CARESS FLOW

OXYGEN FOR VULVO - VAGINAL TREATMENTS

SCIENTIFIC PUBLICATIONS

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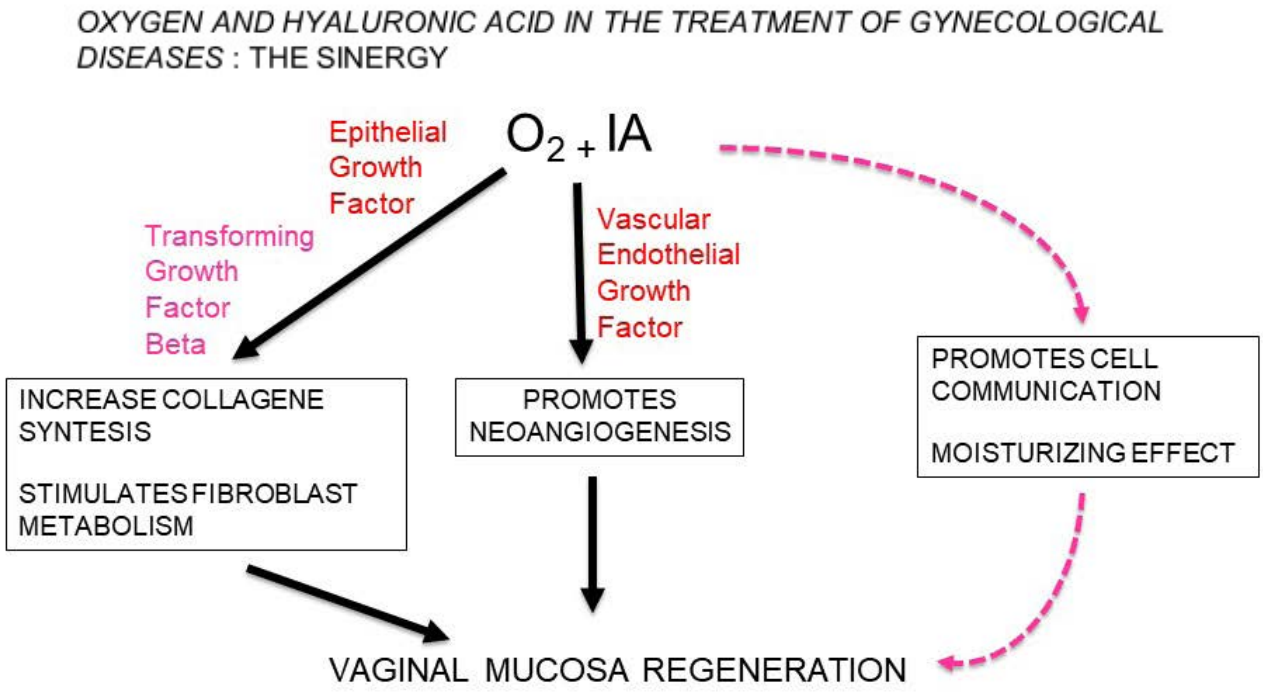
1. SCIENTIFIC RATIONALE

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.



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. SCIENTIFIC PUBBLICATION ON VAGINAL TREATMENTS WITH OXYGEN IN COMBINATION WITH HYALURONIC ACID

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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 therapeutic 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 levels[1]. 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

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affect the quality of life[6]. 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 lubricants[7]. 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 tissue[12], 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

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 ± 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.

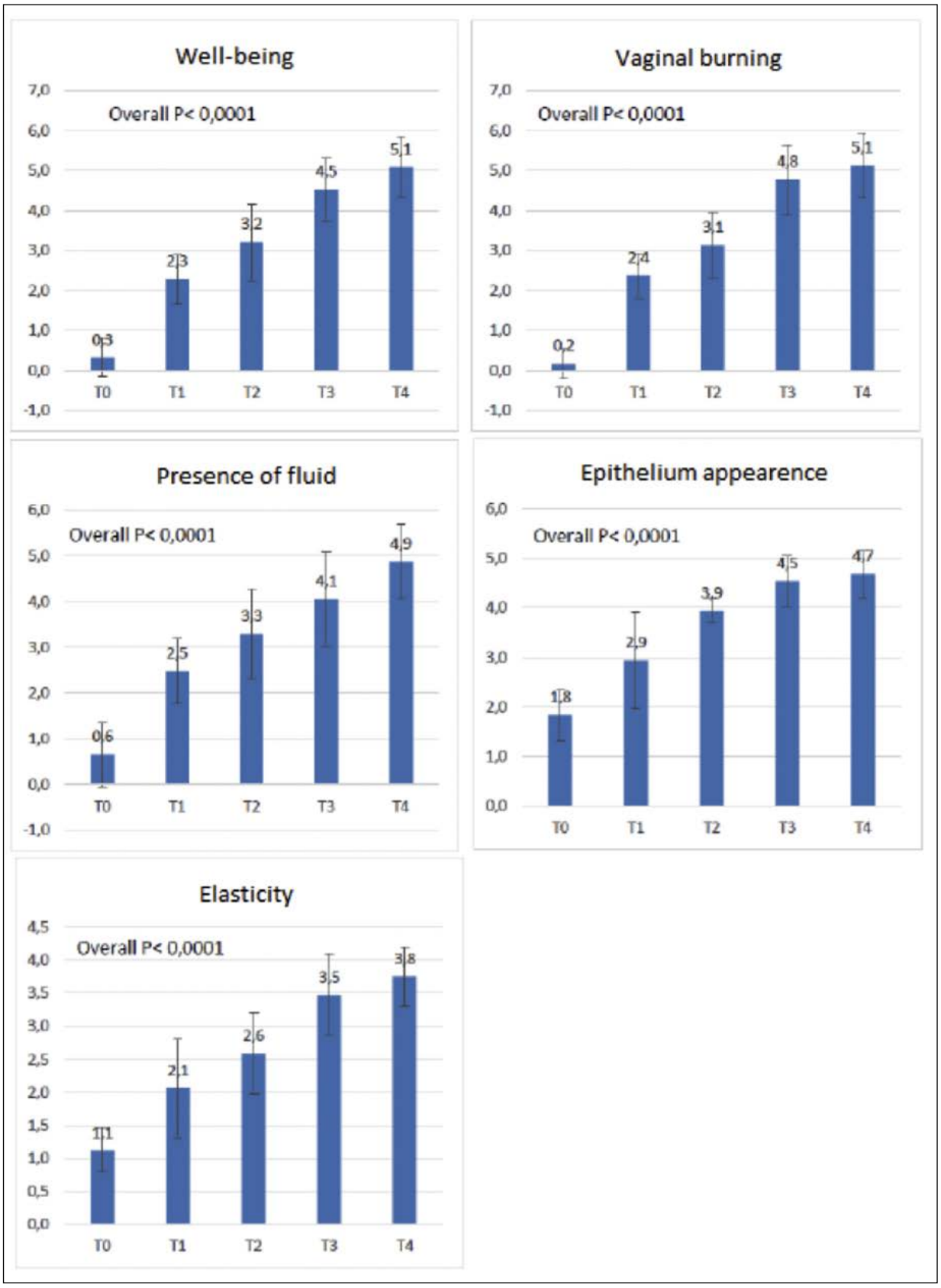

Figure 1. Trend of variation of mean score values between treatment sessions.

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 treatment[13]. 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 studies[14-17] 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 instead[15-17]. Furthermore, there are few safety studies[18] 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 ospemifene[19]. 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 report[21] 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 protein[22]. 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 VEGF[23]. This function is essential for the restoration of the microcirculation in compromised vascular tissues, re-establishing a vascular flow in the hypoxic areas[24]. 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 membranes[25]. 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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LICHEN SCLEROSUS: TREATMENT WITH OXYGEN THERAPY AND HYALURONIC ACID

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INTRODUCTION

Lichen sclerosus 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 sclerosus 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 sclerosus 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 Sclerosus 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

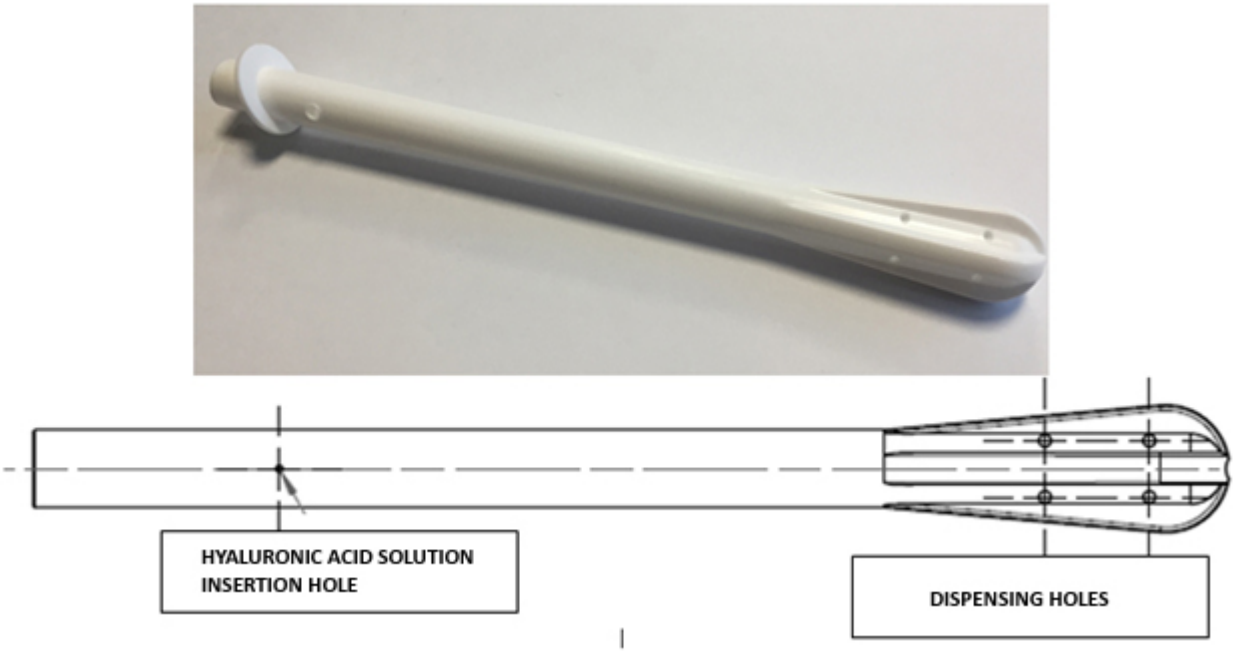
Twenty-five (25) women diagnosed with lichen, carried out by vulvoscopy and biopsy, underwent a weekly session for 5 weeks which included vulvar oxygen therapy for 10 minutes with low molecular weight hyaluronic acid nebulization, at a concentration of 0, 2%, followed by vaginal oxygen therapy combined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

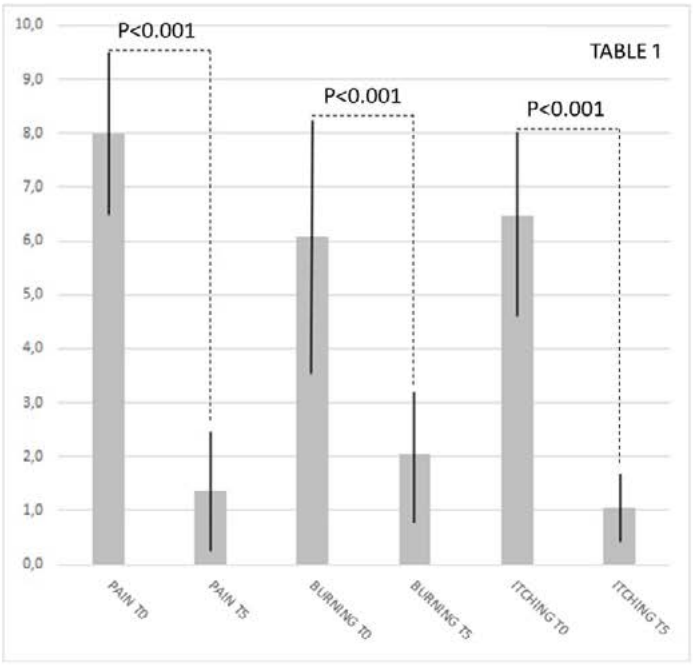
Hyaluronic acid is previously dissolved in distilled water, to form a 0.2% (w / v) solution.

FIGURE 2

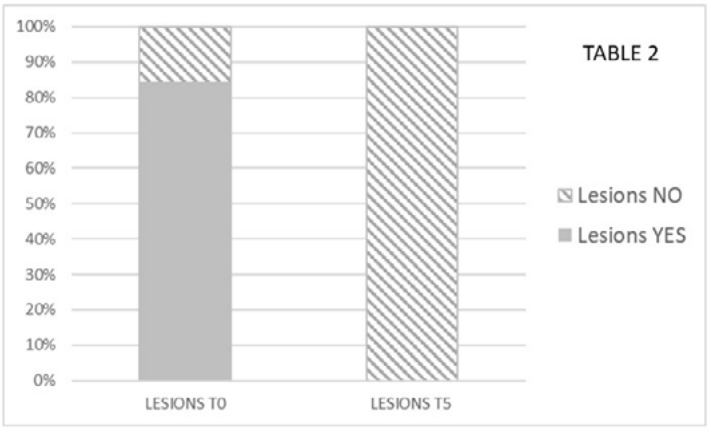
A double evaluation was performed on the treated subjects: one subjective by the patients and one by the doctor. The subjective scale was compiled using an analog graded card that assessed pain during sexual intercourse (dyspareunia), itching and burning with a VAS scale from 0 to 10, where 10 represents 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.

RELAPSING VULVOVAGINAL CANDIDIASIS: TREATMENT WITH OXYGEN THERAPY AND HYALURONIC ACID

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1) 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, 5). About 70-75% of women, especially of childbearing age, have VVC infection at least once in their life and 40-50% relapse (6). In fact, about 20% of acute infections turn in relapsing mycotic vulvar-vaginitis, four or more times in a year (7). 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 (8). 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 (9-11). The main virulence factors include: the morphological transition from yeast to hyphal form (12), the expression of cell surface molecules, such as

adhesins, to help the *Candida* to adhere to epithelial cells (13), directional hyphal growth (thigmotropism) (14), the biofilm formation (15), phenotypic switching, the secretion of extracellular hydrolytic enzymes such as proteinases and phospholipases (16), the ability to change its morphology (17), and its metabolic adaptability (18). 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) (19); auto-induced hyphal formation through selective aminoacid and metals, such as carbon, nitrogen, iron, zinc or copper uptake can influence the fungal pathogenicity (20).

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 (16, 21).

The symptoms are pruritus, soreness, irritation, vaginal discharge, and discomfort, while the clinical signs are introital/vulvar and vaginal erythema, oedema, excoriation, typical leukorrhea, vulva and vaginal burning, dysuria, dyspareunia, and frequent micturition (22-24). The patients also report reduced fitness and physical activities, with psychological involvement in terms of anxiety, depression, and loss of self-esteem (3, 22).

The moderately acidic (range: 3.8-5,0) normal vaginal pH prevents vaginal infections but several factors, such as age, nutrition, vaginal hydration et al. can modify it (25, 26). Consequently, a high vaginal pH level (greater than 5) suggests bacterial vaginosis, trichomonas vaginitis or candidiasis (27, 28).

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 (29).

The relapse episodes can be due to recurrent oropharyngeal candidiasis in patients with advanced and uncontrolled HIV infection, to genetic factors (polymorphism, familial, ethnicity), to immune mechanisms (uncontrolled diabetes, antibiotics, hormone replacement therapy), to behavioral reasons (oral sex, oral contraceptive, corticosteroids, hormonal changes, 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 (30-35).

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 (36, 37), like *C.glabrata* and *C.krusei* (38), which are more pathogenic in terms of oedema, skin irritability and chronic vulvar pain (vulvodynia) (39, 40). In fact, in the last decade, different women with recurrent

VVC appeared fluconazole resistance (41). Some other less used but effective drugs, including polyenes, echinocandins, etc, induce also substantial toxicity (42-44).

Considering these limitations and the continuous increase of Candidiasis incidence, semi-solid mucoadhesive formulations, such as gel with propolis, have been proposed (45, 46), but new more effective, safe and cheaper drugs and strategies are required to treat this infection, especially the recurrent cases.

