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Effects of Cold Atmospheric Plasma on Infectious Diabetic Wound Healing in Rat Models

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Abstract

Objectives: Patients with diabetes often suffer from chronic wounds which can occur due to the impairment of wound healing in these patients. Scientists have been trying to address this issue by using wide spectrum of antibiotics and drugs. However, overusing antibiotics in recent years has led to antibiotic resistant crisis; therefore, there is an urgent need to develop new approaches in order for controlling microbial infections in diabetic patients. This study aimed to determine if cold plasma was effective in wound healing in the infectious diabetic conditions.

Material and Methods: In this experimental study, 40 adult male rats with diabetes infected with *Staphylococcus aureus* were included. The animals were randomly divided into two groups of 20 rats and four sub-groups (i.e., day 3rd, day 7th, day 14th, and day 21st). The rats in the treatment group were exposed to helium plasma irradiated for 3 minutes per day for 21 days. Wound healing in the samples was evaluated using five healing indexes including epithelium formation, Inflammatory cells, new vascularization, fibroblast, and collagen formation.

Results: A significant difference ($P<0.05$) was found between the indexes in the plasma radiation group compared to the control group. The results showed that the speed of wound healing in the group treated with cold helium gas plasma was higher than that in the control group. Collagen formation was always completed faster in the helium plasma group, indicating the positive effects of helium plasma on infected diabetic wounds.

Conclusions: Helium plasma was remarkable effective in healing wound and controlling infections in diabetic rats.

Keywords: Cold atmospheric, Diabetes, Healing, Histopathology, Plasma ultraviolet radiation

Introduction

In addition to neuropathy, vascular disease, and foot deformities, the patients with diabetes have wound healing complications (1). About 15% of diabetic patients experience diabetic foot injuries. A study by Tehran Medical School in 2007 showed that the average cost of a diabetic ulcer is US$ 5000-8000. The spread of diabetic foot amputation is 30% in Iran, which is meaningfully higher than that suggested by the world statistics (2). In diabetics, the immune system is less capable of fighting infections, resulting in diabetes wounds that cannot be treated effectively due to the slow wound healing process, high costs of therapies, and lack of improvement of diabetes-induced disturbed healing processes (3). Cold atmospheric plasma (CAP) has been recently used to treat diseases, and it has been found that treatment with cold plasma at most up to 240 seconds and repeated every 12 hours for 30 seconds is absolutely safe and eliminates the mutation risk (4). Recent studies have also shown that cold plasma significantly decreases the bacterial count of chronic ulcers (5).

in repeated foot infections. *Staphylococcus aureus*, as one of the most important members of the *staphylococcus* family in medicine, is a gram-positive facultative anaerobic coccus. It is also known as "golden staph" because it produces a pigment called staphyloxanthin, which serves as an antioxidant and protects bacteria from oxygen-free radicals produced by white cells in the host (6). Wound healing becomes a complicated and organized process after an injury to the skin or soft tissues. Wound healing occurs in several stages including coagulation, inflammation, granulation, fibroplasia, collagenase wound constriction, and epithelialization (7). *Staphylococcus aureus* causes various infections ranging from simple skin infection to those which endanger life. Golden staph is one of the five most common factors causing hospital infection – post-surgery infectious ulcers, in particular. Annually, 500 000 patients are afflicted with staphylococcus infections in US hospitals (8). A study was conducted in 2013 to ensure that CAP was safe for human skin, the results of which demonstrated that a CAP treatment of up to 240 second repeated for 30 second every 12 hours caused no mutagenicity at the HPRT (hypoxanthine-guanine phosphoribosyltransferase) locus beyond naturally occurring mutations (9). CAP has been employed as a powerful tool for combating nosocomial

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Key Messages

- Helium plasma was remarkably effective in healing wound and controlling infections in diabetic rats.

infections and antibiotic-resistant bacteria. A 'lethal cocktail' of pathogen-killing products is generated by plasma electrons, ions, reactive species, and UV light, which creates new opportunities in hygiene and medicine. Several pre-clinical in vitro studies should be conducted before performing human clinical trials on CAP aiming at, for instance, treating wounds or skin diseases (9). CAP, according to a retrospective study, was also found to be effective in dealing with chronic wounds. Another study has also determined that the combined treatment of Argon and Helium Plasma can accelerate skin and acute wound healing (10). Taking into account the fact that the healing speed positively contributes to the diabetic wound treatment process in these patients, this study aimed to investigate the effectiveness of cold helium plasma in controlling the healing of infected diabetic wounds.

