

Non-invasive Oxygen-Ozone therapy in treating digital ulcers of patients with systemic sclerosis

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ACTA REUMATOL PORT. 2018;43:210-216

ABSTRACT

Background: Digital ulcers (DUs) in systemic sclerosis (SSc) result from recurrent Raynaud's phenomenon (RP) and microtrauma with high impact on quality of life. Medical use of ozone (triatomic oxygen) was initiated in the 19th century. Ozone has multiple therapeutic effects in wound healing due to the property of releasing nascent oxygen, which has been shown to stimulate antioxidant enzymes. We aimed to assess the effects of ozone therapy on the healing of scleroderma DUs and determine levels of expression of vascular endothelial growth factor (VEGF), and endothelin-1 type A receptor (ETAR) autoantibodies in the wounds after treatment.

Subjects and Methods: Fifty SSc female patients with DUs, were randomized into ozone group (I) (n=25) treated with calcium channel blockers plus oxygen-ozone treatment and control group (II) (n=25) treated with calcium channel blockers only. Ozone group received noninvasive oxygen-ozone treatments for 30 minutes per day for 20 days using the ozone generator device. Therapeutic effects were graded into 4 levels according to Zhang and other researchers. The wound sizes were measured at baseline and day 20, respectively. Expressions of VEGF and ETAR autoantibodies proteins were determined by immune-histochemical examination.

Results: Demographics and clinical characteristics of the 2 groups showed no significant differences at baseline. At day 20, the effective healing rate was significantly higher in group (I) than in group (II), where it represented 96% (24/25) in ozone group versus 44% (11/25) in control group ($p=0.007$).

After treatment, the wound sizes in both groups were significantly smaller than before treatment. In group (I), the wound size reduction was significantly more than in group (II) (0.75 ± 0.30 versus 2.44 ± 0.80 mm), ($p<0.001$). At day 20, VEGF was significantly higher in ozone group (I), than in control group (II), (83.96 ± 9.68) versus (67.92 ± 6.55), ($p<0.001$) while, ETAR was significantly lower in ozone group (I), than in control group (II), (3.14 ± 1.12 versus 4.59 ± 1.24), ($p<0.001$).

Conclusion: Ozone therapy may be beneficial tool in the treatment of DUs in SSc patients, where it promotes the wound healing through a potential induction of VEGF and down-regulation of ETAR at sites of the ulcers.

Trial registration: NCT02733978. Registered April 12, 2016

Keywords: Ozone; Systemic sclerosis; Digital ulcers

INTRODUCTION

Systemic sclerosis (SSc) is a chronic connective tissue disabling disease associated with fibrosis, microvascular involvement, immunologic dysfunction, increase of extracellular matrix deposition in the skin and variable internal organs involvement^{1,2}.

Vascular disease displays an essential role in the pathogenesis of SSc, including digital ulcers (DUs)^{3,4}, through involvement of micro-vessels and digital arteries⁵. DUs are responsible for pain, poor quality of life, impaired function and morbidity associated with SSc^{6,7}. Moreover, they are correlated to disease severity and outcome⁷.

About one half of patients with SSc report a history of DUs along the course of their disease⁸. Recurrent DUs occur in approximately 10% of those patients. Three quarters of patients develop their first DUs within 5 years after SSc diagnosis⁹. Patients with anti-Scl-70 antibody develop DUs ~5 years earlier than those with

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positive anti-centromere¹⁰.

Healing process in DUs takes long time, especially if there is an underlying calcinosis¹¹. Amanzi and coworkers (2010) found that the average time to healing was 76.2 days in DUs without calcinosis and 93.6 days in DUs with underlying calcinosis¹². DUs are frequently infected and may be complicated by osteomyelitis¹³. In a retrospective study, 42% infected DUs were associated with osteomyelitis, shown by clinical and radiographic features¹⁴.

Management of DUs is a great challenge for clinicians and often requires the input of a multi-disciplinary team of health professionals⁶. Medical use of ozone (triatomic oxygen) was initiated in the 19th century and was considered to be an oxidant and a disinfectant¹⁵. Currently, ozone is acknowledged as an antiviral and a bactericidal agent^{16,17}, and has been used for treating coronary artery disease, chronic hepatitis and chronic low back pain¹⁸⁻²⁰. Also, its effectiveness was proved in healing chronic wounds such as trophic ulcers, ischemic ulcers and diabetic foot ulcers^{17,21-22}.