Farida et al (47) analysed the beneficial effect of Indonesian propolis wax, as anti-candida agent, in 40 vaginal candidiasis patients. The patients were randomly divided in 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 (48, 49). Other used potential anti-fungal compounds are essential oils, including thymol (found primarily in thyme and oregano) (50), and curcumin (51), 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, nonsulfared, linear glycosaminoglycan, composed of repeated disaccharide units that can reach high length and molecular weight (52): it has been challenged into the vagina due to its moisturizing mucosal healing chemotactic and hydrating properties, significantly contributing to viscoelastic interstitial tissue replacement (53).

Our previous preclinical investigations demonstrated that the high molecular weight HA has antiviral, antibacterial and antifungal activity towards a few *Candida* species (54-59). Many clinical contributions confirm that Hhyaluronic acid gives remarkable adhesive, moisturizing and repairing benefits in the vaginal mucosa (60, 61).

A total of 833 studies, reviewed by Campagnaro et al (62), 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 via hydroxylation of its chains (63-66). It improves the oxygen-dependent transport of some antibiotics, such as tobramycin, across bacterial cell walls (67). Specifically, this treatment can improve the damage to the mucosae after gynaecologic cancer radio-therapy, promoting neo-angiogenesis, enhancing high levels of vascular endothelial growth factor (VEGF) from macrophages and improving local hypoxia (68). 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 (69-71). The hyperbaric oxygen stimulates also induction of collagen synthesis, fibroblast proliferation and antimicrobial defence (72).

Recent in *vitro* and in *vivo* studies have demonstrated that oxygen therapy is an antifungal weapon against *Aspergillosis* and *Zygomycosis* (73, 74). 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 (74).

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 neutrophiles, monocytes and macrophages cytoplasm (74). Gudewicz and coworkers observed the combined effect of oxygen and amphotericin B (antifungal medication) to inhibit the growth of *Candida albicans* (75). 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) (75). 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 (76).

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

2.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. 45 women (30,9+/- 4,4) 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 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 (77-80). 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, 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 seconds, the patients check the tip swab to see if the colour 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).**

The yellow indicator may turn in green/blue, if vaginal pH is anormal (pH>4,7), denoting infections such as candidiasis or don't change colour after 10 seconds, if vaginal pH is normal (pH: 3,8-4,5) (81, 82). A standard vaginal examination to detect the elastic properties of pelvic floor tissues, elastography index (EI), was performed by a practicing gynaecologist, 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 (83-85). 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 (86, 87).

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 (88).

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, androgens) or vaginal hormone products (rings, creams, gel) in the last 2 months before the enrollment. The patients accepted to undergo oxygen/hyaluronic acid therapy treatment, once a week, for a total of five weeks at the outpatient clinic (Poliambulatorio Sirio, Fidenza, Italia).

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 hours 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 minutes) and subsequently combined with sprayed 5 ml low molecular weight hyaluronic acid (for the next 5 minutes) **(Figure 1)**. Post-treatment maintenance of one session per month was recommended. The intravaginal oxygen flow (95% pure oxygen delivered at a rate of 2 lt/minute) 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, 10ml) 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.

Table 1. Patient's demographic characteristics. Data are presented as the mean (SD); BMI, Body mass index.

Figure 1: Vaginal disposable cannulas for injection of hyaluronic acid.

No. of patients (women)	45
White race, No	45
Geographic region, Italy, No	45
Age, years	30,9 +/- 4,4
Weight, kg	61,1 +/- 7,8
BMI, kg/m^2	20,8 +/- 2,3
Candidiasis, No	24
Candidiasis + Cystitis, No	21
Disease duration, years	≥5

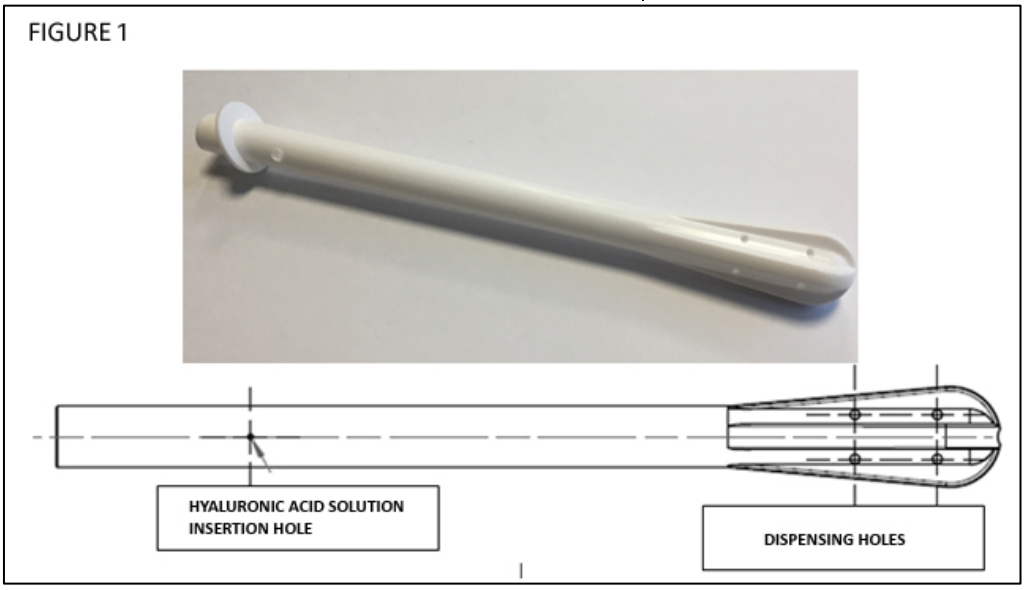


Table 2: The initial self pH readings of patients before the treatment.

Patients'initial self pH readings	No patients (before treatment)
pH: 6-7	21
pH: 7-8	24

3. 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 (colour 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 had only one VVC relapse was 4,44% (2/45), a percentage that increased just to 8,8% at six months (4/45). In the **Figure 4** are reported the relapsing events, for each patient, in the six 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 ± SD, 2,55 ± 0,545 (pre-treatment) vs 4,48 ± 0,505 (post-treatment)] **(Figure 5).**

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

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

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.

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

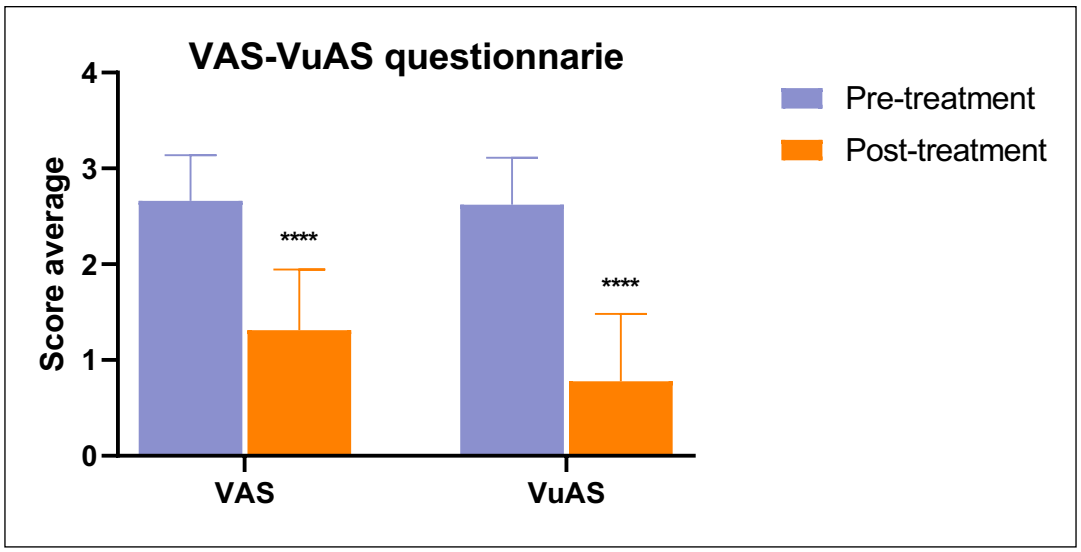


Figure 3: Vaginal pH and swab measurements before and post treatment. There were significant differences. ****P<0.0001 pre- vs. post-treatment.

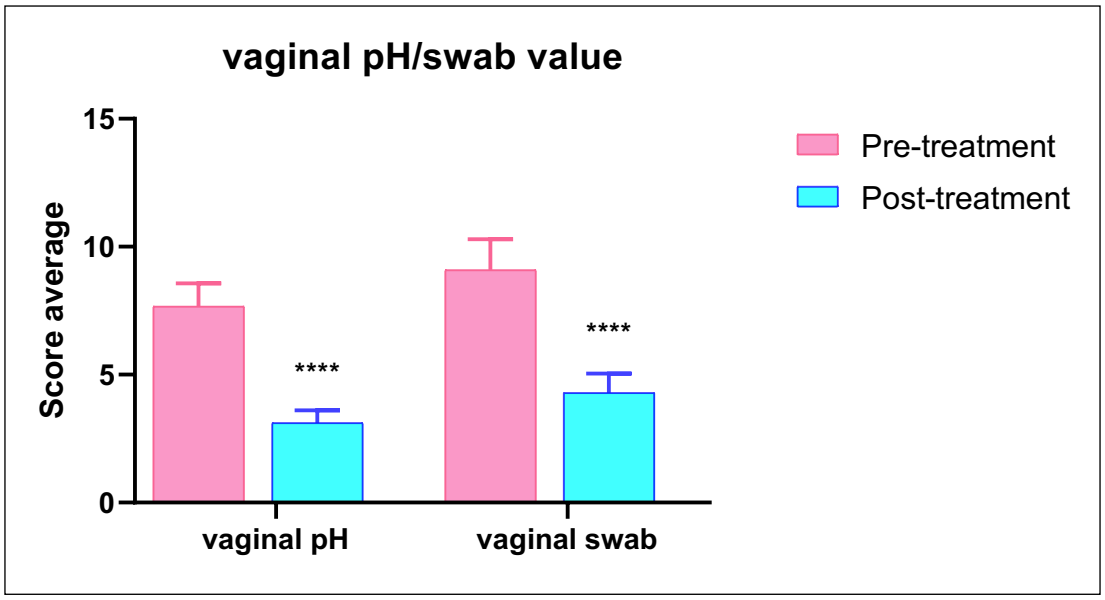


Figure 4: Frequency VVC infection/year pre-treatment and at 3 and 6-month follow up. There were significant differences. ****P<0.0001 pre- vs. post-treatment.

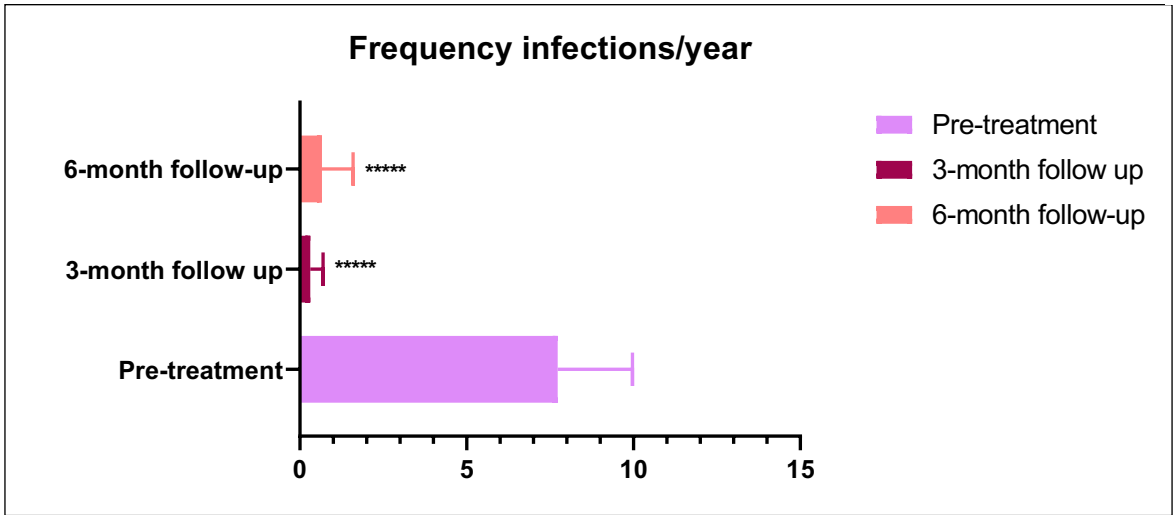
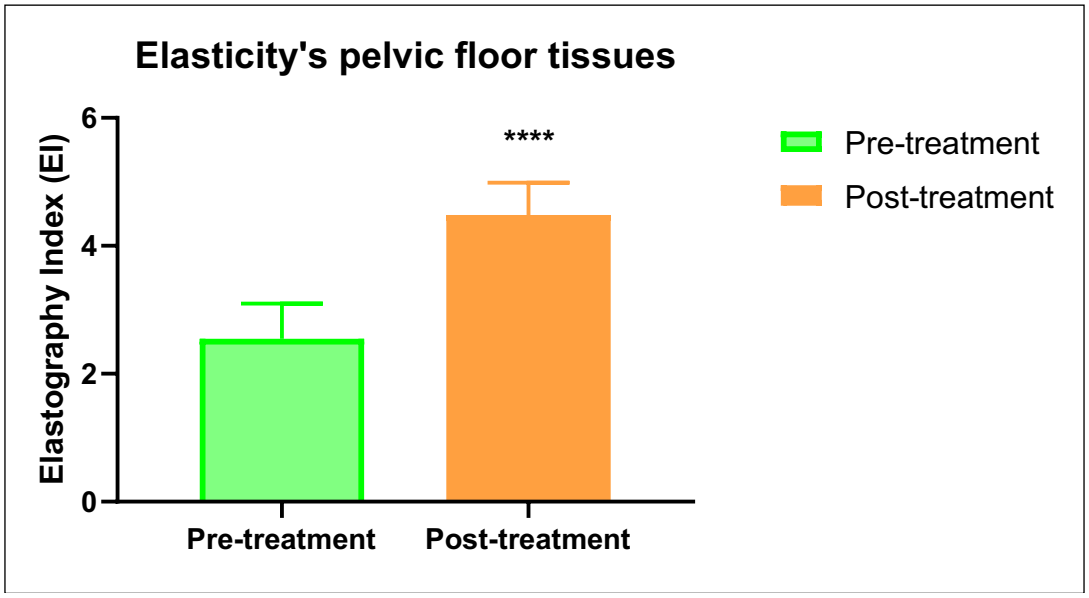


Figure 5: Elastography index (EI) values of pelvic floor tissues pre and post treatment. There were significant differences. ****P<0.0001 pre- vs. post-treatment.



4. 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) (76, 89-91). The amount of oxygen uptaken 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 (92). The safety and effectiveness of HA at different molecular weights in VVA patients has been stated by several studies (93-95).

As to the mechanisms of action, the low molecular weight hyaluronic acid spreads into the deeper vaginal layers (96), and can block the intercellular adhesion molecule (ICAM)-1 receptor, reducing inflammation and secretion of pro-inflammatory cytokines (97).

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 behaviour.

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. But 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.

5.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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URETHRAL SYNDROME: TREATMENT WITH OXYGEN AND HYALURONIC ACID

Submitted to: Gynecology and Pelvic Medicine

Urethral pain syndrome, also called urethral syndrome, is a symptom of pain and / or burning and / or itching of the urethra with urination. It is a very common urinary symptom experienced by most people at least once in their life.

Symptoms of urethral pain syndrome include an increase in urinary frequency[1] and bladder pain that is slightly relieved by urination. There may also be hesitation, slowed urine flow, and a feeling of incomplete emptying of the bladder.

Urine cultures are usually negative, and urinary symptoms are generally worse during the day than at night.

This disorder typically presents with dysuria as one of the key symptoms, the original description of urethral syndrome being urinary frequency and dysuria with no evidence of infection. Dysuria typically occurs when urine comes in contact with the inflamed or irritated urethral mucosa. This symptom is aggravated by contraction of the detrusor muscle and urethral peristalsis, which then stimulates submucosal pain receptors that cause pain or a burning sensation when urinating. Several conditions can cause dysuria through different mechanisms. The problem was thought to be mainly due to treatable urethral stricture with serial urethral dilations. Urethral dilations are now thought to be appropriate in only a very small minority of patients.

