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Evaluation of Wound Healing Activity of Sea Cucumber *Holothuria parva* Hydroalcoholic Extract in Rat

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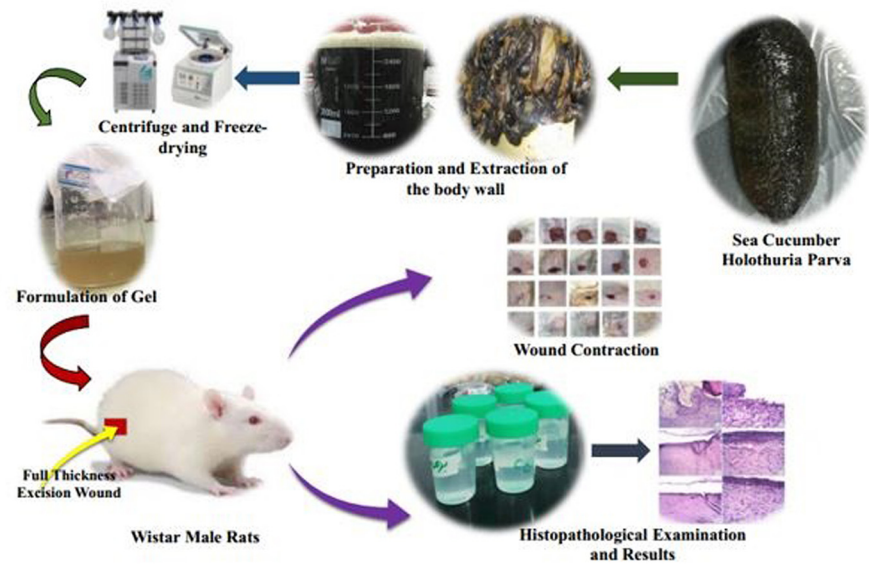
Abstract

A significant challenge in biomedicine is the development of Biomaterials with the potential to accelerate wound healing. The aim of this study was to investigate the effect of the gel prepared from sea cucumber on wound healing in rats. Full-thickness wounds were created in male rats divided into five experimental groups, negative control (NC), positive control (PC), and treatments. The NC and PC groups received respectively gel base and phenytoin cream 1%. Treatment groups were treated topically by gels of 1%, 3%, and 5% *Holothuria parva* extract (HPE) in the gel base. The rats were sacrificed on days 7, 14, and 21. Pathological reports revealed the proliferation of keratinocytes in the borders of the wound in treatment groups and controls. Formation of granulation tissue was seen on day 7 in treatment groups, collagen fibers in granulation tissue were randomly organized, and the rate of fibroblast decreased. Results showed that there were statistically significant differences in wound contraction between all groups in comparison to the NC group ($P < .05$) on day 8. It was concluded that the 1% HPE gel has a good potential for promoting wound healing.



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wound healing, sea cucumber, *Holothuria parva*, skin, rat

Introduction

Wound healing continues to remain a clinical challenge and is essential for proper, effective wound management. Wound healing takes place throughout the body's tissues and organs. Many of these repair procedures are prevalent in all tissues. Although the healing process is constant, it is arbitrarily organized into distinct stages to assist in comprehend the physiological functions that take place in the wound and surrounding tissue.[1] Healing is an intricate mechanism that involves coordinated connections among various immunity mechanisms and biological systems. It consists of a cascade of carefully and precisely controlled steps and events that relate to the appearance of different cell kinds in the wound bed, including populations of migratory cells, extracellular matrix, and soluble mediator action. Process-based mechanisms include (1) inflammatory mediators and growth factors; (2) Interactions of cell-cell and cell-extracellular matrix governing cell proliferation, immigration, and distinction; (3) processes of epithelialization, fibroplasia, and angiogenesis; (4) contraction of wounds; and (5) remodeling. In the case of physical injury, these mechanisms are initiated and continued throughout the repair process.[2–5] Given that marine organisms account portion of the worldwide marine biodiversity, the sea is a vast resource for new compounds.[6] Sea cucumber is a marine invertebrate, belonging to the Holothuroidea class. It generally contains a soft body, mouth, anus, and tube feet. Sea cucumbers were well-recognized for efficacy in high blood pressure, asthma, rheumatism, cuts and burns, sexual dysfunction, and constipation as a tonic and traditional remedy in China and Malaysia.[7] The attendance of essential levels of bioactive compounds such as saponins, chondroitin sulfates, peptides, glycosaminoglycans, sulfated polysaccharides, sterols, phenols, lectin, and cerebrosides, can be ascribed to medicinal advantages and health functions of sea cucumbers.[8] Therefore, in this work, it was decided to formulate a gel from *H. parva* extract and evaluate its wound-healing effect.

