HISTOLOGICAL STUDY OF THE SKIN OF SULAWESI MEDAKA FISH (*ORYZIAS CELEBENSIS*) DURING THE PROCESS OF PUNCTURE WOUND HEALING

Dwi Kesuma Sari*, Kadek Dian Krisna Putri K, Jessica Tania Loto, Andi Azifah Cahyani, Nurul Sulfi Andini Khusnul Yagin and Irma Andriani[1]

Study Program of Veterinary Medicine, Faculty of Medicine, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10, Makassar, Indonesia

Received on: 06.10.2020
Accepted on: 18.11.2020

ABSTRACT

The Sulawesi medaka fish (*Oryzias celebensis*) as an animal model should be done as additional information because this fish is the endemic fish in Sulawesi Island. This study aims to determine the histological picture of the Sulawesi Medaka fish's skin during the wound healing process. This study used the Sulawesi medaka fish which were divided into three groups: K1 as a control group without treatment, K2 a group of the puncture treatment group, and K3 the fish group with puncture wounds and then stored in methylene water. The wound healing process in K2 and K3 was then observed on days 1, 3, 5, and 7. The results show that the redness around the puncture wound decreases every day, and the size is reduced in K2 and K3. The observations on the first day of K2 and K3 showed that there were a lot of inflammatory cells, fibroblasts, and collagen fibers, and there were very few and no granulation cells were found, while on day three, there were still more inflammatory cells, fibroblasts and collagen fibers and the granulation tissue is still not found. Histological observations on the 5th day of K2 showed that there were more fibroblasts, inflammatory cells were decreased, and a large amount of granulation tissue was found, while on the 7th day of K2, fibroblasts were seen but not as much as 3rd and 5th day with high density, inflammatory cells were also still visible, more collagen fibers were found on K2 and K3.

Key words: Histology, skin, Sulawesi medaka fish, puncture wound

Introduction

Indonesia is the second largest fish-producing country globally, providing seafood products for international and domestic markets. In Indonesia, fishery products are estimated to provide 54 per cent of all animal protein consumed in human food, making the country one of the highest resource-dependent countries in the world (USAID, 2018)

Indonesian waters are the "homeland of oryzias" as more than half of the genus Oryzias reportedly inhabits Indonesian waters. The genus Oryzias is better known as rice fish because it generally occupies rice ecosystems, ponds, sewers, and lakes. Spreadrice fish covers fresh water to the sea. Oryzias was a member of the family Adrianicthyidae. The family is widespread in southern and eastern Asia. Various studies related to rice fish (Oryzias sp.) have been widely conducted in East Asian countries such as Japan, Korea, Taiwan, and China. In these countries, the genus Oryzias is better known as medaka fish and has evolved into a model fish or experimental animal in various studies such as organogenesis research, molecular genetics, and molecular evolution (Fahmi *et al*., 2015).

The health of aquatic populations needs to be considered for several reasons. The disease can affect the productivity or overall ability of ecosystems to sustain fish or shellfish populations, reducing the amount of seafood that can be harvested. The general public will strongly feel the impact of animals that are sick, dead, or dying. In particular, certain diseases, such as skin ulcers in fish, can affect aesthetically (Noga, 2000)

Skin diseases and skin sores are common in many species of aquaculture fish. Some studies report the effects of environmental factors, hormones, and food components on deep skin wounds' healing rate. The need can weaken the skin and increase the risk of mechanical damage. Damage to the skin can threaten the body of fish's defense function, thus reducing animal welfare. If the skin is severely injured (epidermis damage), wound healing treatment is done well to restore tissue integrity. Wound healing treatment begins from re-epithelialization, accompanied by inflammation and the onset of tissue repair and re-modeling (Sveen *et al*., 2018; Siswonto *et al*., 2020).