Materials and Methods

Animals

All animal procedures were carried out in accordance with the national animal care guidelines and approved by local ethics committee. A total of 40 Wistar rats were provided by our basic science department. The animals were housed in standard cages and 12-hour light/dark cycles, and were provided with free access to standard pellets and water.

Study Design

In this experimental study, 40 healthy Wistar rats were randomly divided into two groups of 20 rats as control and treatment groups. After induction of diabetes, an excisional wound was created and infected by staphylococcus aureus. Then the rats in the treatment group were exposed to cold plasma. Finally, animals were euthanized by CO_2 inhalation. Skin around the wound area was harvested and compared histologically with those of control rats on days 3, 7, 14, and 21 after the wound creation.

Infectious Diabetic Wound Modeling

Diabetes was induced in the animals by intraperitoneal injection of Alloxan (Sigma-Aldrich, St. Louis, MO) at a dose of 125 mg/kg. After three days, diabetes onset was confirmed in rats by testing blood glucose levels, and animals with a glucose levels above 250 mg/dL were considered diabetic (11).

General anesthesia and analgesia were achieved by intraperitoneal injection of ketamine 10% (50 mg/kg) and xylazine 2% (5 mg/kg). Wound was created on the dorsum of the rats by creating 4 cm^2 full thickness excision. In order to infect the wounds, *Staphylococcus aureus* (ATCC 6538) were purchased from Institute of Immunology and Infectious Diseases (Iran University of Medical Sciences) and, then, the wounds were infected with 108 CFU/mL of staphylococcus aureus suspension (12).

Plasma Treatment

A plasma jet source was used to generate cold plasma radiation, as described in details by He et al (13). The device includes a plasma generation gun, a RF power supply (42 W, 220 V), and a Helium gas supply unit. The wound area was exposed to plasma radiations for 3 minutes from 30 mm distance for 21 days.

Wound Healing Assessment

Skin around the wounded area were harvested and immersed in formaldehyde 10% (Sigma-Aldrich, St. Louis, MO) 7, 14, and 21 days after the surgeries. Samples were embedded in paraffin blocks, and 5μm sections were prepared and stained with trichrome and hematoxylin-eosin (H&E). Semi-quantitative wound healing score was used to evaluate healing effects of the treatment. Briefly, epithelialization, infiltration of inflammatory cells and fibroblasts, neovascularization, and collagen formation were scored in the groups (14). Also, testicular tissues, kidneys, livers, spleens, and hearts were harvested to detect any adverse effects or possible malignancies.

Statistical Analysis

GraphPad Prism was used for statistical analysis and graphing. Table 1 shows values as mean ± standard error (SE). Kolmogorov–Smirnov test was performed to confirm the normal distribution of the data. T-test was used to calculate the main differences between control and the treatment groups, and $P<0.05$ was considered significant.

Results

Diabetes Onset in the Rats

Glucose levels were evaluated in rats and summarized in Table 1. Diabetes incidence was confirmed in all rats three days after the surgeries.

Histological Analysis

According to histological results, significantly poorer inflammatory responses were produced by wounded

Table 1. Glucose Levels in Rats from Control and Treatment Groups

Group Name	Injection Day	3 Days After Surgery	7 Days After Surgery	14 Days After Surgery	21 Days After Surgery
Control	80.7±9.1	361.4±21.2	274±23.2	231.7±22.4	217.7±21.8
Treatment	79.4±9.0	360.7±22	270±22.5	229.3±21.6	215.3±22.1

Mean values in each group ± SE were presented.

skins in treatment group compared to control group. Furthermore, epithelization and fibroblast infiltration were significantly improved in the treatments group compared to the control group. Neovascularization scores indicated that wounds in treatment group showed less vascular reactions (Figure 1). Moreover, pathological evaluation of testicles, liver, kidneys, spleen, and heart revealed no malignancies and abnormality (Figure 2). Trichrome staining was used to evaluate morphological properties of collagen fibers in two groups. Collagen fibers in the treatment group had natural extracellular matrix architecture, whereas destruction was observed in collagen alignments and denaturized collagen fibers in control group (Figure 3). Statistical calculations demonstrated that all wound healing indexes were significantly improved in treatment group compared to the controls (Figure 4).

