Aperio, Vista, CA, USA) were used for all evaluations to quantify changes in levels, using established digital pathology algorithms. Quantitative assessments on digitally scanned slides have been used to quantify immunohistochemistry in several different tissues.¹² All artifacts were manually excluded prior to digital algorithm. *H*-score, a widely used pathology method for quantitatively evaluating staining features is directly related to staining intensity (0, +1, +2 or +3) of the area, cell or object and calculated by the formula: $(3 \times \%3+) + (2 \times \%2+) + (1 \times \%1+)$. Results range from 0 to 300.¹³

Real-time PCR

Tissues were thawed on ice, then homogenized in RLT lysis buffer (Qiagen, Valencia, CA, USA) using an Omni THq rotor stator (Omni International, Kennesaw, GA, USA) for 30 s per sample. Total RNA was isolated with RNeasy Mini Kit (Qiagen). The cDNA was reverse transcribed with the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). cDNA was diluted 2:1 prior to addition to 10 μL PCRs containing 2 $\times$ Taqman Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and amplified on a 7900 Real-Time PCR System (Applied Biosystems). Real-time analysis was performed with the following Taqman probes: IL-6, IL-8, MMP-1 (collagenase), MMP-12 (elastase), TNF- α , TP53, and VEGF and normalized to GAPDH. Samples were analyzed using the $2^{-\Delta\Delta C_t}$ method, using GAPDH as the housekeeping gene to normalize for sample-to-sample variations in RNA/cDNA. Data are presented as "fold change" in active vs. control.

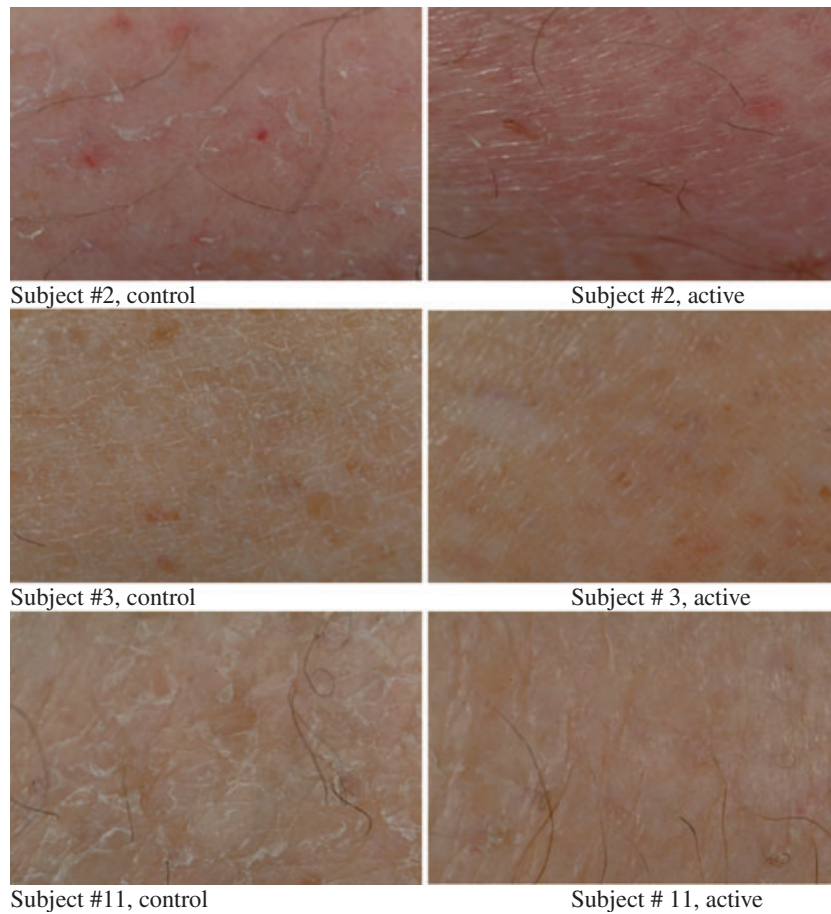


Figure 1 Clinical pictures of control vs. active at the 8-week time point.

Statistical analysis

A paired Student's *t*-test was performed to determine whether differences existed between the active group vs. the control group. A *P*-value < 0.05 was considered to be statistically significant.

Results

Human subjects

A total of 50 subjects completed the study without any major adverse events or deviations from the study design.

Clinical measurements

Skin hydration measurements showed a statistically significant increase in stratum corneum hydration from baseline to 4 weeks, 4–8 weeks, and baseline to 8 weeks (*P* < 0.05 at each time point), with an average increase

in hydration of 41 μ S in active vs. control from baseline to 8 weeks (Table 1). There were no statistically significant changes in skin coloration with the dermospectrophotometer, nor were there significant changes in TEWL, monofilament sensorial measurement, or elasticity measures across all time points (data not shown).

Blinded clinical investigator-measured outcomes of itching, burning, stinging, and erythema were not significantly different between the active vs. control at all time points compared (data not shown). However, the blinded clinical investigator measures of desquamation, roughness, and skin texture showed significant decreases from baseline to 8-week time points only (*P* < 0.05), see Table 1. There were no significant differences for these measures in active vs. control from baseline to 4 week and 4- to 8-week comparisons. Subject assessment of flakiness, roughness, redness, and overall problems were not significantly different between the active group and the control group at all time points (data not shown).

Table 1 Clinical measurements between control and treatment sites

		Control	Treatment	<i>P</i> value
Skin hydration (μ S)	0–4 weeks	-8.18 ± 44.01	15.88 ± 46.17	0.010
	4–8 weeks	-2.04 ± 32.34	15.22 ± 41.96	0.025
	0–8 weeks	-10.22 ± 33.91	31.10 ± 44.71	<0.001
Desquamation	0–4 weeks	-0.27 ± 1.09	-0.49 ± 1.29	0.356
	0–8 weeks	-0.12 ± 0.99	-1.08 ± 1.19	<0.001
Roughness	0–4 weeks	-0.33 ± 1.12	-0.53 ± 1.24	0.396
	0–8 weeks	-0.18 ± 0.99	-1.14 ± 1.15	<0.001
Skin texture	0–4 weeks	-0.33 ± 1.12	-0.53 ± 1.24	0.396
	0–8 weeks	-0.18 ± 0.99	-1.14 ± 1.15	<0.001

Histology

Histopathology comparisons between active vs. control sites at the 8-week time point did not reveal any noticeable differences with respect to acanthosis, spongiosis, chronic inflammation, hyperkeratosis, epidermal mononuclear infiltration, focal acantholysis, or dermal edema. Subtle differences exist in epidermal thickness, vascular prominence, and occasional perivascular mononuclear cells. However, these features were concluded to be consistent of normal human skin. No trend change was observable for any of these characteristics between active and control samples. Representative histopathology is shown in Figure 2. Rete peg analysis showed a slightly lower level in active vs. control sites; however, no statistically significant differences were seen.

Immunohistochemistry

Immunohistochemical (IHC) analysis of coronal, serially sectioned biopsies from the 12 subjects revealed a modest increase in 8-OHdG levels in active vs. control sites, suggesting increased oxygen was penetrating the epidermis through the dressing, resulting in a measurable effect of higher 8-OHdG levels.

The aquaglyceroporin channel, AQP3 showed a slight decrease in active vs. control sites; however,

active sites had a more “circumferential” or membrane-localized staining pattern, suggesting recruitment of AQP3 from the cytoplasm to the membrane to facilitate water and glycerol transport (Fig. 3). IHC analysis revealed an increase in *H*-score of filaggrin, collagen I, and elastin proteins in active vs. control sites. The *H*-score value represents a quantitative measurement of staining intensity (quantity of antibody) from whole-slide digital scans of IHC-reacted slides. Evaluated collectively, while not statistically significant, a trend analysis of these key structural proteins demonstrates that these parameters all increase, suggesting a mechanism of influencing structural organization in the skin (Fig. 4 and Table 2).

Real-time RT-PCR

GAPDH was used as the housekeeping gene to normalize for sample-to-sample variations in mRNA. Analysis was performed on a range of inflammatory, structural, angiogenic, and cellular stress genes. Proinflammatory cytokines IL-6, IL-8, and TNF- α showed a modest decrease in active vs. control sites. MMP-1 (collagenase) and MMP-12 (elastase) showed a more robust decrease. During inflammation or damage, both MMP-1 and -12 are up-regulated to degrade extracellular matrix (ECM). Trend analysis of these markers suggests

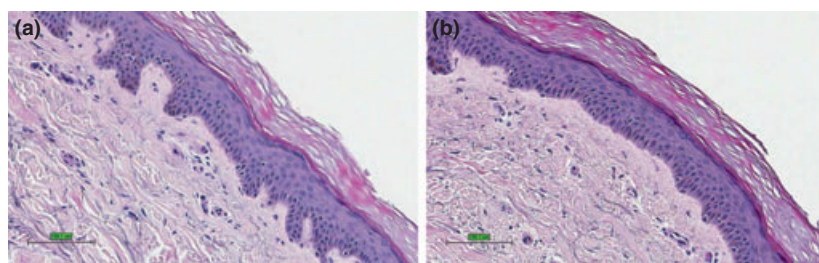


Figure 2 Representative histopathology (hematoxylin and eosin stain). (a) Subject #10, control site at the 8-week time point. (b) Subject #10 active site at the 8-week time point. Scale bars = 100 μ m.

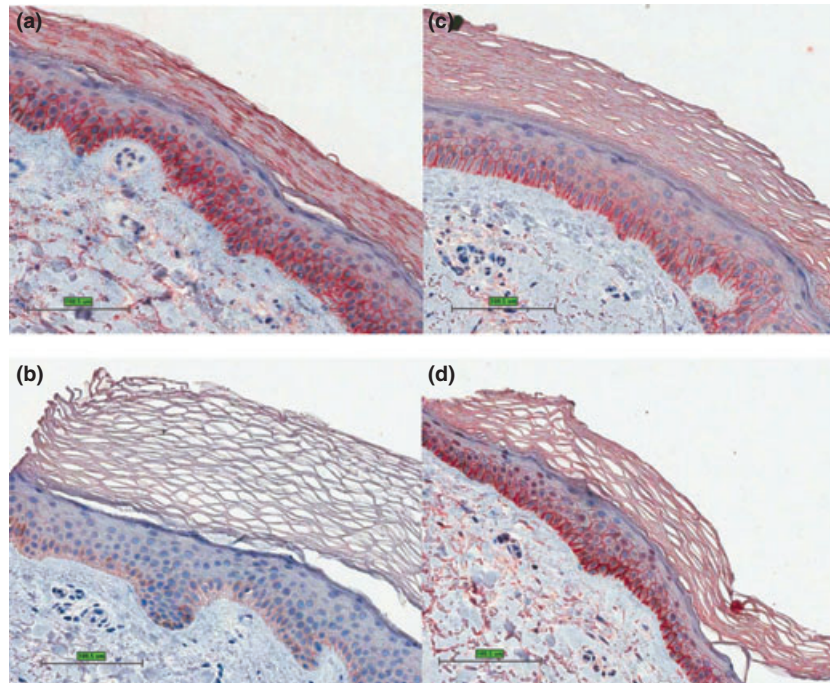


Figure 3 Representative aquaporin-3 levels at 8 weeks. (a) Subject #10, control site. (c) Subject #10, active site. (b) Subject #13, control site. (d) Subject #13, active site.

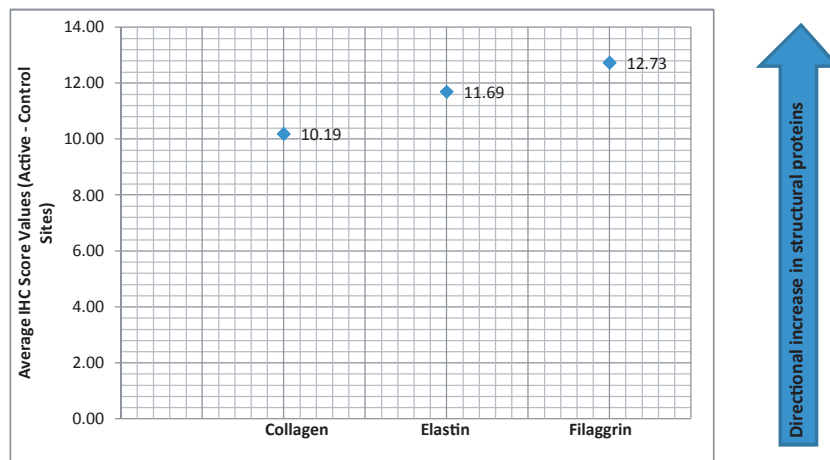


Figure 4 Immunohistochemistry trend plot of structural proteins collagen I, elastin, and filaggrin at 8 weeks.

consistency in the data and a mechanism of down-regulating the expression of inflammatory markers (Fig. 5 and Table 2). VEGF expression levels were unchanged, suggesting the oxygen dressing neither decreased tissue pO_2 levels to hypoxic levels nor increased pO_2 to hyperoxic levels, both of which would lead to an increase in VEGF expression. TP53 (p53) levels were unchanged. As a central monitor of cellular stress and its environment, including sensing reactive oxygen spe-

cies (ROS) levels, the data suggest the topical oxygen dressing-covered skin was in a healthy state with respect to p53 levels (Table 2).

Discussion

In the current study, normal, healthy subjects were enrolled in a pilot clinical evaluation to assess the efficacy and tolerability of a dissolved oxygen dressing in

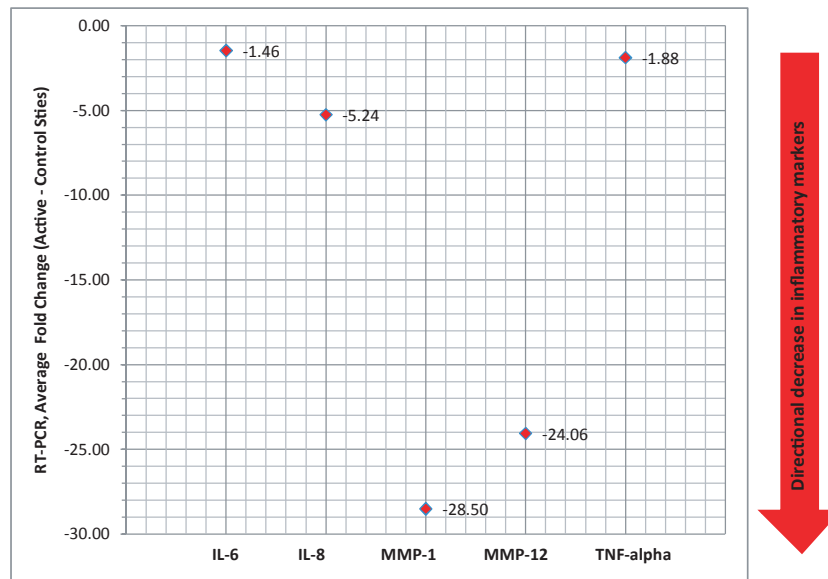


Figure 5 Real-time RT-PCR trend plot of inflammatory markers at 8 weeks.

improving skin health and appearance. The dressing provides an oxygen-enriched environment that may promote a favorable environment for promotion of healing. Clinical analysis was performed on 50 subjects; histological and gene expression analyses were performed on 12 of the 50 subjects. Clinical data showed the dressing was well tolerated, and several measures of skin health and integrity showed improvement compared with the dressing-only control site. No safety issues were seen during the 8-week study period. These data were supported by the immunohistological and gene expression studies, which showed a general reduction in inflammatory response markers and transcription products and a general increase in structural proteins. Additionally, there was a significant decrease in investigator-measured desquamation, roughness, and skin texture in active vs. control sites.

The skin, like all tissues, relies on physiologic levels of oxygen for the maintenance of normal homeostasis. The role of oxygen in maintaining skin homeostasis has been previously described.¹⁴ Numerous homeostatic mechanisms in the skin are dependent on physiologic oxygenation of the skin. When the skin is damaged, wound healing processes are initiated to restore the barrier function, for example, the respiratory burst mechanism leveraged by white blood cells to kill harmful bacteria requires oxygen.⁵⁻⁷ Additionally, physiologic oxygen levels are needed to enable fibroblasts to perform their normal production of structurally critical extracellular matrix proteins.⁵⁻⁷ The delivery of topical

dissolved oxygen to the epidermis and potentially the dermis has many implications, including the potential to enhance the wound healing process.³ While an increase in oxygen is beneficial to the wound healing process, it also increases the amount of ROS-induced physiological changes that may have negative consequences downstream.

Reactive oxygen species are continuously formed in living cells of aerobic organisms as part of normal physiologic processes. Endogenously produced ROS, including H_2O_2 , superoxide anion radical (O_2^-), and the hydroxyl radical ($*OH$), have many important functions in healthy cells and tissues, acting as secondary messengers in the regulation of cell cycle, proliferation, apoptosis, and response to inflammation or damage.¹⁵ ROS levels are managed by glutathione, glutathione peroxidase, superoxide dismutase, and other scavenging systems.¹⁵ The hydroxyl radical is highly reactive, short-lived and can induce damage to purine and pyrimidine bases and the deoxyribose backbone.¹⁵ The most frequently studied reaction of $*OH$ with DNA is the formation of 8-OHdG, which when left intact can result in G:C→T:A transversions.

The overall health of the cells and the skin is continually monitored through a complex series of signals, including degree of DNA damage by the level of 8-OHdG. In healthy cells, the cellular repair system, including base excision repair, nucleotide excision repair and mismatch repair, removes the mutations. However, when ROS are produced in amounts that

exceed the cell's capacity to remove the DNA adducts, a series of responses are induced to recruit additional resources to handle the insult. A critical stress response involves phosphorylation and subsequent stabilization of p53 protein, the guardian of the genome, resulting in two major cell pathways – cell cycle arrest for DNA repair, promoting cell survival or apoptosis, eliminating cells that are damaged beyond repair.¹⁶

In the current study, we observed an increase in 8-OHdG in active vs. control epidermis, suggesting additional oxygen, delivered by the dressing, penetrated the skin and caused an increase in *OH-intermediates. The DNA analogs appear to be managed by normal physiological processes without deleterious consequences. If the DNA damage were beyond the capacity of the cell to manage, we would anticipate seeing an up-regulation of p53 mRNA expression.

Tumor protein 53 (TP53, p53) is the central monitor of stress in the cell and has been described as the guardian of the genome. p53 has a high turnover rate and is present in cells in low levels in a nonphosphorylated, inactive state. In response to DNA damage, p53 is phosphorylated at multiple sites, along with several feedback loop proteins (ATM, Mdm2, Akt, PTEN), initiating a series of transient p53 pulses. The amount of these p53 pulses ultimately determines cell fate to either cell cycle arrest or apoptosis.¹⁶

In the current study, p53 expression levels were slightly down-regulated in active treatment sites. This minor change in p53 message expression in active vs. control sites, in combination with the increased production of 8-OHdG indicates that the topical oxygen therapy is penetrating the skin and resulting in positive changes that are not causing excessive stress to the skin. This strongly suggests an active therapy mechanism is in effect without negatively impacting cell and tissue health. Furthermore, VEGF levels were unchanged in active vs. control sites, suggesting the dissolved oxygen dressing created no hypoxic or hyperoxic states.¹⁷ VEGF expression is extremely sensitive to deleterious physiologic changes in oxygen levels. Therefore, as VEGF expression in the current study was unchanged and p53 levels were effectively unchanged, we can conclude that the therapeutic conditions were safe at the cell and tissue level.

Aquaporin-3, the most abundant aquaglyceroporin in the skin, has been shown to transport both water and glycerol.¹⁸ It is primarily found in the stratum basale and stratum spinosum layers of the epidermis and has been identified to play an important role in epidermis and SC hydration, elasticity, wound healing,

Table 2 Data summary

	Marker	Analysis	Result
Stress monitor	8-OHdG	IHC	Increase
	p53	PCR	No change
	VEGF	PCR	No change
Stratum corneum barrier	AQP-3	IHC	Slight decrease Redistributed to membrane
	Hydration	Clinical	Increase
	Desquamation	Clinical	Decrease
	Roughness	Clinical	Decrease
	Skin texture	Clinical	Decrease
Inflammation markers	IL-6	PCR	Decrease
	IL-8	PCR	Decrease
	TNF- α	PCR	Decrease
	MMP-1	PCR	Decrease
	MMP-12	PCR	Decrease
	Filaggrin	IHC	Increase
Epidermis structure	Elastin	IHC	Increase
	Collagen I	IHC	Increase

enhancing keratinocyte proliferation, migration, and differentiation.¹⁸

Aquaporin-3 levels decreased slightly among subjects in the study. Interestingly, there was an observable change in subcellular distribution of AQP3, shifting from a general cytoplasmic distribution in the control to more of a membrane or circumferential distribution in the active treatment group (Fig. 3). One hypothesis is that active treatment results in a redistribution of AQP3 water channels to the cell membrane to provide a more consistent level of water and glycerol to the epidermis. In the literature, decreased AQP3 levels are associated with dry skin.¹⁸ Additionally, a more cytoplasmic distribution of AQP3 is associated hyperproliferative skin disorders.^{18–20} If subjects did not have clinically significant dry skin at the start of the study, we might conclude that either the reduced level of AQP3 channel in active treatment is not of clinical significance or the redistribution of the AQP3 channels counteracts the decrease in AQP3 protein. Alternatively, if a subject had dry skin at the start of the study, the persistent presence of the dissolved oxygen dressing on the active treatment site for 8 weeks could result in reduced evaporation, which could have signaled a reduced need for AQP3 expression or a redistribution to the membrane to further facilitate water and glycerol transport. The redistribution of AQP3 to the membrane and its likely beneficial effect of providing additional water and glycerol to the epidermal layers is supported by clinical data, specifically the statistically significant increase in skin hydration ($P < 0.001$), and

significant decrease in investigator measures of roughness, desquamation, and skin texture, reflecting an increase in moisture in the stratum corneum.

Filaggrin, a structural protein produced in the epidermis by terminally differentiating keratinocytes, facilitates the organization and condensation of keratinocytes and contributes to the formation of the stratum corneum. Filaggrin is synthesized as a profilaggrin polypeptide, containing 10–12 tandem filaggrin repeats and is stored in intracellular keratohyalin granules in the granular layer of the epidermis. As terminal differentiation continues, Ca^{2+} levels increase, which signal the dissolution of the granules and dephosphorylation and cleavage of profilaggrin into filaggrin monomers. The free filaggrin binds to keratin intermediate filaments, which condense the keratin cytoskeleton, contributing to the cell compaction process that is required for the squamous cell phenotype of the stratum corneum. Filaggrin is further degraded to produce hydrophilic acids, components of natural moisturizing factor, which are significant contributors to hydration and the mildly acidic cutaneous pH.²¹ Reductions in profilaggrin or filaggrin lead to a poorly formed stratum corneum (ichthyosis), which is also prone to water loss (xerosis).²¹ Filaggrin levels were higher in active vs. control sites, suggesting treatment increased the production of filaggrin in the skin, potentially contributing to more natural moisturizing factor and a more structurally sound barrier function of the stratum corneum. Additionally, increased filaggrin levels correlate with the increase in investigator-measured skin hydration and decrease in skin texture, roughness, and desquamation. Collagen and elastin, other well-evaluated skin proteins, demonstrated positive changes in active sites in the current study. Collagen I protein levels were slightly higher in active sites vs. control sites. Greater collagen I presence in the dermis may provide skin with greater structural integrity. The consistency in the data lies in the fact that three major structural skin proteins (filaggrin, elastin, and collagen), all increase in their expression in active sites when compared to control sites in the same subject.

Elastin is a structural protein found in the dermis as well as other critical tissues such as blood vessels, heart, bladder, and ligaments where it provides physiologically relevant elasticity. Elastin levels appear to be higher in active vs. control sites. Greater elastin presence in the dermis may provide skin with greater structural integrity and elasticity. Additionally, a trend analysis was conducted on key structural proteins within the skin: collagen, elastin, and filaggrin. Trend analysis of these three proteins suggests consistency in

the data and a mechanism of positively influencing the expression of key structural proteins (Fig. 4). Because these proteins trend together, we can conclude with a higher level of confidence that the active treatment is stimulating beneficial structural changes within the underlying skin.

Inflammatory markers IL-6, IL-8, TNF- α , and the matrix metalloproteinases MMP-1 (collagenase), and MMP-12 (elastase) are up-regulated when inflammatory processes or mechanisms are in play. They are secreted by leukocytes and regulate a wide range of cellular and tissue responses, recruiting macrophages, neutrophils, inducing angiogenesis and inducing remodeling of damaged tissue.²² In this study, expression of these markers was down-regulated in active vs. control sites, suggesting a decrease in inflammatory pathways or mechanisms and a decrease in breakdown or remodeling of the extracellular matrix within the skin following active treatment.

Conclusions

The dissolved oxygen dressing was applied to normal, healthy, age-appropriate, photoaged skin and compared with a nontreated site on the contralateral limb in the same subject. The data from this study demonstrate that the total dissolved oxygen dressing has no deleterious effects; rather it stimulates beneficial effects on intact, nonwounded skin.

The increase in 8-OHdG levels in active vs. control sites suggests the dissolved oxygen dressing is increasing O_2 levels in the skin. The levels of both VEGF and p53 are unchanged, suggesting the increased O_2 is within acceptable levels within the cells. Decreases in IL-6, IL-8, TNF- α , MMP-1, MMP-12 also suggest insignificant stresses within the cells and skin. The increases in structural proteins collagen I and elastin is likely explained by reduced turnover by a decrease in MMP-1 and MMP-12 mRNA levels (Table 2).

The blinded clinical investigator's measurements of decreased desquamation, roughness, and skin texture in treated vs. control sites correlate with the significant increase in skin hydration. These data are supported histologically by an increase in filaggrin, resulting in increased production of natural moisturizing factor and a redistribution of aquaglyceroporin, AQP3 from the cytoplasm to the membrane.

In summary, the data from this study demonstrate that the dressing has no deleterious effects and appears to stimulate beneficial effects on intact, nonwounded skin.

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Topical Dissolved Oxygen Penetrates Skin: Model and Method

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Background. It has been commonly perceived that skin receives its oxygen supply from the internal circulation. However, recent investigations have shown that a significant amount of oxygen may enter skin from the external overlying surface. A method has been developed for measuring the transcutaneous penetration of human skin by oxygen as described herein. This method was used to determine both the depth and magnitude of penetration of skin by topically applied oxygen.

Material and Methods. An apparatus consisting of human skin samples interposed between a topical oxygen source and a fluid filled chamber that registered changes in dissolved oxygen. Viable human skin samples of variable thicknesses with and without epidermis were used to evaluate the depth and magnitude of oxygen penetration from either topical dissolved oxygen (TDO) or topical gaseous oxygen (TGO) devices.

Results and Conclusion. This model effectively demonstrates transcutaneous penetration of topically applied oxygen. Topically applied dissolved oxygen penetrates through >700 μm of human skin. Topically applied oxygen penetrates better through dermis than epidermis, and TDO devices deliver oxygen more effectively than TGO devices. © 2010 Elsevier Inc. All rights reserved.

Key Words: topical oxygen; dissolved oxygen; transcutaneous; wound healing; hypoxia.

due to poor perfusion, which causes a decreased oxygen tension. Oxygen is necessary for multiple wound healing processing including bacterial killing by leukocytes, synthesis, and hydroxylation of collagen, proliferation of fibroblasts, promotion of wound resurfacing by keratinocytes, oxidative pathways for ATP formation, and nitric oxide dependent signaling pathways [2,3].

There is an association between tissue oxygenation and wound healing in the clinical setting. For example, nonhypoxic wounds with O_2 levels greater than 30 mmHg typically have little or no accumulated necrotic debris, develop normal granulation tissue, and close uneventfully [4]. In contrast, wounds with less than 30 mmHg are termed hypoxic and, depending on O_2 levels, follow predictable courses. Wounds with 13–30 mmHg of O_2 usually have accumulated necrotic deposits over the wound bed, form little or no granulation tissue, and are stalled or very slow to heal. Wounds with less than 13 mmHg of O_2 tension have insufficient oxygen to support even static metabolic activities and become gangrenous.

The goal of an oxygen therapy for wound care is to transfer sufficient oxygen to interstitial tissues to maintain a concentration near the 40 mmHg found in healthy, well perfused tissues. Therapies such as surgical revascularization and hyperbaric oxygen therapy have demonstrated that improved perfusion and oxygenation of the wound accelerates healing. These procedures are expensive and often unavailable to many patients. This report is on a new method for measuring the capacity of topically applied oxygen delivery devices to penetrate through human skin samples. Topical dissolved oxygen (TDO) and topical gaseous oxygen (TGO) devices were analyzed using this *in vitro* model. We hypothesized that topical oxygen devices can effectively deliver oxygen into and through viable human skin and that TDO may be more effective than TGO in doing so.

INTRODUCTION

Chronic wounds are those that “fail to progress through a normal, orderly, and timely sequence of repair [1].” Several key processes in wound healing are dependent upon an adequate supply of oxygen. Chronic wounds are often in need of an adequate supply

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MATERIALS AND METHODS

Model and Apparatus for Transcutaneous Oxygen Transfer

The objective was to determine the rate at which oxygen was transferred from oxygen delivering devices to physiologic saline through test substrates. This was done by using saline (0.9% NaCl) equilibrated to 21% saturation/159 mmHg partial pressure oxygen content of atmospheric air at 37°C in an apparatus designed for measuring dissolved oxygen (Fig. 1). The apparatus consisted of an 11 mL chamber for the containment of fluid (saline) in a closed system with a 2 cm × 2 cm window onto which human skin samples of varying thicknesses were adhered. The apparatus described in Fig. 1 was filled with saline equilibrated with atmospheric oxygen at 159 mmHg and maintained at 37°C. To determine baseline transfer kinetics for oxygen delivery, a support polypropylene mesh was adhered across the sample window and used as control recordings. The topical oxygen devices were placed on the mesh and recordings were made, to assay the effects of 100% gaseous oxygen, the entire apparatus was placed into a Captair airtight glovebox (Terra Universal, Fullerton, CA), and medical grade 100% oxygen was applied at a continuous flow rate of 5 L/min.

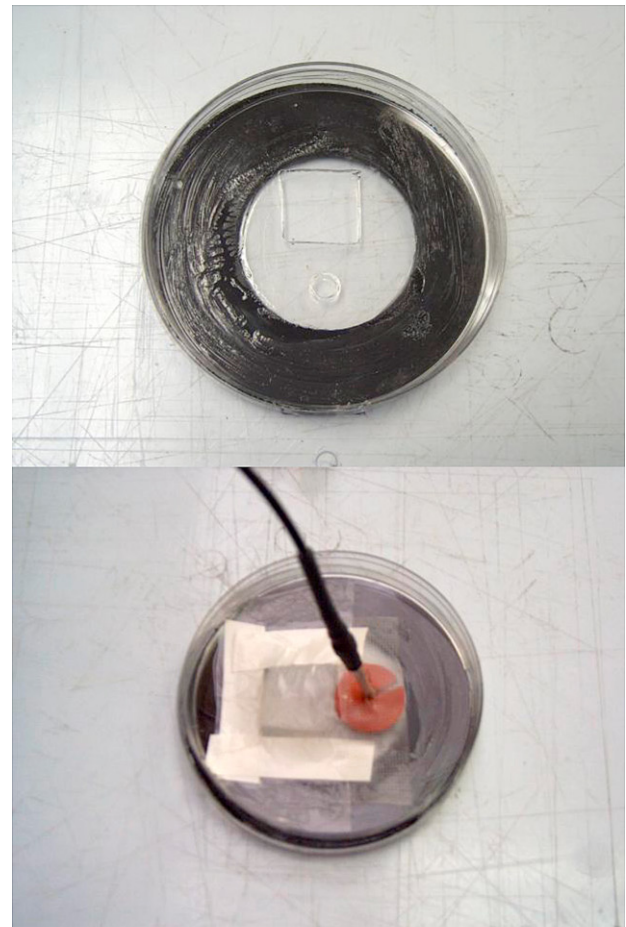
Measurements were taken with a Diamond General Chemical microsensor (Ann Arbor, MI) instrument for the amperometric measurement of dissolved oxygen. A silicone disk was placed around a chemical microsensor, Clark style dissolved oxygen probe. The Clark probe was inserted into fluid chamber and held in place with flexible clear NexCare 3M (St. Paul, MN, USA) medical tape. The final assembly was set in a water bath at 37°C. Measurements were recorded after 5 min intervals for 90 min.

Oxygen Delivery Devices

Two topical dressings were tested, a hydrophilic closed-cell oxygen foam [5], and an alginate-catalyst dressing treated with a 0.3% hydrogen peroxide (Fisher Scientific, Pittsburg PA.) solution [6]. One hundred percent gaseous oxygen was used in some of the testing for comparative purposes. The foam dressing was a polyacrylate polymer specifically modified to enhance flexibility, elasticity, and moisture absorbency. The polyacrylate matrix was initially a semi-solid gel and was transformed to a closed-cell, oxygen rich foam by a proprietary treatment. The alginate-catalyst dressing consisted of the hydrophilic alginate Kaltostat (ConvaTec, Skillman, NJ.) containing ~60–80 µg/cm² of the inorganic catalyst manganese dioxide (Sigma-Aldrich, St. Louis, MO). An aqueous substrate of 0.3% H₂O₂ was added to the dressing and was subsequently decomposed to produce high levels of oxygen and water.

Human Donor Skin Samples

Viable human organ donor skin samples were obtained through Community Tissue Services in Portland, Oregon and designated for research purposes. Donors were Caucasian men and women whose ages were not revealed for privacy purposes. Three skin sample thicknesses were tested. Two dermal thicknesses, intermediate (0.012–0.018 in. or 304–457 µm) and thick (0.018–0.030 in. or 457–762 µm) harvested at the time of organ donation were tested. An intermediate (0.012 in. or 304 µm) split-thickness sample consisting of epidermis and dermis was also tested. It was hypothesized that skin samples with epidermis would present a greater barrier to oxygen penetration than would dermis only samples of equivalent thickness. All samples were excised from the back or thigh using a dermatome. The samples were stored in the cell culture media RPMI-1640 (Invitrogen, Carlsbad, CA.) and refrigerated at 4°C until use. No samples were used after 2 wk in storage. Skin samples were fixed over the window of the test apparatus using veterinary skin adhesive. The saline was equilibrated to atmospheric levels of oxygen, 159 mmHg, using an aerator and room air at 37°C. Topical dressing devices were placed



O₂ Skin Penetration Test Apparatus

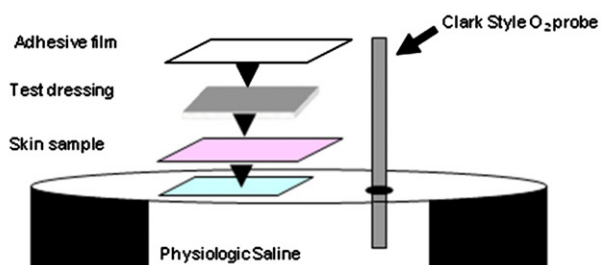


FIG. 1. Photographs and diagram of apparatus for measuring delivery of dissolved oxygen. Constructed from silicone sheeting and polystyrene dish. An 11 mL volume fluid chamber (7 mm depth × 46 mm diameter) within dish filled with saline. A 2 cm × 2 cm sample window over fluid chamber. Oxygen transfer test material (i.e., skin sample) is adhered over window. Oxygen release into a fluid medium was measured using the Diamond General Chemical micro-sensor (Clark Style Electrode).

atop the skin samples and recordings of dissolved oxygen were made for 90 min. Only intermediate dermis was tested using 100% gaseous oxygen, in which case the entire apparatus was placed inside an airtight glove box with continuous 5 L/min flow of 100% oxygen gas.

Calculation of Oxygen Volume

Partial pressure measurements of oxygen were converted to mg/L of oxygen in saline within the apparatus. The value was based on the

solubility of oxygen at 37°C and 1.54 S/m conductivity of a 0.9% saline solution [7, 8], whereas a partial pressure of 159 mmHg is equal to 6.31 mg/L oxygen solubility.

Statistical Analysis

Results are expressed as means $\pm$ standard deviation. Student's *t*-test was used to assess the statistical significance between two means. Differences were considered significant when $P < 0.05$.

RESULTS

Baseline Production of Dissolved Oxygen by TDO and TGO Devices

Initial experiments were performed to determine the transfer kinetics of oxygen from the delivery device to saline without introducing the variable of interposed human skin in the system. As shown in Fig. 2, dissolved oxygen (dO_2) measurements begin to rise immediately with each device. The TDO devices showed an initial high rate of oxygen transfer, which was significantly greater than TGO device at 30 min (P value < 0.005 closed cell foam and P value < 0.05 alginate catalyst), and also at 60 min (P value < 0.005 closed cell foam and P value < 0.01 alginate catalyst) shown in Table 1. The maximum change in oxygen partial pressure reached 231 mmHg for the closed-cell oxygen foam, 152 mmHg for the alginate catalyst dressing, and 109 mmHg for gaseous oxygen. There was a significant difference in peak oxygen level achieved at 90 min comparing a TDO with TGO ($P < 0.05$, closed cell foam).

Transfer of dO_2 Through Human Skin by TDO and TGO Devices

These experiments examine the ability of the TDO and TGO devices to deliver oxygen through interposed viable human skin in the apparatus. As seen in Fig. 3, each device effectively transferred oxygen through intermediate thickness (0.012–0.018 in. or 304–457 μm) dermis samples. TDO devices were significantly more effective in transferring oxygen across viable human dermis than TGO device at 30 min (P value < 0.005 for closed cell foam and P value < 0.01 alginate catalyst), and at 60 min (P value < 0.05 for closed cell foam), as seen in Table 1. There was also a significant difference in peak oxygen level reached at 90 min by a TDO device compared with the TGO device (P value < 0.05 , closed cell foam).

Transfer of dO_2 Through Variable Thickness Human Skin Samples by TDO

Next, the diffusion of oxygen from devices through thick dermis (0.018–0.030 inch or 457–762 μm) was measured. Both TDO devices were able to cause an elevation in the transfer of oxygen, but there was no significant difference in rate between the two TDO

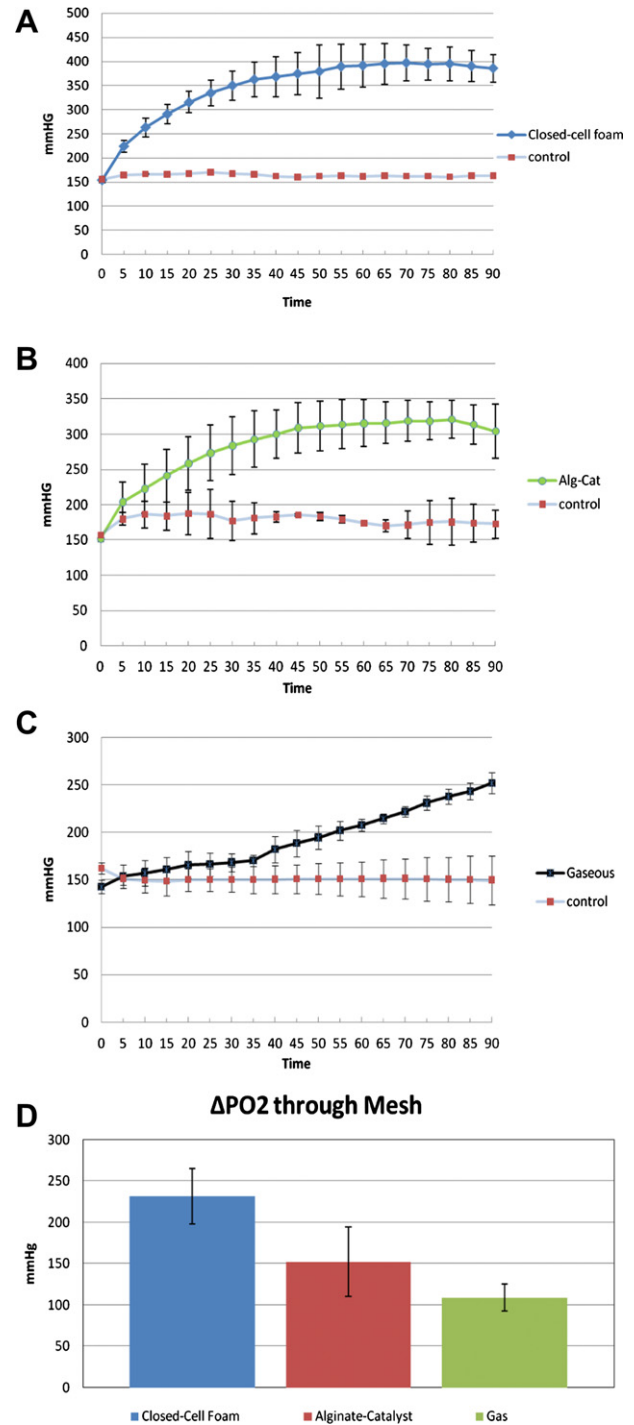


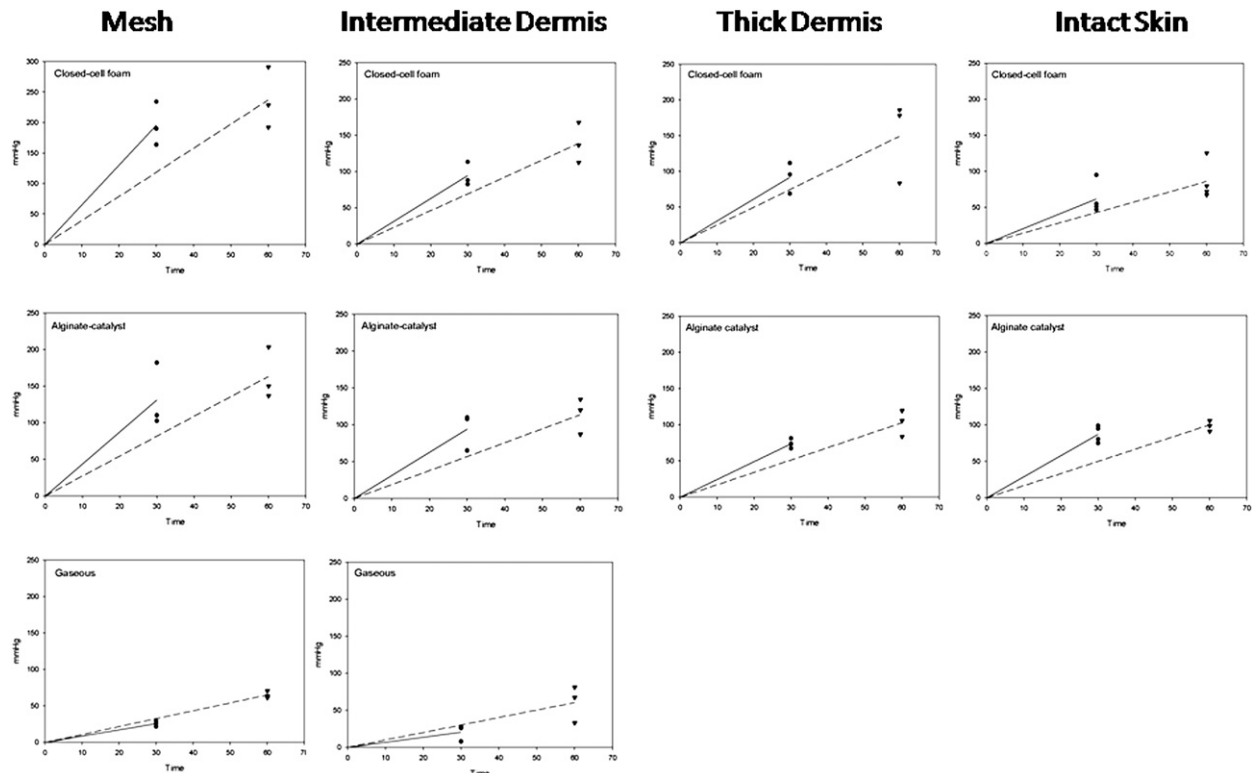
FIG. 2. Transfer of dissolved oxygen for control recording into saline at 37°C; $n = 3$ oxygen delivery devices; $n = 2$ control. (A) Foam dressing; (B) alginate-catalyst dressing; (C) 100% gaseous oxygen; (D) change in partial pressure at 90 min from $T = 0$. Closed cell foam greater than gaseous ($P < 0.05$).

devices. As shown in Fig. 4, the closed-cell foam dressing achieved a change in partial pressure of 148 mm Hg for the closed-cell foam and 111 mm Hg for the alginate-catalyst. The level of partial pressure due to oxygen delivery in the thick dermis was comparable

TABLE 1

Rate of Change in Oxygen Partial Pressure Per Min at 30 Min:60 Min (mmHg/Min)

Topical oxygen device	Skin sample			
	Control	Intermediate dermis 304-457 μ m	Thick dermis 457-762 μ m	Epidermis 304 μ m
Closed-cell foam	6.5*:4.0*	3.2*:2.3*	3.1:2.5	2.1:1.4
Alginate catalyst	4.4*:2.7*	3.1*:1.9	2.5:1.7	2.9:1.7
Gaseous	1.1:0.9	1.0:0.7	na	na



Rate is expressed as change in mm Hg per min, in which starting partial pressure was subtracted from partial pressure at each time point. Values are means of multiple measurements. Both closed-cell foam and alginate catalyst dressings showed significantly ($P < 0.05$) faster rates than gaseous oxygen in controls at 30 and 60 min. Only closed-cell foam showed significantly faster rates through intermediate dermis at 30 and 60 min relative to gaseous oxygen. The alginate catalyst was significantly faster through intermediate dermis at 30 min relative to gaseous (na = not applicable).

*Denotes a significance ($p > 0.05$) relative to gaseous sample.

to that attained in the intermediate dermis, with no significant difference between the two TDO devices.

Transfer of dO_2 Through Human Skin Sample With and Without Epidermis by TDO

The efficiency of oxygen delivery in skin samples with or without dermis was also investigated. An intermediate (0.012 in. or 304 μ m) split thickness skin sample containing the entire epidermis and part of the dermis was used in this investigation. We hypothesized the epidermis would present a greater barrier to oxygen penetration than dermis alone. This was contrasted to a dermis only

sample of equivalent thickness. However, no significant difference in rate of oxygen penetration was observed when TDO devices were applied to epidermal *versus* non-epidermal skin samples. Figure 5 shows that the maximum change in oxygen partial pressure achieved by closed-cell foam dressing was 96 mmHg and the alginate-catalyst dressing reached 102 mmHg in this skin sample with no significance between the two devices.

Table 1 shows a comparison of the rate of oxygen partial pressure change between the different TDO and TGO devices. Both TDO devices demonstrate a faster rate of oxygen penetration than does the TGO device. Rate of oxygen transfer through equal thickness

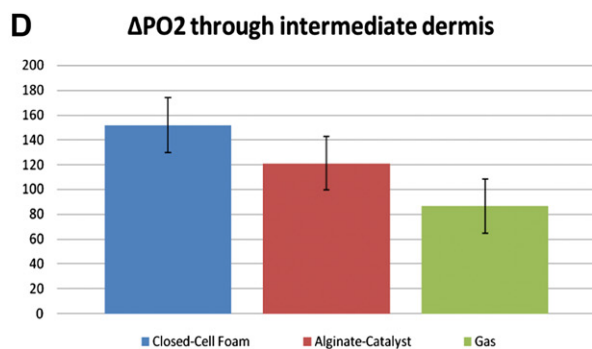
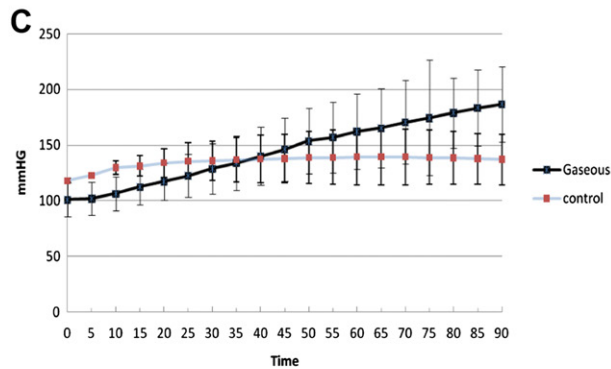
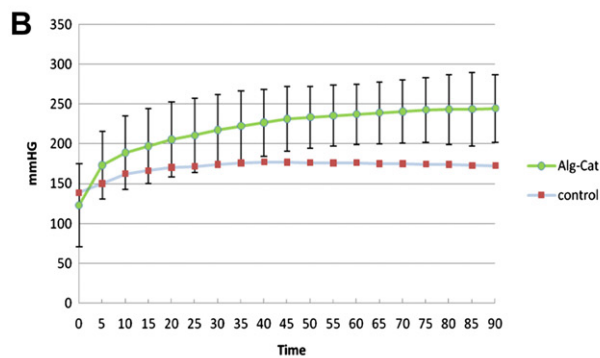
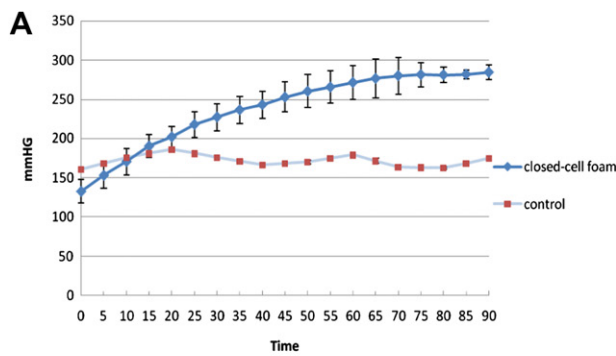


FIG. 3. Transfer of dissolved oxygen through intermediate dermis (304–457 μm) into saline at 37°C; $n = 3$ oxygen delivery devices; $n = 2$ control. (A) Foam dressing; (B) alginate-catalyst dressing; (C) 100% gaseous oxygen; (D) change in partial pressure at 90 min. from $T = 0$. Closed cell foam greater than gaseous ($P < 0.05$).

samples of skin with or without epidermis was different but not significant. Also, alginate-catalyst TDO was more effective through intact skin than closed-cell foam TDO though the difference was not significant.