Efforts to develop Medaka Sulawesi fish (*Oryzias celebensis*) as test animals are considered necessary to be done through research on Sulawesi medaka fish to add information about Medaka fish on Sulawesi island considering this fish is one of the endemic fish on Sulawesi island (Sari *et al*., 2018).

Materials and Methods

Maintenance and Treatment

This research took place from March-July 2020, which included fish sampling activities in Pattunuang River, Bantimurung, Maros Regency of South Sulawesi. Analysis of the histology of the wound healing process in medaka fish was conducted in the Integrated Laboratory, Veterinary Studies Program, Faculty of Medicine, Hasanuddin University. This research has gone through the examination stage of research ethics at the Research Ethics Commission of the Faculty of Medicine, Hasanuddin University, for animal laboratory.

Sulawesi medaka fish are caught directly using traps in Pattunuang River, Bantimurung, Maros Regency. The river is

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10, Makassar.
*Corresponding author email: dwiksari@vet.unhas.ac.id

located close to the local settlements. The sample was taken as many as 18 tails and acclimatized for seven days, and continued with the treatment stage. The medaka fish is then transferred to a prepared container. Further water replacement is carried out every two times per week, and feeding is carried out daily. The feed used is a commercial floating fish feed. The sample was divided into three groups, namely: Control sample (K1), Group 1, as a control group of Sulawesi medaka fish taken as many as six fishes. This group was used as a control group to distinguish the histology of Sulawesi medaka fish that were not stabbed (not wound) with those stabbed (group 2) and who were stabbed and given methylene blue (group 3); Puncture Wound (K2), In group 2 samples were treated with puncture wounds on each fish. The total number of fish injured is 6. First, anesthetized fish by administering clove oil with a dose of 2-3 drops/L of water then carried out stabbing using a 14 gauge needle as deep as approximately 0.05 cm on the body of the fish. Fish was released back into the aquarium and then observed once every two days for seven days; Methylene Blue administration for Puncture Wound Healing (K3), In group 3 samples were given the treatment of stab wounds in each fish as in group 2 and given Methylene blue with a dose of administration of 2 ppm and dripped on the aquarium before the Sulawesi medaka fish was transferred into the aquarium. Methylene Blue includes chemical drugs used in the prevention and treatment of cultivated organisms infected with diseases, including ulcers on the body of fish. Observations are carried out once every two days for seven days.

Macroscopic observations

Comparisons between groups are made with macroscopic observations before microscopic observations are made and described in a table and documentation obtained from observations on the 1st, 3rd, 5th, and 7th days based on inflammatory signs such as redness (color) and wound size.

Microscopic observations

The preparation of histopathology has several stages, according to Marwati *et al*. (2015), whereby fish that have died and preserved using formaldehyde in the body is given the necessary parts then inserted in tissue cassette. Furthermore, processing and embedding by inserting tissue cassette into the tissue processor. The tissue in the cassette tissue is then dehydrated by inserting tissue into a multi-stored alcohol solution 70%, 80%, 90%, 95%, 100% alcohol solution, followed by clearing into xylol, and the next stage is infiltration with liquid paraffin. Furthermore, the process of embedding, cutting with microtome and coloring with eosin hematoxylin. Then this sample was observed under a microscope with an additional 40x magnification.

Data analysis

Analysis of the data is done descriptively qualitatively by comparing the wound healing rate from the first day after the need until the development of the healing process in the wound.

Results and Discussion

Macroscopic overview

Macroscopic observations in the K1 control group without treatment showed no wounds on the skin of the fish (Fig. 1). The fish's skin color is pale yellow-orange and, after soaking formaldehyde for two days becomes gray. This group will be used as a macroscopic and microscopic image control of the wound healing process on the skin (Fig. 2).

K2 group is a group where fish are treated with stab wounds using a 14 gauge needle. The wound healing parameter observed in the puncture wound group (K2) is the skin's redness around the injury, which is one of the signs of inflammation and the size of the wound from the 1st day until the 7th day.

