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Effects of subcutaneous injection of ozone during wound healing in rats

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ABSTRACT

Fibroblast growth factor 2 (FGF2) regulates the wound repair process and it is secreted by inflammatory and endothelial cells, and by myofibroblasts. This study aimed to establish the expression patterns of FGF2 and myofibroblastic differentiation during wound healing in rats treated with subcutaneous ozone injection. We created full-thickness excisional wounds in rats, and the healing process was analyzed through morphometric analyses and digital quantification of immunoreactivity of smooth muscle actin and FGF2. Ozone therapy-treated wounds presented granulation tissue with a reduced number of inflammatory cells and greater dermal cellularity, and intense collagen deposition. FGF2 immunoreactivity, microvessel density, and amount of myofibroblasts were significantly higher in treated wounds compared to controls. In conclusion, it was demonstrated that subcutaneous injections of ozone accelerate and ameliorate wound repairing process. Moreover, injectable ozone therapy's action mechanism may be associated with FGF2 overexpression.

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KEYWORDS
Wound healing; ozone; fibroblast growth factor 2; myofibroblasts

Introduction

Wound repair is a complex process associated with multiple cellular and chemical interactions, resulting in angiogenesis, neocollagenesis, and scar formation (Dorsett-Martin 2004; Enoch and Leaper 2008). Thus, the cells involved in this process, such as inflammatory, endothelial, and mesenchymal cells, produce several chemical mediators for these molecular and cellular regulating events (Barrientos et al. 2008, 2014; Gillitzer and Goebeler 2001; Li et al. 2003). These mediators may influence the healing process positively or negatively. It has been demonstrated that prolonged expression of matrix metalloproteinase-2 (MMP-2) may delay healing (Wetzler et al. 2000). However, this cytokine may be also associated with angiogenesis (de Medeiros et al. 2017) and consequently, enhancing the wound healing.

Since the healing process is compromised in patients with vascular deficiency (e.g. diabetic subjects), several treatment protocols were developed over time (Menke et al. 2007, 2008). Recently, special attention has been directed to therapies able to induce production of angiogenic molecules, such as vascular endothelial growth factor (VEGF) (Zhang et al. 2014) and/or fibroblast growth factor 2 (FGF2) (Bakhshayesh et al. 2012; Eming et al. 2007; Tonnesen et al. 2000). FGF2 is a potent mitogenic and angiogenic molecule. Recently, FGF2 has been described as an important molecule during myofibroblastic differentiation and activation (Strutz et al. 2000; Svystonyuk et al. 2015). Thus, its overexpression may be associated with myofibroblastic differentiation, improving the wound healing process. Its expression in dermis and epidermis has been correlated with re-epithelization, angiogenesis, and neocollagenesis in radiofrequency-treated skin (Meyer et al. 2017). However, few studies have focused on the comprehension of FGF2 expression in inflammatory cells and myofibroblasts during wound healing.

Ozone therapy has been used as an adjuvant treatment in skin wounds, especially in diabetic patients (Zhang et al. 2014). Two ozone-promoted activities, antimicrobial and antioxidant, have been described (Valacchi et al. 2005). Therefore, ozone is used as a clinical therapeutic agent for many acute or chronic inflammatory diseases, burn wounds, diabetic ulcers,

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among others (Degli Agosti et al. 2016). Despite its benefits, few studies have focused on the comprehension of its mechanism of action (Elvis and Ekta 2011); nevertheless, it has been empirically used for decades. Zhang et al. (2014) have described that ozone therapy increases the expression of several growth factors, including VEGF, transforming both growth factor-β (TGF-β), and platelet-derived growth factor (PDGF). However, limited research has been conducted for the establishment of ozone therapy role in myofibroblastic differentiation and in FGF2 expression.

In addition, it has been demonstrated that ozone therapy increased tissue oxygenation and metabolism, interfering in the oxidant/antioxidant balance (Travagli et al. 2010; Viebahn-Hänsler et al. 2012). Based on this mechanism, ozone is capable of regulating a previous disturbed oxidant/anti-oxidant scenario, reducing H_2O_2 levels, and regulating antioxidant molecule levels (Viebahn-Hänsler et al. 2012). Furthermore, ozone-produced peroxides cause an improvement in oxygen release and availability by regulating antioxidant molecules such as SOD and G-6PH in red blood cells, favoring the metabolism and release of cytokines, autacoids, and growth factors, which, together with the antimicrobial activity, are fundamental elements in the treatment of metabolic, inflammatory, and infectious diseases (Travagli et al. 2010). It has also been suggested that the action of ozone therapy may be interpreted as a nontoxic "therapeutic shock" capable of restoring homeostasis as a physiological response modifier (Bocci et al. 2009, 2011; Valacchi et al. 2005). Despite some evidence favoring ozone therapy effectiveness, it is not yet present in the routine of ulcer treatments, which would be an interesting and low-cost adjuvant therapy.

