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Recent Progress in Drug Discovery

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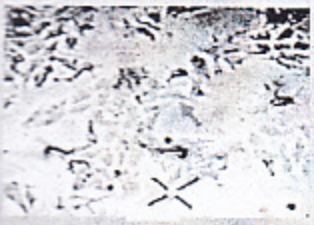

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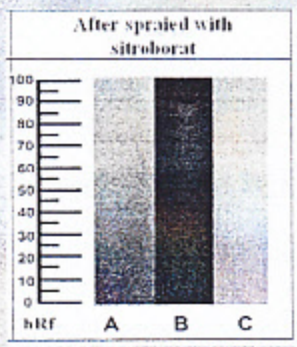

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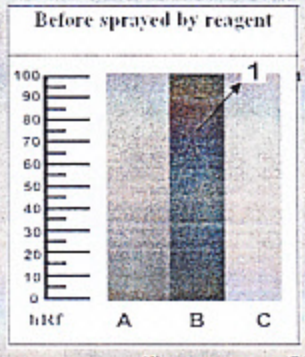

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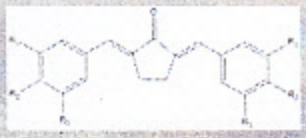

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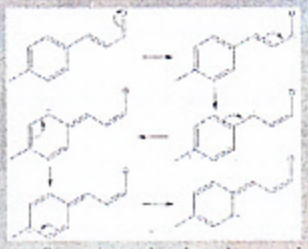

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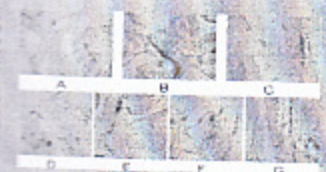

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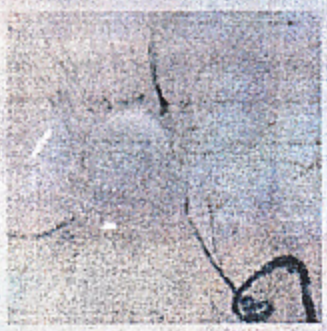

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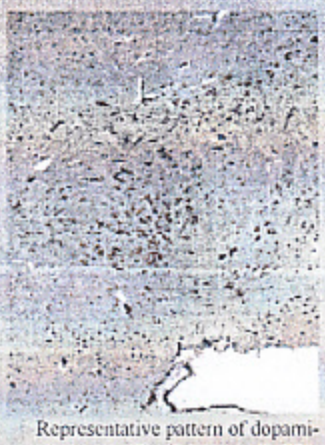

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The effect of Snakehead (*Ophiocephalus streatus*) Fish Water Extract Cream on the Recovery of Rabbit's (*Oryctolagus cuniculus*) Skin Wound Histopathologically

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Abstract

The aims of the study were to discover the effect of cream with several concentrations, duration of wound recovery, effective level needed and skin histological difference of snakehead fish water extract cream and control on wound recovery. The research design used was repeated measure designs. The samples of the study were nine rabbits divided into three groups of treatment : group I (0,5%), group II (1%), and group III (2%). Each rabbit in each group was wounded at the left and right dorsal parts as wide as 4 cm^2 with scalpel to make open wound. Wound at the left dorsal was applied with snakehead fish cream and the right dorsal with basic cream as control according to concentration of each group. The observation was done on day 3, day 6 and day 12 by measuring the size of wound and taking photo at the wounded area. Then on day 12, skin incision was done on each group to observe histopathology. The data were analyzed statistically by applying SPSS 15 program of repeated measures test. The results of the study indicated that group III (2%) had significant effect on recovery of wound by narrowing the size of wound each day ($p<0.05$) and this is proven by observing the skin histopathology showing 2% concentration improves the granulation tissue very quickly on day 12 compared to 0,5% and 1% concentration. From this study, It can be concluded that the application of snakehead fish cream 2% concentration for 12 days can accelerate the wound recovery.