K3 is a group where fish are treated with stab wounds using a 14 gauge needle, and then the fish is kept in a container whose water has methylene blue with a dose of 2 ppm. The wound healing parameter observed in the puncture wound group with the administration of methylene blue (K3) is the skin's redness around the wound, which is one of the inflammatory signs and the injury from the 1st day and the 7th day.

Fish are identified individually; the wound area of each fish is measured at different points in time. Each injured area is determined by the difference in skin color between the injured and the entire region. When the initially injured area is no longer entirely distinguishable *e.g*. regenerated and pigmented, each wound is considered to have healed. Macroscopic observations made in the puncture wound group (K2) obtained results that, on the 1st day until the 7th day there was a change in the wound's color and size. The redness of the skin around the need and the size of the wound is also large (0.056 mm). Very clear on day 1 (Fig. 3 a), on the 3rd day the redness of the skin around the need is more minimal. The wound's size has also started to shrink (0.043 mm) but is still visible (Fig. 3 b). On the 5th day, the wound's redness is no longer visible, the size is also very small and difficult to observe (0.023 mm) (Fig. 3c) and on the 7th day, the redness of the wound is gone. The wound is also closed (Fig. 3d).

Macroscopic observations conducted on the group of puncture wounds and the administration of methylene blue (K3) obtained results that on the 1st and 7th day, there was a difference in skin color around the injury and the size of the wound. The redness of the skin on the 1st-day wound looks red, and the size of the wound is visible (0.068 mm) (Fig. 4a), while on the 7th day, the skin color around the wound is no longer visible, and the wound is also invisible (Fig. 4b).

Observations on puncture wounds in both K2 and K3 show that wound healing in Sulawesi medaka fish (*Oryzias celebensis*) occurs very quickly compared to wound healing in mammals. This is following the statement by Richardson *et al.* (2013) that there are taste differences in mammals and fish's wound healing process. Healing wounds in fish takes place faster because there is a mucosa that helps prevent foreign objects from the outside so that the healing of wounds quickly occurs (Eissa *et al.,* 2013). The epidermis and mucus layer have an essential role during the stable condition and skin repair. The surface of the epithelial is protected by mucus produced and secreted by epidermis cells. The function and dynamic of the mucus layer on the skin of the fish is very Complex. The injured fish also showed excessive mucus secretions. Gelmucus can improve wound healing through hemolytic activity andpromotes vasozone-smooth muscle cell resistance. The cells that secrete the mucus are found in all fish' epidermis, but the number varies significantly with location

and species (Mumford *et al.,* 2007).

Puncture wounds in fish given Methylene blue have a faster wound healing process, this is in accordance with Kurniawan (2012), that farmers widely use methylene blue because it is bactericidal and functional and has extensive uses in both the field of biology and chemistry. Some studies have shown that blue Methylene will not damage body tissue or other histological tissues.

Microscopic observations

The K1 control group observed histologically was a fish that was not given any treatment after acclimatization, so its microscopic picture does not show the absence of inflammatory cells and granulated tissue, but fibroblast cells are found in small amounts, and collagen is found in large quantities (Fig. 5).

Histology observations on Sulawesi medaka fish skin (Oryzias celebensis) showed a difference from the 1st to the 7th day. Observations on day 1 of K2 showed very few inflammatory cells (IC/Inflammatory cells), fibroblast cells (Fb/Fibroblasts) and collagen fibers (CF/Collagen fiber) and no granulation cells (G/Granulation) (Fig. 6a), while on the 3rd day showed many inflammatory cells, fibroblast cells are more numerous than on day 1. In contrast, collagen fibers are seen more than on day one, and granulation tissue is still not found (Fig. 6b). Histology observations on the 5th day of K2 showed more and more fibroblast cells. Inflammatory cells are still there but not as many on the 1st and 3rd days, in addition to being found in large numbers of granulation cells (Fig. 6c). In contrast, on the 7th day found fibroblasts with not as many amounts on the 3rd and 5th days but with a high density, inflammatory cells are also still visible, collagen fibers are increasingly, and many granulation tissues are found (Fig. 6d).

