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Sitthiphon Bunman

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Application of proteoglycans from fish cartilage for the acceleration of burn wound healing

Sitthiphon Bunman[1], Pornanong Aramwit[2], Noppadol Larbcharoensub[3] and Pasarapa Towiwat[1*]

[1]*Department of Pharmacology and Physiology, Drug and Health Products Innovation Promotion Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*Bioactive Resources for Innovative Clinical Applications Research Unit, Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*
[3]*Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand*

Abstract

Cartilage contains a variety of proteoglycans (PGs) that can accelerate wound healing. The aim of this study was to evaluate the efficacy of proteoglycans extracted from fish cartilage for the acceleration of burn wound healing. Second degree burn wounds were induced by placing an electrical hot plate set at 90°C for 10 s on the back of rats. Rats were randomly assigned to receive 1 g of cream base (control), 1 % silver sulfadiazine (SSD), 1 % PG, 2 % PG, a combination of 1 % SSD + 1 % PG, or a combination of 1 % SSD + 2 % PG applied to burn wounds immediately after burning and once daily until day 27 post-burn. Wound healing was evaluated on days 3, 7, 14, 21 and 28. Histological analysis was performed on days 7, 14 and 21. The percentage wound healing after treatment with cream containing PG was significantly ($p < 0.001$) higher than that in the control group on day 7 post-burn and thereafter. Histological analysis showed that the combination of 1 % SSD + 2 % PG had the highest efficacy in increasing re-epithelialization and neovascularization in burn wounds. We conclude that PG extracted from fish cartilage can accelerate and facilitate wound healing in rats. The combination of 1 % SSD and 1 % or 2 % PG has high efficacy in accelerating and facilitating wound healing.

Keywords: Proteoglycan, Fish cartilage, Wound healing, Burn wounds, Rat

Introduction

Burn wounds are common in medicine and result in high treatment costs. Burn causes an estimated 300,000 deaths yearly worldwide [1] and may occur in any age group or sex and in both developing and developed countries [2]. Burn wounds are classified by their depth and area of burn as 1st, 2nd and 3rd degree burns [2-4]. Current treatment of burn wounds depends on the severity of the burn: for example, drug therapy or surgery are used in cases with severe burn, but new approaches such as amniotic membrane therapy and cytokine and gene therapy are increasingly used [3, 5]. Drug therapy is the most common and easiest way to treat burn wounds, and 1% silver sulfadiazine (SSD) cream is the gold standard treatment. The action of SSD is driven by inhibition of respiratory enzymes and components of the microbial electron transport system, as well as partial impairment of DNA function. The inhibitory action of silver can be attributed to its strong interaction with thiol groups present in cell respiratory enzymes in the bacterial cell.

Correspondence to Pasarapa Towiwat, Department of Pharmacology and Physiology, Chulalongkorn University Drug and Health Products Innovation Promotion Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
Email: pasarapa.c@chula.ac.th

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The mechanism of action affects the bacterial cell wall and cell membrane [6, 7]. SSD is a bactericidal agent for many Gram-negative and Gram-positive bacteria, including resistant bacteria, and is a simple and low-cost treatment. However, this treatment has the drawback of delaying the wound healing process [6, 8].

Fish bone cartilage is composed of a variety of proteoglycans (PGs) [9, 10], including chondroitin sulfate and dermatan sulfate [11, 12]. PGs are glycoproteins with a core protein with one or more covalently attached glycosaminoglycans. PGs are essential components of the extracellular matrix and contribute > 90 % of the dry weight of the tissue [11]. PGs in cartilage play an important role in wound healing [9, 11] because the molecular structure is similar to an epidermal growth factor (EGF)-like domain [12-14]. The bioactive mechanism of PGs is also similar to that of EGF with regard to wound healing through promotion of epidermal growth and regeneration of tissues and blood vessels [10, 11]. PGs can also bind to several types of growth factor receptors, including TGF-β, FGF-2, VEGF family and PDGF receptors, to promote cell division, cell proliferation and activation of neovascularization [10, 15].