Keywords: Cream, histopathology, snakehead fish, wound recovery

Introduction

All provinces in eastern part of Indonesia are rich in natural products from sea plants and animals. Gorontalo province is one of them. The snakehead fish is many found in Gorontalo Province especially in marsh and lakes. The snakehead fish is fresh water fish that has many function in improving public health and reported can increase skin wound healing. The snakehead fish have biochemistry component such as amino acids and fatty acids are very important to synthesis of collagen fiber during wound healing process.[1] One problem faced by people and surroundings is less information about the benefit of the snakehead fish. The snakehead fish is other alternative of albumin protein sources. Albumin is one of the greatest number of proteins in plasma liquid that achieves 60% for new tissue cell formation. In medicine, this albumin is used to accelerate restoration of human body cell tissue broken by surgery.[2]

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At the moment, particularly Indonesia, albumin serum often used by patients is hard to come by. For once surgery, by using this serum can reach three times of 100 ml. From the result of Suprayitno's researcher (2003), on some fish in Indonesia, the large number of albumin and amino acids were found on snakehead fish compared to those in other fish such as catfish, goldfish, gurami, etc.[3] Giving albumin therapy with snakehead water extract orally can assist wound healing process faster. Making snakehead fish water extract as wound healing at post-operation is not only cheaper than albumin serum but the wound also recover faster within three days than albumin serum. It takes three bottles of albumin serum that are very expensive. Besides that, the snakehead fish has discomfortablity taken because it smells fishy. Therefore, this is one of appropriate alternative to make snakehead fish water extract in other dosage form.[4]

Cream is semi solid dosage form like thick emulsion containing no less than 60% water and intended to external application.[5] Cream o/w is good cream for delivering active compound to target tissues, gratifying appearance and feeling comfortable after using it. This cream is not greasy and

easy to be cleaned.[6] There are many doctors and patients prefer using cream to using ointment. In this case, cream is a lot easier spread on skin layer than ointment especially cream o/w.[7]

Damage to the skin frequently provokes wounds or extensive loss of tissues and to reestablish functional integrity a complex process of wound healing begins. This sequence involves the migration of cells to the wound, proliferation of different cells types, and changes in the synthetic and secretory cellular activities.[8,9] Arachidonic acid is one of the most prominent polyunsaturated fatty acids in the skin, and its functional role depends largely on the generation of biologically potent oxidative metabolites. Eicosanoid, arachidonic acid derivates, act as inflammatory mediators. They are chemotactic agents for leukocyte, stimulating vessel dilation and induce platelet aggregation. The eicosanoid family consists of prostaglandins, thromboxanes, leukotrienes, and other many physiological and pathological process and are potent regulators of cell function.[10]

The present work evaluates the clinical and histopathological aspects of cutaneous application of snakehead fish cream with three concentrations of cream (0.5%, 1%, 2%) on the healing of surgically created open wounds on rabbits.

Materials and Methods

The study protocol and procedures were reviewed and approved by the health research ethics committee of faculty of Medicine, Hasanuddin University.

Sample extraction

The snakehead fish were bought from traditional market of equal body weight standard. The samples were cleaned by water and cut head and tail then the body of fish cut and taken bones. The sample 5 kg that have been processed and cleaned till cut small box. The samples were put into caldron by using 1 liter of water, after that steamed for 15 minutes and settled 60°C. afterwards gained snakehead fish water extract. It was put into funnel then extracted with hexan (1:1), shaked and rested till formed two layers. The Water extract layer was taken and the hexan liquid layer was thrown. The snakehead fish water extract was steamed till dry with using vacuum evaporator and got dry extract.

Snakehead Fish Cream Preparation

The snakehead fish dry extract was weighed and formulated into cream o/w dosage form in the following way : Oil phases were made by melting some materials that dissolve in oil phase. The temperature was kept at 60°C. Water phases were made by dissolving some materials that dissolve in water phase including the snakehead dry extract. The temperature was kept at 60°C. Emulsion was made by adding oil phase into water phase while mixed with electric mixer for 3 minutes then rested for 20 seconds while back mixed till formed homogenous emulsion. It was put into 10 g cream tube.

Histopathology Examination

Nine clinically healthy young male rabbits 2 – 2.5 kg of weights were used in the experiment. Three rabbits per group were maintained in a box (0.64 m^2) with some vegetables and carrots.

