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A Histological Evaluation of the Effect of Ghrelin on Wound Healing in Rats

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ABSTRACT

Objective: This study aimed to investigate the potential effects of ghrelin on wound healing.

Materials and Methods: Sprague-Dawley rats were divided into 3 groups: Control ($n = 8$), wound-saline (W+S, $n = 16$), and wound-ghrelin (W+Gr, $n = 16$). A wound was created on the cervical back region of rats using an 8 mm biopsy punch tool in the W+S and W+Gr groups. Either saline (1 mL/kg) or ghrelin (10 ng/kg) was administrated intraperitoneally each day to the rats in the non-control groups after the onset of the wound. Rats from the W+S and W+Gr groups were euthanized on the 7th ($n = 8$ from each group) and 14th day ($n = 8$ from each group) of the experiment. The histopathological score was evaluated statistically using one-way analysis of variance and Tukey's multiple comparison tests.

Results: The rats euthanized from the W+S group on day 7 (subgroup W+S_7) showed degenerated epidermis, no hair follicles, presence of granulation tissue, inflammatory cell infiltration, vasocongestion, and increased collagen fibers in dermis. However, all these histopathological findings significantly decreased in the rats euthanized from the W+Gr group on day 7 (subgroup W+Gr_7) compared to the W+S_7 group ($p < 0.05$). The W+S_{14} group showed thick epidermis, a few hair follicles, angiogenesis, and increased collagen fibers in the dermis. Additionally, the histopathological findings decreased significantly in the W+Gr_{14} group compared to W+S_{14} group ($p < 0.05$).

Conclusion: Based on the statistical analysis of the histological findings, the ghrelin treatment appears to have a beneficial effect on wound healing.

Keywords: Wound healing, ghrelin and histology

INTRODUCTION

Wound healing is a complicated process involving crosstalk between cells, growth factors, and inflammatory cytokines in three distinct phases: Inflammation, new tissue formation, and remodeling. The first step is inflammation and takes place within the first 3 days after the injury with the invasion of neutrophils and macrophages. The proliferative phase starts after the inflammatory phase with the formation of new blood vessels (neoangiogenesis), the production of connective tissue, and contraction of the wound at this stage. Lastly, the remodeling phase starts usually from day 21 up to 2 years after the injury, during which the collagen fibers reorganize and the tissue remodels and matures, resulting an overall increase in tensile strength (1).

Improper wound healing is one of the most important factors affecting the convalescence of patients worldwide. Currently, the removal of necrotic tissue, changing of bandages, and local and systemic antibiotic therapies have been commonly used to treat patients with improper wound healing (2). The administration of topical growth factors coupled with several biomaterials have also been reported for overcoming this issue (3).

Ghrelin is a gastrointestinal peptide hormone and has a central function in balancing feeding, adiposity, body

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weight, and glucose metabolism (4). Ghrelin has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHSR) 1a and to stimulate the secretion of growth hormones from the pituitary gland (5). Ghrelin has also been shown to be able to prevent organ injury and to improve survival in irradiated rats with severe sepsis by decreasing the induction of inflammatory mediators (6, 7). This study aims to histologically examine the role of parenteral ghrelin treatment with regard to wound healing in rats.

MATERIALS AND METHODS

Animals

The study obtained 40 adult Sprague-Dawley rats (200-250 g) from Marmara University, School of Medicine's Animal Laboratory. The rats were kept under standard laboratory conditions, including a 12-h light/dark cycle and constant temperature (22 ± 2 °C) and humidity (45–65%). They were also given Ad Lib standard pellet food and tap water. All procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (8). This study procedure was approved by the relevant animal experimental local ethics committee of Marmara University (protocol no. 10.2008.mar).

Experimental Design

The Sprague-Dawley rats were divided into 3 groups: Control (C; $n = 8$), wound+saline (W+S; $n = 16$), and wound-ghrelin (W+Gr; $n = 16$), with no treatment being applied to the control group. The W+S group was given saline (1 mL/kg, intraperitoneal (i.p.)) and the W+Gr group was given ghrelin (10 ng/kg, i.p., Sigma, G8903, St Louis, MO) once a day immediately after the wound was created (9). In both the W+S and W+Gr groups, rats were euthanized on the 7th (subgroups W+S_7 & W+Gr_7, $n = 8$ for each) and 14th (subgroups W+S_{14} and W+Gr_{14}, $n = 8$ for each) days following the wound creation. At the end of the experiment, skin samples were removed for histological examination.

