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Original article

Effect of cold atmospheric plasma/NO gas application with different exposure times on healing in wounds with tissue loss in diabetic rats

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Abstract

Applications of cold atmospheric plasma/nitric oxide (CAP/NO) gas have recently garnered popularity when treating impaired wound healing in patients with diabetes. In this study, we aimed to investigate the effects of NO gas application for 60 and 120 s on wound healing in diabetic rats. A dorsal excision 3 cm in diameter was performed in 15 diabetic rats; these rats were categorized into the following 3 groups: DC (untreated diabetic control); DNO/60 (exposure to 200 ppm NO gas for 60 s/day); and DNO/120 (exposure to 200 ppm NO gas for 120 s/day). Wound contraction on days 0, 3, 7, 11, and 14 and wound contraction rate between days 0 and 14 were evaluated. On day 14, tissue samples were collected for histopathologic assessment of inflammation, epithelial regeneration, angiogenesis congestion, and collagen fiber organization. Normality of distribution was assessed using the Shapiro-Wilk test, and intergroup comparisons were performed using the Mann-Whitney U test (NPar Test) and the Kruskal-Wallis test (non-parametric ANOVA). Wound contraction during treatment days 7-14 was significantly greater in the NO-treatment groups than in the DC group ($p<0.05$). The NO60 s and NO120 s groups showed a significantly higher wound contraction rate than the DC group ($p=0.033$, $p=0.049$, respectively). Significant differences were noted between the control and NO groups in terms of inflammation ($p<0.05$) and between the control group and DNO/60 and DNO/120 groups in terms of collagen organization ($p<0.05$, $p<0.01$, respectively). Evaluation of epithelialization revealed significant intergroup differences between the control and NO treatment groups ($p<0.01$). In this study, the application of NO once a day for 60 seconds and 120 seconds in diabetic wounds contributed equally to wound healing.

Keywords: cold atmospheric plasma, diabetic wound healing, nitric oxide, rat

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Introduction

Diabetic wound healing represents a major clinical problem owing to impairment of all stages of normal wound healing in such chronic wounds. Animal and human studies have demonstrated that diabetic wounds in both humans and laboratory animals elicit a dysregulated inflammatory response and reduced neovascularization compared to non-diabetic wounds (Malone-Povolny et al. 2019). In circumstances where each phase of wound healing is compromised, the presence of high blood sugar levels (hyperglycemic environment) encourages the formation of biofilm. Recently, nitric oxide (NO) has been reported to contribute to the repair of such wounds. The beneficial effects of NO on wound repair have been attributed to its functional effects on angiogenesis, inflammation, cell proliferation, and extracellular matrix deposition and remodeling (Luo and Chen 2005, Caskey and Liechty 2013, Burgess et al. 2021). Diabetic wounds are usually characterized by a pathologic inflammation and, when coupled with infection and hypoxia, this causes delayed healing. NO critically influences the regulation of various processes of wound healing, including inflammatory response, cell proliferation, collagen formation, antimicrobial action, and angiogenesis. The potential role of NO in wound healing has prompted researchers to focus on NO-based wound healing therapies. Therefore, studies have investigated applications of exogenous NO gas (exNO gas) for diabetic wounds and the placement of chitosan hydrogels and polymeric nanoparticles that provide extended NO release in the wound bed to accelerate wound healing. NO administration has garnered increased attention in recent yers as a pharmaceutical strategy to achieve wound healing. (Bryan 2015, Krausz and Friedman 2015, Shekhter et al. 2019, Gronbach et al. 2020, Ahmed et al. 2022). NO gas at concentrations of >160 ppm showed strong antimicrobial properties, while applications at lower concentrations have partial bactericidal and bacteriostatic activity in a dose-dependent manner. Based on these findings, the minimum concentration of NO required for a bactericidal effect was reported to be 200 ppm and continuous exposure to 200-ppm NO gas was shown to create an antimicrobial effect (Ghaffari et al. 2005, Ghaffari et al. 2006). Although NO gas therapies have become prominent, recent research has focused on the duration of delivery of NO gas therapy and the timing of the release mode. (Ghaffari et al. 2007, Malone-Povolny et al. 2019, Wu et al. 2021).