Discussion

According to our study results, significantly poorer inflammatory responses were produced by wounded skins in treatment group compared to control group. Furthermore, epithelization and fibroblast infiltration were significantly improved in treatments group compared to controls. Neovascularization scores indicated that wounds in the treatment group showed less vascular reactions.

Several factors such as poor blood circulation contribute

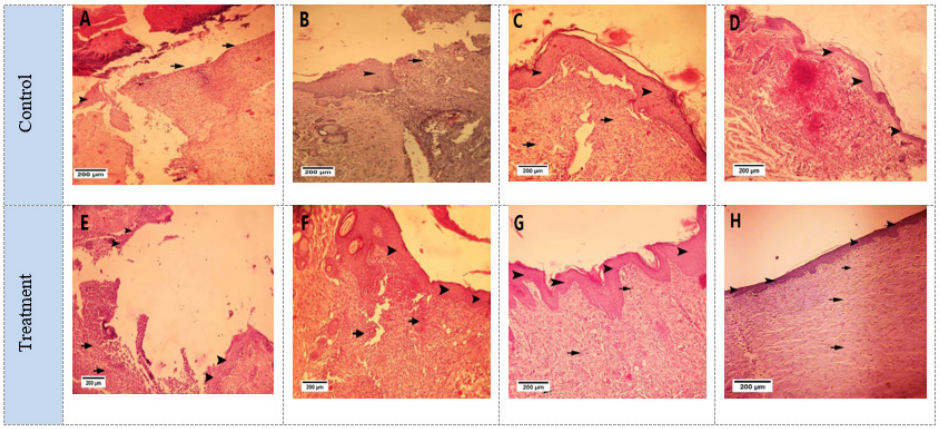

Figure 1. Histopathology of skin wounds in control (A-D) and treatment (E-H) groups. A; skin wound in control group at day 3 after the surgery, note the injured epidermis (arrowhead) in edges and infiltration of inflammatory cells (arrow). B; 7 days after the surgery, migration of epithelial cells (arrowhead) and infiltration of inflammatory cells (arrows) are observable. C; 14 days after the surgery. Beginning of keratinization in epidermis (arrowhead) and excessive infiltration of inflammatory cells (arrows). D; 21 days after the surgery, the entire epidermis underwent keratinization (arrowhead). E; skin wound in treatment group at day 3 after the surgery, note the migration of epithelial cells (arrowhead) and infiltration of inflammatory cells (arrows). F; 7 days after the surgery, remarkable migration of epidermis epithelial cells (arrowhead) and infiltration of inflammatory cells (arrows). G; 14 days after the surgery, the completion of keratinization (arrowhead) in dermis and subtle presence of inflammatory cells (arrows). H; 21 days after the surgery, epithelial layer is complete and well keratinized (arrowhead). (H & E) (100x) (scale bar: 200 µm)

