Electro-Acupuncture Accelerate Pressure Ulcer Healing by Increasing Asic3 Expression in Mouse Model

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Research

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Abstract

Background: The efficiency of insert electro-acupuncture stimulation (EAS) on improving pressure ulcer (PU) healing was still unclear. This study explored the effects of insert EAS on PU and provided the regulation information of acid-sensing ion channel 3 (Asic3) during EAS intervention.

Methods: A total of 32 BALB/c mice were randomly divided into control group, model group, EAS group, EAS + APETx2 (Asic3 inhibitor) group, each for eight mice. The ulcer tissues of all mice were harvested at 14 days after modeling. HE stains and immunohistochemical staining evaluated the pathologic change, Western blot, and RT-PCR evaluated Asic3 expression.

Results: Compared with the model group, EAS group showed alleviated epithelial thickness, increased number of fibroblasts, and reduced inflammatory cell infiltration. Thickened epithelium, decreased number of fibroblasts, and obvious inflammatory cell infiltration in the EAS+APETx2 group were no significant differences with the model group. Asic3 expression in PU tissues in the EAS group was elevated compared with the model group. In addition, Asic3 expression was decreased in EAS + APETx2 group than the EAS group.

Conclusion: EAS can accelerate the PU healing process in mice, and EAS also could improve the expression of Asic3 in PU tissues.

Trial-registration: Not applicable.

Introduction

Pressure ulcer (PU) is a common complication bringing heavy medical resource use and health care costs in various diseases^{1,2}. PU remarkably resulted in impaired function and decreased quality of life of about 1.3 million to 3 million adults in the United States³.

Protein or amino acid supplementation and hydrocolloid dressings were recommended by the American College of Physicians (ACP) to reduce wound size⁴. Besides, electro-acupuncture stimulation (EAS) was recommended as adjunctive therapy to accelerate PU healing by ACP⁵. However, the mechanism and efficiency of EAS on improving pressure ulcer healing were still unclear.

EAS is a kind of physical therapy to deliver different parameters and dosage current to the area of the wound. The electrical signal could affect protein synthesis, cell migration, antibacterial effect to increase wound angiogenesis and sensory recovery in the wound area^{6,7}. One of the biomechanism maybe that EAS could improve the sensory neurons regeneration and growth-associated gene expression^{8,9}. The nerve regeneration may indicate the final state of clinical recovery¹⁰. A critical neuronal sensor, acid-sensing ion channel 3 (Asic3), is a key sensor for extracellular protons expressed in sensory neurons¹¹. Asic3 is a sensitive neuronal mechanosensor to direct pressure and is critical for vasodilation response in

protecting against PU¹². Furthermore, nerve growth factor could increase Asic3 to activate neurons regeneration expression¹³.

Increased Asic3 expression may be an indicator of PU recovery healing under EAS intervention. We hypothesis that EAS also could increase the Asic3 expression in PU tissue and improve PU healing. Therefore, We used HE staining to observe PU tissue pathology change, took western blot and PCR to observe Asic3 expression after EAS intervention in mice PU model group and Asic3 inhibit group.

Material And Methods

Subjects

A total of 32 male BALB/c mice were sacrificed in this study, with 16-week-old and weighing 20 – 25g (Dashuo Co. Ltd, Chengdu, China). All mice were kept under a normal dark-light cycle in a standard environment, with arbitrary access to food and water. This experiment was carried out following the guidelines of the animal ethics committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals¹⁴.

PU Model

PU mice model followed by the well-recognized model of Istvan¹⁵. Fig.1 shows the diagram of PU modeling equipment and method. The dorsal skin was gently pulled up and labeled with the two round ceramic magnetic plates before the PU area hair was shaved. The two magnetic plates could raise 50 mmHg compressive forces, with 12.0 mm in diameter, 5.0 mm in thickness, and 2.4 g in weight. Three ischemia-reperfusion cycles were employed in each animal to initiate the pressure ulcer formation. A single ischemia-reperfusion cycle comprised of a 12h period of magnet placement, followed by a 12h rest period. The stage of the ulcer was graded by using the National Pressure Ulcer Advisory Panel (NPUAP)¹⁶. Three experienced investigators evaluated the ulcer stage. Stage 2 and 3 were considered to be successful in modeling and enrolled in our study.