Due to varying timelines and concentrations reported for NO gas delivery, the purpose of the present study was to investigate the application of CAP/NO gas at a concentration of 200 ppm for 60 and 120 s on wounds in diabetic rats and to determine the effect of this application on wound healing and wound contraction using clinical and histological analysis.

Materials and Methods

A total of 15 female Wistar albino rats (age, 5-6 months) were included in the study and randomly divided into three equal groups. The control group with diabetes (G1-DC) received no treatment. The second group (G2-DNO/60) received NO gas for 60 s and the third group (G3-DNO/120) received NO gas for 120 s once daily for 14 days. The study received ethical approval from the Near East University's Animal Experiments Local Ethics Committee (Decision No: 149).

Diabetes model

Diabetes was induced in rats using a single dose of 50 mg/kg streptozotocin (STZ) dissolved in citrate buffer (0.07 M, pH 6) and administered intraperitoneally (Sigma, St Louis, MO, USA). The rats were fasted 12 hours before and 4 hours after STZ administration and blood glucose levels in all animals were measured with a digital glucometer (HMD Gluco Leader) using blood samples collected from the tail vein on days 1, 2, and 14. During the first two days, the drinking water of all subjects was composed of 15% dextrose. When blood glucose reached a constant level above 200 mg/dL after STZ injection, the rats were considered to have diabetes and a wound model was created (Güngör et al. 2022).

Anesthesia and wound model

The animals were anesthetized intraperitoneally using 100 mg/kg ketamine HCL and 10 mg/kg Xylazine HCL. Meloxicam (METACAM®) 1 ml/kg was administered subcutaneously before surgical excision. An excisional circular wound – 3 cm in diameter – covering the epidermis, dermis, and panniculus carnosus was created in the right dorsal region of the rats.

Cold atmospheric plasma (CAP/NO) applications

A cold atmospheric plasma (CAP) device (Inosante Medical Inc/Turkey) which produces NO from atmospheric air was used. The device is designed to deliver an NO-containing gas mixture to biological tissues in veterinary medicine and produces exogenous NO, which is highly permeable through the cell membrane and acts as a signaling molecule acting on all organs and tissues without the need for cell receptors. In the CAP/NO group, 200-ppm NO gas was applied to the wounds once daily for 60 and 120 s during a period