Materials and Methods

Chemicals

Triethanolamine and propylene glycol (PG) were procured from Merck (Germany), and HPMC was procured from Sigma Aldrich (America).

Table 1. Exhibit the Different Proportions of Compounds Used in Gel Base Production.

Ingredients (in %)	F_1	F_2	F_3	F_4
Triethanolamine	2	5.75	4	4.25
Propylene glycol	9	20	16	9.5
Deionized water	86	70.25	75.5	81.25
HPMC	3	4	4.5	5

F is the abbreviation of the formulation code. F_1 (formulation 3%), F_2 (formulation 4%), F_3 (formulation 4.5%) and F_4 (formulation 5%).

Sample Collection and Identification

This study was conducted at the intertidal zone Bostaneh coasts of Hormozgan Province during summer 2018. An area of 100 m^2 was sampled. As *Holothuria parva* mostly live under the rocks. Thereby the rocks were removed carefully, and the specimens were collected (50 individuals), stored in iceboxes, and transported to the laboratory. The identification of species was based on the external morphology, according to Conand,[9] James,[10] and Aydin.[11]

Extraction

In the laboratory, the body wall tissues were carefully dissected from 50 sea cucumbers, washed with distilled water, and then mixed with methanol 70% for hydro-alcoholic extraction. Components were sorted in the dark condition on a shaker for 72 h and centrifuged at 5000 RPM for 15 min. The top layer of the component was separated and filtered. Removing solvents was done using a rotary evaporator in low-pressure conditions and 40 °C temperature. The resultant extract was dry with a freeze dryer (model Alpha-2) and kept in clean glass vials at −80 °C until use. The prepared powder was used for wound healing in the rat.[12]

Formulation of Gel

To formulate the gel, the hydro-alcoholic extract of *H. parva* was powdered and added to water, and Hydroxy Propyl Methyl Cellulose (HPMC) was used as a gel-forming agent. The different ratios of compounds used in the preparation of the gel base are listed in Table 1.

Physicochemical Evaluation of Gels

Physical appearance: Homogeneity and the presence of any turbidity or particles in the formulated gels were evaluated by visual examination.

pH alteration: To determine the pH of the formulated gels, 1 ml of the gel sample was mixed with 9 ml of deionized water, and the pH was measured using a pH meter (model WGVDC).

Viscosity: The viscosity of samples was determined to utilize a Brookfield viscometer (model LDV-II + Pro) with spindle number 34 at room temperature and differently rated from 0.5 to 3 rpm.

Animal

Wistar males weighing 180 to 200 g were purchased from the animal house of the Ahvaz Jundishapur University of Medical Sciences and were kept rats in a stainless steel cage at a temperature of 23 ± 2 °C under periodic 12-h light/dark cycles. Standard pellet food and tap water were accessible ad libitum. The experimental method has been confirmed by the Animal Ethical Committee of [blinded for peer review]

Wound Procedure and Experimental Design

Animals have divided accidentally into five groups (n = 7).

- Group1: served as test group and treated with gel 1% *Holothuria parva* extract
- Group2: served as test group and treated with gel 3% *Holothuria parva* extract
- Group3: served as test group and treated with gel 5% *Holothuria parva* extract
- Group4: served as positive control and treated with phenytoin cream 1%
- Group5: served as negative control and treated with Only HPMC gel base

The dorsal hair of the rats was shaved and 70% ethanol, and then they were marked and anesthetized using Lidocaine 2%. A full-thickness excision wound of 10 × 10 mm was created along the marking using scalp bland and forceps.[13]

Wound Contraction

For macroscopic examination, the surface area of the wound was measured on days 2, 8, 14, and 21 using a camera that was embedded at a certain distance from the wound; it was shot from the wound in such a way that all rats and photographs had the same conditions.[14] The pictures were then entered into the Digimizer Software to measure the wound surface area; the data were then put into the recovery percentage formula. The percentage of variations in the area of wounds were calculated utilizing the following equation:

$$\text{Wound size reduction } (\%) = (W_0 - W_t)/W_0' \times 100$$

W_0, Initial wound size W_t, Specific day wound size.

Table 2. Viscosity Test Results.

Code	RPM	Viscosity(p)*
F_1	0.5	67.19 ± 0.005
	1	58.79 ± 0.08
	2	44.39 ± 0.05
	3	42.99 ± 0.007
F_2	0.5	18 ± 0.05
	1	17.8 ± 0.005
	2	17.5 ± 0.5
	3	17.1 ± 0.5
F_3	0.5	75 ± 0.5
	1	60.2 ± 0.05
	1.5	48.1 ± 0.15
F_4	0.5	68 ± 0.3
	1	56 ± 0.75
	1.5	39.4 ± 0.05

Data are expressed as mean ± SD.