True dysuria requires differentiation from other symptoms, which can also occur due to pelvic disorders from various bladder conditions such as interstitial cystitis, prostatitis, and suprapubic or retropubic pain[2].

Urethral pain syndrome is found predominantly in women between the ages of thirty and fifty. In this group of women, vaginal pathology (vaginal infections, atrophic vaginitis and similar conditions) should be carefully excluded. It is thought that up to a quarter of all patients, particularly women, with lower urinary tract symptoms without a documented infection may actually have urethral pain syndrome.

Diagnosis is primarily one of exclusion. There is clearly an overlap between urethral pain syndrome and other urogenital disorders, as there is a distinct lack of consensus on specific criteria among these disorders and they may not be mutually exclusive. The exact cause of urethral pain syndrome is unknown; however, certain health conditions and environmental factors can increase the risk of developing urethral syndrome.

Sexually transmitted infections can increase the risk of developing urethral syndrome. Sexually transmitted infections that can lead to urethral syndrome include gonorrhea, chlamydia, and mycoplasma genitalium.

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[1] Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, Van Kerrebroeck P, Victor A, Wein A., Standardisation Sub-Committee of the International Continence Society. The standardisation of terminology in lower urinary tract function: report from the standardisation sub-committee of the International Continence Society. Urology. 2003 Jan;61(1):37-49. [PubMed: 12559262]

[2] Dysuria: What You Should Know About Burning or Stinging with Urination. Am Fam Physician. 2015 Nov 01;92(9): [PubMed: 26554482]

Some foods may contain elements that can irritate the urethra. Foods that can increase the risk of urethral syndrome in some people include caffeine, alcohol and spicy foods.

The chemicals in soaps, personal hygiene products and contraceptives may contain chemicals that irritate the urethra in some people.

People can sometimes develop urethral syndrome after recently having a urinary tract infection. This is because the urethra can be very sensitive during recovery from an infection.

The treatment of urethral syndrome depends on the suspected cause of the condition and may include the use of anti-inflammatories, dietary and lifestyle changes for preventive purposes and also psychological support, being the fundamental psychic component in the course of the disorder. The difficulty in identifying the causes makes it difficult to establish a truly effective therapeutic path.

For the treatment of urethral syndrome, oxygen therapy was chosen as the use of this technique satisfies the treatment of symptoms such as: burning, pain and inflammation that characterize this pathology.

Oxygen therapy has a powerful regenerative, antibacterial and anti-inflammatory effect[3], it is therefore believed that it can also be very useful in the treatment of this pathology for 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 the disposal of pain and inflammation mediators (histamine, serotonin, prostaglandins)[4].

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 repairing properties of the mucosa[5].

The association of high concentration oxygen and hyaluronic acid has been shown to have therapeutic efficacy in the treatment of vulvo-vaginal atrophy[6], in particular in the reduction of painful symptoms associated with this condition, the aim of the study is to use the association between high concentration oxygen and hyaluronic acid for the treatment of urethral syndrome.

[3] Kellar RS et al. Topically delivered dissolved oxygen reduces inflammation and positively influences structural proteins in healthy intact human skin. J Cosmet Dermatol. 2013 Jun;12(2):86-95.
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[6] Condemi L, Di Giuseppe J, Delli Carpini G, Garoia F, Frega A, Ciavattini A. Vaginal natural oxygenation device (VNOD) for concomitant administration of hyaluronic acid and topical hyperbaric oxygen to treat vulvo-vaginal atrophy: a pilot study. Eur Rev Med Pharmacol Sci. 2018 Dec;22(23):8480-8486.

Treatment

Seven weekly oxygen therapy treatments were performed on 20 women diagnosed with urethral syndrome, for a total of five weeks. five minutes of oxygen therapy and 15 minutes of oxygen therapy combined with hyaluronic acid.

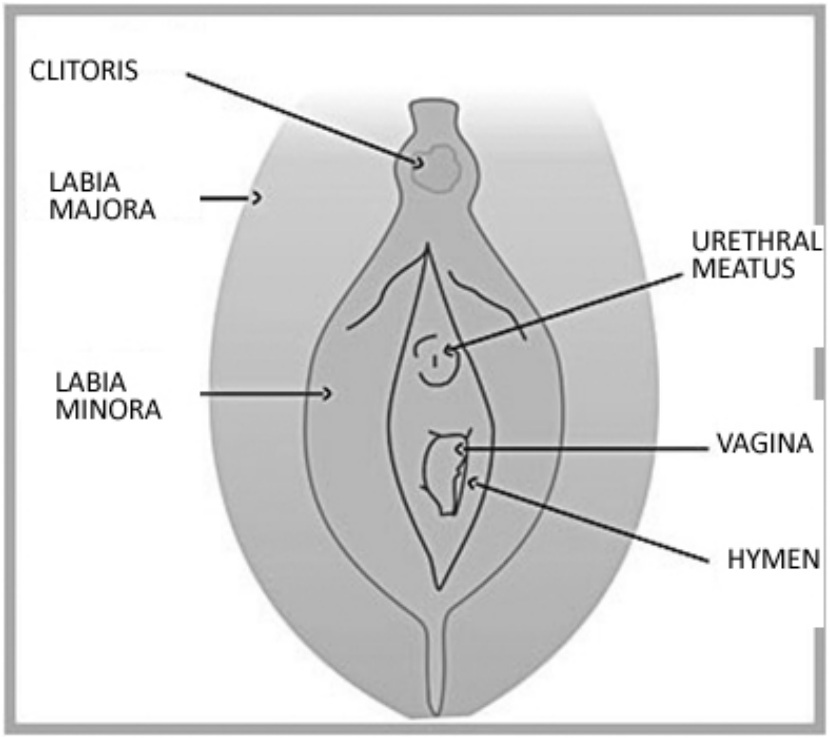

FIGURE 1

Patients are taught home behavior rules, which are necessary to contribute to pelvic health; name that include hygiene, good and healthy nutrition, indications for sexual intercourse.

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 ± 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 ± 3% pure oxygen.

For the application, an airbrush connected to the machine body was used, 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

A questionnaire assessment was performed on the treated subjects including 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 7 sessions (T7).

Results

The patients reported a significant and progressive improvement for all the sessions performed. The improvement is progressive with a decline linked to the accumulation of benefits, with the resolution of the burning sensation (Figure 3 - Kruskal-Wallis test / Two-tailed test P for trend <0.0001). The average VAS value goes from 8.2 in the first session to 1.5 after the seventh application, with a reduction of 82%.

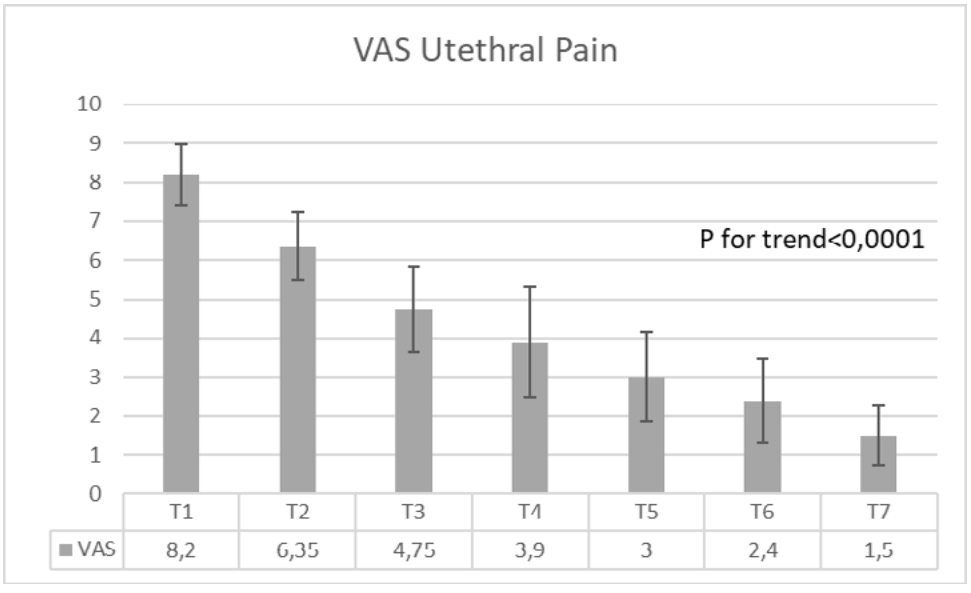

FIGURE 3

The analysis of the treatment values shows how the greatest and statistically significant reduction (from 8.2 to 6.35) occurs between the first treatment session (Figure 3).

The analysis of the progression of the treatment (Figure 4) shows how the improvement of the values is progressively reduced with the accumulation of the induced benefits, which are however progressive.

p-values:

	T1	T2	T3	T4	T5	T6	T7
T1		0,044	0,000	0,000	<0,0001	<0,0001	<0,0001
T2			0,214	0,087	0,001	0,000	<0,0001
T3				0,968	0,224	0,037	0,001
T4					0,945	0,618	0,100
T5						0,984	0,363
T6							0,869
T7							

Figure 4

No side effects associated with the treatment were reported by the patients.

Combined oxygen therapy with hyaluronic acid 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, with no side effects.

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 VV15, 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 data17,18.

• 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.

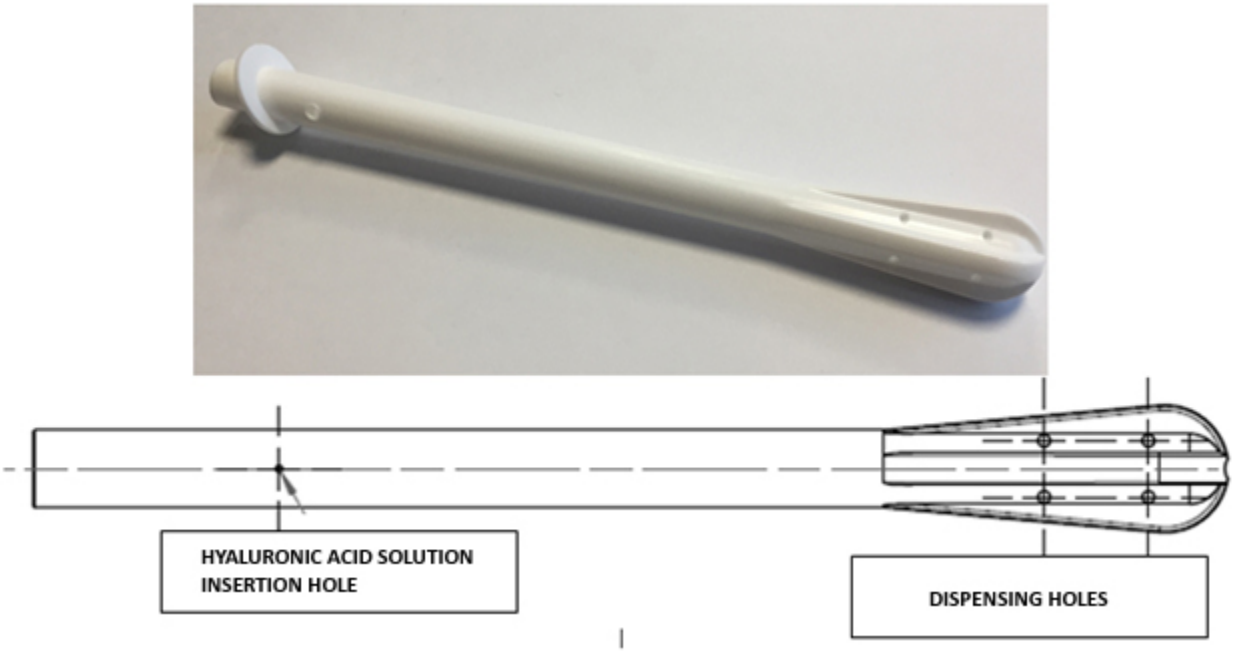
The association of high concentration oxygen and hyaluronic acid has been shown to have therapeutic efficacy in the treatment of vulvo-vaginal atrophy, the aim of the study is to use the association between high concentration oxygen and hyaluronic acid for treatment. of the disorders caused by vestibulitis 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 ± 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 ± 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.

	PH	DYSPAREUNIA	DRYNESS	ITCHING	BURNING	ELASTICITY
T0	7,24	7,2	7,8	6,64	7,28	2,48
T5	4,16	1,5	1,44	1,36	1,48	4,48
P	< 0,0001	< 0,0001	< 0,0001	< 0,0001	< 0,0001	< 0,0001

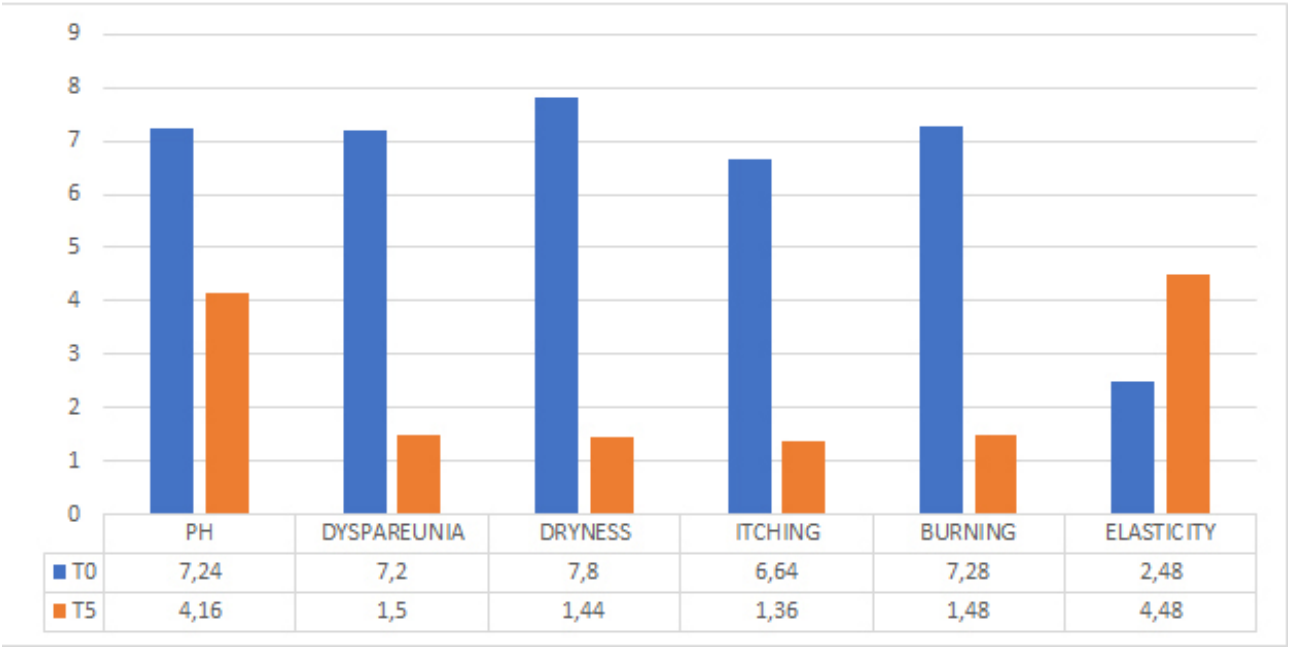


FIGURE 3

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	T0	T5
1	over	normal	Candidiasis	no
2	over	normal	Candidiasis	no
3	over	normal	Candidiasis	no
4	over	normal	Candidiasis/Cystitis	no
5	over	normal	Candidiasis	no
6	over	normal	Candidiasis	no
7	over	normal	Candidiasis	no
8	over	normal	Candidiasis	no
9	over	normal	Candidiasis	no
10	normal	normal	\	\
11	over	normal	\	\
12	over	normal	Cystitis	no
13	over	normal	Candidiasis	no
14	over	normal	Candidiasis/Cystitis	no
15	over	normal	\	\
16	over	normal	\	\
17	over	normal	Candidiasis	no
18	over	normal	Candidiasis	no
19	over	normal	Candidiasis	no
20	over	normal	Candidiasis	no
21	over	normal	\	\
22	over	normal	\	\
23	over	normal	\	\
24	over	normal	Candidiasis	no
25	over	normal	Candidiasis	no

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

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 lubrification 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 ± 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 lubrification and presence of fluid (Figure 1).
For the group B significant variation was reported for the elasticity and the epithelium appareance index, whereas for the group C only the burning index shown a significant improvement (Figure 1).