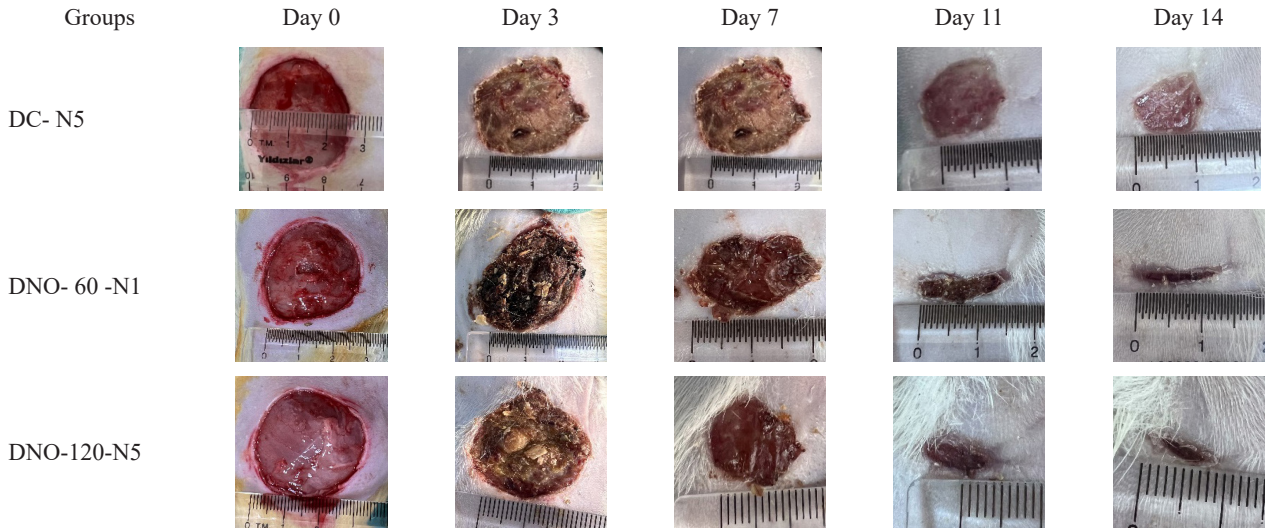

Fig. 1. Intergroup comparison of rat wound contraction rates according to clinical observations.
DC- N5: Untreated diabetic control, DNO- 60 -N1: exposure to 200 ppm NO gas for 60 s/day, DNO-120-N5: exposure to 200 ppm NO gas for 120 s/day

of 14 days. The treatment was delivered using a circular pattern (from the periphery to center and from the center to the periphery of the wound) from a distance of 2 mm.

Calculating wound contraction and wound contraction rate

Photographs taken by the same researcher on the day of wound formation, on days 3, 7, 11, and 14 and were transferred to ImageJ 1.53 k freeware (National Institute of Health, Rockville, MD, USA), and the wound surface areas were recorded in cm^2. Wound contraction rates between days 0-3, 0-7, and 0-14 were calculated and were compared between groups ([Created Wound Area – Measured Wound Area] / Created Wound Area × [100] (Bae et al. 2012, Anuk et al. 2016)).

Histopathological analysis

Once the experiment was completed, tissue samples were collected from the groups and fixed in 10% formaldehyde. After 24-48 hours of fixation, the tissues were subjected to graded alcohol series and xylol and embedded in paraffin. The prepared paraffin blocks were cut into serial sections of 4-5 µm using a microtome (Leica). The sections were stained with hematoxylin and eosin (MERCK HX69657153/MANY) for general evaluation and Masson's trichrome (Bio Optica, 04-010802/Italy) for collagen organization. All sections obtained from the groups were subjected to histopathological evaluation for inflammation, epithelial regeneration, angiogenesis-congestion, and collagen fiber organization (Güngör et al. 2022).

Statistical analysis

Statistical analysis was performed using SPSS software (Statistical Package for the Social Sciences 25.0, SPSS Inc, Chicago, IL, USA). Quantitative variables were expressed as mean±standard deviation (S.D.), and qualitative variables were expressed in percentages. The groups were tested for normality of distribution using the Shapiro-Wilk test. Intergroup differences in terms of wound closure rate, contraction rate, and histopathologic results were statistically analyzed using the Kruskal-Wallis test (non-parametric ANOVA) with Conover post-hoc analysis. Statistical significance was set at $p<0.05$.

Results

Clinical macroscopic results

Macroscopic clinical observations in all groups showed that, on day 3, there was significant crusting and drying in the wound in the NO treatment group and there were biofilms indicative of infection in the control group. On day 7, there were irregular wound surfaces, signs of infection and biofilms in the control group *versus* accelerated wound contraction and disappearance of crusting in the NO treatment group. On days 11 and 14, wound contraction appeared to slow in the control group and accelerated in the NO treatment group (Fig.1). Comparison of the groups revealed that contraction in the wound area from day 7 (to day 11 and day 14) was greater in the groups treated with NO than that in the control group, and the differences were significant ($p<0.05$) (Fig. 2A). However, there were

