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Research Article

Insight into the mechanism of snakehead fish (*Channa argus*) soup promoting skin wound healing using rat and cell models

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Abstract: This study is to explore underlying mechanism of skin wound healing by snakehead fish soup using rat and cell models. We assessed skin wound changes after administering wild and farmed snakehead fish soup intragastrically, then using CCK-8 and scratch tests to evaluate cell proliferation and migration, last assessed vessel tubule formation by HUVEC cells. The results showed that the skin wound healing rates of rats after 14 days of intragastrical administration of farmed and wild snakehead fish soup were 52.71% and 57.76%, respectively, which were substantially greater than the control group (39.84%). With the administration of the soups, the collagen tissue on the skin wound was more homogenous and thicker, and type I collagen was denser. Additionally, interleukin-6 level in rat serum during wound healing was significantly lower, while cyclin-D1 and fibroblast growth factor-2 levels were higher. Furthermore, the soups (particularly at 50 μg/mL) significantly increased the proliferation and migration rates of both the HACAT and NIH3T3 cells, and considerably promoted the tubule formation of HUVEC cells. The findings confirm that the soup can promote skin wound healing and the underlying mechanism involves multiple roles including anti-inflammation, cell proliferation, vessel tubule formation, and collagen expression.

Keywords: snakehead fish soup; wound healing; intragastrical administration; mechanism

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1 Introduction

Snakehead fish (*Channa argus*) is tolerant to environment change, grows fast, and possesses a high economic value. In 2021, the cultivation of snakehead fish in China reached 540 000 tons. The snakehead fish soup contains rich nutritional content, as well as numerous functionalities such as antioxidant[1], wound healing after surgery[2], and so on.

Skin wound healing is a complicated and time-consuming process that relies on the coordinated action of numerous cells (e.g., vein endothelial cells, epidermal cells, and fibroblasts) and growth factors (e.g., interleukin-6 (IL-6), basic fibroblast growth factor-2 (FGF-2), and cyclin-D1)[3–4]. Nowdays, the most common treatments for wound healing are pharmacological therapy, physical therapy, medical dressing therapy, and so forth. Some classical medical works (e.g., Shen Nong's herbal classic, 1616) record that snakehead fish soup can promote wound healing. Both traditional and current clinical practices have demonstrated that snakehead fish soup does have this functionality[1–2], although it lacks theoretical evidence.

The snakehead fish grows in different environment, which influences the nutritional composition of snakehead fish muscle[5]. Previous research has demonstrated that the environment in which food is produced affects its nutritional components, eventually influencing the efficiency of diet therapy[6]. Snakehead fish soup is abundant in unsaturated fatty acids, zinc, peptides, and other nutrients according to our previous study. Except for the slightly lower peptide content, fatty acids and zinc in wild snakehead fish soup are significantly higher than those in farmed snakehead fish soup[5]. However, the effects of soup made from snakehead fish that grow in diverse environment on wound healing are unknown so far.

Therefore, we built a rat model of a skin wound in this study, and later on assessed the changes in the skin wound after administering wild and farmed snakehead fish soup intragastrically, including healing rate, the morphology of the wound tissue, growth factors, the production of collagen. Three types of cells were cultured. There were mouse embryonic fibroblasts (NIH3T3 cells), human umbilical vein endothelial cells (HUVEC), and human immortalized epidermal (HACAT) cells. The CCK-8 technique and scratch test were used to gauge the proliferation and migration of the three different types of cells, respectively. The formation of vessel tubules by HUVEC cells was also evaluated. The aim of this study was to evaluate the effects of snakehead fish soup on wound healing and explore the underlying mechanism. Additionally, a comparison of the two types of snakehead fish soup on wound healing was carried out, which would provide a theoretical foundation for the selection of aquatic food products.

2 Material and methods

2.1 Material

The farmed snakehead fish, which was exclusively farmed in high density (around 350 tails per 100 m^2) and given regular feedings, grew in the fish ponds in Guangzhou, China. The wild snakehead

fish intruded the fish ponds in Guangzhou, China and grew alongside other farmed fish. The wild snakehead fish grew in low density (< 5 tails per 100 m^2) and hunted small fish, frogs, etc. as a diet. The two kinds of fish can be easily distinguished by their morphology[5]. The two kinds of snakehead fish were about one year old, 40 cm in length, and 1 000 g in weight. Market purchases included refined cooking salt from China Salt Industry Group Co., Ltd. (Beijing, China), and peanut oil from Shandong Luhua Group Co., Ltd. (Shandong, China).

SD male rats (initial weight between 160 and 180 g) were obtained from the Hubei Experimental Animal Research Center (Wuhan, China). HACAT cells, HUVEC, and NIH3T3 cells were purchased from Wuhan Warner Biotechnology Co., Ltd. (Wuhan, China). Chueun I composite peptide was purchased from Jiangzhong Pharmaceutical Co., Ltd. (Jiangxi, China).

2.2 Preparation of snakehead fish soup

The method of preparing the soup of snakehead fish was referred to our previous study[5]. Fresh fish meat (about 700 g) with head and bones were washed with tap water, and then the water was drained off. Peanut oil (3% of the weight of the fish) was added into an iron pot and heated by an induction cooker (RT2134, Midea group, Foshan, China). After the water was boiling, the fish meat was dumped into to the pan and stir-fried until the color turn to golden brown. Water (3 times the weight of the fish) was poured into the pan. The mixture was heated up to boiling temperature under 2 100 W power, and the boiling was last for 15 min. Foam in the surface was removed during the heating process. After the pan was covered by a lid, the mixture was heated under mild power (300 W) for 30 min. Salt (1.2% of the weight of the fish) was added prior to turning the induction cooker off. The fish soup was separated by filtering through single layer cheese cloth. The contents of peptide, total unsaturated fatty acids, and zinc in the lyophilized soup of farmed snakehead fish were 12.47%, 9.75%, and 9.04 mg/kg, respectively; 11.69%, 11.55%, and 12.57 mg/kg, respectively for the wild snakehead fish[5].

