EVALUATION OF TILAPIA FISH (OREOCHROMIS NILOTICUS) SKIN COLLAGEN HYDROGEL POTENTIAL AS BURN WOUND HEALING AGENT

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Abstract. Tilapia collagen consists of a complex structural protein that possesses biomaterial to accelerate wound healing activity. The aim of this study is to evaluate the potential effects of Oreochromis niloticus collagen hydrogel, as a burn wound healing agent. Collagen hydrogel of concentrations 0.05 mg/ml and 0.1 mg/ml were prepared via acid soluble collagen extraction and crosslinked with Carboxymethylcellulose. Sprague Dawley rats were used as test subjects and were grouped into negative control (with normal saline), positive control (using 1% silver sulfadiazine cream), two treatment groups (using 0.05 mg/ml and 0.1 mg/ml collagen hydrogel) and a normal group. A second degree burn wound was induced on the rats by using an electrical heater (at 100 °C), and collagen hydrogel of both concentrations were given daily for 14 days. The wound size, gross and histopathological changes were recorded. The burn wound treated with the collagen hydrogel of both concentrations had shown a decrease in size with an average diameter of 4 cm on day 0, and 2 cm at day 14, which was significant with $p<0.05$. The gross examination showed a decrease in diameter of the wound from day 0 to day 14. Histopathology result showed an increase of fibroblast presence, regeneration of hair follicles and sebaceous glands, decrease in inflammatory cells, increase in blood vessels, and restored epidermis layer on the 14th day of the treatment groups compared to the 14th day of the positive control. As a conclusion, the collagen hydrogel extracted from the skin of O. niloticus had displayed its potential as a burn healing agent, primarily by reducing inflammation and facilitating rapid proliferation of fibroblast.

Keywords: Burn wound, *oreochromis niloticus*, collagen hydrogel, acid solubilized collagen

Article Info

Received 4th February 2023
Accepted 26th September 2023
Published 20th December 2023

ISSN: 1823-7010, eISSN: 2600-7444

Introduction

Burn wound healing represents a major health challenge as they require a large amount of healthcare and economic resources to improve the patient's quality of life. In the current healthcare industry, drugs such as silver sulfadiazine and mafenide acetate solution are being used. These drugs have anti-bacterial properties that protects burn injury from infection but may cause allergic reactions to certain individuals [1]. In addition, burn management requires a long hospitalization, overpriced medication, multiple surgery procedures and a longer time to readapt [1].

Nowadays, collagen, especially porcine and bovine has been extensively used for skin wounds and burn healing therapy as they have excellent biocompatibility. However, some cultural and religious like Muslim groups should not use these sources of collagen due to halal issue. Moreover, there are concerns regarding the usage of collagen derived from bovine as it can cause potential spreading of animal diseases such mad cow disease, foot and mouth disease, and bovine spongiform encephalopathy. Researchers from all over the world are drawn to the study of marine produced collagen as an alternative because of the necessity to find an alternative source of collagen. This study was also carried out to get around Muslim community's restrictions on non-halal usage and to prevent the spread of the zoonosis illness [2-4].

Although other studies have shown the beneficial effects of tilapia skin collagen, the collagen hydrogel utilized in this study was unique since it was developed by the self-assembly of collagen and cross-linked with the natural polymer carboxymethyl cellulose (CMC) [5], without the use of any extra chemicals.

Evaluation of the efficacy of collagen hydrogel developed in this study as a halal natural burn wound healing agent will serve as a foundation for further research into fish skin collagen as a new halal source for burn treatment that avoid zoonosis infection while also speeding healing through cellular proliferation, differentiation, and mineralisation [6], all of which are important in the burn wound healing process.