Histopathological Examination

Skin sampling took place 7, 14, and 21 days after wound creation. In the days listed, several rats were murdered. Wound enhanced with a portion of healthy skin around the wound cut and fixed in 10% formalin and used for pathology. After stabilization, isolated tissue samples were prepared and stained with Eosin and Hematoxylin.[15]

Statistical Analysis

Results were presented as mean ± SEM. Data were analyzed using SPSS version 20. One-way ANOVA was used to analyze and compare data with $P < .05$ as the limit of significance.

Results

The results of the viscosity testing of the formulation are shown in Table 2.

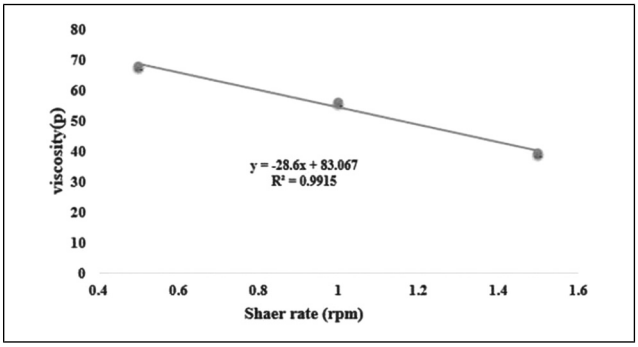

Figure 1. Rheologic behavior of the formulate gel (mean ± SD).

Table 3. pH Test and Physical Appearance Result.

Code	pH*	Physical appearance
F_1	8.69 ± 0.3	Low consistency, homogenous, smooth, and very translucent.
F_2	8.79 ± 0.00	Low consistency, homogenous, smooth, and translucent.
F_3	8.62 ± 0.015	Inappropriate consistency, non-homogeneous, and low translucent.

*Data are expressed as mean ± SD.

As seen, rheological experiments on the final formulation showed that, with increasing rotational speed, the viscosity of the product significantly decreased Figure 1.

The results of physical stability monitoring during six months of storage showed that the exposure of the gels to different temperatures did not cause any changes in their pH. In terms of consistency, except for formulation No.3 that was stored in the incubator at 50 °C, there was no noticeable change in the consistency for other formulations. Also, visual inspection showed no significant change in their color and odor. All air bubbles were disappeared after the time course, except for formulation No.3 that the air bubble was visible. The results of the gel formulation inspection are shown in Table 3.

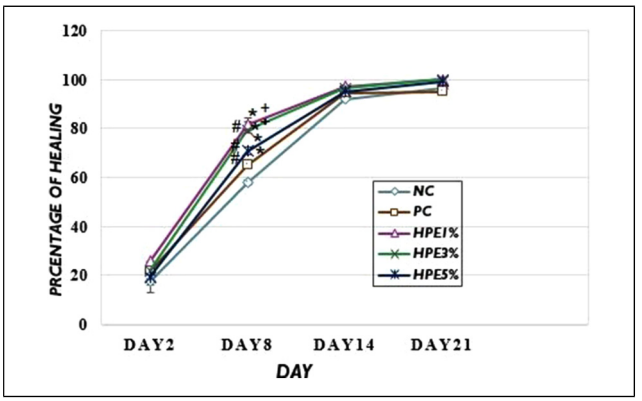

Figure 3. Comparison of the percentage of wound healing in the experimental groups. Data are expressed as mean ± SEM * the difference is significant with the NC group ($P < .05$). # the difference is significant with the PC receiver group ($P < .05$). + the difference is significant with the HPE5% receiver group ($P < .05$).

Macroscopic Examination

Representative pictures of the wound healing process across all experimental groups at specific time intervals are shown in Figure 2. HPE1% treated groups showed a faster wound healing process compared to the control group, in detail the wound regions covering with epidermis and the wound areas closing. During the treatment, the wound area of each group decreased gradually.