2.3 Animal tests

2.3.1 Animal models, experimental groupings, and treatments

The Laboratory Animal Management and Use Committee of the Safety and Assessment Center [Safety Review Center Movement (Fu) No. 202120217] gave its approval to all the laboratory techniques utilized in this investigation. The rats were fed using the approaches described by Shang et al.[7]. The 24 male SD rats, aged 3 to 8 weeks, grew in a special pathogen-free environment. The 6 rats were randomly assigned to each of the 4 groups, which were labeled farmed snakehead fish soup group, wild snakehead fish soup group, positive control group, and control groups. The rats were given 10% chloral hydrate intraperitoneally for anesthesia following a week of adapted feeding. The back hair was shaved and the skin was disinfected. To establish an animal model of a skin wound, the full-thickness skin in a round shape and with a diameter of about 2 cm was cut off from the back of the rat. The rats were housed in a single cage with access to food and water freely. The control (CK) group received normal saline, the positive control (PC) group received the drink of Chueun I composite peptide, the farmed snakehead fish soup (FS) group received farmed snakehead fish soup, and the wild snakehead fish soup (WS) group received wild snakehead fish soup. There was a 1.5 mL/200 g bw intragastrical injection dosage for each group. The rat was subjected to gavage once daily. The lyophilized soup of snakehead fish soup was added with water to a solid concentration of 12.15 g/100 mL prior to the intragastrical administration, which is the same as the drink of Chueun I composite peptide.

2.3.2 Measurement of wound healing rate

On day 1, 3, 5, 7, and 14, the diameter of wound was measured using a vernier caliper to determine the wound healing rate:

$$\text{Wound healing rate}\ (\%) = \frac{D_0 - D_n}{D_0} \times 100 \quad (1)$$

Where D_0 is the diameter of the initial wound (mm); and D_n is the diameter of the wound at day n (mm).

2.3.3 Morphological observation of wound tissue

On the 3rd, 5th, 7th, 14th days of modeling, the skin tissue was separate from the wound, placed in 4% paraformaldehyde, and fixed at 4 ℃ for 48 h, prior to the observing the pathological morphology of the wound[8].

2.3.4 Determination of inflammatory and growth factors

Enzyme linked immunosorbent assay (ELISA) method was applied to determine growth factors. After the rats were anesthetize blood was collected from their orbits in a procoagulant tube. The blood was transferred into a centrifuge tube and centrifuged at a speed of 3 000 r/min for 10 min. The supernatant was taken for serum preparation andstore at –80 ℃ for future use. IL-6, cyclin-D1, and FGF-2 in wound tissue were detected within 30 days[9].

2.3.5 Immunohistochemistry

On the 3rd, 5th, 7th, and 14th days of modeling, immunohistochemistry was performed on the wound tissue after embedding reagent kit staining. After continuous sectioning of paraffin tissue for antigen repair, the sections are placed in hydrogen peroxide solution and kept at room temperature and away from light incubate, and then shake and wash on a decolorization shaker. After blocking with serum, first antibody and secondary antibody were added sequentially. The samples were stained, dehydrated and sealed before positioning under low magnification of an optical microscope[10].

2.4 Cell tests

2.4.1 Cell culture

The method of Zheng et al.[11] with some modifications was referred to for the culture of the cells. HACAT, NIH3T3 cells and HUVEC were cultured in an incubator (INC053, Meintel (Shanghai) Trading Co., Ltd., Shanghai, China) under 37 ℃ and 5% CO_2. The culture medium was changed regularly until the cell density was more than 80%.

2.4.2 Measurement of cell proliferation

It was refered to the method of Zheng et al.[11] for measuring cell proliferation. After digestion, centrifugation, and resuspension, the cells were incubated on a 96 well plate with a density of approximately 1×10^5 cells/mL, and the cell was added with 150 μL of complete culture medium containing the soup of snakehead sample (the concentration was 10, 50, and 100 μg/mL, respectively). After cultivation the OD value was measured using an ELISA reader at a wavelength of 450 nm.

2.4.3 Measurement of cell migration

It was refered to the method of Mouritzen et al.[12] with slight modifications for scratch experiments. A marker was vertically drawn at the center of the outer bottom wall of each hole on a 6-hole plate, with the intersection of the two lines being the midpoint of the mark. Cells at a density of 1×10^5 cells/well were inoculated on a 6-well plate. Culture media containing 0.5% (*V*/*V*) fetal bovine serum and snakehead fish soup (final sample concentration of 10, 50, and 100 μg/mL) was added. After cultivating for 0 and 12 h, the samples were observed under a microscope. ImageJ software was applied to calculate the scratch area.

2.4.4 Observation of vessel tubule formation

Matrigel was added into each hole of the 96-well plate and then horizontally placed in a 37 °C and 5% CO_2 incubator for 2 h to form a solid gel. Cells with a density of 2×10^5 cells/mL were inoculated in each experimental hole. Subsequently, 10 μL of samples with different concentrations of soup (10, 50, and 100 μg/mL) were added. After incubation at 37 °C in a 5% CO_2 incubator for 12 h, the vessel tubule was observed under an inverted microscope. ImageJ software was used to analyze the effects of different samples of snakehead fish soup on the HUVEC cells tubular formation ability[13].

2.5 Statistical analysis

All experiments were performed in triplicate. The results of the experiment were expressed as mean ± standard deviation ($\bar{x} \pm s$). The data obtained were visualized using Origin 2018 software and analyzed using SPSS 26.0 software for significance analysis ($P < 0.05$) via one-way analysis of variance (ANOVA).

3 Results and discussion

3.1 Wound healing and morphology of wound tissue in rats

Figure 1A depicts the progression of rat wounds with various healing days. On day 1, the wounds were round and the subcutaneous tissue was distinctly visible in the four groups. The wounds started to scab on day 3 in both 4 groups. On day 5, the wound area shrank and the scab started to stiffen under the pulling force of new cells at the edge of the wound. On day 7, the margin tissue of the wounds in the FS group, the WS group, and the PC group all grew toward the center, whereas the wound in the CK group was still covered with a scab but the wound area also greatly decreased. On day 14, the wounds of the FS group, the WS group, and the PC group were basically healed, leaving them with smooth skin and more new hair, whereas the wound of the CK group was not healed with less skin and hair.