Materials and Methods

Sample Collection and Collagen Extraction

Fresh tilapias were purchased from the local wet market in Shah Alam, Malaysia. Fish samples were immediately transported to the laboratory by placing in ice after cleaning with running tap water before the skins were removed manually. The collected skins were cleaned with distilled water. The size of the skins was reduced by cutting into small pieces (0.5 cm X 0.5 cm). The extraction of tilapia fish crude collagen was done by using the acid-solubilised collagen (ASC) method [7-9]. All the procedures of collagen extraction were conducted at 4 $^{\circ}$C. This skin was soaked with 0.1 M sodium hydroxide (NaOH) with the ratio of 1:8 (sample to alkali) for 48 hours and was then washed with cold distilled water until its pH became neutral. The sample was soaked in 10% ethanol for overnight (1:10 sample to alcohol), washed with distilled water and soaked in 0.5 M acetic acid for 42 hours with gentle agitation. The samples were then centrifuged at 20 000 x g at 1 hour before the supernatant was collected. Followed by centrifugation at 20 000 x g at 30 minutes, pellet was collected, lyophilized and the final product was collected.

Preparation of Collagen Hydrogel

Collagen hydrogel was developed using crude collagen based on the previous techniques [7]. Crude collagen was weighed and added to 0.1 M acetic acid to dissolve. In a 4:1 ratio, the dissolved collagen was added to cold distilled water and phosphate buffer saline. Using a pH meter, the collagen solution's pH was evaluated (Hanna Instruments, pH211 microprocessor). A neutral pH (7.4 ± 0.2) was obtained by adjusting the pH with 0.1 M sodium hydroxide solution [7]. After the 2% Carboxymethylcellulose (CMC) powder was added (2 mg CMC in 100 ml mixture), the mixture was constantly agitated until a uniform gel consistency was achieved. These procedures were utilized to make *O. niloticus* collagen hydrogel at concentrations of 0.05 mg/ml and 0.1 mg/ml.

Experimental Animal

The animal experiment was approved by the University Ethics Committee of Management and Science University (Code Ethics – MSU-RMC-02/FR01/02/L3/013). The test subjects were 200–250 g male Sprague Dawley rats. They were kept separately in identical climatic circumstances (23 °C with 12 hours of light and 12 hours of darkness, respectively) (five rats per cage) [10]. Water and the standard diet were ad libitum.

Formation of Burn Wound

The work of was referenced in the procedure utilised to identify the burn wound [1,10]. The Sprague Dawley rats had their dorsal shaved. Before applying the burns, ketamine (100 mg/ml) and xylazine (20 mg/ml) were administered intraperitoneally to provide anaesthesia. On their dorsal portions, a second-degree burn wound of 3 cm in diameter was produced using an electric heater set to 100 $^{\circ}$C for 10 seconds.

Experimental Procedure

Male Sprague Dawley rats were divided into five groups (n=5); two treatment groups (0.05 mg/ml and 0.1 mg/ml collagen hydrogel) and each group of normal (without burn infliction and received no therapy), negative control (received normal saline), and positive control (received 1% Silver Sulfadiazine Cream (SSD). For a period of 14 days, collagen hydrogel, SSD, and normal saline were daily topical administered to the burn wound region. Each group's progress toward complete wound healing was monitored and documented. All animals were euthanised and skin tissues were collected at pre-determined days which is 0-, 7- and 14-days post wounding.

Rate of Wound Contraction

By capturing images with a digital camera at the same distance from the burn and at a right angle to the surface, the rate of wound contraction was estimated as the percentage of reduction in the original wound size starting on the second day of therapy. To measure the size of the wound, images were taken using Image Mixle software [1]. The following formula is used to determine the percentage of wound contraction:

Wound contraction (percent) = 100 [(zero day wound size - unique day wound size)/zero day wound size]

Histological Evaluation

The burn wound's skin tissues were harvested and kept in 10% neutral buffered formalin. All tissues had undergone tissue processing and were set in blocks of paraffin. The samples were then manually excised into a 5 μm thickness. Hematoxylin and Eosin (H & E) staining was applied to sections of the tissues after they had been dewaxed and rehydrated. Finally, DPX was used to seal the slides, and light microscope to inspect the histologic changes [7,11].

