

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

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

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## 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 5<sup>th</sup> 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 7<sup>th</sup> day of K2, fibroblasts were seen but not as much as 3<sup>rd</sup> and 5<sup>th</sup> 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 a food 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 Adrianichthyidae. 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 store 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; Siswanto *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

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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 1<sup>st</sup>, 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> 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 1<sup>st</sup> day until the 7<sup>th</sup> 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 1<sup>st</sup> day and the 7<sup>th</sup> 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 1<sup>st</sup> day until the 7<sup>th</sup> 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 3<sup>rd</sup> 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 5<sup>th</sup> 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 7<sup>th</sup> 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 1<sup>st</sup> and 7<sup>th</sup> day, there was a difference in skin color around the injury and the size of the wound. The redness of the skin on the 1<sup>st</sup>-day wound looks red, and the size of the wound is visible (0.068 mm) (Fig. 4a), while on the 7<sup>th</sup> 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 healing 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 and promotes 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 1<sup>st</sup> to the 7<sup>th</sup> 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 3<sup>rd</sup> 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 5<sup>th</sup> day of K2 showed more and more fibroblast cells. Inflammatory cells are still there but not as many on the 1<sup>st</sup> and 3<sup>rd</sup> days, in addition to being found in large numbers of granulation cells (Fig. 6c). In contrast, on the 7<sup>th</sup> day found fibroblasts with not as many amounts on the 3<sup>rd</sup> and 5<sup>th</sup> 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 1<sup>st</sup> to the 7<sup>th</sup> 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 7<sup>th</sup> 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 matrices 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 1<sup>st</sup> 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 3<sup>rd</sup> 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 5<sup>th</sup> day of inflammatory tissue has begun to decrease compared to the 1<sup>st</sup>, and 3<sup>rd</sup> 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 5<sup>th</sup> 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 1<sup>st</sup> to 7<sup>th</sup> day, granulation began to be found on the 5<sup>th</sup> day, fibroblasts were found from the 1<sup>st</sup> to the 7<sup>th</sup> day with a growing number, and collagen fibers were seen more and more on the 7<sup>th</sup> day.

#### Acknowledgment

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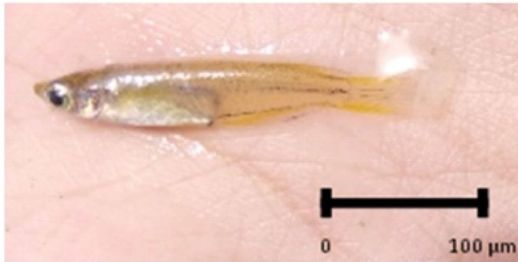


Fig.1: Medaka Sulawesi fish (*Oryzias celebensis*)

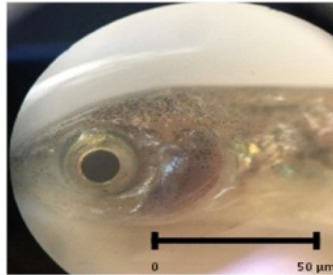


Fig. 2: Macroscopic Overview of Control Groups. Scale: 1:50  $\mu\text{m}$

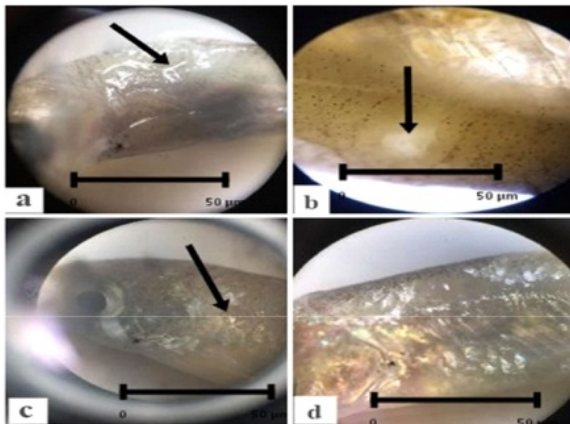


Fig. 3: Macroscopic depiction of the skin of medaka fish in puncture wounds and without administration of methylene blue on the 1st day (a), day 3 (b), day 5 (c), day 7 (d). Scale: 1:50  $\mu\text{m}$