The mechanism through which ozone achieves healing in chronic wounds is not yet well understood. Its therapeutic effect may be related to ozone growth factors stimulation and antioxidant system activation^{21,23}. In addition, it improves microcirculation in the capillary vessels by improving flexibility and stability of the cell membrane and limiting the aggregation and adhesion of platelets²⁴.

To our knowledge, the effectiveness of ozone therapy in SSc DUs was not assessed before. Therefore, this study aimed at assessing the effect of ozone therapy on healing scleroderma DUs. Moreover, we measured the levels of expressions of vascular endothelial growth factor (VEGF), and endothelin-1 type A receptor (ETAR) autoantibodies after treatment.

SUBJECTS AND METHODS

PARTICIPANTS

Fifty SSc female patients with DUs were recruited from Rheumatology and Rehabilitation department at Asyut University Hospital, Asyut, Egypt. SSc was diagnosed and based on the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) Criteria for the classification of SSc²⁵. Skin thickness was evaluated using the modified Rodnan skin score (MRSS)²⁶. DUs were defined according to Amanzi

et al., (2010) as “a loss of epithelialization and tissues involving, in different degrees, the epidermis, the dermis, the subcutaneous tissue and sometimes also involving the bone”¹².

DUs staging was as follow¹²:

1. Superficial: partial skin loss where involvement is restricted to the epidermis. Clinically, the lesion appears as an abrasion, blister or small crater.
2. Intermediate: full thickness skin involvement where damage appears in subcutaneous tissue up to, but not through, underlying fascia. Clinically, there is a deep crater with or without affection of adjacent tissue.
3. Deep: full thickness skin involvement with widespread destruction of the muscle down over the fascia, supportive structures (e.g. tendon) and bone.

Written informed consents were obtained from all patients before entry into the study, according to the Declaration of Helsinki and guidelines of the local ethics committee. The study was approved by Asyut University ethics committee. Trial registration: NCT02733978. Registered April 12, 2016.

Patients were excluded from the study if they had one or more of the following conditions¹⁰: (1) gangrenous ulcers in whole hand, (2) calcinosis ulcer, (3) traumatic ulcer, (4) vasculitis, (5) active osteomyelitis, (6) other connective tissue disease, (7) hyperthyroidism, (8) pregnancy or nursing, (9) a known allergy to ozone.

BASELINE ASSESSMENT

At baseline, both groups were assessed for DUs staging¹², number of Raynaud's attacks /day, duration of Raynaud's attack, ulcer size in mm, ulcer pain assessed by visual analogue scale (VAS) (where 0 = 'no pain' and 10 = 'severe pain').

Laboratory work included autoantibodies, antinuclear antibodies (ANA), anti-centromere antibodies (ACA) and anti-topoisomerase antibody (Scl70). An indirect immunofluorescent assay, for semi-quantitative determination of anti-nuclear IgG antibodies (ANA) in patients' serum NOVA LITE™ IFA HEp-2 ANA Complete Kit was used. Antibodies to extractable nuclear antigens ACA and Scl70 were determined by a commercial clinical enzyme linked immunosorbent assay (ELISA).

Tissue biopsies were obtained from the border area of digital ulcers, including the ulcer edge and part of the surrounding skin at day 0. The tissue was homogenized in 3mL PBS followed by centrifugation. The supernatants were collected for the determination of

VEGF, ETAR autoantibodies proteins by immune-histochemical examinations.

INTERVENTION

Patients who met the criteria were randomly assigned by computer based selection as ratio of 1:1 to one of 2 groups:

Ozone group

It represented the study group that included 25 SSc patients with DUs who received oxygen-ozone treatment in addition to calcium channel blockers (Epilat retard® 40mg /day). Patients received noninvasive oxygen-ozone treatments for 30 minutes per day for 20 days. The treatment session of oxygen-ozone was 52 µg/mL ozone (total volume: 20-50 mL) in a special bag using the ozone generator device (Humazon Promedic, German).