Histology observations on Sulawesi medaka fish skin (*Oryzias celebensis*) showed a difference from the 1st to the 7th day. Statements made on K3 fish showed results that on day 1 there were very many inflammatory cells, fibroblast cells, and tiny collagen fibers, and no granulation cells were found (Fig. 7a). In contrast, on the 7th day only collagen fibers were found (Fig. 7b).

Based on the table above, in group K1, no inflammatory cells and granulated tissues were found, but fibroblasts were found in small amounts, and collagen was found in large quantities. This is in accordance with the assertion by (Mary *et al.,* 2020) that fibroblasts are the most common cell type found in connective tissue. These cells produce a diverse group of products, including type I, III, and IV collagen, proteoglycans, fibronectin, laminin, glycosaminoglycan, metalloproteinase, and even prostaglandins. Fibroblasts remain in silent form until stimuli activate protein synthesis and contractile mechanisms. These cells synthesize and rearrange extracellular matrisks found in the skin, lungs, heart, kidneys, liver, eyes, and other organs; therefore, fibroblasts and collagen remain found in normal tissues.

The results of observations in group K2, on the 1st day, found many inflammatory cells, so given a score of 1, fibroblasts the number is very small, so it is given a score of 1 and no granulation collagen tissue, so the score is four each. The 3rd day K2 group found there were still many inflammatory cells, so given a score of 1, fibroblasts of a large number, and a high density so that the score parameters were 3. In contrast, granulation tissue was not found (score 4), and collagen was already starting to be seen (score 3). Group K2 for the 5th day of inflammatory tissue has begun to decrease compared to the 1st, and 3rd days, while the number of fibroblasts is increasing (score 3), collagen is more than the previous day (score 2). At the same time, granulation cells are very much (score 1). Observations on K2 day 7 obtained the number of inflammatory cells that has begun to decrease (score 3), fibroblasts are not much found (score 1), and collagen and granulation cells are very much found (score 1).

Observations on K3 day 1 showed that many inflammatory cells were found (score 1), fibroblasts were not very large (score 2), while granulation and collagen cells had not been found (score 4). Observations on K3 day -7 showed that no other cells were found other than collagen fibers (score 1). This is under Schmid (2013) statement stating that re-epithelialization of fish skin wounds occurs within a few hours, while granulation tissue does not begin to form until the 2nd day after the wound reaches the most significant level four days. This granulation network contains type I, FN and TN-C collagen. The healing process of wounds in fish and amphibians is with epithelial cells (keratosis) that migrate from the surrounding tissue intact then cover the surface of the wound. The re-epithelialization stage occurs before the epidermis begins producing mucus that serves as a barrier to protecting wounds from the external environment. Small superficial wounds can close within a few hours, but the process of re-epithelialization depends on factors such as temperature, wound dimensions, stress, and nutrition (Sveen *et al.,* 2018).

Conclusion

Based on the results of the research, it can be concluded that there are differences in macroscopic picture and histological picture in the process of healing puncture wounds in Sulawesi medaka fish (*Oryzias celebensis*), wherein macroscopic view found that the more reddish color in the skin around the wound becomes less. The size of the wound is also reduced. In contrast, in the histological picture (microscopic), it is found that inflammatory cells are located on the 1st to 7th day, granulation began to be found on the 5th day, fibroblasts were found from the 1st to the 7th day with a growing number, and collagen fibers were seen more and more on the 7th day.

Acknowledgment

The author would like to thank all the petrified parties in this study, especially the staff at the Integrated Laboratory, Veterinary Studies Program, Faculty of Medicine, Hasanuddin University. The author also thanked the Ministry of Research and Technology for the Competitive Grant of Basic Research No. 1516/UN4.22/PT.01.03/2020.