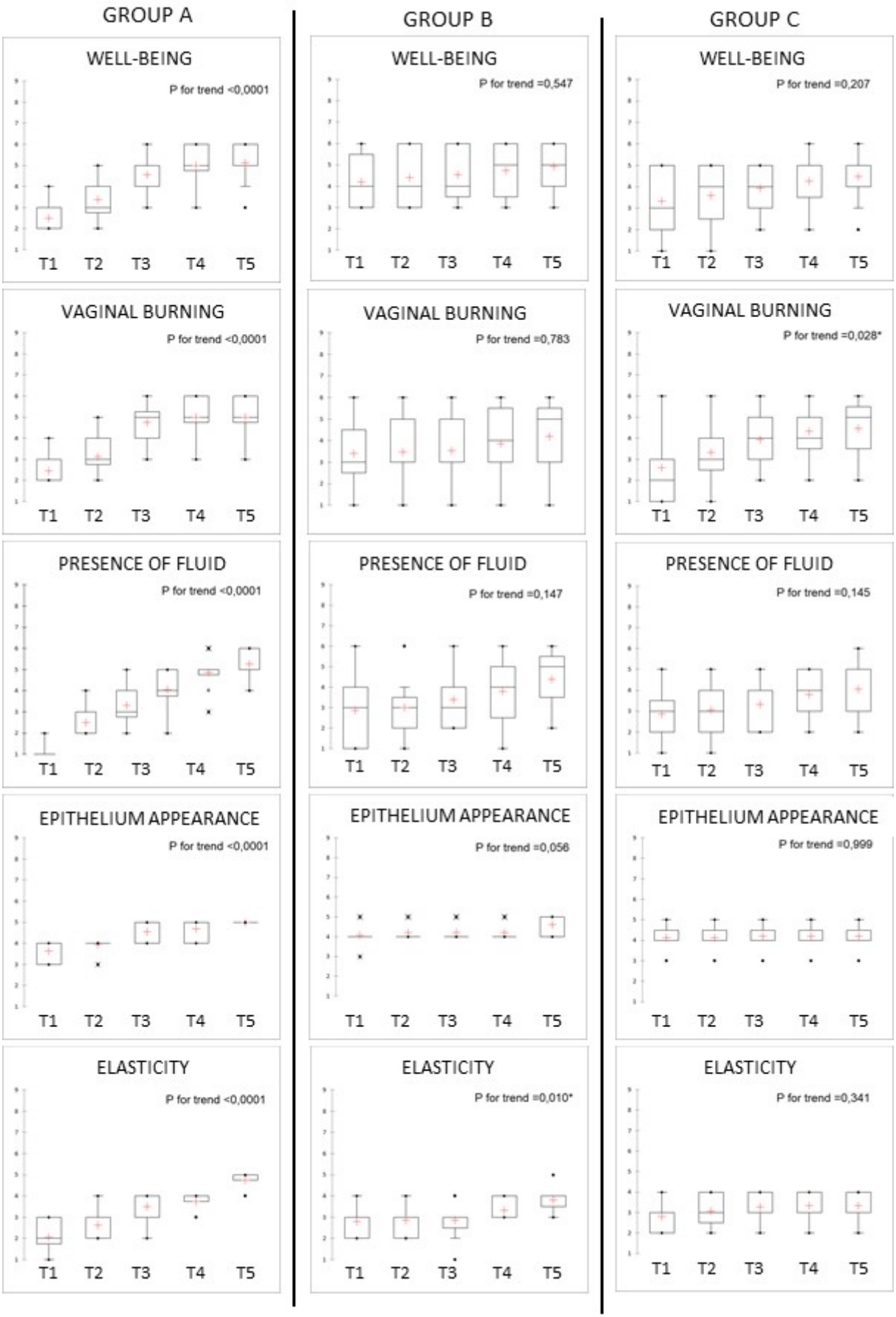


FIGURE 1

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.

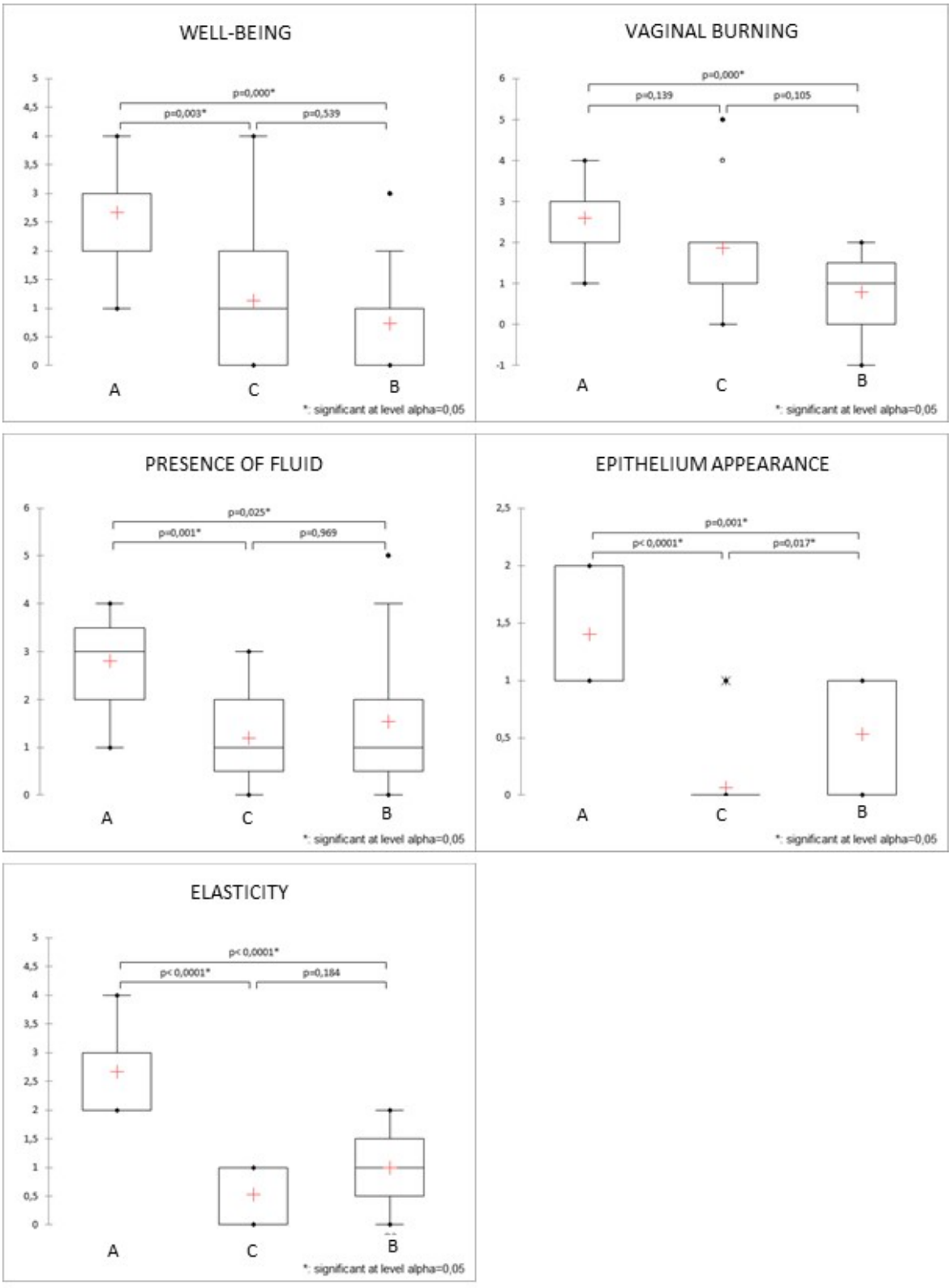


FIGURE 2

TITLE

Vaginal atrophy due to oestrogen deficiency: conjugate treatment with high concentration oxygen and hyaluronic acid.

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ABSTRACT

Background: vulvovaginal atrophy is a condition closely related to circulating oestrogen deficiency with post-menopause being the main cause, but patients of childbearing age may also present with these symptoms due to treatments that reduce oestrogen production. Local oestrogen therapy is the causal treatment of local symptoms; it is not always accepted and often abandoned by patients. In recent years, alternative therapies have been proposed such as the use of fractional CO2 laser or the conjugate treatment with normobaric oxygen and hyaluronic acid, which is the subject of this study.

Objectives: to evaluate the effectiveness of conjugate topical treatment with normobaric oxygen and hyaluronic acid

Materials and Methods: 50 patients were evaluated and treated with 5 applications of 15 minutes each, every 15 days, with Caressflow. All patients presented at least one of the symptoms related to vulvovaginal atrophy: dryness, burning, dyspareunia. In all cases, vulvoscopy, colposcopy, and cervico-vaginal cytology were performed. The patients were interviewed with an analogue scale (VAS) on the symptoms present before and after the treatment. The change in colposcopy and cytology examination results was assessed by VHI at baseline and at the end of treatment.

Results: all patients completed the treatment scheme and presented a significant improvement in subjective symptoms. The colposcopic and cytological control performed 10 days after the end of the last treatment showed a significant change 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.

Conclusion: the data of this study confirm the information in the latest published papers on the effectiveness of treatment with normobaric O2 and hyaluronic acid on vaginal atrophy. Efficacy has been confirmed both in terms of subjective symptoms and objective results.

INTRODUCTION

Vulvovaginal atrophy is a condition closely linked to the deficiency of circulating oestrogen, with post-menopause being the main cause. In fact, more than 50% of women 2-4 years after the end of their fertile period suffer from it and present typical symptoms such as vaginal dryness, burning, itching, dyspareunia, sometimes associated with urinary disorders. Bleeding may also occur in more severe cases, especially after microtrauma. [1, 2, 3,4, 5]

Patients of childbearing age may also present with these symptoms due to treatments that reduce oestrogen production such as bilateral salpingo-oophorectomies, chemotherapy, ovariostatic therapies for endometriosis, but also the use of low oestrogen oral contraceptives.

Oestrogen deficiency not only causes atrophy of the vaginal epithelium but also significantly alters the vaginal environment with decreased lactobacilli, increased pH and reduced blood supply and consequently decreased vaginal fluid secretion. [6]

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 related to the presence of oestrogen. Over the years, a myriad of treatments have been used, including ordinary lubricants/emollients, local or systemic oestrogens, hyaluronic acid, soy and red clover derivatives, vitamins, etc.

Certainly, local oestrogen has been and still is the most effective treatment in terms of results and duration, but not all women willingly accept the use of these hormonal substances, even if systemic absorption is very low. The risk and fear of increased incidence of breast cancer and the need for continued treatment leads many patients to abandon treatment or to reject it altogether. [7]

In recent years, alternative therapies have been proposed such as the use of fractional CO2 laser, or lipofilling with sometimes encouraging results but not without drawbacks such as, for the laser, pain, high costs, and the need for re-interventions. Recently, warnings from American scientific societies, due to the risk of stimulating the progression of pre-cancerous lesions, have reduced its use. [8, 9, 10, 11]

Hyaluronic acid is a natural polysaccharide present in large quantities in the extracellular matrix of the skin and cartilage. By binding to a large quantity of water molecules, it allows adequate hydration of the mucous membranes. Oxygen promotes tissue repair processes and collagen synthesis by stimulating neoangiogenesis processes that promote nourishment of the tissues. [12] The aim of this investigation was to confirm the effectiveness of combined treatment with hyaluronic acid and high concentration oxygen.

MATERIALS AND METHODS

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

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

The treatment comprised five sessions of 15 minutes each every 15 days with oxygen and hyaluronic acid (see device features).

Before starting 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

Table 1 – 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				

The colposcopy and cytological evaluation assessed the status of the cervico-vaginal epithelium with a score from 1 to 4 (Vaginal Health Index: VHI):

1) Reddened with petechiae
2) Bleeding on contact
3) Slightly reddened
4) Normal

Vaginal elasticity was classified with 4 scores:

1) absent
2) very poor
3) average
4) good

The cytological examination defined the presence of atrophy in four classes:

1) intense
2) average
3) scant
4) normal

Table 2 – 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	High	Medium	Poor	Normal trophism

All subjective parameters were reported before each treatment; colposcopy and cervical-vaginal cytological examination were performed 10 to 30 days after the end of the last treatment.
With regard to statistical analysis, the statistical significance of the variance trend in values between treatments was analysed using one-way analysis of variance according to the Kruskal-Wallis test. The significance of pairwise comparisons between treatment sessions was analysed using the Wilcoxon test for non-parametric data.

RESULTS

7 patients were excluded from the study because they had:

- vulvar lichen sclerosus (4),
- abnormal pap smear (3)

The mean age of the patients at enrolment was 55, 5 years +/- 6.4 SD [45-66].

4 patients had a 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, with the highest score in 70% of cases (see table 3).

The colposcopic and cytological control performed 10 days after the end of the last treatment showed a significant change in the appearance and elasticity of the vaginal epithelium (see images 1 and 2) . The cervico-vaginal cytological examination carried out in patients who were checked early (10 days) after the end of the last treatment, showed the disappearance of the findings of atrophy shown on the pre-treatment cytology (image 3) and the presence of cytoplasmic inclusions of hyaluronic acid (image 4).