Results and Discussion

Gross Examination of Burn Wound

Figure 1 displays illustrations of the burn wound healing process at various time points for all experimental groups. Between day 0 and day 14, the wound's diameter shrank on average from 4 cm to 1.5 cm. In comparison to the positive and negative controls, burn wound regions treated with collagen hydrogel at concentrations of 0.05 mg/ml and 0.1 mg/ml on day 7 were noticeably smaller and began to dry out. According to [12], the percentage of the initial wound area that was retained was used to calculate the wound healing rate. Due to the skin's viscoelastic characteristics, all wounds first shrank and then kept shrinking [8,12]. The results of this study were consistent with those of [7], who discovered that collagen hydrogels had sufficient mechanical strength and could hold more moisture, enabling the wound surface to heal in a humid environment and better satisfying the requirements of wet wound healing [8,9].

All these findings were in line with Hu's research, which showed that some healing occurred on day 14 and that mature granulation did not proliferate significantly, and some wounds were partially covered by new epidermis [8,9,14]. Collagen fibers were used to take the place of the damaged granulation tissue. Infiltration of lymphocytes and an abundance of fibroblasts were seen in all groups. In the process of healing a wound, collagen acts as a chemotactic attractant, attracting skin fibroblasts to the wound and promoting fibroblast activity, which leads to the production of granulation tissue.

Burn Wound Diameter

On days 0 through 14 the wound's diameter (cm) was measured. Values for each group of five rats were shown as the mean SD. Table 1 displays the diameter of the burn wounds on the burned rats. Due to the exudation of tissue fluid following the burn infliction, the wound diameter shrinks on days 0 and 7 without any discernible difference between the four groups. On day 14, however, there were noticeable differences across all four groups. In comparison to the positive control group (1.54 ± 0.13, $p < 0.05$), the wound width in the collagen hydrogel treatment group (0.1 mg/ml) group had significantly decreased by day 14 (0.80 ± 0.20, $p<0.05$). On days 0 and 7, the collagen hydrogel burn wound diameter did not substantially differ between the two concentrations; however, the wound size dramatically shrank when compared to the positive control group. Additionally, on day 14, the wound width was larger in the negative control animals (2.00 ± 0.16, $p< 0.05$), but the rats given collagen hydrogel (0.1 mg/ml) treatment had virtually fully recovered. These findings demonstrated that rat burn wound healing was aided by collagen hydrogel made from the skin of tilapia.