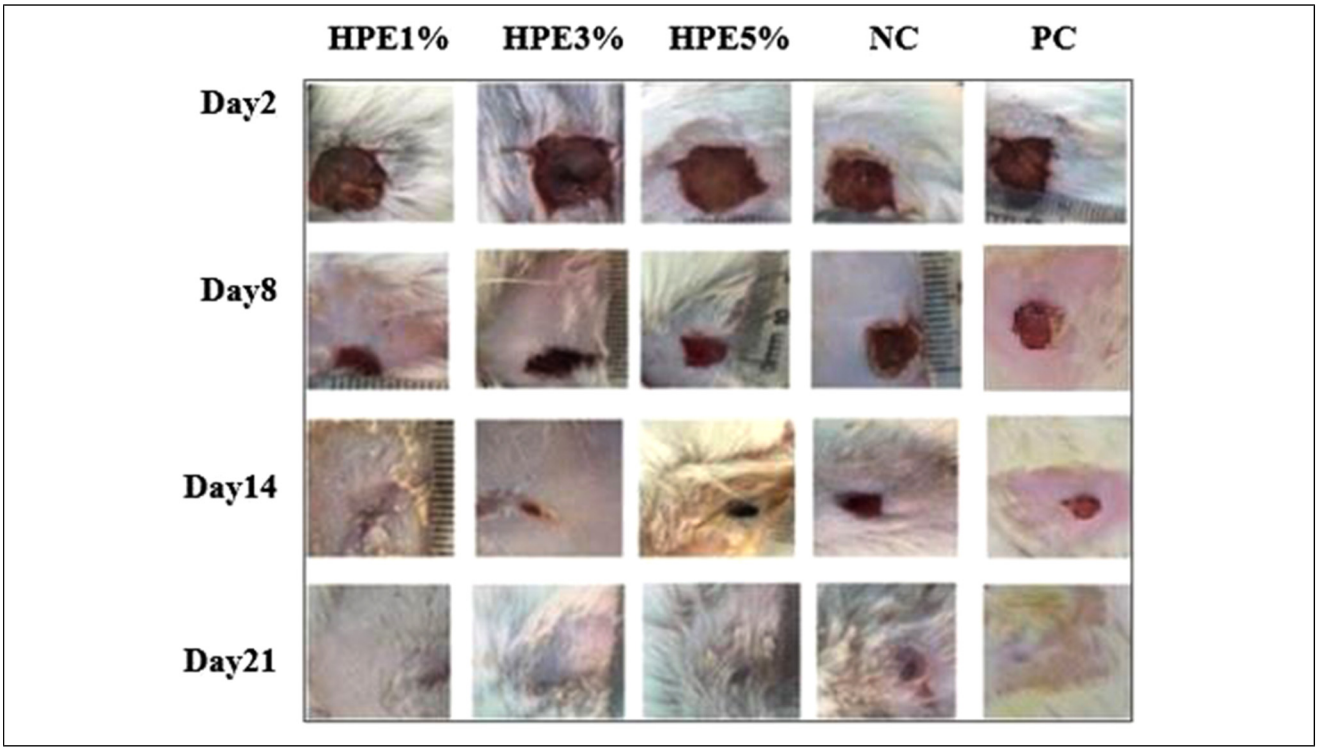

Figure 2. Macroscopic examination of the wound on days 2, 8, 14, and 21. *Holothuria parva* extract (HPE1%), *Holothuria parva* extract (HPE3%), *Holothuria parva* extract (HPE5%), negative control (NC), and positive control (PC).