Control group

It included SSc patients with DUs who received the same standard medical care only in the form of calcium channel blockers (Epilat® 40mg /day)

OUTCOMES

Primary outcomes

Ulcer healing was our primary outcome, which was assessed depending on Zhang and colleagues ulcers grading where they graded ulcers into four levels, Grade 0 (no change), Grade 1 (wound size decreased less than ½); Grade 2 (wound size decreased more than ½) and Grade 3 (wound healing)²⁷. Ozone treatment were considered efficient if patients reached grade 1 to 3.

Secondary outcomes

For our secondary outcomes both group were subjected to the following after 20 days of intervention: they were reassessed for number of Raynaud's attacks/day, duration of Raynaud's attack, ulcer size in mm, and ulcer pain was assessed by VAS.

In addition, at day 20, VEGF, ETAR autoantibodies proteins were reassessed by immune-histochemical examinations.

To maintain the study blind: 1) researcher who conducted 1st clinical assessments were separated from those carrying out and who had access to record, patients' efficacy and safety data; 2) results from the primary histopathological assessments at baseline were recorded on paper, sealed in an envelope and not disclosed to any site staff apart from the physician who

performed it; 3) another physician did the follow up assessments clinical and histopathologic.

STATISTICAL ANALYSIS

Descriptive analysis was presented as mean and standard deviation for quantitative variables and as frequency and percentage for categorical variables. Mann-Whitney U test was used for inter-group comparisons. We used the Chi-square test to compare qualitative variables. P-value equal or less than 0.05 was considered significant in all statistical tests. Statistical analyses were performed using Microsoft Excel and SPSS (SPSS 23.0, IBM, Armonk, NY, USA).

RESULTS

Fifty SSc female patients with DUs were included in the study with a mean age of 40.93 ± 11.71 years. Thirty-one patients had limited SSc while 19 patients had diffuse type. The mean duration of Raynaud's manifestation was 8.13 ± 5.56 years with 38 patients had pitting scars. There were no statistical significant differences between two groups in patient demographics, clinical and laboratory characteristics Table I at baseline.

Results of our primary outcomes are presented in Table II showing the grading of healing of DUs at day 20 in both ozone and control groups. The effective rate was significantly higher in ozone group than in control group, 96% (24/25) versus 44% (11/25), $\chi^2 = 7.26$, $p = 0.007$. Figure 1 reports photo of the digital ulcers before and after ozone treatments.

At day 20, ozone group showed significant improvement in the clinical characteristics of secondary outcomes including number of Raynaud's attacks/day, duration of Raynaud's attack, ulcer size in mm, ulcer pain (Table III). However, the control group showed statistical significant improvement only for ulcer size (Table IV). Ozone group showed greater improvement in clinical characteristics including number of Raynaud's attacks/day, duration of Raynaud's attack, ulcer pain in comparison to control group ($P = 0.02$, 0.001 and <0.001 respectively).

Concerning wound size, there was no significant difference between two groups at baseline (Table I). At day 20 after treatment, the wound size in both groups was significantly smaller than before (P values were <0.001

TABLE I. BASELINE DEMOGRAPHIC, CLINICAL AND LABORATORY CHARACTERISTICS OF THE STUDIED POPULATIONS

Characteristics	Ozone Group No. 25	Control Group No. 25	P value*
Age [Years, mean \pm SD]	38.83 \pm 12.32	44.08 \pm 10.42	0.51
Gender [female, n (%)]	25 (100%)	25 (100%)	–
Type:			
Diffuse [n (%)]	12 (48%)	10 (40%)	0.46
Limited [n (%)]	13 (52%)	15 (60%)	0.71
Onset of Raynaud's [Years, mean \pm SD]	10.72 \pm 4.34	13.33 \pm 3.89	0.54
Disease duration of Raynaud's [Years, mean \pm SD]	6.83 \pm 6.41	6.21 \pm 5.13	0.17
Number of Raynaud's attacks /day [mean \pm SD]	2.90 \pm 1.30	2.93 \pm 1.23	0.94
Duration of Raynaud's attack [mean \pm SD]	10.10 \pm 4.23	11.26 \pm 3.77	0.87
MRSS [mean \pm SD]	15.32 \pm 5.52	13.2 \pm 4.13	0.27
Digital ulcers [present, n (%)]	25 (100%)	25 (100%)	–
Digital ulcers staging:			
Superficial	5 (20%)	8 (32%)	0.36
Intermediate	17 (68%)	15 (60%)	
Deep	3 (12%)	2 (8%)	
Digital pitting scar number [mean \pm SD]	3.08 \pm 1.68	2.06 \pm 2.21	0.31
Ulcer size (mm) [mean \pm SD]	3.61 \pm 0.8	4.18 \pm 0.38	0.27
Ulcer pain VAS [mean \pm SD]	7.98 \pm 0.84	7.41 \pm 1.33	0.06
Autoantibodies#			
ANA [positive, n (%)]	14 (56%)	12 (48%)	0.52
ACA [positive, n (%)]	7 (28%)	7 (28%)	
Scl70 [positive, n (%)]	6 (24%)	7 (28%)	
VEGF	63.48 \pm 6.20	62.80 \pm 6.86	0.76
Anti-ETAR	4.59 \pm 1.31	4.84 \pm 1.33	0.42