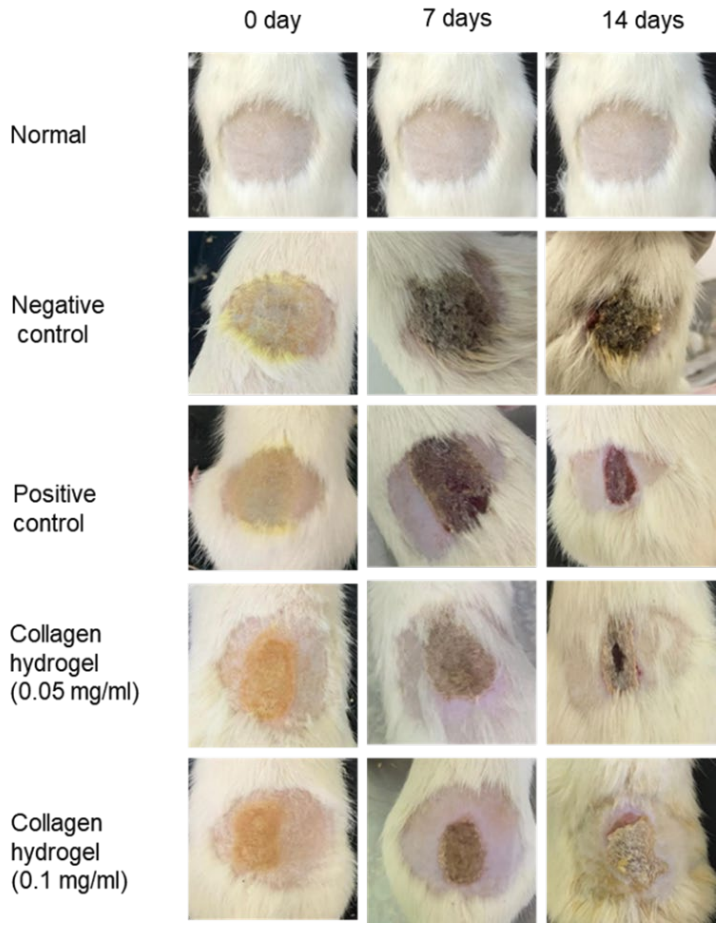

Figure 1: Rat skins with deep second-degree burns were healed using various treatments after 0, 7, and 14 days. The collagen hydrogel group treated with two concentrations (0.05 mg/ml and 0.1 mg/ml); positive control group treated with SSD Cream; negative control group treated with regular saline; normal group untreated for or subjected to burns

Table 1: The burn wound's diameter (in cm) after being treated from day 0 to day 14 with normal saline, SSD cream, and collagen hydrogel at concentrations of 0.05 mg/ml and 0.1 mg/ml.

Group	**0 day**	**7 days**	**14 days**
Negative control (normal saline)	2.50±0.16	2.10±0.19	2.00±0.16[a]
Positive control (SSD)	2.60±0.19	1.90±0.19	1.54±0.13[b]
C. hydrogel (0.5 mg/ml)	2.28±0.19	1.80±0.12	1.30±0.20[c]
C. hydrogel (1 mg/ml)	2.10±0.10	1.90±0.10	0.80±0.20[c]

[a]significantly difference from negative control at 0-day, $p<0.05$
[b]significantly difference from positive control at 7-day, $p<0.05$
[c]significantly difference from SSD at 14-day, $p<0.05$
[c]significantly difference from SSD at 14-day, $p<0.05$

The data is shown as a mean with standard error (SE). A one-way analysis of variance (ANOVA) was used to compare the data, followed by a post-hoc t-test.

Figure 2 shows the percentage of burn wound healing from day 0 to day 1. On day 7, the negative control had a healing percentage of 16.00%, the positive control was at 23.53%, the collagen hydrogel at 0.05 mg/ml was at 29.41%, and the collagen hydrogel at 0.1 mg/ml was at 24.0%. This showed that compared to the control groups, the treatment groups had a higher proportion of wound healing. Between the treatment groups and the control groups, there was a significant difference. On day 14, however, all groups' percentages of wound healing improved noticeably from day 7 to day 14. The highest increases in wound healing were seen in 0.1 mg/ml collagen hydrogel (68.00%), followed by 49.02% in 0.05 mg/ml collagen hydrogel, 39.61% in positive control and 20.00% in negative control.