Wound Creation Protocol

After the application of general anesthesia of ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.), the dorsal area of the animals was shaved and cleaned with 70% alcohol. The wound was created using an 8 mm biopsy punch tool on the shaved cervical back region (10).

Histological Analysis

Skin samples were fixed in a 10% formaldehyde solution and underwent a routine process for embedding in paraffin wax. To identify histological degeneration, 5-6 µm thick paraffin sections were stained with hematoxylin and eosin. Skin sections were also stained with Gomori's one-step trichrome for collagen distribution. All stained sections were evaluated under a light microscope (Olympus BX51, Tokyo, Japan) and photographed with a digital camera (Olympus DP72, Tokyo, Japan). Each sample was examined over at least five microscopic areas for histopathologic scoring. Epithelial and hair follicular degeneration, inflammatory cell infiltration, vasocongestion, and collagen density were assessed for scoring criteria (11). Each criterion was scored semi-quantitatively as follows: 0 = none, 1 = mild, 2 = moderate, and 3 = severe, with the maximum total score being 12.

Statistical Analyses

Statistical analysis was performed using the program GraphPad Prism 9 (GraphPad Software, San Diego, USA). After confirming the normal distribution of the data using the Kolmogorov–Smirnov test, the one-way analysis of variance (ANOVA) test and Tukey's multiple comparison tests were applied, with the data being presented as mean ± standard deviation (*SD*) and a $p < 0.05$ indicating a significant difference.

RESULTS

The histological investigation of skin samples taken from the control groups of rats showed regular epidermis and dermis with a distribution of collagen fibers and hair follicles. The W+S_7 subgroup was observed to have degenerated epidermis, no hair follicles, severe inflammatory cell infiltration and vasocongestion, the presence of granulation tissue, and increased collagen fibers in the dermis. The presence of granulation tissue in the sections from the saline-treated rats indicated the healing to have been impaired, albeit with no obvious retardation in wound healing. The W+S_{14} subgroup was observed to have a thick epidermis, moderate inflammatory cell infiltration and vasocongestion, a small number of hair follicles, angiogenesis, and increased collagen fibers in the dermis. The W+Gr_7 subgroup showed degenerated epidermis, no hair follicles, moderate inflammatory cell infiltration and vasocongestion, the presence of granulation tissue, and increased collagen fibers in the dermis. The W+Gr_{14} subgroup had a regular epidermis, a small number of hair follicles, angiogenesis, mild inflammatory cell infiltration and vasocongestion, and increased collagen fibers in the dermis (Figure 1).

In the semiquantitative analyses, significantly higher histopathological scores were observed for both the saline- and ghrelin-treated wound healing groups compared to the control group. However, the 7-day treatment with ghrelin showed a reduced total histopathological score in the W+Gr_7 subgroup (8.93 ± 0.90) compared to the W+S_7 group (10.19 ± 0.96; $p = 0.028$). Moreover, a similar benefit regarding the total histopathological score was also obtained for the 14-day treatment with ghrelin in the W+Gr_{14} subgroup (4.31±0.53) compared to the W+S_{14} subgroup (5.50±0.92, $p = 0.04$; Figure 2).

DISCUSSION

The study's outcomes demonstrate the ghrelin treatment to have had a prominent impact on the healing process of the rats who'd had wounds induced with the biopsy punch. From a histological point of view, both the 7- and 14-day treatments with ghrelin resulted in significant improvements in the rats' histopathological score compared to the saline treatment. The histopathological findings of the study suggest the potential anti-inflammatory role of ghrelin treatment in the initial stage of wound healing.