Neelam et al. recently studied the effect of extract of *Melampodium divaricatum* (Pers.) leaves on L929 fibroblast cells using a proteoglycan-IPC solution of proteoglycans extracted from nasal cartilage of Oncorhynusketa (Salmon). Epidermal growth factor was used as a positive control. The results showed that crude extract and salmon PG extract promoted fibroblast cell activation by increasing cell proliferation and migration and increasing collagen synthesis, which is involved in wound healing [12].

The aim of this study is to investigate the activity of PG extracted from fish cartilage in facilitation of burn wound healing in an *in vivo* model.

Materials and Methods

Drugs and chemicals: PG solution (Garguar Lab, Co., Ltd., Thailand), SSD powder (Sigma, U.S.A.), Sodium pentobarbital (Nembutal; Tariqbrian Ltd, U.S.A.), Hematoxylin and Eosin (Bio-optica, Italy), Paraffin (Tyco Healthcare Group LP, USA), Formaldehyde (Formalin; Vidhyasom Co., Ltd., Thailand).

Animals: Male Wistar (age 8 weeks, weighing 250-300 g) were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Thailand. The animals were housed in the Laboratory Animal Unit of the Faculty of Pharmaceutical Sciences, Chulalongkorn University under standard conditions of temperature 25 ± 2°C, 50 - 60 % humidity, and a 12 h/12 h light/dark cycle. The rats were kept under laboratory conditions for one week prior to the start of the experiments and allowed food and water ad libitum. At the end of each experiment, the animals were sacrificed with carbon dioxide asphyxiation. Animal experiments in this study were carried out in accordance with the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes of the National Research Council of Thailand. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Protocol Approval No. 13-33-011).

Cream base preparation: Stearic acid, glyceryl monostearate, isopropyl myristate, sodium lauryl sulfate, glycerin, triethanolamine, uniphen P-23 and germaben II-E were dissolved in warm water and then mixed with other ingredients during the cream-forming process.

SSD and PG cream preparation: SSD powder, PG solution, stearic acid, glyceryl monostearate, isopropyl myristate, sodium lauryl sulfate, glycerin, triethanolamine, uniphen P-23 and germaben II-E were used to formulate PG, SSD or SSD + PG creams. SSD powder and/or PG solution were dissolved in warm water and then mixed with other ingredients during the cream-forming process.

Burn wound healing test: The effect of PG on treatment of burn wounds was investigated using the method of Somboonwong [16], which was modified from Zawacki [17]. Rats were randomly divided into 6 groups of 10 rats each: control rats were treated with cream base; positive controls were treated with 1 % SSD cream; and 4 treatment groups received 1% PG cream, 2 % PG cream, 1 % SSD + 1 % PG cream, and 1 % SSD + 2 % PG cream. Rats were anesthetized by intraperitoneal injection with sodium pentobarbital (60 mg/kg body weight). The hair on the back was shaved. Second-degree burn wounds were induced by placing an electrical hot plate of diameter 2 cm set at a temperature of 90°C on a selected skin area of the back for 10 s. The wound area was measured immediately after burning and on days 3, 7, 14, 21 and 28 post-burn. All wounds were cleaned and treated with 1 g of cream base, 1 % SSD cream, 1 % PG cream, 2 % PG cream, 1 % SSD + 1 % PG cream, or 1 % SSD + 2 % PG cream once daily and covered with sterile gauzes. The total body surface area of each burn wound was measured with a Visitrack® (Smith & Nephew, UK). The percentage wound healing was calculated at each time point using the following formula:

$$\% \text{ Wound healing day}_x = \frac{(\text{Area on day}_0 - \text{Area on day}_x)}{\text{Area on day}_0} \times 100$$

Histological analysis: On days 7, 14 and 21 after burning, six rats in each group were sacrificed and skin samples were taken. Tissues were fixed in 10 % buffered formalin and embedded in paraffin. Thin sections (5 μm) were prepared and stained with hematoxylin and eosin dye. Wound-healing was examined histologically using a light microscope (Nikon 516609, Japan) with 40× and 100× objective lenses. The sample was sectioned into 3 pieces and 3 fields per sample were randomly evaluated for re-epithelialization, number of cells/field (including multinucleated giant cells and macrophages) and neovascularization, compared with cream base. The percentage re-epithelialization was calculated at each time point using the following formula: % epithelialization on

day_x = (area of epithelium on day_x/total wound area on day_0) × 100 [18].