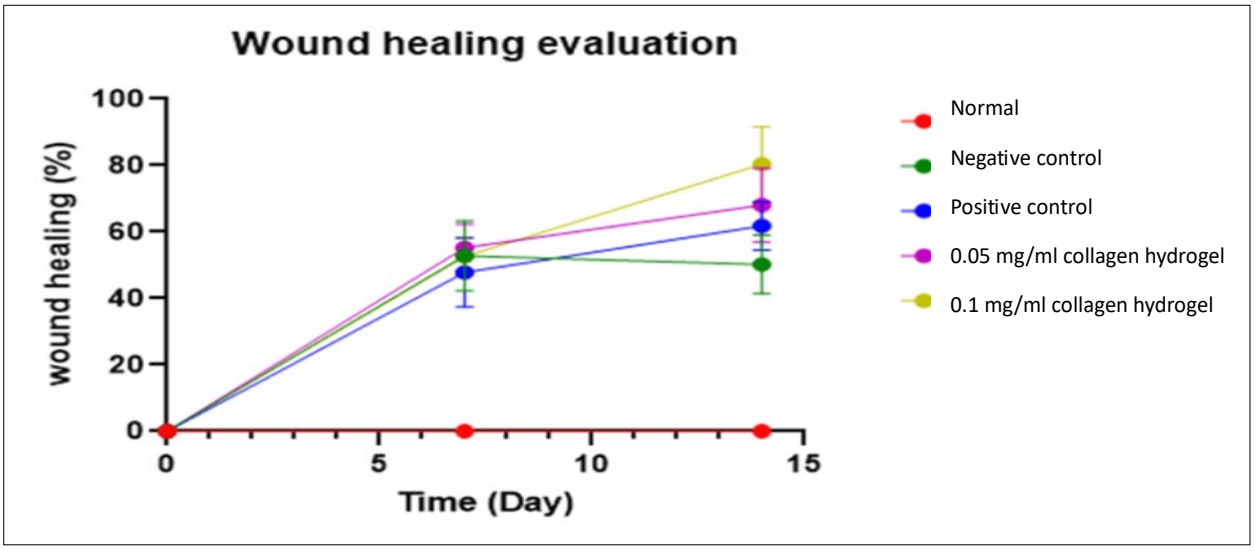

Figure 2: The increase of wound healing percentage from day 0 to day 14

Histological Analysis

H&E staining was used in histological research to further assess the burn healing process (Figures 3 and 4). The dermal-epidermal junction vanished, and the skin appendages were destroyed on day 0 (skin tissue collected roughly 24 hours after burning), as illustrated in Figures 3 and 4, indicating the successful creation of profound second-degree burn lesions. The positive control group (treated with 1% SSD cream) showed the development of hair follicles in the early stages of burn wound healing. The treatment groups receiving collagen hydrogels of both concentrations showed the development of hair follicles, sebaceous glands, and the development of the epidermis layer. It was evident that the negative control groups showed no signs of recovery. On day 14, the treatment groups showed more fibroblasts than the positive control group did, as well as keratinized layer development and dermal layer development. Additionally, inflammatory cell infiltration was reduced in the therapy groups. Collagen hydrogels may have had a higher impact on encouraging burn wound healing than the control groups as seen by the faster epidermis layer creation and high maturity of skin accessories. This may be because collagen promotes the proliferation and differentiation of epithelial cells.