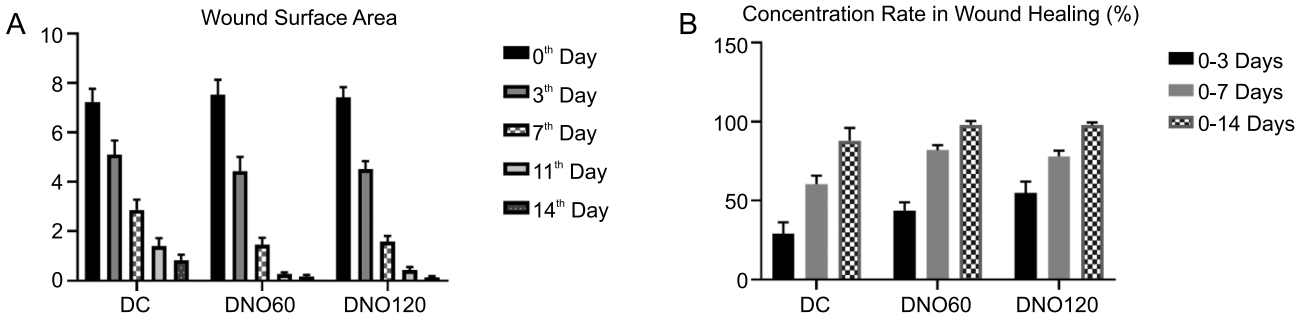

Fig. 2. Differences in the wound surface area (A) and contraction rate (B) in the rat groups. A: In terms of wound surface area; significant differences are present between the treatment group (DNO60 and DNO120) and control group (DC) on days 7 (p=0.028 and 0.040), 11 (p=0.16 and 0.032), and 14 (p=0.006 and 0.024), respectively. B: A significant difference in terms of the contraction rate in wound healing is present between the DNO60 group and DC group (p=0.033) and between the DNO120 group and DC group (p=0.049).