Local subcutaneous anesthesia with lidokain 2%, using the inverted-L technique, was administered on all rabbits. The samples of the study were nine rabbits divided into three groups of treatment : group I (0,5%), group II (1%), and group III (2%). Each rabbit in each group was wounded at the left and right dorsal parts as wide as 4 cm^2 with scalpel to make open wound. Wound at the left dorsal was applied with snakehead fish cream (1 g) and the right dorsal with basic cream as control according to concentration of each group. A bandage was placed around wound of rabbits dorsal perimeter, with the same width as the gauze that covered the wounds. After the surgical procedures, the animals continued their normal diet, and did not receive any antibiotic or anti-inflammatory treatment.

The wounds were rinsed every day observation (day 3, day 6 and day 12) with physiologic saline solution and new rayon gauze containing snakehead fish cream. The basic cream was placed on the control wounds. This procedure was repeated for 3, 6, 12 days for the first, second and third groups respectively.

The observation was done on day 3, day 6 and day 12 by measuring the size of wound and taking photo at the wounded area. Skin biopsies were taken on day 12 postoperatively and in order to take tissue adjacent to the wound, fragments were extended 1 cm from each margin and deepened to the muscular plane. The fragments were immersed in formalin buffer solution. For histological examination, the fixed specimens were processed routinely and embedded in parafin. Sections with 3 micron thickness were stained with hematoxylin and eosin.[11,12,13,14]

The data were analyzed statistically by applying SPSS 15 program of repeated measures test.

Results

Effect of snakehead fish cream of wound areas

On the 3rd,6th,12th day of each group, the experimental wounds treated with snakehead fish cream showed a statistically significant reduction ($p<0.05$) of measured area (Tables 1, 2, 3, 4). This showed that was difference of giving influence snakehead fish water extract cream in several concentration and control on wounds area healing process.

On the other hand, the observation days (on the 3rd, 6th , 12th days) affected wounds area healing. They could be showed by significant value almost approximate 0.000 (table 5). Furthermore, it was also showed that not only the interaction between duration of observation days and cream concentration that used it but also wound area healings on significant value ($p<0.05$). Besides that, very good clinical progress of treated wounds on the 12th day especially cream of 2% concentration had the fastest healing effect in accordance with duration of observation days. Meanwhile control wounds were in the final stage of the

healing process (Table 3) and exhibited small central areas of granulation tissue. However, these control wounds still needed some more time to complete healing. In Figure 1, It described average plot of wound area marginal based on observation result. This showed that cream of 0.5%, 1%, 2% concentration had same pattern in wounds healing on rabbit skin and good design especially cream 2% compared to the other concentrations. Because the result of observation showed the smallest wound area compared to the other concentrations on all of observation days.

Table 1. Area (cm^2) of Wounds on the 3rd day

Rabbits	Experimental groups					
	Control	0.5% (5 mg/1 g)	Control	1% (10 mg/1 g)	Control	2% (20 mg/1 g)
1	4	4	4	4	4	3.2
2	4	3.6	4	2.9	4	1.9
3	4	4	4	3.4	4	2.3

Table 2 . Area (Cm^2) of wounds on the 6th day

Rabbits	Experimental groups					
	Control	0.5% (5 mg/1 g)	Control	1% (10 mg/1 g)	Control	2% (20 mg/1 g)
1	3.6	3.4	3.8	3.2	3.2	2.4
2	4	2.7	3.6	2.5	2.5	1.5
3	3.6	2.9	3.1	3.1	1.8	2.1

Table 3. Area (Cm^2) of wounds on the 12th day

Rabbits	Experimental groups					
	Control	0.5% (5 mg/1 g)	Control	1% (10 mg/1 g)	Control	2% (20 mg/1 g)
1	2.7	0.8	3.1	0.7	2.4	0.7
2	3.1	1.5	2.7	1.9	1.7	0.8
3	1.7	1.4	2.6	1.6	0.9	0.5

Table 4. The influence of giving concentration of snakehead fish cream and control on wounds area

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squares
Intercept	396.365	1	396.365	1947.561		0.994
Konsent	18.963	5	3.793	18.635	0	0.886
Error	2.442	12	0.204		0	