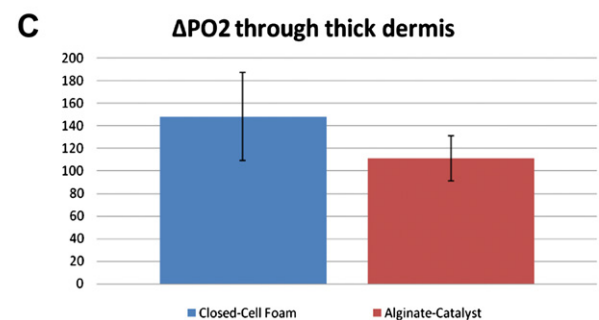
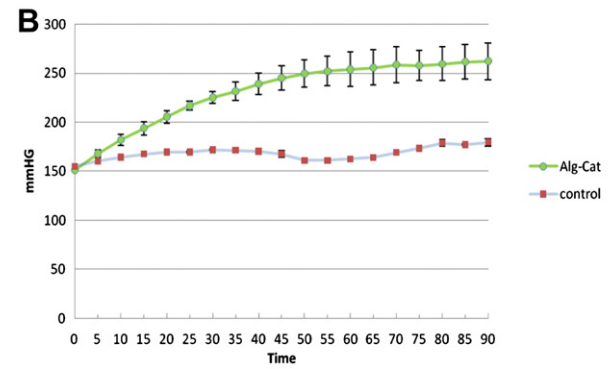
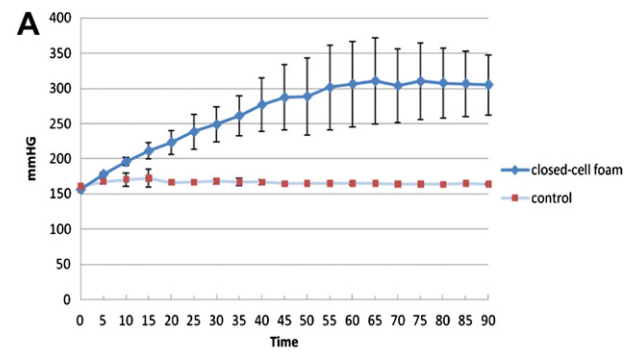


FIG. 4. Transfer of dissolved oxygen through thick dermis (457–762 μm) into saline at 37°C; $n = 3$ oxygen delivery devices; $n = 2$ control. (A) Foam dressing; (B) alginate-catalyst dressing; (C) change in partial pressure at 90 min from $T = 0$.

Table 2 represents the volume of oxygen delivered during the initial 30 min of measurement derived from the Figs. 2–5. Initially, there is a maximal oxygen driving gradient that lessens as the saline reservoir accumulates oxygen. Therefore, the rate of oxygen transfer gradually trends toward a plateau. If the reservoir were living skin, oxygen would be continually extracted and the maximal oxygen driving gradient would likely be preserved.

DISCUSSION

Topical Oxygenation Background

Distribution of oxygen through human dermal tissue is dependent upon oxygen partial pressure gradients and the solubility of oxygen in the tissue. These factors

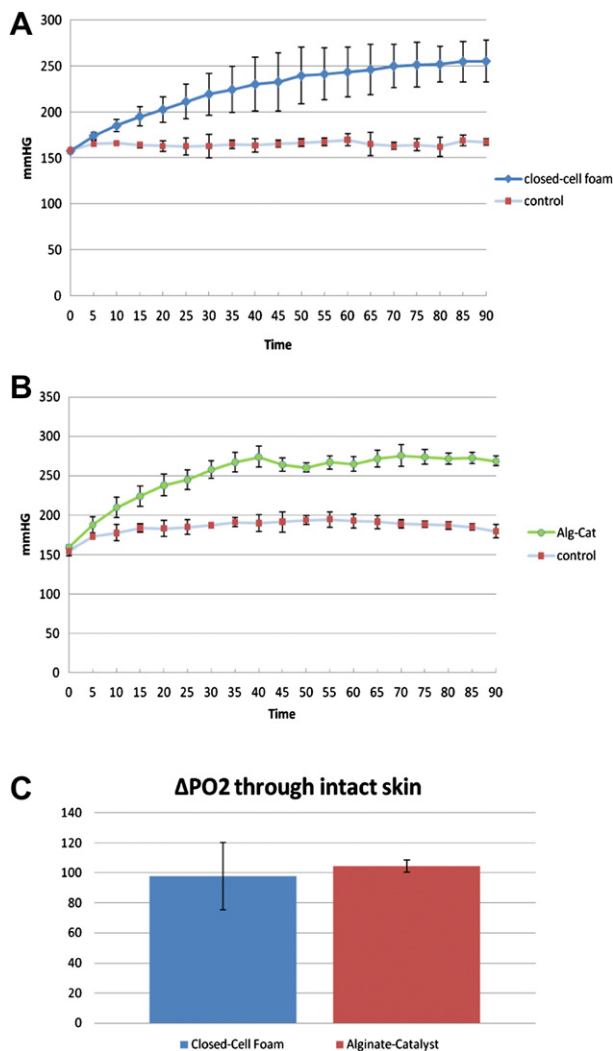


FIG. 5. Transfer of dissolved oxygen through intact skin, epidermis, and dermis (304 μm), into saline at 37°C; $n = 4$ oxygen delivery devices; $n = 3$ control. (A) Foam dressing; (B) alginate-catalyst dressing; (C) change in partial pressure at 90 min from $T = 0$.

determine whether the tissue layers can be supplied by external atmospheric or internal vascular sources of oxygen. This allocation of oxygen involves internal transport by cutaneous circulation *via* dermal papillae and externally by transcutaneous diffusion of atmospheric oxygen. The first evidence of cutaneous respiration occurred when Gerlach (1851) secured a varnished horse bladder to his skin and then measured a decrease in oxygen content and increase in carbon dioxide after 24 h. In terms of the respiratory needs of the individual, the contribution by skin is negligible, providing only ~2% of total respiration needs [9]. However, in several lower vertebrates, cutaneous respiration has been shown to contribute significantly to physiologic needs [10]. Cutaneous respiration in a prenatal marsupial mammal is the primary site of respiration [11]. In humans, cutaneous respiration is seen in preterm infants of <31 wk, where oxygen and carbon dioxide exchange

TABLE 2

Rate of Oxygen Delivered Through Skin During Initial 30 min ($\mu\text{L}/\text{Min}$)

Topical oxygen device	Skin sample			
	Control	Intermediate dermis 304-457 μm	Thick dermis 457-762 μm	Epidermis 304 μm
Closed-cell foam	2.1*	1.0*	0.97	0.63
Alginate catalyst	1.4*	0.97	0.77	0.9
Gaseous	0.27	0.2	na	na

Amount is expressed as the μL of oxygen per min. Values are means of multiple measurements. Both closed-cell foam and alginate catalyst dressings showed significantly ($P < 0.05$) larger volumes than gaseous oxygen through control at 30 min. Only closed-cell foam showed significantly greater volumes through intermediate dermis at 30 min relative to gaseous oxygen (na = not applicable).

*Denotes a significance ($P > 0.05$) relative to gaseous sample.

was 5 to 6 times higher than an adult, and may provide up to 20% of physiologic need [12].

Measurements of Topical Oxygenation

The degree to which cutaneous respiration can contribute to dermal metabolic needs is dependent upon the depth to which topical oxygen penetrates into human skin. Penney *et al.* (1968) took sheets of isolated human stratum corneum and measured the diffusion of oxygen from a chamber of water equilibrated with air to a chamber that contained water deoxygenated by nitrogen [13]. It was shown that oxygen diffused through the stratum corneum, raising the oxygen partial pressure of the de-oxygenated water. The thickness of the stratum corneum was estimated to average 12 μm . Gruber *et al.* (1970) used full-thickness skin samples to demonstrate that gaseous oxygen can penetrate into live dermis taken from the abdomen [14]. Exposing samples to 100% gaseous oxygen at a pressure of 3 atms, oxygen could penetrate to 300–340 μm passing through the epidermis into the dermis. Although, measurements at deeper dermis layers, 1.8–2.2 mm, showed no change in oxygen. Also, oxygen administered at 1 atm was unable to penetrate even at the more superficial layers. Using an oxygen flux optode, a more advanced technique for measuring cutaneous oxygen, Stucker [15] showed that atmospheric oxygen supplies the outer 250–400 μm of human skin *in vivo*.

Our data has confirmed and extended these existing observations. Viable human skin samples demonstrated oxygen penetration by both the TDO dressings and TGO. In most cases, TDO devices deliver oxygen into skin faster and to greater depth than do TGO devices. Our model has demonstrated oxygen penetration into viable skin by TDO devices to beyond 700 μm ,

a depth previously not reported in the literature. In addition, our model is the first to compare viable human skin samples with and without epidermis. It has been felt that the epidermis, including the stratum corneum, provided a significant barrier to transcutaneous oxygenation. Our initial data show that the epidermis is not prohibitive of transcutaneous oxygenation.

The average adult has 2 square meters ($20,000 \text{ cm}^2$) of skin that weighs approximately 4000 g. This gives a single square centimeter of skin a mass of 0.2 g ($4000 \text{ g}/20000 \text{ cm}^2$). It has been reported that skin consumes 5 mL/min of oxygen from circulating blood supply [16] ($5 \text{ mL/min}/4000 \text{ g}$ equals $1.25 \text{ }\mu\text{L/min}$ per g of oxygen consumption). In our model, we used skin tissue samples of 4 square centimeters (0.8 g). This area of tissue would consume generally $1 \text{ }\mu\text{L/min}$ of oxygen. According to Table 2, the TDO devices have a potential delivery capacity of 40–60 μL within the initial 30 min as shown in control recording. When measured through intact skin the TDO devices deliver 20–27 μL of oxygen. This mode of topical oxygen delivery could therefore meet nearly all of the physiologic oxygen requirements of skin.

Topical Oxygenation *via* Dissolved Oxygen or Gaseous Oxygen Sources

For oxygen to become biologically available, it must leave the gaseous phase and enter the liquid phase so that it can diffuse into a cell. Gaseous oxygen must overcome several barriers and partial resistances to enter the liquid phase, including the resistance within the gas film to the phase boundary, penetration of the phase boundary itself, and transfer from the phase boundary to the liquid. A dissolved oxygen source does not have these limitations. It was therefore hypothesized that a dissolved oxygen source would be more effective than a gaseous oxygen source for providing transcutaneous oxygenation. Movement of oxygen within the tissue is governed by Graham's law of diffusion stating that gases move independently and at different rates from areas of high pressure to low pressure. Once in the tissue extracellular matrix, the diffusion of oxygen occurs at a rate dictated by diffusion coefficient, which for human dermis has been estimated at $1.8\text{--}3.1 \times 10^{-5} \text{ cm}^2/\text{s}$ [17]. If one begins with a higher oxygen concentration from the source, there will be greater tissue penetration. Our data confirm that topically applied dissolved oxygen penetrates the human skin at a faster rate and to a greater depth than does topically applied gaseous oxygen. This may be clinically relevant, as oxygen therapy may be achieved to a greater tissue depth and in a shorter time with TDO devices. The transport of oxygen to tissue is faster when the oxygen is dissolved in fluid. The fact that

removal of the lipid rich stratum corneum increases the diffusion of oxygen through the skin suggests the importance of oxygen dissolved in a solution [18]. The removal of the stratum corneum can also increase transcutaneous oxygenation of hemoglobin due to the removal of the corneal lipid rich layer and an increased surface water content of the skin, which allows applied dissolved oxygen to directly enter the skin [19]. Therefore, as expected, in our results, the topical dressings that deliver oxygen dissolved in a fluid phase (TDO) were more capable of oxygen delivery than 100% gaseous oxygen (TGO). Not only is the final partial pressure of oxygen delivered by TDO devices greater than that from TGO after 90 min, but the rate of transfer is faster as well.

There are several ways oxygen may penetrate through skin, which has porous and nonporous regions. Oxygen is a small molecule and can easily pass through skin pores, which span the full thickness of skin layers. Eccrine sweat glands vary in number throughout the body (up to $350/\text{cm}^2$ in the palmer skin), and each gland has a pore $15\text{--}20 \text{ }\mu\text{m}$ in size [20]. Alternatively, oxygen may cross the nonporous portion of the skin, possibly *via* transmembrane proteins such as aquaporin, whose tetrameric structures create oxygen permeable channels that allow passage of oxygen [21]. An earlier study contrasting oxygen passage across sheets of viable and nonviable epidermis showed similar rates, suggesting that the major route of oxygen transmission is mediated by physiochemical structure rather than active cellular function [22].

Other topical oxygen delivery modalities have been developed. A supersaturated oxygen emulsions (SOS) containing perfluorocarbon which, due to its high affinity and carrying capacity, is capable of incorporating high levels of oxygen has been shown to improve wound healing in animal model [23,24]. Recent reports have shown topically applied gaseous oxygen can increase pO_2 of superficial wound tissue and has shown promise clinically [25,26]. Epithelial healing is improved by a transdermal sustained delivery treatment with bubbled gaseous oxygen [27].

This study reports on the transcutaneous delivery of oxygen using an *in vitro* viable human skin penetration model. It has now been shown that the oxygen penetration through $>700 \text{ }\mu\text{m}$ of live human dermis can be achieved with both gaseous and topical dissolved oxygen delivery devices. Our use of dermis only skin samples offers similarity to a partial thickness wound, which lacks epidermis (such as partial thickness burn or excoriation). The ability to deliver topical oxygen to partial thickness wounds may allow the clinician to support the metabolically active wounded tissue, which may be compromised by ischemia. We have demonstrated that TDO devices can provide the necessary

oxygen supply to ensure skin survival, even in the absence of tissue perfusion. This measurement platform offers a reliable tool for the evaluation of topical oxygen delivery to simulated wound environments.

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INVITED REVIEW

Influence of oxygen on wound healing

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Inflammatory phase; Oxygen; Proliferative phase; Reepithelialisation; Wound healing

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Yip WL. Influence of oxygen on wound healing. *Int Wound J* 2015; 12:620–624**Abstract**

Oxygen has an important role in normal wound healing. This article reviews the evidence concerning the role of oxygen in wound healing and its influence on the different stages of wound healing. The evidence reviewed has demonstrated that improving oxygenation may be helpful in limiting wound infection, although there is a lack of good quality studies on the role of oxygen in the proliferative phase and in reepithelialisation. Overall, the relationship between oxygen and wound healing is complex. Knowledge of this aspect is important as many treatment modalities for refractory wounds are based on these principles.

Introduction

The process of wound healing consists of the partially overlapping phases of haemostasis, inflammation, proliferation, epithelialisation and remodelling (1), within which each step requires oxygen (2). The role of oxygen in wound healing has been studied extensively since the 1960s when Hunt *et al.* (3) identified that adequate wound oxygenation could enhance formation of granulation tissues and synthesis of collagen. Oxygen is essential for the production of adenosine triphosphates (ATPs) and other biological energy sources via various metabolic cycles in cellular respiration (4). Furthermore, sufficient oxygenation is especially important for cell proliferation, bacterial defence, angiogenesis, collagen synthesis and epithelialisation (5). The latter are important for proper cellular function, especially during wound healing when there is an increased demand for reparative processes where sufficient tissue oxygenation is required to maintain high energy levels (6). This article reviews the evidence concerning the role of oxygen in wound healing and its influence on different stages of wound healing.

Inflammatory phase

The inflammatory phase of wound healing starts immediately after wounding and may last up to 1 week (1). Bacterial killing by phagocytosis is an important element, which depends on a high partial pressure of oxygen. After engulfing the pathogen, respiratory burst occurs. By transferring electrons from nicotinamide adenine dinucleotide phosphate (NADPH), NADPH oxidase in the neutrophil membrane produces superoxide, which combines with oxygen molecules and undergoes further changes to produce reactive oxygen species (ROS) (7).

ROS subsequently mediates bactericidal killing (8). Common ROS include peroxide anion (HO_2^-), hydroxyl ion (OH^-), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (9).

Allen *et al.* (10) performed an *in vitro* experiment using neutrophils from blood of healthy volunteers and wounds of two patients undergoing mastectomy. The bacterial killing capacity of the neutrophils was measured by oxygen consumption using a Clark-type oxygen polarograph. The authors found that the concentration of atmospheric oxygen was directly proportional to neutrophil oxygen consumption during the respiratory burst, with other confounders such as temperature, pH and glucose concentration being tightly controlled. The half-maximal velocity (K_m) for the NADPH oxidase with oxygen as a substrate was 40–80 mmHg. Clinically, the resistance to infection with reference to the neutrophil activity was expected to be critically impaired by wound hypoxia, but became more efficient with an increase in the tissue oxygen partial pressure, rising up to very high levels (500–1000 mmHg).

This study demonstrated that oxygen tension was an important factor affecting the respiratory burst and antimicrobial

Key Messages

- oxygen has an important role in normal wound healing
- this article reviews the evidence concerning the role of oxygen in wound healing and its influence on different stages of wound healing
- overall, the relationship between oxygen and wound healing is complex

effects of neutrophils, which may in turn affect infection rates and wound healing. However, the study was neither blinded nor randomised, which may limit its reliability and external validity.

Hopf *et al.* (11) conducted a prospective study of 130 surgical patients to determine whether subcutaneous wound oxygen tension could predict the development of wound infection. The authors found that the subcutaneous wound oxygen tension was inversely proportional to the risk of infection as predicted by an anticipated Study on the Effect of Nosocomial Infection Control (SENIC) score, a result that was also statistically significant ($P < 0.03$). This suggests that improving wound oxygen tension may reduce the risk of infection and subsequently promote wound healing. The validity of the SENIC index as a predictor of surgical wound infection risk has been well verified (12); therefore, this was an objective and comprehensive measurement of infection risk, which makes the result more reliable. However, since other potential confounders, such as supplemental oxygen or prophylactic antibiotics, were not controlled, the overall reliability of the study may be questionable.

Greif *et al.* (13) conducted the first randomised controlled trial (RCT) recruiting 500 patients undergoing colorectal surgery to examine whether peri-operative supplemental oxygen reduced the incidence of wound infection. Patients were given either 30% or 80% oxygen during their operation, up to two hours postoperatively. The wound infection rate during the first 15 days postsurgery was halved in the 80% oxygen group compared with the control group ($P < 0.01$). The anaesthetic procedures and surgery were standardised and similar in both groups, which limited potential confounding effects. Moreover, supplemental oxygen was given via endotracheal tubes and non-rebreathing masks to all patients, with subcutaneous oxygen tension (P_{scO_2}) and arterial oxygen partial pressure being measured and documented throughout the process. This ensured correct delivery of the planned oxygen concentration to the different groups and hence improved the validity of the study. However, in contrast to the study by Hopf *et al.* (11) who used a validated tool, in this study a wound was considered to be infected only when there was culture-positive pus. Therefore, the incidence of wound infection may have been underestimated as not all infected wounds would have fulfilled this criterion.

These *in vitro* and *in vivo* studies demonstrated a reduction in the rates of infection with increasing wound oxygenation. Subsequent trials designed with similar objectives demonstrated more heterogeneous results.

A recent RCT conducted by Thibon *et al.* (14) recruited 434 patients to study the effects of hyper-oxygenation with inspired oxygen of 80% compared with 30% oxygen on the frequency of surgical site infection (SSI) within 30 days postoperation in patients who had undergone routine abdominal, gynaecologic and breast surgery. Using a computer-generated allocation list, 226 and 208 patients were randomised into the 80% and 30% inspired oxygen groups, respectively. These oxygen fractions were administered only during intubations, and only the anaesthesiologists were aware of the group to which the patients had been allocated. The baseline characteristics were similar in both groups. In contrast to Greif *et al.*, (13) this study found no

statistically significant difference in the outcome between the two groups.

In this study, the researchers utilised the National Nosocomial Infection System (NNIS) risk index to evaluate SSI, which was different from the one used in the previous two trials. The different results of these studies maybe due to the different types of operations performed on the subjects, the different methods of oxygen administration, or the different ways of result assessment. Moreover, the authors did not measure P_{scO_2} or arterial oxygen partial pressure, which may limit the validity of the findings.

Recently, Kao *et al.* (15) conducted a systematic literature review and Bayesian meta-analysis to determine whether peri-operative supplemental oxygen reduced SSI in patients undergoing surgery. The authors identified a total of eight suitable RCTs to be included in the review and found a 77–85% chance of reduction in SSI with hyperoxia of up to 80% oxygen. Furthermore, they confirmed a higher probability of benefit of hyperoxia in patients with colorectal operations, with an 86–92% chance of reduction in SSI in this patient subset. However, the treatment hazards of hyperoxia were not studied, which may limit its use in clinical practice despite the demonstrated potential benefits in SSI reduction and wound healing.

Proliferative phase

The proliferative phase starts approximately 4–5 days after wounding and may last for a number of weeks (16). It consists of angiogenesis, formation of granulation tissue and extracellular matrix (ECM) and reepithelialisation, processes which Schreml *et al.* (5) suggested required oxygen to progress.

Angiogenesis

Angiogenesis is stimulated by both hypoxia and ROS (17). Hypoxia initiates angiogenesis by activating the transcription factor hypoxia-inducible factor (HIF)-1 α , which in turn upregulates vascular endothelial growth factor (VEGF), the major growth factor of angiogenesis (18). Paradoxically, a review by Chambers and Leaper (19) suggested that VEGF expression is linked to ROS. Sen *et al.* (20) suggested that hyperoxic conditions, for example, by increasing local ROS, could induce a higher degree of angiogenesis.

An animal study by Hopf *et al.* (21) demonstrated stimulation of angiogenesis with hyperoxia. In their experiment, mice were administered a subcutaneous injection of an unsupplemented gel, gel with VEGF or with anti-VEGF antibodies. They were then maintained under various environments of 13% (hypoxia), 21% (normoxia) and 100% oxygen (hyperoxia) at 1 absolute atmosphere (ATA), 2 ATA, 2.5 ATA and 3 ATA. These gels were then explanted, sectioned and graded for the degree of angiogenesis. Angiogenesis was statistically significantly decreased in the hypoxic animals ($P = 0.001$) but increased in the hyperoxic group ($P < 0.05$) with unsupplemented gels compared with normoxic controls. The authors concluded that oxygen was required for angiogenesis. However, it was also found that this significant finding vanished with VEGF-supplemented gel in the hyperoxic mice under

1 ATA and 2 ATA. These findings suggested that the role of VEGF may dominate that of oxygen in angiogenesis under normoxic environments, while the role of oxygen may be significant only under hyperbaric conditions.

Another more recent animal study by Sander *et al.* (22) determined the effects of hyperbaric oxygen (HBO) on wound neovascularisation in mice. HBO treatment was provided to a 'non-impaired healing' group ($n = 8$) and a 'macrophage reduction' group ($n = 8$), with equal numbers of controls. The wounds were measured by photographic images, while neovascularisation was directly visualised and measured using intravital video microscopy and computerised planimetry. Measurement was assessed by blinded investigators unaware of the treatment groups. The results demonstrated that neovascularisation occurred earlier in the HBO treatment groups compared with controls, with faster rates of wound closure observed. This finding was statistically significant ($P < 0.05$). However, photographic measurements rather than histological analysis were used in this study. Such methods may limit the reliability and reproducibility of results, as they may be affected by other confounders such as blood oxygenation, surrounding temperature and hydration status (19).

Extracellular matrix

Angiogenesis and ECM synthesis are interdependent processes (5). The new capillaries that form as a result of angiogenesis branch out and invade the surrounding matrix, which is then replaced by a new ECM produced and deposited by fibroblasts. This ECM consists of collagen fibres, proteoglycans, glycosaminoglycans, fibrin, fibronectin and hyaluronic acid. Hydroxylation of proline and lysine is an important oxygen-dependent step in the production of collagen (6).

Tissue fibroblast growth and collagen biosynthesis are related to oxygen tension (23). Kan *et al.* (24) conducted an *in vitro* study on human fibroblasts. They found that hypoxia was responsible for delayed wound healing with a reduction in the amount of collagen in the wound, which was associated with an increase in MMP-1 synthesis. Moreover, Kang *et al.* (25) conducted another *in vitro* study on HBO treatment on human dermal fibroblasts. They found that daily HBO treatment at 2.0 atmosphere (ATM) selectively stimulated fibroblast proliferation after 7 days, together with an increase in basic fibroblast growth factor (bFGF) production.

A prospective RCT on humans ($n = 29$) was conducted by Hartmann *et al.* (26) to compare the accumulation of collagen in standardised wounds in patients who had abdominal operations and whose postoperative fluid replacement was decided either clinically or by measurements of PscO₂. Silicone rubber catheters were placed in the upper arm to measure PscO₂, while two tubes of expanded polytetrafluoroethylene (ePTFE) were implanted subcutaneously parallel to the silicone rubber catheter to measure the amount of collagen accumulated. They found that the group treated according to PscO₂ measurements received more fluid on the day of operation than the group treated according to clinical criteria ($P < 0.05$); also, more collagen accumulated in their ePTFE tubes by day 7 ($P < 0.05$). Collagen formation in healing wounds seems to be associated with improvement in PscO₂.

However, this trial studied collagen formation as a surrogate outcome, rather than studying wound healing directly, that is, by measuring wound size and depth. Abundant collagen formation secondary to improved tissue oxygenation or hydration may not be equivalent to improved wound healing as it is a complicated process involving other ongoing events, such as migration of various types of cells across ECM. Further studies are required to delineate this issue.

Nakada *et al.* (27) conducted a case series on seven patients with leg ulcers refractory to conventional therapy who were administered a combined therapy of HBO and human bFGF. HBO at 2 ATA for 90 minutes and spray treatment of bFGF to the ulcer bed, both daily, were prescribed for an average of 2.6 months (1.3–4.4 months). Biopsies of ulcers were obtained for histological examination as well as fibrous tissue measurement. Ulcers in five of the patients were completely healed and two showed a reduction in ulcer size macroscopically. Proliferation of connective tissue was found to be induced in the ulcer, with an increased amount of both collagen and non-collagenous proteins. However, the result could be questioned as no control group was recruited for comparison. The improvement could be due to HBO alone, bFGF alone, their combined effects, or other confounders. Moreover, no blinding of patients, medical practitioners and investigators was mentioned, which may impair the internal validity. In general, this study demonstrates the induction of connective tissue proliferation in ECM and enhanced wound healing by the combined effects of improved tissue oxygenation (with HBO therapy) and growth factor application (bFGF).

Reepithelialisation

Parallel to the formation of granulation tissue and underlying ECM, reepithelialisation is initiated to cover the wound surface by a layer of epithelium and is based on differentiation, proliferation and migration of epidermal keratinocytes from the margin of the wound (5).

O'Toole *et al.* (28) conducted an *in vitro* study on the motility of human keratinocytes subjected to either hypoxic (2% oxygen) or normoxic (20% oxygen) conditions, and demonstrated that keratinocytes migrated faster under hypoxic conditions on connective tissues, associated with the increased expression of lamellipodia proteins and collagenase, but decreased expression of laminin-5, which inhibits keratinocyte motility.

However, a more recent study by Loo and Halliwell (29) examined the effects of H₂O₂, one of the common ROS, on a keratinocyte-fibroblast co-culture model of wound healing. The re-epithelialisation rate was measured by taking images of the closure of wounds with a dissection microscope. In contrast to the study by O'Toole *et al.*, (28) the authors found that H₂O₂ increased keratinocyte proliferation and the rate of reepithelialisation.

These contradictory results may be due to the different culture models used as well as the different experimental settings. Oxygen may mediate activities of keratinocytes via other ROS, apart from H₂O₂, giving rise to different effects. An *in vivo* study, if practically possible, might be helpful in delineating such issues. Such information is important for future development of local treatments, such as local oxygen therapy or

various dressing materials, speeding up reepithelialisation of partial-thickness wounds or second-degree burns.

Conclusion

The evidence reviewed here has demonstrated that improving oxygenation may be helpful in reducing wound infection. However, as the majority of research has been undertaken only on surgical rather than other types of wounds, the external validity and generalisability of the studies are limited. Further research is required to identify the fraction of inspired oxygen that is most beneficial for wound healing, and the duration for which supplemental oxygen should be administered for maximum benefit.

There appears to be a lack of good quality human studies on the role of oxygen in the proliferative phase of wound healing, in particular concerning angiogenesis and formation of granulation tissues or ECM. Current evidence is mainly from *in vitro* or animal studies. However, the lack of *in vivo* clinical data is probably due to the ethical issues concerning induction of wound hypoxia in humans.

Moreover, no good quality *in vivo* or human studies are available concerning the relationship of oxygen and reepithelialisation. The studies and findings on this issue to date are contradictory and are mainly from *in vitro* studies. These experiments may oversimplify the situation and neglect the numerous possible interactions of keratinocytes *in situ*, such as the presence of other inflammatory cells, bacterial colonisation and granulation tissue. It may be similar to that of angiogenesis, where hypoxia plays a role in initiating the process, while adequate oxygenation is required for forming a healthy wound bed in order to complete reepithelialisation. Future studies, particularly *in vivo* ones, are surely required to fill this gap in the current knowledge base.

Overall, it seems the relationship between oxygen and wound healing is complex. Additional knowledge regarding this aspect is important as many treatment modalities for refractory wounds are based on these principles, including HBO, local oxygen therapy and other specific dressing materials.

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Commentary

Molecular Biomarkers of Oxygen Therapy in Patients with Diabetic Foot Ulcers

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Abstract: Hyperbaric oxygen therapy (HBOT) and topical oxygen therapy (TOT) including continuous diffuse oxygen therapy (CDOT) are often utilized to enhance wound healing in patients with diabetic foot ulcerations. High pressure pure oxygen assists in the oxygenation of hypoxic wounds to increase perfusion. Although oxygen therapy provides wound healing benefits to some patients with diabetic foot ulcers, it is currently performed from clinical examination and imaging. Data suggest that oxygen therapy promotes wound healing via angiogenesis, the creation of new blood vessels. Molecular biomarkers relating to tissue inflammation, repair, and healing have been identified. Predictive biomarkers can be used to identify patients who will most likely benefit from this specialized treatment. In diabetic foot ulcerations, specifically, certain biomarkers have been linked to factors involving angiogenesis and inflammation, two crucial aspects of wound healing. In this review, the mechanism of how oxygen works in wound healing on a physiological basis, such as cell metabolism and growth factor signaling transduction is detailed. Additionally, observable clinical outcomes such as collagen formation, angiogenesis, respiratory burst and cell proliferation are described. The scientific evidence for the impact of oxygen on biomolecular pathways and its relationship to the outcomes in clinical research is discussed in this narrative review.

Keywords: oxygen; hyperbaric; topical oxygen; continuous diffusion oxygen; diabetic foot ulcer; molecular biomarkers



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1. Introduction

Lower extremity complications in people with diabetes constitute a large worldwide burden within in already burdened population [1,2]. Every 1.2 s, someone with diabetes develops a foot ulcer [1]. More than half of these wounds become infected [3–5], leading to a high rate of emergency department visits, hospitalizations and ultimately amputation [6]. Every 20 s, someone with diabetes undergoes an amputation somewhere in the world [7–9]. Patients with diabetic foot ulcers are at a near three-fold greater risk for death in the year following wounding than patients with diabetes without foot ulcers [10]. This increases with additional comorbidities. Following ulceration, Charcot arthropathy, development of chronic limb threatening ischemia or amputation, 5-year mortality is comparable to most cancers [11–13]. Additionally, the costs for care for patients with diabetic foot ulcers exceed the cost of care most individual cancers [11,14,15].

The role of oxygen in wound healing has long garnered interest among researchers and clinicians alike. This interest has only increased as modalities for delivery of oxygen have evolved from large hyperbaric chambers to portable, direct topical application using localized chambers and more recently to handheld, wearable systems which continuously diffuse oxygen directly into the wound bed. There are distinct differences and advantages

of each modality for oxygen delivery. Although oxygen therapy can be used for a variety of etiologies, the focus of oxygen therapy discussed revolves around its most common usage which is diabetic foot ulcerations due to its element of ischemia, either involving a macro or micro circulatory component. In hyperbaric oxygen therapy (HBOT) a contained chamber is pressurized with 100% oxygen to 2.0–2.4 atmospheres absolute for 90 min 5–7 days per week. HBOT relies on respiration and the circulatory system to deliver oxygen to the wound bed [16]. The increased pressure supersaturates the plasma; however, oxygen delivery relies on local capillary structure to reach injured tissues. Deficient or absent capillary beds may impede the oxygen delivery to ischemic tissues. Traditional TOT uses high flow oxygen concentrators coupled with chambers or bags placed directly over or around the wound. TOT applies oxygen directly to the wound, allowing the oxygen to diffuse directly into the wound and is therefore not reliant on underlying capillary structures. TOT follows an intermittent treatment regimen similar to HBOT. Currently, most recent devices are wearable and continuously generate pure, humidified oxygen from surrounding air using electrochemical oxygen generators. There is no need for an external oxygen source. They continuously diffuse oxygen (CDO) directly into wounds (24 h a day, 7 days a week) using an oxygen diffuser or oxygen diffusion dressings. CDOT, like TOT, does not depend on the underlying capillary structure of the wound bed, however, unlike TOT, the continuous application of oxygen resembles physiologic oxygen delivery. The biomolecular evidence for the effects of oxygen in wound healing including all modalities of delivery are presented. Although delivery mechanisms differ, the effect of oxygen at a cellular level is consistent.

However, the availability of oxygen to injured tissues will depend on the method of delivery. For HBOT, which relies on inspired oxygen, the availability depends on arterial pO₂, vascular supply, local capillary structures and the diffusion distance for the oxygen from the capillaries to the cells. Both edema and necrotic debris increase the diffusion distance. If the local structures are impaired or vasoconstriction is present, wound perfusion can be significantly impaired such that little to no increase in wound pO₂ levels occurs despite breathing supplemental oxygen [17–19]. Hence, there is a need for determining local vascular adequacy using methods such as transcutaneous oxygen pressure measurement prior to initiating HBOT. Modalities that use direct application of oxygen to the wound, such as TOT and CDOT, still require adequate vascular sufficiency, yet are significantly less dependent on local capillary structures. Necrotic tissue increases the diffusion distance to the wound, so debridement is an important step to ensure optimal diffusion of oxygen into the wound bed for topically applied oxygen. Debridement has been shown to have significant benefit when applied to standard moist wound therapies [20–22]. The importance of debridement in topically applied oxygen was recently demonstrated in a double blind, placebo-controlled clinical study, where CDOT showed dramatically higher wound closure rates and overall closure for wounds that were debrided frequently versus those that were not [23] in patients with diabetic foot ulcers.

The molecular processes discussed herein are oxygen dependent and do not occur without oxygen. The reactions are catalyzed by enzymes which typically have about 50% maximum speed at normal tissue pO₂ levels (40–80 mm Hg) and reach 90% of maximum speed at levels varying between about 150 mm Hg to over 400 mm Hg [24,25]. These higher levels can only be achieved with supplemental oxygen. An interesting finding regarding the positive correlation between oxygen concentration and functionality is that the more oxygen there is, the faster and better the outcomes are compared to normal wound healing. The differences are even greater when compared to ischemic wounds which are hypoxic. The definitions of hypoxia and hyperoxia are relative. In the context of this review, they are relative to the levels normally found in healthy tissue surrounding a wound (40–80 mm Hg).

2. Cell Metabolism and Energy

Oxygen plays a crucial role in energy production and cell metabolism. In this role, oxygen is required for intracellular processes such as biosynthesis and transport, not to mention cell survival [26]. Oxygen dependent enzymes include adenosine triphosphate (ATP) for chemical energy and nicotinamide adenine dinucleotide phosphate (NADPH) oxygenase for respiratory burst (reactive oxygen species release). ATP fuels most active cellular processes and the increased energy demand of tissue that is undergoing healing leads to a hypermetabolic state wherein additional energy is generated from oxidative metabolism [27–30]. Other metabolic processes such as aerobic glycolysis, β -oxidation of fatty acids and the citric acid cycle are tightly attached to the energy acquisition by oxidative phosphorylation and are, therefore, oxygen dependent [31]. Conversely, when tissue oxygen levels are consistently too low (<20 mmHg pO₂), cells convert to anaerobic metabolism and go into survival mode in which wound healing activities such as mitotic cell division, and, therefore, re-epithelialization with collagen production are impaired [32–34]. Prolonged exposure to extremely low oxygen levels, if not alleviated by oxygen, can result in cell death and tissue necrosis due to the inability of the cells to repair the spontaneous decay of cell components (DNA, RNA and proteins) and inability to maintain calcium pumps which require ATP to function [35,36].

3. Molecular Biomarkers in Growth Factor Signaling Transduction

Reactive oxygen species (ROS) are essential for the signaling processes of growth factors and processes such as leukocyte recruitment, cell motility, angiogenesis and extracellular matrix formation involved in wound healing [37]. The rate-limiting substrate for ROS production is oxygen. In a wound site, almost all wound-related cells can generate ROS using the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The functionality of NADPH oxidase correlates positively to pO₂ levels, with the maximal function of NADPH oxidase observed at pO₂ > 300 mm Hg, levels only achievable with supplemental oxygen. In wounds deficient of oxygen, such as diabetic or ischemic wounds, NADPH oxidase ceases to function at pO₂ levels below 20 mm Hg. There have been no noted adverse effects or increased reports of safety issues associated with high concentrations of oxygen in wound care. The increased ROS levels appear to accelerate the signaling processes without causing any damage at the cellular level. On a clinical level, studies have shown comparable or decreased adverse events and hospitalizations compared to standard of care with no supplemental oxygen [38–41].

Signal transduction of growth factors and cytokines is stimulated by ROS [42]. ROS, such as superoxide and hydrogen peroxide, increase vascular endothelial growth factor (VEGF) production in macrophages and keratinocytes [43,44]. ROS are also required for platelet-derived growth factor (PDGF) to regulate cell growth and division [45]. Like VEGF, PDGF plays a significant role in blood vessel formation (angiogenesis) [46]. ROS have effects on other processes such as cytokine action, cell motility and extracellular matrix formation [47]. Conversely, tissue hypoxia will limit redox signaling and disable the function of several growth factors such as PDGF, VEGF, keratinocyte growth factor, insulin-like growth factor one (IGF-1), transforming growth factor beta (TGF- β) and numerous molecular mechanisms (e.g., leukocyte recruitment, cell motility and integrin function) which rely on redox signaling [37,48,49]. This positive correlation between pO₂ levels, ROS production, and growth factor promotion of cytokine expression explains why ischemic diabetic wounds, having little to no ROS, fail to heal and why wounds supplemented with oxygen heal faster. Typical molecular biomarkers that are indicative of wound healing are shown in Table 1 along with the processes that they are associated with. These biomarkers and their effects on wound healing will be discussed in greater detail throughout this review.

Table 1. Molecular Biomarkers and Clinical Impact in Wound Healing.

Growth Factors	
IGF-1	protein production and cell proliferation and migration
PDGF	cell growth and division and chemotaxis
TGF- β	angiogenesis, fibroblast proliferation, collagen synthesis and deposition, extracellular matrix (ECM) remodeling, tissue remodeling, granulation tissue stimulant and anti-inflammatory mediator
VEGF	angiogenesis and collagen deposition and epithelialization
IGF-1	protein production and cell proliferation and migration
cytokines	
CXCL8	angiogenesis, epithelialization, fibroblast migration and inflammatory mediator
IL-6	leukocyte infiltration, angiogenesis, collagen accumulation, anti-inflammatory, granulation tissue stimulant and mitogenic
TNF- α	leukocyte recruitment, cell regulator, ECM synthesis and inflammatory mediator

The impact of continuous diffusion of oxygen therapy (CDO) on wound cytokines and growth factors was recently demonstrated in a prospective study of 23 patients with diabetic foot ulcers below the malleolus [50]. Results showed significant increases in growth factors, cytokines and transcutaneous oxygen pressure measurement levels after application of CDO. Growth factors significantly increased from 280% to 820% of base levels in the first week and decreased in subsequent weeks [50] (Figure 1). Cytokines increased significantly (up to 680% compared to baseline levels) in the first two weeks and then decreased. Significant increases in transcutaneous oxygen pressure measurement indicated increased oxygen perfusion in the wound periphery. This is evidence that the topically applied oxygen not only saturated the wound bed, yet also elevated the levels of oxygen in the surrounding tissues.

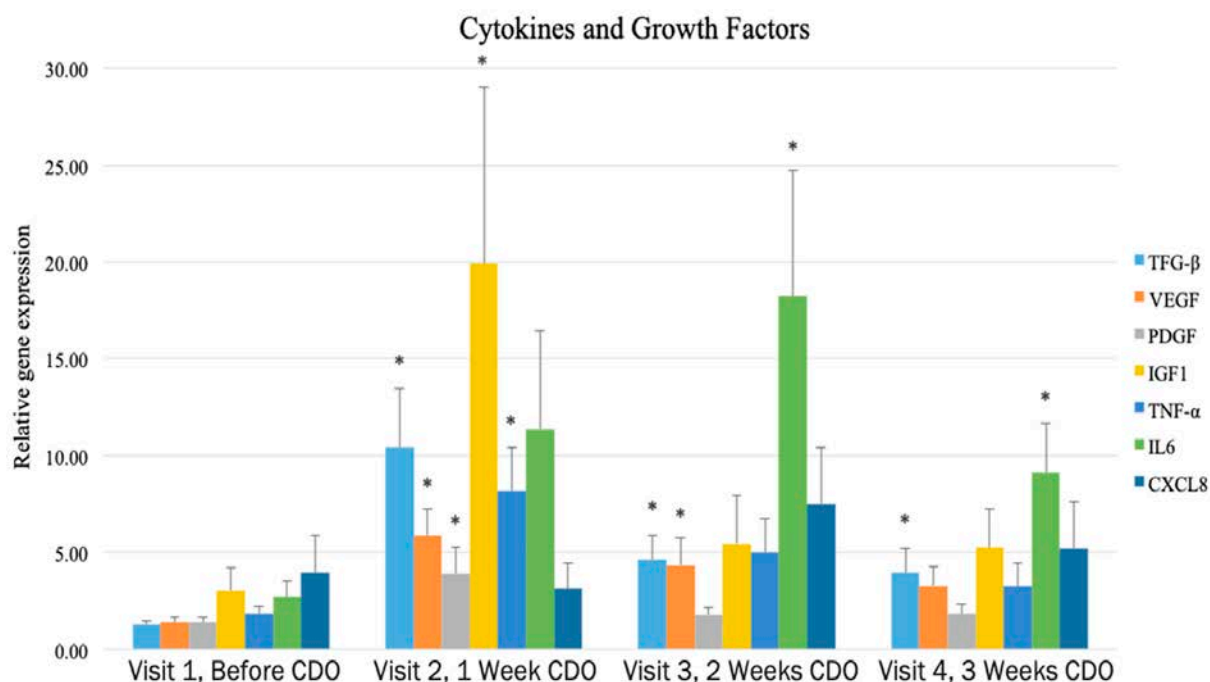


Figure 1. Impact of CDO on gene expression for various cytokines and growth factors at each visit Asterisks indicate significant increase from baseline [50]. Adapted with permission.

4. Collagen Formation

Oxygen is essential to make and properly organize collagen, which is the primary component of skin, accounting for 70–80% of dry weight and acts as the primary structural scaffold of skin and structures the matrix for angiogenesis. Organized collagen is bundled into fibers, which are interwoven and can be stretched in multiple directions without tearing. At the biomolecular level, oxygen is required for the hydroxylation of proline and lysine in procollagen [51]. Several posttranslational steps in collagen synthesis are oxygen dependent. The enzymes prolyl hydroxylase, lysyl hydroxylase and lysyl oxidase all require oxygen [46,52,53]. The formation of cross-linked triple-helices occurs via the oxygen-dependent enzyme prolyl hydroxylase and are excreted as collagen fibers. Collagen fibers are arranged into linear fibrils via cross-linking by lysyl hydroxylase. Linear fibrils are cross-linked by lysyl oxidase—a necessary step to achieve the necessary tensile strength for healed wounds.

Higher oxygen concentrations increase the amount of collagen deposition [54] and tensile strength [55–57]. The rate limiting step is the rate of prolyl hydroxylation [52,53]. The oxygen level required for optimal prolyl hydroxylase activity is at oxygen levels approaching 250 mmHg, exceeding those present in normal wounds and only achievable using oxygen therapy treatment [58,59]. It has been shown that increasing oxygen concentrations above normal physiologic levels enhances collagen synthesis and tensile strength in both animal and human subjects [55–57] and can increase the level of collagen organization [60]. Correction of vasoconstriction and hypoxia can result in a 10-fold increase in collagen deposition in wound repair [17,54,56,61]. The rates of collagen deposition increase as oxygen levels increase, with optimal activity at levels higher than 250 mmHg [62]. Conversely, hypoxic wounds as in patients with diabetes deposit collagen poorly and become infected easily [51,54].

In a study using supplemental oxygen at a rate of 4 L/min through nasal cannula for 12 h a day for 3 days, it was found that three times as much collagen was deposited in patients with well-perfused and oxygenated wounds compared with those with lower oxygenation and perfusion scores [54]. A separate study using direct topical oxygen on chronic diabetic foot ulcers showed significant increases in the expression of genes associated with collagen production (TGF- β , VEGF and IL-6) during weekly follow-up visits after application of CDO in patients with diabetic foot ulcers [50].

5. Angiogenesis Biomarkers

The creation of new blood vessels, angiogenesis, is essential to the growth and survival of repair tissue. Oxygen levels directly affect not only the rate, yet also the quality of new blood vessel growth. Sufficient oxygen levels are required for correct collagen synthesis (posttranslational hydroxylation) [63] without which the new capillary tubes assemble poorly and remain fragile [62,64,65]. Supplemental oxygen has been shown to accelerate blood vessel growth [66]. Moderate hyperoxia increases the appearance of new blood vessels in wounds [67]. Similar to ROS activity, the rate of angiogenesis has been shown to be directly proportional to oxygen levels in damaged tissues [62], with maximum activity levels at pO₂ levels exceeding 250 mm Hg.

VEGF has been shown to be a major long-term angiogenic stimulus at the wound site and is believed to be most prevalent and efficacious signal for angiogenesis. Oxygen treatment induces VEGF mRNA levels in endothelial cells and macrophages [68–70] and VEGF 121/165 protein expression in wounds [71]. Oxygen has also been shown to facilitate the release of VEGF165 from cell-associated stores [72].

Hyperbaric and topical oxygen therapy have been shown to increase VEGF expression in wounds [73] and induce angiogenesis [74]. More recently, a clinical study on gene expression of multiple factors involved in angiogenesis (VEGF, TGF- β , IL-6 and CXCL8) showed significant increases upon continuous application of oxygen (Figure 1) [50]. The expression levels over time are similar to gross observations of their effect in the field: increased redness within the first week and exudate levels that peak within the first

two weeks and then subside, both indicators of new capillary formation. Furthermore, the curved response shown in Figure 1 reflects what would be expected of a chronic wound “reawakening” and entering the inflammatory stage.