Table 1 shows that on day 3, the wound healing rates for the CK, PC, FS, and WS groups were 3.94%, 1.37%, 1.12%, and 2.64%, respectively. On day 5, the wound healing rates were 4.29%, 7.69%, 4.27%, and 9.58%, respectively. There was no significant difference in the wound healing rates among the four groups on both day 3 and 5. On day 7, the wound healing rates of the PC (17.63%) and WS (19.33%) groups were higher than those of the CK (10.96%) and FS (9.78%) groups ($P > 0.05$). On day 14, the wound healing rates of the PC, FS, and WS groups reached 59.22%, 52.71%, and 57.76%, respectively, which were 1.48, 1.32, and 1.45 folds of that of the CK group (39.84%). These findings suggested that snakehead fish soup promoted wound healing, with WS having a more favorable impact than FS.

Table 1 Effect of different snakehead fish soups on skin wound healing rate in rat (%).

Group	Time (day)			
	3	5	7	14
CK	3.49 ± 1.38[a]	4.29 ± 1.66[a]	10.96 ± 2.05[a]	39.84 ± 0.45[b]
PC	1.37 ± 0.86[a]	7.69 ± 2.19[a]	17.63 ± 5.20[a]	59.22 ± 7.44[a]
FS	1.12 ± 0.82[a]	4.27 ± 2.39[a]	9.78 ± 5.54[a]	52.71 ± 4.84[ab]
WS	2.64 ± 1.69[a]	9.58 ± 4.16[a]	19.33 ± 3.37[a]	57.76 ± 8.22[a]

Note: CK, control; PC, positive control; FS, farmed snakehead fish soup; WS, wild snakehead fish soup. Different lowercase letters in the same column indicate significant differences in wound healing rates between different treatment groups ($P < 0.05$).

In Figure S1, the morphology of the wound tissue in various groups is displayed. The epidermis was thin, and the collagen fibers were disorganized and sparse in the four groups on day 3. In comparison to day 3, the collagen organization on day 5 was thicker and more orderly, and the inflammatory cells in the four groups increased. On day 7, the epidermis became thicker, the collagen fibers became denser, and the number of inflammatory cells decreased. On day 14, the tissues become more compact and homogeneous as compared to day 7. It was noticed that there was much less tissue in the CK group as compared to the other three groups on day 14.

Skin wound healing is an intricate process that goes through several stages, including hemostasis/inflammation, proliferation, and remodeling[14]. After the skin is traumatized, the actions such as vasoconstriction, platelet aggregation, and fibrin clot formation occur immediately and participate in wound hemostasis. The development of fibrin clots and the degranulation of aggregated platelets result in an inflammatory response. Within a few days after trauma, the inflammatory cytokines began to decline, accompanied by apoptosis of inflammatory cells. In the process of healing a skin wound, the stage of cell proliferation and differentiation is primarily the creation of granulation tissue, angiogenesis, and re-epithelialization. A crucial step in wound healing is the change from the inflammatory to the proliferative stage[15]. Finally, the continual synthesis and catabolism of collagen is the primary characteristic of the re-modeling stage. These three stages, which each include diverse cell proliferation, differentiation, and migration as well as the involvement of different growth factors, do not appear to have a time limit. Numerous growth factors are necessary for wound healing at the molecular level, and these growth factors have various biological effects via various routes. Among them, IL-6, cyclin-D1, and FGF-2 are essential to the healing of wounds. In the meantime, collagen, the primary component of the extracellular matrix, is crucial for preserving tissue stability and structural integrity, accelerating wound healing[16].

The main effects of snakehead fish soup on promoting wound healing are related to anti-inflammation, cell proliferation, angiogenesis, and expression of type I collagen. Research shows evidence that nutrition like carbohydrates, lipids, and protein are essential for wound healing. Wound healing is significantly impacted by specialized dietary interventions. Malnutrition or nutrient deficit can impair wound healing by lowering fibroblast proliferation, extending the inflammatory stage, and changing collagen synthesis[17]. Due to the inclusion of peptides in the sample, the PC group effectively promoted wound healing. This finding is comparable with the findings reported by Mistry et al.[18], who found that oral nutritive peptides could improve wound healing. Besides