Table 3

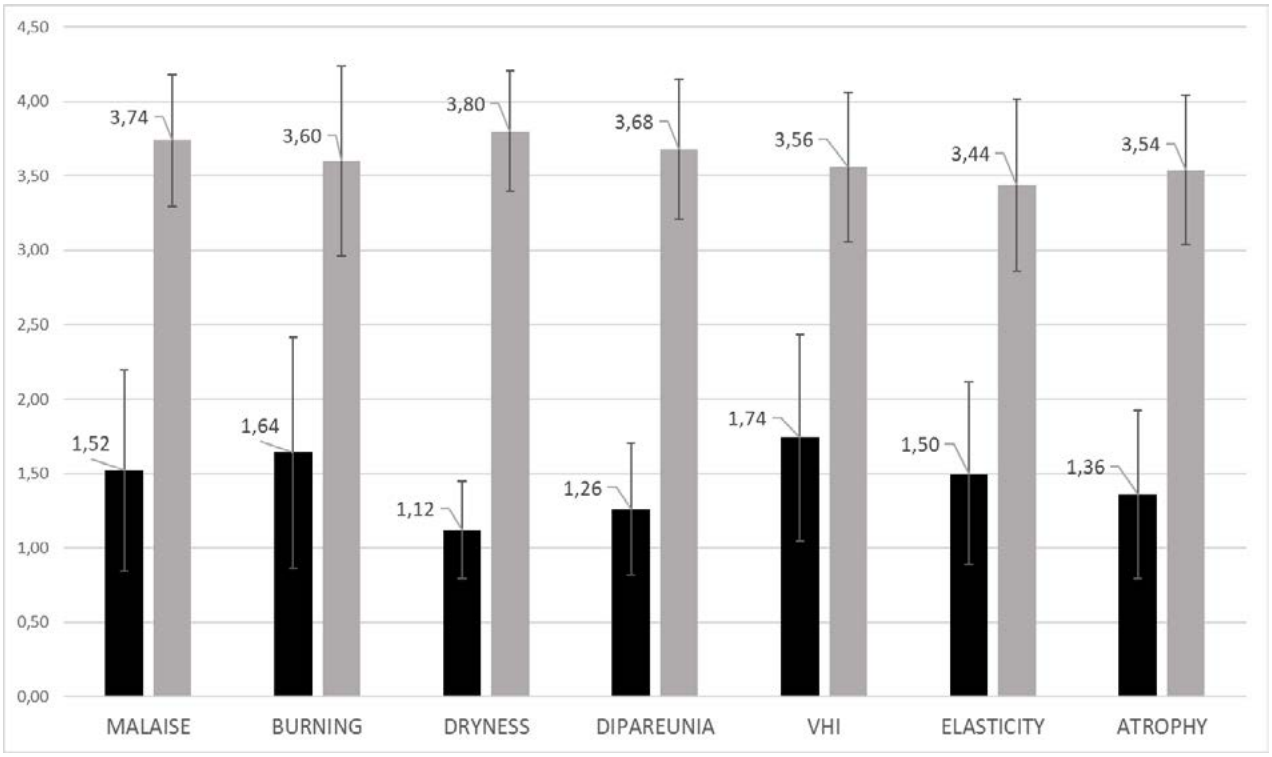


Image 1 – Intense atrophy, reddened epithelium with petechiae

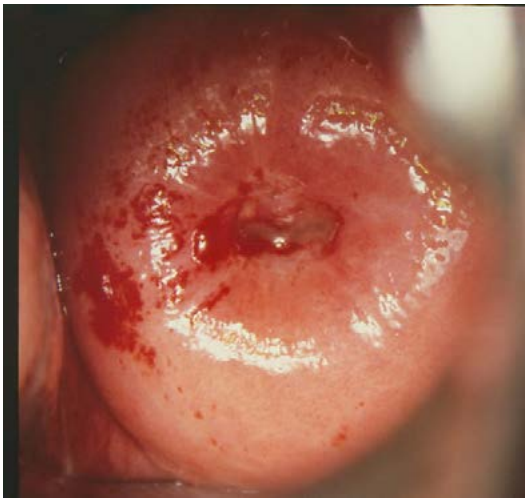


Image 2 – Normal epithelium after treatment

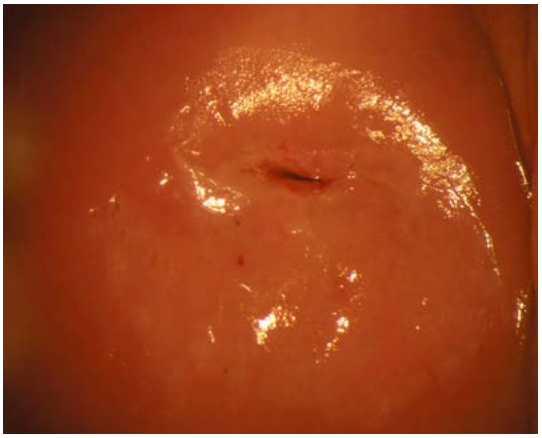


Image 3 – Cytological atrophy

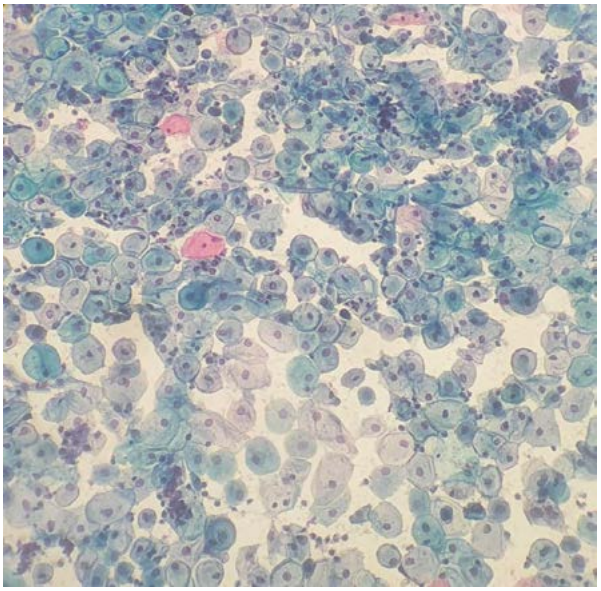
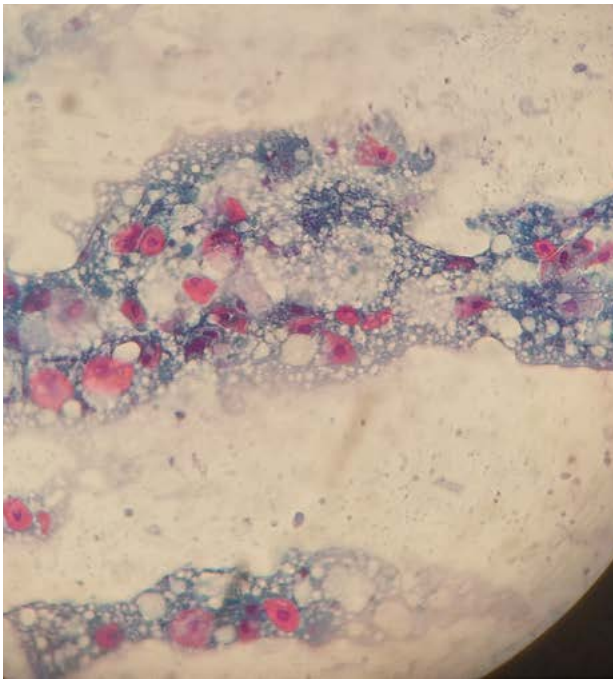


Image 4 – 10 days after the end of the last treatment



DISCUSSION

Vaginal atrophy is undoubtedly a very frequent condition in post-menopausal women (about 50%) or women with oestrogen deficiency induced by other causes such as surgical castration, chemotherapy or radiation treatments. This percentage is certainly underestimated as many women do not report some of the disorders associated with vaginal atrophy, especially those related to the sexual sphere. [12]

Hormone replacement therapy post-menopause can certainly prevent the onset of this situation 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 flushes, insomnia, decreased libido, anxiety, etc.

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

Local oestrogen therapy has played and plays an important role in maintaining good trophism of the vaginal mucosa, but even in these cases not all patients accept it and in some situations the therapy is not very effective.

Many topical, non-hormonal products on the market often demonstrate a positive action in promoting lubrication but are often not sufficient to solve all the problems related to alterations of vaginal trophism and above all allow transient benefits with the consequent need for repeated

treatments especially at the time of sexual intercourse. Even the most recent treatments with fractionated CO2 laser and radiofrequency have given significant improvements in the management of the pathology, but some difficulties persist related to the aspect of pain during the procedures. [13, 14, 15] The use of the device that is the subject of this study, which delivers normobaric O2 and hyaluronic acid, is a valid alternative to the devices that have been available up to now and, according to the data that have emerged from previous studies [16, 17] it is one of the best in terms of the results obtained both in the resolution of subjective symptoms: burning, itching, dryness, discomfort, dyspareunia (VAS scale) and objective results: elasticity, trophism of the vaginal epithelium, cervico-vaginal cytology (VHI). The differences proved to be statistically significant in all the areas analysed, with the greatest benefits being observed in the reduction of vaginal dryness and the disappearance of dyspareunia, thus allowing a clear increase in quality of life in over 98% of cases.

The most relevant data not yet highlighted in the publications currently available was found on the cytology performed after the end of the treatments; the presence of hyaluronic acid vesicles in the cytoplasm of the cells observed confirms the regenerative action at tissue level, thus surpassing the surface action of many other methods and thus suggesting a serious possibility that the improvement in subjective and objective parameters may be lasting over time. This aspect will be demonstrated in the long-term follow-up of the treated patients; in any case, out of 10 patients reviewed after 6 months, 8 maintained the improvements achieved, 2 reported a slight increase in vaginal dryness that had appeared in the last month before the check-up and underwent two further cycles at 15-day intervals that brought the situation back to its previous level.

CONCLUSIONS

The data from this study confirm the information in recent published papers on the effectiveness of treatment with normobaric O2 and hyaluronic acid on vaginal atrophy. Efficacy has been confirmed both in terms of subjective symptoms and objective results.

The demonstration of the integration of hyaluronic acid in microvesicles in the cytoplasm of vaginal cells constitutes further scientific confirmation of the regenerative activity of hyaluronic acid associated with normobaric O2.

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3.
OXYGEN AND HYALURONIC ACID IN TISSUE REGENERATION

European Review for Medical and Pharmacological Sciences 2016; 20: 4190-4195

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 Dryiness, 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 condition[4]. 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 absorption[5]. 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 patients[6]. 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 symptoms[7].

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 tissues[8].

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 intercourses 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 Bachmann[9] 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 intercourses. 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.

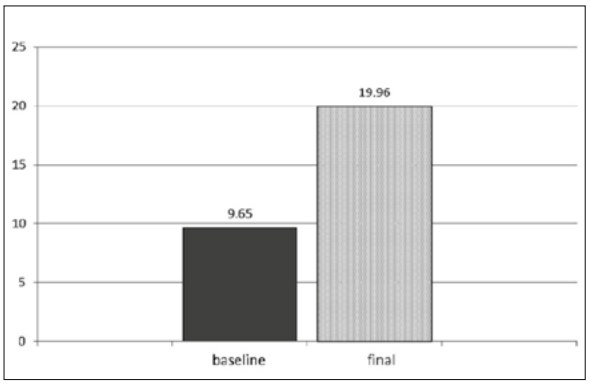

Figure 1. Vaginal Health Index (VHI) mean scores at baseline and after treatment (Wilcoxon signed-rank test: $p < 0.0001$).

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 painful intercourses (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 dryiness	7.65	3.95	< 0.0001
Vaginal burning	5.15	2.93	< 0.0001
Genital itching	2.21	1.13	< 0.0001
Painful intercourses	5.63	3.54	< 0.0001

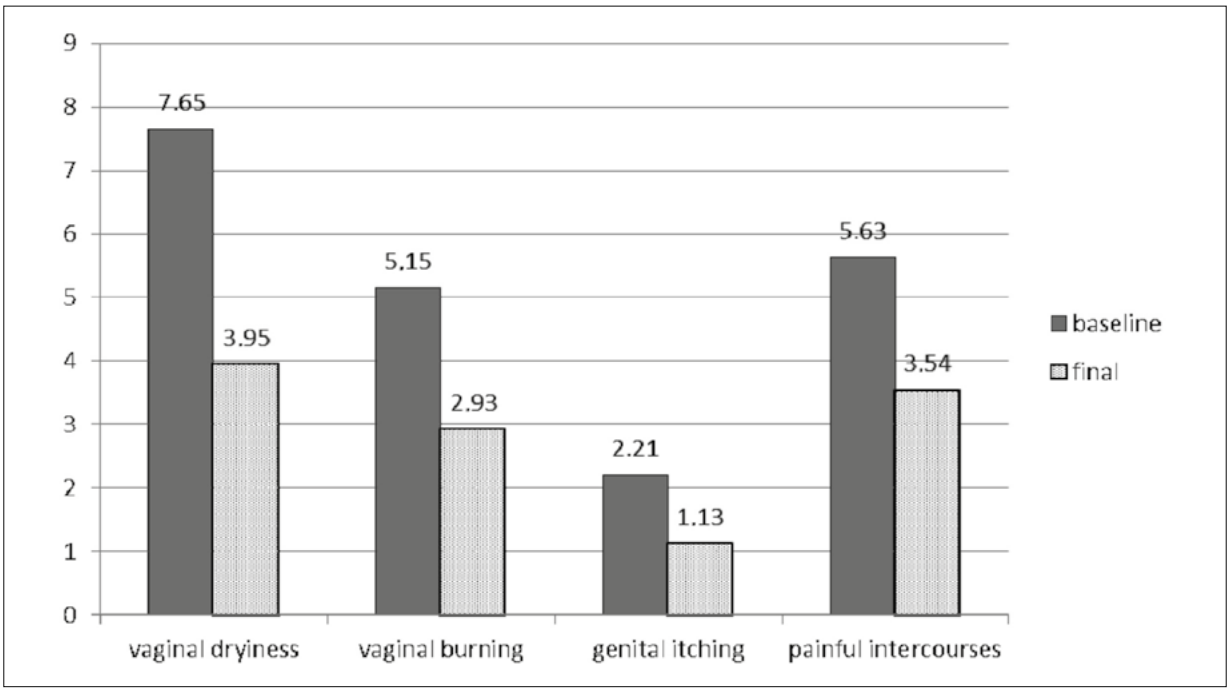

Figure 2. Visual Analogic Scale (VAS) mean scores of symptoms at baseline and after treatment (Wilcoxon signed-rank test: $p < 0.0001$).

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 undertreated[10]. 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 CO_2 laser treatment of VVA symptoms has been reported in the literature[13-16]. 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 results[17-19]. 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 women[20-22] are available in literature and, even when compared with vaginal estrogen, hyaluronic acid demonstrated similar effectiveness[19]. 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 discontinuation[10]. 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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ORIGINAL ARTICLE

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 Kingerberg 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

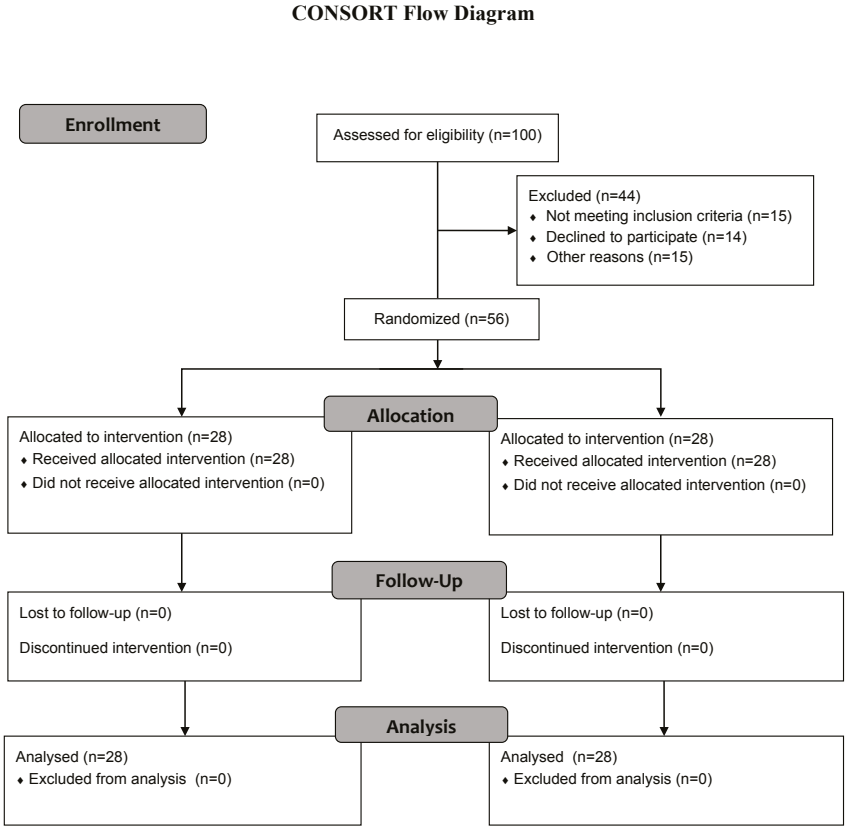

Figure 1: Consort flow diagram of participants

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,sevear 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±.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) Mean±SD	P-value within groups	Hyaluronic acid group (n=28) Mean±SD	P-value within groups	P-value between groups
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

comparedwith 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 g roups treated before and after the intervention

VCMI[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)		

[a]Vaginal 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 researc-, the results f 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 drugs but and doesn't match in terms of drug form and comparison with vitamin E.

Another researcher studied the effectiveness of three drugs, i.e. hyluronic 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 menopaused 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 hyluronic acid that is similar to the results obtained by Castantino and Guaraldi's study. But is 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 hyaloronic 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 hyaloronic 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 increase[5].

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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 atrophy[6]. 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 2005[9], 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 therapy[13].