Grouping

All mice randomly assigned into four groups: control group, model group, EAS group, ESA+APETx2 (Asic3 inhibitor) group, each for eight mice. The control group received no PU modeling and no EAS intervention. The model group received PU modeling, needle insertion, and without electricity. EAS group received PU modeling with EAS. The EAS+APETx2 (Asic3 inhibitor) group received PU modeling, with EAS and APETx2 injection. EAS was applied to the mice after pressure ulcer formation. APETx2 was subcutaneously injected into mice from the EAS+APETx2 group at 20 min before EAS intervention.

EAS intervention

Considering the results of the clinical trials[17, 18], we applied the widely used high-voltage pulsed current to avoid electrolysis. Minimal acupuncture entailed the superficial insertion of fine needles (0.30×25mm, made by Beijing Han Yi Medical Instrument Co., Ltd). The acupuncture point was around the scope of wound edges about 1 cm. We applied an insulating coating within 5 mm of the front end of the needle to avoid damage to the deep tissue and reduce the risk of infection (Fig.1). The EAS detail parameters were as follows: Fixed frequency output 0.5 Hz, 300 μ A, 250 μ s pulse width, three sec ON, five sec OFF, duration of 30 min, twice/day, and for 14 days. The amount of water intake exuded, and necrotic wounds were observed daily.

HE staining

All the mice sacrificed by cervical dislocation. Wound surface area was assessed in all four groups. Mouse skin PU tissues were fixed with 4% polyformaldehyde for 24 h. Tissues were embedded in the waxed. Then we placed the waxed in the frozen machine at -20°C. Paraffin sections 4 μ m in thickness conventionally stained with haematin dyes. The sections stained with hematoxylin-eosin after paraffin slicing. Finally, image acquisition and analysis by Image-Pro Plus 6.0 software.

Immunohistochemical Staining

A total of 4 μ m longitudinal sections of the paraffin-embedded were kept at 60°C for two h. After successively incubating with antigen, drop endogenous peroxidase blocker, soak in 3% H₂O₂ for 20 min at room temperature. The primary antibody was configured according to the dilution ratio of 1:200. Each section was added with 50 L primary antibody and incubated at 4 °C. 50 μ L of secondary antibody (Asic3, Abcom china Company; ab49333) working fluid was added to each section and incubated at room temperature for 10-15 min. The DAB staining was analyzed by Image-Pro Plus 6.0 software.

Western blot

The collected PU tissues were mashed and added lysate. Subsequently, the supernatant collected for determining protein concentration by using a BCA kit. The proteins separated by SDS-PAGE and transferred onto a PVDF membrane, then incubation with Asic 3 anti-mouse antibodies.

The qRT-PCR

RNA extracted by using the classic Trizol (Invitrogen) method. PCR template was generated in a PCR instrument using a reverse transcription kit. Real-time fluorescent quantitative PCR machine (ABI, V7) completed PCT according to the instructions of the SYBR Green qPCR kit. Samples were divided into three groups and detected using the following primers: Asic3, CCTCAGACATCCGGGTGTTC (forward), GGGAAGGTAAGCTGGTGGC (reverse); GAPDH, AACAGCGACACCCACTCCTC (forward), GGAGGGGAGATTCAGTGTGGT (reverse).

Statistical analysis

All statistics were analyzed using SPSS19.0. All data expressed as mean $\pm$ standard deviation (M $\pm$ SD). Differences between samples were analyzed using the least significant difference (LSD) method of repeated measures ANOVA, with a significance level of $p < 0.05$.

Results

1. Change of PU area

Fig.2 shows the PU wound surface area in mice from each group after 7, 14 days of treatment. As could be observed from the above-described results, dorsal skin in mice from the control group was healthy without PU formation. In contrast, dorsal PU was observed in mice from the model, EAS, and EAS + APETx2 groups. Dorsal PU areas in mice from the EAS group had significantly decreased compared with the model group ($p < 0.05$) and the EAS + APETx2 groups. There was no significant difference between the model group and the EAS + APETx2 group. Notably, dorsal PU areas in mice had significantly decreased gradually with treatment in the model, EAS, and EAS + APETx2 groups ($p < 0.05$). Collectively, these results suggested that EAS exerted a therapeutic effect on PU in mice through activating Asic3.

2. PU histopathological changes detected by HE staining.

Fig.3 shows the results of HE staining of mouse PU from each group. The control group characterized by stratified squamous epithelium, clear skin structure, and scattered with numerous fibroblasts. No inflammatory cell infiltration was in the control group. In contrast, the model group showed distinctly thickened epithelium, evidently decreased fibroblasts and inflammatory cell infiltration in dorsal skin PU tissues. Compared with the model group, the EAS group showed alleviated epithelial thickness, increased number of fibroblasts, and reduced inflammatory cell infiltration. Pathological changes of dorsal skin PU tissues from the EAS + APETx2 group were similar to those from the model group. These results implicated that EAS had a therapeutic effect on PU in mice.