Statistical analysis: Results are expressed as means ± SD. Data were analyzed using one-way analysis of variance (ANOVA), followed by a Bonferroni *post hoc* test using SPSS for Windows, ver. 17. Values of $p < 0.05$ were considered to be significant.

Results and Discussion

Burn wound healing test

Results are shown in **Table 1**. On day 3 post-burn, rats treated with 1 % PG and 2 % PG had significantly ($p < 0.05$, $p < 0.01$, respectively) higher % wound healing compared to controls (cream base). Those treated with 2% PG had a higher % wound healing compared to the 1% PG group. The effect of 1% PG on the increase in % wound contraction was comparable to that of rats treated with the standard drug (1 % SSD). On day 7 post-burn, the effects in all groups treated with PG were higher than those with the standard drug alone. From day 7 to day 28 post-burn, all groups (1 % SSD, 1 % PG, 2 % PG, 1 % SSD + 1 % PG, and 1 % SSD + 2 % PG) had a signify-cantly higher % wound healing compared to the control group ($p < 0.05$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively, for day 7; and $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively, for days 14, 21, and 28). The effects of 1 % PG, 2 % PG, 1 % SSD + 1 % PG, and 1 % SSD + 2 % PG were comparable to that of 1 % SSD. Wound healing was complete for all treated groups on day 28, but not for the control group.

Histological analysis

Re-epithelialization: On days 14 and 21 post-burn, the re-epithelialization of wounds treated with 1 % PG, 2% PG, 1 % SSD + 1 % PG and 1 % SSD + 2 % PG was well developed. Keratinocytes proliferated and migrated across the wounds. On day 21 post-burn, the skin surface of all rats treated with 1 % SSD + 2 % PG was almost completely covered with new epidermal cells (data not shown).

Number of multinucleated giant cells: On days 7, 14 and 21 post-burn, the number of multinucleated giant cells/field from wounds treated with 1 % SSD, 1 % PG, 2% PG, 1 % SSD + 1 % PG, and 1 % SSD + 2 % PG did not differ significantly from that in the control group. The number of multinucleated giant cells in all groups was highest on day 7 and then decreased on days 14 and 21 (**Figure 1**).

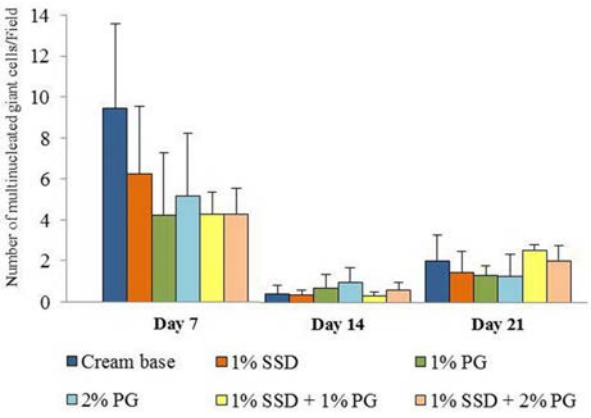

Figure 1 Number of multinucleated giant cells/field on days 7, 14 and 21 post-burn. Data are shown as means ± SD, N = 6 for all groups.

Number of macrophages: On days 7, 14 and 21 post-burn, the number of macrophages/field from wounds treated with 1 % SSD, 1 % PG, 2 % PG, 1 % SSD + 1 % PG, and 1 % SSD + 2 % PG did not differ significantly from that in the control group. The number of macrophages was highest on day 7 and then decreased on days 14 and 21 (**Figure 2**).

Neovascularization: On day 7 post-burn, the number of vessels/field from wounds treated with 1 % SSD + 2 % PG was significantly higher than those in the control, 1 % SSD, 1 % PG, and 2 % PG groups ($p < 0.05$, $p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively). On day 14 post-burn, the numbers of vessels/field from wounds treated with 1 % SSD + 1 % PG and 1 % SSD + 2 % PG were significantly higher compared to the control and 1 % SSD groups ($p < 0.01$ and $p < 0.01$, respectively). The number of vessels/ field was highest on day 14. On day 21 post-burn, the percentages of vessels/field in all groups tended to decrease and did not differ significantly with that in the control group (**Figures 3**, **4** and **5**).