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 elastic[15]. 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 vaginosis[18]. 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 vulvovaginitis[19]. 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 vulvoscopie, vaginoscopie, cervix, ovarian, uterus and tubal analysis. Registration of objective vaginal symptoms (inflammation, edema, vulvo-vaginal abrasions) and of subjective vaginal symptoms (dryness, hitching, 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 (hitching, 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 ± 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 ± SD, 51.6 ± 7.6), with a BMI between 18.1 and 37.47 (mean ± SD; 24 ± 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 ± 7.6 (min 44 max 64)
BMI (Body Mass Index)	24 ± 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

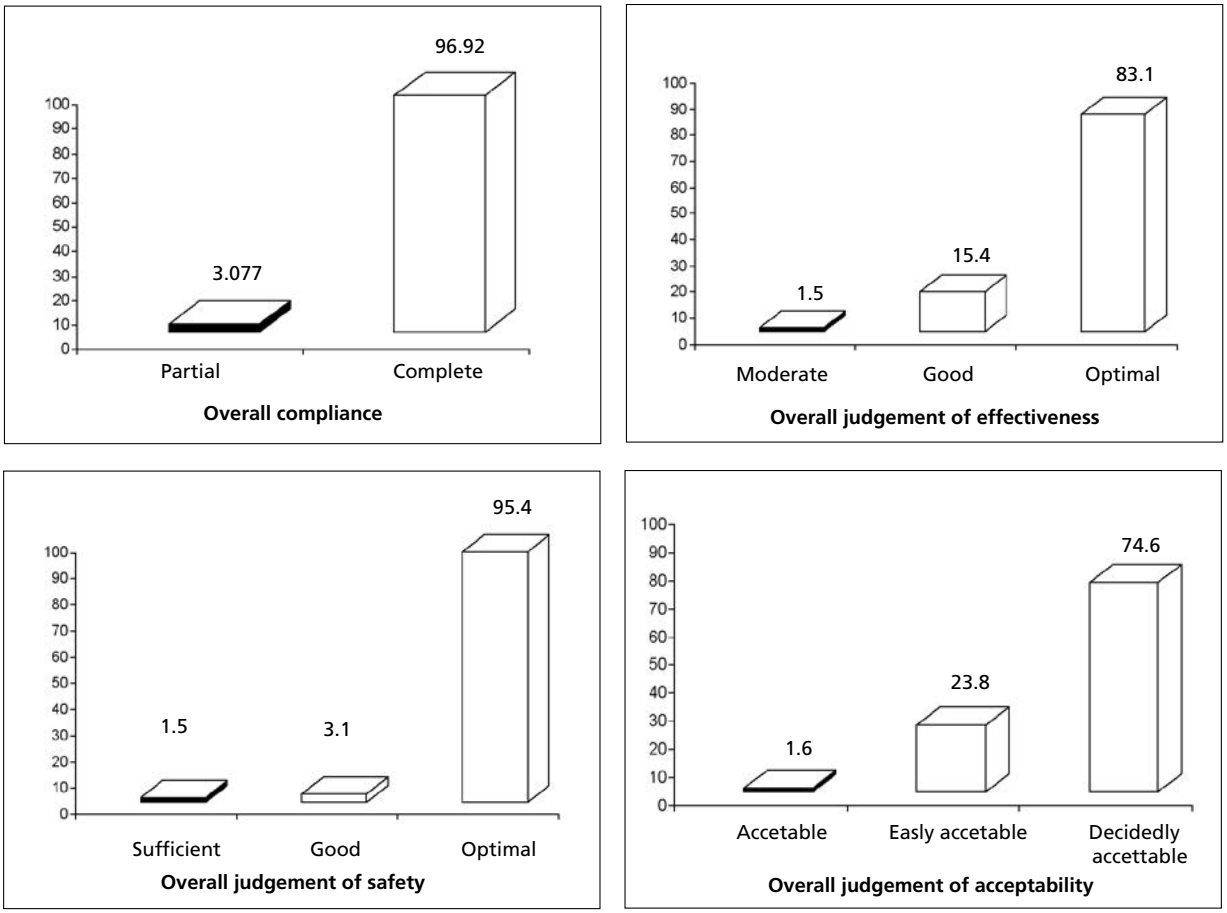

Figure 2. A-D, Overall judgement and compliance in post-menopausal women after 28 days of treatment (data expressed in percentage).

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 results[14].

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 O_2 is known to benefit wound healing. Beyond its role as a nutrient and antibiotic, O_2 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) O_2-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 O_2 benefits wound healing in the peri-operative and outpatient settings [1]. Clinical trials have shown that keeping patients warm and administering supplemental O_2, 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, O_2 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 O_2 to promote wound healing began in the 1960's with administration of systemic HBO to treat wounds [7]. 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 stress[8]. 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 disease [9]. 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 toxicity [13]. 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 wounds [17]. 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. At 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 CACGTCTGCGGATCTTGTACAAACA

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) guidelines [18]. 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 previously [19]. 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 TGFβ-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 cm^3, 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 polynomials [27]. 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 would 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 VEFG, TGFβ1, 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 polynomials [27]. 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, TGFβ1 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 TGFβ1 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 a 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, TGFβ1, 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 edema [35]. In addition, HBO therapy may have important effects on the biology of cytokines and other mediators of inflammation [36]. In patients whose wounds were favorably affected by HBO therapy, increased levels of nitric oxide were observed in response to HBO treatment [37]. 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 disease [9]. 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 exudation [38]. 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 therapy [8].

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 designs [47] and insufficient number of clinical trials [48,49]. The high cost of providing HBO has also raised concerns [47]. In that vein, the cost of not accepting a potentially productive treatment modality may be also counter-argued [50]. 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 outcomes [57]. 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 tissue [15]. 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 clinical [13] as well as pre-clinical [15] 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 process [59] while chronic hypoxia impairs wound angiogenesis [60]. 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 expression [61]. 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 angiogenesis [66].

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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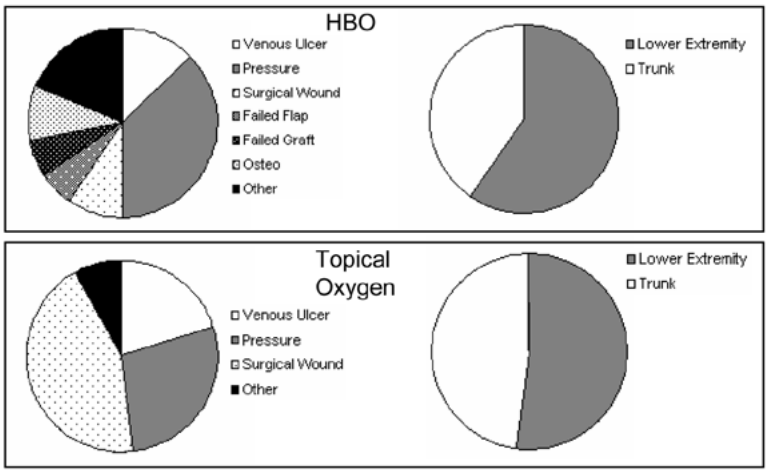

Figure 1.
Wound etiology and locations for patients enrolled in the HBO and topical oxygen studies.

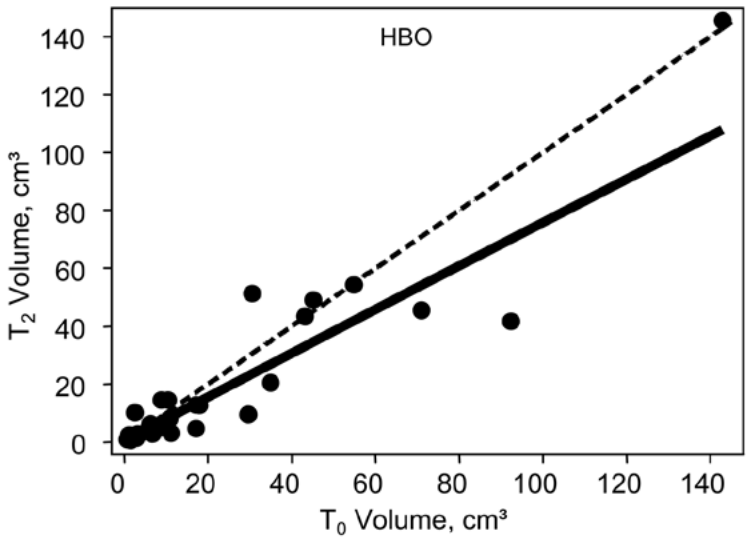

Figure 2. Wound closure in response to HBO treatment

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)

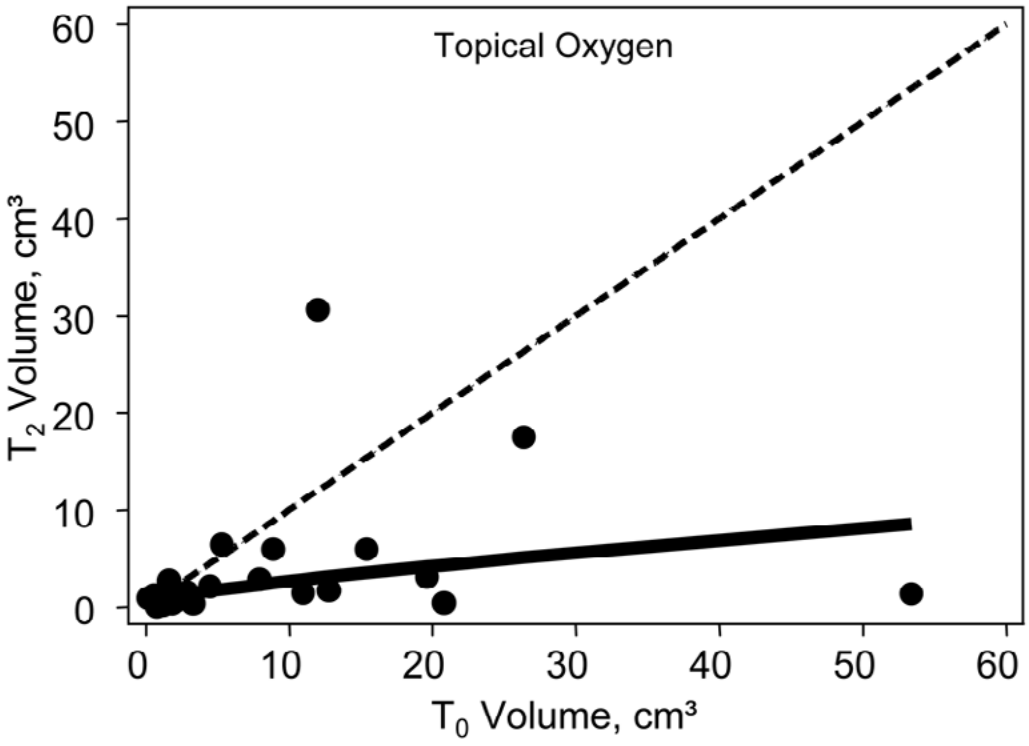

Figure 3. Wound closure in response to topical oxygen treatment

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.

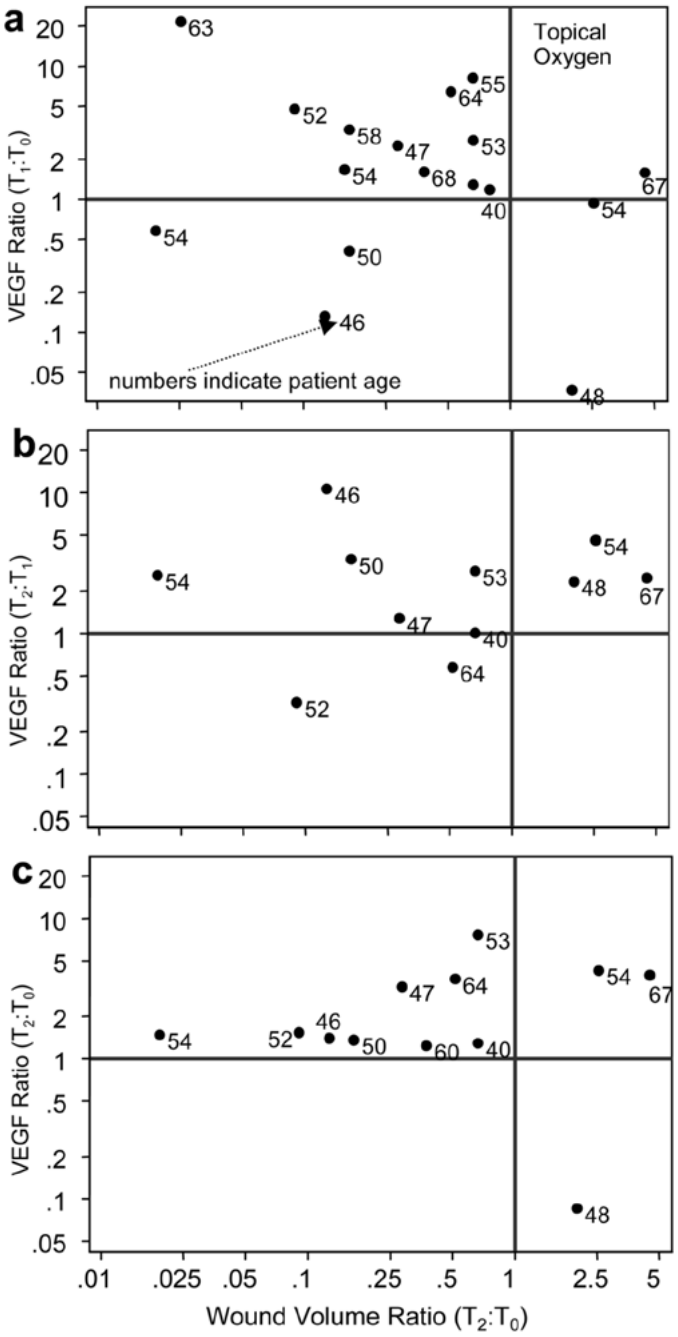

Figure 4. Scatter plot illustrating individual data points plotting topical oxygen induced changes in VEGF gene expression in the time period specified on the y-axis against changes in wound volume over the entire study period

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.

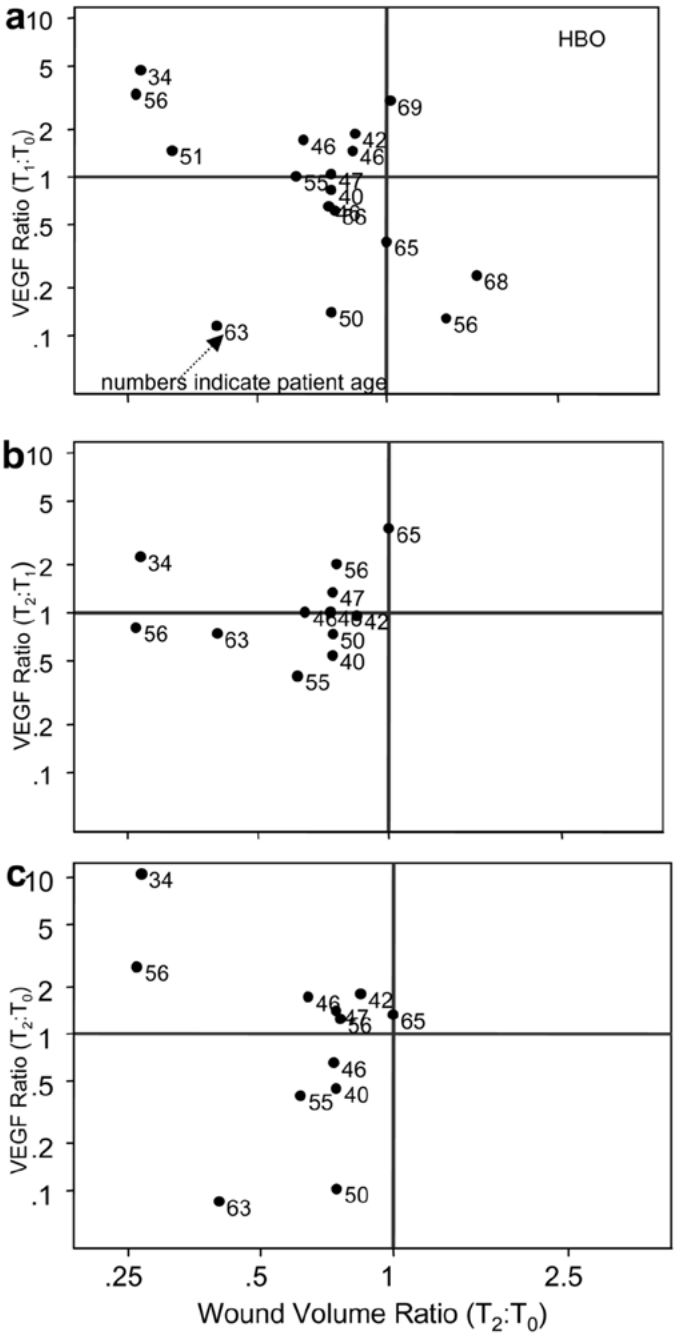

Figure 5. Scatter plot illustrating individual data points plotting changes in HBO-induced VEGF gene expression in the time period specified on the y-axis against changes in wound volume over the entire study period

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 Not Healing	Wounds Healing	Wounds Healing to not healing Ratio	*p*- value[1]
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 T_0 *versus* T_1 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] T_0= baseline measurement; T_1 = midpoint of therapy; T_2 = 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 Not Healing	Wounds Healing	Wounds Healing to not healing Ratio	*p*- value[1]
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 T_0 *versus* T_1 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] T_0= baseline measurement; T_1 = midpoint of therapy; T_2 = 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.