In the current study, the effects of injectable ozone therapy (IOT) in wound healing were systematically evaluated, focusing on the comprehension of FGF2 expression in inflammatory cells, keratinocytes, and myofibroblasts. Furthermore, a possible mechanism of action of IOT during wound healing was established.

Materials and methods

Subjects and experimental groups

All animal experiments were approved by the Institutional Ethics Committee of Potiguar University (Natal, Brazil), protocol number #08877, and followed the statements of the Guide for the Care and Use of Laboratory Animals. Twenty-four male Wistar rats (Rattus norvegicus) with an average age of 12 weeks (body weight, 300 g ± 50 g) supplied by Potiguar University Animal Facility (Natal, Brazil) were kept in a controlled light (cycles of 12 h light/dark), ventilation, and temperature (24 °C) environment. Subjects were fed on a balanced diet (Labina™ Purina) and water *ad libitum*.

Instruments and materials

The following materials were used: a digital caliper, sterile surgical towels, sterile gloves, procedure gloves, scalpel, sterile gauze, crepe tape, scissors, syringes, insulin needles, a digital camera, eyedropper, Zoletil™ 50, chlorhexidine, sodium dipyrone, and IONEX™ ozone therapy equipment from Medite™, Argentina.

Wound induction and experimental groups

The wounds were surgically induced in all subjects. After disinfection, the back of all animals was shaved, and a wound measuring 2 cm in diameter was performed with a stainless-steel dermatologic punch. The animals were also treated with a single dose of dipyrone for analgesic effect (after wound induction). The first group CG (control group with lesion) had 12 animals submitted only to injury, which were euthanized after 7 days ($n = 6$) and 14 days ($n = 6$) from injury induction. The second group IOTG (group treated with IOT) had 12 animals, which received a daily subcutaneous injection of ozone, which were euthanized after 7 days ($n = 6$) and 14 days ($n = 6$) of treatment.

Ozone therapy protocol

The animals were immobilized under a square field with a central opening, where the lesion area was positioned. A syringe was filled with ozone in its gas form, and then 0.1 ml was subcutaneously injected in the four sides of the lesion border. Animals in the control groups were treated with vehicle injections. The lesion was photographed with a digital camera after each session of ozone therapy. All animals were accommodated in individual cages and received dipyrone analgesic drops 500 mg/ml (Medley Laboratories, Brazil) dissolved in water. Following the treatment period, the animals were euthanized.

Histopathological analysis

Briefly, skin wound tissues were immersion-fixed in 10% NBF (neutral-buffered formalin) for 24 h at RT. Then, the specimens were processed by routine histotechnique procedures for hematoxylin and eosin (HE), Masson's Trichrome (MT), and Picrosirius Red staining.

Morphometric analyses

Pictures of the wounded area were taken with a digital camera daily during the experimental period and measured using specific photographic software (Photoshop 10.0.1 for Windows, USA). The epithelization rate was calculated as follows: ([original wound area – current wound area]/[original wound area]) × 100% as described in the studies carried out by Huang et al. (2016).

For histomorphometric analyses, photomicrographs of various microscopic fields were obtained at different magnification levels (40×, 100×, or 400×), as previously described by de Medeiros et al. (2017). Digital images were obtained with a binocular light microscope (Olympus CX31, Hamburg, Germany) with an attached camera and then the images were analyzed with Image J software (NIH, Bethesda, MD, USA). Inflammatory cells (neutrophils, lymphocytes) were quantified in 10 fields, considering the [absolute number] of cells per 400× magnification field. The total area of collagen was evaluated by the blue aniline staining in MT slides, whereas Type I collagen content was evaluated by the PR staining (considering the orange-red fibers after light polarization), both automatized analyses with ImageJ software. The area of collagenization or Type I collagen index was calculated as follows: ([area of blue staining in MT sections] or [area of orange-red staining in PR sections]/[total area of field]) × 100%).

Growth factors screening

Initially, two rats were selected, the tissue around the wound was collected and processed to evaluate mRNA expression. A pool of growth factors was screened, including epidermal growth factor (EGF), PDGF, FGF2, TGFβ, and VEGF. FGF2 levels were significantly elevated in the treated group when compared to the control, which led us to investigate this marker.