References

Eissa AE, Zaki MM, Saeid S, Abdelsalam M, Ali HM, Moustafa AA, Ibrahim TB and Abumhara AA (2013) *In vitro* evaluation of the efficacy of hemodialysate (Solcoseryl®) as a wound healing agent in Nile tilapia (*Oreochromis niloticus*). *Int. J. Vet. Sci. and Med.* 1:57-64. https://doi.org/10.1016/j.ijvsm.2013.09.003.

Fahmi MR, Prasetyo AB and Vidiakusuma R (2015) Potential of Medaka fish (*Oryzias woworae*, *O. javanicus* and *O. profundicola*) as Ornamental fish and model fish. *Prosiding Seminar Nasional Ikan. Ke.***8**: 8.

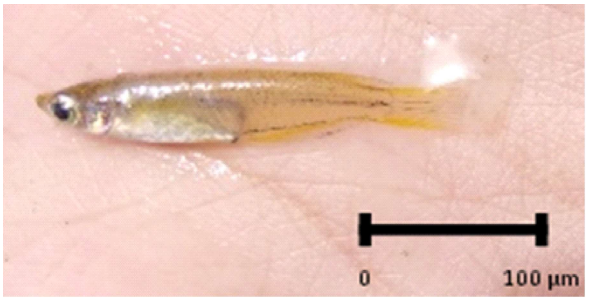

Fig.1: Medaka Sulawesi fish (*Oryzias celebensis*)

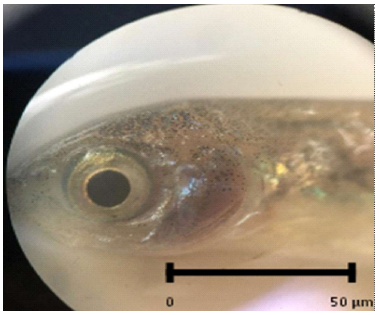

Fig. 2: Macroscopic Overview of Control Groups. Scale: 1:50 µm

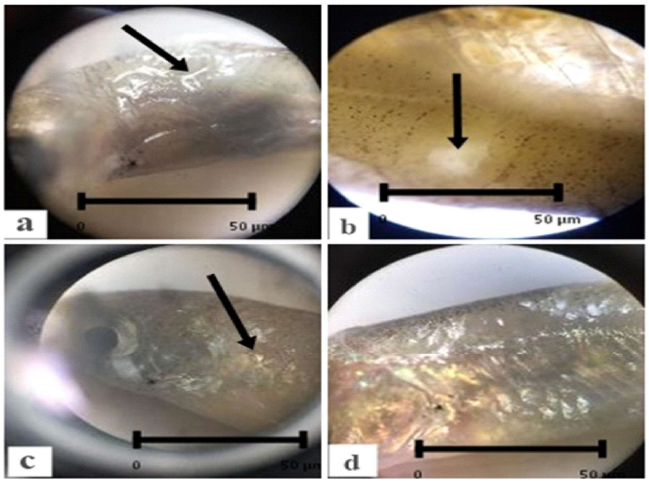

Fig. 3: Macroscopic depiction of the skin of medaka fish with puncture wounds and without administration of methylene blue on the 1st day (a), day 3 (b), day 5 (c), day 7 (d). Scale: 1:50 ìm

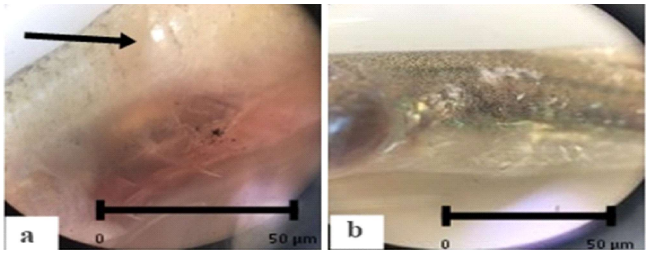

Fig. 4: Macroscopic depiction of the skin of medaka fish with puncture wounds with methylene blue administration on the 1st day (a) and day 7 (b). Scale: 1:50 µm

Kurniawan A (2012) *Penyakit Akuatik*. UBB Press.