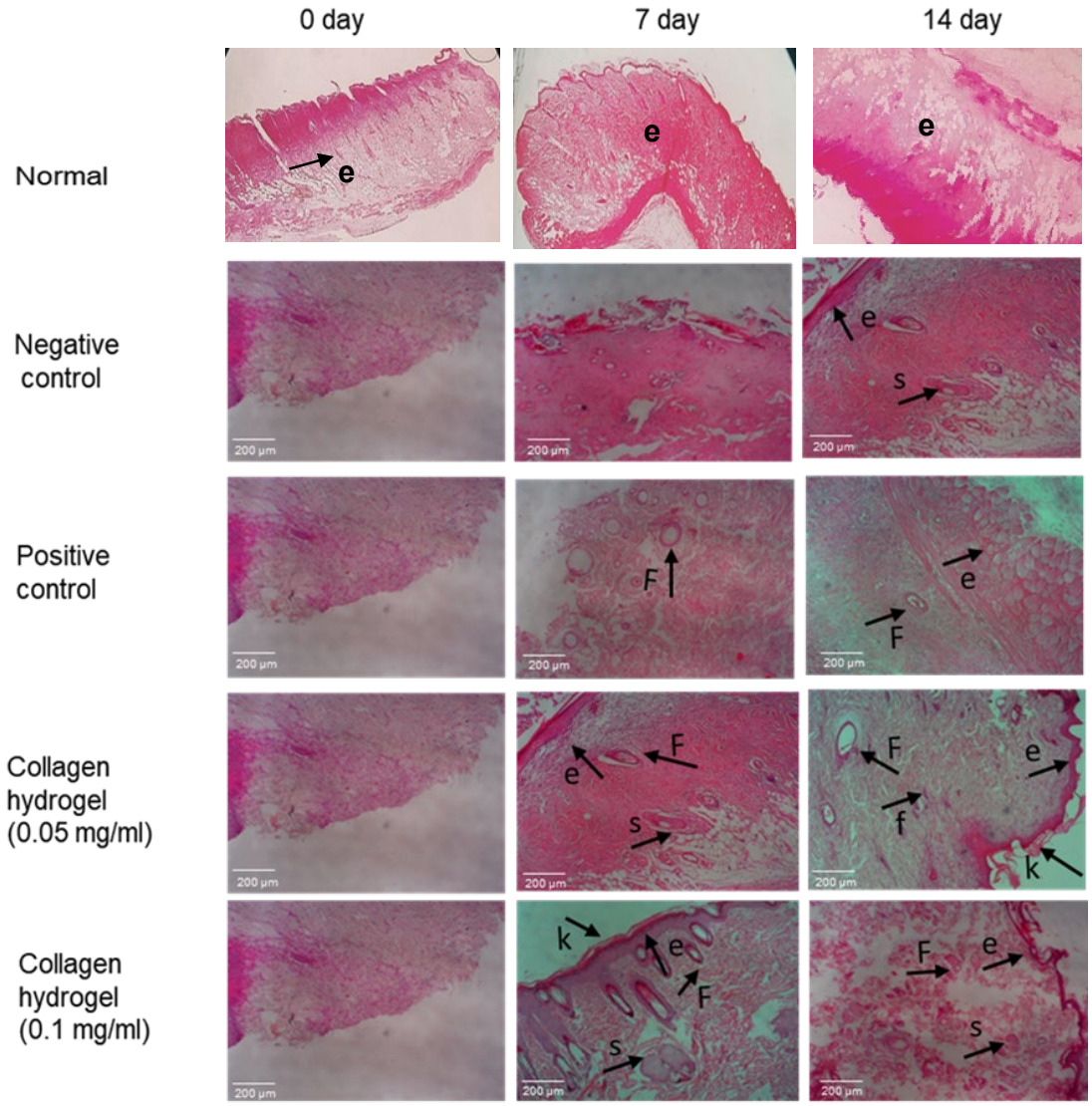

Figure 3: H and E-stained histologic sections of burn wound at 0,7 and 14 days**.** Normal group without any burn infliction and treatment; Positive control group treated with SSD Cream; Negative control group treated with normal saline; Collagen hydrogel group treated with two concentration (0.05 mg/ml and 0.1 mg/ml). Abbreviations: F: follicle; S: sebaceous gland; k: keratinized layer; e: epidermis; f: fibroblast. (H&E, 10X)