Immunohistochemical procedures

Immunohistochemistry (IHC) was performed for quantification of myofibroblasts, vessels, and FGF2 expression. Briefly, 3 µm-thick sections were mounted on silanized slides, deparaffinized with xylene, cleansed in alcohol, and rinsed with PBS. After this, the sections were incubated with 0.3% hydrogen peroxide for 15 min to allow the quenching of the endogenous peroxidase activity. Diluted primary antibodies SMA (anti-mouse, clone 1A4, 1:400, Dako) and FGF2 (anti-mouse, Polyclonal, 1:100, Santa Cruz Biotechnology) were applied in the slides and incubated at room temperature for 120 min, followed by the EnVision + Dual Link System-HRP (Dako, Carpinteria, CA, USA). The reactions were revealed by incubation with DAB chromogen (Dako) and then the sections were counterstained with Carazzi' hematoxylin.

Digital evaluation of immunoreactivity

The smooth muscle actin (SMA) and FGF2 immunoexpression was evaluated by a calibrated oral pathologist, and the number of SMA-positive myofibroblasts per field was counted at 400× magnification ($n = 10$ fields for each slide/subject). Microvessel density (MVD) was assessed in a similar manner, considering the SMA-positive vessels per field counted at 400× magnification ($n = 10$ fields for each slide/subject). FGF2 staining quantification was performed based on intensity using IHC profiler plugin in ImageJ software (NIH, Bethesda, MD, USA), as described previously (Varghese et al. 2014). A quantitative scale was proposed ranging from 100 to 300, in which 100 corresponds a weak or absent immunostaining and 300 marked maximum immunostaining (obtained from the internal positive control – striated muscle). Similar to the other analyses, 10 random fields were considered.

The data were obtained and organized in Excel worksheets. Numerical data are presented as mean ± SD. Briefly, data were submitted to normal distribution tests (D'Agostino–Pearson and Shapiro–Wilk). Next, Student's *t*-tests for dependent and independent variables were performed and results were subjected to the sequential Bonferroni–Holm correction. All analyses were performed using the GraphPad Prism software (v6.0, GraphPad Software, Inc., La Jolla, CA, USA), and the considered significance level was 5% ($p < .05$) for all tests.

Results

Injectable ozone therapy accelerates wound repair at 7 and 14 days' post-wounding

Wounds of control group showed a granulation tissue with predominance of inflammatory cells and discrete collagen deposition. At 7 days, IOT-treated wounds revealed granulation tissue with a reduced number of inflammatory cells and greater dermal cellularity (fibroblasts and endothelial cells, predominantly), and moderate/intense collagen deposition.

We first measured the wound area and the epithelization rate. The mean wound area was significantly smaller in the treated group (IOTG) than in control group (CG) at 7 (Figure 1(A), $p = .0020$) and 14 days (Figure 2(A), $p = .0002$) post-wounding. Similarly, the number of neutrophils was significantly smaller in IOTG than in CG at 7 ($p = .0016$) and 14 days ($p = .0004$). However, no difference was observed in the number of lymphocytes at any time ($p > .05$). No adverse effects (e.g. body weight, condition, or behavior change) were observed during the complete treatment course.

As observed in Figures 1(B) and 2(B), after 7 and 14 days of wound induction, collagen deposition was more evident in the IOTG ($p = .0027$ and $p = .0015$, respectively). Higher levels of Type I collagen were present in IOT-treated wounds, especially at 7 days ($p = .0044$), when compared to control group.

Injectable ozone therapy increases myofibroblastic differentiation and microvessel density

Immunohistochemical staining for SMA was performed to quantify myofibroblasts and vessels in the wounds. It was observed that IOT treatment induced myofibroblastic differentiation and proliferation, while the control group showed smaller amount of myofibroblasts during wound healing. These cells, as revealed by SMA expression, were encountered in higher quantity at 7 and 14 days after treatment than in non-treated groups ($p < .05$). The MVD was significantly higher in the IOT-treated group than in control group ($p < .05$), 14 days after wound induction (Figure 3).

Injectable ozone therapy induces FGF2 overexpression in the epidermis and dermis during wound healing

FGF2 expression in wounds treated or not with ozone was also evaluated. In the control group, this protein was expressed mainly in the cytoplasm of macrophages and other inflammatory cells, but rarely in myofibroblasts. In the treated group, FGF2 was found in the cytoplasm of inflammatory cells and in keratinocytes (Figure 4) At 7 days, only dermal expression was significantly higher in IOT-treated group ($p < .05$), while at 14 days, the immunoexpression of FGF2 was significantly higher in epidermis and dermis of IOT-treated group than in control group ($p < .05$).