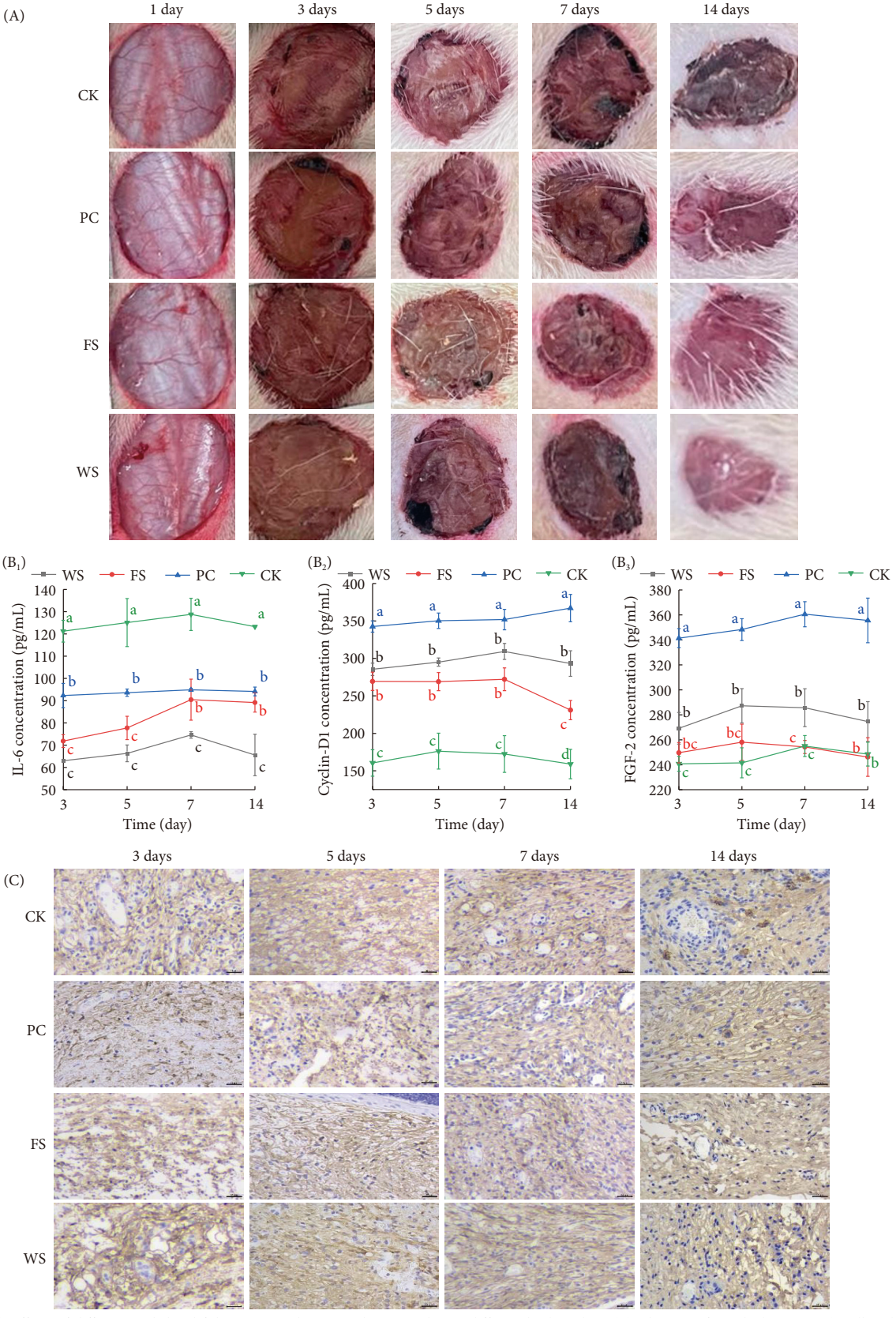

Figure 1 (A) Effects of different snakehead fish soups on skin wounds in rats during different healing days. (B) Changes of interleukin-6 (IL-6), cell cycle protein D1 (cyclin-D1), and basic fibroblast growth factor-2 (FGF-2) concentrations in rats with different snakehead fish soups and healing days. (C) Immunohistochemical observation of collagen I expression in rats with different snakehead fish soups and healing days (400 ×). CK, control; PC, positive control; FS, farmed snakehead fish soup group; WS, wild snakehead fish soup group. Different lowercase letters indicate significant differences in wound healing rates between different treatment groups at same time ($P < 0.05$).

peptides, the snakehead fish soup also contains zinc and unsaturated fatty acids[5], which can help with wound healing. Due to its greater amount of unsaturated fatty acids and zinc, which has anti-inflammatory and cell proliferation effects, the WS healed wounds more pronouncedly than the FS.

3.2 Serum levels of IL-6, cyclin-D1, and FGF-2 in rats

IL-6 has the ability to limit the generation of pro-inflammatory cytokines and the bactericidal activity of macrophages while promoting the alternatively activating macrophage phenotype linked to wound healing[19]. Cyclin-D1 is a cyclin G1 that regulates the cell cycle and is connected to the nuclear factor κB. Numerous studies have demonstrated that cyclin-D1 can promote cell migration in the healing of wounds[20]. Epithelial reformation begins a few hours after injury, and FGF release stimulates epithelial cell migration and proliferation. Several processes are involved in the family of cell signaling proteins known as FGF. FGF-2 rises in acute wounds and plays a role in granulation tissue formation, re-epithelialization, and tissue remodeling[21].

As seen in Figure 1B$_1$, the level of IL-6 displayed a trend of first rising and then falling, peaking on day 7. The WS group had the lowest peak value, followed by the FS group, the PC group, and the CK group with the highest peak value. On day 3 and 5, IL-6 expression was considerably higher in the CK group than in the PC group ($P < 0.05$), and it was significantly higher in the PC group than in the FS and WS groups ($P < 0.05$). Between the FS and the WS groups, there was no significant difference ($P > 0.05$). IL-6 expression of the FS group was significantly higher than the WS group on day 7 and 14 ($P < 0.05$). IL-6 is a biologically active pro-inflammatory cytokine that can directly stimulate the growth of T lymphocytes and B cells to produce cytotoxic T immunological activation and take part in the body's transformation of inflammation and particular immune responses[3–4]. It can be deduced from Figure 1A that both the PC group and fish soup groups showed an anti-inflammatory effect, which might be attributed to the components of Zn, *n*-3 polyunsaturated fatty acid (PUFA), and polypeptide[22]. Since the WS group has a higher concentration of *n*-3 PUFA and Zn than FS group[5], it has a greater capacity to inhibit inflammation. Snakehead fish soup had a stronger anti-inflammatory impact because it included more anti-inflammatory ingredients (peptide, *n*-3 PUFA, and Zn) than the PC sample (only peptide), which might have a synergistic effect.

As seen in Figure 1B$_2$, the concentration of cyclin-D1 increased slightly and then declined during the healing process. Cyclin is a pro-cell cycle regulator, and an increase in it can be utilized to detect mitotic activity in all cell type[20]. The increased expression of cyclin-D1 can, in conclusion, promote cell division and proliferation, which aids in wound healing. Cyclin-D1 plays a crucial regulatory role in the normal cell cycle when the G1 phase transitions to the S phase. Uncontrolled cell division and proliferation will result from abnormal cyclin-D1 expression[23]. Therefore, the decline of cyclin-D1 expression (Figure 1B$_2$) led to cell division. At the four time points, the PC group had the greatest expression level of cyclin-D1, followed by the WS group, the FS group and the CK group with the lowest expression level. From day 3 to 7, there was no significant difference in the expression of cyclin-D1 between the FS and WS groups ($P > 0.05$), whereas the expression was significantly higher in the WS group on day 14 ($P < 0.05$).