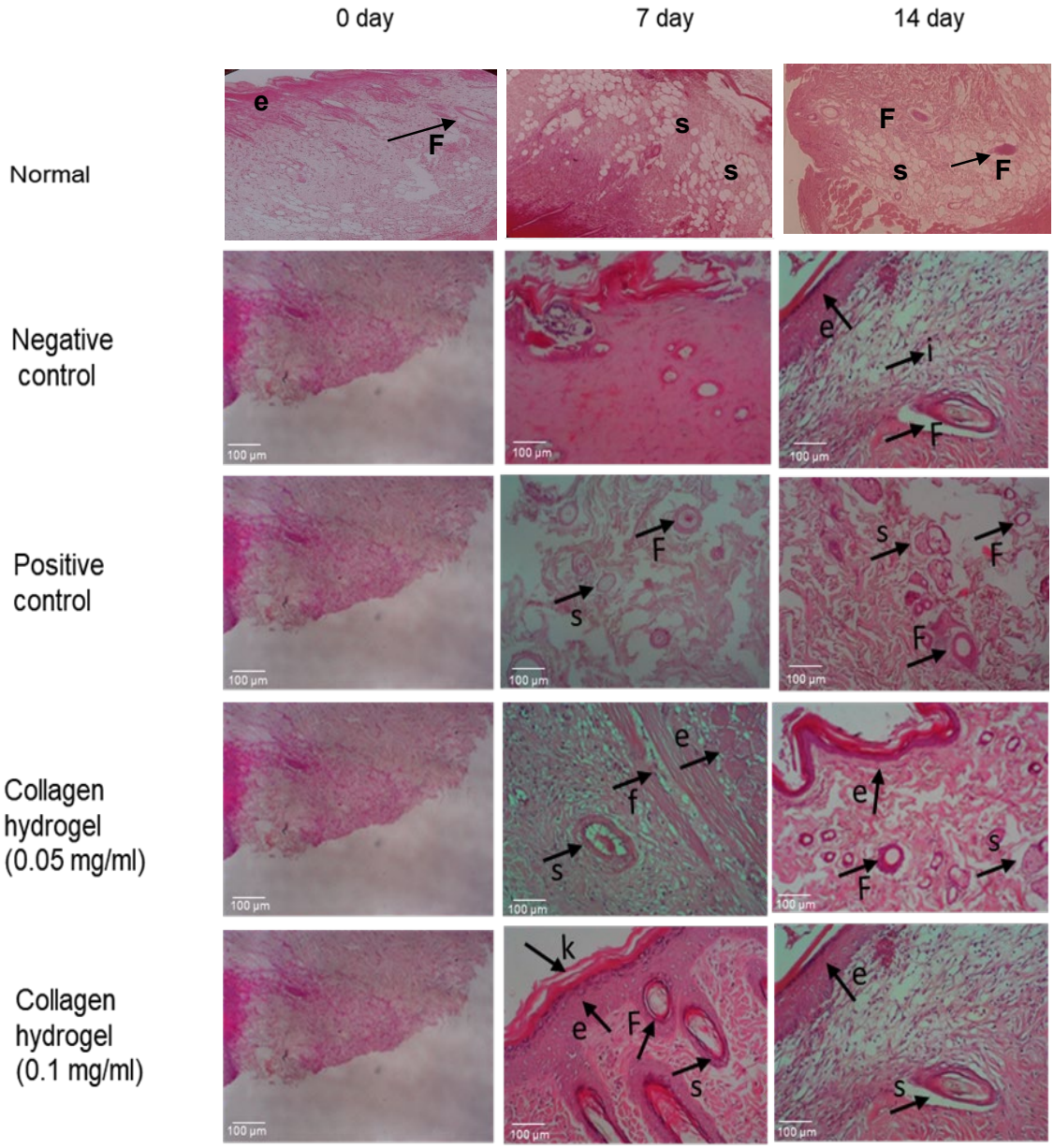

Figure 4: H and E-stained histologic sections of burn wound at 0,7 and 14 days. Normal group without any burn infliction and treatment; Positive control group treated with SSD Cream; Negative control group treated with normal saline; Collagen hydrogel group treated with two concentration (0.05 mg/ml and 0.1 mg/ml). Abbreviations: F: follicle; S: sebaceous gland; k: keratinized layer; e: epidermis; d: dermis; i: inflammatory cells; f: fibroblast. (H&E, 40X)

Conclusions

By reducing inflammation, encouraging the formation of granulation tissue, and facilitating the quick proliferation of epithelial, endothelial, and fibroblast cells, we found that collagen hydrogel from tilapia fish skin could both accelerate and improve the healing of skin scald wounds in rabbits. The underlying chemical mechanism is not yet understood, though.

Our results suggest that collagen hydrogels from tilapia fish skins may be used as wound dressings for the management of severe second-degree burns. The results obtained above allow us to draw the conclusion that the groups treated with collagen hydrogel had greater healing potential than the other groups.

Acknowledgments

Authors would like to thank Management and Science University for funding this research via SG 032-022018-FHLS and the Ministry of Health Malaysia for granting access to secondary data for this research.

Author Contributions

Haniza Harun, the lead researcher and owner of the grant for this project, oversees designing and planning the framework, writing the research, managing the lab activity, and performing the data analysis. Renisha Nair, Vausalya Taumany, and Nurul Farah Nadhirah Mohd Asri oversee managing the lab work and doing the literature search. Haniza and Nurzafirah Mazlan worked on the paper's critical revision, drafting, and data analysis during this time. Eddy Yusuf and Fadli Asmani served on the supervision committees and contributed general comments and constructive criticism to the study's result.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards. The animal experiment was approved by the University Ethics Committee of Management and Science University (Code Ethics – MSU-RMC-02/FR01/02/L3/013).

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