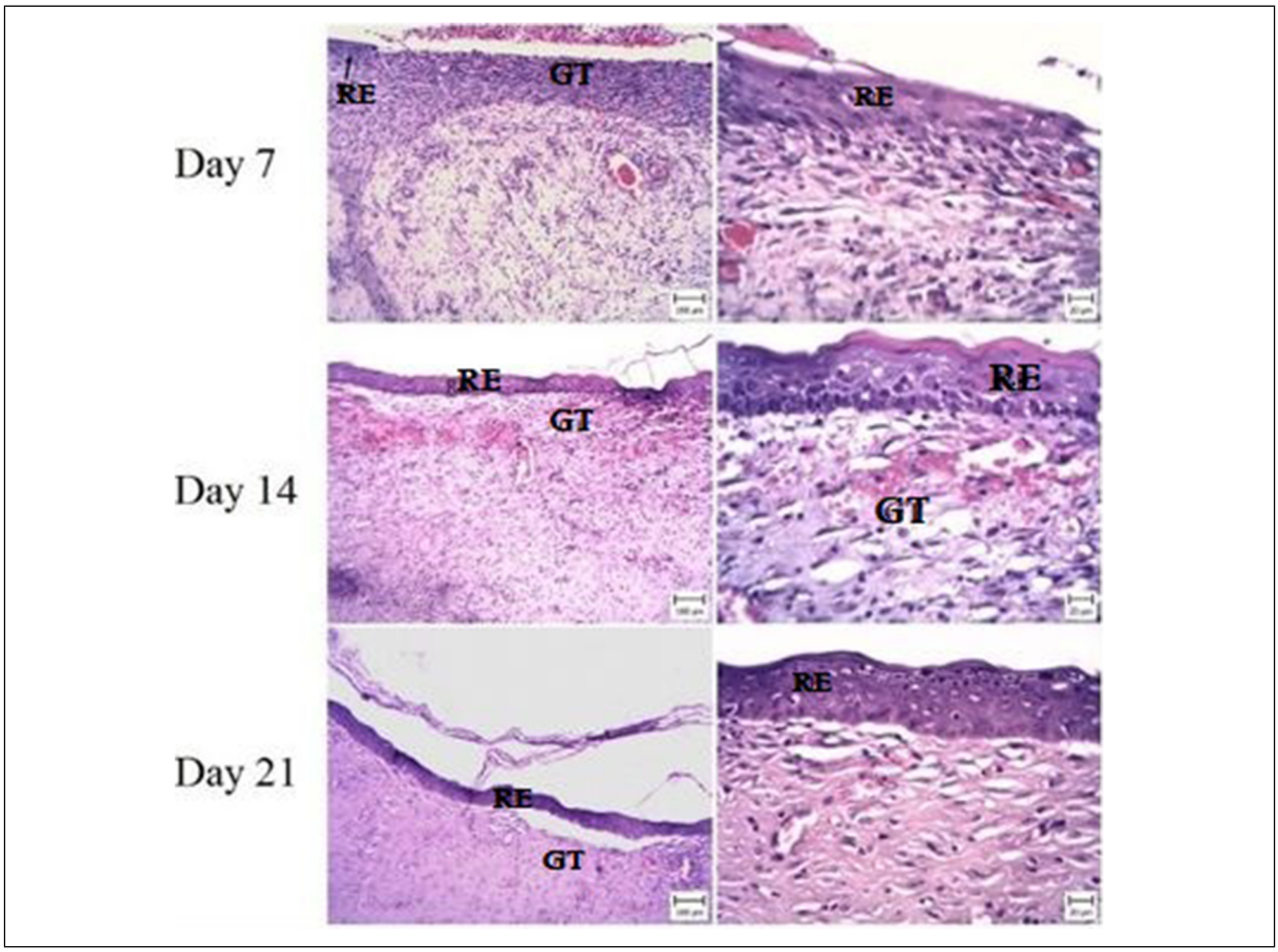

Figure 4. Photomicrograph of skin in HPE 1% group days 7, 14, and 21. Re: reepithelialization, GT: granulation tissue (H&E).

Wound Contraction

Results showed that there were statistically significant differences in wound contraction between all groups compared with negative control groups ($P < .05$) on day 8. All HPE groups with the positive control group showed a significant difference on day 8. Group HPE5% has a significant difference with HPE 3% and HPE1% on day 8. There was no significant difference on other days. Show the percentage of wound healing in all groups on days 2, 8, 14, and 21 in Figure 3.

Histopathology Results

Microscopic examination of sections on day 7 revealed the proliferation of keratinocytes in the borders of the wound in treatment groups and controls. Migration of keratinocytes beneath the scab and bridging on the wound was completed in treatment groups on day 14, whereas in controls, keratinocytes proliferated and migrated. On day 21, the wound was entirely covered by keratinized epithelium, and multiple layers of keratin were seen on the surface of the healed area in treatment groups. Formation of granulation tissue with noticeable collagen formation was seen on day 7 in treatment groups. On day 21, collagen fibers in granulation tissue were randomly organized, and the rate of fibroblast decreased. Photomicrographs of wound area in treatment groups on different days are shown in Figures 4 to 6. Mentioned characteristics were more evident in HPE 1% and 3% in comparison with HPE 5%. Microscopic examination of hypoderm revealed granulomatous dermatitis in 6 rats in treatment groups Figure 7. Healing process characteristics such as keratinocyte proliferation and decrease of inflammatory cells were more brilliant in 1% HPE.

Discussion

Wound healing is a series of cellular and molecular events that involve the absorption of cells into the wound,