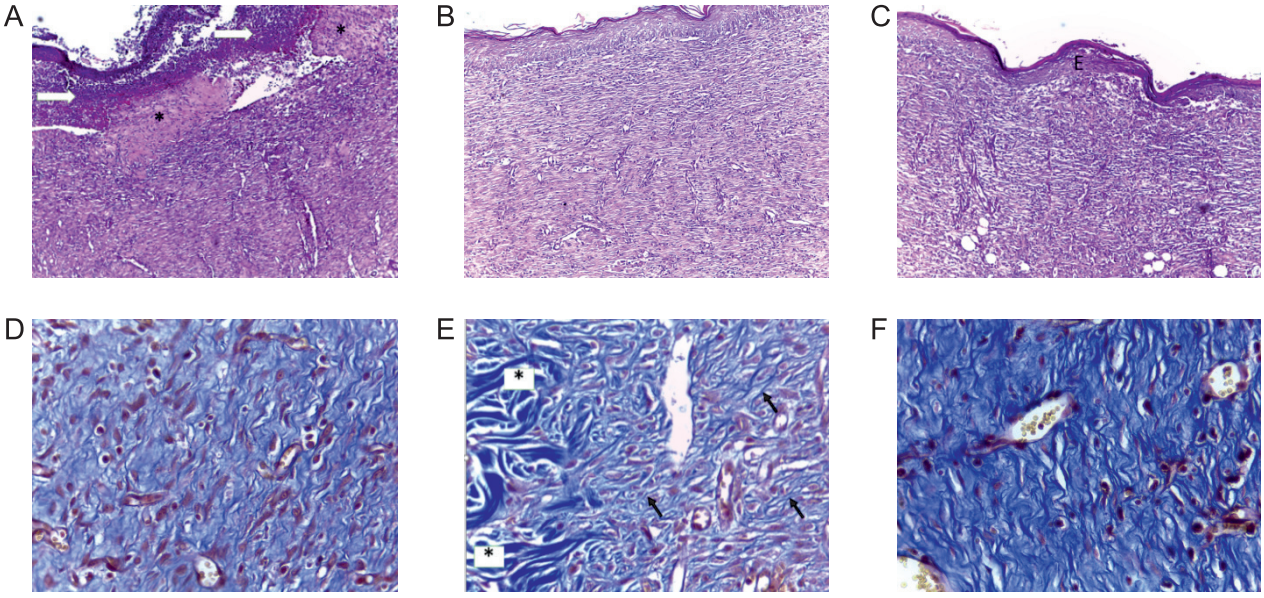

Fig. 3. Evaluation of inflammation, collagen organization, epithelialization in control and NO treatment rat grıups. A: The control group shows no epithelialization in the wound area and shows an increase in neutrophil-dominated inflammatory cells (arrows) and necrotic areas (*). D (dermis). ×10; hematoxylin and eosin (H&E) stain. B: The DNO/120 Group, epithelialization appears to be complete. ×10; H&E stain. C: The DNO/60 group, epithelium (E) appears to be regenerated, showing closure of the wound area; furthermore, congested capillaries perpendicular to the surface and inflammatory cells seem to be decreased compared with the control group. ×10; H&E stain. D: The DNO/60 group, the wound area shows a higher concentration of collagen fibers than the control group, and collagen bundles are present in some areas, although not widespread. ×40 Masson's trichrome stain. E: The control group, collagen fibers are thin and scattered and show no bundling (arrows). Collagen fibers in the intact area (*) ×40; Masson's trichrome staining. F: The DNO/120 group, collagen organization shows the formation of bundles similar to those in Group 2. ×40; Masson's trichrome stain.

no differences between the groups treated with NO. The analysis found a significant difference between day 0 and day 14 in wound contraction rate in the DNO/60 and DNO/120 groups compared to the control group (p=0.033, p=0.049, respectively) (Fig. 2B).

Histopathologic results

In the control group, Masson Trichrome staining clearly showed the absence of epithelialization in the wound area, and the presence of increased neutrophil-dominated inflammatory cells and necrotic areas (Fig. 3A), In the DNO/120 group, epithelialization was complete (Fig. 3B). In the DNO/60 group, the epithelium regenerated and closed the wound area, there were fewer congested capillaries perpendicular to the wound surface and fewer inflammatory cells compared to the control group (Fig. 3C), there were more collagen fibers in the subepithelial region than in the control group, and collagen bundles were present in some areas, although not widespread (Fig. 3D). inadequate fibroblast activity in the dermis layer, thin and scattered collagen fibers, and complete absence of bundling (Fig. 3E) and collagen organization was bundled similar to that in Group 2 (Fig. 3F). NO resulted in an equal level of contribution

Table 1. Statistical analysis of rat groups; G1 (Control: Untreated diabetic control), G2 (DNO/60 s: exposure to 200 ppm NO gas for 60 s/day) and G3 (DNO/120 s: exposure to 200 ppm NO gas for 120 s/day) in terms of inflammatory cells, angiogenesis, collagen organization, and epithelialization.

	Inflammatory cell	Angiogenesis	Collagen organization	Epithelialization
Control	3.0±0.0$^{(a)}$	3.00±0.00	0.50±0.54$^{(a)}$	0.16±0.40$^{(a)}$
DNO/60 s	2.0±0.63$^{(b)}$	2.66±0.51	1.83±0.40$^{(b)}$	1.50±0.83$^{(b*)}$
DNO/120 s	1.83±0.75$^{(b)}$	2.83±0.40	1.83±0.75$^{(b*)}$	1.83±0.98$^{(b*)}$
	a:b $p<0.05$	$p>0.05$	a:b $p<0.05$ a:b* $p<0.01$	a:b* $p<0.01$

Control – Untreated diabetic control, DNO/60 s: exposure to 200 ppm NO gas for 60 s/day, DNO/120 s: exposure to 200 ppm NO gas for 120 s/day

to diabetic wound healing in both treatment groups compared to the control group.

Evaluation of inflammatory cells showed a difference between the control and NO treatment groups ($p<0.05$). Evaluation of angiogenesis demonstrated no difference between the control group and DNO/60 and DNO/120 groups ($p>0.05$). Evaluation of collagen organization revealed significant differences between the control group and DNO/60 group and between the control group and DNO/120 group ($p<0.05$, $p<0.01$, respectively). Evaluation of epithelialization found a significant difference between the control group and NO treatment groups ($p<0.01$) (Table 1).