Table 5. The influence of day on wound healing that was spread snakehead fish water extract cream compared to control

Effect		Value	F	Hypothesis df	Errof df	Sig.	Partial Eta Squared
Days	Pillai's Trace	0.931	73.657	2.000	11.000	0.000	0.931
	Wilk's Lambda	0.069	73.657	2.000	11.000	0.000	0.931
	Hotelling's trace	13.392	73.657	2.000	11.000	0.000	0.931
	Roy's Largest Root	13.392	73.657	2.000	11.000	0.000	0.931
Day + Konsent	Pillai's Trace	1.051	2.656	10.000	24.000	0.024	0.525
	Wilk's Lambda	0.171	3.125	10.000	22.000	0.012	0.587
	Hotelling's trace	3.562	3.562	10.000	20.000	0.008	0.640
	Roy's Largest Root	3.151	7.562	5.000	12.000	0.002	0.759

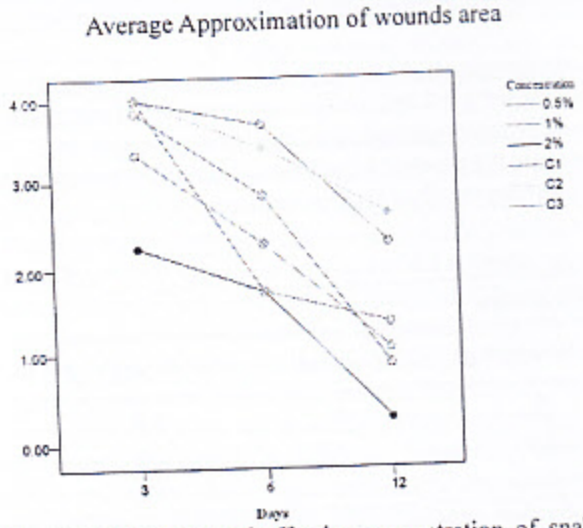


Figure 1. The Profil of effective concentration of snakehead fish water extract cream Clinical Evaluation

After skin excisions on day, it was possible to observe control wounds with hyperemia, necroses and highly vascularized granulation tissue. Although the wound borders were geometrically well defined, edema and crust surrounding the wound margins were generally observed. In this experimental stage, the most striking feature of the treated wounds was a well-preserved vascular bed with discreet hyperemia and also the presence of edema in granulation tissue (Figure 1A).

On the 3rd day, the treated wound (Cream 0.5%) showed alteration of wound form that was marked by fibrin yarns young yellow color which covered into wounds (Figure 1B). In contrast, control wounds were still visible necrosis and inflammation that marked still much blood around the wounds and produced hyperemia (Figure 2A) whereas result of wound area measuring showed no alteration of wound areas (Table 1). The treated wound (Cream 1%) showed alteration of wound form for the third rabbits that marked growing of new granulation tissues on wound margins (a little crust area) (Figure 3A). Whereas result of wound area measuring showed alteration of wound areas on the third rabbits that marked alleviation of wound areas (Table 1). The treated wound (Cream 2%) showed that alteration of wound form faster than cream 0.5% and 1% treatment (Table 1, figure 5A).

On the 6th day, the control wounds (Cream 0.5%, 1%, 2%) showed that a high quantity of crust was detected surrounding the control wounds' margins, which described granulation tissue in the central area, hyperemia and edema (Figures 2B, 4B, 6B). At the same time, treated wounds not only had no crust but also presented well-developed granulation tissue. In addition, no secretion in the treated wound bad was observed, although the presence of a mild hyperemia and few edema areas were detected (Figures 1C, 3B, 5B). Treated wounds had a geometrical alteration characterized by approximation of borders. This process was strengthened by significant reduction in the wound area (Tables 2).

On the 12th day, the control wounds were still visible hyperemia, crust and edema on the wounds especially control wound of cream 0.5% and 1% (Figure 2C, 4C). Whereas control wound of cream 2% had small areas of central granulation tissue, though it needed more time for total healing resolution (Figure 6C). At the same time, the treated wounds showed complete epithelial resurfacing with hair follicles lining the borders of wounds characterizing a centripetal evolution of contraction and presented better clinical evolution than the control wounds (Figures 1D, 3C, 5C and Table 3).