FGF-2 displayed a similar pattern to the cyclin-D1 as seen in Figure 1B$_3$. The PC group had the highest expression level on day 3 and 5, followed by the WS group, the FS group, and the CK group. On day 7 and 14, the PC group had the highest expression, followed by the WS group, the CK group, and the FS group had the lowest expression. According to the results, the fish soup could enhance the secretion of the two growth factors. These findings suggested that snakehead fish soup can promote cell migration and proliferation, which is consistent with the results of cell tests.

3.3 Immunohistochemistry of wound tissue in rats

The comparison of immunohistochemical results of rat wound tissue in different treatment groups is shown in Figure 1C. The collagen fibers in the four groups were scarce and disordered on day 3, and their positive expression was less pronounced. The collagen fibers gradually became neatly and densely arranged from day 5 to 14, and positive expression increased.

Type I collagen is crucial for recovery from skin injuries. The fibers it produces make up about 70% of the dermal extracellular matrix and are the fundamental structural elements of the dermis. To control cell migration, survival, and growth[24], type I collagen can also function as a ligand of receptor-mediated signal transduction. Type I collagen mainly plays a role in the re-modeling stage of wound healing. As noted in Figure 1C, the generation of type I collagen was promoted by snakehead fish soup and the chueun I composite peptide. The expression of type I collagen was not visually different between the FS and WS groups, indicating that the two snakehead fish soups have equivalent effects during the traumatic re-modeling stage.

3.4 Cell proliferation

In Figure S2, the cell morphology and proliferation rate of HACAT cells, NIH3T3 cells, and HUVEC under various treatments are displayed. HACAT cells in all 7 treatment groups adhered to the wall and grew steadily to form an oval shape, which is consistent with the report of Gao et al.[25]. NIH3T3 cells in all the 7 groups attached to the wall and formed a spindle shape with distinct boundaries, which is the same as the result reported by Song et al.[26]. Similar to the research of Yang et al.[27], HUVEC cells in all groups were strongly bound to the wall and developed in either a spindle-like or goose-egg-like shape. In conclusion, the cells treated with various concentrations and sources of snakehead fish soup showed no appreciable alterations in cell shape compared to the CK group.

The OD value of the HACAT cells in the CK group was 0.966, as shown in Figure 2A, The OD value of the FS group at 10 μg/mL was 1.171, which was considerably higher than that of the CK group ($P < 0.05$). The OD value initially climbed and subsequently fell as the soup concentration was raised. The OD value of the FS at 50 μg/mL was 1.36 times as that of the CK group. The OD value dropped to 1.130 at a concentration of 100 μg/mL, and there was no significant difference between this group and the CK group ($P > 0.05$). The OD values of the WS groups at concentrations of 10, 50, and 100 μg/mL were significantly increased to 1.277, 1.318, and 1.244, respectively ($P < 0.05$). At the same concentration, the OD value of the FS group was lower than that of the WS group ($P > 0.05$).

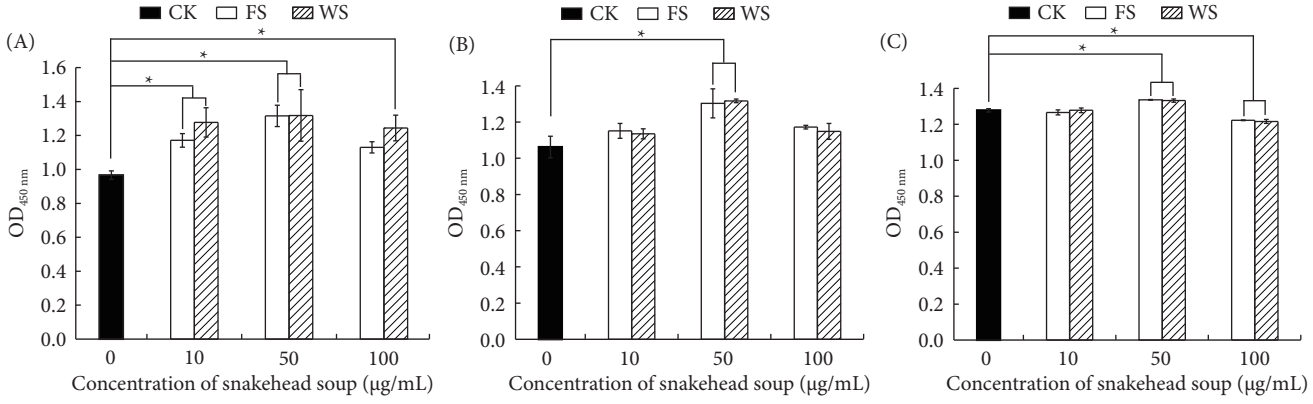

Figure 2 The proliferation rate of HACAT cells, NIH3T3 cells, and HUVEC with different snakehead fish soup and concentrations. (A) HACAT cells; (B) NIH3T3 cells; (C) HUVEC. CK, control; FS, farmed snakehead fish soup group; WS, wild snakehead fish soup group. * Indicates significant difference between the treatment group and the control group ($P < 0.05$).

The OD value of the NIH3T3 cells in the CK group was 1.062, as shown in Figure 2B. The OD values of the FS group at 10 and 100 g/mL were 1.151 and 1.172, respectively, which were slightly higher than that of the CK group ($P > 0.05$). The OD value of the FS group at 50 μg/mL was 1.303, which was significantly greater than that of the CK group ($P < 0.05$). The effect of WS on NIH3T3 cells proliferation was comparable to that of the FS.

The OD value of the HUVEC in the CK group was 1.278 (Figure 2C). The OD value of the FS group at 50 μg/mL was significantly higher than that of the CK group ($P < 0.05$), while the FS group at 100 μg/mL was significantly lower ($P < 0.05$). There was no statistically significant difference between the 10 μg/mL FS group and the CK group ($P > 0.05$). The effect of the WS on the proliferation of NIH3T3 cells was comparable to that of the FS.

