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Original Article

Effect of a nonthermal-atmospheric pressure plasma jet on wound healing: An animal study

Yi-Wen Hung [a,b], Li-Tzu Lee [c], Yen-Chun Peng [d], Chiou-Tuz Chang [c], Yong-Kie Wong [e,f], Kwong-Chung Tung [g,*]

[a] *Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan, ROC*
[b] *Department of Medicine Research, Taichung Veterans General Hospital, Taichung, Taiwan, ROC*
[c] *Department of Stomatology, Taichung Veterans General Hospital, Taichung, Taiwan, ROC*
[d] *Division of Gastroenterology, Taichung Veterans General Hospital, Taichung and School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC*
[e] *Show Chwan Memorial Hospital, Chang Hwa, Taiwan, ROC*
[f] *School of Dentistry, National Yang-Ming University, Taipei, Taiwan, ROC*
[g] *College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan, ROC*

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Abstract

Background: The use of a nonthermal plasma (NTP) jet in the treatment of living tissue has been the subject of considerable interest in the field of medical technology, and has the potential to reduce the recovery time of open wounds. We aimed to investigate the wound-healing process by clinical observation, blood tests, and expression of cell adhesion markers and reactive oxygen species in NTP jet-treated rats.
Methods: This study utilized Sprague-Dawley (SD) rats as experimental subjects, and wounds measuring 2 cm × 2 cm were produced on the animals' backs. The experimental group was treated with NTP for 5 min/d for 4 weeks. The NTP was injected in a diffused manner into the cage housing the rats. The SD rats that had not received plasma treatment were designated as the control group. Blood was drawn on Postoperative Day 2, Day 4, and at 3 months. An immunohistochemical stain of E-cadherin and 4-hydroxy-2-nonenal (4-HNE), a reactive oxygen species marker, were evaluated and quantified for analysis using a CMYK color model.
Results: A total of 35 SD rats were included in the study (25 in the NTP group and 10 in the control group). Low dose plasma treatment shortened the wound-healing time without damaging organs. In the NTP group, the white blood cell counts at Day 2 post-NTP treatment was not increased significantly more than that in the control group. After quantification of immunohistochemical staining, 4-HNE was increased at Day 14 compared with Day 7 (16.16 ± 12.81% vs. 55.11 ± 8.11%, $p < 0.001$), and E-cadherin was also increased (52.17 ± 14.96% vs. 70.46 ± 12.78%, $p = 0.04$) in the NTP group. After comparison of NTP and the control, it was observed that 4-HNE and E-cadherin were increased in the NTP group on Day 14.
Conclusion: Short-term, low-dose NTP wound treatment was demonstrated to accelerate wound healing in SD rats without vital organ toxicity.

Keywords: 4-hydroxy-2-nonenal; E-cadherin; nonthermal plasma; wound healing

1. Introduction

Caring for wounds and the process of wound healing are important issues in healthcare. A period of prolonged wound healing may cause patient discomfort, increased medical costs, and can even endanger lives. For the purpose of improving wound healing, “plasma” is a common tool that

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* Corresponding author. Professor Kwong-Chung Tung, College of Veterinary Medicine, National Chung Hsing University, 250, Kuo-Kwun Road, Taichung 402, Taiwan, ROC.
E-mail address: kctung1234@gmail.com (K.-C. Tung).

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has the capacity to potentially benefit patient wound healing.[1] Plasma is defined as ionized gas. In terms of applications, plasma can be divided into thermal and nonthermal plasma (NTP), and is generated at atmospheric pressure with low electrical power (tens of watts). Besides the treatment of wounds, nonthermal-atmospheric pressure plasma has wide applications, such as disinfection by topical treatment of skin diseases with microbial involvement, local treatment for erosion in cancer diseases, or hemostasis for bleeding mucosa. There are several molecular mechanisms wherein NTP could improve wound healing by its antiseptic effects, by stimulation of proliferation and migration of wound relating skin cells, by activation or inhibition of integrin receptors on the cell surface, or by its proangiogenic effect.[1] In a clinical setting, NTP is widely used for a variety of purposes that include coating an implant surface with a biocompatible layer,[2] hemostasis,[3] and sterilization of surgical tools.[4] There are also several therapeutic effects of plasma including destruction of micro-organisms, acceleration of blood coagulation, and regulation of cell surfaces in the wound-healing process.[5,6] NTP may also induce neutral and charged particles, electric fields, radicals, e.g., reactive oxygen species (ROS) and other reactive molecules, such as hydrogen peroxide and nitric oxide.[7−9] Because electrons are extremely light, they can move quickly and have no heat capacity. Therefore, NTP has become a promising medical option without adverse effects.[10−14]