Discussion

Although the effects of different ozone therapy protocols on the repair process of various tissues have been investigated (Degli Agosti et al. 2016; Elvis and Ekta 2011; Valacchi et al. 2005; Zhang et al. 2014), few studies have focused in the comprehension of its mechanism of action. Furthermore, the biological effects of ozone therapy found in the literature are controversial. In the present study, the authors aimed to specifically evaluate the effects of IOT during wound healing in rats. In summary, it was demonstrated that IOT promotes increased epithelialization, increased matrix deposition, and enhanced cellular proliferation during wound healing.

Histologically, IOT-treated wounds demonstrated a granulation tissue with fewer inflammatory cells and a higher number of fusiform cells, compatible with myofibroblasts. Furthermore, the number of vessels and collagen deposition were significantly higher in IOT-treated than in control group. These findings indicate a more advanced and controlled healing process than in control group. Quantification of myofibroblasts during wound healing has been previously described by SMA expression (André-Lévigne et al. 2016). A number of SMA-positive myofibroblasts were 200% higher in the treated than in the control group at 7 days, and up to 400% higher at 14 days. This is an important finding of this study, indicating a possible mechanism of action, in which IOT might increase myofibroblastic proliferation, improving the wound healing process.

FGF2, also known as basic FGF, is an important protein that activates pathways associated with myofibroblastic proliferation and angiogenesis (Svystonyuk et al. 2015). Moreover, FGF2 expression was associated with fibroblast activation resulting in higher production of collagen in tissues. In the present study, FGF2 was overexpressed in macrophages and myofibroblasts, which might indicate improved wound healing events (e.g. neocollagenesis and re-epithelization). These