Original Contribution

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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-alpha, 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 by 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; g/m^2 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 x %3+) + (2 x %2+) + (1 x %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× 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.

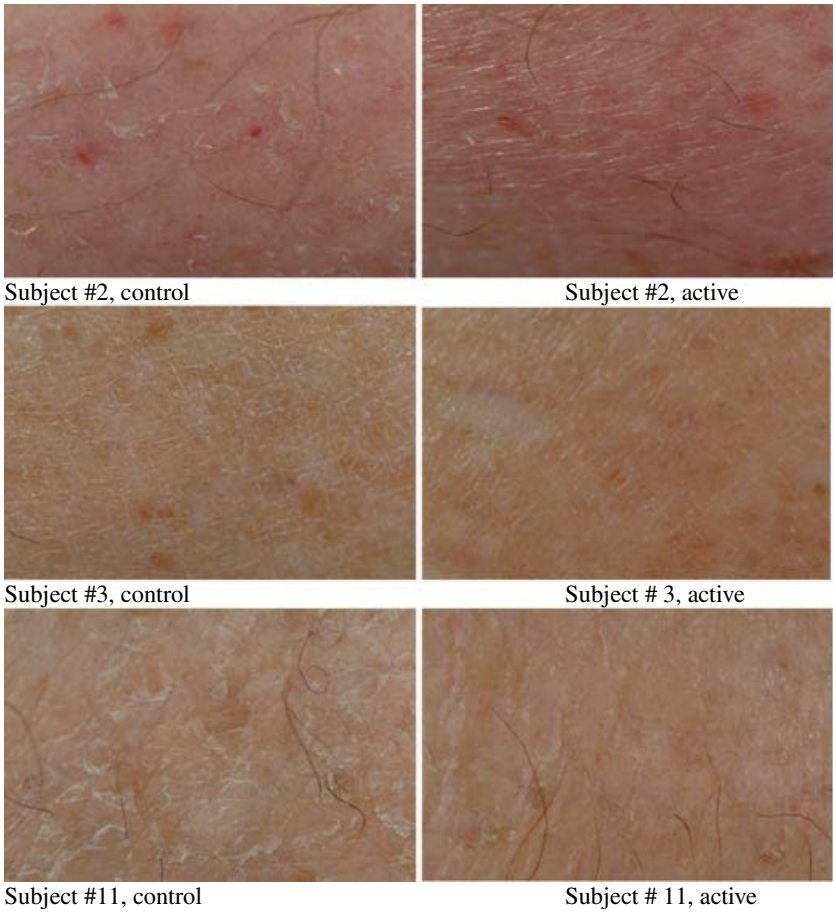

Figure 1 Clinical pictures of control *vs.* active at the 8-week time point.

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 μS 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	*P* value
Skin hydration (μS)	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-alpha 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

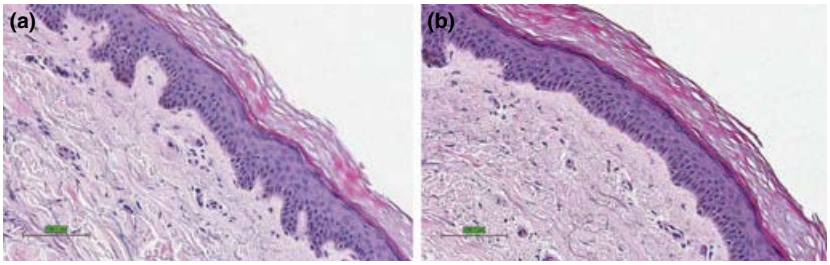

Figure 2 Representative histopathology (hematoxylin and esosin stain). (a) Subject #10, control site at the 8-week time point. (b) Subject #10 active site at the 8-week time point. Scale bars = 100 μm.

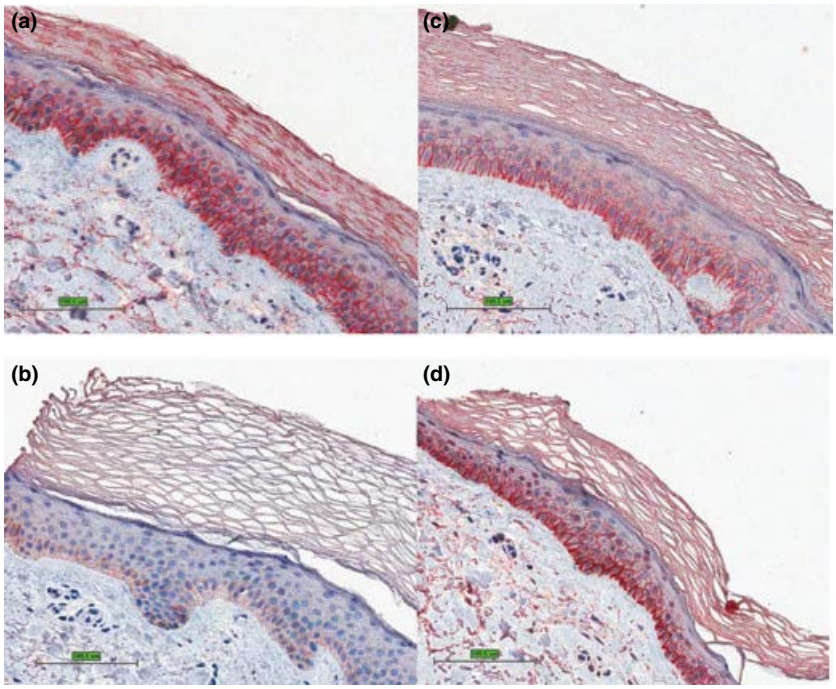

Figure 3 Representative aquaporin-3 levels at 8 weeks. (a) Subject #10, control site. (c) Subject #10, active site. (b) Subject #13, control site. (d) Subject #13, active site.

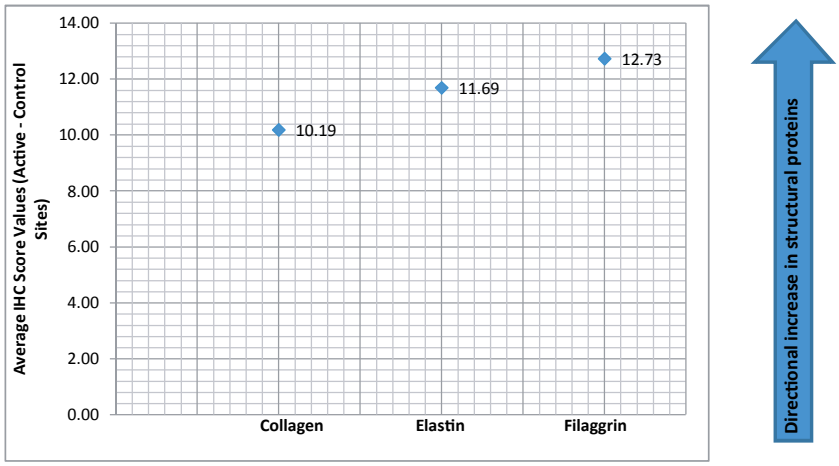

Figure 4 Immunohistochemistry trend plot of structural proteins collagen I, elastin, and filaggrin at 8 weeks.

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 species (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

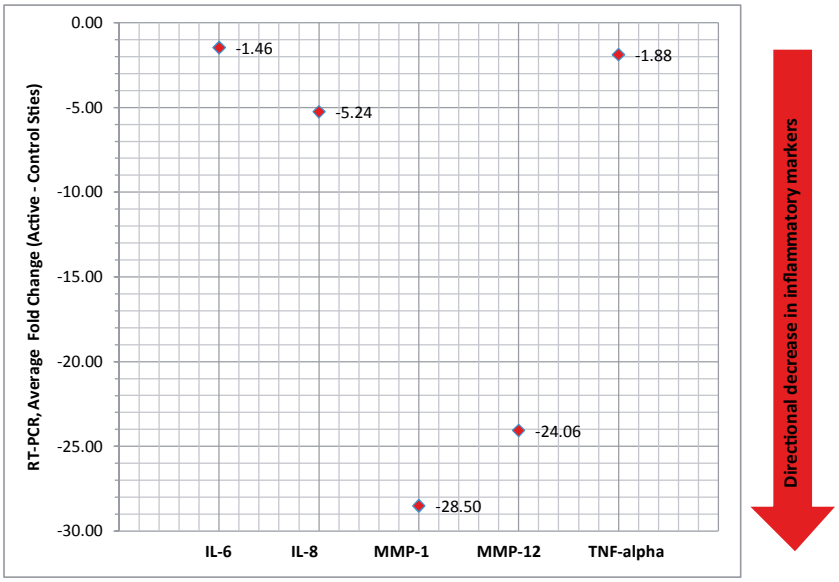

Figure 5 Real-time RT-PCR trend plot of inflammatory markers at 8 weeks.

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, enhancing keratinocyte proliferation, migration, and differentiation.[18]

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-a	PCR	Decrease
	MMP-1	PCR	Decrease
	MMP-12	PCR	Decrease
Epidermis structure	Filaggrin	IHC	Increase
	Elastin	IHC	Increase
	Collagen I	IHC	Increase

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 though dermis than epidermis, and TDO devices deliver oxygen more effectively than TGO devices.**

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

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 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.

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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 $\mu g/cm^2$ of the inorganic catalyst manganese dioxide (Sigma-Aldrich, St. Louis, MO). An aqueous substrate of 0.3% H_2O_2 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 304–457μm) 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 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.

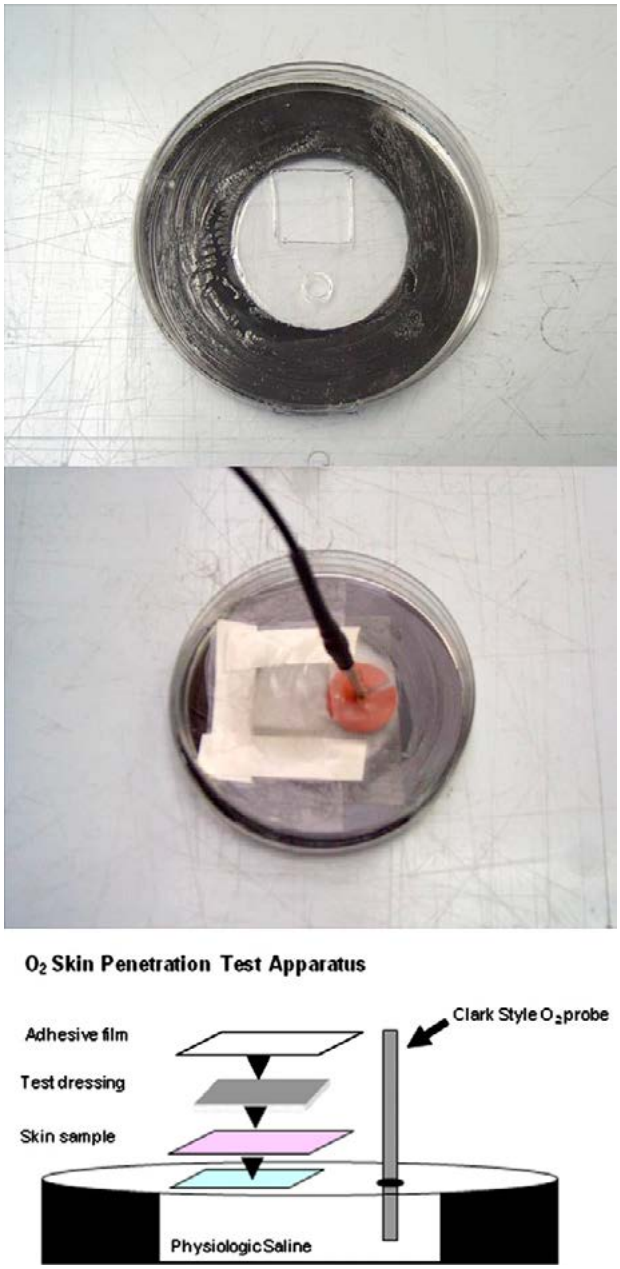


FIG. 1. Photographs and diagram of apparatus for measuring delivery of dissolved oxygen. Constructed from silicone sheeting and polystyrene dish. An 11 mL volume fluid chamber (7 mm depth × 46 mm diameter) within dish filled with saline. A 2 cm × 2 cm sample window over fluid chamber. Oxygen transfer 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).

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 ± 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 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

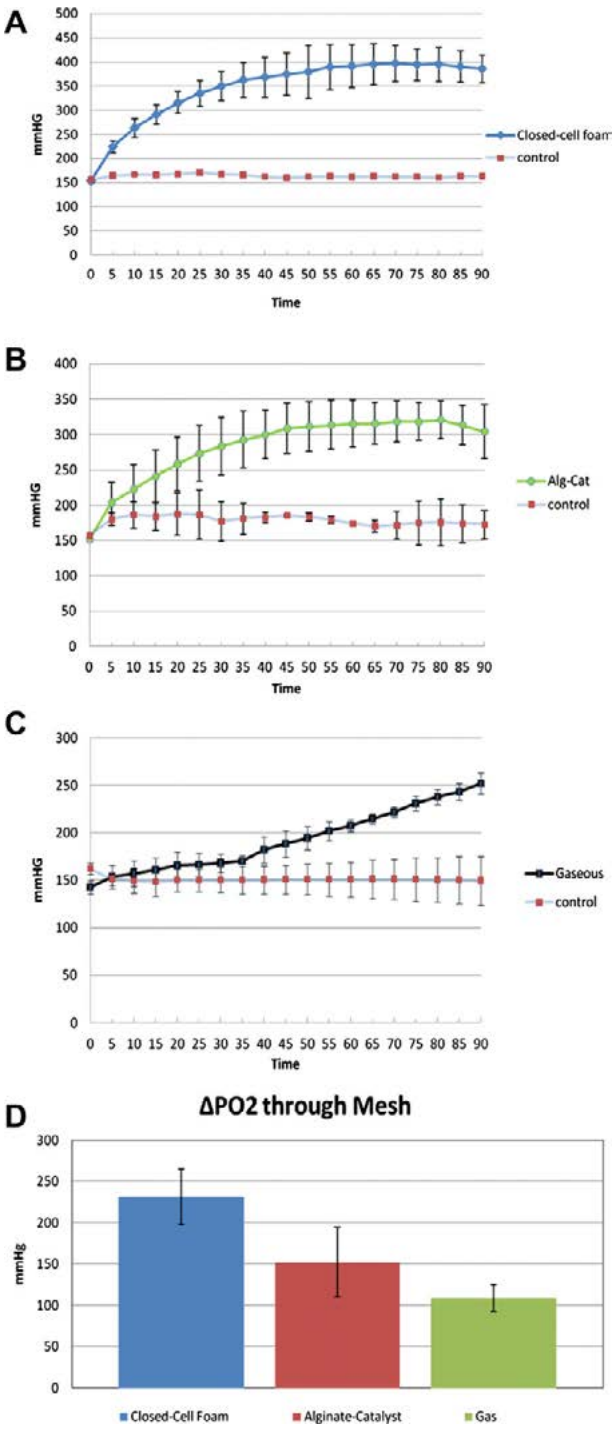
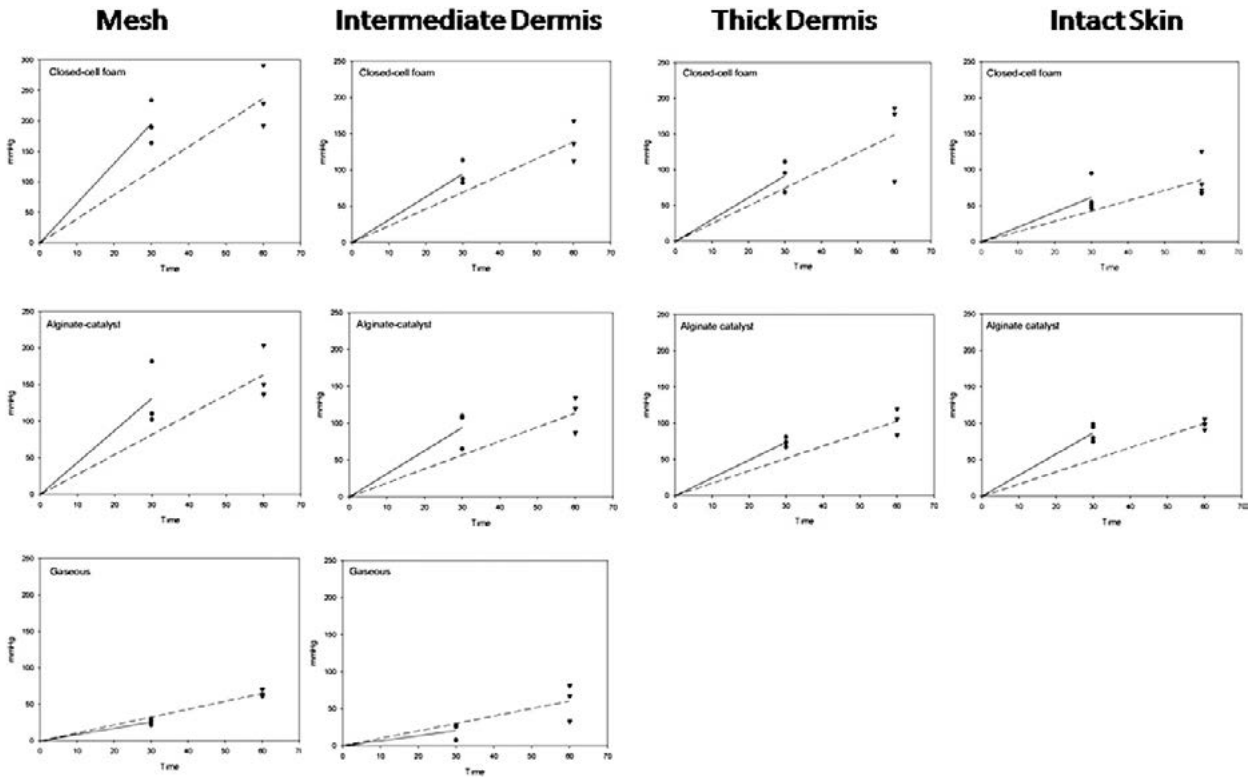