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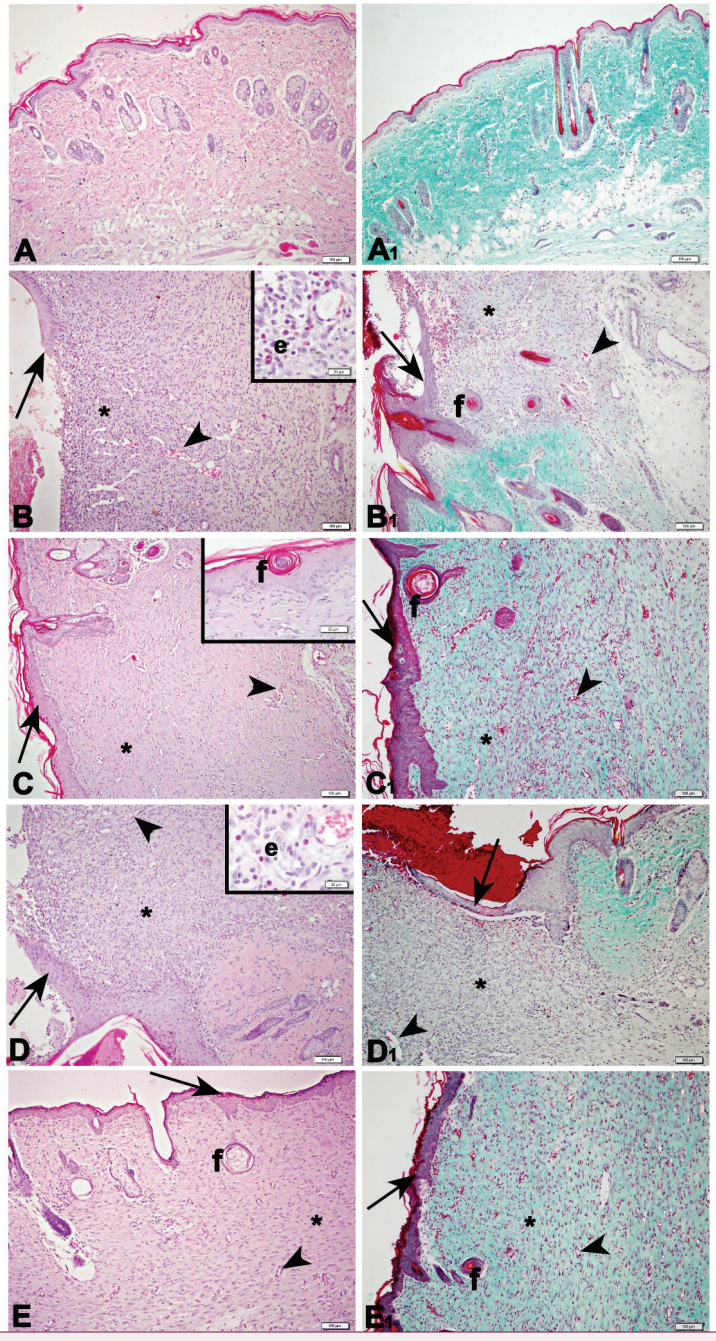

Figure 1. Representative photomicrographs of skin tissue samples from the experimental groups. The control group (A and A1) shows regular epidermis, collagen fibers and hair follicles in the dermis. The W+S_7 subgroup (B and B1) shows degenerated epidermis morphology (arrow), inflammatory cell infiltration (e, B), localized increase in collagen fibers (asterisks), hair follicles (f, B1), and the formation of blood vessels (arrowhead) in the dermis. The W+S_{14} subgroup (C and C1) shows thick epidermis morphology (arrow), localized increase in collagen fibers (asterisks), hair follicles (f, C), formation of new blood vessels (arrowhead) in the dermis. The W+S_7 subgroup (D and D1) shows degenerated epidermis morphology (arrow), inflammatory cell infiltration (e, D), localized increase in collagen fibers (asterisks), formation of new blood vessels (arrowhead) in the dermis. The W+S_{14} subgroup (E and E1) shows quite a regular epidermis morphology (arrow), localized increase in collagen fibers (asterisks), hair follicles (f), and formation of new blood vessels (arrowhead) in the dermis. A-E: Hematoxylin and eosin staining; A1-E1: Gomori's one-step trichrome staining. Original magnifications are x100 and x400 (inset).