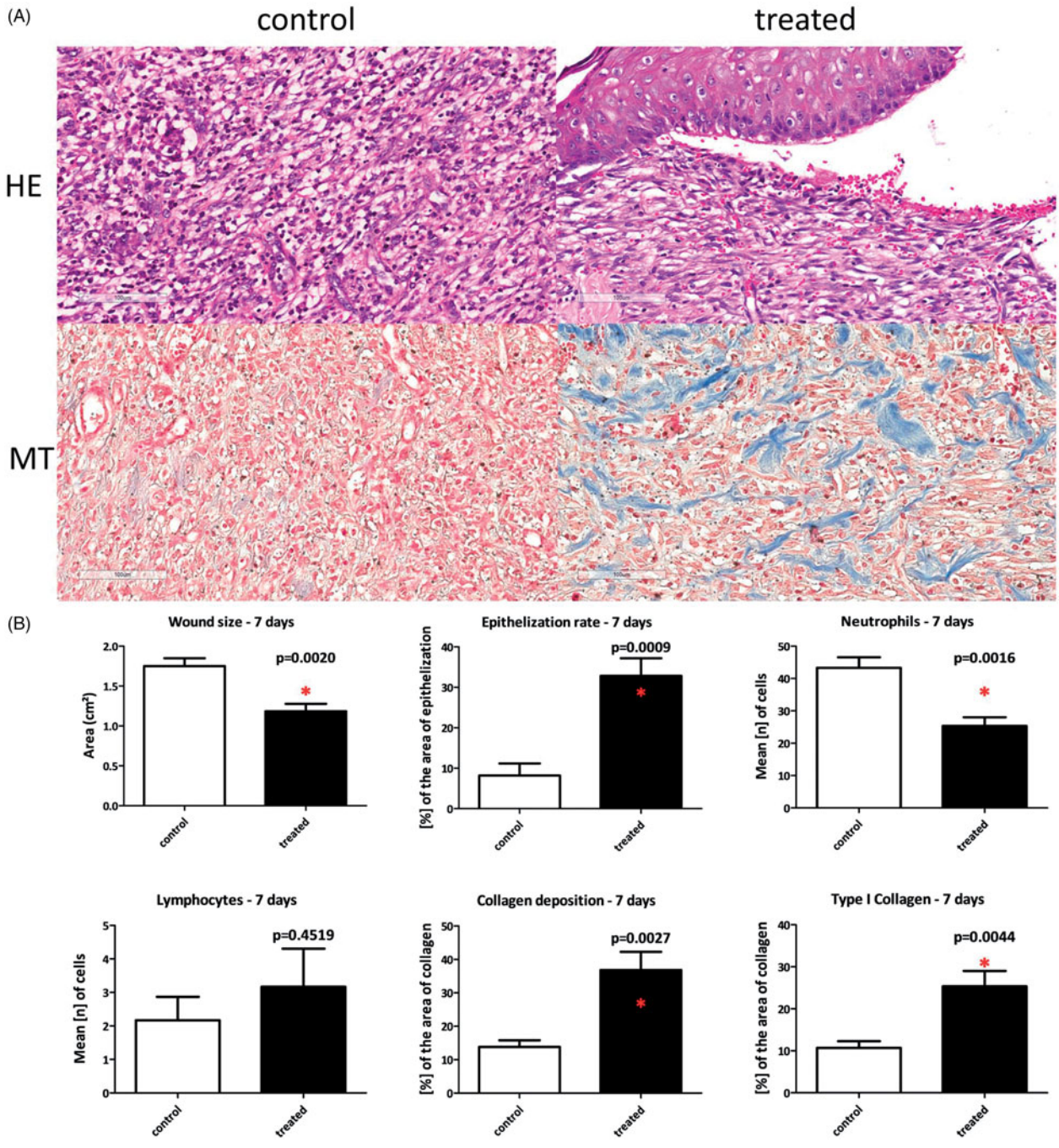

Figure 1. Main morphological and morphometric findings in the wounds 7 days' post-wounding. (A) Treated wounds demonstrated a granulation tissue with intense fibroplasia and endothelial cell proliferation, whereas, in control group, higher amount of inflammatory cells was observed (polymorph nuclear neutrophils). The collagen deposition was more evident in the treated group. (B) Graphic representation of wound size, epithelization rate, neutrophils, lymphocytes, collagen deposition, and Type I collagen. Error bar: SD, Student's *t*-test, *p*-values $<.05$ were considered statistically significant. *means significant difference with the control group. HE: hematoxylin and eosin; MT: Masson's Trichrome.

findings corroborate with the histological findings of re-epithelialization via induction of collagen synthesis and matrix regeneration.

After 7 days from wounding, a higher number of macrophages FGF2-positive seen in the control and treated groups were also registered. Overall, there was an increase of FGF2-positive macrophages and myofibroblast in IOT-treated animals, when compared with controls. This finding corroborates with the hypothesis that these cells secrete important growth factors and cytokines, which are essential for wound healing. The exact role of macrophages in wound healing has