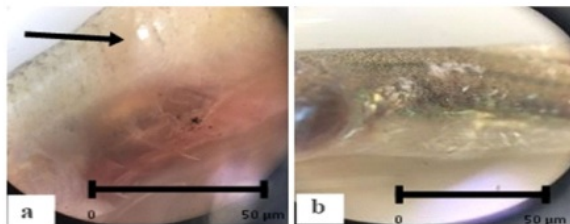


Fig. 4: Macroscopic depiction of the skin of medaka fish with puncture wounds with methylene blue administration on the 1st day (a) and day 7 (b). Scale: 1:50  $\mu\text{m}$

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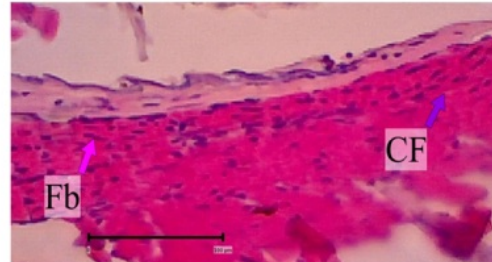


Fig. 5: Control group overview (without treatment). Ket: (Fb) Fibroblasts Cells (Fibroblasts), and (CF) Collagen fiber (HE 40x). In microscopic observations, the control group of fibroblast cells and collagen fibers was seen (HE 40x).

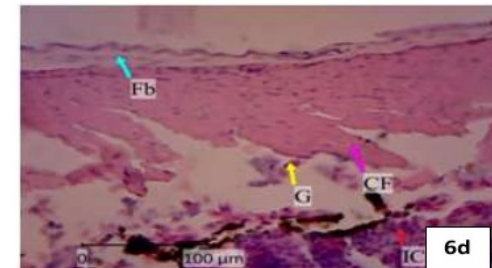
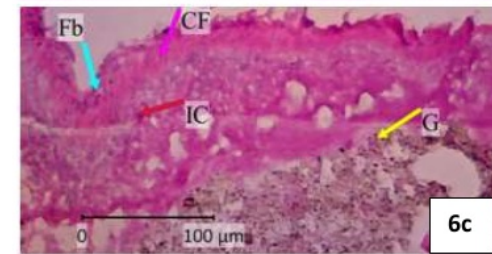
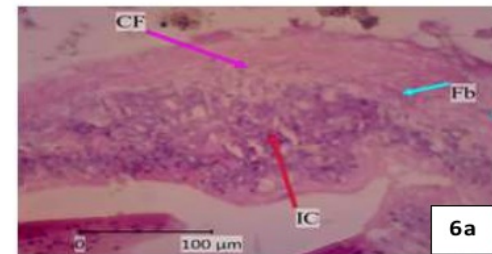
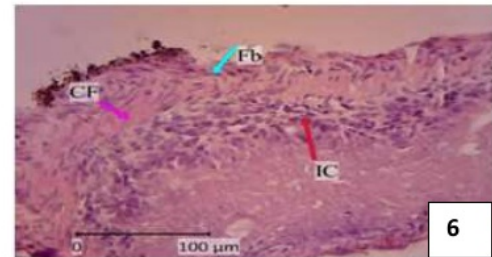


Fig. 6: Group K2. Histology of fish skin medaka Sulawesi (*Oryzias celebensis*) on day 1 (6a), day 5 (6c), and day 7 (6d). Ket: (IC) Inflammatory cell, (Fb) Fibroblasts, and (CF) Collagen fiber (HE 40x).

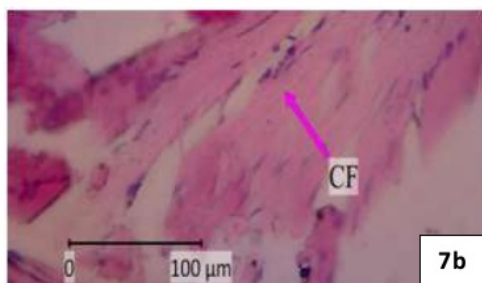
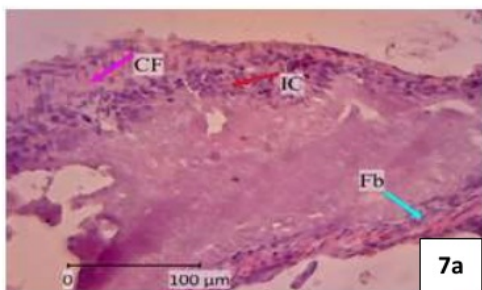


Fig. 7: Group K3. Histology of fish skin medaka Sulawesi (*Oryzias celebensis*) on day 1 (7a) and day 7 (7b). Ket: (IC) Inflammatory cell, (Fb) Fibroblasts, and (CF) Collagen fiber (HE 40x).

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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)

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