The three different cells treated with the appropriate concentrations (particularly at 50 μg/mL) of the farmed or wild snakehead fish soup had significantly higher ($P < 0.05$) OD values than these of the CK group, demonstrating the promotion of cell proliferation. It might be attributed to the nutrients (fatty acids, zinc, peptides, etc.)[22], which enhanced the expression of cell growth factors (Figure 1B). Regardless of cell type, the OD value increased first and then decreased with the concentration. When the snakehead fish soup concentration was too low, the content of the corresponding functional substance was also too low, as a result, the promotion effect was weak. When the concentration was too high, the proportion of culture medium and serum content greatly decreased, which might cause the decline of cell proliferation. Our results were consistent with the findings of Taniguchi et al.[22], which work on rice bran peptide. In contrast to HUVEC, snakehead fish soup showed more pronounced effects on promoting the proliferation of HACAT and NIH3T3 cells. It might be that HUVEC has a limited capacity for proliferating, which requires large amount of nutrition[28]. The effect of the WS on cell proliferation is slightly greater than that of the FS at low concentrations, which might be owing to the higher zinc and unsaturated fatty acid content[5].

3.5 Cell migration

In Table 2 and Figure S3, the migration rates of HACAT cells, NIH3T3 cells, and HUVEC cells under various treatments, as well as their migratory state at 0 and 12 h, are displayed. As can be seen from Figure S3, the scratched areas of the three types of cells decreased after a 12 h culture, demonstrating that the three types of cells migrated under various treatments. It is clear, though, that the three types of cells in the various groups had different migratory regions. After 12 h of migration, HACAT cells still maintained ordered edges, whereas NIH3T3 cells and HUVEC cells migrated irregularly.

The migration rate of the HACAT cells in the CK group was 32.13%, as shown in Table 2. The migration rates of the FS group at 10 and 50 μg/mL were 37.17% and 36.58%, respectively. There was no statistically significant difference between them and the CK group ($P > 0.05$). However, the migration rate of the FS group (42.29%) at 100 μg/mL was considerably greater than that of the CK group ($P < 0.05$). Migration rates in the WS groups at 10 and 50 μg/mL were 41.81% and 51.66%, respectively, which were substantially higher than those of the CK group ($P < 0.05$). In comparison to the FS group, the migration rate of the WS group at

Table 2 The migration rates of HACAT cells, NIH3T3 cells, and HUVEC cells with different snakehead fish soups and concentrations.

Group	Concentrations of snakehead soups (μg/mL)	Migration rate (%)		
		HACAT cells	NIH3T3 cells	HUVEC
CK		32.13 ± 2.80^{c}	27.57 ± 2.43^{e}	12.25 ± 0.32^{b}
FS	10	37.17 ± 1.19^{bc}	36.17 ± 0.98^{bc}	20.63 ± 3.30^{a}
	50	36.58 ± 2.35^{bc}	33.64 ± 0.92^{cd}	20.21 ± 3.81^{a}
	100	42.29 ± 1.10^{b}	32.62 ± 0.17^{d}	19.79 ± 2.57^{a}
WS	10	41.81 ± 2.56^{b}	40.02 ± 1.87^{a}	20.85 ± 1.93^{a}
	50	51.66 ± 8.28^{a}	39.85 ± 1.29^{a}	19.64 ± 3.46^{a}
	100	39.22 ± 4.56^{bc}	39.00 ± 1.55^{ab}	20.01 ± 1.40^{a}

Note: CK, control; PC, positive control; FS, farmed snakehead fish soup; WS, wild snakehead fish soup. Different lowercase letters in the same column indicate significant differences in wound healing rates between different treatment groups ($P < 0.05$).

50 μg/mL was significantly higher ($P < 0.05$), while at 10 μg/mL, it was slightly higher ($P > 0.05$).

In the CK group, the migration rate of NIH3T3 cells was 27.57%. Migration rates were 36.17%, 33.64%, and 32.62% for the FS group at 10, 50, and 100 μg/mL, and 40.02%, 39.85%, and 39.00% for the WS group, respectively. In comparison to the CK group, the NIH3T3 cells migration rate of either FS or WS soup was considerably greater ($P < 0.05$). The migration rate of the WS group was substantially higher than that of the FS group at the same concentration ($P < 0.05$).

In the CK group, HUVEC migrated at a rate of 12.25%. Migration rates of the FS group at 10, 50, and 100 μg/mL were 20.63%, 20.21%, and 19.79%, respectively, and 20.85%, 19.64%, and 20.01% for the corresponding WS group. When compared to the CK group, the migratory rate of either the FS or WS group was considerably greater ($P < 0.05$). There was no discernible difference between the FS and WS groups at the same concentration ($P > 0.05$).

Cell migration is crucial to wound healing. Although the cells showed different migration states, the migrations were steadily promoted by snakehead fish soup. It might be mainly due to the increases in growth factors (Figure 1B). Zheng et al.[29] discovered that peptides derived from sea cucumber could stimulate cell migration by boosting the production of specific proteins or growth factors in cells. The migration rates of the three cells did not show concentration dependence. Horikoshi et al.[30] demonstrated that the promoting effect of α-tocopherol on cell migration was not concentration-dependent, correlating with the findings of this study. The effects of snakehead fish soup on HACAT and NIH3T3 cells migration were more pronounced than on HUVEC, which was consistent with the effect of cell proliferation (Table 2). The higher migration rate of the WS group than the FS group could be attributed to the higher zinc and unsaturated fatty acid content[5].

3.6 HUVEC tubule formation

As can be seen in Figure 3A, HUVEC form tubules under various treatments. In conjunction with Figure 2B, it is clear that the concentration of FS and WS have different effects on the formation of the HUVEC tubule.