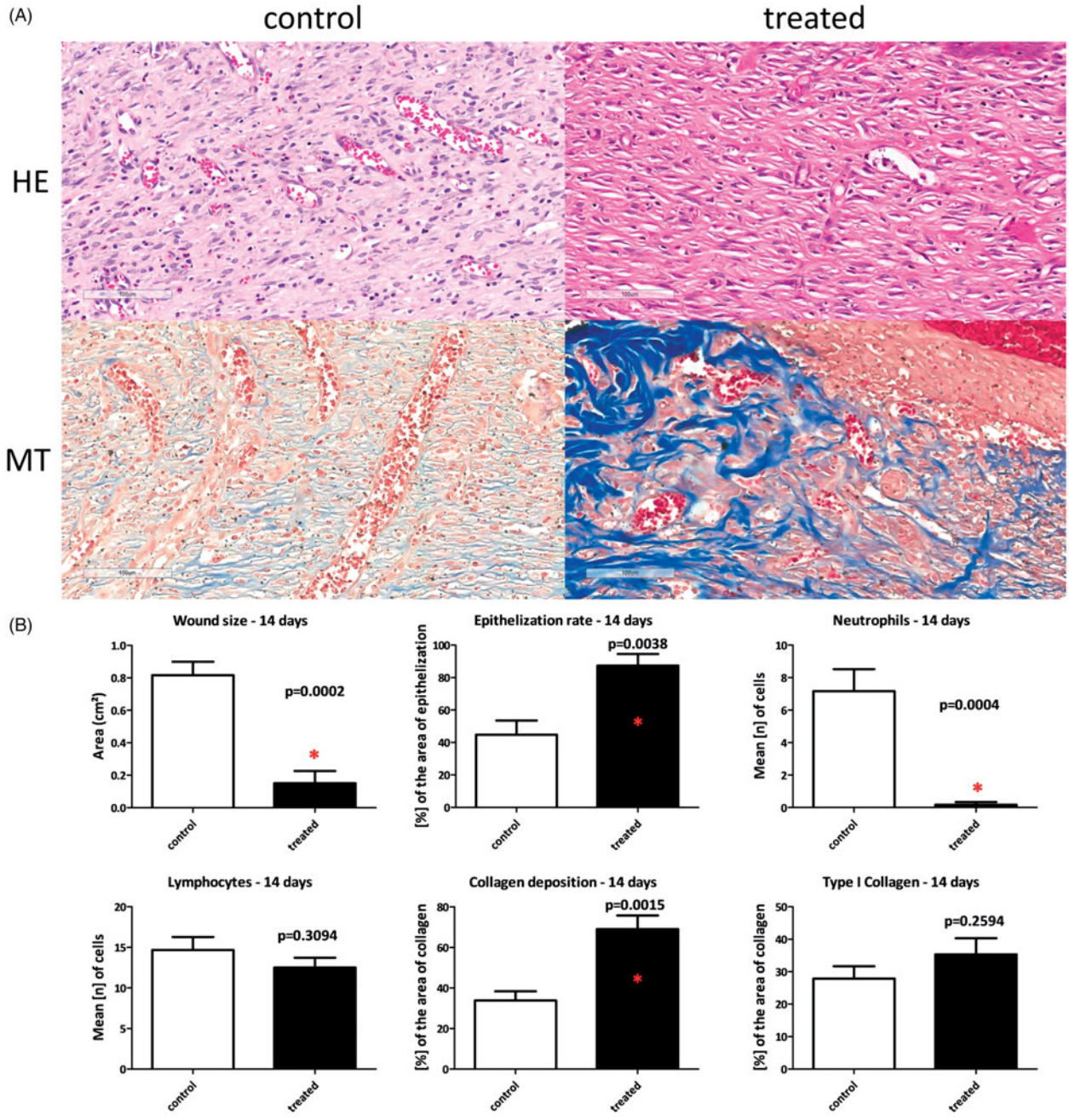

Figure 2. Main morphological and morphometric findings in the wounds 14 days' post-wounding. (A) Control wounds demonstrated the proliferation of fusiform cells compatible with fibroblasts, and persistent inflammatory infiltrate in the wounds. Similar morphological aspects were found in treated wounds; however, the collagen deposition (with large amounts of collagen highlighted by Masson's Trichrome) and re-epithelization were more evident, and the absence of inflammatory cells. (B) Graphic representation of wound size, epithelization rate, neutrophils, lymphocytes, collagen deposition, and Type I collagen. Error bar: SD, Student's *t*-test, *p*-values $<.05$ were considered statistically significant. *means significant difference with the control group. HE: hematoxylin and eosin; MT: Masson's Trichrome.

been thoroughly studied (Snyder et al. 2016). Higher number of these cells associated with a greater number of myofibroblasts in treated group induces fibroblast and myofibroblastic proliferation at the wound, as well as neovascularization in the proliferative phase and vascular regression in the remodeling phase in wound of the animals treated with ozone. Additional studies should be carried out to highlight the phenotype of macrophages that produce and secrete FGF2 during wound healing (M1 or M2 macrophages). Characteristically, M1 macrophages know which cells that up-regulate the early healing process by producing IL-12. However, M2-macrophages down-regulate the healing process, in a process mediated by IL-10