6. Respiratory Burst Process and Cytokine Production

Oxygen is essential for respiratory burst, or the production of reactive oxygen species (ROS), used by phagocytes such as neutrophils and macrophages in bactericidal activity and the removal of necrotic cellular debris. NADPH oxidase, also known as leukocyte oxidase, has been shown to support macrophage survival, a delay of apoptosis and enables dead cell cleansing by phagocytosis [75]. NADPH oxidase in wound phagocytes, such as neutrophils and macrophages, produces superoxides (O_2^- and H_2O_2) for bactericidal activities [76]. It has been shown that up to 98% of oxygen consumed by these cells is used to produce ROS during phagocytosis [24]. Leukocyte activity, which involves the production of ROS which enables oxidative killing, is directly proportional to local oxygen concentration [77,78]. The optimal ROS production is seen at oxygen levels of greater than 300 mmHg, levels which can only be achieved with supplemental oxygen [79]. ROS activity is not restricted to phagocytes. At the wound site, ROS are generated by almost all wound-related cells [46]. The efficacy of supplemental oxygen has been shown to be similar to antibiotic administration and has additive effects when used together [80,81].

Interleukins are a type of cytokine protein that play important roles in the differentiation/activation of immune cells in addition to their proliferation, maturation, migration and adhesion. [<https://www.ncbi.nlm.nih.gov/books/NBK499840/> StatPearls Publishing; 31 January 2021]. The addition of continuous oxygen therapy directly to a wound has been shown to increase IL-6 significantly (up to 680% relative to baseline) in clinical studies [50,82]. IL-6 has been shown to induce chemotaxis of leukocytes into a wound [83,84]. As inflammation progresses, IL-6 signaling is responsible for the switch to a reparative environment.

7. Cell Proliferation Molecular Markers

Increasing oxygen levels results in faster cell proliferation, re-epithelialization and collagen formation. Fibroblast proliferation and protein production have been reported to be optimal at 160 mmHg, i.e., at pO_2 levels two-fold to three-fold higher than those found in healthy tissues [85], indicating that supplemental oxygen increases the rate of wound repair. Endothelial progenitor cells (EPCs) are essential in wound healing, but their circulating and wound level numbers are decreased in diabetes. Elevated oxygen levels (hyperoxia) reverse the diabetic defect in EPC mobilization [86]. EPC mobilization into circulation is triggered by hyperoxia through induction of nitric oxide with resulting enhancement in ischemic limb perfusion and diabetic wound healing [87–89].

Matrix metalloproteinases (MMPs) are a group of enzymes responsible for degrading a majority of extracellular matrix proteins during tissue development, growth and turnover [90,91]. MMPs have diagnostic, predictive and indicative power for wound healing and can be measured from wound fluid. They are required for a wound to heal properly, at a suitable level, in the correct position and for a certain length of time. Excess activity may lead to a chronic non healing wound. Chronically increased levels of MMPs and reduced levels of TIMPs (MMP regulators), or just abnormalities in their ratio, are associated with non-healing. Studies show that medical interventions which aid in lowering MMP activity will promote the healing of stalled wounds and that decreasing MMP-2 tissue levels will result in wound healing. In one study elevated MMP-1 and TIMP-1 levels were noted in on oxygen treatment group, yet not in the control group [82].

At the clinical level, the cumulative effects of oxygen in all the various aspects discussed herein result in significant real-world results. In a clinical study which analyzed VEGF expression versus wound size reduction using TOT, a significant correlation between wound closure and VEGF expression was found [73]. Recent results using topically applied oxygen therapy, both continuously and intermittently, on diabetic foot wounds

has been shown to increase the rate of wound closure, by as much as 460% relative to moist wound therapy in several double-blinded trials, two of which had placebo control groups [23,38–41]. As would be predicted by the positive correlation of various mechanisms of action to the relative concentration of oxygen, wounds that were larger, deeper, more chronic and weight-bearing had improved responses relative to controls than those that were smaller, shallower, less chronic or non-weight-bearing, respectively [38].

8. Summary

Biomolecular pathways associated with wound care have been shown to be positively correlated to local tissue oxygen concentration. Maximal activity levels of the related enzymes, growth factors and cytokines, as well as the associated physiological processes, are significantly above the levels normally found in healthy tissues. The positive, differential effect from supplemental oxygen has been shown to be even higher for tissues with compromised blood supply leading to ischemic diabetic wounds. Increasing the levels of oxygen in afflicted tissues significantly increases not only the rate, yet also the quality of tissue repair. These elevated levels of oxygen can only be achieved through supplemental oxygen therapy, whether it be respiratory based (HBOT) or directly applied to the wound (TOT, CDOT), all of which are reliant on diffusion gradients. The recent research on the scientific basis and clinical outcomes of oxygen therapy lays a foundation for further research in molecular biomarkers utilizing oxygen therapy.

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3.
SCIENTIFIC
PUBBICATION ON
VAGINAL TREATMENTS
WITH OXYGEN IN
COMBINATION WITH
HYALURONIC ACID



3.1. PUBLISHED STUDIES



Treatment With Vaginal Oxygen Therapy in Patients With Genitourinary Syndrome of Menopause Symptoms: An Observational Trial

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Abstract

Genitourinary Syndrome of Menopause (GSM) GSM is a chronic and progressive condition that can have many medical consequences (vaginal dryness, vulvar pain, dyspareunia, and dysuria) that may affect quality of life. The objective of our study was to evaluate the efficacy of vaginal hyperbaric oxygen treatment on GSM. Thirty patients received six weekly sessions of 95% pure oxygen that was delivered at a flow of 2 lt/minute for 15 minutes using a cannula designed for vaginal therapy, with a subsequent follow-up after two months. There was a significant improvement in vaginal dryness (before treatment VAS 7.52 versus 3.52 after treatment -p = 0.05). The same improvement was observed for vulvar burning/pain and dyspareunia, but these were not statistically significant. VHI before treatment was 15.1 versus 19.59 after treatment (p = 0.05). All the parameters maintain their value at 2 months of follow-up. No adverse effects were observed.

Introduction

The International Society for the Study of Women's Sexual Health (ISSWSH) and the North American Menopause Society (NAMS) defined the Genitourinary Syndrome of Menopause (GSM) in May 2013 as the clinical and functional consequences of vulvovaginal and lower urinary tract changes associated with menopause [1]. The definition includes cellular, histological, anatomical, and functional changes due to estrogen deficiency [1–3]. GSM is a chronic and progressive condition that can have many medical consequences that may affect relationships, daily activities, quality of life and enjoyment of sex [4]. Despite being highly prevalent it is frequently underdiagnosed, and thus remains untreated [5]. Women with GSM are often managed with hormonal treatments by the systemic (menopausal hormone therapy (MHT)) or local (local estrogen) routes: MHT improves vaginal lubrication, blood flow and trophicity of genital tissues [5, 6], and small doses of intravaginal estrogen increase vaginal pH and the epithelial maturation index and restore vaginal flora with minimal systemic effect [7, 8]. The Women's EMPOWER Survey conducted in 2017 to evaluate women's perception of the different therapeutic alternatives of GSM showed that approximately 35% of women refuse hormonal treatments, whether systemic or local, for fear of adverse effects [9]. Non-hormonal

alternatives in this context are therefore essential and currently include lubricants, moisturizers, hyaluronic acid, and physical methods such as perineal rehabilitation. Furthermore, due to contraindications to hormonal treatments (hormone-dependent cancer) and the constraints of use and variable effectiveness of local treatments, other therapeutic options have been developed such as vaginal therapy with hyperbaric oxygen. The application of oxygen therapy determines the increase of the reparative processes of the tissues and increases the synthesis of collagen which is defective in GSM [10]. The objective of our study was to evaluate the efficacy of vaginal hyperbaric oxygen treatment on GSM in a prospective approach based on quality of life related to symptoms and signs in treated patients..

Methods

Women included in the study were postmenopausal with at least 1 year of amenorrhea and at least one moderate or severe symptom of GSM (vaginal dryness, burning, and dyspareunia). Key exclusion criteria included the presence of clinically significant abnormal gynecological findings

other than signs of vaginal atrophy; use of concomitant hormonal medications, SERMs, or products expected to have estrogenic and/or antiestrogenic effects or use of hormone therapy (unless a sufficient washout period preceded any procedure). Review Board

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approval for the study was obtained and all participating individuals gave written informed consent. At the first assessment, symptoms of dryness, burning, and dyspareunia were evaluated on a 10 cm visual analog scale (VAS), and vaginal trophism through Vaginal Health Index was also collected. For the treatment, the Caressflow® device (Caress Flow srl - Via dei Giudei 39 Funo di Argelato 40050 -BO-Italy) was used composed of a compressor unit and a base unit (generator) able to deliver up to 6 l/minute of 95% pure oxygen. The 95% pure oxygen was delivered at a flow of 3 lt/ minute for 15 minutes using a single use cannula specifically designed for vaginal therapy. At visit 0, an accurate collection of patient history and a complete clinical examination was performed to evaluate eligibility criteria. The full treatment cycle included six weekly sessions, with a subsequent follow up after 60 days. The statistical significance of the trend of variation in values between treatment sessions was analyzed using the one-way variance analysis according to the Kruskal-Wallis method. The significance of couples' comparisons between treatment sessions was analyzed using the Wilcoxon test for non-parametric data. A $p < 0.05$ was considered statistically significant.

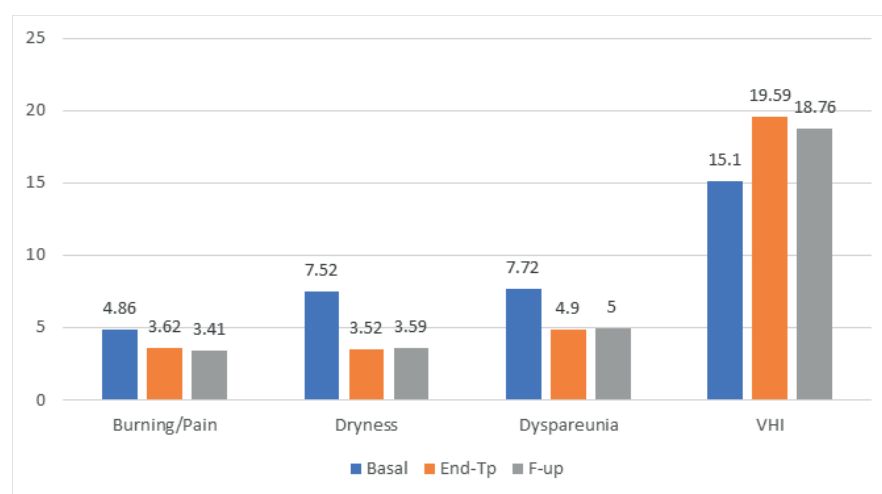
Results

Thirty patients enrolled in the study completed the full treatment protocol and attended the follow up consultation at 2 months. The patients had a mean age of 57.3 years (± 11.1 years). There was a significant improvement in vaginal dryness; the mean VAS before treatment was 7.52 (± 3.14) versus 3.52 (± 3.12) after treatment ($p = 0.05$). The same improvement was observed for vulvar burning/pain and dyspareunia, but these were not statistically significant. Instead, there was an improvement with a significant difference for vaginal trophism; the mean VHI before treatment was 15.1 versus 19.59 after treatment ($p = 0.05$). All the parameters maintain their value at 2 months of follow-up (Fig.1). No adverse effects were observed.

Discussion and Conclusion

Vaginal hyperbaric oxygen treatment appears to have a beneficial impact on the quality of life and management of patients with GSM. In our study, all symptoms and signs

improved, and a significant change was demonstrated in vaginal dryness, one of the main symptoms of GSM. One study demonstrated the efficacy of vaginal hyperbaric oxygen plus hyaluronic acid in patients with GSM, in which a significant improvement in well-being, vaginal burning, presence of fluid, vaginal epithelium appearance, and vaginal elasticity was observed between the first and the last therapy session [11]. We also confirmed the efficacy of vaginal hyperbaric oxygen treatment, but it was used alone without the adjunct of hyaluronic acid, proven the "strength" of vaginal oxygen in GSM. We can say that vaginal oxygen could be a valuable alternative or adjunct to others GSM treatments. Such device is particularly interesting for patients being treated for cancer and who suffer the long-term consequences of the treatments received or who present a contraindication to hormonal treatments. Oxygen therapy has a powerful regenerative and bio stimulating effect, promotes the increase in tissue repair processes and increases the synthesis of collagen allowing normal hydroxylation of this protein [10]. In addition, oxygen induces a neo-angiogenic stimulus by releasing factors such as the Vascular Endothelial Growth Factor (VEGF) [11]. Our results were good but not-so-bright on vulvar pain and dyspareunia. It is our experience that many menopausal women with complaints of dyspareunia have vestibular tenderness with more pronounced atrophic changes in this region rather than the vagina. Furthermore, it is likely, as Kao et al. suggest, that vulvovaginal atrophy alone does not adequately explain the findings of vestibular pain [12]. In 95% of her postmenopausal cohort with vestibular pain and dyspareunia, women used a variety of estrogen supplements, and atrophy varied but vestibular pain persisted. A rich nerve plexus within the vaginal submucosa was identified, but it is composed only of sympathetic and parasympathetic axons with smaller contributions by sensory fibers; in the vulvar vestibule, the sensory nerve endings are dense and shallow, making this region physiologically more sensitive [13]. We can therefore hypothesize that vaginal oxygen is not enough to re-establish a normal vestibular trophism. However, the results are encouraging and should stimulate the research regarding this innovative therapy, using the device also in vestibular area.



Data are expressed as mean

Figure 1. Results before and after vaginal oxygen therapy

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Vaginal Natural Oxygenation Device (VNOD) and Lichen: A Pilot Study on the Assessment of Effectiveness of the Simultaneous and Local Administration of High Concentration Oxygen and Hyaluronic Acid to treat Vulvar Lichen Scleroatrophicus

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Abstract

Background

This is a pilot study to assess the effectiveness of simultaneous and local administration of high concentration oxygen and hyaluronic acid by a specific medical device (VNOD) to improve vulvar lichen symptoms healing vulvar lesions.

Methods

17 women with histological diagnosis of vulvar lichen were included. Five weekly administrations of concomitant oxygen-hyaluronic acid therapy with VNOD were performed. The severity of the pathology was evaluated using the Vaginal Health Index (VHI). Patients' symptoms were evaluated with two questionnaires: VAS and FSFI. VAS, FSFI and VHI were evaluated at the start and at the end of the therapy. The statistical analysis was performed

according to the Kruskal-Wallis method and using the t-test calculator.

Results

The study has showed that each subsequent treatment determined a significant increase in all parameters, especially for VHI hydration index, VAS fluidity index and FSFI lubrication index, however all the patients reported a recovery of their sexuality at the end of the five treatment sessions. All the parameters maintain their value at six weeks of follow-up. No adverse effects were observed.

Conclusions

Combined topical oxygen therapy with hyaluronic acid by VNOD seems to be a valid and totally painless treatment for vulvar lichen scleroatrophicus, improving symptomatology, and sexual wellbeing.

Keywords: Vulvar Lichen Scleroatrophicus, Topical Oxygen Therapy, Hyaluronic Acid, Sexual Wellbeing

Introduction

Lichen Scleroatrophicus is a cutaneous-mucosal dermatosis characterized by chronic changes in the trophism of epithelial maturation together with connective tissue alterations. According to others, it is a chronic inflammatory disease manifested by sclerosis, atrophy and ulceration of the involved tissues and leading to the formation of scarring outcomes of varying degrees accompanied by more or less disabling symptoms.

In 80% of cases, it is localized in the ano-genital region with a higher incidence in the female sex (ratio 6 to 1). [1-2].

In the ano-genital area, the clitoris and labia minora are mainly affected by the disease; this is followed by the labia majora and the perineal area. Lichen Sclerosus can occur at any age although it prefers the peri- and post-menopausal period [3].

The causes are not entirely clear to date, but correlations have been noted with hormonal trends, especially in women, especially after menopause and with hyperactivity of the immune system [4].

Lichen is part of unobligated precancerous, meaning that not all patients with the disease will develop a neoplastic form,

however, statistics show that 6-10% of untreated severe forms evolve into vulvar cancer. The predominantly reported symptom is itching; this is followed by burning, spontaneous pain, dyspareunia (painful or impossible intercourse), dysuria (difficulty urinating) and skin-mucosal dryness with recurrent fissures^[5].

The vulva is pearly white in colour with an atrophic surface; the labia minora may show varying degrees of atrophy up to total fusion with hooding of the clitoris. The vestibule is rigid and sclerotic; the vaginal introitus appears reduced to the point of sometimes not allowing vaginal exploration. Fibrosis can involve the periurethral region with dislocation of the urethra and cause so-called vaginal urination. Tissues so sclerotic go into ulceration causing intense pain and burning to the point of difficulty with even simple intimate hygiene manoeuvres or contact of the mucosa with clothing^[6].

Materials and methods

17 women with histological vulvar diagnosis of Lichen were considered. The mean age at diagnosis of the 17 included cases was 56.5 years (range 48 - 65).

Patients were identified and recruited from investigators clinics and referring physicians, with privacy protection and avoiding undue influence. Each included patient provided an informed consent that allowed treatments, that certified the comprehension of the information provided, with voluntary agreement of the subject, free from coercion. The treatments were administered at Antonio Cardarelli Hospital in Napoli (AORN "A. Cardarelli").

Every patient was submitted to a colposcopy and histological biopsy to confirm lichen diagnosis. Topical corticosteroid therapy performed in the previous 30 days was considered as an exclusion criteria from the study.

The clinical study was approved by the local institutional review board (Comitato Etico Campania 3 Prot. N. 00014304-14/06/2024) and conducted in accordance with the ethical standards of the Declaration of Helsinki.

Five weekly administrations of concomitant oxygen-hyaluronic acid therapy with VNOD were performed.

The severity of the pathology was evaluated using the Vaginal Health Index (VHI) which includes three parameters: Vaginal elasticity, vaginal secretions, and vaginal hydration. The degree of the health of the vagina was defined by the final score obtained after the evaluation of each parameter based on a five-point scale: Score 1 = absence of elasticity, secretions, hydration, score 2 = weak elasticity, secretions, hydration intensity, score 3 = mild elasticity, secretions, hydration intensity, score 4 = good elasticity, secretions, hydration intensity, score 5 = excellent elasticity, secretions, hydration intensity.

Patients' symptoms were evaluated with two questionnaires: VAS and FSFI.

VAS studies the intensity of the four following symptoms: (I) well-being such as absence of dyspareunia, (II) vaginal dryness, vaginal lubrication and presence of fluid, (III) vaginal burning, (IV) vulvar or vaginal itching. Symptoms were collected from patients based on a six-point scale: Score 1 = maximum intensity, score 2 = strong intensity, score 3 = average intensity, score 4 = mild intensity, score 5 = weak intensity and score 6 = absence of symptom).

The Female Sexual Function Index (FSFI) based on clinical interpretations of a principal components analysis, included desire, subjective arousal, lubrication, orgasm, satisfaction

and pain and were evaluated based on a six point score scale: Score 0 = absence of symptoms, scores 1 = weakness of symptoms, score 2 = mild symptoms intensity, score 3 = medium symptoms intensity, score 4 good symptoms intensity; score 5 =excellent symptoms intensity.

VHI, VAS and FSFI were evaluated at the start and at the end of the therapy. The final administration was performed after 5 weeks. The vaginal well-being and symptoms were determined and recorded by the clinician with a numerical score according to the scales described above.

The statistical significance of the trend of variation in values between treatment sessions was analysed using the one-way variance analysis according to the Kruskal-Wallis method. The significance of couples' comparisons between treatment sessions was analysed using the t-test calculator for two independent means. A $p < 0.05$ was considered statistically significant. Statistical Analysis was performed using SOCS statistics. After 6 weeks since the last treatment a follow up was performed, and the three most statistically relevant parameters maintained their value.

For the treatment, the self-cooling Caress Flow (Caress Flow- srl, Casette di Funo, Bologna, Italy) device was used composed of a compressor-base unit (generator) able to deliver up to 2+/- 0,2 L/minute of 93% +/- 3% pure oxygen provided at a pression of 1+/- 0,1 bar.

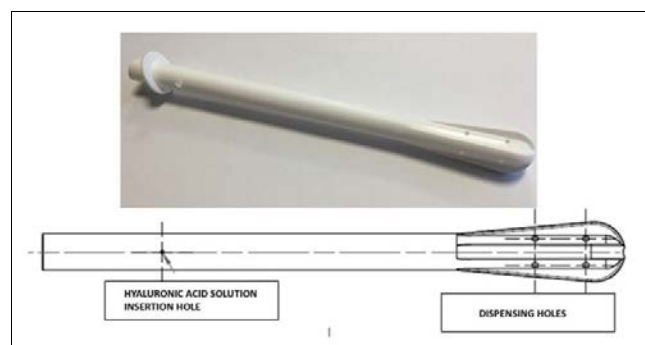


Fig 1: ↓ Cannula

Two dispensers were used, the first consisting of a vaginal cannula, connected to the machine body. The cannula is equipped with outlet holes for the delivery of oxygen and hyaluronic acid, which is inserted through a special insertion hole located in the upper part of the cannula (Fig 1). The vaginal cannula is used for treatment inside the vaginal canal.



Fig 2: ↓ Airbrush

In addition to the cannula, an airbrush was used (Fig 2), always connected to the machine body, capable of delivering oxygen in combination or not with the hyaluronic

acid solution. The airbrush is used for the treatment of external genitalia, nebulizing the combination of oxygen and hyaluronic acid.

The full treatment cycle includes five weekly sessions, during which the treatment was performed for 20 minutes, and the high purity oxygen and the hyaluronic acid were administered in a contemporary way. The treatment began with the delivery of the solution through an airbrush for vulvar therapy. 3ml hyaluronic acid was delivered with oxygen at a flow of 2 L/ minute for 15 minutes; for 5 minutes 12ml of low molecular weight sodium hyaluronate and high purity oxygen, at a concentration of 0.2%, was administered through a cannula specifically designed for vaginal therapy. At visit 0, an accurate collection of patient history and a complete clinical examination was performed to evaluate eligibility criteria.

Results

The analysis of the comparisons between the first and last treatment have showed that each subsequent treatment determined a significant increase in all parameters, especially for VHI hydration index, VAS fluidity index and FSFI lubrication index, however all the patients reported a recovery of their sexuality at the end of the five treatment sessions.

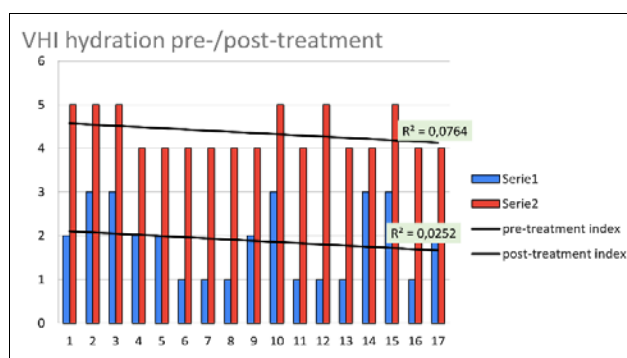


Fig 3: ↓ Diagram - VHI hydration pre-/post-treatment

VHI: The intensity of the three symptoms reported according to the 5-point VHI scale showed an average increase of the elasticity index from 1.8 to 4.2 - $p < 0.00001$, an average increase of the secretion index 1.7 to 4.1 and average increase of the hydration index from 1.9 to 4.9 (Fig 3). The analysis of the comparisons between the start and the end of the therapy have shown that each subsequent treatment determined a significant increase in all parameters, including the vulvovaginal elasticity and the vulvovaginal wall epithelium hydration.



Fig 4: ↓ Photos of the patient's genitals pre-/post treatment



Fig 5: ↓ Photos of the patient's genitals pre-/post treatment



Fig 6: ↓ Photos of the patient's genitals during the 5 weekly therapy session

Below are shown as an example the photographic images of three of the patients in which it is possible to appreciate the differences between the appearance of the genitals before and after the treatment (Figures 4, 5) and also the five stages of the treatment (Fig 6): All initially show an absent or poor overall elasticity and an absent hydration with the mucosa vulvar predominantly white and ulcerated, while at the end good or excellent elasticity with modest/normal hydration with many islands of pink mucosa.

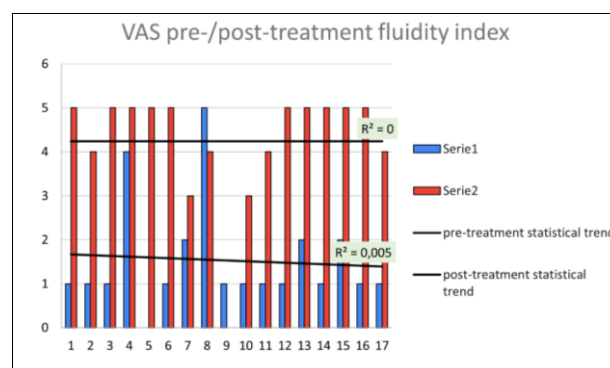


Fig 7: ↓ Diagram - VAS fluidity index pre-/post-treatment

VAS: The results shown a significant improvement of all the mean scores analysed: Well-being such as absence of dyspareunia, vaginal dryness, vulvar and/or vaginal itching, vaginal burning. The intensity of the symptoms reported according to the 6-point VAS scale showed an average increase of the well-being index from 2.1 to 4.7, an average increase of the burning index 1.9 to 5.3, an average increase of the fluidity index from 1.6 to 4.5 (Fig 7) and an average increase of the itching index 2.2 to 5.4.

FSFI: The results shown a significative improvement of all the mean scores analysed: Increasing sexual desire, excitement, satisfaction, raised vaginal lubrication and ability to join the orgasm and decreased vulvar or vaginal pain.

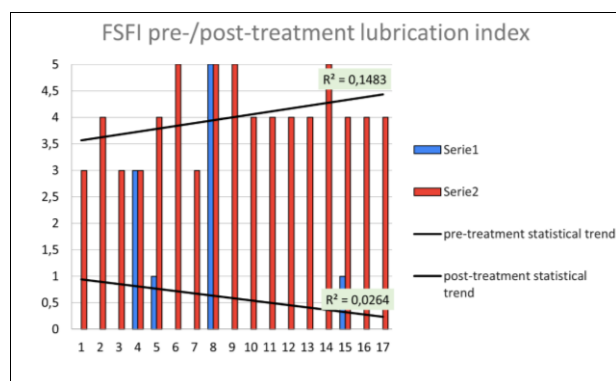


Fig 8: ↓ Diagram - FSFI lubrication index pre-/post-treatment.

The intensity of the symptoms reported according to the 6-point FSFI scale showed the following average improvements: Sexual desire index from 1.5 to 3.5, excitement index from 1.3 to 3.7, satisfaction index from 1.2 to 3.8, vaginal lubrication index from 0.6 to 4 (Fig 8), ability to join the orgasm index from 1.8 to 3.6 and decreased vulvar or vaginal pain index from 4.1 to 1.

Discussion

In this pilot study we found that the combined topical oxygen therapy with hyaluronic acid by VNOD is a valid and totally painless treatment for vulvar lichen scleroatrophicus, improving symptomatology and sexual wellbeing.

Lichen sclerosus is a chronic disorder of the skin and mucosal surfaces, and is most commonly seen on the female genital skin. It also occurs in other areas of the body. Any age group may be affected, although it is seen more often in elderly women [1, 2, 3, 4, 7].

The exact cause of lichen sclerosus is unknown. There have been reports of family members with lichen sclerosus; thus, it may have a genetic link [8]. There is also the possibility of an autoimmune connection [9].

Currently ultra-potent topical corticosteroids (TCS) are the medical treatment of choice: Clobetasol propionate and mometasone furoate [10, 11, 12].

The risk of developing squamous cell carcinoma of the vulva approaches 5% in women with vulvar lichen sclerosus, and therefore close surveillance by the healthcare provider and patient is needed [14, 15, 16].

Long-term TCS has a protective effect from malignant evolution: Carcinoma developed only in nontreated or irregularly treated vulvar lichen sclerosus lesions [17]. It could be interesting to evaluate the efficacy of simultaneous and local administration of high and hyaluronic acid on the malignant evolution of the vulvar lichen in vulvar carcinoma. A long-term monitoring is necessary to set the timing for another session with VNOD therapy and to monitor the evolution of patients' symptomatology and the state of vaginal elasticity and hydration.

Oxygen therapy determines the increase of the reparative processes of the tissues and increases the synthesis of collagen allowing a normal hydroxylation of this protein [18].

In fact, at oxygen tensions lower than normal, the collagen is not correctly synthesised, delaying the healing of the wounds. Furthermore, oxygen induces a neo-angiogenic stimulation through the release of VEGF [19]. This function is essential for the restoration of the microcirculation in compromised vascular tissues, re-establishing a vascular flow in the hypoxic areas [20]. Hyaluronic acid is a natural polysaccharide and that can bind a large quantity of water molecules contributing to the maintenance of water balance, proper hydration and structure of skin and mucus membranes [21].

VNOD therapy increases the elasticity of the tissues and improves the VVA (Vulvo-Vaginal Atrophy) and the genitourinary symptoms. The therapy has been well tolerated without side effects [22, 23, 24].

Our findings must be confirmed in a well-designed randomised controlled trial.

Conclusions

Combined topical oxygen therapy with hyaluronic acid is a fast, repeatable, and totally painless treatment with no side effects for the patients. It proved to be a valid method for healing vulvar lesions and improving lichen symptoms with excellent compliance by patients.

The results of the study demonstrate that this therapy increases the vulvovaginal mucosa hydration, fluidity and lubrication improving women's sexual health and wellbeing also after 6 weeks from the last treatment.

Conflicts of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Authors' contributions

Raffaella De Simone, Maria Di Pietro and Rosa Elena Frulio have given substantial contributions to the conception and the design of the manuscript and acquisition of the data, Eleonora Russo to analysis and interpretation of the data. All authors have participated to drafting the manuscript, author Gianpaolo Mainini, Flavio Garoia and Claudio Santangelo revised it critically. All authors read and approved the final version of the manuscript.

All authors contributed equally to the manuscript and read and approved the final version of the manuscript.

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Topical high concentration oxygen with hyaluronic acid: A safe and effective treatment for vaginal atrophy and sexual function improvement

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Abstract

Background: Genitourinary syndrome of menopause (GSM) is a condition affecting many postmenopausal women. Among the available treatments, the vaginal natural oxygenation device (VNOD) is a new promising option.

Aim: To evaluate the safety and the effectiveness of vaginal high concentration (HC) oxygen (O₂) and hyaluronic acid (HA) in postmenopausal women with vaginal atrophy (VA).

Methods: Study 1: Ten subjects received HA and O₂ at a concentration of 21% (sham group) and ten at 95% (treatment group). All subjects completed five sessions of VNOD. Evaluations and questionnaires were completed at baseline, before each session and at one-month follow-up: Visual Analogue Scale, Vaginal Health Index (VHI), Vaginal Maturation Value (VMV), Menopause Quality Of Life (MeNQoL), Female sexual function index (FSFI) and Female sexual distress scale (FSDS). Study 2: 52 postmenopausal women with VA completed the same protocol of study 1 with HC O₂ and HA.

Results: Treatment was well-tolerated. In study 1, in the treatment group we recorded significant improvements in VHI score (*p*-value 0.001), VMV (*p*-value 0.005) and in the psychosocial, physical and sexual domains of the MeNQoL compared to sham group. In study 2, all women experienced a significant improvement in VHI (*p*-value <0.001), FSFI total score (*p*-value 0.007), desire (*p*-value 0.004), lubrication (*p*-value 0.006), satisfaction (*p*-value 0.042), pain (*p*-value 0.001) domains, FSDS score (*p*-value 0.003) and MeNQoL sexual domain (*p*-value <0.001).

Conclusion: The preliminary results of this study suggest that VNOD is a safe and effective treatment for VA in postmenopausal women, and improves sexual function.

Keywords

VNOD, Vaginal atrophy, Female sexual function, Vaginal health index, Hyaluronic acid, oxygen

Introduction

The Genitourinary Syndrome of Menopause (GSM) is a chronic condition which affects over 50% of postmenopausal women according to different study samples,^{1–3} resulting from ageing and the physiological changes associated with menopause, primarily the decline in estrogen levels in the vaginal epithelium, urethra, and bladder. The estrogen deprivation leads to thinning and atrophy of the vaginal tissues, decreased vaginal lubrication, and alterations in the pH of the vaginal environment, predisposing women to a constellation of symptoms such as vaginal dryness, itching, burning, dyspareunia, and dysuria. Despite the high prevalence, the GSM is still under-reported, under-diagnosed and under-treated.^{4,5}

International guidelines suggest lubricants and moisturizers as a first line treatment for women suffering GSM, being free from significant contraindications and side effects.^{6–8} In women

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without contraindications, the second line therapy is represented by hormonal therapy, which can be administered locally or, especially in case of coexisting menopausal symptoms, systemically. Local estrogen treatment (LET), dehydroepiandrosterone (DHEA) or testosterone formulations directly target the vaginal tissues while minimizing systemic absorption, reducing the risk of adverse effects. All have shown successful improvement of vaginal health and sexual function in postmenopausal women.^{6,7}

The Selective Estrogen Receptor Modulator (SERM) Ospemifene, is another non-hormonal alternative which has proven to be highly effective and has been approved for use also in women with breast cancer who have completed adjuvant treatments.^{9–11}

Other non-hormonal treatments that have been demonstrated to be effective in women who cannot (or do not want) use hormones include energy-based devices such as laser therapy.^{8,12–15} Laser therapy is not usually suggested in all women as a first line because it is not always well-tolerated,¹⁶ has quite high costs and its safety is under scrutiny.¹⁷

A recently developed and promising option is a vaginal natural oxygenation device (VNOD) which allows concomitant administration of hyaluronic acid (HA) and topical high concentration (HC) oxygen.^{18–20} Oxygen (O₂) has a well-known effect on epithelialization, angiogenesis, collagen formation and granulation.²¹ Many authors reported the role of O₂ therapy in enhancing skin rejuvenation and wound healing, highlighting its potential to promote tissue repair.²² The penetration of O₂ delivered at high concentration with the VNOD device is facilitated by the histology of the vagina mucosa which lacks stratum corneum and hydro-lipidic film. The addition of a liquid-based solution of hyaluronic acid (HA) may further enhance the passage of O₂ at the epithelial level.^{23,24}

HA is a component of the extracellular matrix and is involved in several physiological processes, including tissue homeostasis and regeneration, wound healing, inflammation, cell migration and proliferation, and embryonic development. HA plays a relevant role also in the urogenital system as, under physiological conditions, it helps to maintain water balance and tissue integrity.²⁵

The HA low molecular weight form is also able to penetrate into the deeper layers of the vaginal epithelium and facilitate the diffusion of essential nutrients, including O₂, glucose, and vitamins.^{26,27}

Preliminary data suggest that the synergic use of O₂ and low molecular weight HA may indeed alleviate symptoms of the vaginal atrophy (VA) included in GSM.^{18–20}

In this study, we aimed to extend previous preliminary data on the safety and effectiveness of HC O₂ administered through a VNOD in postmenopausal women with VA.

Methods

Our study consists of two parts: the first part (study 1) was a longitudinal sham-controlled trial, consisting of two samples, the sham group and the treatment group; the second part (study 2) is a prospective longitudinal study, performed to expand the treatment group enrolled in study 1.

The study 1 period ranged from June 2021 to December 2021. The study 2 was conducted from June 2021 to November 2023.

The protocol of the studies was approved by the Human Subject Committee Area Vasta Emilia Centro (CE-AVEC) of Emilia-Romagna and written informed consent was obtained from all participants in accordance with the 1975 Helsinki Declaration.

Subjects

All participants were recruited at the Outpatient Clinic of Gynecology and Physiopathology of Human Reproduction Unit, IRCCS Azienda Ospedaliero Universitaria of Bologna, Sant'Orsola University Hospital.

Menopausal state was defined as absence of menstruation for at least 12 months.²⁸

The inclusion criteria, which were the same for both studies, were: no treatment for VA in the previous 6 months, menopausal status as described, and Vaginal Health Index (VHI) score <15, which is a cut-off used in literature to define the presence of VA.²⁹

Exclusion criteria, which were the same for both studies, included: concomitant clinically important medical disease; undiagnosed vaginal bleeding; liver or kidney disorder; thromboembolic disorders; cerebrovascular accident, stroke, or transient ischaemic attack; myocardial infarction or ischaemic heart disease; endocrine disease. Women who were using or had used oral products containing hormones or selective estrogen receptor modulators (SERMs) within the previous 12 weeks were also excluded.

Study design and objectives

The design of both part of our study (study 1 and study 2) included five VNOD sessions performed one week apart. At the first visit, details of medical history, previous therapies for VA, sociodemographic characteristics, personal clinical history, the presence of symptoms of vaginal atrophy and full gynaecological examination (GE) were collected, inclusion and exclusion criteria were checked.

Once subjects were considered eligible for the study, they signed the consent form and were scheduled for the VNOD sessions. Before each VNOD session (T0, T1, T2, T3, T4) and one month after the last VNOD session (T5) all women underwent GE to assess the presence of signs of VA and to calculate the scores of VHI (Vaginal Health Index).

Further symptoms of GSM were not assessed as not related to the aim of this study.

The five VNOD sessions were performed at 7-day intervals. Prior to each session, all women completed the Female Sexual Function Index (FSFI), the Female Sexual Distress Scale (FSDS) questionnaire and MENopause-specific Quality of Life (MeNQoL) questionnaire.

At the end of each session visual analogue scale (VAS) scores for discomfort during VNOD session were checked [scale from zero (none) to ten (maximum discomfort)]. A vaginal smear was also performed before the first session and at the follow-up visit for evaluation of vaginal maturation value (VMV)²¹ in the first 20 participants enrolled (Study 1).

Primary objectives: monitor the safety parameters and pain levels during the procedure and improvement of VA assessed with the VHI and, only for study 1, VMV. Secondary objectives: changes in sexual function assessed by subjective questionnaires (FSFI, FSDS and MeNQoL).

Evaluation of study's parameters

The VHI is a tool that, by evaluating five parameters (elasticity, secretions, pH, epithelial mucosa and hydration) enables the determination of an overall score which defines the degree of vaginal atrophy, assigning to each parameter a score from one to five. The total score can range from five to twenty-five and a value below 15 is indicative of VA.²⁹

The sexual function and distress caused by sexual dysfunctions were evaluated through the FSFI and FSDS questionnaires^{30,31} which were administered at each session and one month after last session (t5). A FSFI cut-off score of 26.5, was used to differentiate between women with and without sexual dysfunction³² and a cut-off score of 15 for the FSDS.³³

The FSFI questionnaire explores female sexual function by evaluating six domains of sexuality: desire, arousal, lubrication, orgasm, sexual satisfaction and pain. The questionnaire consists of 19 items, each of which can be assigned a score ranging from zero (lack of sexual activity) or one (very dissatisfied with one's sex life) to five (very satisfied with one's sex life). The maximum score obtainable is 36, while the minimum is two.

Quality of life of the enrolled women was assessed by completing MENQOL questionnaire, which was administered at each VNOD session and one month after last session (t5).

The MENQOL consists of 29 items related to four areas: vasomotor (items 1–3), psychosocial (items 4–10), physical (items 11–26) and sexual (items 27–29). Symptoms may be present or absent; if present the subject evaluates the related discomfort on a scale from zero (no discomfort) to six (substantial). Each MENQOL domain has a separate score ranging from one to eight.^{34,35}

VAS scores for discomfort during VNOD treatment were checked at each session, asking the subjects to evaluate using a zero (symptoms absent) to ten (maximum discomfort) scale.³⁶

We also recorded any possible adverse effect, defined as vaginal burning, bleeding, abnormal genital discharges, low urinary tract infection, pain with a VAS > 4, genital infections.

Vnod device

The VNOD device (Caress Flow Bologna, Italy ®)³⁷ was used: a compressor unit with a generator able to deliver up to 3 L/minute of 95% pure O₂. The purified O₂ is obtained using a compressed air generator, equipped with zeolite molecular sieves. It exploits the principle of the different absorption of gas molecules by different surfaces, allowing O₂ to pass through and retaining other gases present in the air, such as nitrogen, argon, helium and hydrogen. The dispenser is a vaginal cannula, connected to the machine body. It is equipped with outlet holes for the delivery of O₂ and HA, which is inserted through a special injection valve, located in the upper part of the cannula.

A special disposable cannula is inserted at the opening of the vagina and O₂ at the defined concentration and HA solution are delivered. [Figures 1 and 2.](#)

VNOD is a medical device approved by the EU according to the Directive 93/42/EEC on medical devices.³⁸ ([Supplemental materials S0](#)).

The use of the device is extremely simple. The treatment involves the following steps:

1. Turn on the device using the ON/OFF button.
2. Insert the cannula into the vaginal canal.
3. Perform 10 min of pure oxygen delivery.
4. Continue for an additional 5 min with oxygen delivery combined with the HA solution.
5. Remove the cannula and dispose of it as household waste.

As reported by the device instructions and protocol for GSM, the complete treatment cycle included five weekly sessions lasting 15 min. The first 10-min, O₂ was dispensed alone followed by the addition of HA delivered over the next 5 min. The O₂ was administered at a concentration of 21% (air concentration) in the sham group of study 1 and of 95% (HC) in the treatment group and in study 2.

A gel solution of low molecular weight sodium hyaluronate, at a concentration of 0.2% in pre-filled applicators (1020 mOsm/Kg), was administered during the last 5 min of treatment in both studies (Hyalogyn® vaginal gel by Fidia Farmaceutici S.p.A.). This HA is commercially available, has a CE marking and was approved by the United States Food and Drug Administration in 2010.³⁹ Hyalogyn® acts as a moisturizer and lubricant because of the strong hydrophilic characteristics, that allows retaining water molecules forming



Figure 1. VNOD components kit (Caress Flow®).

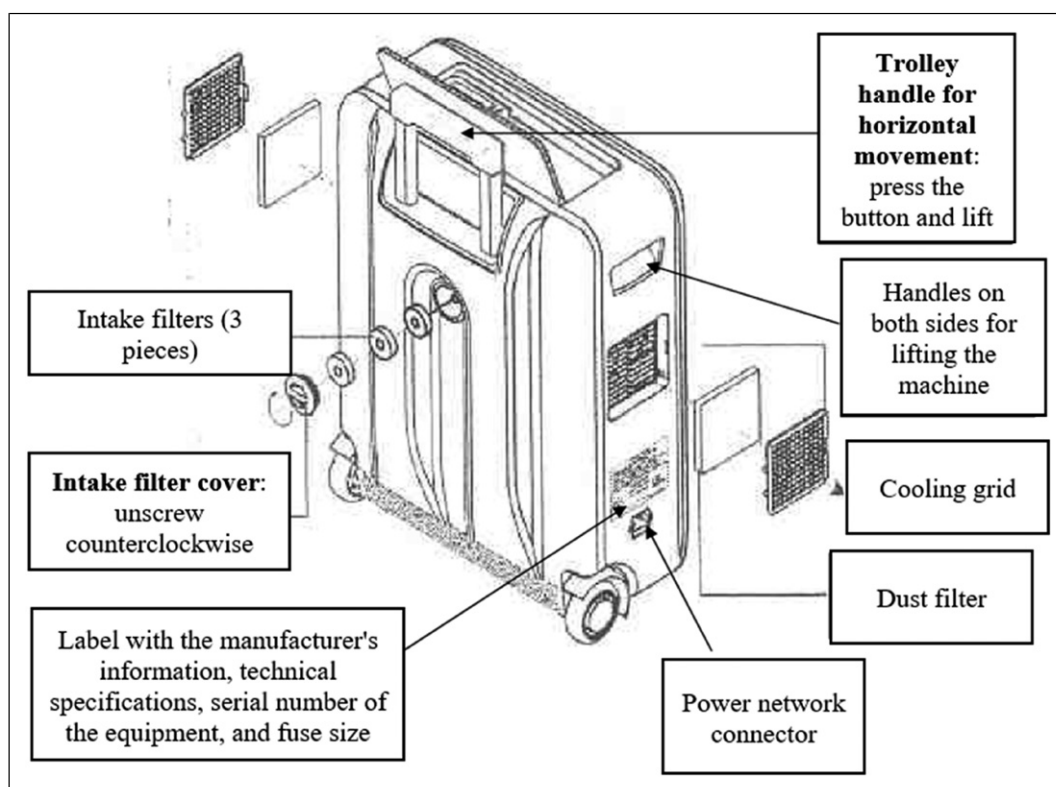


Figure 2. VNOD back and maintenance.

a hydrated matrix which determines a release of a constant aliquot of principle component and assures a notable and prolonged hydrating effect at the vaginal tissue level. It has mucoadhesive properties and adheres to the vaginal mucosa, exerting a prolonged hydrating action on this tissue and favouring the natural healing of microlesions.²⁵

In the first part of this study (study 1), by mistake, the first 10 women were treated with O₂ at air concentration (later, we decided to consider them the sham group). Neither the participants nor the physicians were aware of the incorrect set-up of the machine. The settings were corrected and the session with HC O₂ was performed in the second group of treated women (treatment group).

During study 2, all women underwent HC O₂ treatment with the same set-up.

Statistics

Descriptive statistics for continuous parameters were reported as mean and standard deviation (SD) or median and interquartile ranges (IQ 25th – 75th) upon verification of the normality of the distribution through the Shapiro-Wilk test. Categorical variables were expressed as absolute frequencies and percentages. Pairwise comparisons of independent observation were performed by the Student t test using the Welch correction where appropriate. Not normally distributed data were compared by the Mann Whitney U test. The Chi square test was used to compare proportions between the sham and treatment groups. The sample size was determined based upon data from a previous study evaluating the impact of VNOD device on several symptoms related to VVA [7]. A sample of 10 subject in the treatment group of study 1 was calculated after the correction of the VNOD set-up used for the first 10 women, to provide 90% power to detect a statistically significant improvement of the VHI score (two-sided $\alpha = 0.05$). Changes of the recorded scores from baseline to the last visit were evaluated using the Friedman test followed by the Wilcoxon rank test for pairwise comparisons). Based on these preliminary data, a sample of minimum 47 subjects in study 2 was calculated to provide 90% power to detect a statistically significant improvement of the VHI score (two-sided $\alpha = 0.05$). We also hypothesized a 10% exclusion rate and therefore decided to include 52 women in study 2, including the 10 women enrolled in the treatment group of study 1.

All tests were two-sided and p -values <0.05 were considered statistically significant. Data analysis was performed using the Statistical Package for Social Sciences (SPSS) version 25 (IBM corp).

Results

Study 1: Sham-controlled trial

Twenty subjects were enrolled progressively between June 2021 and December 2021. Ten subjects received treatment

with HA and 21% O₂ concentration (air O₂ concentration) (sham group). At the end of the treatment of this group, the flow of O₂ adjusted to deliver 95% O₂ concentration (HC O₂) (treatment group). The ten subjects in the treatment group received HA and 95% O₂ concentration.

Study 1: Subjects

In both groups, all subjects completed the full treatment cycle of five weekly sessions.

Sociodemographic and clinical characteristics of women enrolled are reported in Table 1. The two groups were comparable in terms of demographic, anthropometric parameters, and time since the onset of amenorrhea. Moreover, 1 (10%) subject in the sham group and 3 (25%) subjects in the treatment group had never received any other treatment for VA. No differences were detected concerning cardiometabolic risk factors (Table 1).

At the end of the treatment, no adverse events related to the procedure were recorded. No subject dropped out of the study because of pain or discomfort at sessions. Baseline and end-of-treatment VAS scores for pain/discomfort during the procedure were between zero and one in all subjects of both groups.

Study 1: Impact of VNOD on VA

Baseline VHI scores were 11 (IQ 11–13) (median and interquartile range) and 10 (IQ 9–11), in the sham and treatment group, respectively ($p = .075$). All women in both groups had VA at baseline (VHI < 15). Figure 3 shows the VHI data recorded from baseline to the last visit in the sham and treatment group. Only 3 out of 10 subjects in the sham group improved their VHI score above 15 (Figure 3, Panel A), whereas a VHI score above 15 was observed in 7 out of 10 subjects in the treatment group. Overall, no significant changes of the median VHI score were observed in the sham group (Figure 3, panel B) whereas a progressive increase of the score was observed in the treatment group (Figure 3, panel D) in which the VHI score significantly improved compared to baseline from T3 to T5 (Figure 3, panel D). In the treatment group, significant improvements were observed in all domains of the VHI score (elasticity, secretion, mucosae, hydration) except for vaginal pH ($p = .288$) which remained stable (Table 2). In particular, the score almost doubled for the ‘secretion’ and ‘mucosae’ domains between the baseline and end-of-treatment evaluations. No significant changes in any of the parameters of the VHI were observed in the sham group (Table 2).