Marwati, Riauwaty M and Syawal H (2015) Histopathology Of Gill And Brain of Tilapia (*Oreochromis niloticus*) That were Infected with Streptococcus iniae. *Jurnal Online Mahasiswa*

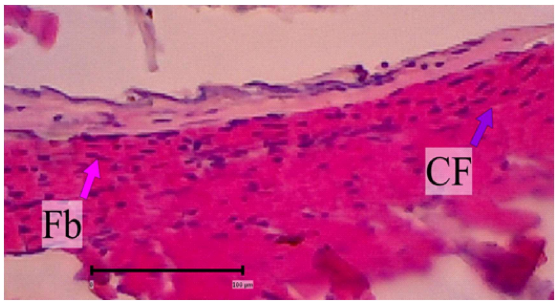

Fig. 5: Control group overview (without treatment). Ket: (Fb) Fibroblasts Cells (Fibroblasts), and (CF) Collagen fiber (HE 40x). In microscopic observations, the control group of fibroblast cells and collagen fibers was seen (HE 40x).

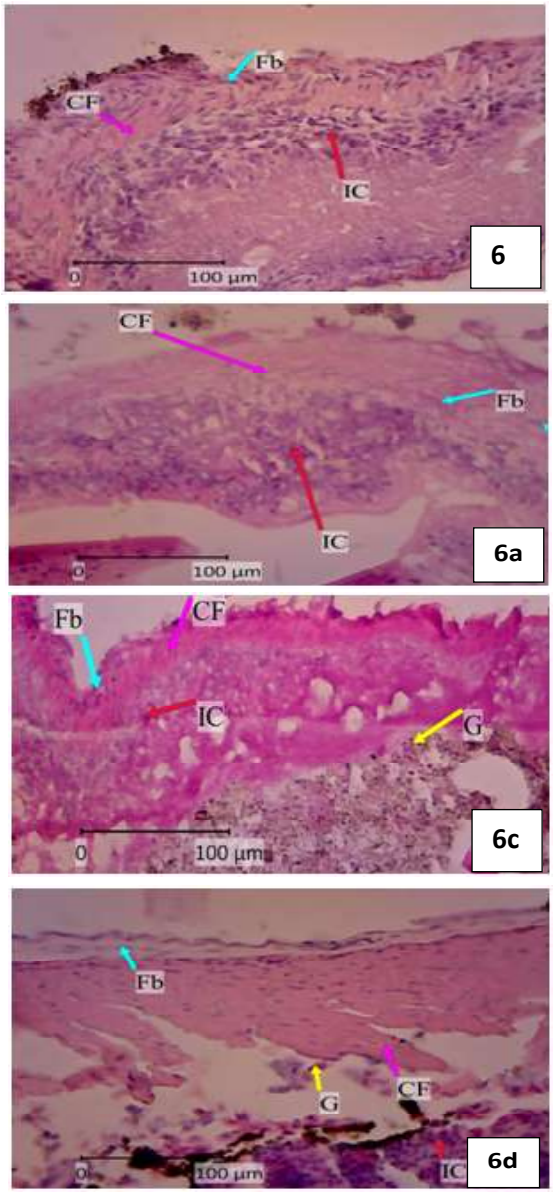

Fig. 6: Group K2. Histology of fish skin medaka Sulawesi (*Oryzias celebensis*) on day 1 (6a), day 5 (6c), and day 7 (6d). Ket: (IC) Inflammatory cell, (Fb) Fibroblasts, and (CF) Collagen fiber (HE 40x).