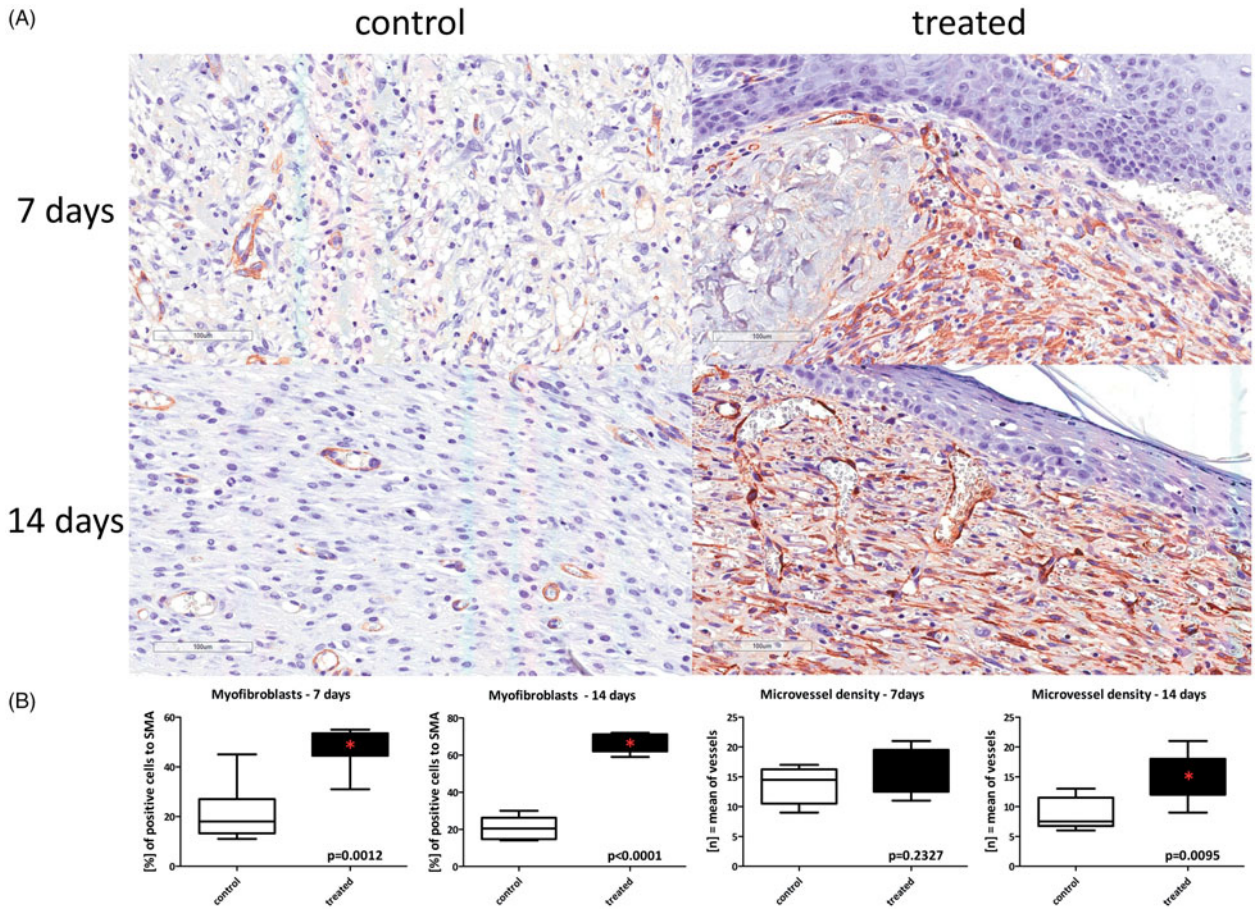

Figure 3. The expression of smooth muscle actin (SMA) was delayed in control group after 7 and 14 days compared to treated group (A). The graphs represented the higher number of myofibroblasts (7 and 14 days) and SMA-positive vessels (14 days) in treated than in control group. All photomicrographs with 200× magnification. Error bar: SD, Student's *t*-test, *p*-values <.05 were considered statistically significant. *means significant difference with the control group.

secretion. The expression of FGF2 by alveolar macrophages has been related in the fibroproliferative disorder of intraalveolar fibrosis following acute lung injury (Henke et al. 1993); however, we provide additional information of this correlation during wound healing. Other positive aspect of the study was the correlation of FGF2 with activated myofibroblasts, and an insight that these cells, when expressing FGF2, may demonstrate a more effective phenotype for wound healing acceleration. This was proved by the increased amount of collagen in within the samples from the treated group at 7 and 14 days than in the ones from the control group and, surprisingly, by the higher number of myofibroblasts in the IOT-treated group.

The complex keratinocyte–fibroblast interaction during wound healing has been well related in the literature (Werner et al. 2007). In addition, in the current study, information about the FGF2 expression in epithelial cells and its interactions were provided, with dermal cells inducing high levels of myofibroblastic differentiation. In fact, the necessity to carry out additional studies that evaluate the expression of other growth factors in epithelial cells during wound healing is known, such as keratinocyte growth factor (KGF)/fibroblast growth factor 7 (FGF7), IL-6, and GM-CSF. Some growth factors and its receptors of the FGF family have been reported in the keratinocyte–fibroblast interactions, FGF22, FGF7, and FGFR2IIIb receptor (Beyer et al. 2003; Zhang et al. 2006). These interactions are fundamental for a complete re-epithelization within a shorter period for good healing. However, in this study new insights about enhanced healing induced by FGF2 epidermal expression were provided.