Discussion

Numerous topically or systemically applied therapeutic agents are being investigated for their role in wound regeneration process and wound closure, with the aim of reducing healing times. Given the alarming increase in the prevalence of diabetes and associated diabetic wounds, future treatment strategies should target the impaired healing of diabetic wounds (Burgess et al. 2021, Mieczkowski et al. 2022). Diabetic wounds are characterized by microangiopathy and neuropathy, which inhibits cell proliferation and collagen organization under the influence of hyperglycemia, which leads to a decrease in growth factors for fibroblastic and angiogenic activity and increases susceptibility to bacterial infection by inhibiting phagocytic activity, this delaying wound healing (Powers et al. 2016, Merviş 2018). Exogenous NO gas, a regulator of inflammation in chronic wound healing, is used in wound care as a non-resistant antibacterial agent and is reported to be effective in all regenerative processes (Shekhter et al 2005, Fridman et al. 2008, Forstermann and Sessa 2012, Pekshev et al. 2018, Malone-Povolny et al. 2019). The discovery of devices that generate exogenous NO from atmospheric air (CAP/NO) using a chemical method has laid the foundation for a new therapeutic strategy in the treatment of wound pathologies as well as acute and chronic inflammatory processes (Pekshev et al. 2018). In one study, NO gas was delivered daily for 60 s at a concentration of 500 ppm on purulent and aseptic 3-cm^2 wounds in rats using a Plason device that produces NO from atmospheric air; it was found to contribute to the healing of skin wounds and shorten the healing time by about one third. The same study also used NO gas at concentrations up to 500 ppm once daily for 6 days to reduce toxicity concerns, and reported a reduction in tissue hypoxia and microbial infection in infected or clean wounds (Shekter et al. 2005). This result was supported by another study that reported that NO gas is safe to use in a mouse lymphocyte model at a concentration of 200 ppm for 8 hours. It was reported that NO used at concentrations of 200 ppm did not damage the extracellular matrix (ECM) and increased lymphocyte proliferation (Rezakhanlou et al. 2011).

These results, achieved with the reported device seem to be supported by the present study, which used a device that produced NO gas from atmospheric air (Inosante Medical Inc/Turkey) and delivered the generated gas at a dose of 200 ppm for 60 and 120 s/day from a distance of 2 mm; this contributed significantly to wound healing in diabetic rats compared to the control group. Clinical macroscopic observations in this study found that there were biofilms indicative of infection in the control group from day 3 onwards, but there were no signs of infection in both NO treatment groups and wound contraction continued and increased in NO treatment groups from day 7 to day 14. This was subjectively attributed to the antibacterial activity of NO gas. The results also showed a significant difference between the control group and NO groups in terms of wound contraction from day 7 to day 14 of the treatment ($p<0.05$) and a significant difference between the control group and DNO/60 and DNO/120 groups in terms of wound contraction rate between day 0 and day 14 ($p=0.033$, $p=0.049$, respectively). This showed that the application of NO contributed to healing of diabetic wounds.