FIG. 2. Transfer of dissolved oxygen for control recording into saline at 37°C; $n = 3$ oxygen delivery devices; $n = 2$ control. (A) Foam dressing; (B) alginate-catalyst dressing; (C) 100% gaseous oxygen; (D) change in partial pressure at 90 min from T = 0. Closed cell foam greater than gaseous ($P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

TABLE 1

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

	Skin sample			
Topical oxygen device	Control	Intermediate dermis 304-457 μm	Thick dermis 457-762 μm	Epidermis 304 μm
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 oxgyen. 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 μm) 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 then does the TGO device. Rate of oxygen transfer through equal thickness

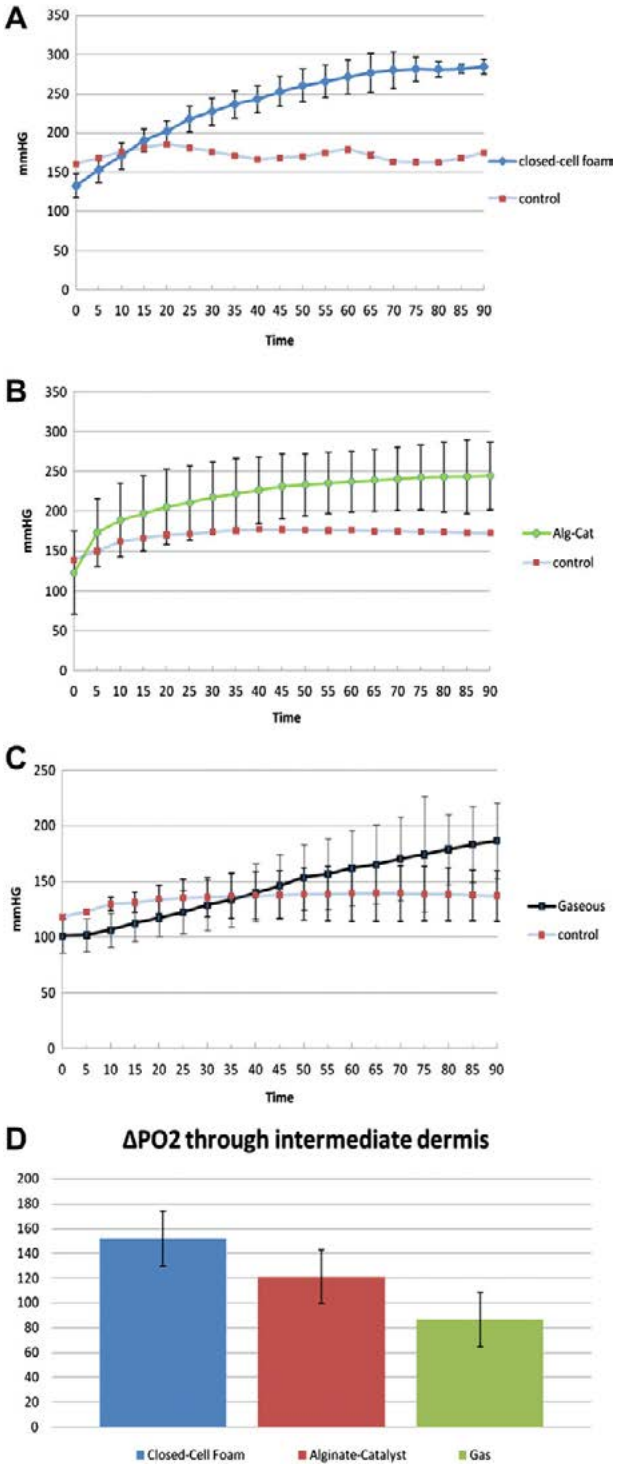


FIG. 3. Transfer of dissolved oxygen through intermediate dermis (304–457 μm) into saline at 37°C; $n = 3$ oxygen delivery devices; $n = 2$ control. (A) Foam dressing; (B) alginate-catalyst dressing; (C) 100% gaseous oxygen; (D) change in partial pressure at 90 min. from T = 0. Closed cell foam greater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

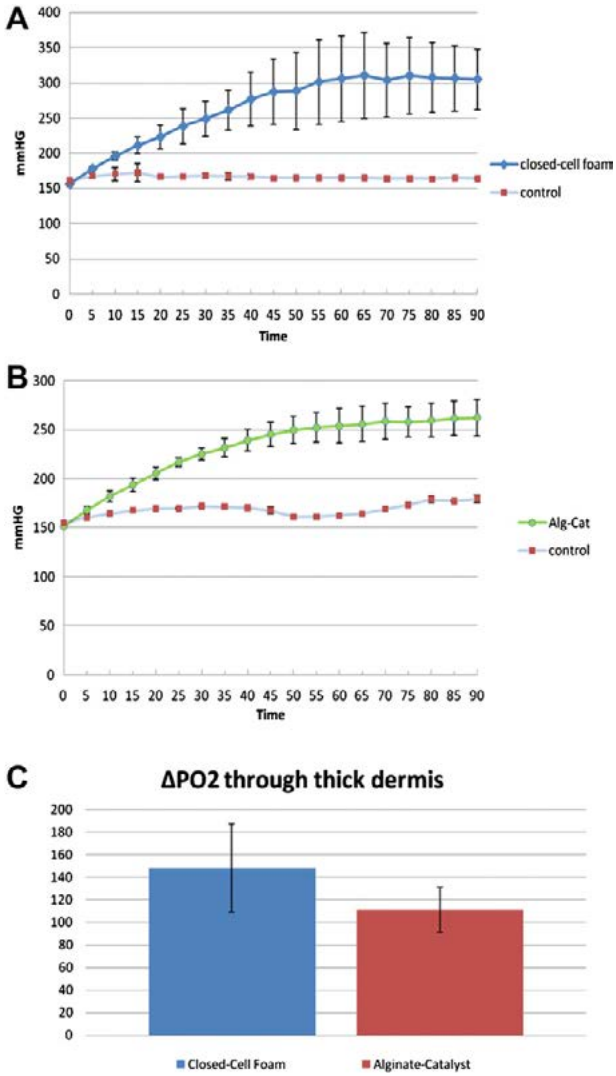


FIG. 4. Transfer of dissolved oxygen through thick dermis (457–762 μm) into saline at 37°C; $n = 3$ oxygen delivery devices; $n = 2$ control. (A) Foam dressing; (B) alginate-catalyst dressing; (C) change in partial pressure at 90 min from T = 0.

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

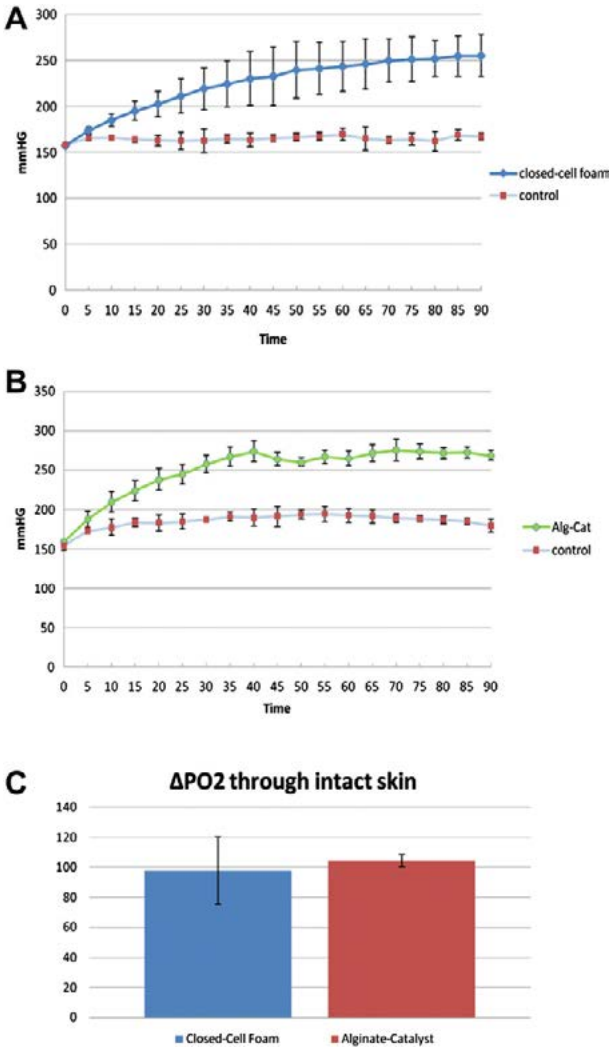


FIG. 5. Transfer of dissolved oxygen through intact skin, epidermis, and dermis (304 μm), into saline at 37°C; $n = 4$ oxygen delivery devices; $n = 3$ control. (A) Foam dressing; (B) alginate-catalyst dressing; (C) change in partial pressure at 90 min from T = 0.

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 (μL/Min)

	Skin sample			
Topical oxygen device	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 oxgyen (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 cm^2) of skin that weighs approximately 4000 g. This gives a single square centimeter of skin a mass of 0.2 g (4000 g/20000 cm^2). It has been reported that skin consumes 5 mL/min of oxygen from circulating blood supply [16] (5 mL/min/4000 g equals 1.25 μL/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 μL/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 $1.8–3.1 \times 10^{-5}$ cm^2/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/cm^2 in the palmer skin), and each gland has a pore 15–20 μm in size [20]. Alternatively, oxygen may cross the nonporous portion of the skin, possibly *via* transmembrane proteins such 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 pO2 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 μm 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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Key words
Inflammatory phase; Oxygen; Proliferative phase; Reepithelialisation; Wound healing

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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 (Km) 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 ($P < 0{\cdot}03$). 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 ($P < 0{\cdot}01$). 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 ($PscO_2$) 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 $PscO_2$ 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)-1a, 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 ($P = 0{\cdot}001$) but increased in the hyperoxic group ($P < 0{\cdot}05$) 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 ($P < 0{\cdot}05$). 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, glycosaminglycans, 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 $PscO_2$. Silicone rubber catheters were placed in the upper arm to measure $PscO_2$, 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 $PscO_2$ measurements received more fluid on the day of operation than the group treated according to clinical criteria ($P < 0{\cdot}05$); also, more collagen accumulated in their ePTFE tubes by day 7 ($P < 0{\cdot}05$). Collagen formation in healing wounds seems to be associated with improvement in $PscO_2$.

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 H_2O_2, 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 H_2O_2 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 H_2O_2, 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, ß-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 (TFG-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 (CDOT) 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 CDOT. 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.

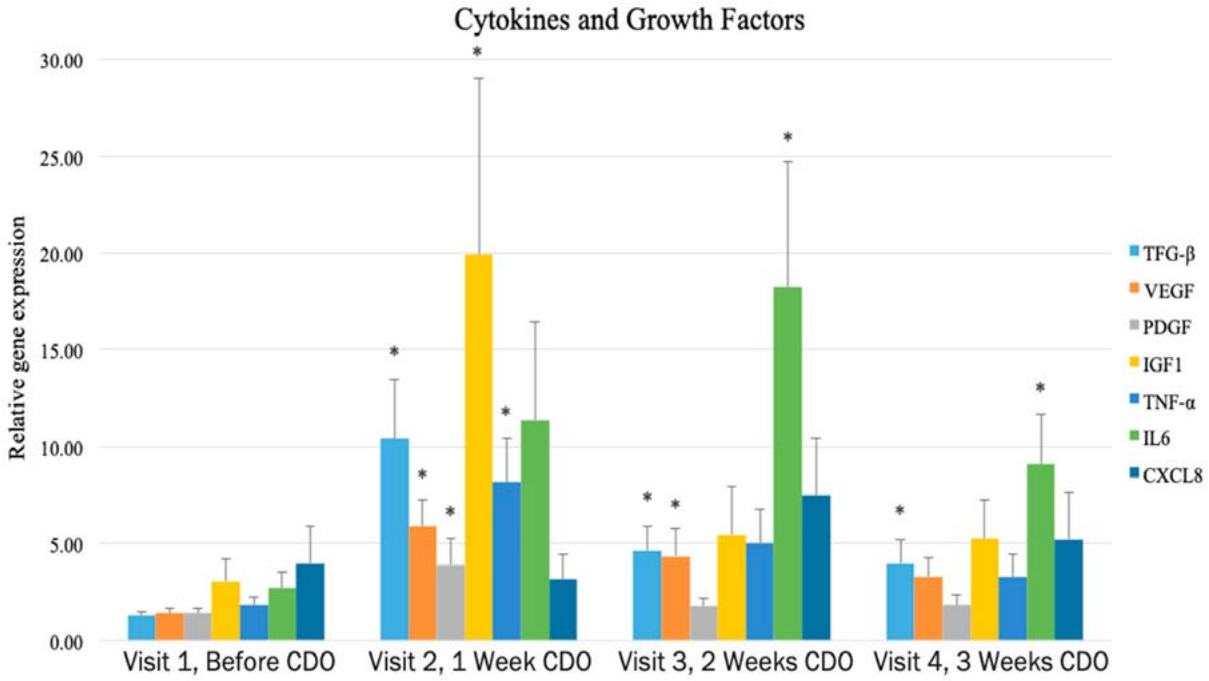

Figure 1. Impact of CDO on gene expression for various cytokines and growth factors at each visit Asterisks indicate significant increase from baseline [50]. Adapted with permission.

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 hydryoxlase 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 (O2- and H2O2) 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 increaseIL-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 pO2 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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4.
UNDERGOING STUDIES

STUDIES IN COURSE OF 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	S. Orsola Hospital – Bologna Prof. Maria Cristina Meriggiola
Prospective observational study of symptoms and signs in women with genitourinary **syndrome of menopause after a period (six treatments)** of endo-vaginal application of high concentration oxygen via the Caress Flow device.	V. Buzzi Hospital Milan Prof. Filippo Murina
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
Vaginal natural oxygenation device coupled with hyaluronic acid to treat genital symptoms of iatrogenic menopause in **breast cancer patients**	S. Martino Hospital Genoa Prof. Angelo Cagnacci
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

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