Treated wounds (Cream 0.5%)

Figure 1A (the 1st day)

1B (the 3rd day)

1C (the 6th day)

1D (the 12th day)

Control Wounds (Cream 0.5%)

Figure 2A (the 3rd day)

2B (the 6th day)

2C (the 12th day)

Treated wounds (Cream 1%)

Figure 3A (the 3rd day)

3B (the 6th day)

3C (the 12th day)

Control wounds (Cream 1%)

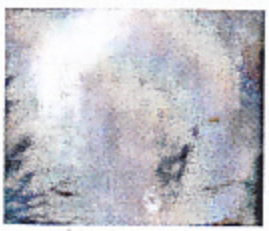
Figure 4A (the 3rd day)

4B (the 6th day)

4C (the 12th day)

Treated wounds (Cream 2%)

Figure 5A (the 3rd day)

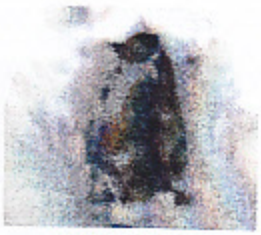


5B (the 6th day)

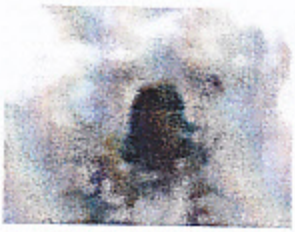
5C (the 12th day)

Control Wounds (Cream 2%)

Figure 6A (the 3rd day)



6B (the 6th day)



6C (the 12th day)

Histological evaluation

On the 12th day after excision, the control wounds had a great presence of crust, highly infiltrated granulation tissue with inflammatory cells, and many active spindle-shaped cells, which were fibroblasts (Figure 7B). It showed a mild process of epithelialization beneath an area of crust, and highly infiltrated connective tissue with polymorphonuclear leukocytes (Figure 7B). This process needed more time to conclude epithelial resurfacing due to the evident area of epithelium discontinuity. This also could be compared to normal skin (Figure 7A), and the other treated wounds (cream of 0.5%, 1%, 2% concentrations) histopathologically (Figures 7C, 7D, 7E).

On the 12th day after excision, The treated wound (Cream 0.5%) showed that the presence of epidermal crusts between the transitional area of hyperplastic epithelium and healing tissue. Granulation tissue was also more developed with minor inflammatory infiltration, the presence of plasma cells, active fibroblasts around new formed capillaries, and abundant extracellular matrix (Figure 7C).

On the 12th day after excision, the treated wound (Cream 1%) presented a strong epidermic hyperplasia, though no epidermic crests moving towards the subjacent connective tissue were detected. Furthermore, fibroblasts exhibited a high activity of synthesis and a mild inflammatory infiltration in the subepidermic tissue next to the transitional area between the skin and cicatricial tissue. In treated wounds the epithelium still presented a hyperplastic appearance, and fibroblasts with characteristic arrangement of miofibroblasts were observed. Also, the treated wound (Cream 1%) presented a reduction of inflammatory cells in ground substance and in the newly formed vascular bed (Figure 7D).

On the 12th day after excision, the treated wound (Cream 2%) described a better clinical progress and microscopic healing process, than those of control wounds and treated wounds (Cream 0.5% and 1%) at 12 days. Wound treated with cream 2% showed total epithelial resurfacing

and subjacent connective tissue very active fibroblastic cells in the synthesis of extracellular matrix, especially collagen fibers. In addition, the amorphous ground substance had a mild quantity of inflammatory cells (Figure 7E).

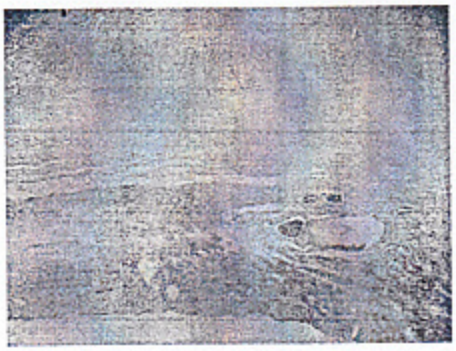


Figure 7A. The Normal Skin consists of epidermis layer (arrow), dermis layer (star), and sub-cutan tissue (arrowhead)

Figure 7B. The Control Wound (12th day), observe extensive area of crust (large arrow) over the granulation tissue. The granulation tissue presenting several active fibroblasts (arrow) and inflammatory cells (arrowheads) surrounding capillaries (star).