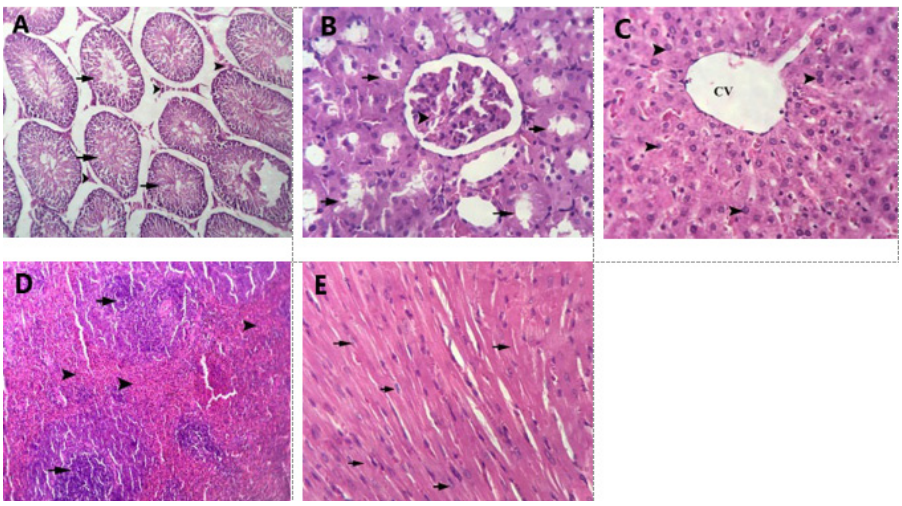

Figure 2. Histopathology of Visceral Tissues in Plasma Treated Animals. A: testicular tissue with interstitial cells (tip of arrow) and sperm tubes (the arrows) with no pathological abnormality. B: kidney tissue, the Malpighian tubules (tip of the arrow) and the urinary tract (arrow) were observed with no malignancy. C: The liver tissue is normally seen with central veins (CV) and hepatocytes (tip of the arrow) D: normal spleen tissue with white pulp (arrow) and red pulp (tip arrow) E: The cardiac tissue is normally seen with cells (arrows). (H & E) (100x) (scale bar: 200 µm).

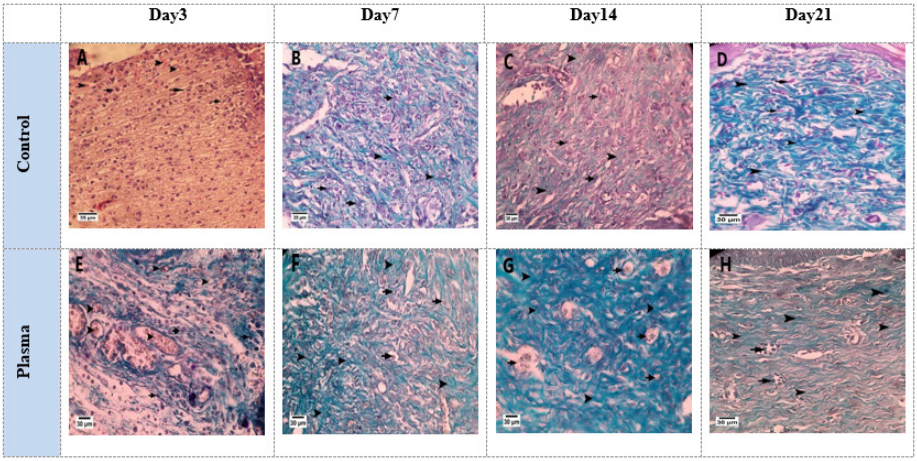

Figure 3. Histological Assessments With Trichrome Staining in Control (A-D) and Treatment (E-H) Groups. A: skin wound in control group at day 3 after the surgery. Remarkable infiltration of polymorph nuclear inflammatory cells (arrow), and new vascularization (arrows) is observable. B: 7 days after the surgery, fibroblasts penetration (arrow) and newly formed collagen (arrowhead) are detectable. C: 14 days after the surgery, there are numerous fibroblasts (arrow) and newly formed collagen (arrowhead) and abundant mononuclear inflammatory cell are noticeable. D; 21 days after the surgery, there are numerous fibroblasts (arrow) and newly formed collagen (arrowhead). E: skin wound in treatment group at day 3 after the surgery. Note the penetrated fibroblasts (arrow) and new collagen forming and extensive neovascularization (arrowhead). F: 7 days after the surgery, numerous fibroblasts (arrow) newly formed collagen (arrowhead) are noticeable. G; 14 days after the surgery, there are remarkable fibroblast infiltration, neovascular lumens (arrow) and newly formed collagen (arrowhead) are observable. H; 21 days after the surgery, considerable neovascularization and blood vessels, subtle presence of mononuclear inflammatory cells (arrow) and organized collagen framework (arrow) are seen. (Trichrome) (100x) (scale bar: 200 μm).