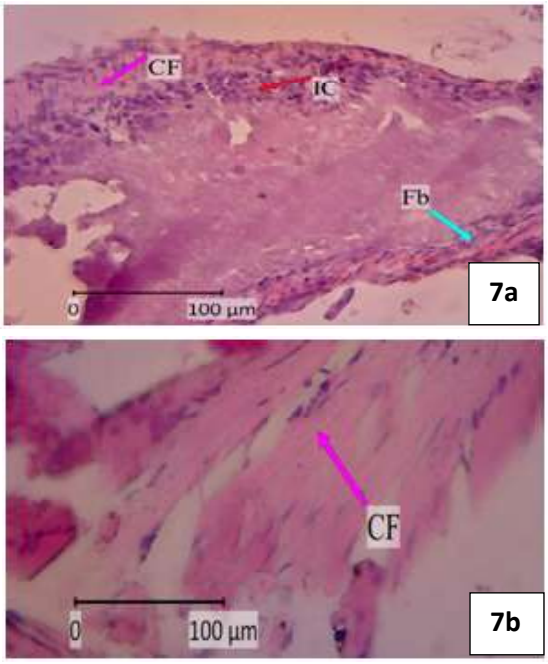

Fig. 7: Group K3. Histology of fish skin medaka Sulawesi (O*ryzias celebensis*) on day 1 (7a) and day 7 (7b). Ket: (IC) Inflammatory cell, (Fb) Fibroblasts, and (CF) Collagen fiber (HE 40x).

Table 1: Microscopic observation

Day-	Treatment	Inflammatory Cells	Fibroblast	Granulation Tissue	Collagen
	Control (K1)	4	1	4	1
1	Puncture wound group (K2)	1	1	4	4
3		1	3	4	3
5		2	3	1	2
7		3	1	1	1
1	Group Puncture Wounds and Methylene Blue Administration (K3)	1	2	4	3
7		3	0	4	1

Description: Inflammatory Cells: Many (1), Medium (2), Some (3), None (4)
Fibroblasts: Many (3), medium (2), some (1), none (0)
Granulation: lots (1), medium (2), slight (3), none (4)
Collagen: lots (1), medium (2), minimal (3), none (4)

Fakultas Perikanan dan Ilmu Kelautan Universitas Riau. 13 pages.

Mary KD, Julia HM and Limaiem F (2020) Histology, Fibroblast, *Stat Pearls*. https://www.ncbi.nlm.nih.gov/books/NBK541065/

Mumford S, Heidel J, Smith C, Morrison J, Macconnell B and Blazer V (2007) Fish Histology and Histopathology. *J. Geoscience and Environ. Protection*.

Noga EJ (2000) Skin ulcers in fish: Pfiesteria and other etiologies. *Toxicol. Pathol.*

Richardson R, Slanchev K, Kraus C, Knyphausen P, Eming S and Hammerschmidt M (2013) Adult zebrafish as a model system for cutaneous wound-healing research. *J. Investigative Dermatol.* **133(**6):1655-1665.

Sari DK, Andriani I and Yaqin K (2018) Histological study of the circulatory system of Sulawesi Medaka fish (*Oryzias celebensis*) for animal model research. *J. Physic.* 1028.

Schmidt JG (2013) Wound healing in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) with a focus on gene expresion and wound imaging. pp. 194.

Siswanto MM, Pradhany RC, Yenniastoeti BP and Pangkahila A (2020) Ethanolic extract of black grapes (*Vitis vinifera*) ameliorates overtraining-induced pancreatic β-cells and muscle damage. *Jurnal Riset Veteriner Indonesia.* **4**(2): 69-75.

Sveen LR, Timmerhaus G, Krasnov A, Takle H, Stefansson SO, Handeland SO and Ytteborg E (2018) High fish density delays wound healing in Atlantic salmon (*Salmo salar*). *Scientific Reports*.

USAID (2018) *T*he State of The Sea: Indonesia. Volume One An Overview of Marine Resource Management for Small-Scale Fisheries and Critical Marine Habitats in Indonesia. USAID.