Figure 7C. The Treated Wound (Cream 0.5%), note the hyperplasic epithelium near the transitional region of the wound bad and the presence of epidermal crusts (large narrow). The granulation tissue exhibiting intense production of extracellular matrix (arrow). Deep areas of granulation tissue with capillaries (star) surrounded by plasma cells.

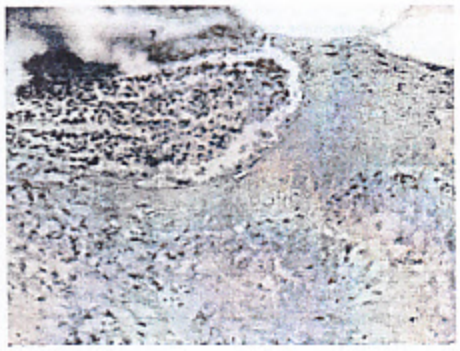


Figure 7D. The Treated wound (Cream 1%), healing transitional area showing epidermal crest of hyperplasic epithelium migrating to the connective tissue. The hyperplasic epithelium showing intense inflammatory infiltration (large arrow). The connective tissue presenting miofibroblastic cells organized in the traction area (arrows), under the hyperplasic epithelium (large arrow). Deep areas of granulation tissue with capillary (star) surrounded by fibroblasts (arrow).

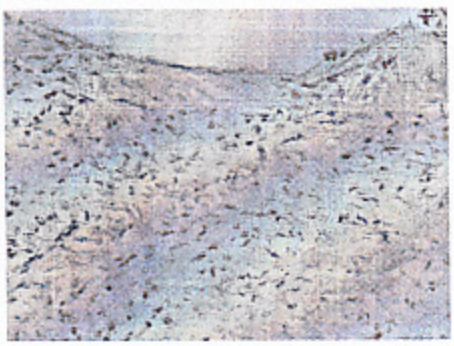


Figure 7E. The Treated Wound (Cream 2%), the epithelialization region (large arrow) and subjacent connective tissue in remodeling process (star). Detail of hyperplasic epithelium without crests (large arrow) and ground substance of connective tissue. Detail of connective tissue showing active fibroblast and few polimorphonuclear cells (arrows).

Discussion

Essential fatty acids (EFA) effectiveness on problems related to skin lesions has been studied since 1929, when the first observation of skin lesions provoked by a shortage of EFA levels in foods were made.[15,16] Linn and shepered[17] described the cure of those alterations by topical application of EFAs. Essential fatty acid, linolenic acid and arachidonic acid are polyunsaturated vegetable lipids that can not be synthesized by the animal organisms, necessitating renewal by diet.[18]

According to Prottey[19] linoleic and arachidonic acids are important in the maintenance of a cutaneous barrier to water loss and as precursor of prostaglandins, which are thought to be involved in both the regulation of cell division and differentiation of epidermis and consequently in

the control of skin scaliness. Elias and Brown[20] showed that EFAs are precursors of pharmacologically active substances such as thromboxanes, prostaciclins, prostaglandins and others that are involved in cellular division regulation and epidermic differentiation.

The snakehead fish is fresh water fish that contain many proteins such as albumin and amino acids i.e. glisin, metionin, isoleusin, triptofan and lisin. Besides that, they also have unsaturated fatty acids such as arachidonic acid, eicosapentanoic acid and minerals such as vitamin A, Ca, Mg, Cu, Fe, Mn, Ni, Co and Zn. All of biochemistry component from the snakehead fish can increase wound healing process on the skin.[1,2,21]

The arachidonic acid and eicosapentanoic acid are a powerful pro-inflammatory mediator that causes a migration of granulocytes and macrophages as well as important changes in granulation tissue.[1,2] The arachidonic acid is a precursor from prostaglandin that important act out in wound healing with increasing of prostaglandin synthesis.