Similar results emerged from the analysis of vaginal cytology. The VMV significantly improved in the treatment group whereas no changes were detected in the sham group (Figure 4).

Furthermore, we did not report any adverse effect.

Table 1. Baseline characteristics of women enrolled in the sham and treatment groups of Study I. Data is reported as mean ± standard deviation, median and 25th – 75th percentiles or absolute frequencies and percentage.

	Sham group (n = 10)	Treatment group (n = 10)	p value
Age (years)	54 ± 6	55 ± 9	0.350
Age at menopause (years)	49 ± 6	49 ± 5	0.500
Months since LMP	48 (36 – 72)	48 (24 – 132)	0.912
BMI (Kg/m ²)	23 ± 2	24 ± 4	0.341
VA treatment naive	1 (10%)	3 (30%)	0.582
Cigarette smoker	0 (0%)	3 (25%)	0.211
Hypertension	1 (10%)	4 (40%)	0.303
Dyslipidemia	1 (10%)	6 (60%)	0.057
Type 2 diabetes	0 (0%)	2 (20%)	0.474
Breast cancer	2 (20%)	3 (30%)	1.000

Abbreviations: BMI, body mass index; VA, vaginal atrophy; LMP last menstrual period.

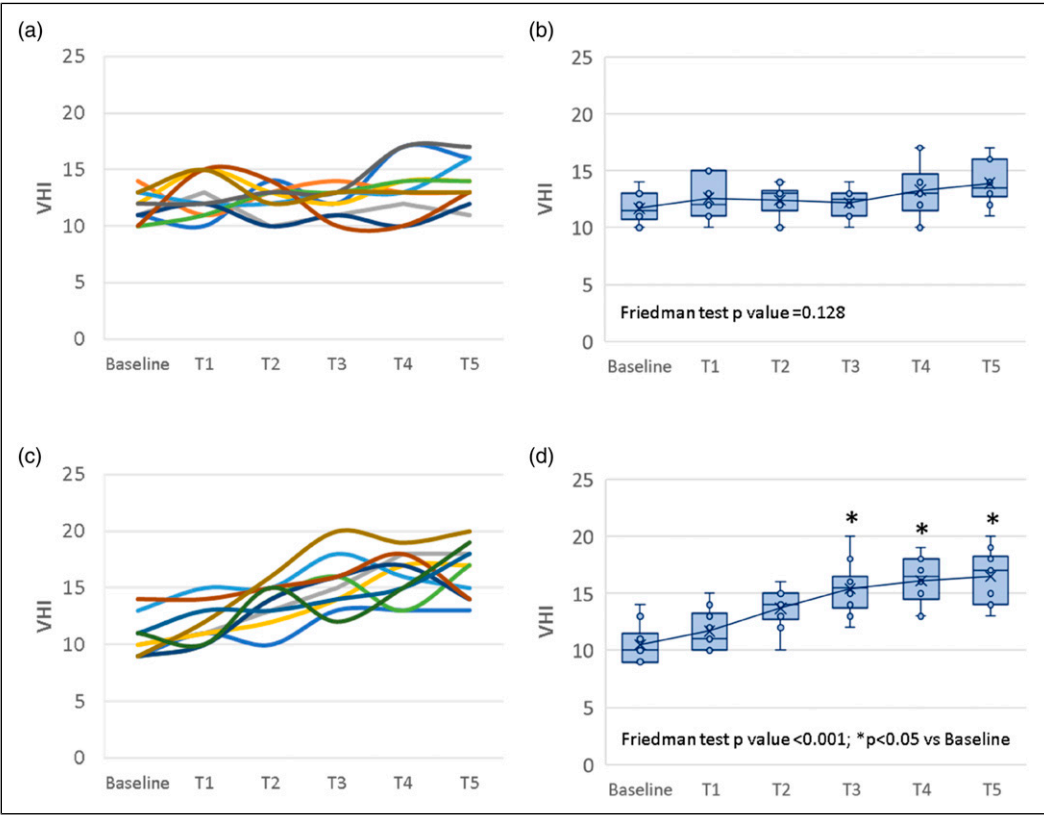


Figure 3. Individual data and box plot of vaginal health index (VHI) score recorded at each visit in women from the sham (Panels A and B) and treatment groups (Panels C and D) of Study I.

Study I: Impact of VNOD on sexual function

Five ($n = 5$) and ten ($n = 10$) women were sexually active in the sham and treatment groups, respectively. [Figure S1](#) shows the FSFI scores in the sexually active women in the two groups. Overall, no significant improvement in the global FSFI score was observed in the sham group

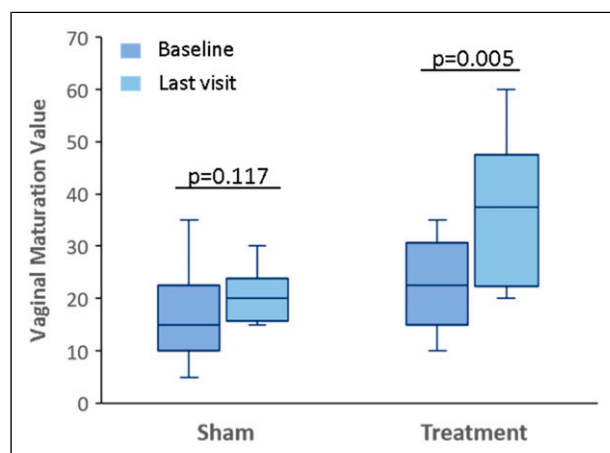
([Supplemental material, Figure S1, panel A](#)) nor in the treatment group ([Supplemental material, Figure S1, panel B](#)).

Analyzing the individual domains of the FSFI score, significant improvements in the desire and pain were detected in the treatment group while no significant changes were observed in the sham group ([Table 3](#)).

Table 2. Median and 25th – 75th interquartile range of individual domains of the VHI scores from baseline up to the last visit in women from the sham and treatment groups of Study 1.

	T0		T5		Δ (T5-T0)
	Median	IQ	Median	IQ	p value
Elasticity					
Sham	2.00	2.00–2.00	2.00	2.00–3.00	0.247
Treatment	2.00	2.00–2.00	4.00	3.00 – 4.00	<0.001
Secretion					
Sham	2.00	1.00–2.00	2.00	2.00–3.00	0.066
Treatment	2.00	1.00–2.00	3.00	3.00 – 3.00	<0.001
pH					
Sham	2.00	2.00–2.00	3.00	2.00–3.00	0.138
Treatment	2.00	2.00–3.00	2.00	1.00 – 4.00	0.288
Mucosae					
Sham	4.00	3.00–4.00	4.00	4.00 – 4.00	0.647
Treatment	2.00	2.00–3.00	4.00	4.00 – 4.00	<0.001
Hydration					
Sham	2.00	2.00–3.00	3.00	2.00–3.00	0.082
Treatment	2.00	2.00–3.00	4.00	3.00–4.00	<0.001

Abbreviations: IQ, interquartile range; VHI, vaginal health index.

**Figure 4.** Mean $\pm$ standard deviation (SD) of vaginal maturation value (VMV) at baseline and at the last visit in women from the sham and treatment groups of Study 1.

Analysis of the FSDS score revealed no statistically significant improvement in either group (sham group $n = 5$, $p = .486$; treatment group $n = 10$, $p = .098$) although there seemed to be a trend towards a decrease of FSDS score in the treatment group ([Supplemental material, Figure S1](#), panels C and D).

MeNQoL questionnaire showed no significant changes in vasomotor, psychosocial, physical and sexual symptom domains in the sham group ([Supplemental material, Table S1](#)). No improvement in the vasomotor symptoms' domain was also observed in the treatment group, whereas a significant improvement from baseline

to the last visit were found in the psychosocial, physical and sexual symptom domains in the treatment group ([Supplemental material, Table S1](#)).

Study 2: Expansion of the sample in treatment

Given the promising results of Study 1 on VNOD, we decided to expand the treatment group of study 1 by recruiting additional subjects and conducting a second statistical analysis on four parameters (VHI, FSFI, FSDS, MeNQoL sexual domain). In this second part of the study, a control group was not used; a comparison of the parameters was made between baseline (before first session) (T0) and the follow-up visit (T5).

Study 2: Subjects

Between June 2021 and November 2023 therefore we enrolled 52 postmenopausal women with VA, who followed the same treatment protocol as in Study 1. In this sample we also included the 10 women of the 'treatment group' of study 1. Sociodemographic and clinical characteristics of the sample of study 2 are reported in [Table 4](#).

Only ten subjects had never received any other treatment for VA.

All newly enrolled subjects completed the 5-session cycle of VNOD, and no treatment-related adverse effects were observed. The VAS score values remained consistently low (0–1) in all women at each session, demonstrating the high tolerability of the treatment. None of the examined participants discontinued the treatment prematurely due to

Table 3. Median and 25th – 75th interquartile range of FSFI individual domains in sexually active women of the sham ($n = 5$) and treatment ($n = 10$) groups of Study 1.

	T0		T5		Δ (T5-T0)
	Median	IQ	Median	IQ	p value
Desire					
Sham	2.4	1.8 – 3.6	2.4	1.8 – 3.6	0.471
Treatment	3.6	2.4 – 3.6	3.6	3.6 – 3.6	0.015
Arousal					
Sham	2.1	2.1 – 2.7	2.4	1.8 – 3.9	0.716
Treatment	4.5	3.9 – 5.4	4.2	3.6 – 5.4	0.546
Lubrication					
Sham	1.8	2.1 – 2.7	3.0	1.8 – 3.6	0.395
Treatment	3.6	3.3 – 4.8	4.8	3.6 – 5.1	0.111
Orgasm					
Sham	2.4	1.2 – 3.6	2.8	1.2 – 3.2	0.898
Treatment	4.8	3.6 – 4.8	4.4	3.6 – 5.2	0.778
Satisfaction					
Sham	2.4	2.0 – 4.4	2.4	2.4 – 4	0.850
Treatment	4.4	3.6 – 4.8	4.8	4.0 – 4.8	0.509
Pain					
Sham	1.2	0.0–1.2	2.0	1.6 – 2.8	0.641
Treatment	3.2	1.6 – 3.6	4.4	3.2 – 4.4	0.023
FSFI total score					
Sham	15.60	2.30–18.10	17.60	5.15–23.75	0.670
Treatment	14.80	3.75–18.25	15.50	7.45–21.05	0.350
FSDS score					
Sham	9.00	6.50–18.00	8.00	0.00–12.00	0.486
Treatment	16.00	9.50–30.50	2.00	0.00–8.00	0.098

Abbreviations: IQ, interquartile range; FSFI, female sexual function index; FSDS, female sexual distress score.

Table 4. Baseline characteristics of women enrolled in the Study 2 ($n = 52$). Data is reported as mean $\pm$ standard deviation, median and 25th – 75th interquartile range or absolute frequencies and percentage.

	Treatment group ($n = 52$)
Age (years)	56.9 $\pm$ 8.0
Age at menopause (years)	47.3 $\pm$ 5.3
Months since LMP	48 (24 – 132)
BMI (Kg/m ²)	22.5 $\pm$ 3.0
VA treatment naïve	10 (19.2%)
Cigarette smoker	3 (5.8%)
Hypertension	5 (9.6%)
Dyslipidemia	14 (26.9%)
Type 2 diabetes	2 (3.9%)
Breast cancer	18 (34.6%)

Abbreviations: BMI, body mass index; VA, vaginal atrophy. LMP last menstrual period.

discomfort, pain, or other issues. These findings are consistent with those obtained in Study 1, supporting the idea that VNOD is a well-tolerated treatment without side effects.

Study 2: Impact of VNOD on VA

In the 52 women enrolled in the study 2, VNOD showed a marked and statistically significant improvement in VHI, with a median score from baseline (T0) of 10.00 (IQ 9.00–12.75) to a median value of 16.50 (IQ 13.00–18.00) at the follow-up visit (T5) with a p -value T0–T5 <0.001 (Table 5). Thirty out of 52 (57.7%) women improved their VHI score above 15.

We observed a progressive and significant increase in all domains of the VHI score (elasticity, secretion, mucosae, pH, hydration) and in the overall VHI score from T0 to T5, but the improvement was already significant at T2 compared to baseline for all the parameters (each p -value <0.001 at T2) except for vaginal pH, in which a significant increase was reported at T3 and T4 (respectively, $p = .049$ and $p = .001$). (Table 5).

We did not report any adverse effect.

Study 2: Impact of VNOD on sexual function

VNOD has proven to be effective on the FSFI as well, promoting an overall score increase from T0 to T5 with a p -value of 0.007 (Table 6). The improvement was

Table 5. Median and 25th – 75th interquartile range of VHI and its domains at baseline (t0) and one month after last VNOD application (t5) in Study 2 (N = 52).

	T0		T5		Δ (T5-T0)
	Median	IQ	Median	IQ	p value
Elasticity					
Treatment	2.00	2.00–3.00	4.00	3.00 – 4.00	<0.001
Secretion					
Treatment	2.00	1.00–2.00	3.00	2.75 – 4.00	<0.001
pH					
Treatment	2.00	1.00–3.00	2.50	1.75 – 4.00	0.012
Mucosae					
Treatment	3.00	2.00–3.00	4.00	3.00 – 4.00	<0.001
Hydration					
Treatment	2.00	1.00–3.00	3.00	2.75 – 4.00	<0.001
VHI total score					
Treatment	10.00	9.00–12.75	16.50	13.00–18.00	<0.001

Abbreviations: IQ, interquartile range; VHI, Vaginal Health Index.

Table 6. Median and 25th – 75th interquartile range of FSFI individual domains, FSFI total score and FSDS in sexually active women at baseline (t0) and one month after last VNOD application (t5) in Study 2.

	N = 52	T0		T5		Δ (T5-T0)
		Median	IQ	Median	IQ	p-value
Desire						
Treatment		2.40	1.20–3.00	2.40	1.80–3.60	0.004
Arousal						
Treatment		2.40	0.15–4.20	2.85	0.00–3.90	0.345
Lubrication						
Treatment		2.10	0.00–3.30	3.00	0.00–4.28	0.006
Orgasm						
Treatment		3.20	0.00–4.70	3.20	0.00–4.40	0.286
Satisfaction						
Treatment		3.20	0.80–4.70	4.00	0.80–4.80	0.042
Pain						
Treatment		1.60	0.00–3.10	2.80	0.00–4.40	0.001
FSFI total score						
Treatment		14.90	3.75–22.98	19.60	3.05–23.53	0.007
FSDS score						
Treatment		24.00	14.25–39.75	18.50	4.00–36.00	0.003

Abbreviations: IQ, interquartile range; FSFI, Female Sexual Function Index; FSDS, Female Sexual Distress Score.

significant only at T5 compared to baseline, while we observed a precocious improvement in the objective vaginal indexes (significant at T2–T3).

In conclusion, unlike what was described in Study 1, with the expansion of the sample in the second study, we also obtained a statistically significant FSFI T0-T5 variation, but without reaching the cut-off of normality. Only 17 subjects (32.7%) reached normal sexual function scores. A statistically significant improvement from T0 to T5 in the desire (p -value 0.004), lubrication (p -value 0.006),

satisfaction (0.042), and pain (p -value 0.001) domains were also detected (Table 6).

A significant improvement (p -value 0.001) was observed from T0 to T5 of the FSDS score, with a median value of 24.00 (IQ 14.25–39.75) at T0 to a median value of 18.50 (IQ 4.00–36.00) at T5, still not reaching the cut-off of 15 (Table 6). Also in this case, the improvement was significant only at T5 compared to baseline.

MeNQoL questionnaire showed no significant changes in vasomotor, psychosocial, and physical symptoms. A

statistically significant improvement in the MeNQoL sexual domain was observed (p -value <0.001) between T0 and T5 (Supplemental material, Table S1).

Discussion

In this study, we evaluated the safety and efficacy of topical O₂ administered at high concentration (treatment group) or air concentration (sham group) together with HA in improving VA in postmenopausal women (study 1). In the treatment group, VHI, pain and desire domains of the FSFI questionnaire and the psychological, physical, and sexual domains of MeNQoL significantly improved, while no changes were recorded in the sham group. VMV also significantly improved in the treatment group whereas it did not vary in the sham group. Given these preliminary promising results, we expanded the treatment group of study 1 to 52 postmenopausal women with VA (study 2). In this second part of the study, statistically significant improvements were recorded in VHI, FSFI total score, FSDS, MeNQoL sexual domain score, and in the desire, lubrication, satisfaction and pain domains of FSFI at the end of treatment in the women treated with VNOD compared to baseline. None of the subjects reported any adverse events.

The VNOD is a recently employed device in the gynaecological field. Currently, there is limited published scientific evidence regarding its validity for the treatment of VA, but the available data are highly promising, particularly in terms of reduced invasiveness, excellent tolerance, and absence of contraindications.^{18–20}

Therefore, the aim of this study was to gain more data on the safety and efficacy of this treatment in postmenopausal women, with the comparison to a sham group and a larger treatment group.

In the first part of this study (study 1), the first 10 women were treated with O₂ at air concentration (sham group) and no improvement in any subjective and objective parameter was detected. Neither the participants nor the physicians were aware of the different set-up of the machine. The settings were changed and an improvement of many parameters of VA was achieved in the second group of 10 women (treatment group). All parameters of VHI, except for pH, showed a statistically significant improvement in the treatment group achieving significance already at the fourth session (T3). It should be noted that 4 subjects of the treatment group of study 1 did not reach a VHI score higher than 15 and remained mildly atrophic at the end of the study, although they reported a statistically significant improvement. This data may suggest that this treatment is effective in most women but there may be subjects who require more sessions to reach a normal vaginal health. In our study, five weekly sessions and a follow-up visit at one month were performed. Our protocol was based on previous studies; the number of sessions needed to manage the symptoms of VA

is an aspect that needs to be studied further. In addition to studying the number of treatment sessions, it would also be interesting to understand if and when it is appropriate to perform further maintenance sessions after the first cycle.²⁰

Condemi et al.¹⁸ were the first to perform 5 VNOD sessions on 25 postmenopausal women and evaluated the presence and intensity of two subjective GSM symptoms (vaginal burning and well-being). They also evaluated the presence of lubrication, vaginal elasticity and vaginal wall epithelium appearance with two not validated numerical scores, demonstrating significant improvements, but without reliable objective methods.

In our study 1, the vaginal cytology (VMV) confirmed the improvement of vaginal health. At baseline all participants had a prevalence of parabasal cells compared with intermediate and superficial cells, which confirms VA. At the end of treatment, a statistically significant reduction in the percentage of parabasal cells and intermediate cells was detected that resulted in a significant increase in the VMV.

A similar analysis was conducted by Barbero et al.,¹⁹ who studied 50 women with VVA also with cervical-vaginal cytological examination. They demonstrated significant improvements in objective and subjective symptoms of VVA, but also the integration of HA in microvesicles in the cytoplasm of vaginal cells, which constitutes further scientific confirmation of the regenerative activity of HA associated with normobaric O₂. Nevertheless, they did not have a control group and conducted this analysis at 6 months, on only 10 out of 50 participants in the study.

Massarotti et al. did²⁰ perform 5 sessions of VNOD on 40 breast cancer survivor women with VA and found an improvement of VHI similar to our results. They also described an improvement in FSFI global score, nevertheless without reaching normal values, as in our sample. In study 1 we detected a positive impact in the treatment group only on the pain and desire domains of the FSFI questionnaire and a not significant downward trend in the FSDS total score in neither group, probably due to the limited sample size of the groups. On the other hand, in study 2, we observed a significant improvement of desire, lubrication, satisfaction and pain domains. The improvement in vaginal trophism and lubrication (as evidenced by the improvement in all VHI domains) may have led to an increased ease in sexual intercourse, as suggested by the higher satisfaction, reduced pain during intercourse, and increased desire of the FSFI domains.

Despite a statistically significant improvement (p -value 0.007) in the comparison between T0 and T5, the mean value of FSFI total score at T5 was below the cut-off for normal sexual function (26.5). However, the failure to surpass the cut-off does not negate the described clinical and subjective improvements; instead, it may suggest the need for a higher number of treatment sessions in a sample of women, as suggested by the VHI results. Only a limited

number of women (17 out of 52 subjects, 32.7%) reached normal sexual function scores (FSFI > 26), still underscoring the need for further investigation about new protocols with tailored number of VNOD sessions.

It is remarkable that VNOD represents a new alternative therapy for VA which can be offered to women who cannot or do not want to use local or systemic hormonal therapies.^{7,8,40} This treatment has no contraindications, except for the presence of ongoing vaginitis or undiagnosed bleeding.

Our study aimed also to investigate the tolerability and safety of VNOD. As reported by previous authors, our findings show that the VNOD treatment was well-tolerated, with a VAS score for pain/discomfort during therapy ranging from 0 to 1; furthermore, no participant prematurely discontinued the treatment. We also did not observe any adverse effects during and after VNOD therapy.

These are important advantages over laser treatment, the other available local treatment, which can cause pain, itching, swelling, oedema, inflammation, burning, vaginal bleeding, lacerations and burning.¹⁷ Therefore, consistent with results from other authors, our study showed that VNOD is a safer and less invasive treatment compared to laser therapy. However, we emphasize that most performed studies on VNOD including our study, were designed with short-term follow-ups, approximately one month after the last session of O₂ therapy.

The VNOD is a device that is easier to handle than the laser because it is small, does not expose personnel to radiation, and does not require personal protective equipment either for the doctor or the subject. Another advantage of the VNOD is the use of disposable materials (connection tube and probe) which reduce the cost of sterilizing probes, as is the case with the laser.

The main strength of our study is that it evaluates HC O₂ in combination with HA on multiple parameters of VA and sexual function with validated scales, comparing the results with those of a sham group in study 1 and comparing the results from the first session to the one-month follow-up examination in a larger group of postmenopausal women (52) diagnosed with VA in study 2. An additional strength of our study is represented by the objective results of the effectiveness of VNOD obtained through cytological analysis (study 1).

However, our study has also a few limitations. The sham group was not randomized with the treatment group (study 1); nevertheless, the enrolment of the women was progressive and the medical staff performing the procedure and administering the VNOD was not aware of the different set-up of the machine during the sessions. Further limitations of the study include the short follow-up time (study 1 and 2). Another limitation is intrinsic to the device and is represented by the localized action of VNOD on the vagina and not on the vulva, due to the only internal application of the probe. In our sample we did not report any adverse effect and the overall satisfaction was high. Unfortunately we

don't have data about the women's prospective, but we plan to add these information in further studies.

Conclusion

Our study demonstrates that VNOD is an effective and safe treatment, which significantly improves VA signs and symptoms in postmenopausal women and has also a positive impact on their sexual function. This new technique is safe and may represent an alternative option in postmenopausal women with contraindications or poor compliance to other treatments. Studies with long-term follow-up and a larger sample size are needed to confirm and extend these results with VNOD.

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Contributorship

All authors contributed to the study conception and design. The first draft of the manuscript was written by Alessandra Lami and Catalina Manta and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. AL, MCM: Validation, Writing- Reviewing and Editing, Visualization. AL, GL, AC: Data curation. AL, MB: Formal Analysis. AL, VA, CM: Writing- Original draft preparation. AL, SA, MCM, AC, VA, GL: Investigation. MCM, RS: Supervision.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: MCM declares fees for lectures, membership of advisory boards, and consultancy from Gedeon Richter, MSD, Shionogi, Italfarmaco and Theramex. MB have had financial relationships (lecturer) with Shionogi Ltd. AL declares fees for lectures for Funzionia SRL, Exeltis and Theramex.

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Ethical statement

Ethical approval

The protocol of the studies was approved by the Human Subject Committee Area Vasta Emilia Centro (CE-AVEC) of Emilia-Romagna.

Informed consent

Written informed consent was obtained from all participants in accordance with the 1975 Helsinki Declaration.

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Supplemental material

Supplemental material for this article is available online.

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Vaginal oxygen plus hyaluronic acid on lower urinary tract symptoms in breast cancer survivors

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ORIGINAL ARTICLE



Vaginal oxygen plus hyaluronic acid on lower urinary tract symptoms in breast cancer survivors

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ABSTRACT

Objective: This study aimed to evaluate the effect of vaginal oxygen and hyaluronic acid on lower urinary tract symptoms (LUTS) in breast cancer survivors.

Method: A prospective trial was conducted at a university hospital's Menopause Outpatient Clinic. Breast cancer patients experiencing LUTS received intravaginal natural oxygen for 15 min, coupled with a 2% hyaluronic acid solution during the last 5 min. This treatment was performed five times at 15-day intervals. Assessments were repeated at each application and 60 days after the last application. Scores on the Urogenital Distress Inventory-6 (UDI-6) short form and its single items assessing different LUTS aspects were determined.

Results: Treatment in 85 breast cancer patients decreased the UDI-6 total score (from 24.8 ± 19.9 to 17.5 ± 16.2 ; $p < 0.001$) and subscales evaluating irritative ($p = 0.001$), stress ($p = 0.006$) and obstructive discomfort ($p = 0.001$) symptoms. In multivariate analysis, aromatase inhibitors were independently associated with a greater improvement of LUTS (coefficient of regression [CR] -8.05 , 95% confidence interval [CI] -13.41 , -2.64 ; $p = 0.004$). The use of a gonadotrophin-releasing hormone (GnRH) analog was associated with a greater improvement of irritative symptoms (CR -2.42 , 95% CI -4.75 , -0.08 ; $p = 0.042$), while tamoxifen reduced the improvement of stress symptoms (CR 2.49 , 95% CI 0.10 , 4.90 ; $p = 0.041$).

Conclusion: Short-term treatment with vaginal oxygen and hyaluronic acid appeared to improve LUTS of breast cancer patients.

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Introduction

Breast cancer is the most common cancer in women, with 2.3 million new cases being recorded in 2020 [1]. Over the past years, irrespective of menopause status at diagnosis, adjuvant endocrine treatment of hormone receptor-positive breast cancers has shifted from the preferential use of tamoxifen to the use of aromatase inhibitors, eventually combined, in premenopausal women, with a gonadotrophin-releasing hormone (GnRH) agonist [2].

Adjuvant endocrine treatments are aimed to reduce estrogen stimulation on the breast and are inevitably associated with endocrine symptoms [3], including those of the genitourinary syndrome of the menopause [4], a chronic, progressive disorder characterized by a variety of unpleasant genital, sexual and urinary symptoms [5]. Systemic or local estrogens are considered the best treatment for the genitourinary syndrome of the menopause [6]. However, systemic estrogens are contraindicated in breast cancer patients [7], while

vaginal estrogens can be used only for a short period of time in women on tamoxifen and preferably not in those on aromatase inhibitors [8,9]. Prasterone (vaginal dehydroepiandrosterone [DHEA]) has been shown to be safe also in women on aromatase inhibitors [10–12]. Lubricants or moisturizing agents may induce symptom relief but are characterized by poor adherence and continuation rates [13]. Attention has also been given to the use of vaginal CO₂ lasers, whose efficacy in breast cancer survivors is still unproven [14,15].

The intravaginal administration of oxygen plus hyaluronic acid seems to improve disturbances of postmenopausal vaginal atrophy [16]. We recently reported that it also improves the clinical symptoms of the genitourinary syndrome of the menopause in breast cancer survivors [17]. However, we did not strictly evaluate the results on specific lower urinary tract symptoms (LUTS). The bladder, just like other parts of the urogenital system, is rich in estrogen receptors and a low estrogen stimulus is a cause of urinary symptoms and urinary incontinence [18]. This is possibly aggravated in breast cancer

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survivors by adjuvant endocrine therapy [19]. Estrogen deprivation reduces vascular endothelial growth factors, vascularization and oxygen delivery [20,21] to the genito-urinary system, causing reduced tissue trophism, thinning and increased permeability of bladder mucosa. Oxygenation of tissues can be achieved by hyperbaric dives [22], by local application of oxygen [23], local oxygen delivering methods [24] and changes of ambient oxygen concentration [25]. An increase of partial tissue oxygen content stimulates vascular endothelial growth factor (VEGF) and cytokines useful to reduce inflammation and increase tissue vascularization and repair [22,26–28]. Hyaluronic acid binds water and stimulates fibroblast collagen synthesis, ultimately leading to better tissue trophism and a reduction of vaginal symptoms [29,30]. In this study, we investigated more in depth whether the intra-vaginal application of pure oxygen and hyaluronic acid improves LUTS in a population of breast cancer survivors.

Materials and methods

Patients were recruited from July 2021 to January 2024 at the Menopause Outpatient Service of a single university hospital. The inclusion criteria were breast cancer survivors in natural or induced menopause, either surgical or induced by the administration of a GnRH agonist. Only patients with urinary symptoms of any intensity were included in this study. Negative cervical cytology was a prerequisite for treatment initiation. Exclusion criteria included the presence or resumption of menstrual cycles, vulvovaginal infections, existing endometrial hyperplasia or cancer and unexplained vaginal bleeding, previous use of laser therapy and previous use in the last 3 months of vaginal moisturizing agents or local hormones (estrogens or prasterone). All patients provided informed consent for the utilization of their anonymized data for clinical research, and the study received approval from the local ethics committee (CER n. 484/21).

Treatment

The treatment was performed with a specialized device (CaressFlow Srl, Bologna, Italy) developed for the treatment of vaginal atrophy in postmenopausal women [16,17]. The procedure involved the administration of 95% pure oxygen through a vaginal cannula. Oxygen was administered at a rate of 2l/min for a duration of 15 min. In the last 5 min, a low molecular weight and low viscosity solution containing sodium hyaluronate at a concentration of 0.2% (Fidia Farmaceutici, Padova, Italy) was introduced through a small opening at the base of the cannula and conveyed into the vagina by oxygen. This 15-min session was repeated every 2 weeks, for five times in total. During treatment and in the follow-up period, women did not receive any other type of therapy nor underwent pelvic floor exercises.

Evaluations

Evaluations were performed at baseline, prior to any application at visit0 to visit4. Visit5 was performed 15 days after the

last application to document the effect of the fifth application. A subsequent follow-up visit was performed after an additional 45 days, that is, 60 days from the last application. Demographic and clinical data were collected before the start of treatment (visit 0), including age, age at menarche, pregnancies, body mass index, urinary incontinence, diagnosis of endometriosis, age at diagnosis of breast cancer, menopausal status at the time of diagnosis, use of chemotherapy, use of radiotherapy and use of adjuvant endocrine therapy (tamoxifen or aromatase inhibitors with or without a GnRH analog). At any evaluation, the Vaginal Health Index Score (VHIS) was computed, by evaluating the appearance of the vaginal epithelium, the elasticity of the vaginal wall, the presence of moisture and the pH value [31]. Concomitantly, the Urogenital Distress Inventory-6 (UDI-6) short form was completed to assess the presence, severity and nature of LUTS [32]. The UDI-6 is a short version of a condition-specific quality of life instrument introduced in 1994 (the UDI). In particular, the questionnaire is divided into three symptom subscales: items 1 ('frequent urination') and 2 ('urine leakage related to feeling of urgency') refer to irritability symptoms; items 3 ('leakage related to physical activity') and 4 ('small amounts of urine leakage – drops') refer to stress symptoms; and items 5 ('difficulty emptying the bladder') and 6 ('pain or discomfort in the lower abdomen/genitalia') refer to obstruction or pain/discomfort symptoms of the lower abdominal pelvic or genital area. For each question, the score varies from 1 ('not at all') to 3 ('greatly'). The individual scores are converted to make their total sum range from 0 to 100 [20]; the higher the score, the more bothersome the symptoms. All evaluations along with treatment-related side effects were carried out at any visit. Women with a baseline UDI-6 score of 0 were not eligible for inclusion in the study.

Sample size

The sample size was based on a previous study in which we assessed the effect of vaginal oxygen on symptoms of vaginal atrophy, with a pre–post-treatment difference effect size of 0.4 for the UDI-6 [17]. Based on these findings, a paired *t*-test was designed to detect an effect size of Cohen's $d=0.4$, with a significance level (α) of 0.05 and a desired statistical power of 0.90. To account for potential non-parametric distribution and dropouts, we increased the sample size by 25%. According to this analysis, a minimum of 85 participants was required to achieve adequate power for this study.

Statistical analysis

Data were analyzed as collected or after categorization by symptom severity. In this regard, each item was categorized in accordance with the severity score as none/mild, moderate and severe. Considering that the sum of the six items should give a total UDI-6 score of 100, each item should have a maximal value of 16.66; and within each item, categorization as 'mild' should give a value of 5.55, 'moderate' a value of 11.11 and 'severe' a value of 16.66. For the total UDI-6 score, the category 'none/mild' should not exceed 5.55 per six items

(i.e. 33.3). This category also included women who did not suffer from all of the symptoms (some item = 0), but that was included because of a total UDI-6 score > 0 (i.e. with values >0 for other items). Women with severe UDI-6 scores should have values exceeding 16.66 (moderate symptom) per six items (i.e. 66.6).

The UDI-6 scale contains subscales evaluating irritative (items 1 and 2), stress (items 3 and 4) and obstructive/discomfort symptoms (items 5 and 6). For each subscale, women who suffered from no or mild symptoms should not exceed a score value of 11.11, and those with severe symptoms should have values >22.22 .

Continuous data were expressed as mean and standard deviation, while categorical data were reported as frequencies. Comparison of values obtained at different time points was performed by analysis of variance for repeated measures, followed when significant by Fisher's post-hoc test. The Friedman test followed by the chi-squared test was used to perform the same comparisons for categorical values. Multiple regression analysis was used to evaluate factors independently related to the baseline values and to the modifications with treatment of the UDI-6 scale score or of its subscales. For these analyses, categorical data were entered as dummy variables, with 0 indicating the absence and 1 the presence of the specific factor. Backward regression analysis was used, by progressively removing the variables less significantly related to the dependent variable, leaving in the models only those variables that at the end were independently related to the dependent variable.

For all analyses, $p < 0.05$ was considered statistically significant.

Results

Subject characteristics

A total of 85 breast cancer patients were included in the study, all of whom completed the cycle of five treatments. The demographic and clinical characteristics are presented in Table 1. Chemotherapy was administered to 55 women (64.7%) and radiotherapy to 54 women (63.5%). Fifteen women (17.6%) received no adjuvant endocrine therapy, while the remaining

70 (82.4%) received either tamoxifen ($n=18$, 25.7%) or aromatase inhibitors ($n=52$, 74.3%). A total of 40 (57.0%) women were premenopausal and they all received a GnRH agonist to induce a medical menopause. Adjuvant endocrine therapy was started 3.1 ± 3.0 years before the first visit (range 6 months–6 years). The baseline UDI-6 score was 28.4 ± 19.9 .

No women reported side effects from vaginal treatment with oxygen and hyaluronic acid and no women discontinued the study.

Main results

The UDI-6 score decreased after five treatments from baseline values to 17.5 ± 16.2 ($p < 0.001$) (Figure 1). At the 45-day follow-up, the mean UDI-6 score was 18.1 ± 16.9 . This score was significantly lower than at baseline ($p = 0.001$) although it had slightly increased from that evaluated at the end of treatment at visit5, that is, 15 days after the last administration. The same statistically significant improvement was observed for irritative ($p = 0.001$), stress ($p = 0.006$) and obstructive/discomfort ($p = 0.001$) symptoms (Table 2).

The percentage of women with severe LUTS defined by a UDI-6 value >66.6 at baseline decreased but not significantly at visit5 (8.2% vs. 2.3%, $p = 0.087$) (Figure 2). Yet the percentage of women with severe irritative symptoms significantly decreased (14.3% vs. 3.8%, $p = 0.002$) (Figure 2). Consistently, the percentage of women with a none/mild UDI-6 value (value of 33.3) increased from baseline (76.5%) to visit5 and follow-up (86.7%; $p = 0.024$). Also women with none/mild irritative symptoms increased from baseline (55.9%) to visit5 (84.9%, $p = 0.001$) to follow-up (82.1%; $p = 0.003$) (Figure 2).

Upon multiple regression analysis no factor among age, body mass index, parity, number of pregnancies, presence of urinary incontinence prior to treatment, endometriosis, time between diagnosis and treatment, age at diagnosis, length of menopause (natural or induced), chemotherapy, radiotherapy, GnRH agonist, aromatase inhibitors and tamoxifen was related to the baseline UDI-6 score or to that of its subscales.

Table 1. Demographic and clinical data of the group under study.

Characteristic	Mean $\pm$ SD or n (%)
Age (years)	48.7 $\pm$ 8.9
Body mass index (kg/m ²)	22.7 $\pm$ 3.6
Parity	51/85 (60%)
Number of pregnancies	1.0 $\pm$ 1.1
Urinary incontinence	18/85 (21.1%)
Diagnosis of endometriosis	7/85 (8.2%)
Time from breast cancer diagnosis (years)	3.1 $\pm$ 3.0
Age at diagnosis (years)	44.9 $\pm$ 7.9
Previous chemotherapy	55/85 (64.7%)
Previous radiotherapy	54/85 (63.5%)
Type of endocrine adjuvant therapy	
No therapy	15/85 (17.6%)
Adjuvant therapy	70/85 (82.4%)
Tamoxifen	18/70 (25.7%)
Aromatase inhibitors	52/70 (74.3%)
GnRH analog	40/70 (57.0%)

GnRH, gonadotrophin-releasing hormone.

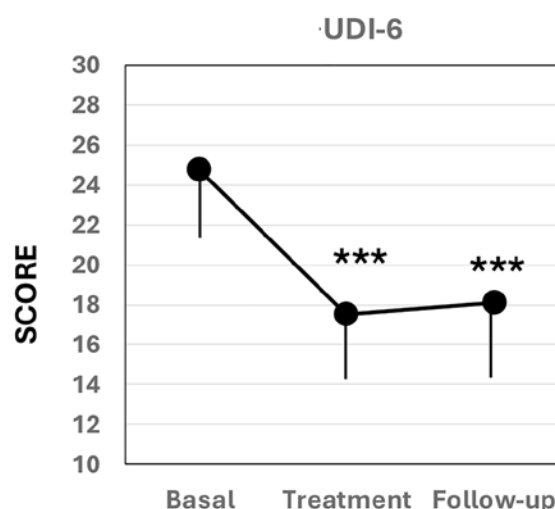


Figure 1. Mean values of the UDI-6 total score evaluated at baseline, at the end of treatment and at follow-up (45 days after the last treatment). UDI-6, Urogenital Distress Inventory-6. *** $p = 0.001$.

Table 2. Mean $\pm$ standard deviation of the UDI-6 evaluated before treatment (visit 0), halfway through treatment (visit 2), at the last treatment (visit 5) and at follow-up.

Parameter	Visit 0	Visit 2	Visit 5	Follow-up	p-Value (visit5 vs. visit 0)	p-Value (visit 5 vs. visit 2)	p-Value (follow-up 1 vs. visit 0)
UDI-6	24.8 $\pm$ 19.9	19.0 $\pm$ 18.3	17.5 $\pm$ 16.2	18.1 $\pm$ 16.9	0.001	0.167	0.001
Irritative symptoms	9.2 $\pm$ 8.4	6.7 $\pm$ 7.7	5.5 $\pm$ 6.8	6.6 $\pm$ 7.3	0.001	0.047	0.001
Stress symptoms	7.1 $\pm$ 8.5	6.2 $\pm$ 8.3	6.1 $\pm$ 7.5	5.7 $\pm$ 7.5	0.065	0.791	0.006
Obstructive/discomfort symptoms	2.6 $\pm$ 4.7	2.5 $\pm$ 4.4	2.2 $\pm$ 4.0	2.1 $\pm$ 3.6	0.001	0.702	0.001
VHIS	9.73 $\pm$ 2.44	13.9 $\pm$ 2.1	16.2 $\pm$ 2.8	15.5 $\pm$ 2.4	0.001	0.001	0.001

UDI-6, Urogenital Distress Inventory-6; VHIS, Vaginal Health Index Score.

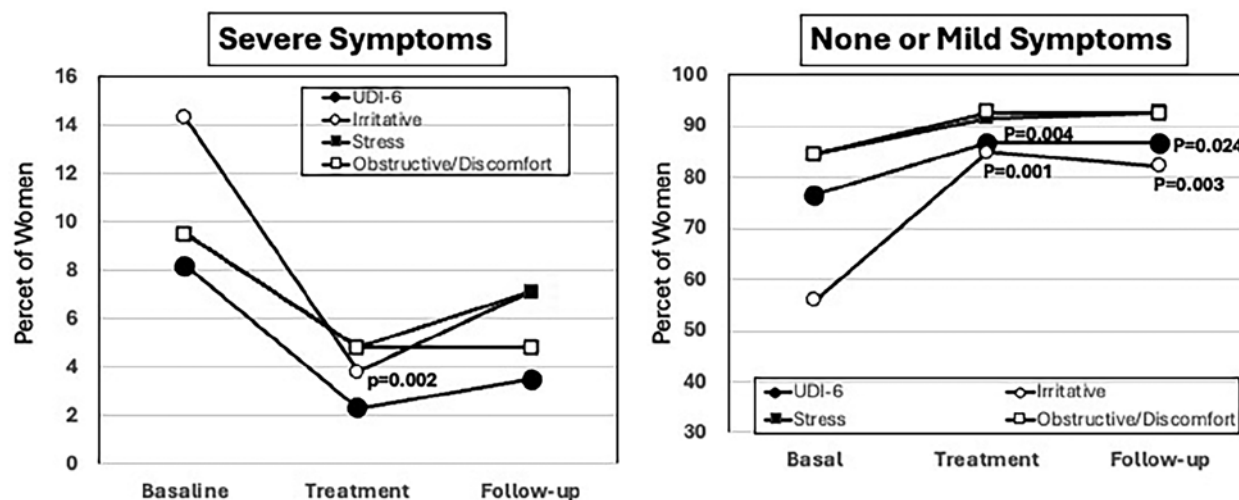


Figure 2. Percentage of women with either severe (left) or none to moderate (right) symptoms of the total UDI-6 score, and its subscale of irritative, stress and obstruction/discomfort symptoms evaluated at baseline, at the end of treatment and at follow-up. UDI-6, Urogenital Distress Inventory-6.

Table 3. Multiple regression analyses of factors independently related to modifications induced by treatment for the UDI-6 short-form score and its subscales investigating irritative, stress and obstructive/discomfort symptoms.

Relation	Coefficient of regression	95% CI	p-Value
UDI-6 total score ($R^2 = 0.309$; $p = 0.001$)			
Baseline UDI-6 score	-0.33	-0.47, -0.19	0.001
Aromatase inhibitors	-8.04	-13.41, -2.64	0.004
Irritative symptoms ($R^2 = 0.328$; $p = 0.001$)			
Baseline value	-0.51	-0.66, -0.36	0.001
GnRH analog	-2.42	-4.75, -0.08	0.042
Stress symptoms ($R^2 = 0.261$; $p = 0.001$)			
Baseline value	-0.31	-0.44, -0.18	0.001
Tamoxifen	2.49	0.10, 4.90	0.041
Obstructive/discomfort symptoms ($R^2 = 0.281$; $p = 0.001$)			
Baseline value	-0.406	-0.54, -0.27	0.001

CI, confidence interval; GnRH, gonadotrophin-releasing hormone; UDI-6, Urogenital Distress Inventory-6.

The use of aromatase inhibitors was associated with a greater decrease of the UDI-6 score (coefficient of regression [CR] -8.04, 95% confidence interval [CI] -13.41, -2.64; $p = 0.004$) (Table 3). At the end of treatment, being on aromatase inhibitors was associated with a lower UDI-6 score (CR -9.2, 95% CI -17.4, -0.89; $p = 0.031$).

The extent of the decrease of each UDI-6 subscale was based on baseline values. The decrease of the irritative symptom subscale was also influenced by the use of a GnRH

analog. Vice versa, the decrease of stress symptoms induced by treatment was reduced using tamoxifen (Table 3).

Urinary symptoms and vaginal atrophy

The VHIS at time0 was 9.73 ± 2.44 and was not correlated with either the UDI-6 total score or its subscales except for the obstructive/discomfort subscale (CR 0.63, 95% CI 0.02, 1.23; $R^2 = 0.038$; $p = 0.042$). At the end of treatment, the VHIS score increased ($p = 0.001$) to a value of 16.2 ± 2.8 . The modifications of the total UDI-6 score or of its subscales were not related to VHIS modifications.

Discussion

Our study shows the benefit of vaginal oxygen plus hyaluronic acid on LUTS of breast cancer survivors. At the end of treatment, the mean UDI-6 total score was significantly lower than the baseline value. Irritative symptoms appeared the most affected, but significant and slightly delayed improvements were observed also for stress and obstructive/discomfort symptoms.

Oxygen can be absorbed by the mucosa [25,27] and its partial increase in tissues stimulates VEGF synthesis and reduces inflammatory cytokines [22,26–28]. It may stimulate collagen synthesis and in this activity is corroborated by hyaluronic acid [29]. The close relation between the bladder and the vagina, and their partially common vascularization, may explain the concomitant improvement of vaginal symptoms and LUTS. The improvement in bladder trophism may reduce sensitivity to irritative

symptoms, and the increase of vascularization, particularly around the urethra, may tend to reduce stress symptoms [33].

The included women were on adjuvant hormone therapy. Although not consistently [34], it has been reported that urinary incontinence can occur more with the use of tamoxifen [35] than aromatase inhibitors [36]. In our study, urinary disturbances of women on tamoxifen were similar to those of women on aromatase inhibitors at baseline, but the response to treatment was better in women on aromatase inhibitors, the improvement of stress symptoms being notably mitigated in women on tamoxifen.

The impact on irritative symptoms was more pronounced and immediate than on stress or obstructive/discomfort symptoms. The effect on stress and obstructive/discomfort symptoms were clearer at the follow-up investigation, 60 days after the last treatment. Possibly, the mechanisms leading to these symptom improvements, like collagen formation or peri-urethral vascularization enhancement [33], require a longer period of time to become clinically meaningful. At the 45-day follow-up – that is, 60 days after the last application – the UDI-6 total score overall slightly worsened, mainly due to a slight increase of irritative symptoms. We cannot rule out the possibility that the benefit may gradually diminish over time, and it might be useful to establish a longer follow-up period to determine if and when a treatment cycle should be repeated.

Strengths and limitations

The most important strengths of this study include the standardization of the procedure and the excellent treatment compliance of the patients. Notably, no participant was lost to follow-up.

The main limitations are the relatively small sample size and the lack of a control group. Furthermore, the study was limited in time, and how long the benefits on LUTS can last cannot be defined. Furthermore, time-divergent effects may emerge between irritative and stress or obstructive/discomfort symptoms.

Generalizability

The data were obtained in breast cancer survivors, mostly those on adjuvant endocrine therapy. The number of treated subjects was relatively small, and comparisons among the responses of women on different adjuvant treatments were mostly underpowered, even if better responses were observed in women on aromatase inhibitors. The study was performed in a single center and mostly on white women. In addition, it was performed in breast cancer survivors and not in women in natural menopause. Accordingly, the results should be replicated in other settings and populations. We applied only a treatment protocol, and we cannot exclude that protocol variations – such as a length of vaginal oxygen application different from 15 min, a number of applications different from five applications or intervals between applications different from 15 days – may lead to different results.

Conclusion

The administration of pure oxygen together with a hyaluronic acid solution every 15 days for a total of five sessions appears

to significantly alleviate LUTS in breast cancer survivors, especially in patients on adjuvant endocrine therapy with aromatase inhibitors.

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Disclosure statement

A. Cagnacci received speaking honoraria from Caress Flow.

M. Lambertini reports an advisory role for Roche, Lilly, Novartis, AstraZeneca, Pfizer, Seagen, Gilead, MSD, Menarini, Daiichi Sankyo and Exact Sciences; receiving speaker honoraria from Roche, Lilly, Novartis, Pfizer, Sandoz, Libbs, Daiichi Sankyo, Takeda, Knight, Ipsen, Gilead and AstraZeneca; receiving travel grants from Gilead, Roche and Daiichi Sankyo; receiving research funding (to his institution) from Gilead; and having non-financial interests as the chair of the European Society for Medical Oncology (ESMO) Young Oncologists Committee (YOC) and as a member of the national council of the Italian Association of Medical Oncology.

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Vaginal natural oxygenation device (VNOD) for concomitant administration of hyaluronic acid and topical hyperbaric oxygen to treat vulvo-vaginal atrophy: a pilot study

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Abstract. – OBJECTIVE: This is a pilot study to evaluate the effectiveness of concomitant administration of hyaluronic acid and topical hyperbaric oxygen therapy (THOT) by a specifically designed medical device (vaginal natural oxygenation device, VNOD) in improving the symptomatology of postmenopausal patients with vulvo-vaginal atrophy (VVA).