In conclusion, IOT accelerated and improved skin wound healing in a Wistar rat model. Both the acute and chronic phases of wound healing were enhanced by IOT. The mechanism of action of IOT may be associated with FGF2 overexpression and myofibroblastic differentiation. Moreover, higher epidermal cell proliferation and thickness, higher number of

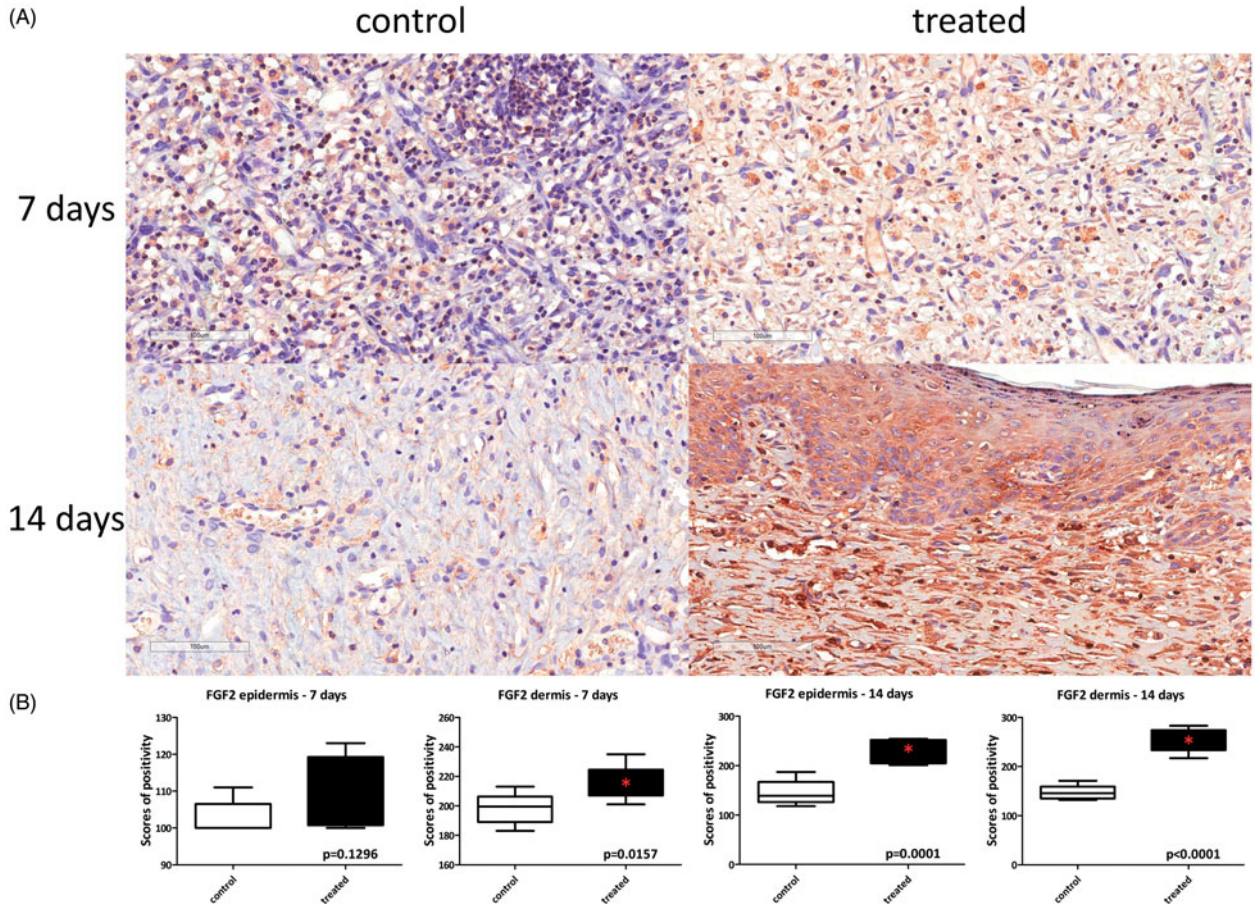

Figure 4. Patterns of FGF2 immunohistochemical expression in control and treated wounds (A). At 7 days, control and treated groups demonstrated macrophages FGF2-positive. At 14 days, a higher amount of myofibroblasts FGF2-positive were observed in treated than in control group, besides that, in the re-epithelization areas, keratinocytes demonstrated a higher FGF2 expression. Control group after 14 days presenting FGF2-positive macrophages. All photomicrographs with 200× magnification. Error bar: SD, Student's *t*-test, *p*-values $<.05$ were considered statistically significant. *means significant difference with the control group.

myofibroblasts, macrophages FGF2-positive, and MVD (neovascularization) indicate IOT effectiveness in chronic wounds healing.

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Disclosure statement

The authors have no conflicts of interest or financial ties to declare.

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