Table 1 Percentage wound healing on days 3, 7, 14, 21 and 28 post-burn

Groups	% Wound healing				
	Day 3	Day 7	Day 14	Day 21	Day 28
Creambase	4.51 ± 1.69	14.06 ± 4.25	35.00 ± 10.23	66.80 ± 8.50	92.83 ± 19.71
1 % SSD	6.71 ± 3.09	38.68 ± 18.30*	69.33 ± 8.55***	88.45 ± 4.96***	100.00 ± 0.00***
1 % PG	5.80 ± 4.09*	40.89 ± 10.93***	69.25 ± 7.59***	89.78 ± 4.05***	100.00 ± 0.00***
2 % PG	11.60 ± 7.70**$	42.47 ± 11.55***	74.04 ± 6.71***	93.28 ± 1.81***	100.00 ± 0.00***
1 % SSD + 1 % PG	8.30 ± 2.00	45.63 ± 13.63***	77.39 ± 6.69***	92.39 ± 4.65***	100.00 ± 0.00***
1 % SSD + 2 % PG	9.39 ± 3.73	54.01 ± 13.96***	80.22 ± 10.70***	95.62 ± 2.85***	100.00 ± 0.00***

N = 10 for all groups
Data are shown as means ± SD
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. cream base; $^{\$}p < 0.05$ *vs*. 1 % PG

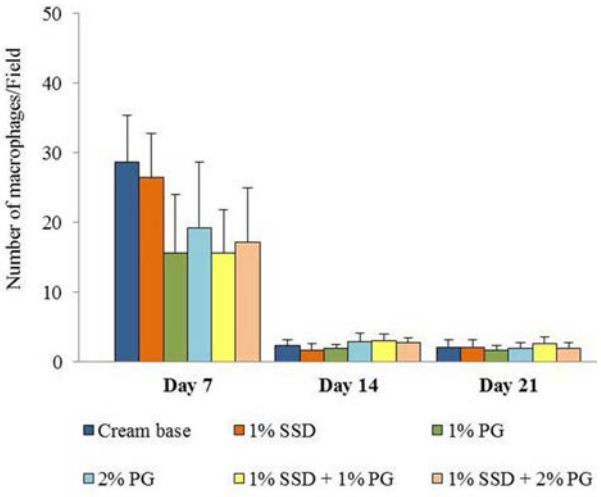

Figure 2 Number of macrophages/field on days 7, 14 and 21 post-burn. Data are shown as means ± SD, N = 6 for all groups**.**

Wound healing is a process in which the skin repairs itself after injury. This process involves soluble mediators such as cytokines or growth factors, blood cells such as platelet and macrophages. Wound healing has four phases: hemostasis, inflammation, tissue proliferation and tissue maturation or remodeling, which overlap in time [6, 19]. In this study, epidermal regeneration and keratinocyte migration were analyzed histologically, and calculated as the percentages of re-epithelialization and wound healing on days 3, 7, 14, 21, and 28 post-burn. On day 3, rats treated with PG cream had a faster wound healing rate than those treated with 1 % SSD as a standard positive control. On days 7 and 14 post-burn, re-epithelialization was well developed in rats treated with 1% SSD or PG. On day 21 post-burn, the percentages of re-epithelialization of rats treated with 1 % SSD, 1 % PG, 2% PG, 1 % SSD + 1 % PG, and 1 % SSD + 2 % PG were significantly higher than the cream base group. The skin surface of rats treated with 1 % SSD + 2 % PG which had the highest percentage of re-epithelialization was almost completely covered with new epidermal cells and new hair (data not shown). The percentages of wound healing in all PG groups were comparable to that in the 1 % SSD group. On day 21, rats treated with 1 % SSD + 2 % PG cream had the fastest wound healing rate. Application of 1% SSD or PG facilitated and accelerated healing of burn wounds, as observed on the last day of the study (day 28). Results from histological observation were in accordance with the % wound healing obtained by calculation.