PATIENTS AND METHODS: Women with diagnosis of severe VVA from September 2017 to May 2018 were included. Five biweekly administration of THOT and concomitant of hyaluronic acid were performed with a specifically designed medical device. In each occasion, the intensity of patient's symptoms (well-being such as absence of dyspareunia, vaginal dryness, vulvar and/or vaginal itching; vaginal burning; presence of fluid) was determined with a graduated scale from 1 to 6 and the vaginal elasticity and the vaginal wall epithelium appearance were also determined with a graduated scale from 1 to 5. The change in all parameters from baseline to end of therapy was evaluated.

RESULTS: Twenty-five patients were considered for the final analysis. A significant improvement in well-being (0.3 vs. 5.1, $p < 0.001$), vaginal burning (0.2 vs. 5.1, $p < 0.001$), presence of fluid (0.6 vs. 4.9, $p < 0.001$), vaginal epithelium appearance (1.8 vs. 4.7, $p < 0.001$), and vaginal elasticity (1.1 vs. 3.8, $p < 0.001$) was observed between the first and the last therapy session. All the patients reported a recovery of their sexuality at the end of the five treatment sessions.

CONCLUSIONS: In this pilot study, the use of VNOD seems to be a valid treatment of VVA, resulting in a completely natural type of therapy well accepted by patients with immediate ther-

apeutic effects and without side effects; these findings must be confirmed in a well-designed randomized controlled trial.

Key Words:

Vulvo-vaginal atrophy, Topical hyperbaric oxygen, Hyaluronic acid, Dyspareunia, Genitourinary syndrome of menopause.

Introduction

Menopause is commonly associated with somatic symptoms, including hot flashes, night sweats and fatigue, but women are less frequently aware of vulvovaginal symptoms, including vulvo-vaginal dryness and atrophy, recurrent urinary tract infections, and dyspareunia. Postmenopausal vulvar and, vaginal atrophy (VVA) is characterized by the thinning, drying, and loss of elasticity of the vaginal epithelium associated with the reduction in serum estrogen levels¹. VVA can be diagnosed because of symptoms reported by the patient and by clinical examination^{2,3}. These symptoms are presents up to 50% of postmenopausal women, including vaginal dryness, irritation, itching, dysuria, and pain or bleeding with sexual activity^{4,5}. Based on the increase in life expectancy, most of the women can live almost 40% of life after menopause, and because the VVA is progressive without treatment, it can negatively significantly

affect the quality of life⁶. The loss of estrogenic production by the ovary is associated with the onset of vaginal atrophy and results in decreased vaginal lactobacilli, increased pH, alteration of the epithelium morphology, reduction of blood flow and vaginal fluid secretion. The loss of vaginal wrinkled folds and the thinning of the epithelium occurs about 2-3 years after menopause with a variable onset of these physical signs. The loss of roughness is the consequence of an alteration of the collagen supporting the vaginal epithelium. The first-line therapies recommended by the North American Menopause Society include vaginal moisturizers, continuous sexual activity and lubricants⁷. When symptoms persist after taking first-line therapies, vaginal local estrogenic therapies (LET) are considered effective and well tolerated for the treatment of moderate to severe symptomatic VVA due to minimal systemic absorption, and are currently recommended compared to systemic estrogenic therapy when VVA is the only pathology. However, estrogenic therapy is used and continued by only about 2% of women. Recently, various solutions have been proposed such as hyaluronic acid in cream^{8,9}, vaginal laser^{10,11}, or injection of autologous adipose tissue¹², with varying results in terms of clinical effectiveness. To develop new therapeutic approaches to the treatment of VVA, molecular oxygen could have promising characteristics to counteract the main modifications of tissue atrophy and hypoxia-related to this condition. Oxygen therapy increases the availability of oxygen to the tissues, promotes the increase of the reparative processes of the tissues, increases the synthesis of collagen and induces a neo-angiogenetic stimulation through the release of the Vascular Endothelial Growth Factor (VEGF). The hyperbaric oxygen can be administered topically, directly on the skin or mucous membranes affected, using particular devices (topical hyperbaric oxygen therapy, THOT). The THOT could be used in combination with hyaluronic acid, in patients who have contraindications for hormone treatment or in women who wish to use non-hormonal and non-invasive methods.

The aim of this pilot study was to evaluate the effectiveness of concomitant administration of hyaluronic acid and THOT by a specifically designed medical device (Vaginal Natural Oxygenation Device, VNOD) in improving the symptomatology of postmenopausal patients with VVA.

Patients and Methods

Patients

A series of women with diagnosis of VVA based on symptoms reported and clinical examination from September 2017 to May 2018 were considered. The severity of vaginal atrophy was assessed using a Visual Analogue Scale (VAS), based on a four-point scale. Patients indicated the intensity of the perceived symptom using a score variable from 0 (no symptom) to 3 (presence of the symptom with maximum intensity). The present study included only patients reporting a score of 3 (maximum intensity).

Women who had not performed a cervical cytology in the last year, those with presence of vaginal infections and patients who had concomitant clinically important medical disease (endometrial hyperplasia or cancer; undiagnosed vaginal bleeding; liver or kidney disorder; thromboembolic disorders; cerebrovascular accident, stroke, or transient ischemic attack; myocardial infarction or ischemic heart disease; malignancy; endocrine disease or any clinically important abnormalities on screening physical examination, assessments, mammogram, electrocardiogram (ECG), or laboratory tests), were excluded. Women who used oral products containing estrogens, progestins, androgens, or selective estrogen receptor modulators (SERMs) within 8 weeks, transdermal hormone products within 4 weeks, vaginal hormone products (rings, creams, gels) within 4 weeks, intrauterine progestins within 8 weeks, progestin implants/injectables or estrogen pellets/injectables within 6 months, an intrauterine device within 12 weeks before screening, vaginal lubricants and moisturizers within 8 weeks, were excluded.

For the treatment, the self-cooling X2 (Exea - MDM Industrial srl, Padulle, Bologna, Italy) device was used composed of a compressor unit and a base unit (generator) able to deliver up to 6 l/minute of 95% pure oxygen. The base unit was equipped with an on-board computer and a graphic-touchscreen interface that allowed the flow modulation. The full treatment cycle includes five biweekly sessions, during which the single use dispenser was inserted through the vagina for 15 minutes, and the hyperbaric oxygen and the hyaluronic acid were administered at first alternately and subsequently in a contemporary way. The treatment began with the delivery of 95% pure oxygen through a cannula specifically designed for vaginal therapy. The oxygen was

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delivered at a flow of 2 lt/ minute for 15 minutes; in the last 5 minutes a solution of low molecular weight sodium hyaluronate, at a concentration of 0.2%, was administered. At visit 0, an accurate collection of patient history and a complete clinical examination was performed to evaluate eligibility criteria. The intensity of the three following symptoms (1. well-being such as absence of dyspareunia, vaginal dryness, vulvar and/or vaginal itching, 2. vaginal burning 3. vaginal lubrication and presence of fluid) were collected from patients with a VAS based on a six-point scale: score 1 = maximum intensity, score 2 = strong intensity, score 3 = average intensity, score 4 = mild intensity, score 5 = weak intensity and score 6 = absence of symptom (Table I). Before the treatment, the vaginal elasticity and the vaginal wall epithelium appearance were determined by the clinician with a numerical score as reported in Table II. The first 15 minutes session was performed and the occurrence of any discomfort or adverse effect was recorded. The next four administrations (visit 1, 2, 3 and 4) were performed after 14 days. On every occasion, the intensity of patient's symptoms, the vaginal elasticity, and the vaginal wall epithelium appearance were determined and recorded with the method described above, as well as the occurrence of any adverse event. Follow-up visit was performed after 30 days from the last administration and even on those occasions the vaginal elasticity, the vaginal wall epithelium appearance and the intensity of patient's symptoms were determined and recorded. Patients were identified and recruited from investigators clinics and referring physician, with privacy protection and avoiding undue influence. Each included patient provided an informed consent that allowed treatments, that certified the comprehension of the information provided, with voluntary agreement of the subject, free from coercion. The treatments were administered at Ospedale Civile Urbino – SSD Oncologia Ginecologica; the Local Ethical Committee approval was obtained.

Statistical Analysis

Statistical analysis was performed using IBM SPSS version 22.0 (IBM Corporation, Armonk, NY, USA). The statistical significance of the trend of variation in values between treatment sessions was analyzed using the one-way variance analysis according to the Kruskal-Wallis method. The significance of couples' comparisons between treatment sessions was analyzed

using the Wilcoxon test for non-parametric data. A $p < 0.05$ was considered statistically significant.

Results

The mean age at diagnosis of the 25 included cases was 56.6 years $\pm$ 6.8 SD (range 44 - 66). Ten patients were excluded for the presence of at least one of the factors reported above. Five included patients underwent previous hysterectomy (in one case for ovarian cancer and in four cases for benign diseases). Three patients reported previous surgical and medical treatments for breast cancer. No patient discontinued therapy, performing less than five sessions. One patient reported a slight bleeding after the first treatment following sexual intercourse, probably due to neo-vascularization of the tissue. This side effect was not reported after subsequent treatments. The results shown a significative improvement of all the mean scores analyzed: well-being such as absence of dyspareunia, vaginal dryness, vulvar and/or vaginal itching, vaginal burning, vaginal lubrication and presence of fluid (Figure 1). The intensity of the three symptoms reported according to the 6-point VAS scale (Table I) showed an average increase of the well-being index from 0.3 to 5.1, an average increase of the burning index 0.2 to 5.1 and of the fluidity index from 0.6 to 4.9. On a 5-point scale (Table II), the average epithelial appearance index increased from 1.8 to 4.7 and the average elasticity index from 1.1 to 3.8. The analysis of the comparisons between the different phases of therapy have showed that each subsequent treatment determined a significant increase in all parameters, except for the vaginal elasticity and the vaginal wall epithelium appearance for the

Table I. VAS scale for the intensity of the three following symptoms.

Symptoms	
Score 1	Maximum intensity
Score 2	Strong intensity
Score 3	Average intensity
Score 4	Mild intensity
Score 5	Weak intensity
Score 6	Absence of symptom

(1) well-being such as absence of dyspareunia, vaginal dryness, vulvar and/or vaginal itching, (2) vaginal burning (3) vaginal lubrication and presence of fluid.

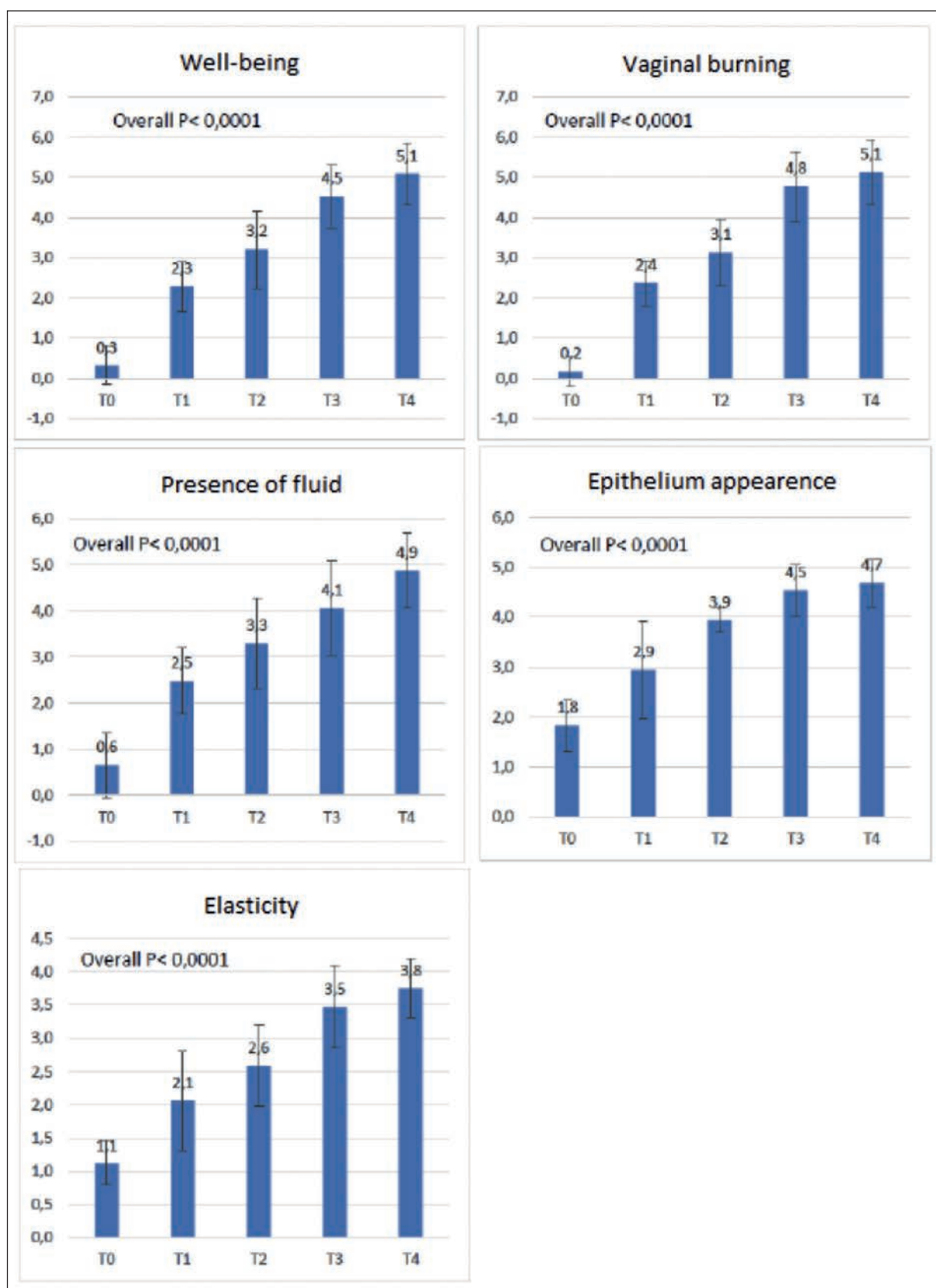


Figure 1. Trend of variation of mean score values between treatment sessions.

Table II. Vaginal elasticity and vaginal wall epithelium appearance determined with two numerical scores.

Vaginal elasticity		Vaginal wall epithelium appearance	
Score 1	Absent	Score 1	Petechiae
Score 2	Poor	Score 2	Contact bleeding
Score 3	Average	Score 3	Scratching bleeding
Score 4	Good	Score 4	Erythema
Score 5	Excellent	Score 5	Normal

last treatment (interval T3-T4) (Table III). All the patients reported a recovery of their sexuality at the end of the five treatment sessions.

Discussion

Despite the high prevalence and the substantial effect on quality of life, VVA often remains underestimated and not subjected to treatment¹³. LET is the effective standard therapy, but many women in this condition refuse its use because of negative publicity in recent years related to the side effects. In fact, while less recent studies¹⁴⁻¹⁷ showed an increased breast cancer risk with estrogen and progestin combination, which has led to the perception of estrogen as a harmful treatment, later publications showed that the risk has been overestimated and limited to selected types of combinations, whereas estrogens alone seem to be protective instead¹⁵⁻¹⁷. Furthermore, there are few safety studies¹⁸ supporting the use of LET in breast cancer survivors, and this therapy is considered contraindicated. The SERM ospemifene is currently indicated in Europe for the “Treatment of moderate to severe symptomatic VVA in post-menopausal women who are not candidates for LET”. Although women with contra-indications to LET are clearly ‘not candidates for LET’. It is ultimately at the discretion of the treating physician whether to prescribe ospemifene in that case or not^{19,20}. Patients who have survived breast cancer and have completed their adjuvant

treatment are not candidates for LET due their history of breast cancer but can use ospemifene¹⁹. Patients who have not completed follow-up or who are performing adjuvant therapy can only use the non-hormonal vaginal moisturizers, lubricants designed to treat VVA symptoms. These treatments address vaginal dryness and reduce burning dyspareunia and hitching, but they have no effect on the loss of elasticity and compliance of vaginal walls, and their effect is only transitory. A recent report²¹ by the North American Menopause Society has not taken a position on how to treat women with early menopause in cases of breast, ovarian or endometrial cancer, suggesting that the management of the problem should be left to the oncologist, considering the potential risk of hormone treatment in these subjects. In this context the therapy with hyperbaric oxygen in combination with hyaluronic acid could be proposed in the future to the patients. Oxygen therapy determines the increase of the reparative processes of the tissues and increases the synthesis of collagen, allowing a normal hydroxylation of this protein²². In fact, at oxygen tensions lower than normal, the collagen is not correctly synthesized, delaying the healing of the wounds. Furthermore, oxygen induces a neo-angiogenic stimulation through the release of VEGF²³. This function is essential for the restoration of the microcirculation in compromised vascular tissues, re-establishing a vascular flow in the hypoxic areas²⁴. Hyaluronic acid is a natural polysaccharide, which is an important part

Table III. Comparisons between the different phases of therapy in relation to the intensity of the three following symptoms and of the vaginal elasticity and the vaginal wall epithelium appearance

	T0-T1	T0-T2	T0-T3	T0-T4	T1-T2	T1-T3	T1-T4	T2-T3	T2-T4	T3-T4
Well-being	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$
Vaginal burning	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.05$
Presence of fluid	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.05$
Epithelium appearance	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	NS
Elasticity	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	NS

of the extracellular matrix of skin and cartilage. This substance can bind a large quantity of water molecules contributing to the maintenance of water balance, proper hydration and structure of skin and mucous membranes²⁵. Various researches^{26,27} of hyaluronic acid in VVA therapy have shown that this compound has been well tolerated without side effects. Our pilot study has shown very high efficacy of the VNOD, both in the analysis of subjective and objective data. The data showed a reduction of burning symptoms already after the first session, reaching a value of 5.1 (on a scale of 6) at the end of the therapy. One of the most immediate effects was the increased elasticity of the tissues, already after the first session, reaching a value of 3.8 (on a scale of 5) at the end of the therapy. All the indexes used show a statistically significant improvement at each treatment except for the epithelium and elasticity parameters in the T4-T5 (although the data showed an improvement), suggesting that for these scores, 4 sessions are sufficient to achieve the maximum result with this therapy. VVA is often a cause of urinary tract disorders. Some patients reported an improvement of the genitourinary symptoms such dysuria, pollakiuria and mild incontinence. Our pilot study was not aimed at the evaluation of these disorders. Further studies could confirm the effect on urinary symptoms. Moreover, to demonstrate the effectiveness of this combined therapy, a double-blind, randomized, controlled, trial is required. This study will be completed with the analysis of the degree of restoration of the mucosa (epithelium and connective tissues) by histological and immunohistochemical analyses. The role exerted by Mesenchymal Stem Cells (MSCs) in the reparative process will also be studied.

Conclusions

We observed that the use of VNOD has proven to be a valid treatment of VVA, resulting in a completely natural type of therapy well accepted by patients with immediate therapeutic effects and without side effects. A following larger pivotal trial may support such data and study other beneficial effects of such therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Relapsing Vulvovaginal Candidiasis: Treatment with Oxygen Therapy and Hyaluronic Acid

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Abstract

BACKGROUND: Vulvovaginal candidiasis (VVC) is a common fungal infection caused by *Candida* species. It affects about 138 million women per year worldwide. Several clinical studies confirmed that hyaluronic acid administration has vaginal estrogens-like effects, relieving the typical vaginal atrophy symptoms, including dryness, itching, and dyspareunia. Local oxygen therapy, on the other hand, has a powerful regenerative, antibacterial, and biostimulating effect.

AIM: The aim of our open, anecdotal, retrospective, and spontaneous trial has been to evaluate the efficacy of the association between high concentration oxygen and hyaluronic acid for treatment of relapsing candidiasis.

MATERIAL AND METHODS: Forty-five women (30.9 ± 4.4 years) with relapsing candidiasis, and complaining of dryness, dyspareunia, pain, appealing to our Second Opinion Medical Consultation Network, signed an informed consent form and were treated with oxygen/hyaluronic acid therapy treatment, once a week, for total of 5 weeks at the outpatient clinic (Healthy Center, Sirio, Fidenza, Italy). The physicians of the Second Opinion Network followed up weekly from remote (WhatsApp, Skype) each treated patient as to state the effectiveness, tolerability, and side effects of the treatment.

RESULTS: The mean VAS and VuAS scores measured at first visit were 2660 and 2622 and significantly ($p < 0.0001$) reduced to 1,311 and 0.77 at last visit. The measurements of the vaginal pH and of the vaginal swab after the last treatment session confirmed significantly ($p < 0.0001$) the absence of candidiasis. Three months later in the follow-up, the percentage of patients who had had only one VVC relapse was 4.44% (2/45), a percentage that increased just to 8.8% at six months (4/45). The elastography index was significantly ($p < 0.0001$) increased after the last treatment session (2.55 ± 0.545 vs. 4.48 ± 0.505).

CONCLUSION: The combined oxygen therapy with hyaluronic acid gave definite therapeutic benefits in this cohort of relapsing candidiasis in the acute phase of the infection. The 6-month follow-up, also detected a lower reinfection rate compared with the historical available data. The procedure is totally painless with excellent compliance by patients and no untoward effects.

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Introduction

Vulvovaginal candidiasis (VVC) is a common fungal infection caused by *Candida* species [1], [2]. It affects about 138 million women (between 25 and 34 years old) per year worldwide [3], [4]. The number of women with recurrent disease is supposed to increase to almost 158 million in 2030 [3]. About 70–75% of women, especially of childbearing age, have VVC infection at least once in their life and 40-50% relapse [5]. In fact, about 20% of acute infections turn in relapsing mycotic vulvar-vaginitis, four or more times in a year [6]. The *Candida albicans* strain affects 85% of the women, relapsing in about 5% of the infections [1]; however, in the last years further pathogenic species, such as *Candida glabrata*, *Candida krusei*, and *Candida tropicalis*, have emerged [7]. All these strains in a balanced saprophytic environment are normal commensals of the microbiota either in the vaginal

cavity or in other warm-humid body surfaces [1]. In fact, the *Candida* species, in particular *Candida albicans*, are ubiquitous pathobiotic microorganisms, members of commensal flora, which can cause infections in healthy and immunocompromised people when an imbalance between microbial-related factors (virulence factors) and host-related factors occurs [8], [9]. The main virulence factors include: the morphological transition from yeast to hyphal form [10], the expression of cell surface molecules, such as adhesins, to help the *Candida* to adhere to epithelial cells [11], directional hyphal growth (thigmotropism) [12], the biofilm formation [13], phenotypic switching, the secretion of extracellular hydrolytic enzymes such as proteinases and phospholipases [14], the ability to change its morphology [15], and its metabolic adaptability [16]. Through these virulence factors, *Candida* spp. can adapt to different host niches and cause infections. Other factors include rapid adaptation to fluctuations in environmental pH, mediated by heat shock proteins

(Hsps) [17]; auto-induced hyphal formation through selective amino acid and metals, such as carbon, nitrogen, iron, zinc, or copper uptake can influence the fungal pathogenicity [18].

Diagnosis of relapsing candidiasis can be quite puzzling due to the non-striking symptoms, the inconspicuous objectivity, and the positivity of the culture examination only in 20–30% of cases but being a widespread public health problem, it has a heavy medical and socio-economic burden [14], [19].

The symptoms are pruritus, soreness, irritation, vaginal discharge, and discomfort, while the clinical signs are introital/vulvar and vaginal erythema, edema, excoriation, typical leukorrhea, vulva and vaginal burning, dysuria, dyspareunia, and frequent micturition [20], [21], [22]. The patients also report reduced fitness and physical activities, with psychological involvement in terms of anxiety, depression, and loss of self-esteem [3], [20].

The moderately acidic (range: 3.8–5.0) normal vaginal pH prevents vaginal infections but several factors, such as age, nutrition, and vaginal Hydration *et al.* can modify it [23], [24]. Consequently, a high vaginal pH level (greater than 5) suggests bacterial vaginosis, trichomonas vaginitis, or candidiasis [25], [26].

Candidiasis is also frequent in pregnant women and can be transmitted to the new-born during delivery or in the postnatal phase, requiring clinical protocols for prevention [27].

The relapse episodes can be due to recurrent oropharyngeal candidiasis in patients with advanced and uncontrolled HIV infection, to genetic factors (polymorphism, familial, and ethnicity), to immune mechanisms (uncontrolled diabetes, antibiotics, and hormone replacement therapy), to behavioral reasons (oral sex, oral contraceptive, corticosteroids, hormonal changes, and intercourse frequency), to psycho-emotional changes, to inadequate intimate hygiene or clothes inducing sweat and local heat in the vulvovaginal area, and to idiopathic factors [28], [29], [30], [31], [32], [33].

The use of topic and oral antifungals, such as nystatin, fluconazole, azole and triazole derivatives, is generally safe and well tolerated, but can promote, especially fluconazole, in the long run, progressive drug resistance by the *Candida* strains [34], [35], such as *C. glabrata* and *C. krusei* [36], which are more pathogenic in terms of edema, skin irritability, and chronic vulvar pain (vulvodynia) [37], [38]. In fact, in the last decade, different women with recurrent VVC appeared fluconazole resistance [39]. Some other less used but effective drugs, including polyenes and echinocandins induce also substantial toxicity [40], [41], [42].

Considering these limitations and the continuous increase of Candidiasis incidence, semi-solid mucoadhesive formulations, such as gel with propolis, have been proposed [43], [44], but new more effective, safe and cheaper drugs and strategies are

required to treat this infection, especially the recurrent cases.

Farida *et al.* [45] analyzed the beneficial effect of Indonesian Propolis wax, as anti-candida agent, in 40 vaginal candidiasis patients. The patients were randomly divided into two groups: (1) Treated with suppositoires containing propolis wax from *Tetragonula* sp. (5%) and Oleum Cacao (n = 20) and (2) treated with the standard antifungal agent, nystatin vaginal tablet (n = 20); administered one at night before sleep for seven days. The results showed that Propolis can improve the immune response against *C. albicans* by inhibiting the biofilm production and increasing the microbial activity of neutrophils [46], [47]. Other used potential anti-fungal compounds are essential oils, including thymol (found primarily in thyme and oregano) [48], and curcumin [49], that perform a cleansing, refreshing and lenitive action, promoting the tissue repairing and the cellular microenvironment recovery by acting as mucosal protection elements.

Another potentially useful natural mucopolysaccharide, is the hyaluronic acid (HA), a uniform, anionic, nonsulfated, linear glycosaminoglycan, composed of repeated disaccharide units that can reach high length and molecular weight [50]: It has been challenged into the vagina due to its moisturizing mucosal healing chemotactic and hydrating properties, significantly contributing to viscoelastic interstitial tissue replacement [51].

Our previous preclinical investigations demonstrated that the high molecular weight HA has antiviral, antibacterial and antifungal activity toward a few *Candida* species [52], [53], [54], [55], [56], [57]. Many clinical contributions confirm that hyaluronic acid gives remarkable adhesive, moisturizing, and repairing benefits in the vaginal mucosa [58], [59].

A total of 833 studies, reviewed by Campagnaro *et al.* [60], confirmed topical hyaluronic acid gel administration has vaginal estrogens-like effects, relieving the typical vaginal atrophy symptoms, including dryness, itching, and dyspareunia, in postmenopausal women. It improves the vaginal microenvironment allowing for a better migration and proliferation of mesenchymal and epithelial cells involved in tissue repair process and has a hydrating effect due to persistent adhesion to the mucosa, with recovery of cutaneous microlesions caused by friction due to vaginal dryness.

Local oxygen therapy, on the other hand, has a powerful regenerative, antibacterial and biostimulating effect; in fact, it increases the availability of oxygen to the tissues, promotes the tissue repair processes and enhances the synthesis through hydroxylation of its chains [61], [62], [63], [64]. It improves the oxygen-dependent transport of some antibiotics, such as tobramycin, across bacterial cell walls [65]. Specifically, this treatment can improve the damage

to the mucosae after gynecologic cancer radiotherapy, promoting neo-angiogenesis, enhancing high levels of vascular endothelial growth factor (VEGF) from macrophages and improving local hypoxia [66]. Especially, hyperbaric oxygen increases the VEGF and activates capillary endothelial cells to migration, forms tubules off-post-capillary venules, and connects to existing blood supplies [67], [68], [69]. The hyperbaric oxygen stimulates also induction of collagen synthesis, fibroblast proliferation, and antimicrobial defense [70].

Recent *in vitro* and *in vivo* studies have demonstrated that oxygen therapy is an antifungal weapon against *Aspergillosis* and *Zygomycosis* [71], [72]. It is supposed that the oxygen decreases acidosis of the inflamed areas, reducing the anaerobic metabolism improving the cell mediated reaction against the infection and phagocytosis [72].

By increasing the partial pressure of oxygen in ischemic tissues, anaerobic metabolism is reduced, and local pH may improve and promote the intracellular killing of fungi into neutrophils, monocytes and macrophages cytoplasm [72]. Gudewicz *et al.* observed the combined effect of oxygen and amphotericin B (antifungal medication) to inhibit the growth of *Candida albicans* [73]. Oxygen tensions of 1800 mm Hg for 90 min in the presence of amphotericin B showed an enhancement of both minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) [73]. Condemi *et al.*, in a pilot trial, showed that the concomitant administration of topical hyperbaric oxygen and hyaluronic acid can have therapeutic efficacy in the treatment of vulvar-vaginal atrophy in 25 post-menopausal women [74].

On the basis of this literature contribution, the aim of our anecdotal, retrospective, spontaneous trial has been to evaluate the efficacy of the association between high concentration oxygen and hyaluronic acid for treatment of relapsing candidiasis.

Materials and Methods

The clinical study was approved by the local institutional review board and conducted in accordance with the ethical standards of the Declaration of Helsinki.

Patients

Forty-five women (30.9 ± 4.4 years) with relapsing candidiasis, and complaining of dryness, dyspareunia, pain, vaginal itching, and burning; appealed to our Second Opinion Medical Consulting, from January 2019 to January 2020, and were included in the present protocol (Table 1). The Second Opinion Medical Network is a consultation

Table 1: Patient's demographic characteristics

Patient's characteristics	Value
Number of patients (women)	45
White race, n	45
Geographic region, Italy, n	45
Age, years	30.9 ± 4.4
Weight, kg	61.1 ± 7.8
BMI, kg/m ²	20.8 ± 2.3
Candidiasis, n	24
Candidiasis+cystitis, n	21
Disease duration, years	≥ 5

Data are presented as the mean $\pm$ SD. BMI: Body mass index, SD: Standard deviation.

referral web and medical office system enclosing a wide panel of specialists, to whom any patient with any illness or syndrome not adequately satisfied with diagnosis or therapy can ask for an individual clinical audit [75], [76], [77], [78]. After signed the informed consent form, all the participants answered a life quality questionnaire describing the clinical symptoms, frequency of relapses and personal history.

Each participant has been also required to fill in before and after the treatment, the Vaginal Assessment Scale (VAS) and a modified version of the VAS, the vulvar assessment scale (VuAS), identifying the vulvar symptoms (dryness, itching, burning, and pain), and to undergo to a self-administered vaginal swab for microbiological analysis at baseline and at the end of therapy. The patients were instructed to insert the vaginal swab (Canestest[®] self-test for vaginal infections, Bayer S.p.A., Milan, Italy) 1–2-inches into the vagina, rotating the swab to collect sample on all sides of the tip, keep the swab in the vagina for 10 s, and then remove the swab and place it in a sterile tube. After 10 s, the patients check the tip swab to see if the color has stayed yellow or changed to blue-green (indicating vaginal infections). In addition, each woman self-tested the vaginal pH, always before and after the treatment, using a vaginal applicator (Gyno-Canestest[®] Vaginal Ph Self-test swab, Bayer S.p.A., Milan, Italy) that includes a pH indicator embedded into a biocompatible grip. It detects vaginal pH, providing information regarding vaginal infections such as VVC, vaginosis and trichomoniasis (Table 2).

Table 2: The initial self pH readings of patients before the treatment

Patients' initial self pH readings	Number of patients (before treatment)
6–7	21
7–8	24

The yellow indicator may turn in green/blue, if vaginal pH is a normal (pH > 4,7), denoting infections such as candidiasis or do not change color after 10 s, if vaginal pH is normal (pH: 3.8–4.5) [79], [80]. A standard vaginal examination to detect the elastic properties of pelvic floor tissues, elastography index (EI), was performed by a practicing gynecologist, at baseline and at the end of the last treatment section, using an transvaginal elastography, also known as elasticity imaging -EI-, (EPIQ7C Ultrasound System, Philips Medical Systems, Andover, MA, USA) consisting of C10–3v PureWave 10MHz vaginal probe [81], [82], [83]. It products 3-D tactile images based on generating a

stress in the tissues using various static or dynamic means and on measuring their consequent elastic properties by ultrasound or MRI [84], [85].

The VAS and VuAS are each 4-item questionnaires that determine the severity scale (range 0–3 points: 0 = none, 1 = mild, 2 = moderate, and 3 = severe) of dryness, soreness, irritation, and pain (dyspareunia or painfulness to touch with external stimulation) for both the vaginal and vulvar areas. In particular, the VuAS focuses on the external genitalia, including the tissue surrounding the vaginal opening, the labia minora, labia majora, clitoral hood, clitoris, and perineum. Lower scores indicate better health conditions [86].

Inclusion and exclusion criteria

The inclusion criteria were as follows: Diagnosis of VVC by, one or more than one molecular swab, such as microbiome-based polymerase chain reaction (PCR) assay, or by blood test, including IgA, IgM, and IgG antibodies test; clinical history of a least 6-year candidiasis relapses. The exclusion criteria were pregnant women, patients with sexually transmitted diseases, women that used hormone therapy (estrogens, progestins, and androgens) or vaginal hormone products (rings, creams, and gel) in the past 2 months before the enrollment.

The patients accepted to undergo oxygen/hyaluronic acid therapy treatment, once a week, for a total of 5 weeks at the outpatient clinic (Healthy Center, Sirio, Fidenza, Italy).

Detailed instructions about healthy hygienic and sexual behavior were given before and after the treatment: The patient should avoid intravaginal medications, vaginal douching, and sexual intercourse within 24 h of her clinical procedure and also then it.

The physicians of the Second Opinion Medical Consulting Network followed up weekly from remote (telemedicine), each treated patient as to state the effectiveness, tolerability, and side effects of the treatment, through WhatsApp and Skype or visit when required.

Treatment protocol

It included one/weekly session (total 5 sessions) with a specific device for gynecological practice (Caressflow®, Caress Flow Srl, Bologna, Italy): A vaginal disposable cannula, connected to the machine body. Each patient is treated, inserting the vaginal cannula equipped with outlet holes, and releasing molecular oxygen at 1 Atm alone (for the first 10 min) and subsequently combined with sprayed 5 ml low molecular weight hyaluronic acid (for the next 5 min) (Figure 1). Post-treatment maintenance of one session per month was recommended. The intravaginal oxygen flow (95%

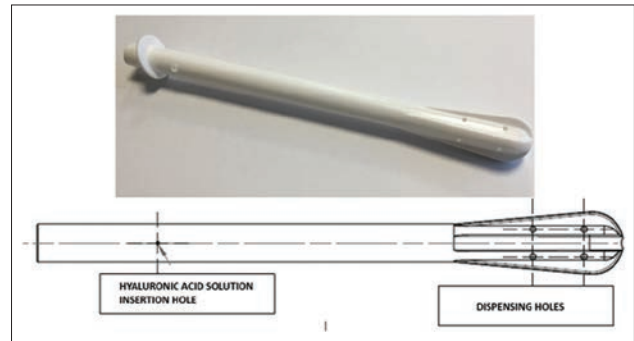


Figure 1: Vaginal disposable cannulas for injection of hyaluronic acid

pure oxygen delivered at a rate of 2 lt/min) has been chosen to optimize the best absorption by the vaginal mucosa. Highly concentrated oxygen spreads easily through the vaginal mucosa, counteracting the critical hypoxia of microcirculation impairment and recovering the superficial cells metabolism. After the oxygen session, hyaluronic acid solution (0.2% concentration, 10 ml) is sprayed through a special injection hole located in the upper part of the cannula (Figure 1). Due to its low molecular weight, is easily absorbed by the mucosa preconditioned with the pure oxygen flow. The low molecular weight hyaluronic acid penetrates easily in the mucosa prepared by the action of oxygen. All the patients were evaluated for the presence of candidiasis at the end of the last treatment session, with follow-up at 3 and 6 months from the end of treatment.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). The data were analyzed using an unpaired t-test with Welch's correction. $p < 0.05$ was considered significant.

Results

The Caress flow® protocol delivering HA and oxygen in the vagina of the women affected by candidiasis gave satisfactory results in terms of symptoms relieve and *Candida* disappearance at the end of the treatment. The mean VAS and VuAS scores measured at first visit were 2,660 and 2,622 and significantly ($p < 0.0001$) reduced to 1.311 and 0.77 at last visit (Figure 2). The measurements of the vaginal pH (value < 5) and of the vaginal swab (color yellow) after the last treatment session confirmed significantly ($p < 0.0001$) the absence of bacterial vaginosis, trichomonas vaginitis, or candidiasis (Figure 3).

Three months later in the follow-up, the percentage of patients who had only one VVC relapse was 4.44% (2/45), a percentage that increased just

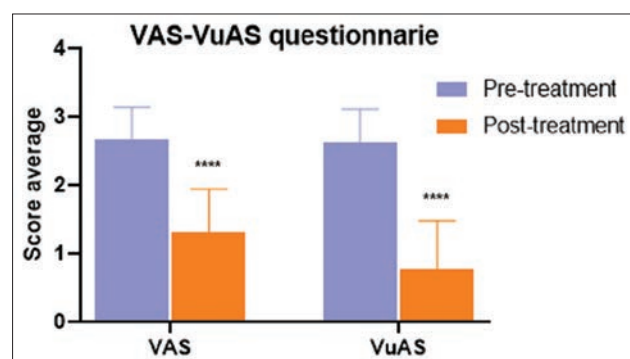


Figure 2: VAS and VuAS values pre- and post-treatment. There were significant differences. **** $p < 0.0001$ pre- vs. post-treatment

to 8,8% at six months (4/45). Figure 4 reported the relapsing events, for each patient, in the 6 months before treatment and in the 2 follow-up after treatment (3 and 6 month-follow up). The elastography index (EI) was significantly ($p < 0.0001$) increased after the last treatment session [mean $\pm$ SD, 2.55 ± 0.545 (pre-treatment) vs. 4.48 ± 0.505 (post-treatment)] (Figure 5).

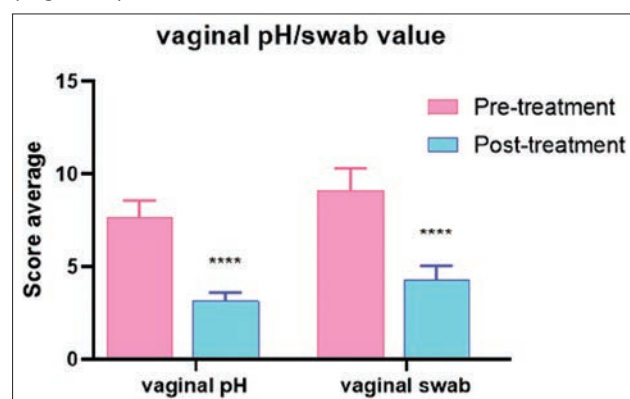


Figure 3: Vaginal pH and swab measurements before and post-treatment. There were significant differences. **** $p < 0.0001$ pre- versus post-treatment

The microbiological analysis of the secretions, in the 3 and 6-month follow-up, showed total elimination of fungal colonization in all the patients.

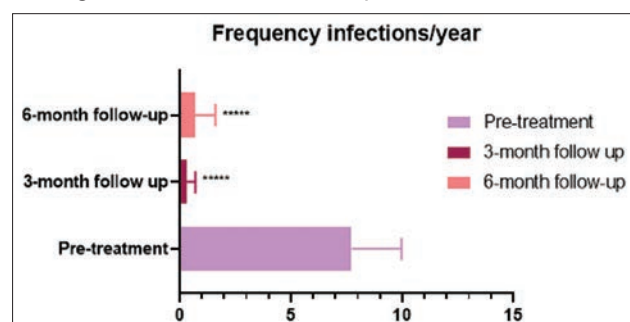


Figure 4: Frequency VVC infection/year pre-treatment and at 3 and 6-month follow-up. There were significant differences. **** $p < 0.0001$ pre- versus post-treatment

The tolerability was excellent: Indeed, no side effects of the treatment were reported by the patients that described the protocol "pleasant and refreshing."

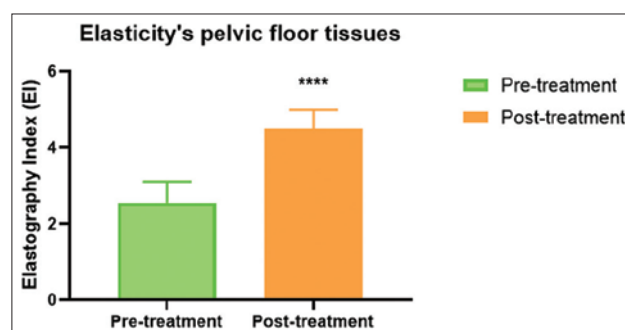


Figure 5: Elastography index (EI) values of pelvic floor tissues pre- and post-treatment. There were significant differences. **** $p < 0.0001$ pre- versus post-treatment

Combined oxygen therapy with hyaluronic acid has proved to be a valid method for treating the symptoms associated with relapsing candidiasis, capable of resolving both the acute phase of the infection and effectively preventing its reappearance. It is a totally painless therapy, with excellent compliance by patients.

Discussion

Our observation suggests that the Caressflow® protocol combining hyaluronic acid and oxygen has antimicrobial, antifungal, antioxidative, and also anti-inflammatory properties. Selectively the oxygen enhances the reparative processes of the inflamed mucosa and the collagen synthesis by the hydroxylation pathway and induces a neo-angiogenetic stimulation through the release of the vascular endothelial growth factor (VEGF) [74], [87], [88], [89]. The amount of oxygen up taken by plasma is 10–20 times higher than normal, and oxygen diffusion into tissues is strongly increased.

On the other side, hyaluronic acid, a natural polysaccharide, binds large amount of water molecules rehydrating, and re-structuring the inflammation-injured skin and mucous surfaces [90]. The safety and effectiveness of HA at different molecular weights in VVA patients have been stated by several studies [91], [92], [93].

As to the mechanisms of action, the low molecular weight hyaluronic acid spreads into the deeper vaginal layers [94], and can block the intercellular adhesion molecule (ICAM)-1 receptor, reducing inflammation and secretion of pro-inflammatory cytokines [95].

The individual interviews with the patients by mean of Skype or physician's visit showed improvement in life quality, a notable goal especially for recurrent VVC, source of discomfort in the social daily life relationships, in the partner relationships and sexual intercourse. In fact, the life quality scores improved

progressively during the treatment in terms of intimacy and sexual intercourse fitness but also of mood and social behavior.

The main limitations of our study were (1) the small clinically cohort of patients and (2) the absence of a control group. Given the small size of the patient sample, we cannot exclude error rates (Type 1 and Type 2 errors) and cannot ensure that our results may be replicated in future research with larger sample size. However, this preliminary observation and the positive outcome are very promising and recommend a further major evidence based clinical trial. The identification of control group was also difficult because Caressflow[®] is a specific combination of gas and a natural mucopolysaccharide; in a possible next study a single- or double-blind placebo effect might be evaluated by a sham treatment with air flow and saline being our study just a basic reference of the procedure.

Conclusion

The combined oxygen therapy with hyaluronic acid gave definite symptomatic benefits in this cohort of relapsing candidiasis in the acute phase of the infection. The 6-month follow-up, also detected a lower reinfection rate compared with the historical available data. The procedure is totally painless with excellent compliance by patients and no untoward effects.

The Caressflow[®] device is non-invasive and repeatable treatment and can thus be considered a profitable hygienic procedure to be periodically repeated for optimal environmental conditions and functions of the vulvar-vaginal apparatus.

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INTRODUCTION

Lichen sclerosis is a chronic inflammatory dermatitis, with a predilection for the anogenital area predominantly female (female / male ratio 6 to 1), which in some cases can be seriously affected (atrophy of the labia minora, phimosis, introital stenosis, etc.). Most cases are diagnosed in postmenopausal women, but it can affect women and men of any age. Lichen sclerosis is usually an itchy condition, although it can also be asymptomatic.

The pathology of lichen is characterized by symptoms such as burning and itching that can afflict the woman in her daily life. These symptoms can lead to dyspareunia and vulvar lesions, both caused by scratching and by tissue fragility. In the advanced stages there is a radical change in the anatomy of the genitals: disappearance of the clitoris, incorporated in fibrosis, disappearance of the labia minora and labia majora, reduction of vulvar intake to the point of impossibility of having sexual intercourse and in extreme cases not even undergoing at gynecological examination. In addition, fibrosis can involve the peri-urethral region with displacement of the urethra and cause the so-called vaginal urination. Such sclerotic tissues undergo ulceration causing intense pain and burning. In women, Lichen sclerosis is considered a rare disease (1.7% of gynecological patients) due to the low incidence, but it is also an underestimated disease because it is hidden or not recognized: patients, in fact, sometimes consider the symptoms of Lichen Sclerosis related to menopause.

In men, phimosis or fusion of the foreskin over the coronal sulcus may occur. Those affected perceive a progressive discomfort.

The treatment of lichen, aimed at reducing the disabling symptoms, is mainly carried out using cortisone for topical use. However, prolonged use can lead to drug resistance and cause important

side effects such as aggravation of atrophy. Furthermore, cortisones have no effect on the repair of the scarring of the disease.

To overcome the problems associated with the prolonged use (often for a lifetime) of corticosteroids, new approaches have been tried to reduce symptoms.

Hyaluronic acid is a natural polysaccharide that forms a fundamental part of the extracellular matrix of the skin and cartilage, has remarkable adhesive, moisturizing and repairing properties of the mucous membranes and skin. The use of hyaluronic acid for topical use has been used with positive results in the treatment of lichen planus, suggesting that even non-pharmacological active ingredients can have a significant effect in reducing the symptoms of this type of chronic dermatosis. Oxygen therapy has a powerful regenerative, antibacterial and biostimulating effect, it is therefore believed that it can also be very useful in the treatment of this pathology for the healing of lesions and the treatment of symptoms such as itching and burning. Oxygen therapy increases the availability of oxygen to the tissues, promotes the increase in tissue repair processes and increases the synthesis of collagen allowing normal hydroxylation of this protein. In fact, at tissue oxygen tensions lower than normal, collagen is not synthesized correctly, slowing the healing of ulcers and wounds. In addition, oxygen induces a neo-angiogenic stimulus by releasing factors such as the Vascular Endothelial Growth Factor (VEGF). This function is essential for the restoration of the microcirculation in compromised vascular situations, re-establishing a vascular flow in the hypoxic areas that guarantees correct tissue regeneration. With regard to dermatological pathologies, topical oxygen therapy has proven effective in the treatment of psoriasis, as well as atopic dermatitis and acne, suggesting the usefulness of the regenerating and anti-inflammatory capabilities of oxygen in this therapeutic area.

The combination of high concentration oxygen and hyaluronic acid has proven to have therapeutic efficacy in the treatment of vulvo-vaginal atrophy, a condition characterized by atrophy of the vaginal mucosa and alterations in tissue regeneration, and which presents symptomatic aspects similar to lichen.

The goal of the study is to use the association between high concentration oxygen and hyaluronic acid for the treatment of disorders caused by lichen and to improve the quality of life of people affected by this disease.