MRSS: modified Rodnan skin score, VAS: visual analogue scale, ANA: antinuclear antibody; ACA: Anti-centromere antibodies, Scl70: anti-topoisomerase antibody. # = patient may be positive for more than one autoantibody. VEGF: Vascular endothelial growth factor, Anti-ETAR: endothelin-1 type A receptor autoantibodies, * $p < 0.05$ adjusted by Bonferroni test

TABLE II. DIGITAL ULCER GRADING AT DAY 20 IN BOTH OZONE AND CONTROL GROUPS

Digital grade	Ozone Group No. 25	Control Group No. 25	P value
Grade 0	1 (4%)	14 (56%)	0.001*
Grade 1	7 (28%)	3 (12%)	0.032*
Grade 2	10 (40%)	5 (20%)	0.018*
Grade 3	7 (28%)	3 (12%)	0.032*

* $p < 0.05$ adjusted by Bonferroni test

and 0.042 respectively). In ozone group the wound size reduction was significantly higher than in control group (0.75 \pm 0.30 versus 2.44 \pm 0.80 mm), ($p < 0.001$).

At baseline, there was no statistical significant difference in the expressions of VEGF and ETAR protein be-

tween two groups (Table I). But after treatment at day 20, they were significantly higher in ozone group than in control group (Table V). Also, there were highly significant differences in the expressions of VEGF and ETAR protein in the ozone group after treatment than before treatment (83.96 \pm 9.68 versus 63.48 \pm 6.20), ($p < 0.001$); (3.14 \pm 1.12 versus 4.59 \pm 1.31), ($p < 0.001$). In the control group there was only a statistical significant difference in the expressions of VEGF between baseline and after 20 days (62.80 \pm 6.86 versus 67.92 \pm 6.55), ($P = 0.04$).

DISCUSSION

DUs are one of the most common complications of SScs at digital level, which has a great impact on patients' quality of life¹². Different researches have shown that



FIGURE 1. Systemic sclerosis digital ulcers before (A1) and after ozone treatments (A2) for the same patient and (B, C) for other patients.

TABLE III. CLINICAL CHARACTERISTICS FOLLOW UP AT DAY 20 IN OZONE GROUP

Characteristics	Baseline	Day 20	P-Value
Ulcer size (mm) [mean ± SD]	3.61±0.8	0.75±0.30	0.00*
Number of Raynaud's attacks/day	2.90±1.30	1.64±1.07	0.00*
Duration of Raynaud's attack	10.10±4.23	5.00±2.88	0.00*
Ulcer pain VAS [mean ± SD]	7.98±0.84	4.04±0.93	0.00*

VAS: visual analogue scale, *p<0.05 adjusted by Bonferroni test

the healing process in the wounds is delayed due to hypoxia, reactive oxygen species, and pro-inflammatory cytokines²⁸.

For more than 100 years, O₃ has been used in dif-

TABLE IV. CLINICAL CHARACTERISTICS FOLLOW UP AT DAY 20 IN CONTROL GROUP

Characteristics	Baseline	Day 20	P-Value
Number of Raynaud's attacks /day	2.93±1.23	2.68±1.02	0.12
Duration of Raynaud's attack	11.26±3.77	9.80±3.67	0.64
Ulcer size (mm)	4.18±0.38	2.44±0.80	0.042*
Ulcer pain VAS	7.41±1.33	7.02±1.30	0.16

VAS: visual analogue scale, *p<0.05 adjusted by Bonferroni test

ferent ways to treat diseases, infections, and wounds through increasing collagen contents inside the wounds and levels of VEGF, Transforming growth factor beta (TGF-β), and Platelet-derived growth factor (PDGF) in wound exudates³⁰⁻³².