3. Expression of Asic3 in PU tissues.

Fig.4 shows the Asic3 expression results of western blot, RT-PCR, and immunohistochemical staining in PU tissues of each group. The Asic3 protein and mRNA expression in the model group and EAS group were distinctly higher than the control group. Moreover, Asic3 in the EAS group had elevated compared with that in the model group, and Asic3 in the EAS + APETx2 group had outstandingly decreased. Asic3 expression decreased in EAS + APETx2 group than that in the EAS group. Compared with the model group, the immunohistochemical staining of Asic3 was increased in the EAS group and decreased in the EAS+APETx2 group. Compared with the EAS+APETx2 group, the Asic3 expression was increased in the EAS group.

Discussion

This study explored the effect of EAS on the BALB/c mice PU model. Our results indicate that EAS could accelerate PU healing and observe the enhanced Asic3 expression in PU skin tissue. In the microscopic evaluation of PU healing, our results of HE stain showed a better structure of epidermal and increased fibroblast in the EAS group. These histological changes may explain by cellular behavior change. Human cells could be activated by EAS, such as keratinocyte and fibroblast. Sebastian A found EAS can evoke epidermally proliferation in human cutaneous wounds¹⁷. Yet, EAS could enhance the directional migration fibroblasts growth¹⁸, and increase the activity of skin fibroblasts through the Smad signaling FGF2 secretion¹⁹. EAS has found to increase fibroblasts regeneration to promote wound healing in the last year²⁰. The spinal cord injury pig and rat experiment also indicated the effectiveness of accelerates the healing process EAS on PU²¹⁻²³. Likewise, the same observation was from our ischemia-reperfusion BALB/C mice model. Asic3 as a neuronal mechanosensor could improve the release of the vasodilator co-express calcitonin gene-related peptide (CGRP) from afferent nerve terminals¹². CGRP can interact with nitric oxide, which can induce vascular smooth muscle relaxation to change localized ischemia²⁴. Localized ischemia resulted from mechanical loading associated with tissue necrosis^{25, 26}. Asic3 is a sensitive neuronal mechanosensor involved in vasodilation response to pressure¹². Additionally, NGF likely increased ASIC3 expression in neurons and could activation neuron regeneration¹³. Therefore, the upregulation of Asic3 may indicate the recovery of PU tissue. Studies have demonstrated that EAS enhances the recovery of sensory nerve⁷⁻⁹.

This PU model, made by the compression pressure from two external magnets, was noninvasive with high clinically relevant²⁷. According to our knowledge, this kind of ischemia-reperfusion mice model was first used in EAS research in this study. There are four stages of PU based on NPUAP. We evaluated the effects of EAS on 9 days post modeling in BALB/C mice. We designed the study to mimic clinical PU features that full skin thickness loss and necrosis of subcutaneous tissue. Diverse stimulating, including pulse width, waveform, frequencies, intensities, electrodes have been used in a different clinical situation. Based on the result of the case report²⁸, the insert needle electrode may transfer electro signals into the deep PU tissue and could promote wound healing of PU. KAMBIC found there is no significant difference between direct current and alter the current in pig PU model²⁹. However, in human body study, the alter current, High-voltage pulsed current (HVPC) is recommended as a better choice in a systematic review of Lisa Kawasaki³⁰. The reasons were described by the review as follows: first, clinical studies found to support PU healing; second, HVPC shows a lower risk of skin burn³¹ and a greater depth of penetration³². Third, HVPC more closely mimics the cutaneous bioelectric currents^{33, 34} to trigger tissue healing. Considering these former results, we used HVPC in EAS intervention.

The limitation of using EA on a PU could be on infected wounds where the insertion of the needles could propagate further in-depth, involving the skin layers, underlying tissues, and muscles. Therefore, We made an insulating coating needle to avoid deep tissue damage. To rule out an infected skin wound, we found that insert needles on 1 cm borderline have a low infection risk based on former clinical study³⁵. This clinical study used 0.5 Hz, 500 μ A, duration of 30 min electroacupuncture in the PU treatment.

Considering the different skin tissue of the mouse, we choose intensity with output 0.5 Hz, 300 μ A, duration of 30 min.