MATERIALS AND METHODS

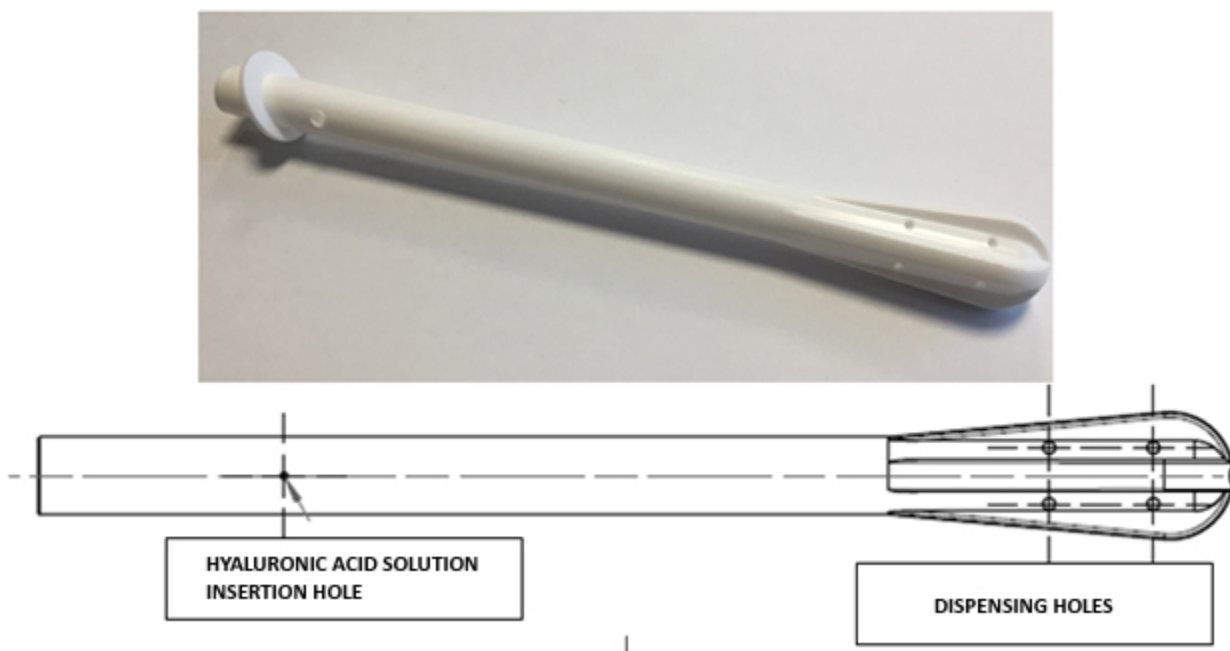
Twenty-five (25) women diagnosed with lichen, carried out by vulvoscopy and biopsy, underwent a weekly session for 5 weeks which included vulvar oxygen therapy for 10 minutes with low molecular weight hyaluronic acid nebulization, at a concentration of 0, 2%, followed by vaginal oxygen therapy combined with hyaluronic acid for 5 minutes.

For the treatment, the Caress Flow system was used, an oxygen therapy device for gynecological use that allows the topical administration of oxygen with a high degree of purity up to $93 \pm 3\%$, at a flow of 1-6 l / minute.

The device consists of a compressor that generates compressed air by sucking air from the external environment, filtering and compressing it. Inside the machine body there are zeolite molecular sieves that exploit the principle of the different absorption of gas molecules, letting the O₂ pass and retaining the other gases present in the air, such as nitrogen, argon, helium and hydrogen. The machine body transforms the outside air into $93 \pm 3\%$ pure oxygen.

Two dispensers were used, the first consisting of a vaginal cannula, connected to the machine body. The cannula is equipped with outlet holes for the delivery of oxygen and hyaluronic acid, which is inserted through a special insertion hole located in the upper part of the cannula (Figure 1). The vaginal cannula is used for treatment inside the vaginal canal.

FIGURE 1



In addition to the cannula, an airbrush was used (Figure 2), always connected to the machine body, capable of delivering oxygen in combination or not with the hyaluronic acid solution. The airbrush is used for the treatment of the external genitalia, nebulizing the combination of oxygen and hyaluronic acid directly on the areas affected by the lesions.

Hyaluronic acid is previously dissolved in distilled water, to form a 0.2% (w / v) solution.

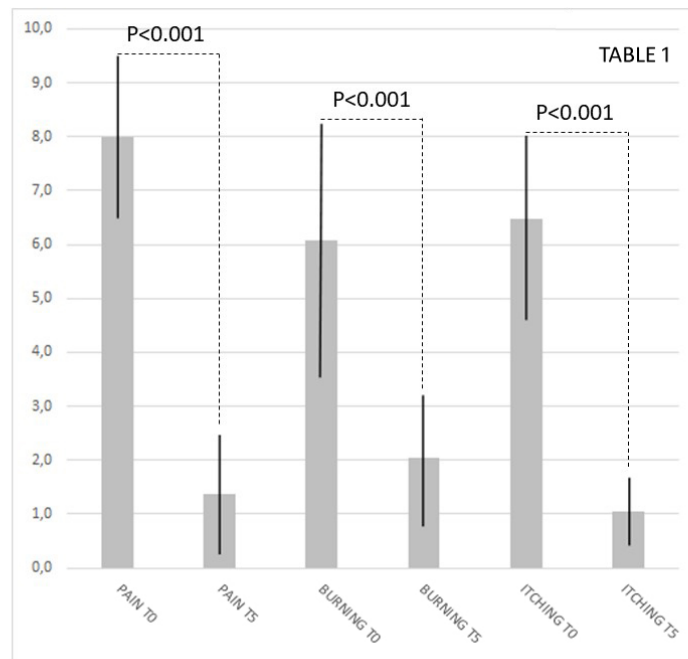
FIGURE 2



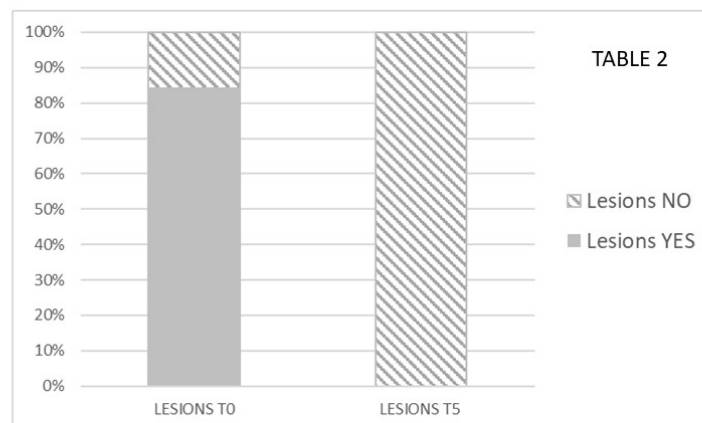
A double evaluation was performed on the treated subjects: one subjective by the patients and one by the doctor. The subjective scale was compiled using an analog graded card that assessed pain during sexual intercourse (dyspareunia), itching and burning with a VAS scale from 0 to 10, where 10 represents the maximum intensity and 0 the absence of the disorder, analyzing symptoms at T0 (before the first treatment session) and at the end of the 5 sessions (T5). Finally, the presence or absence of lesions was assessed by the doctor.

RESULTS

Thanks to the qualitative improvement of the tissues, the patients reported a significant improvement of all the indexes analyzed (Table 1), with the greatest effect regarding pain reduction (VAS T0 = 8.0; VAS T5 = 1.4, Wilcoxon signed-rank test $P < 0.0001$), but also with regard to burning (VAS T0 = 6.1; VAS T5 = 2.0, Wilcoxon signed-rank test $P < 0.0001$) and pruritus (VAS T0 = 6.5; VAS T5 = 1.1, Wilcoxon signed-rank test $P < 0.0001$).



The fundamental observation for this study was the evaluation of the presence of vulvar lesions at T0 and at the end of the treatment. All women who initially had lesions (84%), particularly in the fork area, no longer presented this problem, demonstrating the complete resolution of the problem (Table 2). No side effects associated with the treatment were reported by the patients.



Lichen is a very complex pathology, with autoimmune aetiology, the treatment used does not aim to have a therapeutic effect but to improve the symptoms that afflict women and that affect their quality of daily and sexual life.

Combined oxygen therapy with hyaluronic acid has proven to be a valid method for healing vulvar lesions and improving lichen symptoms. It is a totally painless therapy, with excellent compliance by patients. It is a fast, non-invasive and repeatable treatment, with no side effects.

Conjugate treatment with high concentration normobaric oxygen and hyaluronic acid for vaginal atrophy: a prospective study

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Abstract. – OBJECTIVE: Vulvovaginal atrophy is a condition closely related to low circulating estrogen levels, with post-menopause being the main cause. However, patients of childbearing age may also present with these symptoms due to treatments that reduce estrogen production. Local estrogen therapy is the causal treatment of local symptoms, but it is not always accepted and is often abandoned by patients. In recent years, alternative therapies have been proposed: fractional CO₂ laser or the conjugate treatment with normobaric oxygen and hyaluronic acid, the latter being the subject of this study.

The study aimed to evaluate the effectiveness of conjugate topical treatment with normobaric oxygen and hyaluronic acid.

PATIENTS AND METHODS: 50 patients were evaluated and treated with 5 applications of 15 minutes each, every 15 days, with Carressflow®. All patients presented at least one of the symptoms related to vulvovaginal atrophy: dryness, burning, and dyspareunia. In all cases, vulvoscopy, colposcopy, and cervicovaginal cytology were performed. The patients were interviewed with an analogic scale (VAS) concerning the severity of symptoms before and after the treatment. Colposcopy and PAP-smear were assessed by mean of Vaginal Health Index Score (VHI) at baseline and at the end of the treatment.

RESULTS: All patients completed the treatment scheme and presented with a significant improvement in subjective symptoms. The colposcopy and PAP-smear performed 10 days after the end of the last treatment showed a significant improvement in the appearance and elasticity of the vaginal epithelium and the cytological picture, which showed, in the sample taken after treatment, hyaluronic acid vesicles within the cell cytoplasm.

CONCLUSIONS: This study corroborates the data presented in the latest published papers on the effectiveness of treatment with normobaric O₂ and hyaluronic acid on vaginal atrophy. Effi-

cacy has been confirmed both in terms of subjective symptoms reported by the patients and objective improvement at colposcopy and PAP-smear cytology.

Key Words:

Vulvovaginal atrophy, Hyaluronic acid, Normobaric oxygen, Non-hormonal treatment, Menopause, Carressflow®.

Introduction

Vulvovaginal atrophy (VVA) is a condition closely related to the deficiency of circulating estrogen, with post-menopause being the main cause. More than 50% of women 2-4 years after the end of their fertile period present with this condition and report typical symptoms such as vaginal dryness, burning, itching, and dyspareunia, sometimes associated with urinary disorders. Bleeding may also occur in more severe cases, especially after microtrauma¹⁻⁵.

VVA can also affect women suffering from premature interruption of ovarian production of estrogens. Examples include cancer treatments, such as surgery and pelvic radiation therapy, that remove ovaries or make them inactive, either temporarily or permanently. Although in the recent years a great effort has been made to promote fertility-sparing surgery in young patients with gynecological malignancies⁶⁻⁸, some of them may still develop atrophy-related symptoms due to subsequent treatments that reduce estrogen production such as chemotherapy or endocrine therapies. Also, the therapies for endometriosis and the use of low estrogen oral contraceptives have been shown to induce, in some patients, vaginal discomfort.

Estrogen deficiency not only causes atrophy of the vaginal epithelium but also significantly alters the vaginal environment by decreasing the count of lactobacilli, increasing the pH, reducing blood supply, and consequently decreasing the vaginal fluid secretion⁹.

All these alterations mainly affect the vaginal and vestibular mucosa, and much less the entire vulvar region, which is more than 60% covered by skin and therefore less influenced by the presence of estrogens.

Hormonal replacement therapy (HRT), be it topical or oral, is considered the gold standard treatment for VVA. However, many women decline the use of these hormonal substances due to the risk and fear of increased incidence of breast cancer and the need of a prolonged treatment. Moreover, HRT is contraindicated in oncological patients, even if some data show good results on VVA with little systemic absorption of local estrogens¹⁰. A recent report of The North American Menopause Society was inconclusive on the modality of treatment for women affected by premature menopause after breast, ovarian or endometrial cancer, suggesting that for these women the management depends on individual preference or need, and it must follow a consultation with oncologist considering the potential risks of hormonal treatment choices¹¹.

Over the years, many non-hormonal treatments have been used, including ordinary lubricants/emollients, local hyaluronic acid, soy and red clover derivatives oral compounds, vitamins, etc.

In recent years, alternative therapies have been proposed, such as the use of fractional CO₂ laser¹² or lipofilling with sometimes encouraging results. However, the pain associated with the procedures,

the high costs, and the need for reinterventions are major drawbacks to the widespread use of these methods. Recently, warnings from American scientific societies, due to the risk of stimulating the progression of pre-cancerous lesions, have induced a reduction in the use of CO₂ laser¹³⁻¹⁶.

Hyaluronic acid is a natural polysaccharide present in large quantities in the extracellular matrix of the skin and cartilage. By binding to many water molecules, it allows adequate hydration of the mucous membranes. Oxygen promotes tissue repair processes and collagen synthesis by stimulating neoangiogenesis, promoting a good nourishment of the tissues¹⁷.

Our investigation aimed to confirm the effectiveness of combined treatment with hyaluronic acid and high concentration normobaric oxygen administered intravaginally through the Caress-flow® device (Bologna, Italy).

Patients and Methods

Fifty-seven patients who had been menopausal (spontaneous/surgical/chemotherapy) for at least 6 months were enrolled during the period March 2021-March 2022.

All patients had vulvovaginal atrophy (VVA) and presented at least one of the following symptoms: vaginal dryness, vaginal burning, and dyspareunia unrelated to infectious/inflammatory pathology. In all cases, the following investigations were performed: vulvoscopy, colposcopy, and sampling for cervical-vaginal cytological examination (PAP-smear).

Table I. VAS scale of assessment of subjective symptoms (scores from 1 to 4).

Subjective Symptoms	1 Maximum intensity	2 Medium intensity	3 Low intensity	4 Absent
Malaise				
Burning				
Dryness				
Dyspareunia				

Table II. Healthcare provider rating scale of VHI symptoms (scores from 1 to 4).

Objective signs	1	2	3	4
Vaginal epithelium	Reddened with petechiae	Bleeding on contact	Slightly reddened	Normal
Elasticity	Absent	Poor	Medium	Good
Atrophy on cytology	Intense	Medium	Poor	Normal trophism

Patients with vulvar dermatological changes, signs of vulvovaginal infections, or pre-neoplastic/neoplastic changes in the lower genital tract were excluded from the study.

The treatment consisted of five sessions of 15 minutes each, every 15 days of oxygen and hyaluronic acid administration using Caressflow®.

Before starting the treatment, patients filled out a form (Visual Analogic Score: VAS scale) in which the following symptoms were scored from 1 to 4 (1 Highest intensity, 2 Medium intensity, 3 Low intensity, 4 No symptom):

- Malaise
- Burning
- Dryness
- Dyspareunia

The colposcopy and PAP-smear cytology performed to assess the status of the cervicovaginal epithelium were evaluated with a score called Vaginal Health Index (VHI). The appearance of the epithelium and the cells was graded from 4 to 1 according to the severity of the alteration observed:

- Epithelium
 - 1) Reddened with petechiae
 - 2) Bleeding on contact
 - 3) Slightly reddened
 - 4) Normal
- Vaginal elasticity:
 - 1) absent
 - 2) poor
 - 3) medium
 - 4) good
- Atrophy at cytology:
 - 1) intense
 - 2) medium
 - 3) poor
 - 4) normal

All subjective parameters were reported before and after the end of the treatment; colposcopy and cervical-vaginal cytological examination were performed 10 to 30 days after the end of the last treatment.

Statistical Analysis

The statistical significance of the variance trend in values between treatments was analyzed using the Kruskal-Wallis' test for one-way analysis of variance. The significance of pairwise comparisons between treatment sessions was analyzed using the Wilcoxon test for non-parametric data.

Two-sided p -values <0.05 were considered statistically significant.

Results

Seven patients were excluded from the study because they had:

- Vulvar lichen sclerosus (4)
- Abnormal PAP-smear (3)

The mean age of the patients at enrollment was 55.5 years $\pm$ 6.4 SD [45-66].

Four patients had chemotherapy-induced menopause for breast cancer.

All patients completed the treatment regimen and showed a marked and statistically significant ($p<0.001$ for all symptoms) improvement in subjective symptoms, achieving their complete disappearance in the 70% of cases (Figure 1).

The colposcopy and PAP-smear performed 10-30 days after the end of the last treatment showed a significant change in the appearance and elasticity of the vaginal epithelium (Figures 2 and 3).

Figure 1. Subjective symptoms and objective evaluation before and after treatment: comparison.

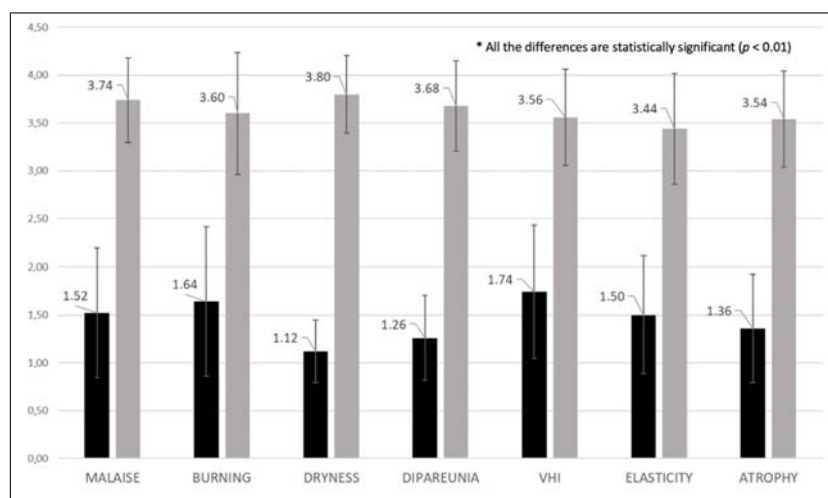




Figure 2. Intense atrophy, reddened epithelium with petechiae.



Figure 3. Normal epithelium after treatment.

The cervicovaginal cytological examination carried out in patients at the end of the last treatment, showed the disappearance of the atrophy which was present in the pre-treatment cytology (Figure 4), and the presence of cytoplasmic inclusions of hyaluronic acid (Figure 5).

So far, 10 patients have been resubmitted to colposcopy and PAP-smear 6 months after the last treatment: of them, 8 maintained the improvements achieved both in the subjective symptoms and at colposcopy and cytology, while 2 reported a slight increase in vaginal dryness.

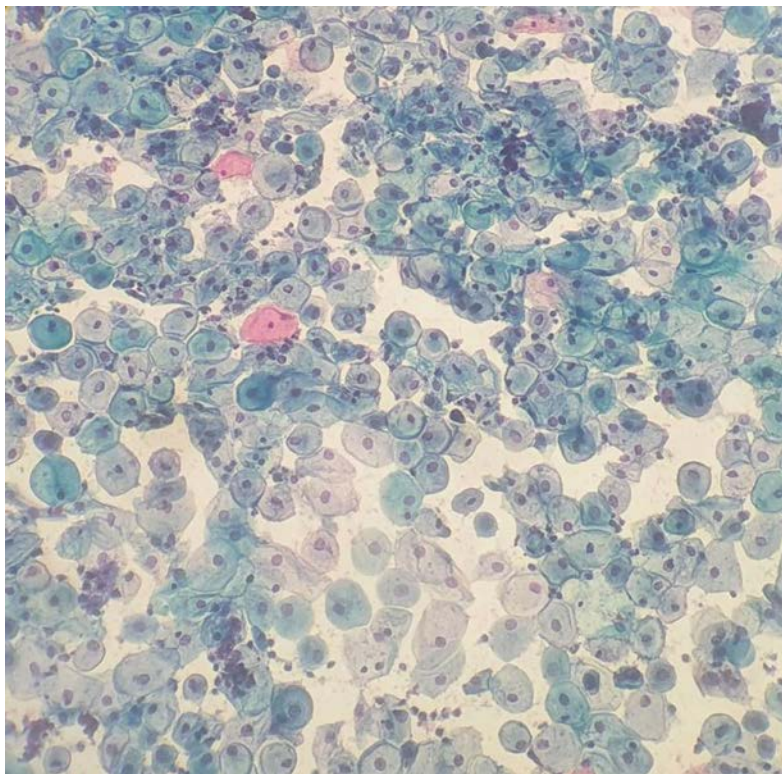
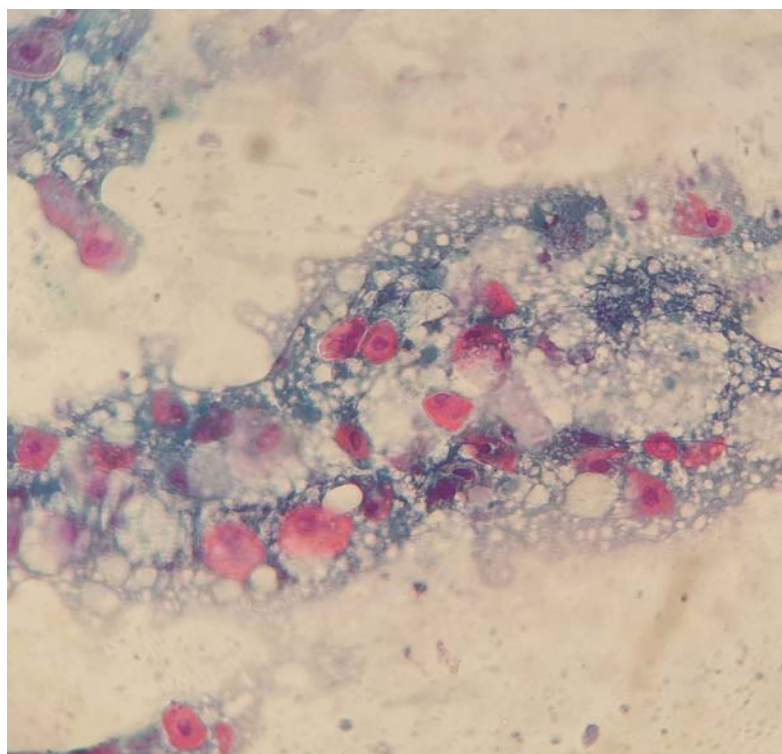


Figure 4. Pap smear cytology showing mostly immature basal cells, typical for vaginal atrophy.

Figure 5. Pap smear cytology 10 days after the end of the last treatment: prevalence of mature superficial cells showing intraplasmic inclusions of hyaluronic acid.



Discussion

Vaginal atrophy is a very frequent condition in post-menopausal women (about 50%) or women with estrogen deficiency induced by other causes such as surgical castration, chemotherapy, or radiation treatments. This percentage is possibly underestimated as many women do not report some of the disorders associated with vaginal atrophy, especially those related to the sexual sphere⁴.

Post-menopausal hormone replacement therapy can prevent the onset of this condition or at least delay or mitigate it significantly. This option remains, at least in Italy, limited to a few cases, especially in patients with intense autonomic symptoms such as hot flashes, insomnia, decreased libido, anxiety¹⁸.

Furthermore, there are many situations in which replacement therapy is contraindicated as it represents an important risk factor for metabolic, thromboembolic, and oncological problems, especially in the field of breast cancer¹⁹.

Local estrogen therapy has played and plays an important role in maintaining good trophism of the vaginal mucosa, but even in this case not all patients accept it, and, in some situations, the effectiveness might not be optimal²⁰.

Many topical, non-hormonal products on the market have demonstrated a positive action in promoting lubrication but are often not sufficient to solve all the problems related to alterations of vaginal trophism and allow only transient benefits, with the consequent need for repeated treatments, especially at the time of sexual intercourse²¹.

Even if the most recent treatments with fractionated CO₂ laser and radiofrequency have given significant improvements in the management of the pathology, some difficulties persist regarding the aspect of pain during the procedures²²⁻²⁴.

The use of the Caressflow[®] device, which delivers normobaric highly concentrated O₂ and hyaluronic acid, is an alternative to the devices that have so far been available. According to the data emerged from previous studies^{25,26}, it appears to be one of the most performing methods to alleviate VVA symptoms. Our study corroborates the previous findings, as we observed a significant improvement both in the subjective symptoms reported by the patients and in the cytology of PAP-smear, the elasticity, and the trophism of the vaginal epithelium. The greatest benefit involved the reduction of vaginal dryness and the disappearance of dyspareunia, thus allowing an increased reported quality of life in over 98% of cases.

Our study is the first, to our knowledge, to have evaluated the actual presence of changes at PAP-smear cytology after the completion of the treatments: the presence of hyaluronic acid vesicles in the cytoplasm of the cells confirms the regenerative action at the tissue level, thus surpassing the surface action of many other methods and suggesting a serious possibility that the improvement in subjective and objective parameters may be lasting over time. This aspect will be analyzed by prolonging the period of follow-up of the treated patients, but the data we have obtained so far by retesting the patients 6 months after the end of treatment is promising.

Conclusions

The data from this study confirm the information in recently published papers on the effectiveness of treatment with normobaric, highly concentrated oxygen, and hyaluronic acid on vaginal atrophy. Efficacy has been confirmed both in terms of subjective symptoms and at colposcopy plus cytology.

The demonstration of the integration of hyaluronic acid in microvesicles in the cytoplasm of vaginal cells constitutes further scientific confirmation of the regenerative activity endowed by Caressflow®.

Ethics Approval

All procedures performed in this study involving human participants followed the ethical standards of the institutional, national research committee and were in accordance with the 2013 Helsinki declaration or comparable ethical standards. All the patients participated to the present study on a volunteer basis. According to the study design, the Interprovincial CN-AT Ethical Committee gave exempt to ethical approval with decision protocol number 43/2021 on the 17th of February 2021.

Informed Consent

All the patients included in the study signed an informed consent prior to the procedure and agreed to the anonymous use of clinical data for research purposes.

Funding

This research received no external funding.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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Urethral Pain Syndrome: Treatment with Oxygen and Hyaluronic Acid

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Abstract

AIM: The aim of our clinical study has been to evaluate the efficacy of the association between high concentration oxygen and hyaluronic acid (HA) for the treatment of urethral pain syndrome (UPS).

MATERIALS AND METHODS: Twenty women (45–65 years old) with suspected UPS diagnosis appealing to our Second Opinion Medical Consultation Network signed an informed consent form and were treated with oxygen/HA therapy treatment, 7 times a week, for a total of 5 weeks at the outpatient clinic (Clinic Ester Veronesi, Modena, Italy). The physicians of the Second Opinion Network followed up weekly from remote (WhatsApp, Skype) each treated patient as to state the effectiveness, tolerability, and side effects of the treatment.

RESULTS: We observed significant reductions in number of urgency urinary incontinence (–25.37 vs. –12.01 mean value), nocturia episodes (–24.01 vs. –11.23 mean value), volume voided in each micturition (–127.79 vs. –98.20 mean value), and micturitions per 24 h (–44.01 vs. –20.12 mean value). Analyses of the pre- and post-treatment scores showed a statistically significant improvement in Vaginal assessment scale, pain urgency frequency, and patient perception of bladder condition scale scores ($p < 0.0305$, $p < 0.0001$, and $p < 0.0001$, respectively). No side effects associated with the treatment were reported by the patients.

CONCLUSIONS: We can highlight that Caressflow® is effective and very well tolerated in UPS syndrome: The oxygen flow mixed with HA gives immediately a sense of freshness and urethro-bladder relaxation that lasts several hours. Further studies including larger sample sizes, placebo, and or challenge with other local and systemic treatments and different administration schedules versus longer follow-up are recommended.

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Introduction

The female urethral pain syndrome (UPS), also formerly defined urethral syndrome, is identified by recurrent urethral daily pain, emptying the bladder and nocturia, without infectious agents such as the urinalysis or any inflammatory background [1], [2], [3]. Accordingly with the European Urology Association, UPS is defined by chronic or recurrent episodic pain fixed in the urethra, lasting for more than 6 months, in the absence of proven infection or other obvious local pathology [4], [5]. Thus, it cannot be identified with the classic urethritis, rather is enclosed into the “genitourinary pain syndromes” with complaints of lower urinary tract, sexual, intestinal, or gynecological disorders [1], [5].

The exact UPS incidence and prevalence is unknown because of a lack of consensus in the method of diagnosis and overlap with other clinical conditions, including early interstitial cystitis or urethral spasm [6]. Different clinical trials confirmed that 15–30% of women with lower urinary tract symptoms were diagnosed with UPS [7], [8]. The syndrome is most frequent in women

(typically 13–70 years of age), in white women in westernized societies than in women of other races but is also seen in men and children [6], [9]. This syndrome has also a high rate of spontaneous remission [10].

According to the classification of the International Association for the Study of Pain, 2019, several concurrent causes of UPS have been identified such as vascular lesions, inflammation of skene gland or paraurethral glands (female prostatitis), urethral spasm and/or stenosis, subclinical early cystitis, oestrogen impairment, dysfunction of the pelvic floor musculature, environmental chemicals (bubble bath, soaps, contraceptive gels, Condoms *et al.*), food that can irritate the urethra (caffeine, alcohol, and spicy food), and psychological distress involving reactions of anxiety, depression, dysphoria, and even hostility, with cognitive, behavioral, sexual, or emotional consequences [4], [6], [11], [12], [13], [14], [15]. Parson [16] supposed, as UPS main cause, a dysfunction of the mucosal barrier layer, that allows to solutes/metabolites (e.g., potassium which is normally present in urine at levels that are toxic to the bladder interstitium) in the urine to seep through the urothelial barrier leading to inflammatory changes, spasm, and fibrosis. Stamm

et al., and coworkers asserted that the syndrome could be caused by a long standing low-grade bacterial infection (bacterial counts $<10^2/\text{mL}$) in the urine [17].

Symptoms: Dysuria is the commonest symptom in 40% of the UPS cases [18] but very often no real physiopathological cause can be identified and the drug therapy is based on different approaches: Anti-inflammatory painkillers, such as non-steroidal anti-inflammatory drugs (NSAIDs), local anesthetics, steroids and estrogens, antibiotics, alpha-blockers, topical urethral dilation, epitheliotropic compounds, acupuncture, antidepressants, bladder training, dietary and lifestyle changes, and rehabilitation therapy [6], [19], [20], [21]. The antibiotic treatment empirically administered in the out-patient clinics, in the absence of a true bacterial infection, should be avoided, because of the risk of resistance and toxicity [22]. In the absence of approved guidelines of UPS syndrome therapy, each specialist gives a proper prescription tailored on the psycho-physical complaints of the patient, and often a multimodal therapy gives some temporary positive results [21].

The difficulty in identifying the pathophysiology of the syndrome makes it difficult to plan an effective therapeutic strategy.

Recently, a new cosmetic and hygienic gynecological protocol gained our attention because of the synergic action of a pure oxygen flow (intended as drug delivery system) and low molecular weight hyaluronic acid (HA), a natural mucopolysaccharide sprayed over the mucosa: It penetrates into the submucosal spaces to modulate the connective tissue matrix elasticity, hydrating, and wandering cells chemotaxis.

The oxygen has a powerful regenerative, antibacterial, and anti-inflammatory effect [23]; increases the availability of oxygen to the tissues, promoting the increase in tissue repair processes and the disposal of pain and inflammation mediators (histamine, serotonin, and prostaglandins) [24]. HA has remarkable adhesive, moisturizing, and repairing properties of the mucosa [25].

The rational of UPS treatment hypothesis with Caressflow® (Caress Flow Srl, Bologna, Italy) is substantiated by the clinical study of Streltsova *et al.* [26]: Accordingly with her observation, the urethra and the bladder are embryologically and physiologically joined in “the painful bladder syndrome” [27] and the common connective tissue matrix of both the structures with its protective and trophic functions, displays a mechanosensitive signalling system [28], [29] and might be responsible of the disease. The authors in their utmost interesting paper describe also the routinary diagnostic methods, such as ultrasound (US) and uroflowmetry, but also US elastography and cross-polarization optical tomography (CP OCT); the last one seems be the most appealing method to detect UPS, on an investigational basis, being able to explain the fine neurovascular

disorder of urethra [30], [31], [32], [33], [34]. Streltsova *et al.* compared transvaginal US (TVUS) with compression elastography and CP OCT in 55 patients with UPS against 14 control healthy patients [26]. TVUS showed enlargement of the internal lumen of the upper third of the urethra. Compression elastography detected fibrotic stiffness (in urethral and periurethral tissues). The CP OCT in UPS confirmed some hypertrophy or atrophy of epithelial mucosa and increased connective tissue density [35]. The proximal urethra of UPS and the bladder neck above showed overlapping pathological changes. Oxygen together with HA is supposed to relieve the UPS symptoms acting specifically on the soft-tissue matrix, and carefully also stimulating the urethra channel with the gas flow, to help it to increase the tone and reduce its enlarged diameter [26].

The UPS clinical stages are usually defined by the classification (Urinary, psychosocial, organ specific, infection, neurologic/systemic, and tenderness of skeletal muscles) [30] and the bladder voiding diary, reaching only the diagnosis of exclusion after cystoscopy, urethroscopy, cytologic, and bacteriologic all negative findings.

As to our study, an open spontaneous trial, it has mainly been focused on the life quality score before and after Caressflow® treatment, being this original cosmetic approach never previously attempted in the UPS therapy.

Materials and Methods

The clinical study was approved by the local institutional review board and conducted in accordance with the ethical standards of the Declaration of Helsinki.

Patients

The protocol provided for enrollment of 20 women (45–65 years old) with suspected UPS diagnosis appealed to our Second Opinion Medical Consulting, from March 2019 to March 2020 (Table 1). The Second Opinion Medical Network is a consultation referral web and medical office system enclosing a wide panel of specialists, to whom any patient with any illness or syndrome not adequately satisfied with diagnosis or therapy can ask for an individual clinical audit [36], [37], [38], [39]. The UPS diagnosis was made

Table 1: Characteristics and baseline values of study population

No patients	20
Age (years)	37.2 ± 8.2
Symptoms history (months)	18
Number of micturition per 24 h (Mean ± SD)	14.01 ± 0.29
Volume voided in each micturition during 3-day micturition diary (mL) (mean ± SD)	127.79 ± 3.78
Number of nocturia episodes during a 3-day micturition diary (mean ± SD)	4.01 ± 1.45
Number of urgency episodes during 3-day micturition diary (mean ± SD)	5.37 ± 1.28

accordingly with the presence of clinical symptoms, including frequency, urgency and suprapubic discomfort, no abnormal findings at gynecological examination with negative neurologic and pelvic findings, and no inflammation at all at the urinalysis and no infections at the urine culture. The examination and enclosed bimanual pelvic examination, to evaluate the cervical motion adnexal masses and/or uterine enlargement with tenderness in each explored district; echography, cystoscopy, and urodynamic test were required to each patient by the same gynecologist, before the treatment, to exclude bacterial infections or interstitial cystitis with micturition problems.

After signed the informed consent form, all the participants answered a life quality questionnaire describing the personal history.

Inclusion and exclusion criteria

Women, age of 18-years-old or above, with self-reported UPS syndrome that had failed at least 6 months of conventional treatments, including NSAIDs, were enrolled, as well as patients complaining of pollakiuria (bladder voiding 6–8 times daily and 2–4 times nightly), painful intercourse and/or painful pollakiuria soon after. We also included women with common unpleasant symptoms (pain, pressure, and discomfort), of the urinary bladder lasting more than 6 months, in the absence of infections, bladder lesions, coagulopathy, or positive cystoscopy findings.

Criteria of exclusion

We excluded patients with interstitial cystitis, tuberculosis, and active sexually transmitted diseases (e.g., chlamydia, genital herpes, or human papilloma virus), or with positive findings of glomerulation or Hunner's ulcer, or with previous vaginal surgery or toning therapy, or with conservative pelvic floor treatment (e.g., pelvic floor exercises and estrogenic cream) in the past 6 months. We also ruled out women with urethral anatomical pathology or with neurological disorder, or psychopathology, as well as pregnant or lactating volunteers and women with diabetes, history of cancer, in chemo or radiotherapy or with obesity.

The patients accepted to undergo oxygen/HA therapy treatment, 7 times a week, for a total of 5 weeks at the outpatient clinic (Clinic Ester Veronesi, through Giardini 470, Modena, Italy).

Detailed instructions about healthy hygienic, good, and healthy nutrition, and sexual behavior were given before and then the treatment: The patient should avoid intravaginal medications, vaginal douching, and sexual intercourse within 24 h of her clinical procedure and also then it.

The physicians of the Second Opinion Medical Consulting Network followed up weekly from remote

(telemedicine), each treated patient as to state the effectiveness, tolerability, and side effects of the treatment, through WhatsApp and Skype or visit when required.

This study did not utilize a separate control group, but it compared the patient outcomes with their baseline clinical conditions through the previous attempts to be cured.

Treatment protocol

It included seven/weekly sessions (total 5 weeks) with a specific device for gynecological practice (Caressflow®, Caress Flow Srl, Bologna, Italy): An airbrush connected to the machine body, capable to carry and to spray an oxygen-HA combined solution (Figure 1). The instrument transforms the outside air into $93 \pm 3\%$ pure oxygen, by the action of zeolite molecular sieves with non-uniform electrostatic fields and absorb molecules with higher polarizability or higher quadrupole moments, such as nitrogen, argon, helium, and hydrogen [40], [41].



Figure 1: Description of the airbrush connected to the machine body

Each patient is treated, inserting the vaginal cannula equipped with outlet holes, and releasing molecular oxygen alone at a flow of 1–6 l/min (for the first 5 min) and subsequently combined with sprayed 10 mL of low molecular weight HA- (for the next 15 min). This HA has been previously dissolved in distilled water, to form a 0.2% (w/v) solution. Highly concentrated oxygen spreads easily through the vaginal mucosa, counteracting the critical hypoxia of microcirculation impairment and recovering the superficial cells metabolism. After the oxygen session, HA solution is sprayed through a special injection hole located in the upper part of the cannula (Figure 1). Due to its low molecular weight, it is easily absorbed by the mucosa preconditioned with the pure oxygen flow. The low molecular weight HA penetrates easily in the mucosa prepared by the action of oxygen.

The efficacy of the treatment was evaluated, before and after, by (1) micturition diaries and (2)

scores of three self-reported questionnaires: Vaginal assessment scale (VAS), patient perception of bladder condition scale (PPBC), and pain urgency frequency (PUF) scale. The micturition diary included 3-day S recordings of urine output per 24 h, number of urgencies, incontinence, and nocturia episodes per 24 h, intensity of urgency using the five-point URS (1 = no urgency, 2 = mild urgency, 3 = moderate urg, 4 = severe urg, and 5 = UII), and additionally the volume voided during each micturition at timeline and after the five session's treatment. The VAS questionnaire identifies the vulvar symptoms (dryness, itching, burning, and pain) and consists in 4-item questionnaires that determine the severity scale (range 0–3 points: 0 = none, 1 = mild, 2 = moderate, and 3 = severe) of dryness, soreness, irritation, and pain (dyspareunia or painfulness to touch with external stimulation) for both the vaginal and vulvar areas. In the validated PPBC scale, each participant filled in his perceived bladder condition on a 6-point scale (1 point = no problems and 6 points = many severe problems) [42]. Finally, the valid and reliable PUF scale measures urinary symptoms, for example, pain and urgency, and symptoms related to sexual intercourse, consisting of eight items related to urinary frequency daily and nightly, symptoms associated with sexual intercourse or with the bladder, and discomfort caused by these symptoms (range score = 0–35 points, a score ≥ 5 = urinary symptoms) [43].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). The data were analyzed using an unpaired t-test with Welch's correction. $p < 0.05$ was considered significant.

Results

All patients successfully completed this study. We observed significant reductions in number of urgency urinary incontinence (–25.37 vs. –12.01 mean value), nocturia episodes (–24.01 vs. –11.23 mean value), volume voided in each micturition (–127.79 vs. –98.20 mean value), and micturitions per 24 h (–44.01 vs. –20.12 mean value). There was statistically significant difference ($p < 0.0001$) between the baseline and post-treatment values in any parameter (Figure 2).

There was a decrease in all the scores evaluated at the end of the treatment compared with the pre-treatment. In fact, analyses of the pre- and post-treatment scores showed a statistically significant improvement in VAS, PUF, and PPBC scores ($p < 0.0305$, $p < 0.0001$, and $p < 0.0001$, respectively). The VAS value scale showed that pain was reduced after the treatment.

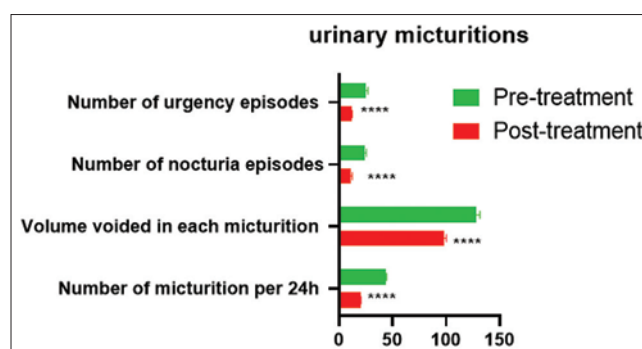


Figure 2: Urinary micturition's parameters values pre- and post-treatment. There were significant differences. **** $p < 0.0001$ pre- versus post-treatment

In fact, the mean value of VAS = 0.6 after treatment (mean value VAS, before treatment = 2.65) confirmed an improvement of symptomatology. The PPBC scores were linearly correlated before ($p < 0.0001$) and after treatment ($p < 0.0001$). At the end of therapy, 13/20 participants (65%) had PPBC-score < 10 with significant reduction of urinary urgency rate and of nocturnal micturition episodes and resolution of burning sensation. Statistical analysis showed, from baseline to 150 days of treatment, significant improvements ($p < 0.0001$) in all PPBC parameters. PUF mean value at 5 weeks after the last treatment PUF symptom scores decreased by 10.45 points ($p < 0.0001$) (Figure 3). No side effects associated with the treatment were reported by the patients. Combined oxygen therapy with HA has proven to be a valid method for treating symptoms associated with urethral syndrome. It is a totally painless therapy, with excellent compliance by patients. It is a fast, non-invasive, and repeatable treatment.

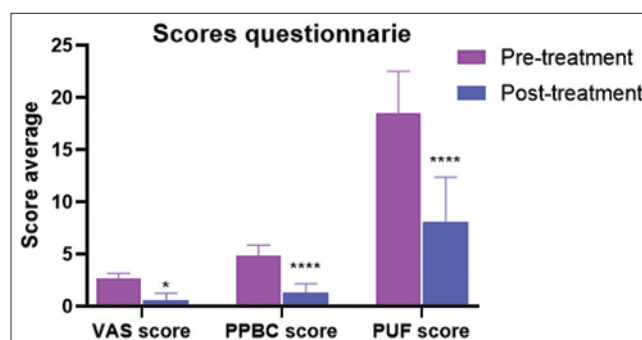


Figure 3: VAS, PPBC, and PUF values questionnaires pre- and post-treatment. There were significant differences. **** $p < 0.0001$ pre- versus post-treatment

Discussion

UPS is an hard challenge for the practicing physician due to some gaps of evidence-based treatments, and of standardized diagnostic guidelines ("diagnosis of exclusion" is mandatory). Petros *et al.* (Petros, 2010) showed that the urothelium

controls the activity of the afferent nerves, acting as a mechanoreceptor and contributing to chronic pelvic pain, typical symptom of urethral syndrome. Findings confirmed by Streltsova and coworkers that reported in UPS women thinning of urothelium and an enhance in the thickness of the connective tissue matrix of the bladder neck (Streltsova, *et al.* 2020). Furthermore, Kiseleva *et al.* confirmed that urethral tissues in UPS can be linked to chronic bacterial inflammatory processes (Kiseleva *et al.* 2020). In addition, Gittes and Nakamura affirmed that a local infection within the short urethra segment can be the cause of the syndrome, since the paraurethral gland, within the prevaginal zone, is located at 1/3 of the distance from the urethra (an identical prostatic homologue tissue in the men, stained similarly with prostate specific antigen (Gittes and Nakamura 1996); a paraurethral gland inflammation promoting tenderness in the urethra through the anterior vaginal wall might be responsible of UPS.

Different types of antibiotic therapy have been proposed for the treatment of female urethral syndrome; however, the results of these treatment methods are disappointing, as they might be not necessary or might cause side effects or induce resistance (Llor and Bjerrum 2014). Ivarsson *et al.* (Ivarsson *et al.* 2019) analyzed the UPS treatment schedules of 137 Swedish hospital clinics and showed that this syndrome is treated at all medical specialty departments (urology, gynaecology, gynecologic oncology, venereology, and primary care), with different methods: For example urologists and gynecologists primarily use local corticosteroids, alone or in combination with urethral dilation; general practitioners, oncologists, and venereologists use instead estrogens and oral/local antibiotics. In our study, the efficacy of Caressflow® was associated with a statistically significant decrease in VAS, PPBC, and PUF questionnaires at the end of treatment. These effects are due to the oxygen-HA combination: The oxygen enhances the reparative processes of the inflamed mucosa and the collagen synthesis by the hydroxylation pathway and induces a neoangiogenetic stimulation through the release of the vascular endothelial growth factor [44], [45], [46], [47]. On the other side, HA binds large amount of water molecules rehydrating and restructuring the inflammation-injured skin and mucous surfaces [48]. We recorded a high patient satisfaction grade (97%) among patients after the five sessions' therapy that declared improvement in life quality, in particular in social daily life relationships, partner relationships, and sexual intercourse.

The limitation of this study is the lack of a control arm and small sample size. Consequently, we cannot exclude error rates (Type 1 and Type 2 errors) and cannot ensure that our results may be replicated in the future research with larger sample size. However, this preliminary observation and the positive outcomes are very promising and recommend a further major evidence-based clinical trial.

Conclusions

In this study, we highlight that Caressflow® is effective and very well tolerated in UPS: The symptomatic benefit is perceived by the treated patients since the first session; the oxygen flow mixed with HA, gives immediately a sense of freshness and urethro-bladder relaxation that lasts several hours; the reasonable hypothesis explaining the action mechanism of HA across the periurethral soft tissue is that this highly hydrophilic molecule mobilizes CD44 cells into the lymphoreticular network that crosstalk with local inflammatory cells to modulate the lymphokine cascade; in the following sessions increased softness and elasticity of the urethral segment is steadily achieved, accordingly with the persisting benefit several weeks after the conclusion of the treatment schedule. In order to provide more clear-cut evidence-based data, further studies including larger sample sizes, placebo and or challenge with other local and systemic treatments, and different administration schedules versus longer follow-up are recommended.

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Vaginal supplementation of high concentration Oxygen for the treatment of vaginal infections: A pilot, controlled trial

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ABSTRACT

Background: Vaginal discomfort (VD) due to infections remains a challenge in clinical practice. Since many microbial agents grow in anaerobic conditions, a novel approach using oxygen supply has been proposed.

Objective: The aim of this pilot study was to verify the clinical effects of vaginal oxygen insufflation in women presenting with vaginal infections.

Methods: Fertile age women with a diagnosis of vaginal infections of different etiology were enrolled in a randomized, controlled, open label, monocentric trial. Women with vulvo-vaginal atrophy, amenorrhea since at least 3 months and exposure to antibiotics in the previous 30 days were excluded. Gynecological examination and vaginal swabs were performed. Women were randomized to 3 treatment groups. Group A received vaginal normobaric, high concentration oxygen supply (O₂ concentration >95%, 3 lt/min for 20 minutes), twice/week for 2 weeks; group B received vaginal oxygen as Group A added to a targeted antibiotic or antimycotic treatment for 5-7 days; Group C received only the targeted antibiotic or antimycotic treatment for 5-7 days. Subjects rated vaginal discharges, vulvovaginal itching/burning and dyspareunia before and 10-14 days after end of treatment on a Likert scale. VD score was obtained with the sum of single symptoms.

Results: Out of 36 randomized women 32 were analyzed. Bacterial vaginosis ($n=14$), candidiasis ($n=7$) and aspecific vaginal infections ($n=11$) were equally distributed among groups. Baseline VD, as well as single symptom scores were not different among groups. At the end of the treatments, the VD score was significantly reduced by $89.2 \pm 7.1\%$ in Group A, by $85.1 \pm 10.1\%$ in group C and to a lesser extent by $52.8 \pm 9.2\%$ in group B, with significant reduction occurred in single symptoms.

Conclusions These findings demonstrate that the vaginal supply of high concentration oxygen has proven efficacy in patients presenting with vaginal infections. The effect is similar to the one obtained through targeted antimicrobial therapy.

KEYWORDS

Oxygen treatment, vaginal infections, vaginosis, candidiasis.

Introduction

During reproductive life vaginal discomfort (VD) is frequently claimed by women as a factor worsening their quality of life. VD is mainly induced by vaginal discharges, burning and itching of genitalia, and it represents the primary reason for gynecological consultation, in different medical settings [1]. Causes of VD are recognized under the umbrella diagnosis of vaginal infections which could be sustained by bacteria, fungi and protozoa local infection. Bacterial vaginosis (BV) is the most prevalent while either vulvovaginal candidiasis (VVC) and *Trichomonas* infections account for the vast majority of the remnant cases [2].