Wound healing requires the migration of epithelial cells. Several markers can detect cell surface adhesions, e.g., integrins, cadherins, etc.[7,15] An *in vitro* study demonstrated that plasma treatment influenced the growth and differentiation of keratinocytes and fibroblasts.[7] Cells showed a dramatic loss of E-cadherin within 24 hours after the plasma treatment, leading to loss of cell−cell contact.[7] E-cadherin was shown to be significantly reduced by the ROS-inducing treatment.[16,17] ROS changed the DNA integrity, and the effectiveness of cellular defense mechanisms characterizes the interaction of NTP and eukaryotic cells. Hence, a stimulation of eukaryotic cells using short-term NTP treatment seems possible, e.g., in the context of chronic wound care.[9] Long-term plasma treatments stopped cell proliferation and apoptosis activities, which could be relevant in controlling neoplastic conditions. It is widely believed that ROS can disrupt cell−cell adhesion, leading to various biological responses which include cell migration and proliferation.[18]

Plasma could produce ROS in cells or tissue and generate results in oxidative stress to the microenvironment.[9,19,20] One of the important products of ROS is 4-hydroxy-2-nonenal (4-HNE), an oxidized lipid, which could contribute to the disruption of the cell membrane structure and break down the protein or enzyme activity.[21−23]

The aim of this study was to investigate the effect of NTP-treated wounds. To achieve this, we compared wound size, blood data, and immunohistochemical (IHC) staining of 4-HNE and E-cadherin on rats.

2. Methods

2.1. Animals

Thirty-five Sprague-Dawley (SD) rats weighing 250−350 g were used in this study. Permission was obtained from the Institutional Animal Care and Use Committee of Taichung Veterans General Hospital (Taichung, Taiwan; La-101937). The rats were separated into a control (10 rats) and an experimental (25 rats) group, and were anesthetized with 4% isofluorane for induction, followed by a maintenance dose (1−2%). A 2 cm × 2 cm skin wound was produced on the back of the SD rats under anesthesia (Fig. 1A and B). In the experimental group, all rats received NTP jet diffusely to the carriage 5 min/d for 4 weeks. The control group received no treatment. The size of each wound was calculated daily by multiplying the long and short axial lengths of the rats. In addition, in both the control and experimental group, 0.5 mL of blood was drawn at 2 days, 4 days, 1 week, 2 weeks, 1 month, and 3 months. The blood was sent for analysis of white blood cell counts (WBC), red blood cell counts, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine. This study has been reviewed by our institution review board for animal study (La-101937).

2.2. Experimental equipment

The NTP apparatus generates plasma in a dielectric barrier discharge configuration, manufactured by the Taiwan Yih Dar Technologies (Changhua, Taiwan; Fig. 2). Aluminum tape electrodes were fitted onto a quartz tube with an inner diameter of 4 mm and separated by 10 mm of space. Then, argon and oxygen were released at the rate of 1.8 L/min and 0.01 L/min, respectively. High voltage mono-polar square pulses were applied using the powered electrode with a repetition rate between 0.5 kHz and 4 kHz, which provides a stable and high energy plasma.

2.3. IHC staining

In order to detect the expression of the 4-HNE and E-cadherin in the wound tissue after plasma application, antibodies were used for IHC stain. First, tissue samples were obtained from euthanized rats. The slices were fixed in 10% buffered formalin for 24 hours and then processed with conventional histopathological techniques. Thereafter, the tissue samples were stained with hematoxylin eosin and other IHC stains.

The IHC studies were performed using the Bond-Max Autostainer (Leica Microsystems, Wetzlar, Germany). Slides were stained with 4-HNE polyclonal antibody as well as E-cadherin monoclonal antibodies. These immunomarkers, including methods of pretreatment for antigen retrieval, are shown in Table 1. In short, the formalin-fixed and paraffin-embedded tissue array specimens were added to Tris-buffered saline and Tween 20, rehydrated through serial