The NO molecule is known to have anti-inflammatory effects (Young et al. 2001), and controlling inflammation should be a priority in the treatment of diabetic wounds, as the persistence of chronic inflammation prevents transition from the inflammatory phase to other phases of wound healing (Miller et al. 2004). The study found a significant increase in the number of inflammatory cells in the control group subjects compared to the NO treatment group. This suggests that application of NO for both 60 s and 120 s was effective and modulated inflammation ($p<0.05$). Evaluation of epithelialization found a significant difference between the control group and NO treatment groups ($p<0.01$); the control group had no epithelialization in the wound area and had increased neutrophil-dominated inflammatory cells and necrotic areas, which suggests that the application of NO with both exposure times was effective in epithelialization. At low concentrations, NO gas has a stimulatory effect on collagen synthesis of the skin, whereas at high concentrations, this effect has been reported to disappear. The biphasic effects of NO gas and its metabolites on proliferation of keratinocytes were investigated and NO gas at low concentrations was shown to increase proliferation of keratinocytes but this effect changed at high concentrations. NO gas applications have been shown to elicit important mechanisms in ECM gene expression and to increase intracellular cGMP levels and the cGMP signaling pathway involved in collagen expression (Miersch et al. 2008). This study found that the application of NO gas for 60 and 120 s did not have a negative effect on collagen organization, and there was a significant difference between the control group and DNO/60 and DNO/120 groups ($p<0.05$, $p<0.01$, respectively). With NO application at a concentration of 200 ppm, there was no significant difference in mean angiogenesis levels in two NO groups (Ghaffari et al. 2007) and NO administration did not affect angiogenesis. Exogenous NO gas delivered at a concentration of 200 ppm for 8-12 hours is known to exhibit potent antimicrobial properties, but delivery of NO gas at a dose of 400 ppm for 24 hours has been shown to significantly reduce proliferation of human dermal fibroblasts. For NO gas to be used in skin infections, its cytotoxic effects on dermal cells must be minimized. To minimize the cytotoxic effects of NO gas, cells were exposed to atmospheric air and NO gas for 8 hours daily, and dermal cells showed strong resistance to the cytotoxic and cytostatic effects of 200-ppm NO gas. Although not statistically significant, keratinocytes were found to be more sensitive to NO treatment compared to other skin cells (Ghaffari et al. 2006, Ghaffari et al. 2007). The present study took into consideration the risks with certain times of exposure to NO and tested NO application for 60 and 120 s in order to determine the most effective and shortest possible exposure time. Both exposure times were found to contribute equally to diabetic wound healing. One clinical study used NO gas for diabetic wound healing in a patient who had had a chronic diabetic foot ulcer for more than 2 years and found that NO gas delivered at 200 ppm on the diabetic wound for 14 days enabled the infected wound to heal rapidly, albeit malodorously, and that healthy granulation tissue was formed on day 3, the ulcer area decreased by 70% on day 14 and the wound healed by 90% at six weeks (Miller et al. 2004). This promising preliminary case study was supported by statistical analysis of the efficacy of gaseous NO on an animal model (Rezakhanlou et al. 2011). A mouse lymphocyte model showed that gaseous NO can be safely used at concentrations of 5, 25, 75 and 200 ppm for 8 h and indicated that it can be used safely with concentrations up to 200 ppm. Authors interested in the antibacterial effect of NO (Ghaffari et al. 2007) reported that administration of 200 ppm NO for 8 h improved collagenation in rabbits with wounds heavily infected with Staphylococcus aureus and that gaseous NO is a potent antibacterial agent.

Another study seeking to reduce toxicity concerns investigated NO administered at concentrations up to 500 ppm on rats with both infected and clean wounds for only 60 s once daily for 6 days and reported reduced tissue hypoxia and microbial infection, increased angiogenesis, significantly reduced healing time and improved tissue morphology. Specifically, the authors found that exposure to gaseous NO increased peroxynitrite levels in wound tissue, that peroxynitrite, a cytotoxic free radical, triggered protective mechanisms, including NO production and that gaseous NO treatment elicited increased NO levels in tissue, regulating the healing process in impaired chronic wounds (Shekhter et al. 2005). Although NO has been shown to reduce bacterial load in infected wounds, more research is needed to understand the direct effect of gaseous NO on inflammatory processes (Yang et al. 2015).

There is no experimental diabetic rat model that has previously investigated the effect of NO applications on wound healing. The present study has thus objectively observed that NO applied at concentrations of 200 ppm and different exposure times significantly reduced the bacterial load and prevented wound infection. Moreover, statistical analyses also showed that NO applications modulated inflammation, supported epithelialization and collagenation, and contributed to wound contraction and contraction rate. However, the study found no significant difference in wound healing with NO delivery for 60 s and 120 s.

Conclusion

Based on clinical, histopathological and statistical analysis results, this study objectively found that application of exogenous NO gas at a concentration of 200 ppm for 60 s and 120 s from a distance of 2 mm on wounds in diabetic rats provided an antibacterial contribution to wound healing without forming a biofilm. NO applications contributed to healing through increased wound contraction, greater wound contraction rate, epithelialization and collagen organization. Given the negative effects of long-term inflammation on healing, particularly in diabetic wounds, NO delivery was found to contribute to wound healing by reducing the number of inflammatory cells and application of NO for 60 s and 120 s/day contributed equally to healing.

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