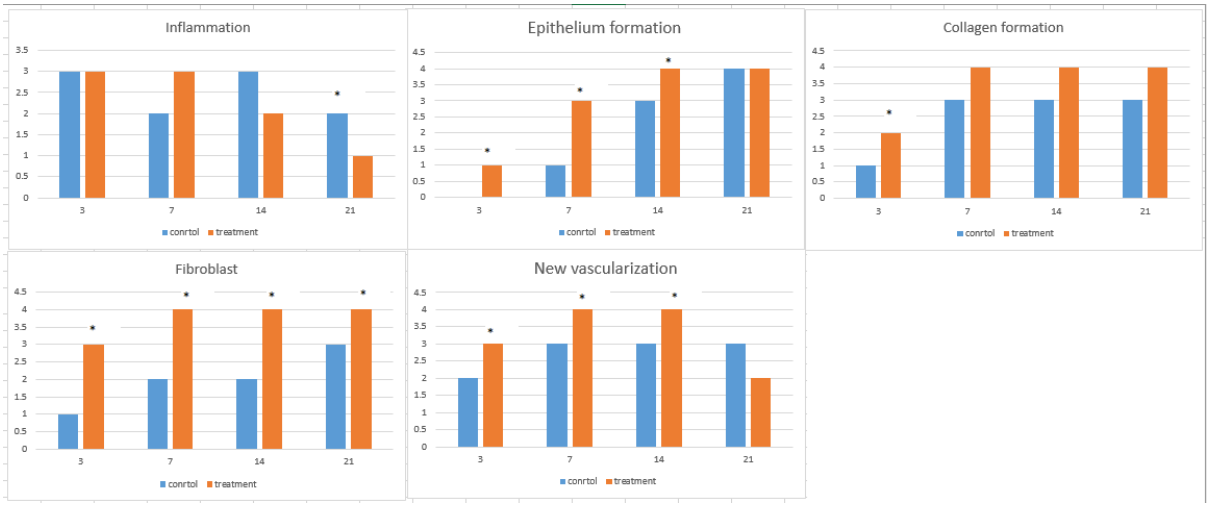

Figure 4. Average Scores of Epithelialization, Infiltration of Inflammatory Cells and Fibroblasts, Neovascularization, and Collagen Formation in Treatment and Control Groups 3,7, 14, and 21 Days After Surgeries (*P<0.05).

to the slow healing of diabetic wounds. Wounds are deprived of oxygen and nutrients due to this factors (15). In diabetic wound healing, neovascularization plays a key role (16). The rate of new vascularization was increased in the wounds in plasma treatment group compared to control group.

Ye et al discovered the dynamic particles in plasma actuate angiogenesis-induced components during development in 2015. They also found that plasma-treated wounds showed keratinocyte cell displacement after a few days, which was not seen in control rat. According to this study, high glucose levels in diabetic patients' blood resulted in overexpression of the FOXO1 gene (17). This process increases the amounts of CCL20 protein instead of transforming growth factor beta (TGF-β) which frustrates the migration of keratinocyte cells in diabetic wounds. Free radicals are produced as a result of plasma production. The interaction between plasma and cells is largely influenced by reactive ions. Activation of TGF-β by NO has been reported to occur via S-Nitrosylation of latency-associated peptide and mitogen-activated protein kinase (MAPK) pathways (18,19). Wound healing and inflammation proliferation are both mediated by MAPK pathways. According to Veith et al, reactive free radicals

activate the vascular endothelial growth factor during angiogenesis. Furthermore, reactive spices are used for wound sterilization (20-22). In a study by Isbary et al exploring argon plasma in chronic wound healing, no abnormal side effects were observed in tissues (23) and, similar to our study, visceral tissues were not damaged by helium plasma radiation during histopathological examination.

Conclusions

Helium plasma was found remarkably effective in healing wound and controlling infections in diabetic rats. Helium plasma had also great potential for improving harmful side effects and carcinogenic complications. Therefore, it was recommended that plasma Helium should be used to control and heal infectious wounds.

Authors' Contribution

Conceptualization: Alireza Jahandideh, Ahmad Asghari.
Methodology: Pouria Dehghanpisheh, Alireza Jahandideh, Pejman Mortazavi, Mahmood Ghoranneviss.
Data curation: Pouria Dehghanpisheh, Mahmood Ghoranneviss.
Formal analysis: Pouria Dehghanpisheh.
Writing – original draft: Pouria Dehghanpisheh.
Project administration: Alireza Jahandideh.
Validation: Pejman Mortazavi.
Supervision: Alireza Jahandideh, Pejman Mortazavi, Ahmad Asghari.
Investigation: Pejman Mortazavi.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Issues

All animal procedures carried out under the national animal care guidelines and approved by the ethics committee of Islamic Azad University Science and Research Branch (IR.IAU.SRB.REC.1398.1096).

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