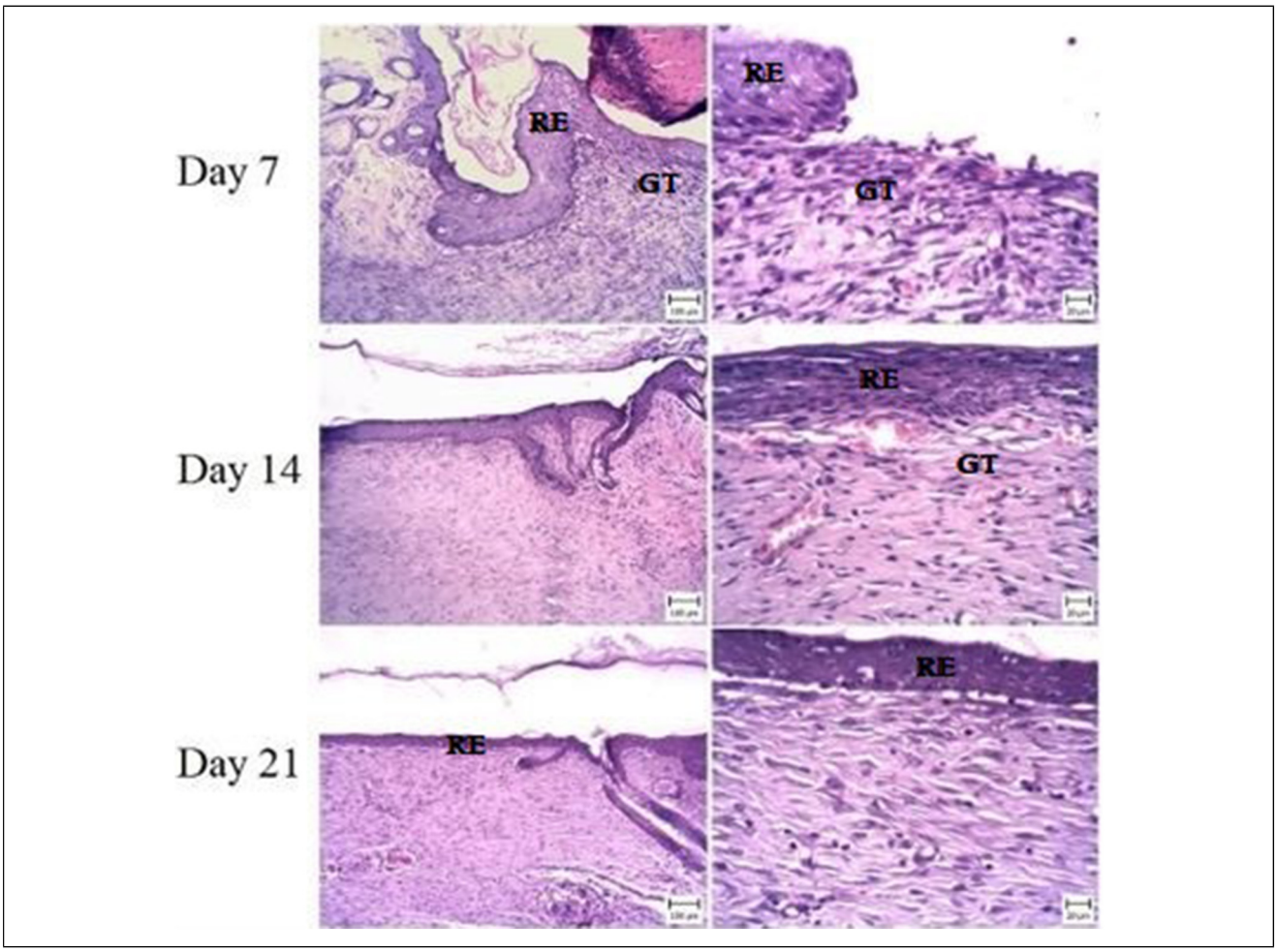

Figure 5. Photomicrograph of skin in HEP 3% group days 7, 14, and 21. Re: reepithelialization, GT: granulation tissue (H&E).

proliferation, synthesis, and aggregation of new connective tissue material. Although this process begins naturally in the scars and continues, in terms of the quality of tissue healing and velocity, this natural process is not always desirable so much of the research and studies are done on the positive and negative effects of the healing process of wounds.[16] Sea cucumber's dried body wall contains many bioactive materials and nutrients, such as polysaccharides, protein, and lipids.[17] Also, it includes multivitamins and minerals.[8] Such composition makes potential candidates for numerous beneficial biological activities and clinical nutrition.[17] In this research, the effect of sea cucumber extract on wound healing was studied in male rats. The results demonstrated that there was the significant formation of granulation tissue and collagen content after 7 days of treatment with gels containing 1% and 3% hydroalcoholic extract of sea cucumber in comparison with the control group.

Yong Li *et al*, in 2018, isolated the small oligopeptide molecules from sea cucumbers, and the results illustrated that the administration of sea cucumber significantly reduced levels of IL-8, IL-6, TNF-a, CCL2, and CRP while IL-10 levels were increased in diabetic rats. Sea cucumber may improve the healing of the wound in diabetic rats. The reason may be due to its multiple therapeutic properties, including reducing inflammatory responses, improving angiogenesis, collagen sedimentation, and antioxidant activities. Current results suggested a new therapeutic concept for diabetic wounds in clinical practice.[18] Yong Li *et al*, in 2011, showed that salmon gelatin extract improves diabetic wounds in rats, suggesting that it reduces inflammatory responses, improves wound contraction, collagen sedimentation, angiogenesis.[6] Fredalin *et al,* in 1999 stated that Stichopus chloronotus, which is a species of sea cucumber, is beneficial for the treatment of various internal and external ulcers. They observed that EPA and DHA fatty acids sea cucumber are bioactive compounds that contribute to wound healing.[7] Zohdi *et al*, in 2011, prepared a hydrogel formulation from Malaysian *Stichopus hermanii* and used it to treat burns wounds in rats. They stated that the topical application of hydrogels