The hemostasis phase in wound healing is followed by an inflammation phase involving inflammatory mediators. The inflammation phase includes activation of the mediator combined with peptides called cytokines, which function as cellular immune response enhancers that responding to the antigen. White blood cells such as macrophages, neutrophils and monocytes are then driven to the interstitial space surrounding the wound. Neutrophils destroy dead cells using protease enzymes, which also degrade the white blood cells. The monocytes are transformed to macrophages, which are then transformed to multinucleated giant cells, which have defensive roles against dead cells and bacteria [20, 21]. The macrophages and multinucleated giant cells also release angiogenesis factors that stimulate the endothelial cells of capillaries around the wound, resulting in generation of new capillaries and tissue to promote wound healing; and growth factors such as PDFG, EGF and TGF-β, which promote synthesis of fibroblasts that then move to the center of the wound for proliferation [22, 23]. The levels of macrophages and multinucleated giant cells increase in the inflammatory phase and decrease in the proliferative and remodeling phases [19]. The number of multinucleated giant cells and macrophages of all treated groups were not significantly different from control, indicating that all treatment cream did not induce more inflammation of burn wounds. In this study, the number of multinucleated giant cells and macrophages started to decrease after day 14 post-burn, indicating reduced inflammation of the wounds.

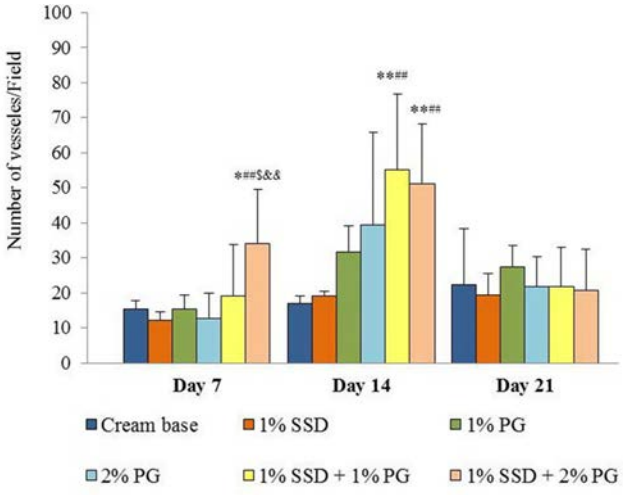

Figure 3 Number of vessels/field on days 7, 14 and 21 post-burn. Data are shown as means ± SD, N = 6 for all groups; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. cream base group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* 1 % SSD; $^{\$}p < 0.05$ *vs.* 1 % PG; $^{\&\&}p < 0.01$ *vs.* 2 % PG.

The inflammatory phase in wound healing is followed by a proliferation phase involving neovascularization and collagen synthesis, and PG promotes neovascularization by binding to VEGF receptors [15]. The number of vessels/field in all treated groups reached its highest level on day 14 and then decreased on day 21. Rats treated with 1 % SSD + 1 % PG cream and 1 % SSD + 2 % PG cream had the highest number of vessels/field on day 14, compared to the other groups, indicating the efficacy of PG in promoting neovascularization. The remodeling phase starts after the proliferation phase, as indicated by a decrease of collagen and fiber synthesis and blood vessel regeneration [22, 24]. Our results showed that the remodeling phase is present on day 21 and wound healing was nearly completed at this time. Overall, the study showed that PG can accelerate and facilitate burn wound healing in rats, partly by stimulating neovascularization. A combination of 1 % SSD + 1 % or 2 % PG