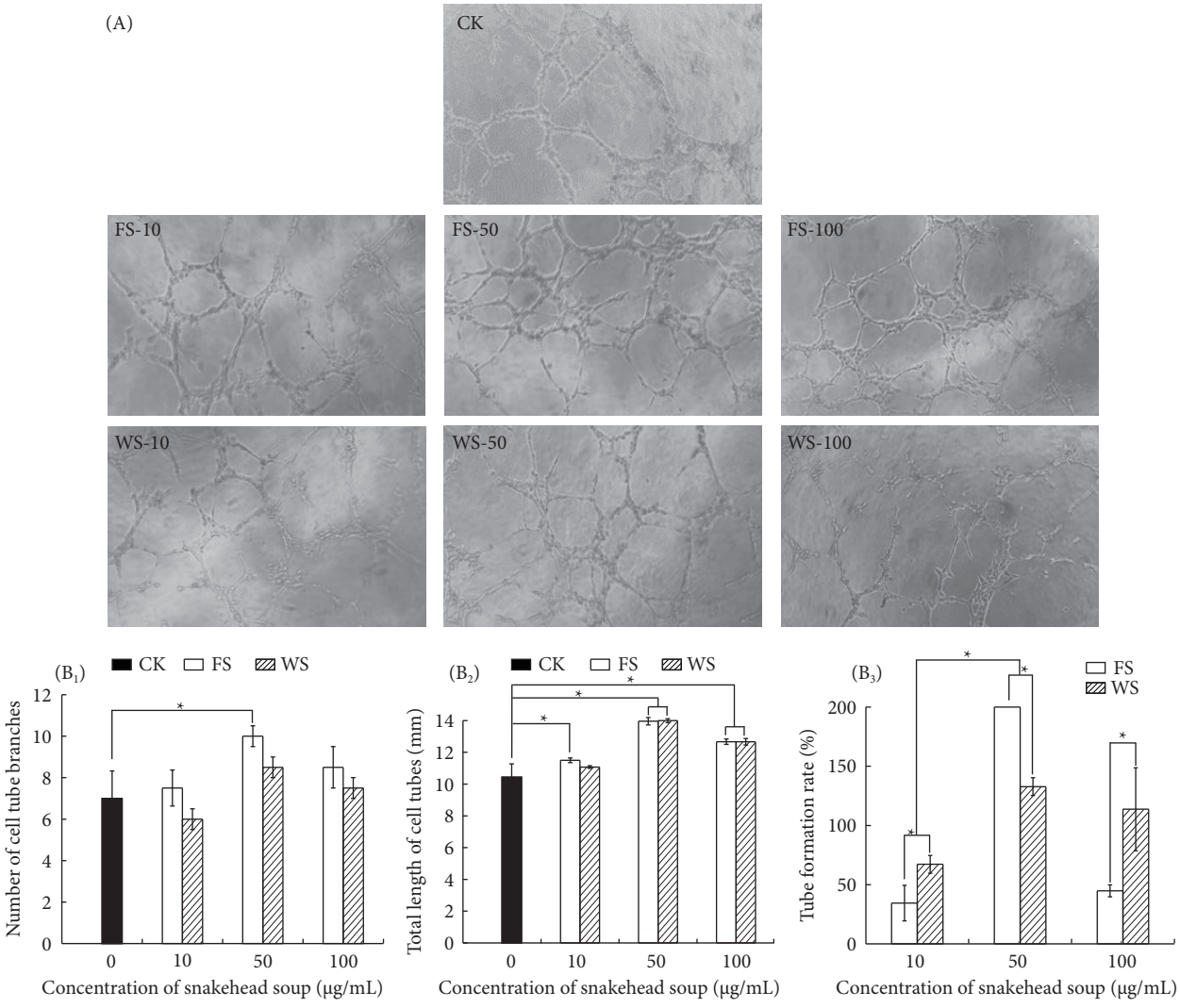

Figure 3 (A) The formation of vessel tubules by HUVEC cells with different snakehead fish soups and concentrations. (B) Analysis of the formation of tubules by HUVEC cells with different snakehead fish soups and concentrations. (B_1) Number of cell tube branches; (B_2) Total length of cell tubes; (B_3) Tubule formation rate. CK, control; FS, farmed snakehead fish soup group; WS, wild snakehead fish soup group. * Indicates significant difference between the treatment group and the blank group ($P < 0.05$).

As shown in Figure 3B$_1$, the average number of cell tubule branches in the CK group was 7. When compared to the CK group, it was not significantly different from the FS groups at 10 and 100 μg/mL ($P > 0.05$) but was substantially higher at 50 μg/mL ($P < 0.05$). Regardless of concentration, there was no significant difference between the CK group and the WS group ($P > 0.05$). The mean value of cell tube branches in the WS group was slightly lower than that in the FS group at the same concentration ($P > 0.05$).

The total length of the cell tubule in the CK group was 10.44 mm as can be seen in Figure 3B$_2$. Total cell tube lengths of the FS groups at 10, 50, and 100 μg/mL were 11.50, 13.96, and 12.67 mm, respectively, which were substantially longer than that of the CK group ($P < 0.05$). The total cell tubule length of the WS group at 10 μg/mL was 11.07 mm, which was not significantly different from the CK group ($P > 0.05$). The total cell tubule length of the WS groups at 50 and 100 μg/mL were 14.00 and 12.67 mm, respectively, and these values were substantially higher than that of the CK group ($P < 0.05$). At the same concentration, the total length was not different between the FS and WS groups ($P > 0.05$).

The tubule formation rates for the FS groups at 10, 50, and 100 μg/mL were 34.44%, 200.00%, and 44.81%, respectively, as shown in Figure 2B$_3$. And for the WS groups, they were 67.22%, 132.78%, and 113.70%, respectively. The rate of tube formation in the WS group was higher than that in the FS group at concentrations of 10 and 100 μg/mL ($P < 0.05$).

The main prerequisite for skin wound healing is capillary regeneration because they are the most fundamental structures that support metabolism[31]. Endothelial cell migration and proliferation are promoted during capillary development, resulting in the creation of blood vessels. And it can also indirectly act on other cells and stimulate the secretion of growth factors[32]. Normal physiological processes including wound healing and embryonic development depend on angiogenesis. Endothelial cells from different tissues can promote angiogenesis, among which HUVEC is the most widely used for the tubule formation assay. HUVEC helps to mediate angiogenesis while minimizing hypoxia and apoptosis[33]. Our results indicated that snakehead fish soup promoted tubule development. The tubule branches, length, and formation rate all showed the change trends of first increasing and then decreasing with the concentration, which was similar to that of cell proliferation. Although the branches and length of the cell tubule between the FS and WS soups were not significantly different ($P > 0.05$), the tubule formation rate was significantly higher for the WS group ($P < 0.05$).