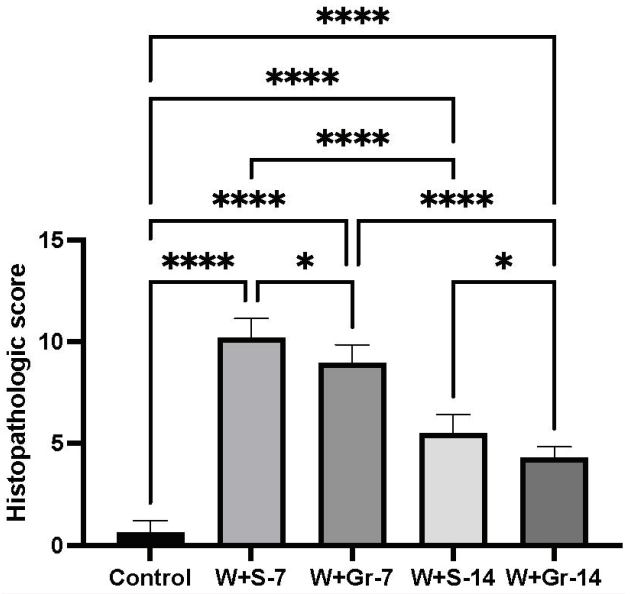

Figure 2. The histopathologic score of the experimental groups. *$p < 0.05$; **** $p < 0.0001$ shows a significant difference between the experimental groups. Each group consists of 8 rats.

Improper wound healing is an important health issue that affects patient convalescence and is known to occur under many clinical conditions such as surgical procedures, diabetes mellitus, and cancer. Apart from being a health problem, delayed wound healing also increases the cost of hospitalization. Moreover, it leads to social isolation, especially for patients with diabetes and obesity (12, 13). The inability to re-epithelialize has been known to dictate the development of improper healing and to lead to chronic non-healing wounds. The disturbance of re-epithelization paves the way for the impairment of the normal phases of wound healing in an orderly and timely manner (14). The current study found the ghrelin treatment in the $W+Gr_{14}$ subgroup to have improved epidermis and hair follicle formation in wound healing compared to the $W+S_{14}$ subgroup.

In addition to inducing the release of growth hormones, ghrelin has multiple functions such as modulating the secretion of several growth factors and affecting the mechanisms of cellular migration, proliferation, and angiogenesis (15). Moreover, recent evidence has suggested ghrelin as having anti-inflammatory and antioxidant effects, with its potential role in oxygen free radical homeostasis being claimed to facilitate the healing process in regard to several tissues (14-18). Sehirli et al.'s study (19) showed ghrelin to have a prominent role in recovering from burn-induced skin and remote organ damage in rats due to its anti-inflammatory and antioxidizing effects. The decreased histopathological score and decrease of inflammatory cell infiltration in the ghrelin-treated wound 7- and 14-day subgroups show ghrelin to possibly regulate wound healing by regulating inflammation through the inhibition of oxidative stress.

The impact of the ghrelin treatment on wound healing has been studied with regard to several tissue types. Cieszkowski et al.'s study (20) showed ghrelin treatment (i.p) to increase mucosal blood flow and decrease local inflammation by reducing mucosal interleukin-1β concentrations in an experimental rat model with mucosal gingival damage. In addition to oral mucosa, ghrelin has been shown to also exert a positive effect on healing other parts of the gastrointestinal tract. Lyra, Jr. et al.'s study (21) studied the potential role of postoperative intraperitoneal ghrelin therapy (i.p, 23 μg/kg/d) on healing colonic anastomosis. They concluded that, due to its anti-inflammatory and antioxidizing effects, ghrelin had increased both the resistance and the hydroxyproline content of colonic anastomosis postoperatively. The current study has found a 14-day ghrelin treatment (10 ng/kg, i.p.) on wound healing to have quite improved the epidermis and dermis injury through epithelial and hair follicle regeneration, collagen distribution, and a decrease of inflammatory cell infiltration.

Several studies have also examined the impact of ghrelin on skin wounds. Liu et al.'s study (22) investigated the protective role of ghrelin with regard to impaired wound healing from radiation exposure. Histologically, they found ghrelin (100 nmol/kg and 200 nmol/kg) to decrease the average wound healing time by about 3-5 days and ghrelin to also boost the expression of vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) through higher collagen content and enhanced neovascularization. The current study found both the 7- and 14-day application of ghrelin (10 ng/kg, i.p.) to improve the biopsy punch wound healing by modulating the inflammatory response.

This study has a few limitations. It did not biochemically examine the anti-inflammatory or antioxidant parameters regarding the skin samples, which may have helped to understand ghrelin's role in wound healing with detail.

CONCLUSION

Based on the statistical analysis of this study's histological findings, ghrelin may have a potential role in accelerating the healing of wounds through its potential anti-inflammatory effects.

Ethics Committee Approval: This study procedure was approved by the relevant animal experimental local ethics committee of Marmara University (protocol no. 10.2008.mar).

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