Although BV can be efficaciously treated with proper antimicrobial agents, metronidazole representing the gold standard [3], this condition is characterized by frequent relapses and the recurrence of VD requires different approaches. Among them, pH changes and biofilm disruption have provided moderate quality of evidence in terms of benefit, in addition to either barrier or hormone contraception [4].

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On the other hand, few attention has been focused on the fact that most of the bacterial species sustaining BV (*Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera*, *Prevotella* and *Sneathia*) are classified as anaerobia bacteria since their proliferation could take place only in the absence of oxygen. Indeed, the presence of oxygen in the environment induced a block of bacterial growth [5]. Although *Lactobacillus spp* are included among anaerobia, they are still aero-tolerant and insensitive to oxygen exposure [6].

Vaginal oxygen supplementation has recently been proposed as a treatment for some gynecological disorders. Once coupled with hyaluronic acid, cycles of vaginal oxygenation gave definite therapeutic benefits in a cohort of patients with relapsing episodes of VVC [7]. Similarly, the same combo improved vaginal atrophy in postmenopausal women [8] as well as sexuality and urinary symptoms in breast cancer survivors, both in fertile or postmenopausal age [9].

On these grounds, we performed a pilot, randomized, controlled trial aiming to verify the effects of vaginal oxygen supplementation in a group of women affected by VD due to vaginal infections.

Methods

This was a pilot, randomized, controlled, open label, monocentric trial in which women of fertile age presenting for VD were screened and those reaching a diagnosis of vaginal infections were enrolled, after informed consent was obtained. Inclusion criteria were: age 18-45 years, diagnosis of vaginal infections of different etiology, ability to fill-in the symptom's sheet and the comprehension of the informed consent sheet. Exclusion criteria were: vulvo-vaginal atrophy, amenorrhea since at least 3 months and exposure to antibiotics in the previous 30 days.

Each woman received a general physical and gynecological examination at entry to the study, together with a vaginal swab. Moreover, the subjects rated their VD on a Likert scale evaluating vaginal discharges, vulvovaginal itching/burning and dyspareunia. Once inclusion and exclusion criteria were met, subjects underwent a randomization (1:1:1) in three different groups of intervention. Randomization was managed by a midwife who used sealed envelopes containing treatment allocation that was performed through a random series of numbers. Treatment was disclosed after the patient's consent was signed.

Group A received vaginal high concentration oxygen supply (O₂ concentration >95%, 3 lt/min for 20 minutes), twice/week for 2 weeks; group B received vaginal oxygen as Group A added to a targeted antibiotic or antimycotic treatment for 5-7 days; and Group C (control group) receive the targeted antibiotic or antimycotic treatment for 5-7 days.

The oxygen treatment was carried out using the Caress Flow system (Caress Flow - Bologna, Italy), an oxygen therapy device for gynecological use that allows the topical administration of oxygen through a vaginal cannula with a high degree of purity and a flow rate of 3 lt/min.

Both vaginal swab and symptoms ratings were performed 10-14 days after the end of treatment. Women self-rated vaginal itching/burning, vaginal discharges and dyspareunia on a Likert scale from 0 (absent) to 10 (maximal discomfort), as performed at entry in the study.

The primary outcome was a composite VD score obtained from the sum of vaginal discharge, vaginal itching/burning and dyspareunia. Secondary outcomes were the individual components of the VD score.

Due to the explorative nature of the study, the sample size was determined *a priori* as being composed by 36 women, 12 each group.

Statistical analysis was performed by comparing Groups through parametric and non-parametric tests when appropriate, according to the distribution of data. Percent variations were also calculated and compared through ANOVA. A *p* value of <0.05 was considered statistically significant.

Results

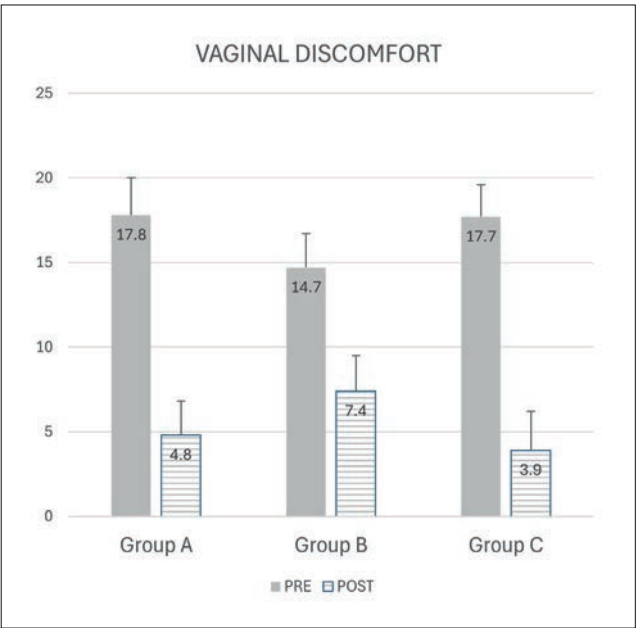
Out of 36 randomized women 32 were analyzed. Two women immediately retired consent and further two were lost after first contact not presenting to follow-up.

The clinical features were not different among the randomized women (Table 1). At baseline, the total VD score was similar among the 3 groups (Figure 1). Similarly, there were no differences in the rating of baseline symptoms such as vaginal itching/burning (Kruskall-Wallis [KW]=1.696, d.f. 2.32; *p*=0.428), vaginal discharge (KW = 1.266, df. 2.32; *p*=0.531 and dyspareunia (KW=0.500, d.f. 2.32; *p*=0.779) among the 3 groups.

Table 1. Clinical characteristics of the patients participating in the 3 groups of treatment (Data are presented as mean ± standard deviations or number of cases.

	Group A Oxygen (n=11)	Group B Oxygen + Antimicrobial therapy (n=11)	Group C Antimicrobial therapy (n=10)
Age (years)	31.3 ± 3.1	33.6 ± 4.2	32.2 ± 2.9
Household	2	3	3
Education > 8 years	7	8	6
Aspecific Vaginal infections	5	3	3
Vaginal Candidiasis	2	3	2
Bacterial vaginosis	4	5	5

Figure 1. Vaginal Discomfort (VD) score changes (mean ± standard deviation) before and after treatments in women randomized to the three groups described in the text.



VD was significantly reduced by treatment in all the 3 groups (Figure 1). Indeed, VD score was significantly reduced by $89.2 \pm 7.1\%$ in Group A, by $85.1 \pm 10.1\%$ in group C and to a lesser extent by $52.8 \pm 9.2\%$ in group B.

Vaginal symptom scores before and after treatment are presented in Table 2. Vaginal discharge and itching/burning score were significantly reduced in every group after treatment. Dyspareunia was not significantly reduced in women receiving both oxygen and antimicrobial therapy (Table 2).

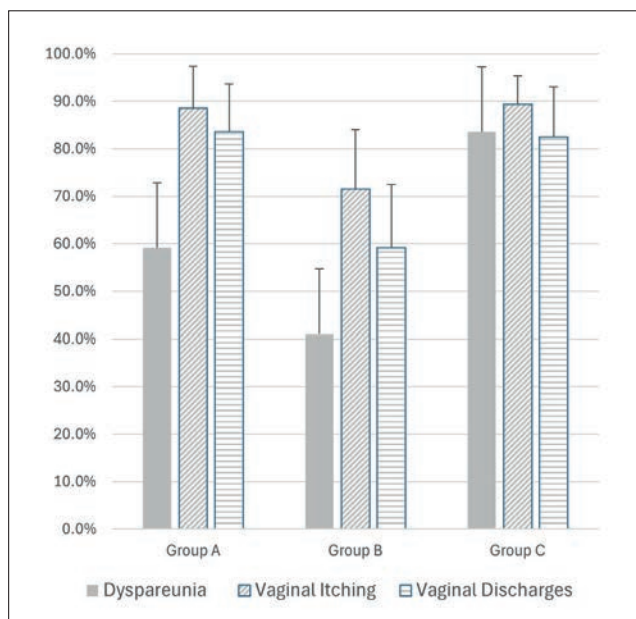
Table 2. Vaginal symptom scores before (top value) and after treatment (below) in the three groups.

Vaginal symptom	Group A Oxygen (n=11)	Group B Oxygen + Antimicrobial therapy (n=11)	Group C Antimicrobial therapy (n=10)
Vaginal discharges	5.82 $\pm$ 2.96 1.27 $\pm$ 2.41 $p=0.005$	4.64 $\pm$ 3.64 1.82 $\pm$ 2.27 $p=0.018$	6.60 $\pm$ 1.90 1.30 $\pm$ 2.83 $p=0.007$
Vaginal itching	6.09 $\pm$ 2.81 1.18 $\pm$ 2.31 $p=0.005$	5.00 $\pm$ 2.79 2.09 $\pm$ 3.39 $p=0.023$	6.10 $\pm$ 2.73 1.10 $\pm$ 2.23 $p=0.005$
Dyspareunia	5.91 $\pm$ 3.02 2.36 $\pm$ 2.50 $p=0.008$	5.09 $\pm$ 3.36 3.46 $\pm$ 3.61 $p=0.058$	5.00 $\pm$ 3.43 1.50 $\pm$ 2.51 $p=0.011$

Data are presented as mean $\pm$ standard deviations. Statistically significant differences within group are reported (Wilcoxon test).

Percent changes (mean $\pm$ standard deviation) of assessed vaginal symptoms occurring in women after treatment in groups A, B and C are presented in Figure 2. No significant differences were demonstrated.

Figure 2. Percent changes (mean $\pm$ standard deviation) of assessed vaginal symptoms occurring in women after treatments in groups A, B and C. No significant differences were demonstrated.



Discussion

This pilot, controlled trial demonstrated that vaginal oxygen delivery is associated with a reduction of the main vaginal discomfort symptoms, i.e. discharges, itching as well as dyspareunia, in women affected by vaginal infections of diverse etiologies. The efficacy extent of vaginal oxygen was not different respect to the standard antimicrobial approach alone or in combination with oxygen.

In the present series the most part of the patients either presented with BV or with candidiasis. Our data confirmed that targeted treatment (antibiotics or antimycotics) is able to reduce symptoms and restore vaginal health [10]. However, literature has reported that there are several limitations in the use of antimicrobial agents. Firstly, vaginal infections frequently relapse, hence requiring recurrent treatments, with over exposure to the drugs [11]. Secondly, the use (and abuse) of these agents is associated with the development of microbial resistances that now become a public health issue on a global scale [12].

For the above reasons it is of paramount importance to look for alternative treatments. Among tested interventions, there are pH modulators such as lactic acid <[13], with few, if any effects, antiseptics like boric acid [14], also with few, if any effects), changing diet [15], with few benefits, and probiotics. These last products have gained attention in the last years when added after the antimicrobial agent [16], or alone [17,18]. Unfortunately, results are still heterogeneous to guide an evidence-based approach being closely depending on the quality and quantity of bacterial strains of each product.

Anaerobic bacteria, namely those sustaining BV, are naturally vulnerable to oxygen. Indeed, aerobiosis stimulates the formation of reactive oxygen species, interferes with the redox processes necessary for their physiology and ultimately arrest their proliferation [19]. For those reasons, hyperbaric oxygen was established as a treatment in cases of clostridial myonecrosis, necrotizing soft-tissue infections, myocarditis and so on [20]. Moreover, oxygen promotes tissue repair, thus stimulating angiogenesis through vascular endothelial growth factor [21].

Just recently the interest towards a local oxygen treatment has arisen. In vitro, antimicrobial oxygen activity on *P. gingivalis* has been reported in patients affected by periodontitis where the treatment could foster eubiosis [22]. On the other hand, the application of local normobaric high concentration oxygen through vaginal insufflation was previously reported useful for relieving the urogenital syndrome once associated with hyaluronic acid, in postmenopausal breast cancer survivors [9].

In view of previous clinical experiences and experimental observations we could hypothesize that oxygen action is exerted at different levels, i.e. inhibition of microbial growth, removal of biofilms and promotion of reparative processes.

This study has some limitations inherent to the small sample and to the fact the sample size was not calculated due to the pilot nature of the investigation. Moreover, different types of vaginal infections were included not allowing to draw conclusion inherent to each one of them. However, we could foresee that oxygen effect would be better exploited in cases of BV to avoid relapses.

In conclusion, the efficacy and tolerability of vaginal oxygen insufflation in the treatment of vaginal infections is here reported for the first time. The clinical short-term effect is not different to the conventional antimicrobial approach. Larger studies with longer follow-up are required to confirm these first pilot observations.

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Caress Flow (Bologna, Italy) provided hardware as well as disposable material for the oxygen treatments.

Conflicts of interest

FF received a consultancy grant.

Innovative Approaches to Postpartum Urogenital Health: A Pilot Study on the Therapeutic Effects of Topical Oxygen and Hyaluronic Acid

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Abstract

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BACKGROUND: The postpartum period challenges women with a variety of urogenital disorders, which significantly impact their physical and psychological well-being. Traditional treatments often fall short in addressing the complexities of these conditions, which include urinary incontinence and sexual dysfunction.

AIM: This study explores the effectiveness of a novel treatment—high-concentration oxygen combined with hyaluronic acid—aimed at improving postpartum urogenital health.

METHODS: This clinical study enrolled 41 postpartum women aged 18 to 42, undergoing a four-week treatment regimen with high-concentration oxygen combined with hyaluronic acid. This device delivers high-concentration oxygen and hyaluronic acid directly to the vaginal area. The severity of symptoms such as vulvar laxity, urogenital triangle lowering, and urethral orifice laxity was assessed at baseline and post-treatment using a four-point scale.

RESULTS: Treatment resulted in significant improvements in all targeted symptoms, with the most notable reductions observed in muco-epithelial inflammation scar (60% reduction) and vulvar laxity (54% reduction). These improvements were statistically significant ($p < 0.0001$), indicating a robust response to the therapy. The study also noted enhancements in general well-being, although these were not statistically significant.

CONCLUSIONS: The treatment with high-concentration oxygen and hyaluronic acid offers a promising new approach to postpartum rehabilitation. By significantly reducing key symptoms of urogenital dysfunction, this treatment has the potential to enhance the quality of life for postpartum women. Future studies should investigate long-term outcomes and the integration of this therapy into broader postpartum care practices.

Introduction

The postpartum period presents unique health challenges for women, prominently featuring urogenital disorders such as urinary incontinence and sexual dysfunction. These conditions not only affect physical health but also impact psychological well-being and quality of life.

Sexual health, crucial for the quality of life of women, is defined by the World Health Organization as "a state of physical, emotional, mental, and social well-being related to sexuality; it is not merely the absence of disease, dysfunction, or infirmity". Considering sexual health as an indicator of well-being, it is possible that sexual dysfunction may be a consequence of maternal morbidity.

Vaginal and perineal pain are predominantly considered nociceptive pain, but neuropathic pain due to obstetric trauma, such as pudendal neuropathy, is

also a possible contributor. Perineal and vaginal pain after vaginal delivery is associated with tissue trauma related to operative vaginal delivery, perineal laceration, and the use of episiotomy, although some studies report its occurrence even after cesarean delivery with or without labor.

Sexual activity typically resumes six to eight weeks postpartum [1]. However, the return to regular sexual activity often only begins to stabilize six months postpartum. This delayed resumption can be attributed to both physical discomforts, such as pain and fatigue, and psychological factors including anxiety and altered body image perceptions post-delivery. These challenges highlight the need for a more open discussion and proactive management of sexual health in postpartum care.

Moreover, as outlined by Fallowfield [2] the quality of life is a crucial measure that should encompass all aspects of health, including sexual health. The neglect of sexual health issues can lead to

long-term consequences, affecting a woman's quality of life and well-being. Studies, such as the systematic review by Jawed-Wessel and Sevvick, have shown that pregnancy and childbirth profoundly impact sexual behaviors, yet the topic remains under-researched and under-discussed in clinical settings.

In addition to the hesitancy in discussing sexual health issues, many women experience significant psychological distress due to changes in their sexual function and body image postpartum. This distress can be compounded by the lack of information and guidance provided on navigating these changes, underscoring the necessity for healthcare providers to initiate conversations about sexual health as a routine part of postpartum care.

Other studies [3], [4] highlighted the high prevalence of urinary incontinence postpartum, affecting up to 50% of women, with significant repercussions on sexual function and overall life satisfaction. In most cases, urinary incontinence appears for the first time during pregnancy and persists into the postpartum period. Urinary incontinence during pregnancy, multiparity, and constipation are risk factors for the persistence of incontinence.

Furthermore, Diz-Teixeira et al. [5] emphasized the effectiveness of physiotherapy in managing urinary incontinence, suggesting that targeted treatments can significantly alleviate symptoms and improve recovery. Despite these advances, many women continue to experience persistent symptoms that are inadequately addressed by conventional therapies.

In response to these challenges, innovative treatments have been explored to provide more effective solutions. One such approach is the concomitant administration of hyaluronic acid and topical oxygen therapy (TOT) using a specifically designed medical device, the Vaginal Natural Oxygenation Device (VNOD). This pilot study [6] evaluated the effectiveness of VNOD in improving the symptomatology of vulvo-vaginal atrophy (VVA) in postmenopausal women—a condition that shares common pathophysiological features with postpartum urogenital disorders.

The VNOD method integrates hyaluronic acid's moisturizing and epithelial barrier enhancing properties with the regenerative capabilities of high concentration oxygen, promoting tissue repair and reducing symptoms such as vaginal dryness, burning, and itching [7], [8]. The study demonstrated significant improvements in vaginal health metrics, suggesting a promising approach for extending these benefits to postpartum women suffering from similar urogenital issues.

Given the parallels between postmenopausal VVA and postpartum urogenital disorders, our research applies the VNOD methodology to a postpartum cohort, hypothesizing that it could similarly alleviate symptoms

and improve the quality of life in this demographic. This study aims to fill the gap in effective postpartum care solutions, providing evidence for a potentially transformative approach to managing postpartum urogenital health.

The aim of the study was to observe the effects of treatment with high concentration oxygen and hyaluronic acid on patients with the following gynaecological post-partum conditions:

- Vulvar laxity;
- Urogenital triangle lowering;
- Urethral orifice laxity;
- Muco-epithelial inflammation scar;
- Mucosal redness;
- Stress urinary incontinence episodes;
- Well-being;
- Burning;
- Dyspareunia;
- Scar pain.

Materials and Methods

The clinical study was approved by the local institutional review board and conducted in accordance with the ethical standards of the Declaration of Helsinki.

Forty-one women between the ages of 18 and 42 who had given birth four to five months earlier were enrolled in the study.

Patients with vulvar dermatological changes, signs of vulvovaginal infections, or pre-neoplastic/neoplastic changes in the lower genital tract were excluded from the study.

Treatment

Treatment was performed by an appropriate device (CaressFlow Srl, Bologna, Italy) recently commercialized for the treatment of VA in postmenopausal women.

The device consists of a compressor that produces compressed air by taking in air from the outside, filtering it and compressing it. Inside the body of the machine, there are zeolite molecular sieves that use the principle of different absorption of gas molecules, allowing O₂ to pass and retaining the other gases present in the air, such as nitrogen, argon, helium and hydrogen. The body of the machine transforms the outside air into 93 ± 3% pure oxygen at a flow rate of 2 l/min.

The first part of the treatment is carried out by an airbrush connected to the machine body (Figure 1A). The airbrush is used for the treatment of external genitalia, nebulizing the combination of oxygen and hyaluronic acid (0,2% solution) for 5 minutes.

The second part of the treatment consists of the use of a vaginal cannula (Figure 1B) through which a flow of pure oxygen is delivered for 5 minutes, followed by the simultaneous delivery of oxygen and hyaluronic acid for 10 minutes.

The same 20-min session was repeated every week four times.

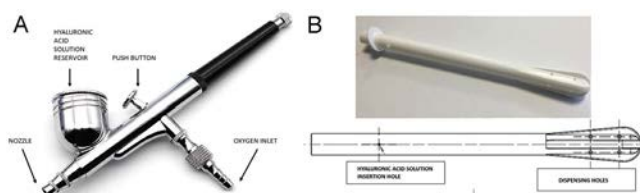


Figure 1: Airbrush (A) and vaginal cannula (B)

Evaluation

At visit 0, an accurate collection of patient history and a complete clinical examination was performed to evaluate eligibility criteria. The intensity of the following symptoms:

- Stress urinary incontinence episodes;
- Well-being;
- Burning;
- Dyspareunia;
- Scar pain;

were collected from patients with a VAS based on a four-point scale: score 1 = absence of symptom, score 2 = weak intensity, score 3 = mild intensity, score 4 = maximum intensity.

Before the treatment, the following symptoms:

- Vulvar laxity;
- Urogenital triangle lowering;
- Urethral orifice laxity;
- Muco-epithelial inflammation scar;
- Mucosal redness;

were determined by the clinician and evaluated on a four-point scale: score 1 = absence of symptoms, score 2 = weak intensity, score 3 = mild intensity, score 4 = maximum intensity.

All patients were assessed for the presence of symptoms from the second treatment session, with follow-up at 7 days after the end of treatment, for a total of 4 assessments. This study did not utilize a separate control group, but it compared the patient's outcomes with their baseline clinical conditions.

Statistical Analysis

Statistical analysis was performed using IBM SPSS version 22.0 (IBM Corporation, Armonk, NY, USA). The significance of pairwise comparisons between treatment sessions was analyzed using the

Wilcoxon test for non-parametric data. Two-sided p-values < 0.05 were considered statistically significant.

Results

The results showed a significant improvement in all mean scores analyzed (Figure 2), except for well-being, which was not significant. The analysis of the comparisons between the different phases of therapy has shown that each subsequent treatment determined a significant improvement in all parameters.

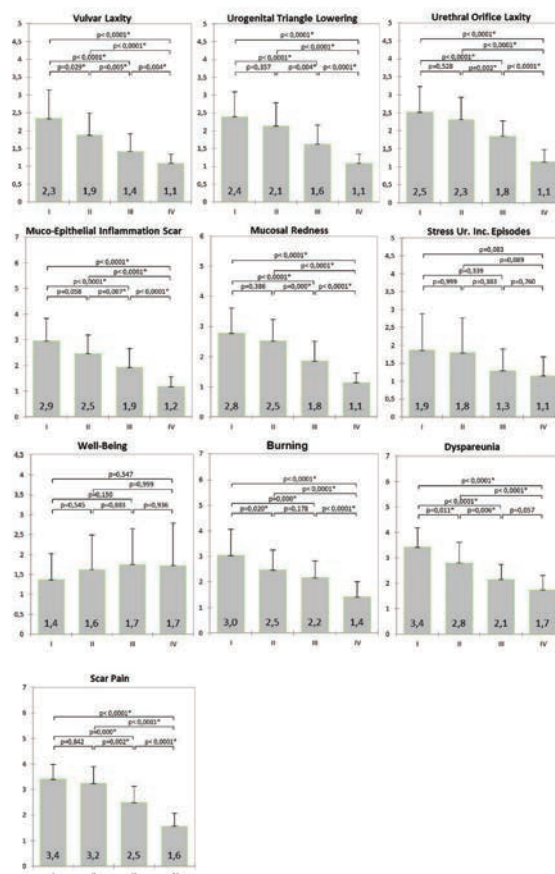


Figure 2: Analysis of Symptom Improvements in Postpartum Urogenital Health. *Significant at level alpha = 0.05

Figure 3 illustrates the percentage changes in various postpartum urogenital symptoms experienced by participants after undergoing the specified treatment. The percentages reflect the extent of symptom improvement or deterioration, with negative values indicating a reduction in symptom severity and positive values indicating an increase.

For all the parameters analysed, except for well-being, which showed a non-significant increase of 21%, the reduction in symptoms ranged from 38% for stress urinary incontinence episodes to 60% for muco-epithelial inflammatory scar.

All patients successfully completed this study. The therapy was well accepted by patients with immediate therapeutic effects and without side effects.

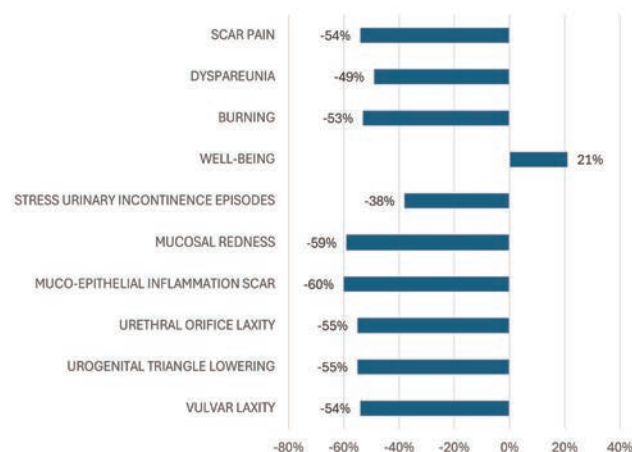


Figure 3: Mean Percentage Changes in Postpartum Urogenital Symptoms Following Treatment

Discussion

The treatment of postpartum complications holds a critical place in enhancing the quality of life and ensuring the psychological well-being of new mothers. The transitional phase postpartum presents numerous health challenges that, if inadequately addressed, can lead to significant long-term physical and psychological issues.

Rehabilitation during the postpartum period, therefore, is not merely a healthcare routine but a crucial intervention that ensures the holistic recovery of women.

Postpartum rehabilitation encompasses a diverse spectrum of practices and techniques aimed at addressing the multifaceted health issues faced by women after childbirth. These issues range from physical injuries such as perineal tears to emotional and psychological stresses that can impact a woman's return to her pre-pregnancy state. Each woman's journey through postpartum recovery is unique, influenced by the nature of the delivery, the presence of pre-existing conditions, and her overall health.

The rehabilitation process is inherently multidisciplinary, involving obstetricians, physiotherapists, psychologists, and other specialists who work together to tailor rehabilitation plans that address individual needs effectively. Techniques used in postpartum rehabilitation include but are not limited to physical therapy for strengthening pelvic floor muscles, psychological counseling to address postpartum depression and anxiety, and nutritional counseling to help women recover from dyspareunia, or painful intercourse, is a common postpartum complaint. The condition can stem from natural changes during childbirth, such as hormonal fluctuations and physical

injuries like perineal tears and episiotomies. Multidisciplinary approaches to treat dyspareunia involve physical therapies that focus on pelvic floor muscle training, psychological counseling to address anxiety associated with painful intercourse, and sometimes medical interventions such as topical estrogen therapies. These interventions aim to not only alleviate pain but also improve the quality of sexual relations, which is crucial for emotional bonding and personal well-being [9].

Intravaginal transcutaneous electrical nerve stimulation (TENS) and pelvic floor muscle training are proven to be effective in reducing symptoms of pelvic floor dysfunction, including urinary incontinence and pelvic pain. These methods enhance muscle strength and alleviate symptoms by improving neuromuscular control and reducing inflammation and pain through nerve stimulation [9]. [10].

This involves exercises aimed specifically at strengthening the pelvic floor muscles which are crucial for urinary continence and sexual function. Rehabilitation starts with simple exercises and progressively increases in intensity as the patient recovers. This method has been shown to be essential, especially in women experiencing persistent symptoms three months postpartum [11].

Against the backdrop of traditional therapies, the innovative treatment involving high-concentration oxygen and hyaluronic acid represents a significant advancement. This combination therapy leverages the healing properties of oxygen and the moisturizing effects of hyaluronic acid to promote tissue regeneration and repair. The introduction of this treatment has marked a novel approach in postpartum care, offering rapid amelioration of symptoms like vaginal dryness, inflammation, and pain, which are often significant barriers to a woman's postpartum recovery.

Recent studies have documented the effectiveness of this treatment in significantly improving the symptoms associated with postpartum urogenital disorders. For instance, clinical trials have shown marked improvements in vaginal health, with reductions in dryness, inflammation, and pain within just four weeks of treatment initiation. The implications of these findings are profound, suggesting that combining high-concentration oxygen with hyaluronic acid can expedite the healing process, thereby reducing the overall recovery time and improving the quality of life for new mothers [9], [12], [13].

Moreover, the broader implications of these improvements extend beyond physical health, influencing psychological and emotional well-being. Enhanced recovery from physical symptoms facilitates better overall mood, reduces stress, and improves the capacity for mothers to bond with their infants and resume their social and professional lives.

The rehabilitation of pelvic floor health postpartum is crucial for restoring function and enhancing quality of life, particularly in addressing conditions like vulvar laxity, urogenital triangle lowering, and urethral orifice laxity. The treatment involving high-concentration oxygen and hyaluronic acid has demonstrated significant improvements across these symptoms, which are often overlooked in typical postpartum care. As illustrated in our results, each of these conditions showed a marked improvement over the course of the treatment, which can be attributed to the regenerative properties of the therapy employed.

Vulvar laxity, which affects the structural integrity and functionality of the vulvar region, showed a progressive decrease in symptom severity across the treatment phases. This improvement is critical as it directly enhances the structural support of the pelvic floor, contributing to better overall pelvic health and functionality. Similarly, the lowering of the urogenital triangle, a common issue that can lead to discomfort and urinary incontinence, was significantly mitigated, suggesting an effective restoration of the pelvic anatomy, which is essential for maintaining urinary continence and sexual function [14].

Moreover, the urethral orifice laxity, which can significantly impact urinary control and sexual satisfaction, also saw considerable improvements. By reducing laxity in this area, the treatment helps in tightening the urethral closure mechanism, thereby enhancing urinary continence and reducing the risk of related complications. The statistical significance of these improvements ($p < 0.0001$) across the initial and final phases of treatment underscores not only the efficacy but also the potential of this therapeutic approach to serve as a cornerstone in postpartum pelvic rehabilitation [15].

These findings are pivotal as they provide empirical evidence supporting the use of high-concentration oxygen and hyaluronic acid in the targeted rehabilitation of the pelvic floor postpartum. By addressing these specific aspects of pelvic health, the treatment offers a comprehensive approach that significantly improves the structural and functional recovery of the pelvic floor, which is vital for the long-term health and well-being of postpartum women [12].

Despite the promising results, the adoption of high-concentration oxygen and hyaluronic acid treatment in routine postpartum care faces challenges. These include variability in treatment response, the need for specialized equipment, and potential accessibility issues. Future research should focus on addressing these challenges, exploring the long-term effects of the treatment, and expanding its application to a broader range of postpartum conditions.

Conclusions

In summary, the integration of high-concentration oxygen and hyaluronic acid into postpartum rehabilitation protocols offers a promising avenue for enhancing maternal health care. As evidence of its efficacy grows, it is imperative for healthcare providers to consider these innovative treatments as part of standard care practices to ensure that every woman can achieve the best possible postpartum recovery.

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3.2. SUBMITTED STUDIES

Evaluation of Topical Administration of Low Molecular Weight Hyaluronic Acid (LMWHA) Solution and High Concentration Oxygen (HCO) versus Hyaluronic Acid (HA) Alone in the Improvement of Urinary Incontinence: A Double Center Retrospective Study

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Keywords: hyaluronic acid and high concentration oxygen; menopause; genitourinary syndrome; stress incontinence; mixed urinary incontinence



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Article

Evaluation of Topical Administration of Low Molecular Weight Hyaluronic Acid (LMWHA) Solution and High Concentration Oxygen (HCO) versus Hyaluronic Acid (HA) Alone in the Improvement of Urinary Incontinence: A Double Center Retrospective Study

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Abstract: Objectives: To rate the effects of low molecular weight hyaluronic acid (LMWHA) solution and high concentration oxygen (HCO) through a specific medical device (Caress Flow®), compared to topical administration of hyaluronic acid (HA) alone on symptoms of mild and moderate stress and mixed urinary incontinence in women with genitourinary syndrome of menopause (GSM). **Methods:** A total of 68 postmenopausal women were enrolled (from October 2021 to September 2023). Patients were separated in two groups: one group used therapy with LMWHA solution and HCO combined and the second group used therapy with HA alone. Patient Global Impression of Improvement (PGI-I) as the first outcome was used. As secondary outcomes International Consultation on Incontinence Questionnaire - short form (ICIQ-SF) questionnaire and International Quality of Life (IQoL) questionnaire were used. **Results:** Sixty-eight (68) women, 35 (51%) and 33 (49%), were treated with LMWHA solution and HCO combined and HA alone for 10 weeks, respectively. 28 (80%) versus 7 (20%) women reported an improvement (PGI-I score ≤3) of the symptoms in LMWHA solution and HCO combined group versus HA alone group. According to the ICIQ-SF, a statistically significant difference was recorded after treatment between the two groups (median, 7 [5–11] vs 10 [8–11]; $P = 0.03$). According to the IQoL questionnaire, a statistically significantly lower median score was recorded in the LMWHA solution and HCO combined compared with the HA group, before (71 [IQR 55–81] vs 89 [IQR 67–94]; $P < 0.01$) and after (78 [IQR 65–86] vs 88 [IQR 72–99]; $P = 0.04$) treatment. **Conclusions:** LMWHA solution and HCO combined might improve the mild and moderate stress and mixed urinary incontinence in this set of women and that it is superior to HA therapy alone. However, these results need to be confirmed in further studies with a controlled design and a larger population.

Keywords: hyaluronic acid and high concentration oxygen; menopause; genitourinary syndrome; stress incontinence; mixed urinary incontinence

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1. Introduction

“Genitourinary Syndrome of Menopause” (GSM) is a chronic condition characterized by the set of signs and symptoms associated with absence of estrogens (ER) and other sex steroids caused by the cessation of ovarian function, involving lower genital structures (labia majora/minora, clitoris, vestibule, vagina and vulva) and the lower part of the urinary tract (specifically bladder trigone and proximal urethra) [1]. GSM is a common disorder (affecting approximately 50 to 70% of post-menopausal women), but at the same time it is frequently under-diagnosed and consequently under-treated. Symptoms related to GSM, especially those at the vulvo-vaginal level, interfere with sexual satisfaction (74%), sexual spontaneity (70%) and relationship with one's own partner (66%) [2], therefore they are the main cause of discomfort in sexual intercourse, which consequently are often avoided, with further worsening of the condition atrophy and consequent negative impact on the couple's relationship, with a strong impact on life quality [3]. In Italy the prevalence is comparable to the average of other European countries. In “The AGATA Study” by F. Palma and collaborators, 79.1% of post-menopausal women involved in the study is affected by GSM, with a prevalence varying from 64.7% one year from menopause, to 84.2% in the following six years [4]. Hypoestrogenism following menopause represents the main pathogenetic cause of GSM, but there are several concomitant risk factors [5]. Regarding the pathophysiology of urinary signs and symptoms of GSM, it was seen that the urethra becomes atrophic and prominent with a relative increase of transitional cells and a reduction of intermediate and superficial squamous cells. There is a remodeling of the bladder with reduction of the tropism of smooth muscle tissue and collagen. This involves a reduction in the contractile force of the detrusor, bladder capacity and sensitivity, urethral closure pressure and urinary flow [6,7]. The absence of ER receptors at the trigone level determines a reduction in the bladder sensory threshold with a reduction in pressure to the point of leakage, leading to urgency incontinence and bladder hyperactivity [8]. Furthermore, the loss of collagen is also responsible for the greater laxity of the structures support of the pelvic floor thus contributing to the genesis of the clinical pictures of prolapse genitals and urinary incontinence characterizing GSM. There are currently various therapeutic options to choose, based on the patient's clinical history and the severity of the symptoms: non hormonal therapy, hormonal therapy, and laser therapy [9]. Several studies have highlighted how topical oxygen therapy is useful in counteract inflammatory dermatological diseases, androgenic alopecia, and dry eyes [10–12]. In the literature it is indicated that oxygen, in addition to having an anti-inflammatory and antibacterial, would appear to induce the stimulation of collagen synthesis and would promote hydroxylation. Increasing oxygen could promote release of VEGF (Vascular Endothelial Growth Factor)², increasing the angiogenic stimulus and tropism of tissues [13,14]. Hyaluronic acid is a polymer that is normally found in the connective tissue known for its supporting, hygroscopic, re-epithelizing, viscoelastic, antioxidant, bacteriostatic and anti-inflammatory. Its administration has proven effective in improving dryness, dyspareunia and tropism of the vaginal epithelium in women affected by GSM has also proven to be a valid alternative to conjugated estrogen therapy also having effects on the symptoms of incontinence [15].

2. Materials and Methods

2.1. Study Design

The current study is a double center, retrospective study conducted at the gynecological clinic of the Hospital “Pugliese-Ciaccio” in Catanzaro and “Umberto I ” in Enna. The protocol was approved by the Ethical Committee of the Calabria Region (nr 342, October 2021). Menopausal patients suffering from genitourinary syndrome of menopause with mild and moderate stress and mixed urinary incontinence were enrolled from October 2021 to September 2023. Patients were divided into two groups. The first group received combined therapy with low molecular weight hyaluronic acid (LMWHA) solution and high concentration oxygen (HCO) through a specific device (Caress Flow©). The second group had received topical daily therapy with vaginal tablets of hyaluronic acid (HA) for 10 weeks daily.

2.2. Inclusion Criteria and Validated Questionnaire

We enrolled only women with mild or moderate stress and mixed urinary incontinence, after verifying inclusion criteria (good general health; aged between 45 and 70 years; natural or surgical menopause at least 12 months before enrollment, absence of lower urinary tract infections (UTI); absence of pre-existing prolapse; absence of incontinence due to neurological damage; absence of neoplasm affecting the genitourinary system; absence of contraindications to local therapy with high oxygen concentration and hyaluronic acid) and exclusion criteria (prolapse; incontinence due to neurological damage; genitourinary cancer; previous radio/brachytherapy; systemic intake of estrogens, progestins, androgens, selective modulators of estrogen receptor (SERM), intrauterine progestins or vaginal lubricants and moisturizers within 8 weeks, alternatively taking transdermal or vaginal hormonal products within 4 weeks; signs and symptoms of severe urinary incontinence or absence of signs and/or symptoms of urinary incontinence).

The patients were selected based on the score obtained on the questionnaires administered during the first outpatient visit. The questionnaires that we used were approved by the International Consultation on Incontinence (ICI), validated for the Italian language. With the Overactive Bladder Screener (OAB-S) we evaluated the presence of urge incontinence symptoms and with the International Consultation on Incontinence Questionnaire- Short Form (ICIQ-SF) we identified the frequency and entity of urinary leakages, how much these leaks impacted daily activities, and when they appeared. It is composed of three questions plus a multiple-choice question about urine leaks. The total score allows classifying incontinence as mild (scores 1-5), moderate (scores 6-12), severe (scores 13-18), and very severe (scores 19-21). Only women with mild or moderate stress or mixed incontinence (ICIQ-SF score ≤ 12) were enrolled. The OAB-S is composed of eight questions, with scores ranging from 8 to 48, with a score > 8 representative of the presence of incontinence. This questionnaire was repeated at the end of the 5 sessions treatment to verify symptomatic improvements. Also, we administered the International Quality of Life (IQoL) questionnaire. The IQoL is a validated tool composed of 22 questions that evaluate the impact of incontinence on quality of life. Total scores range from 22 to 110. The lower score indicates the stronger impact of urinary incontinence on quality of life. At the end of the 5 sessions of treatment, the Patient Global Impression of Improvement (PGI-I) questionnaire was administered. The PGI-I questionnaire is composed of a single question evaluating the perceived improvement by the patient of a specific symptom after treatment, with scores ranging from 1 (meaning "very much better") to 7 (meaning "very much worse"). An improvement is defined when the PGI-I score is ≤ 3 . At this point, the women were also asked to answer the ICIQ-SF and the IQoL questionnaires again.

2.3. Treatment Administration

The patients underwent, under medical prescription, combined therapy with low molecular weight hyaluronic acid (LMWHA) solution and high concentration oxygen (HCO) for 15 minutes a session, for a total of 5 sessions at 14-day intervals (for a total of 10 weeks), using the X2 self-cooling device (Caress Flow©) or hyaluronic acid (HA) vaginal tablet therapy (dose is the same of one vaginal tablet each evening for 10 weeks).

2.4. Effectiveness Evaluation Procedures

For both groups, LMWHA solution and HCO combined and HA alone, 30 days after the treatments a follow-up visit was done and in that occasion the following questionnaires were administered to evaluate the possible improvement of urinary symptoms: the first outcome was used Patient Global Impression of Improvement (PGI-I) to investigate the degree of improvement perceived by the patient following the 5-session treatment in the LMWHA solution and HCO combined group in the comparison with local HA group. As secondary outcomes, Overactive Bladder Screener - (OAB-S); International Consultation on Incontinence Questionnaire Short Form (ICIQ-SF); International Quality of Life Questionnaire (IQoL) were administered to both groups. During each session, any adverse event was noted and reported according to the criteria CTGAE.

2.5. Statistical Analyses

The primary endpoint was the difference in the proportion of women who reported improvements (PGI-I scores ≤ 3) between the two groups. Secondary endpoints were ICIQ-SF and IQoL scores at the end of the 5 sessions of treatment. Descriptive statistics were presented as means and SDs, medians, and interquartile ranges (IQRs) for continuously coded variables or counts and percentages for categorically coded variables. t Test, Kruskal-Wallis test, and χ^2 test examined the statistical significance in differences between means, medians, and proportions. Wilcoxon signed ranks test for the paired sample was used to compare continuous nonparametric variables. In all statistical analyses, the R software (University of Toronto, Canada) environment for statistical computing and graphics (R version 4.0.0) was used. All tests were two-sided with a level of significance set at $P < 0.05$.

3. Results

From October 2021 to September 2023 a total of 68 postmenopausal women were enrolled. Among them, 35 (51,5 %) had started and finished combined therapy with LMWHA solution and HCO combined and 33 (48,5 %) had used therapy with HA alone. In the overall population (Table 1), the median age was 59,7 years (IQR, 45-66 years), and the median menopause age was 58,5 years (IQR, 45-64 years). There was no statistically significant difference in age ($P = 0.4$) and age at menopause ($P = 0.4$) between the two groups. All women enrolled reported an OAB-S score >8 (overall median score, 28 [IQR, 22.5-34], and no statistically significant difference was recorded between the two groups, $P = 0.9$). All women were Caucasian of Italian ancestry.

Table 1. Selection criteria and study population characteristics.

	HCO + LMWH combined	HA topically alone
Age	59,7	58,5
BMI	24,7	25,0
Parity	2,1	1,8
LMP (y)	50,2	51,2
Distance from LMP (y)	5,5	6,0
IQoL score pre-treatment	73	89
OAB score pre treatment	27	26

3.1. Primary Endpoint: PGI-I Questionnaire at 10 Weeks after Treatment

According to the PGI-I questionnaire (Figure 1), a significantly higher percentage of women reported an improvement (score ≤ 3) in the LMWHA solution and HCO combined group than in the hyaluronic acid (HA) alone group ($P < 0.001$). Indeed, 28 women (80%) in LMWHA solution and HCO combined group reported an improvement at the end of treatment (2 [7,1%] very much better, 7 [25%] much better, 19 [67.9%] a little better). Conversely, 18 (55%) women in the HA group reported no improvement at the end of the treatment (15 [83,4%] no change, 2 [11,1%] a little worse, 1 [5,5%] much worse).

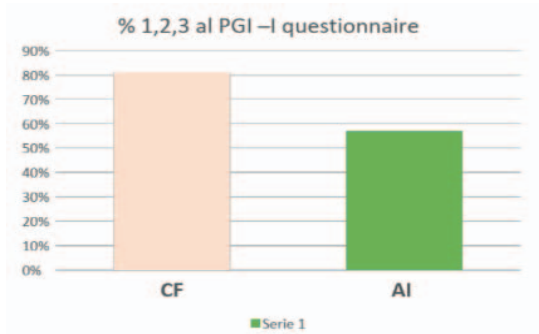


Figure 1. The Patient Global Impression of Improvement (PGI-I) questionnaire was administered to 35 women (51%) who received LMWHA solution and HCO combined and 33 women (49%) who

received HA alone after 10 weeks of treatment. (A) The PGI-I questionnaire stratified in ≤ 3 and >3 scores. (B) The PGI-I questionnaire reports all possible answer results.

3.2. Secondary Endpoint I: ICIQ-SF Questionnaire

According to the ICIQ-SF questionnaire (Figure 2), in the between group analysis, no statistically significant difference was recorded before treatment administration between the LMWHA solution and HCO combined and HA alone groups (median score, 12 [6–12] vs 10 [8–12]; $P = 0.8$). Conversely, a statistically significant difference was recorded after treatment administration between the two groups (median score, 7 [5–11] vs 10 [8–11]; $P = 0.03$).

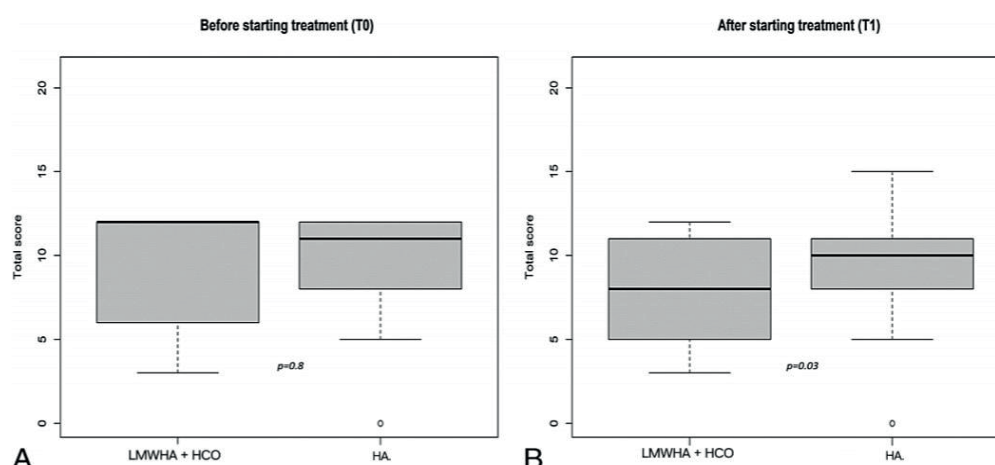


Figure 2. The International Consultation on Incontinence Questionnaire - short form (ICIQ-SF) questionnaire before (T0, A) and after (T1, B) starting treatment, between LMWHA solution and HCO combined and HA group. Boxes denote the interquartile range. The solid black horizontal bar denotes the median score. Whiskers denote 95% range of the distribution of the ICIQ-SF score. The open circles denote outlier values.

3.3. Secondary Endpoint II: IQoL Questionnaire

According to the IQoL questionnaire (Figure 3), in the between group analysis, a statistically significantly lower median score was recorded in the LMWHA solution and HCO combined group than the HA alone group, before (71 [IQR 55-81] vs 89 [IQR 67-94]; $P < 0.01$) and after (78 [IQR 65-86] vs 88 [IQR 72-99]; $P = 0.04$) treatment.

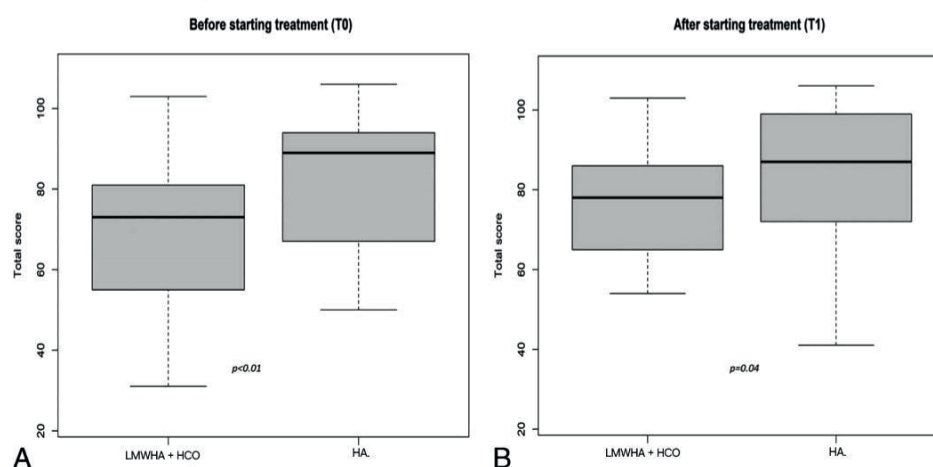


Figure 3. The International Quality of Life (IQoL) questionnaire before (T0, A) and after (T1, B) starting treatment, between LMWHA solution and HCO combined group and HA group. Boxes

denote the interquartile range. The solid black horizontal bar denotes the median score. Whiskers denote 95% range of the distribution of IQoL score.