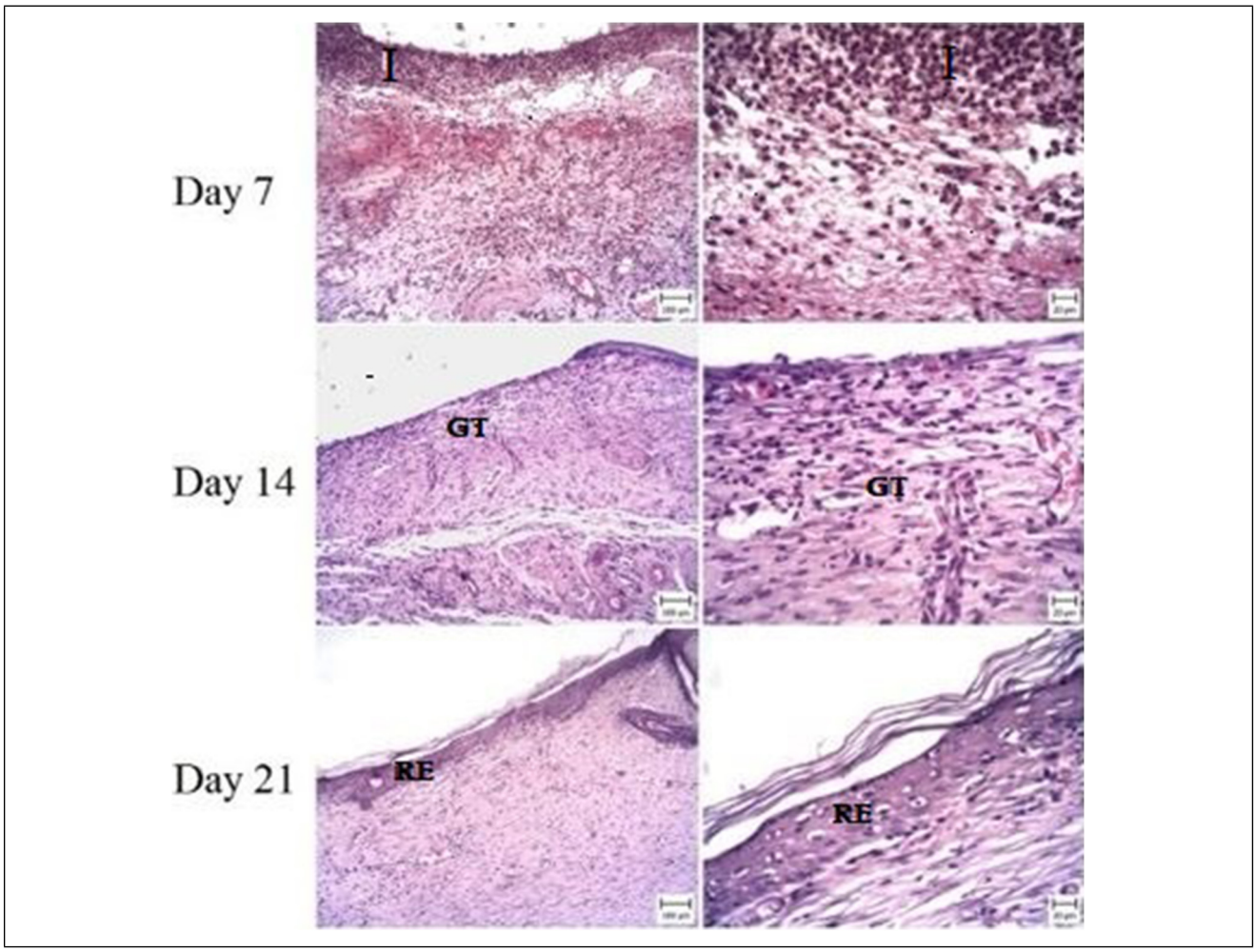

Figure 6. Photomicrograph of skin in HEP 5% group days 7, 14, and 21. Re: reepithelialization, GT: granulation tissue, I: inflammatory cells (H&E).