Our results revealed the possible effectiveness of a

TABLE V. TISSUE BIOPSY DAY 20 FOLLOW UP IMMUNE-HISTOCHEMICAL EXAMINATIONS

	Ozone Group No. 25	Control Group No. 25	P-Value
VEGF	83.96±9.68	67.92±6.55	0.00
Anti-ETAR	3.14±1.12	4.59±1.24	0.00

VEGF: Vascular endothelial growth factor, Anti-ETAR: endothelin-1 type A receptor autoantibodies.

*p<0.05 adjusted by Bonferroni test

short-term treatment with oxygen-ozone into healing in SSc DUs. In 20 days, we noticed a significant improvement in the clinical characteristics in comparison to control group. Our study was the first to investigate the effect of oxygen-ozone in treating SSc DUs.

Other studies reported the efficacy of oxygen-ozone on different ulcers. One randomized controlled clinical trial in diabetic patients showed better glycemic control, reduced ulcers size, inhibited oxidative stress products and decrease number of amputations in patients treated with ozone for 20 days via rectal insufflation than in the control group³³. Wainstein et al. (2011) reported that when ozone therapy was added to conventional treatment for 24 weeks, it promoted complete wound healing than conventional treatment alone for Diabetic foot ulcers (DFUs)³⁴. Another research showed that oxygen-ozone treatment for DFUs significantly promoted early effective rate of the wound healing at day 20²⁷. Degli Agosti and other researchers (2016) suggested in their case report that oxygen-ozone therapy would have a positive role in helping wound healing and reducing pain of complicated wounds³⁵.

We also reported significantly higher expressions of VEGF in the ozone group than in the control group. Multiple growth factors, such as VEGF and TGF- β , have been proved to play an important role in wound healing and lack of their upregulation may explain the poor formation of granulation tissue and chronicity of ulcer epithelialization³⁶. This could explain that the efficacy of ozone therapy for healing of DUs in SSc may be partially to increase endogenous growth factors in local ulcer. These results were similar to Zhan et al., (2014) who observed a higher expressions of VEGF, TGF- β , and PDGF in ozone group compared to control group³⁴.

Moreover, our results revealed downregulation of ETAR autoantibodies at local wound site in ozone group compared to control group. Agonistic autoanti-

bodies against ETAR were recently identified in the sera of SSc patients and are considered to contribute to the pathogenesis of the disease. Anti-ETAR could exacerbate the inflammatory response in SSc patients through promoting recruitment of immune cells to inflamed tissues³⁷. In addition, in vitro studies reported that anti-ETAR autoantibodies promote the production of collagen by skin fibroblasts, the release of reactive oxygen species by neutrophils, and affect endothelial repair; these processes lead to progressive fibrosis of the skin and vasculopathies³⁸⁻³⁹. All these effects indicate that these autoantibodies may have a role in the pathogenesis of SSc DUs and that downregulation of these autoantibodies may play a role in promoting DUs healing.

The present study has some potential biases including small sample size, non-blinded patients and outcome assessors and long-term follow up.

In conclusion, oxygen-ozone treatment significantly stimulated early effective wound healing at day 20 in DUs of SSc patients. We also reported that there were significantly higher expressions of VEGF and downregulation of anti-ETAR in the ozone group than in the control group. These results show that the efficacy of the ozone treatment for the healing of DUs may be partially due to increasing endogenous growth factors and decreasing autoantibodies in the local wounds. However, due to several potential biases oxygen-ozone treatment should be tested more carefully in large, blinded and placebo-controlled studies. Also, Further studies are required to assess the long term effects of ozone therapy on DUs.

ETHICS, CONSENT AND PERMISSIONS

Written informed consents were obtained from all patients before entry into the study, according to the Declaration of Helsinki and guidelines of the local ethics committee.

AVAILABILITY OF DATA AND MATERIALS

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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