4. Discussion

The prevalence of bladder symptoms, such as frequency, urgency, and incontinence, tends to increase around the menopause, although it is still debated whether these disorders are due to menopause, or aging, or a combination of the two. Urinary symptoms are associated with the genitourinary syndrome of menopause (GSM) which is characterized by physical and hormonal modifications. It has been seen that hormonal therapies improve the symptoms of GSM, but to date their time of use/dose and the type of effect on long-term systemic levels are still unclear. Local estrogen therapy has played and plays an important role in maintaining good tropism of the vaginal mucosa, but even in this case not all patients accept it, and, in some situations, the effectiveness might not be optimal. Furthermore, it must be considered the impossibility of hormonal therapies' use in some categories of women with high risk factors like previous breast cancer or thromboembolic diseases [16]. The use of non-hormonal therapies that significantly improve the symptoms of GSM and the quality of life of affected patients is successful, but many topical, non-hormonal products on the market have demonstrated a temporary positive action in promoting only lubrication but are often not sufficient to solve all the problems related to mild and moderate stress or mixed urinary incontinence of GSM but allow only transient benefits about vaginal tropism, with the consequent need for repeated treatments [17,18]. Several studies support the use of non-hormonal therapies for the treatment of stress and mixed incontinence in postmenopausal women, particularly, several studies have highlighted how topical oxygen therapy is useful in counteract inflammatory diseases, because the oxygen reactivates the microcirculation of the urogenital epithelium favoring neo angiogenesis, stimulates cellular turnover, improving trophism and conveys nutrients to the epithelial cells. A study published in 2018 by Condemi et All. demonstrated how the treatment with LMWHA solution and HCO combined by a specific medical device is useful in the treatment of urinary symptoms related to GSM [11]. In the diagnosis and management of mild and moderate stress or mixed urinary incontinence of GSM, the gynecological evaluation includes accurate medical history and the use of validated questionnaires that investigate the presence, frequency, and the impact of the symptoms on the quality of life, accurate objective examination that searches for signs at the vulvar, vaginal level, request for tests and/or diagnostic procedures that confirm the diagnosis. Once the patients were selected, they were followed for 10 weeks of treatment and at the end they were re-evaluated with the same questionnaires administered at the beginning of the treatment. In this retrospective study, we did not consider patients with severe stress urinary incontinence. The use of the medical device Caress Flow© appears to constitute a valid treatment for GSM, especially useful for urinary symptoms of mild and moderate stress and mixed incontinence, being completely natural, well tolerated by patients with immediate therapeutic effects, with statistically significant improvement in symptoms before and after treatment, with recovery of one's sexuality, urinary control and without side effects. In our study, the fist endpoint, according to the between-group analyses of the PGI-I questionnaire postmenopausal women with vulvovaginal atrophy, suffering from symptoms of mild and moderate stress or mixed urinary incontinence, reported a subjective improvement of their symptoms at a significantly higher percentage when treated with LMWHA solution and HCO combined than only HA. In our opinion, as there is no biological or pharmacological reason for believing that hyaluronic acid could exert any significant positive or negative effect on stress and mixed urinary incontinence symptoms, its use alone for this indication may be comparable to a placebo, unless it is associated with a combined treatment with HCO, in which an improvement in all questionnaires score was seen used in the post-treatment. Therefore, our data suggest that Caress Flow© device might exert a beneficial effect on the symptoms of urinary urge incontinence in postmenopausal women with vulvovaginal atrophy. In our experience the medical device Caress Flow© had no contraindications or immediate side effects, had effective results perceivable from the first treatment, was pleasant and non-invasive device, the sessions were very lasts (15 minutes) and did not require recovery time. The evaluation of our secondary endpoints led

to interesting observations. No difference in the AOB-S and ICIQ-SF total score was detected between the two groups at the beginning of the study, whereas after 10 weeks it was significantly lower for the ICIQ-SF in the LMWHA solution and HCO combined group and HA group. Women using combined LMWHA and HCO solution had a significantly better quality of life at the end of the study. However, this observation is hardly relevant, as the IQoL questionnaire score was already significantly higher at the beginning of the study in women who would use LMWHA and HCO combined solution. In conclusion, our study showed a significantly superior effect of LMWHA solution and HCO combined with the medical device Caress Flow© over the use only HA in improving the symptoms of mild and moderate stress or mixed urinary incontinence, the functional and inflammatory state of the vaginal mucosa and above all the quality of life, which agrees with the previous literature. Also, the union of LMWHA solution and HCO represents one of the better alternatives for patients who do not want to take hormones, which they have contraindications or who have had negative experiences with hormone therapies.

Furthermore, patients must be enrolled to increase the sample number and increase the statistical power of our preliminary data or confirmed in a controlled design study, but these first results are encouraging.

5. Conclusions

The current multicenter retrospective study supports the hypothesis that combined therapy with LMWHA solution and HCO with Caress Flow© device might improve the severity of mild or moderate stress and mixed urinary incontinence in this set of women. However, these results need to be confirmed in further studies with a controlled design and a larger population.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

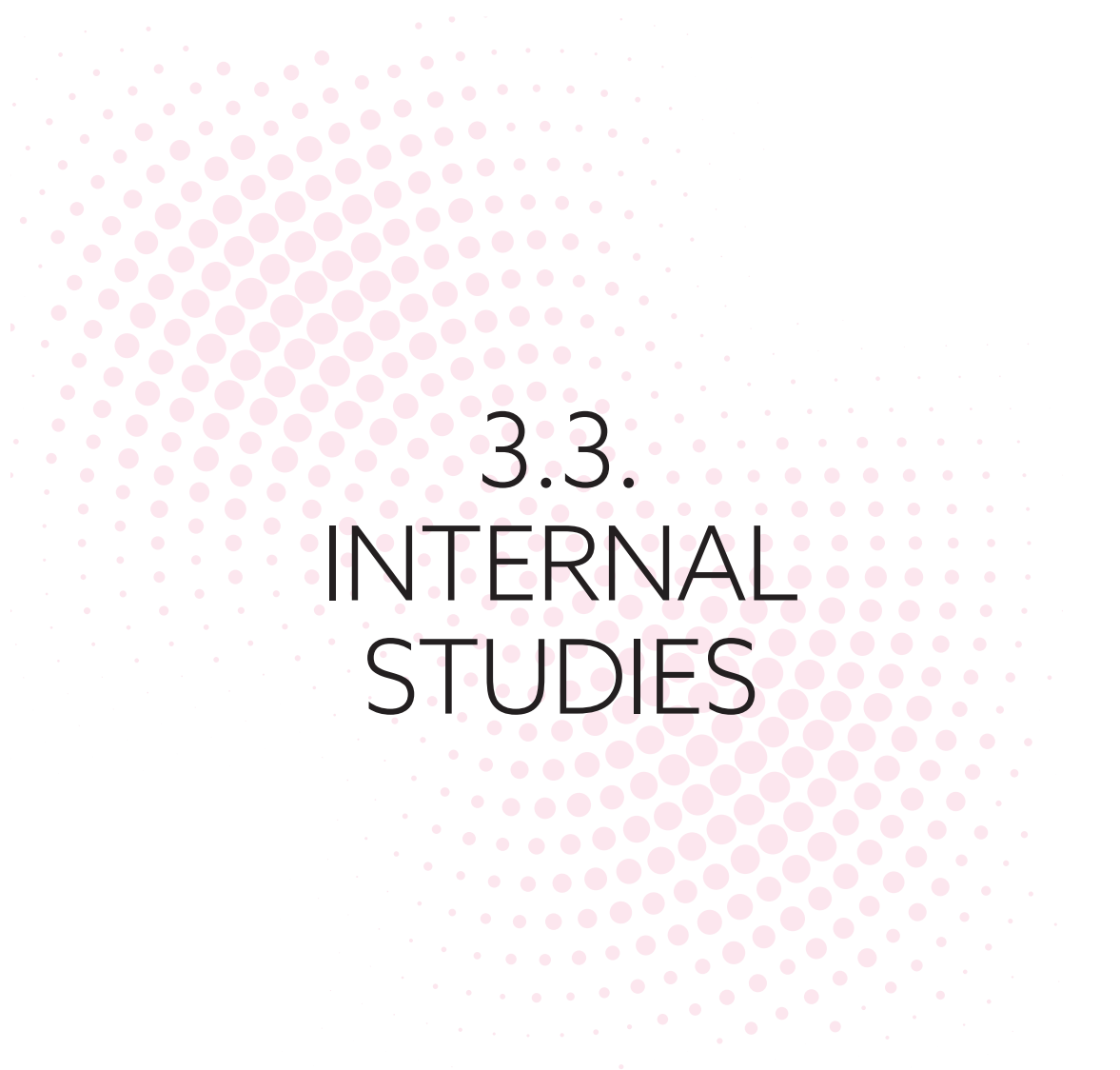
Conflicts of Interest: The authors declare no conflicts of interest.

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3.3. INTERNAL STUDIES

VULVAR VESTIBOLITIS: TREATMENT WITH OXYGEN THERAPY AND HYALURONIC ACID

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Vestibulitis Vulvar (VV) is a chronic, heterogeneous, multifactorial and multi-systemic disease. Friederich first described it in 1987 as a disease characterized by the presence of three particular symptoms and signs:

1. severe pain upon contact with the vaginal vestibule and dyspareunia;
2. fragility of the vestibular tissue, evident on contact;
3. objective finding of vestibular erythema of different degrees.

It is the main cause of dyspareunia in women of childbearing age and affects 8-12% of women who go to a gynecological clinic.

As a multi-systemic disease, VV has a complex pathophysiology. Bornstein demonstrated that the mucosal structure of the vestibule is susceptible to a mast cell-mediated inflammatory response. In the population affected by VV, in fact, a significantly higher number of mast cells can be found in the superficial layers of the vestibular mucosa than in the deep ones and, in parallel, a significant increase in the production, deposition and release of multiple inflammatory mediators. Chronic inflammation could therefore be responsible for the thinning and friability of the introital mucosa typical of VV.

The up-regulation of mast cells increases the production of cytokines, substance P and other inflammatory mediators that cause vasodilation, transudation, edema, swelling, redness and pain. The production of Nerve Growth Factor (NGF) by the hyperactive mast cell stimulates the proliferation of pain nerve fibers, which by extending to the most superficial layers of the mucosa cause both an increase in pain perception (hyperalgesia) and a perceptual change in pain tactile type to one characterized by burning (allodynia).

The activation of mast cell degranulation is mediated in different ways and times by different agonist stimuli that contribute to the heterogeneity of the clinical presentation. Infections (primarily from *Candida albicans*, present in 58.1% of women with VV in personal cases, against a prevalence of 5-8% in the general population), mechanical trauma from rubbing during intercourse, if lubrication was insufficient, estrogen, chemical and physical stimuli, allergens can all activate the release of mast cell mediators. They can promote inflammation by showing its typical clinical

characteristics (rubor, tumor, calor, dolor et functio laesa, understood as the impossibility of sexual intercourse).

The first symptom of VV is acute (nociceptive) pain, caused by the intercurrent inflammation and the injury from which the body tries to react and adapt. Over time, the pain can become chronic and itself become a disease (neuropathic), being generated by the nerves themselves or by the higher centers. The up-regulation of the pain system stimulates the adrenergic system by inducing the activation of the autonomic nervous system responsible for pain hyperesthesia, any defensive posture and changes in the pain threshold.

The pain often causes a defensive contraction of the muscles in the affected area to minimize further injury. The contraction of the levator ani may also pre-exist VV, if associated with vaginismus as a predisposing factor, or acquired in response to persistent introital pain. Recent research suggests that the difficulty in inserting internal tampons, in adolescents, could be the symptom of an hyperactivity of the levator ani muscle, which causes an excessive contraction of the same such as to restrict (reversibly) the vaginal entrance. This hypertonicity would become a predisposing factor both to make penetration persistently painful from the beginning of sexual life ("lifelong", or primary dyspareunia) and to cause microtrauma of the vestibule in the event of sexual intercourse, then precipitating the VV.

As for the involvement of the hormonal system, VV is a typical disorder of the fertile age. Premenstrual pain attacks ("flares") are typical in some women. Hypersensitivity of the mast cell to estrogen has been hypothesized. Estrogens are in fact agonists of mast cell degranulation. After menopause, recurrences of VV were found during vaginal and / or systemic hormone replacement therapy, in synergy with recurrent candidiasis. This is due to the interaction between estrogen and vulnerability to candida infections, and between estrogen and mast cell degranulation.

The vascular system is activated during chronic inflammation. Vestibular erythema is the epiphenomenon of superficial vasodilation mediated by the peptide of the calcitonin gene (CGRP) released by nociceptors C which can cause vasodilation and activation of the axonal reflex even at low levels of activity. Neurogenic inflammation describes acute vasodilation mediated by nerve signals that move retrograde along the sensory nerves and could activate both degranulation and vasodilation.

Diagnosis

VV should be suspected: a) when a woman complains of superficial dyspareunia, with burning and / or acute pain, on introital contact; b) when the three signs described by Friederich are found on clinical examination. Instrumental examinations are currently mainly used in research.

Anamnesis

1) General medical evaluation, which includes, in addition to the traditional medical history, particular attention to recording:

- pelvic medical comorbidities: urological disorders of the lower urinary tract, relapsing cystitis, interstitial cystitis, urinary urgency, enuresis; iatrogenic vaginal pains (outcomes of episiorraphy, colpoplasty, especially if posterior, of radical vaginal surgery and / or pelvic radiotherapy); constipation, fissures, hemorrhoids;
- systemic comorbidities: for example diabetes, as it favors relapsing candida infections; infections, also in other organs, which require repeated antibiotic therapies, which often result in recurrent candida infections; allergies and atopies; depressions¹⁵;
- previous or current vaginal infections, or sexually transmitted diseases such as candida, gardnerella, HPV and genital herpes, which deserve specific attention from the doctor, as predisposing or precipitating factors for VV. If so, the vaginal environment should then be evaluated with a vaginal swab and a culture test, especially in the suspected infectious;
- all current or previous pharmacological treatments aimed at treating both other conditions and VV. Any ongoing hormonal treatment (oral contraceptive therapy, hormone replacement, etc.), and all systemic and local treatments should therefore be documented.

2) Sexological anamnesis focused on any dysfunctions prior to the appearance of VV^{15, 16}. A woman's sexual practices, and the coexistence of any sexual dysfunction, primary ("lifelong") or acquired (secondary), concerning possible dysfunctions of desire, excitement, orgasm and dyspareunia must be investigated and recorded. In young women, the possible symptoms associated with VV, including difficulty in using vaginal tampons and fear of penetration, as well as any post-coital urinary symptoms (cystalgia and / or postcoital urethralgia, dysuria, urge urination) deserve particular attention. ;

3) A woman's perception of the duration and characteristics of the symptoms, that is, how the disease is experienced both as physical suffering and as an interaction with affective, work and social activities.

Physical examination

It should document:

- the presence of general defensive postures (ie characterized by an excessive increase in general muscle tone and the refusal of any genital contact, including vaginal physical examination) and primary dyspareunia. These signs and symptoms may be associated with primary vaginismus concomitant with VV.
- The evaluation of the vaginal pH, tested with a vaginal stick during the gynecological examination, since it must always be recorded in the file.
- The quality of the pain. During the gynecological examination, three basic questions should be reformulated regarding: "Where does it hurt?", "When does it hurt?" and "What symptoms accompany the pain?". These three questions are essential: location and onset characteristics of pain are in fact the strongest predictors of the biological etiology of pain itself and should introduce the next step regarding the objectification of clinical data^{17,18}.

- Pain mapping: precise identification of points of tenderness at the level of the external genitalia, the middle and distal vaginal third 17,18. The precise localization of pain, its beginning, its characteristics are the main predictive signs of its organicity.
- The quantization (score) of pain. It involves bilateral quantification of the perception of pain intensity using the Likert visual analog scale (from 0: no pain to 10: pain of a more intense degree).

COMBINED USE OF OXYGEN AND HYALURONIC ACID IN THE TREATMENT OF VESTIBOLITIS: CASE STUDY

For the treatment of vestibulitis, oxygen therapy was chosen as the use of this technique satisfies the treatment of symptoms such as: recurrent infections, burning, pain and dyspareunia that characterize this disease.

Oxygen therapy has a powerful regenerative, antibacterial and biostimulating effect, it is therefore believed that the treatment of symptoms such as itching and burning can also be very useful in the treatment of this pathology. Oxygen therapy increases the availability of oxygen to the tissues, promotes the increase in tissue repair processes and increases the synthesis of collagen allowing normal hydroxylation of this protein. In fact, at tissue oxygen tensions lower than normal, collagen is not synthesized correctly, slowing the healing of ulcers and wounds. In addition, oxygen induces a neo-angiogenic stimulus by releasing factors such as the Vascular Endothelial Growth Factor (VEGF). This function is essential for the restoration of the microcirculation in compromised vascular situations, re-establishing a vascular flow in the hypoxic areas that guarantees correct tissue regeneration.

Hyaluronic acid is a natural polysaccharide that forms a fundamental part of the extracellular matrix of the skin and cartilage. Hyaluronic acid: has remarkable adhesive, moisturizing and reparative properties of the vaginal mucosa.

The association of high concentration oxygen and hyaluronic acid has been shown to have therapeutic efficacy in the treatment of vulvo-vaginal atrophy, the aim of the study is to use the association between high concentration oxygen and hyaluronic acid for treatment. of the disorders caused by vestibulitis and the improvement of the quality of life of people affected by this pathology.

Treatment

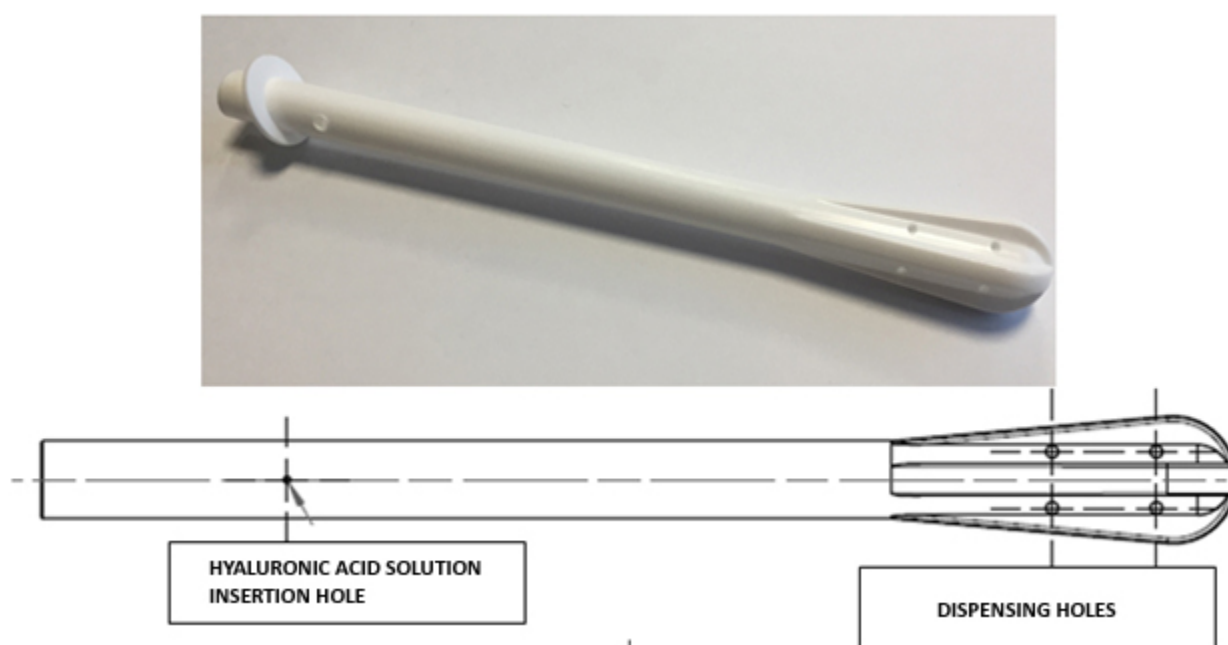
Five weekly oxygen therapy treatments were performed on 25 women diagnosed with vestibulitis, for a total of five weeks. La terapia ambulatoriale era così composta: erogazione di ossigeno e acido ialuronico nebulizzato con applicatore vulvare, per una durata di cinque minuti. Successivamente è stata applicato ossigeno vaginale per 10 minuti e erogazione di ossigeno e acido ialuronico per ulteriori 5 minuti.

For the treatment, the Caress Flow system was used, an oxygen therapy device for gynecological use that allows the topical administration of oxygen with a high degree of purity up to $93 \pm 3\%$, at a flow of 1-6 l / minute.

The device consists of a compressor that generates compressed air by sucking air from the external environment, filtering and compressing it. Inside the machine body there are zeolite molecular sieves that exploit the principle of the different absorption of gas molecules, letting the O₂ pass and retaining the other gases present in the air, such as nitrogen, argon, helium and hydrogen. The machine body transforms the outside air into $93 \pm 3\%$ pure oxygen.

Two dispensers were used, the first consisting of a vaginal cannula, connected to the machine body. The cannula is equipped with outlet holes for the delivery of oxygen and hyaluronic acid, which is inserted through a special insertion hole located in the upper part of the cannula (Figure 1). The vaginal cannula is used for treatment inside the vaginal canal.

FIGURE 1



In addition to the cannula, an airbrush was used (Figure 2), always connected to the machine body, capable of delivering oxygen in combination or not with the hyaluronic acid solution. The airbrush is used for the treatment of the external genitalia, nebulizing the combination of oxygen and hyaluronic acid.

Hyaluronic acid is previously dissolved in distilled water, to form a 0.2% (w / v) solution.

FIGURE 2



All patients have pelvic muscle hypertonus, in addition to vulvar and vaginal oxygen therapy, a manual physiokinesitherapy treatment was applied after the delivery of oxygen and hyaluronic acid with the aim of treating pelvic contracture.

A double evaluation was performed on the treated subjects: one subjective by the patients and one by the doctor. The subjective scale was compiled using an analog graded card that assessed pain during sexual intercourse (dyspareunia), dryness, itching and burning with a VAS scale from 0 to 10, where 10 represents the maximum intensity and 0 the absence of the disorder, analyzing the symptoms before the first treatment session (T0) and at the end of the 5 sessions (T5). Instead, the pH, elasticity, tone and the presence or absence of infections were evaluated by the doctor (Table 1).

TABLE 1

	Absent	Mild	Moderate	Strong
SCORE	0	1--2--3	4--5--6	7-8-9-10

	Absent	Poor	Mean	Good	Excellent
ELASTICITY	1	2	3	4	5

Results

Patients reported significant improvement in all indices analyzed (Figure 3), with the greatest effect in pain reduction (VAS T0 = 7.2; VAS T5 = 1.5, Wilcoxon signed-rank test $P < 0.0001$), but also with regard to burning (VAS T0 = 7.28; VAS T5 = 1.48, Wilcoxon signed-rank test $P < 0.0001$) and pruritus (VAS T0 = 6.64; VAS T5 = 1.36, Wilcoxon signed-rank test $P < 0.0001$).

As regards the elasticity index, the variation between T0 and T5 appears highly significant (Wilcoxon signed-rank test $P < 0.0001$) with an increase in the index from 2.48 to 4.48.

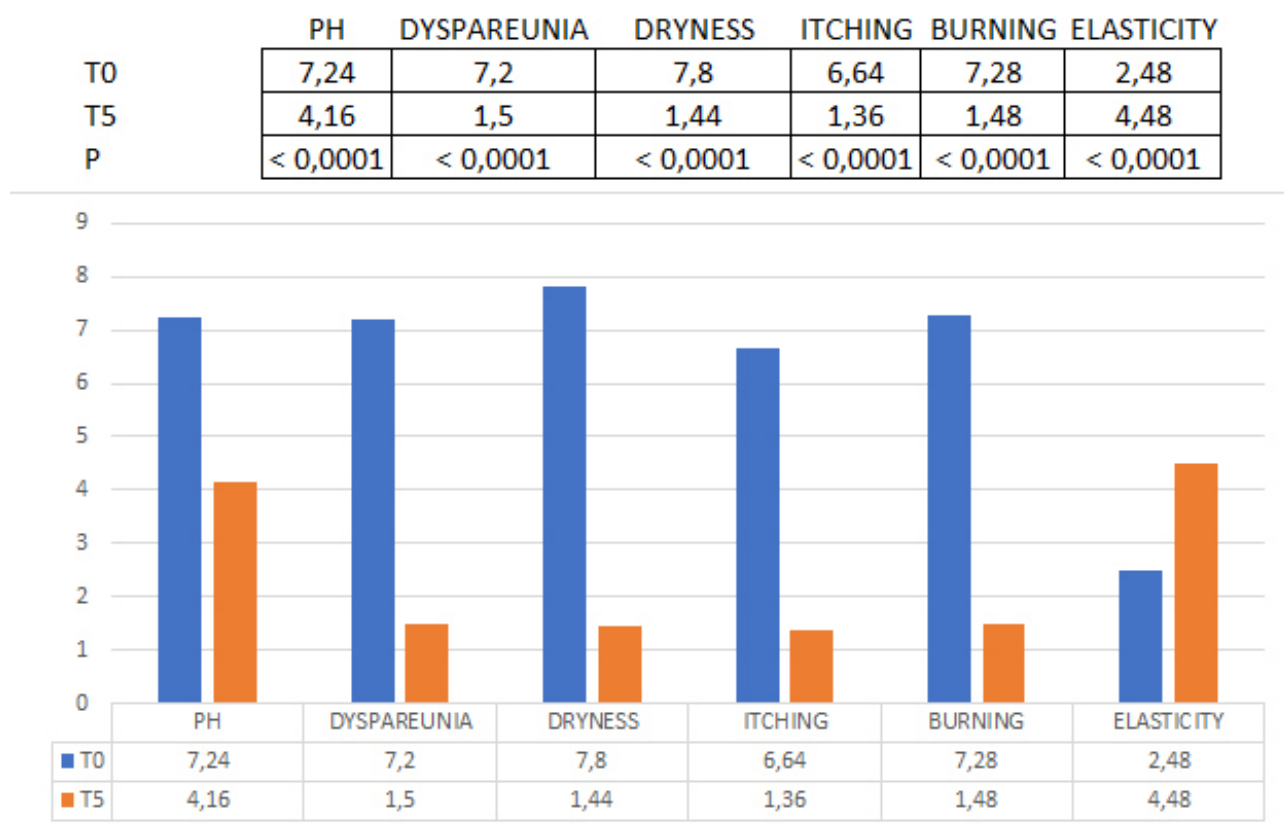


FIGURE 3

The data regarding the effect of the treatment on tone show that in all cases there was an improvement in symptoms (Table 2).

Of the 25 women included in the trial, 18 had T0 infections, confirming the association between vestibulitis and vulnerability to candidiasis. At the end of treatment (T5) all detected infections were resolved (Table 2), demonstrating the effectiveness of the combined treatment of high concentration oxygen and hyaluronic acid.

No side effects associated with the treatment were reported by the patients.

TONE		INFECTIONS	
	T0	T5	
1	over	normal	Candidiasis
2	over	normal	Candidiasis
3	over	normal	Candidiasis
4	over	normal	Candidiasis/Cystitis
5	over	normal	Candidiasis
6	over	normal	Candidiasis
7	over	normal	Candidiasis
8	over	normal	Candidiasis
9	over	normal	Candidiasis
10	normal	normal	\
11	over	normal	\
12	over	normal	Cystitis
13	over	normal	Candidiasis
14	over	normal	Candidiasis/Cystitis
15	over	normal	\
16	over	normal	\
17	over	normal	Candidiasis
18	over	normal	Candidiasis
19	over	normal	Candidiasis
20	over	normal	Candidiasis
21	over	normal	\
22	over	normal	\
23	over	normal	\
24	over	normal	Candidiasis
25	over	normal	Candidiasis

TABLE 2

Combined oxygen therapy with hyaluronic acid has proven to be a valid method for treating the symptoms associated with vestibulitis. It is a totally painless therapy, with excellent compliance by patients. It is a fast, non-invasive and repeatable treatment, with no side effects.

VAGINAL NATURAL OXYGENATION DEVICE (VNOD): A RCT STUDY PROTOCOL OF CONCOMITANT ADMINISTRATION OF HYALURONIC ACID AND TOPICAL OXYGEN TO TREAT VULVO-VAGINAL ATROPHY

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This is a pivotal, open-label, randomized, controlled, three-arm, evaluation of effectiveness and adverse effects of the administration by a specifically designed medical device of hyaluronic acid in combination with topical oxygen for the treatment of VVA compared with topical oxygen alone or hyaluronic acid alone.

Participants were be randomized to the group A received concomitant administration of hyaluronic acid and topical oxygen; participants randomized to the group B received administration of topical oxygen alone, and participants randomized to the group C received administration of hyaluronic acid alone.

The duration of treatment was three months, with a total of five (the first at time 0) out-patient sessions every 14 days lasting 15 minutes each. The follow-up was conducted at 30 days from the last administration. The study is designed to determine the superiority of the administration of hyaluronic acid in combination with topical oxygen with respect to the topical oxygen alone or the hyaluronic acid alone in the improvement of the symptomatology of patients affected by VVA.

The primary objective of this study is to evaluate the impact of administration of hyaluronic acid in combination with topical oxygen by a specifically designed medical device for the treatment of vulvo-vaginal atrophy (VVA), compared with administration of topical oxygen alone or hyaluronic acid alone. The secondary objective is to determine the safety and tolerability of administration of hyaluronic acid in combination with topical oxygen in women affected by VVA.

SUBJECT ENROLLMENT

The sample size was determined to detect an improvement of 30% in severity of vaginal dryness from baseline to last follow-up. Considering an alpha value of 0.05 and a beta value of 80 a sample size of 15 patient per arm will be needed (45 patients in the entire study population).

Patients were identified and recruited from investigators clinics and referring physician, with privacy protection and avoiding undue influence. Each included patient provided an informed consent that allows treatments, that certifies the comprehension of the information provided, with voluntary agreement of the subject, free from coercion.

Patients were randomly assigned to one of the three study group only if they meet all the inclusion criteria and none of the exclusion criteria.

Inclusion criteria

1. Has age higher than 18 years
2. Has a diagnosis of VVA, with onset of symptoms from at least one year, with self-assessed “severe” grade of the most bothersome symptom (see further).
3. Has negative cervical cytology executed in the last three years or is outside the Italian cervical cancer screening age range (with a last negative cervical cytology).
4. Is able to understand and sign the informed consent document

Exclusion criteria

1. Has concomitant clinically important medical disease (endometrial hyperplasia or cancer; undiagnosed vaginal bleeding; liver or kidney disorder; thromboembolic disorders; cerebrovascular accident, stroke, or transient ischemic attack; myocardial infarction or ischemic heart disease; previous gynecological malignancy; or endocrine disease; or any clinically important abnormalities on screening physical examination, assessments, mammogram, electrocardiogram (ECG), or laboratory tests).
2. Has used oral products containing estrogens, progestins, or androgens within 8 weeks; transdermal hormone products within 4 weeks; vaginal hormone products (rings, creams, gels) within 4 weeks; intrauterine progestins within 8 weeks; progestin implants/injectables or estrogen pellets/injectables within 6 months; an intrauterine device within 12 weeks before screening.
3. Assumes the following concomitant medications: estrogen, progestin, androgen, SPRM, vaginal lubricants, and moisturizers.

TREATMENT

Patients randomized to the group A will receive combined treatment with hyaluronic acid and topical oxygen, dispensed by a medical device, while patients randomized to the group B will receive the topical oxygen alone dispensed by the same medical device, without administration of hyaluronic acid, and patients randomized to the group C will receive hyaluronic acid alone.

The treatment involves a specifically designed medical device (Caress Flow) consisting of an oxygen generator of oxygen (95%) from air and a monousage kit including a dispensing handpiece with an ergonomic design and a sodium hyaluronate row. The full treatment cycle includes four monthly sessions, during which the dispenser is inserted through the vagina for 15 minutes, and the oxygen and the hyaluronic acid are administered at first alternately and subsequently in a contemporary way. At visit 0, an accurate collection of patient history and a complete clinical examination was performed to evaluate eligibility criteria. The intensity of the three following symptoms (1. well-being such as absence of dyspareunia, vaginal dryness, vulvar and/or vaginal itching, 2. vaginal burning 3. vaginal lubrication and presence of fluid) were collected from patients with a VAS based on a six-point scale: score 1 = maximum intensity, score 2 = strong intensity, score 3 = average intensity, score 4 = mild intensity, score 5 = weak intensity and score 6 = absence of symptom (Table I).

Table I. VAS scale for the intensity of the three following symptoms.

Symptoms	
Score 1	Maximum intensity
Score 2	Strong intensity
Score 3	Average intensity
Score 4	Mild intensity
Score 5	Weak intensity
Score 6	Absence of symptom

(1) well-being such as absence of dyspareunia, vaginal dryness, vulvar and/or vaginal itching, (2) vaginal burning
(3) vaginal lubrication and presence of fluid.

Before the treatment, the vaginal elasticity and the vaginal wall epithelium appearance were determined by the clinician with a numerical score as reported in Table II.

Table II. Vaginal elasticity and vaginal wall epithelium appearance determined with two numerical scores.

Vaginal elasticity		Vaginal wall epithelium appearance	
Score 1	Absent	Score 1	Petechiae
Score 2	Poor	Score 2	Contact bleeding
Score 3	Average	Score 3	Scratching bleeding
Score 4	Good	Score 4	Erythema
Score 5	Excellent	Score 5	Normal

The first 15 minutes session was performed and the occurrence of any discomfort or adverse effect was recorded. The next four administrations (visit 1, 2, 3 and 4) were performed after 14 days. On every occasion, the intensity of patient's symptoms, the vaginal elasticity, and the vaginal wall epithelium appearance were determined and recorded with the method described above, as well as the occurrence of any adverse event. Follow-up visit (T5) was performed after 30 days from the last administration and even on those occasions the vaginal elasticity, the vaginal wall epithelium appearance and the intensity of patient's symptoms were determined and recorded.

Statistical analysis was performed using IBM SPSS version 22.0 (IBM Corporation, Armonk, NY, USA). The statistical significance of the trend of variation in values between treatment sessions was analyzed using the one-way variance analysis according to the Kruskal-Wallis method. The significance of couples' comparisons between treatment sessions was analyzed using the Wilcoxon test for non-parametric data. A $p < 0.05$ was considered statistically significant.

The treatments were administered at Ospedale Civile Urbino – SSD Oncologia Ginecologica. The Ethical Committee approval was obtained (Comitato Etico di Area Vasta Emilia centro (AVEC) 24/03/2020, protocol number 27/2020/Disp/AOUBo).

RESULTS

The mean age at diagnosis of the 45 included cases was 54.9 years $\pm$ 7.8 SD. No patient discontinued therapy, performing less than five sessions. No side effects were reported.

The results for the group A shown a significative improvement of all the mean scores analyzed: well-being such as absence of dyspareunia, vaginal dryness, vulvar and/or vaginal itching, vaginal burning, vaginal lubrication and presence of fluid (Figure 1).

For the group B significant variation was reported for the elasticity and the epithelium appearance index, whereas for the group C only the burning index shown a significant improvement (Figure 1).

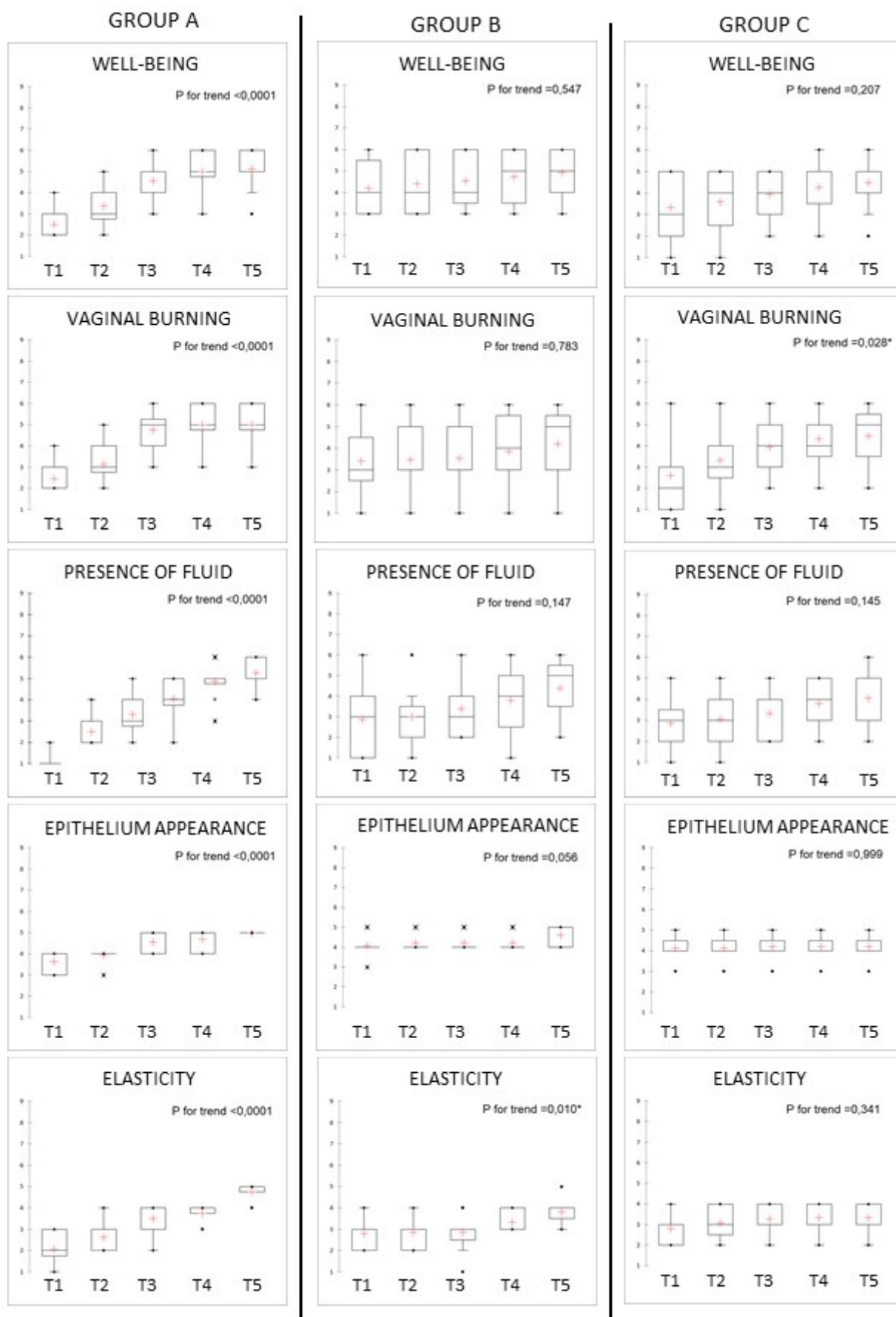


FIGURE 1

The changes in the indices between T1 and T5 were compared for each treatment group. For all the indexes examined (Figure 2) the group treated with the combination of oxygen and hyaluronic acid (A) showed a highly significant improvement compared to the group treated with oxygen only (B). As regards the comparison with the treatment with the Hyaluronic Acid group (C), all the indices were significantly improved except for the burning index.

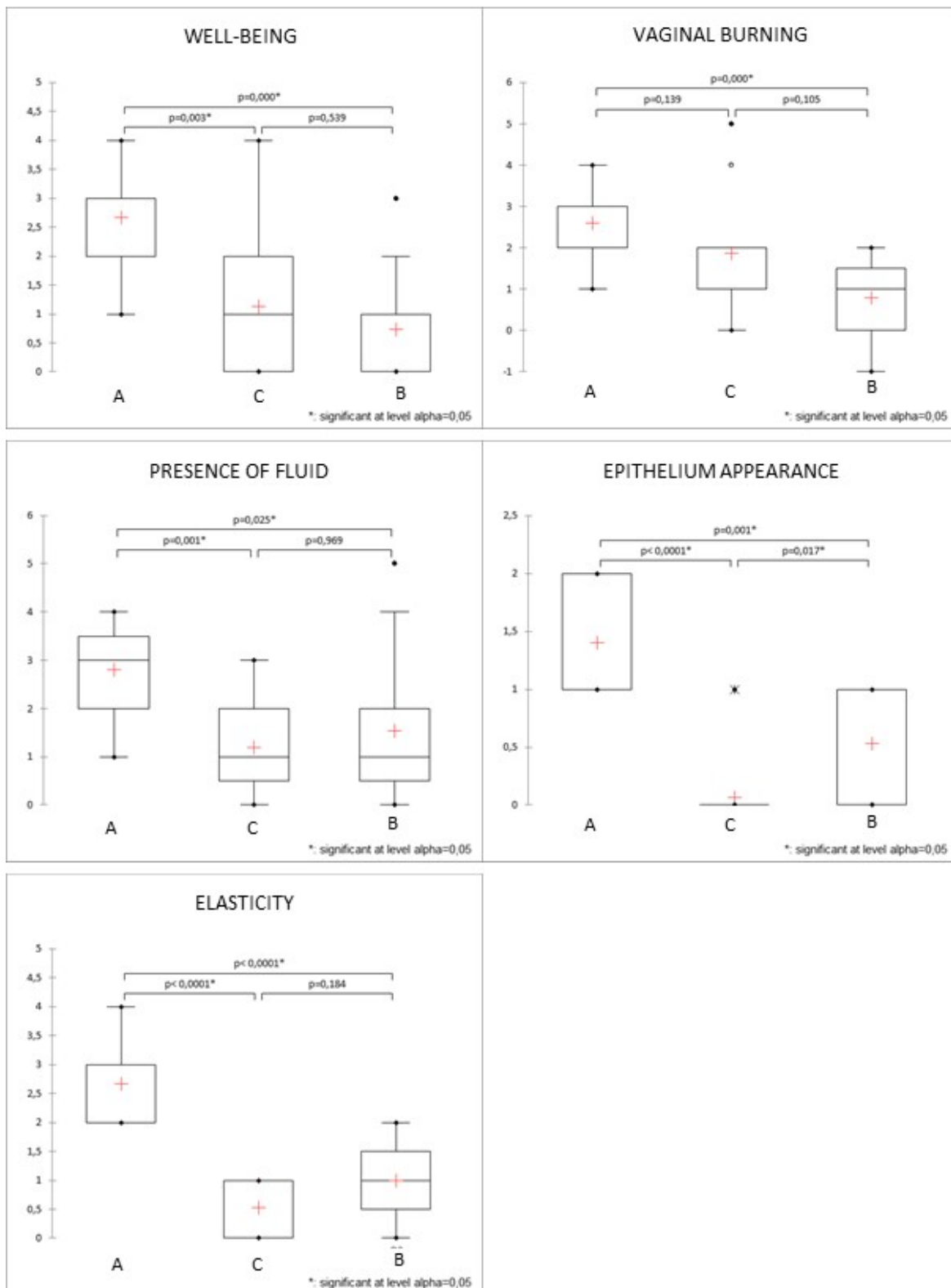
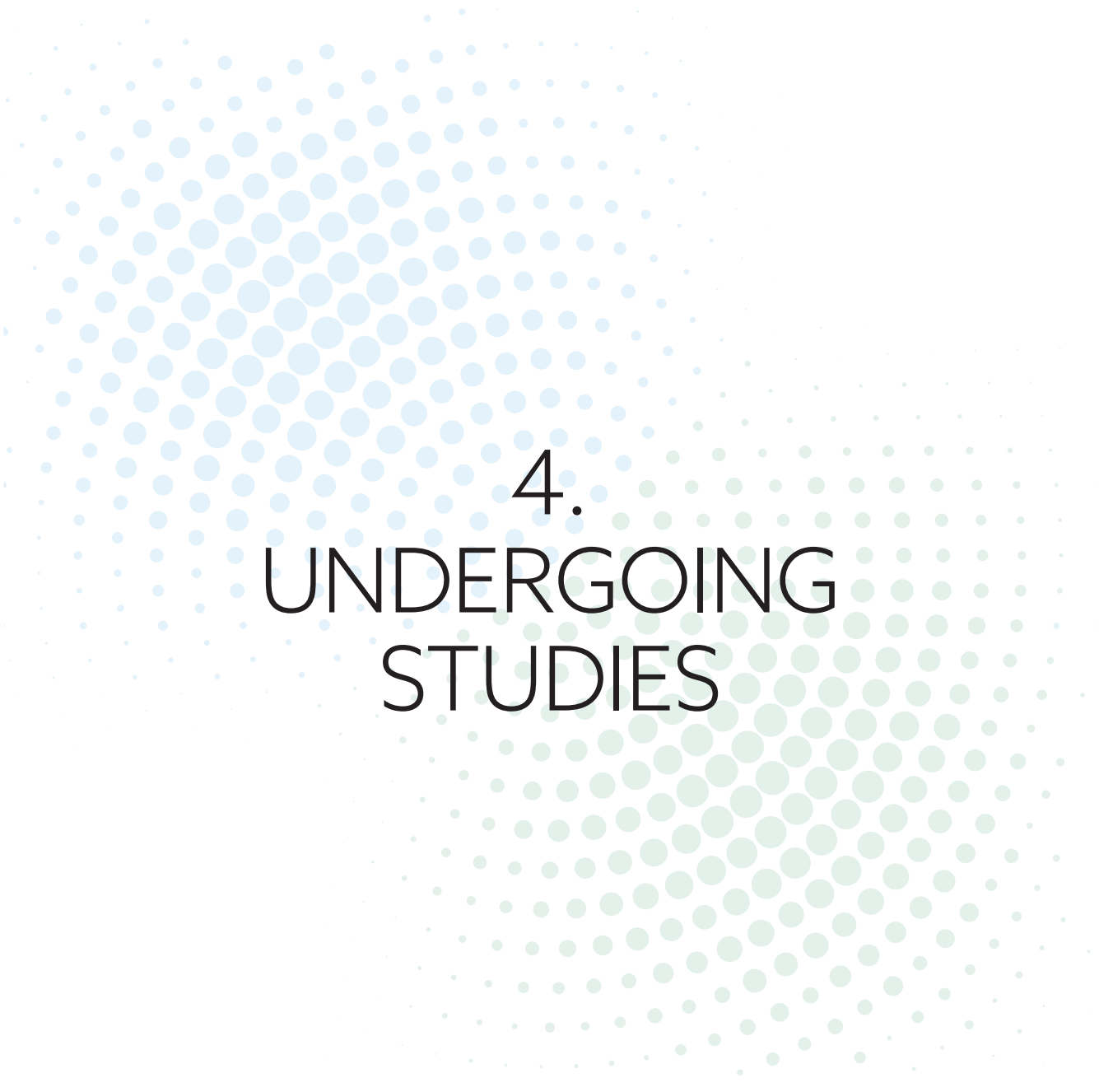


FIGURE 2



4. UNDERGOING STUDIES

UNDERGOING STUDIES AND COMPLETED STUDIES IN PUBLICATION

UNDERGOING STUDIES

Effectiveness of concomitant administration of hyaluronic acid and topical hyperbaric oxygen therapy via a vaginal probe in improving symptoms of vulvovaginal atrophy

BostonUroGyn, Wellesley, MA
[Dr. Neeraj Kohli, BA, MD, MBA](#)

Impact of Caress Flow treatment on vaginal microbiota

S. Orsola Hospital – Bologna
[Prof. Maria Cristina Meriggiola](#)

Evaluation of the **effectiveness of topical administration of acid hyaluronic and hyperbaric oxygen through a specific medical device, compared to the topical administration of hyaluronic acid alone** in the improvement of urgency, stress and mixed incontinence in patients with genitourinary syndrome of menopause (GSM): a prospective study single-blind, multicentre, randomized

Department of Clinical Medicine and Experimental, Unit of Obstetrics and Gynecology, University of Studies Magna Graecia of Catanzaro, Italy.
[Prof. Costantino Di Carlo](#)

Evaluation of the effects of therapy with molecular oxygen and hyaluronic acid (Caress Flow) on the persistence of HR-HPV infection and on the vaginal microbiota in patients with CIN1 HPV related: randomized perspective study.

Department of Morphology, Surgery and Experimental Medicine, Section of Obstetrics and Gynecology, Azienda Ospedaliero-Universitaria S. Anna, University of Ferrara
[Prof. Pantaleo Greco](#)

Concomitant treatment with topical hyaluronic acid and topical oxygenotherapy in the improvement of symptoms related to genito-urinary syndrome in women in physiological and iatrogenic menopause

Fondazione IRCCS Policlinico San Matteo di Pavia
[Prof. Rossella Nappi](#)

COMPLETED STUDIES IN PUBLICATION

Vaginal Natural Oxygenation Device (VNOD): a controlled, randomized study on concurrent administration of hyaluronic acid and topical oxygen for the treatment of vulvo-vaginal atrophy

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