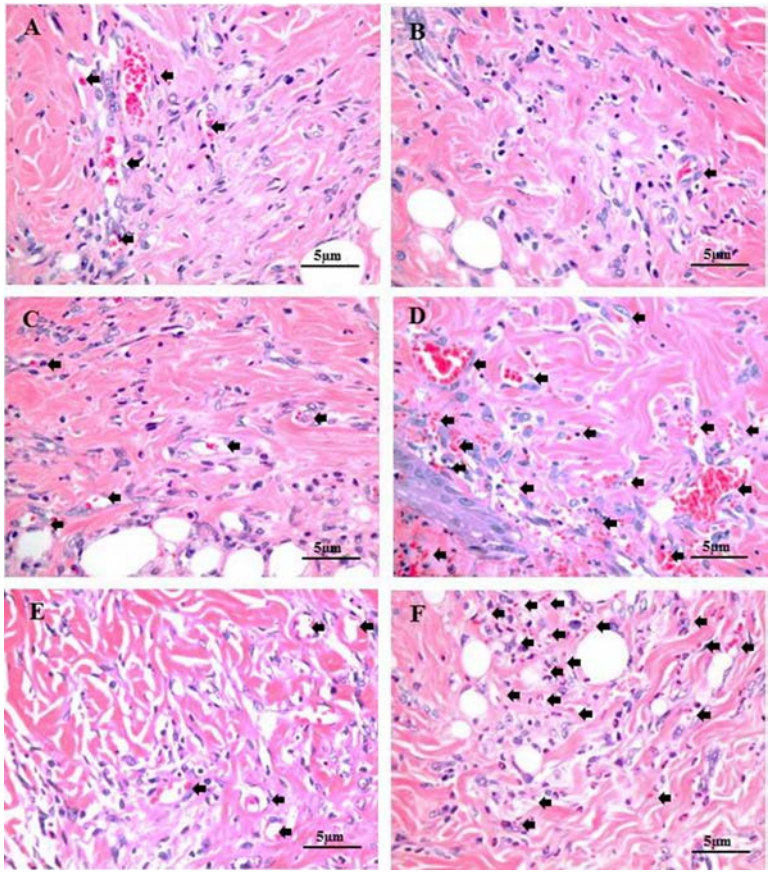

Figure 4 Histological appearance on day 7 post-burn. Arrows show the number of vessels. Hematoxylin and eosin stains of samples of wounds treated with (A) cream base (control); (B) 1 % SSD cream; (C) 1 % PG cream; (D) 2 % PG cream; (E) 1 % SSD + 1 % PG cream; and (F) 1 % SSD + 2 % PG cream**.**

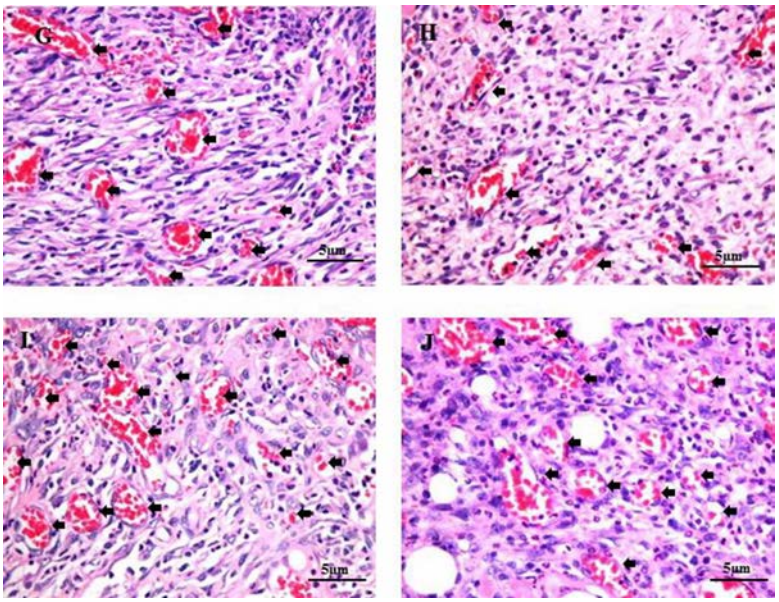

Figure 5 Histological appearance on day 14 post-burn. Arrows show the number of vessels. Hematoxylin and eosin stains of wounds treated with (G) cream base (control); (H) 1 % SSD cream; (I) 1% SSD + 1 % PG cream; and (J) 1 % SSD + 2 % PG cream.

cream seems to be the effective formulations for treating burn wounds.

Conclusion

The results suggest that PG extracted from fish cartilage can accelerate and facilitate wound healing in rats. The combination of 1% SSD and 1 % or 2 % PG seem to have high efficacy in accelerating and facilitating wound healing in rats. The combination of 1 % SSD and 1% or 2 % PG is safe for long term use. Further investigation of the safety profile of PG and the combination of 1 % SSD and PG in detail is required.

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