In open wounds, epithelialization occurs after the development of a granulation bed[22], which is also formed when the repair process of connective tissue is necessary. By definition, this is an inflammatory growth of connective tissue characterized by synthesis and deposition of products of fibrogenic cells that can be divided in three phases : (a) cell mobilization and proliferation; and (b) synthesis and deposition of collagen products, glycosaminoglycans and extracellular matrix, and (c) a final organization or remodeling of the wound scar.[1,9]

This paper evaluated the evolution of the wound healing process in adult rabbits utilizing cutaneous application of snakehead fish cream with designing of snakehead fish water extract in cream dosage form with three concentrations i.e. cream 0.5%, 1% and 2%, describing a simple methodology for wound management and the progression of wound healing.

By the twelfth day after excision, the treated wound of cream 0.5% was more evolved macroscopically than the control wounds due to a great development of granulation tissue, contraction in the outer edge of borders and absence of secretion. On the other hand, the control wounds had extensive areas of crust, hyperemia and edema. Histopathological data in treated wound of cream 0.5% indicated a more advanced repair process than control wounds, due to fewer inflammatory infiltration and presence of active fibroblasts around newly formed vessels.

While evaluating wounds on the twelfth day of cream 1% had well arranged miofibroblastic cells in the contraction area while in the control wounds these cellular types were in high synthesis activity of matrix components showing little organization in wound borders.

By the twelfth day after excision, the treated wound of cream 2% showed effective concentration in accelerating of wound healing process statistically. In this case, the acceleration of the inflammatory process can be explaned by biological and biochemical features of the arachidonic acids and amino acids. This polyunsaturated fatty acid is changed by desaturation and elongation of its molecule to arachidonic acid which is metabolized via the 5-lipoxygenase and cyclooxygenase pathway in leukotrienes (LTB_4, LTC_4 and LTD_4). prostaglandins (PGE_2, PGF_2, PGD_2 and PGI_2) and thromboxane A2 by polymorphonuclear cells. These substances produced by arachidonic acids have pro-inflammatory properties that can stimulate new vascularization locally, cell migration, proliferation and fibroblastic differentiation as well as extracellular matrix synthesis.[1,23,24]

On the area of wound contraction, miofibroblast cells had very important role in closing wound from outer margin to wound center. In this case, it can be showed by cream 2% that indicated the importance of cytokines and growth factors in the wound healing process. Many of these factors, such as the epidermal growth factor (EGF), fibroblastic basic growth factor (bFGF); platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF-α) and interleukin-6 (IL-6) are cytokines that stimulate keratinocyte proliferation. The topical use of bFGF, PDGF and TGF-α in wounds not only facilitates the migration of monocytes, neutrophils, macrophages and fibroblasts, but also stimulates the proliferation of the granulation tissue.[25] Histological analysis of the treated tissue showed that the fish oil acted as a local immunogen causing the migration of both polymorphonuclear leucocytes and macrophages, and resulting in proliferative activity during the appearance of granulation tissue.

In addition, In the snakehead fish contain many fish oils such as arachidonic acid, eicosapentanoic and high protein such as albumin and amino acids especially glisin, lisin and minerals especially Zn, Fe. The all of above can accelerate wound healing process.

The present work showed by morphometrical, clinical and histological assays that snakehead fish cream improved the granulation tissue formation and epithelial resurfacing of wounds. Therefore, the snakehead fish cream containing high concentration of unsaturated fatty acids and amino acids, can be indicated as a therapeutic alternative for wound healing process.

Conclusion

1. The giving of snakehead fish water extract cream with 0.5%, 1%, 2% concentrations and control gave significant influence of wound areas healing.
2. The duration of giving snakehead fish water extract cream on all of cream concentration affected wound areas healing significantly and occurred interaction between observation days and concentrations of cream on wound areas healing on significant value $\alpha = 5\%$.
3. The effective level of snakehead fish water extract cream that could accelerate wound healing process was 2% concentration.
4. The histological pictures of rabbit skin on the 12th day gave significant difference on 0.5%, 1%, 2% concentrations and control concerning wound healing process.

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