There were some limitations to this study. Our parameter of EAS has to consider the results of human and pig research but may lead to different results in the mice model. The Asic3 mostly expressed in peripheral sensory nerve, so to explore EAS influence on the nerve will be more precise. The purpose of our study was to explore the short effects of EAS on PU, and we only observed two weeks of intervention effects. It is still unclear which is better in direct current and alters current, pulsed or continuous current. So, the next study should observe these different parameters. Moreover, the mouse is not related to humans as species to be able to transpose all this study into the clinical practice.

Conclusion

EAS can accelerate the PU healing process in mice, and EAS also could improve the expression of Asic3 in PU tissues.

Abbreviations

Pressure ulcer (PU), American College of Physicians (ACP), electro-acupuncture stimulation (EAS), acid-sensing ion channel 3 (Asic3), Pressure Ulcer Advisory Panel (NPUAP), least significant difference (LSD), National Pressure Ulcer Advisory Panel (NPUAP), calcitonin gene-related peptide (CGRP), High-voltage pulsed current (HVPC), High-voltage pulsed current (HVPC).

Declarations

Ethics approval and consent to participate

The Ethics committee has approved the work of southwest medical university (Number: 201912-14).

Consent for publication

All authors have provided consent for publication in the journal of Chinese Medicine.

Availability of data and material

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Author contributions

All the authors have contributed accordingly, we can provide a file about the specific contributions if necessary.

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Figures

Figure 1

PU modeling method, PU: Pressure ulcer; Fig.1A Experimental device mode diagram; Fig.1B Two magnets squeeze the subcutaneous tissue; Fig.1C The total length of the needle is 25mm, the inserted tissue is about 5mm and the front end of the needle is about 2mm insulation coating.

Figure 2

PU in mice of each group after 7 days and 14 days of treatment. Fig.2 showed the PU area changes in PU area in mice from each group after 7, 14 days of treatment. As could be observed from the above-described results, dorsal skin in mice from the control group was healthy without PU formation. In contrast, massive areas with dorsal PU in mice from the model, EAS, and EAS + APETx2 groups. Besides, dorsal PU areas in mice from the EAS group had decreased compared with those from the model group. EAS + APETx2 group were similar to those in the model group. Notably, dorsal PU areas in mice had decreased gradually with treatment in the model, EAS, and EAS + APETx2 groups. Control group: no PU modeling or EAS; Model group: PU modeling, electroacupuncture needle insertion and without electric,

with saline injection ; EAS group: PU modeling, with EAS and saline injection; EAS + APETx2 group: PU modeling, with EAS and APETx2 injection for twice. PU: Pressure ulcer; EAS: Electrical acupuncture stimulation;

Figure 3

HE staining of dorsal skin of mice from each group The control group characterized by stratified squamous epithelium, clear skin structure and scattered with numerous fibroblasts. No inflammatory cell infiltration was in the control group. In contrast, the model group showed distinctly thickened epithelium, evidently decreased fibroblasts and inflammatory cell infiltration in dorsal skin PU tissues. Compared with the model group, the EAS group showed alleviated epithelial thickness, increased number of fibroblasts, and reduced inflammatory cell infiltration. Pathological changes of dorsal skin PU tissues from the EAS + APETx2 group were similar to those from the model group. Control group: no PU modeling or EAS; Model group: PU modeling, electroacupuncture needle insertion and without electric, with saline injection ; EAS group: PU modeling, with EAS and saline injection; EAS + APETx2 group: PU modeling, with EAS and APETx2 injection for twice. PU: Pressure ulcer; EAS: Electrical acupuncture stimulation;

Figure 4

Asic3 expression in PU tissues of mice from each group ** $p < 0.01$.vs. Control group; ## $p < 0.01$.vs. Model group; and ^## $p < 0.01$.vs. EAS group. Fig.4 showed the Asic3 expression results of western blot (Fig.4A), RT-PCR (Fig.4B), and immunohistochemical staining (Fig.4C) in PU tissues of each group. The Asic3 protein and mRNA expression in the model group and EAS group were distinctly higher than the control group. Moreover, Asic3 in the EAS group had elevated compared with that in the model group, the EAS + APETx2 group had outstandingly decreased. Asic3 expression decreased in EAS + APETx2 group than that in the EAS group. Compared with the model group, the immunohistochemical staining of Asic3 was increased in the EAS group and decreased in the EAS+APETx2 group. Compared with the EAS+APETx2 group, the Asic3 expression was increased in the EAS group. Control group: no PU modeling or EAS; Model group: PU modeling, electroacupuncture needle insertion and without electric, with saline injection; EAS group: PU modeling, with EAS and saline injection; EAS + APETx2 group: PU modeling, with EAS and APETx2 injection for twice. PU: Pressure ulcer; EAS: Electrical acupuncture stimulation;