3.7 Mechanistic explanation

Figure 4 explains how snakehead fish soup promotes wound healing. Inflammation, proliferation, and re-modeling are the three key stages of the skin wound healing process. Different cytokines are involved at different stages in controlling cell migration and proliferation. The macroscopically observed effects of these cellular processes include tissue development and wound healing. Epidermal cells and fibroblasts are the primary skin cells involved in wound healing[34]. Likewise, the quantity and quality of new blood vessels have a direct impact on how quickly wounds heal[32]. The most prevalent protein in mammals' skin is type I collagen. The primary structural elements of the dermis are the fibers created by collagen. It can promote fibroblast proliferation, angiogenesis, and extracellular matrix re-modeling, which speed up wound healing and increase hemostasis[16].

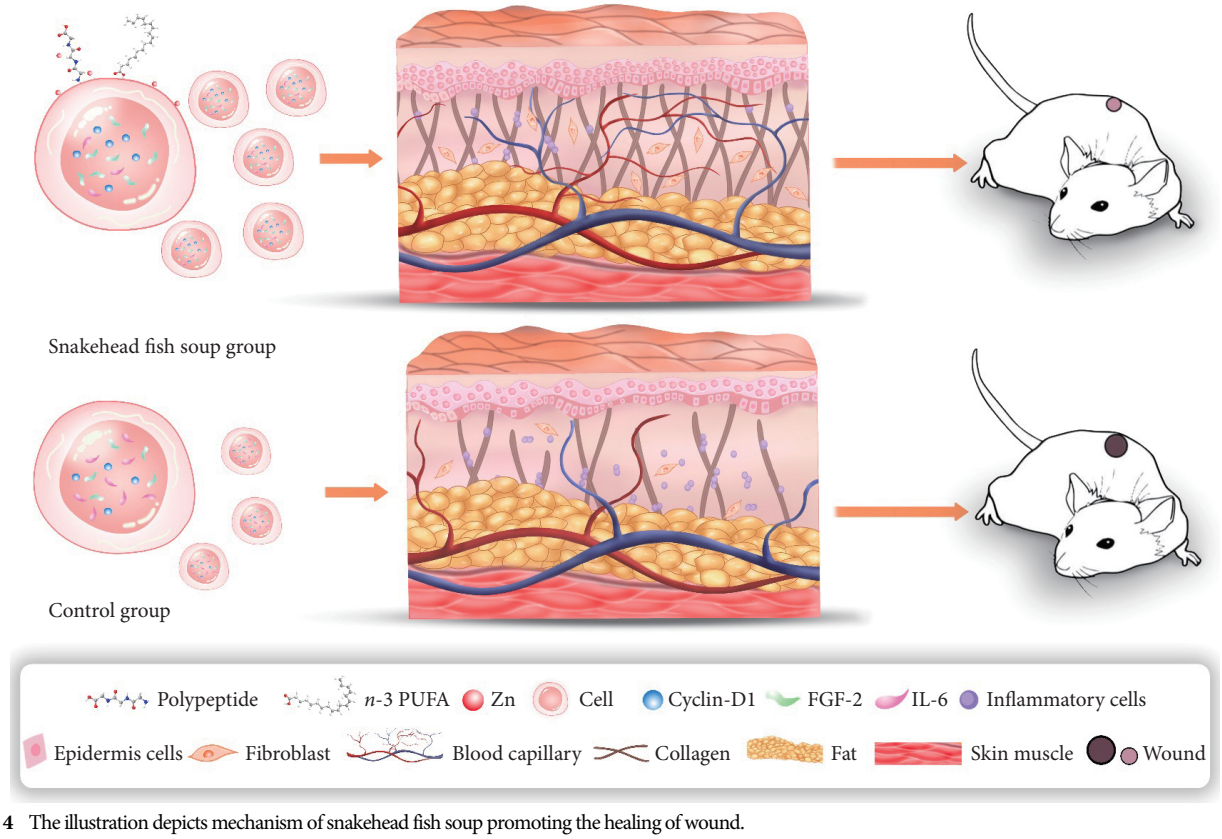

Figure 4 The illustration depicts mechanism of snakehead fish soup promoting the healing of wound.

The inflammatory response, cell proliferation, and re-modeling that occur during wound healing are all significantly influenced by snakehead fish soup. Nutrients like Zn, *n*-3 PUFA, and polypeptides are present in snakehead fish soup[5]. These nutrients might have an anti-inflammatory effect by decreasing the expression of the pro-inflammatory cytokine like IL-6 during the inflammatory phase (Figure 1B$_1$), speeding up the healing of wounds by reducing the duration between the inflammatory and proliferative stages[35]. In the cell proliferation stages, a rise in growth factors (cyclin-D1 and FGF-2) (Figure 1B$_2$, B$_3$) promoted the proliferation of epidermal cells and fibroblasts (Figure 1C), thickening and densifying the newly created epidermis. The nutrients in the snakehead fish soup also promoted cell migration (Table 2) and tubule formation by endothelial cells (Figure 3), which contributed to the growth of blood vessels. The collagen expression was enhanced during the trauma re-modeling period by the snakehead fish soup, and the fiber organization became denser and more orderly. Taken together, snakehead fish soup promoted wound healing.

4 Conclusion

It was demonstrated through animal and cell studies that snakehead fish soup could promote the healing of skin wounds. The snakehead fish soup inhibited inflammation and promoted the growth factors (cyclin-D1, FGF-2), thus promoting cell migration, proliferation, and vascular formation. And the soup promoted the production of type I collagen. The polypeptide, fatty acid, and Zn in snakehead fish soup might be responsible for the promotion of wound healing, but further research is required to verify. Wild snakehead fish soup had a slightly better impact on wound healing than farmed snakehead.

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Conflict of interest

The authors have declared no conflict interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.26599/FSAP.2024.9240065.

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