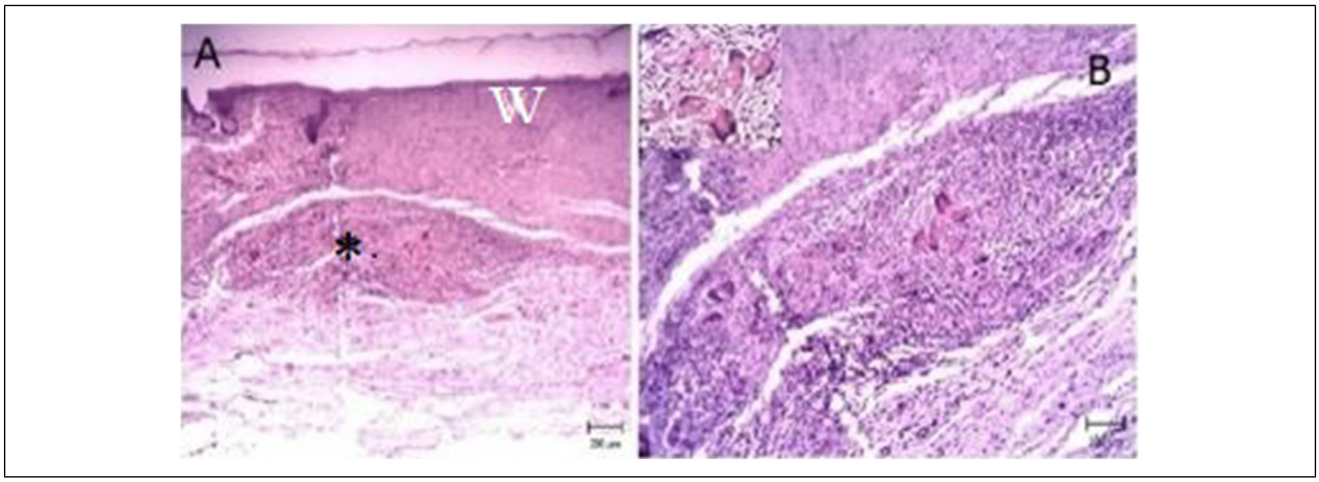

Figure 7. Photomicrograph of rat skin in HPE5% group (H&E). A: Note the existence of granulomatous dermatitis (asterisk) in the beneath of wound area (W) (Bar: 200 μm). B: multiple giant cells (Inset figure) is seen in the center of granuloma (Bar: 100 μm and Inset, Bar: 20 μm).

significantly stimulated the amount of contraction and wound healing.[19] Siti *et al*, in 2010, showed that glycosaminoglycan (GAG) extracted from *Stichopus hermanii* and *Stichopus vastus* species accelerated the wound healing process in rats compared to control groups.[20] Pishehvarzadeh *et al,* In 2014, revealed levels of natural antioxidants in two species of Persian Gulf sea cucumbers *Holothuria parva* and *Holothuria leucospilota*, among which the polar compounds in the aqueous-methanolic extracts have the higher ability in inhibition of free radicals than non-polar extracts and semi-polar. Therefore, isolation and identification of the active compounds present in these extracts are recommended for the achievement of marine anti-agents with antioxidant properties.[21] Fahmy *et al*, in 2015 in a study on gastric ulcers, reported that *Holothuria arenicola* sea cucumber reduces lipid peroxidation, improves antioxidant status, and activates free radical killer enzymes in rates so that it can be a suitable treatment for stomach ulcers. The study also showed that a combination of sea cucumber and ranitidine has a more potent therapeutic effect than someone who uses sea cucumber or ranitidine.[22] Mazliadiyana M *et al*, in 2017, conducted a study on the impact of an ointment prepared from an aqueous extract of *Stichopus Chloronotus* sea cucumber on wound healing. They observed that they have effective anti-oxidant and anti-inflammatory attributes in wound healing.[23] However, more studies are needed to fully understand and discover the mechanisms of the sea cucumber effect on the wound healing process.

In the current research, macroscopic findings demonstrated that topical use of sea cucumber 1% gel increased the contraction of the wound compared with control groups. There were no signs of swelling and infection in animals in the studied groups. The top of the healed wound was entirely covered by keratinized epithelium, and pathological evaluation of hypoderm revealed granulomatous dermatitis in treatment groups. Therefore, due to the creation of granulomatous dermatitis in the treatment groups, it is suggested that the effect of hydroalcoholic extract of sea cucumber be examined more closely.

Conclusions

From the results of the current study, it may be concluded that 1% gel of hydro-alcoholic extract of *Holothuria parva* gel demonstrated better concentration for wound healing in a rat model.

Ethical approval

The project was approved by the Animal Ethical Committee of Ahvaz Jundishapur University of Medical Sciences (approval code no. IR.AJUMS.ABHC.REC.1397.068).

Declaration of Conflicting Interests

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

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