Expression of Toll-Like Receptors (TLR2 AND TLR4) In The Wistar Dental Pulp After Being Given GIC (Glass Ionomer Cement) With Addition Of Gouramy Fish Scales

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***Abstract*: Gouramy fish scales have a similar composition with the teeth and alveolar bones. Material must be able to recognize microorganisms via TLR receptors. TLR2 and TLR4 have outstanding abilities in identifying a variety of pathogenic ligands. Aim. Analyzing the expression of TLR2 and TLR4 on dental pulp after being given GIC with addition of Gouramy fish scales. 24 male Wistar rats were grouped into: K (rats made holes in their teeth), P1 (rats made holes in their teeth + GIC, P2 (rats made holes in their teeth + GIC + Gouramy fish scales). Day 3, 7 were made of tooth for TLR2 and TLR4 analysis using immunohistochemical methods. Cells were counted under a light microscope with magnification of 1000X and 400X. The data obtained were analyzed using ANOVA followed by LSD test. The result showed that the scales of Gouramy and GIC were able to reduce the expression of TLR2 and TLR4, caused by antibacterial content in both GIC and Gouramy fish scales. Gouramy fish scales also contain bioactive components that can act as anti-inflammatory. Conclusion. The addition of Gouramy fish scales to GIC decreased the expression of TLR2 and TLR4 in the dental pulp of Wistar rats.**

***Keywords*: Anti-inflammatory; bioactive components; immunohistochemistry; immunomodulators; receptor; restorative**

1. Introduction

Dental restoration not only removes caries and improves tooth function, but also aims to prevent caries from occurring again. Therefore, a dental caries management pattern is needed which is not only in the form of restorations, but also preventive efforts, so that the demineralization process caused by cariogenic bacteria stops [1]. Therefore, a dental filling material that has antibacterial and anti-inflammatory properties is needed. Restoration materials that are still frequently used are tooth-colored restorative materials, namely composite resin and Glass Ionomer Cement (GIC). The advantages of GIC are able to prevent secondary caries, good esthetics, biocompatible, low solubility, good translucency and antibacterial properties. The drawbacks are its brittle nature and resistance to fracture and low wear, so it cannot be used for restoration of teeth with high chewing pressure. Several studies have added to the GIC filling material with a certain composition ratio to increase inhibition against cariogenic bacteria such as the addition of propolis and chitosan [2,3]. In this study, we added Gouramy fish scales, because their composition is similar to teeth and bones. Fish scales consist of collagen type-I fibrils, partially mineralized with hydroxyapatite (mineral content 16-59%). The outer layer of fish scales is significantly more mineralized and is often referred to as the bone layer, while the inner layer (basalt or collagen layer) [4]. Gouramy scales contain 29% protein, 8-40.9%, carbohydrates by differences 2.0-5.7%, calcium 5.0-8.6%, and chitin as much as 0.4-3.7% [5]. The content of collagen, flavonoids, catechins from Gouramy fish scales, it is thought to have an antibacterial role [6]. Therefore, besides chitosan, collagen has been developed for antibacterial applications, wound coverings, and regenerative medicine [7]. Fish is one type of food that is widely consumed by Indonesians to meet the need for animal protein compared to other products. The increase in production and consumption certainly has an impact on the large amount of waste it produces. Fish waste consists of heads, bones, guts, fins, and scales. One of these wastes is often found and is thought to have the potential to be used in the field of dentistry [6]. Increasing of production and consumption of gouramy is of course accompanied by the accumulation of gouramy waste. If this waste is not handled properly or is not utilized, it will cause environmental pollution. The waste produced by the fishing industry in Indonesia ranges from 25-30% or around 3.6 million tons per year. Fish waste can be in the form of leftover pieces of meat, skin, head, offal, bones, scales and fins (KKP, 2007) in [6].

Research on the use of fish scales has existed, such as the addition of 2, 5 and 8% by weight of tilapia fish scales hydroxyapatite to GIC can reduce solubility [8]. Media Indonesia wrote that Diana Fitri Muslimah and Adityakrisna Yoshi Putra Wigianto used Gouramy fish scales to form a nanocalcium paste as an ingredient for remineralizing whitespot lesions [9]. Our previous research proved that Gouramy fish scales increase the viability of salivary leukocyte and monocyte cells against Streptococcus mutans [10]. In addition, thorns of Osphronemus gouramy inhibited inflammation in dental pulp of wistar rats through decreasing IL-1β and the number of inflammatory cells [11].

Antibacterial and anti-inflammatory can occur when microorganisms enter the body and cause an immune response. Microorganisms will be recognized by a receptor, including TLR. The majority of microorganism infections were mediated by TLR2 and TLR4. These two TLRs are the most characteristic of recognizing PRR (Pattern Recognition Receptors) of hosts which not only identify pathogens that attack outside the cell, but also intracellular pathogens that are captured in the endosome or lysosomes. TLR2 and 4 can sense PAMP pathogen associated molecular patterns from various kinds of infectious micro and macro

organisms [12]. So, it can be understood, that the prevention of a disease symptom starts from the recognition ability carried out by TLR.
Based on the foregoing, the researcher wanted to analyze the expression of TLR2 and TLR4 in the dental pulp after being given GIC with the addition of Gouramy fish scales.

2. Materials and Methods

2.1 Animal model
We used 24 male Wistar Rats, male, weight 250 gr BW, age 12-14 weeks from the Animal House at Biomedic laboratory Faculty of Dentistry Jember University. Before being used as research subjects, they passed the ethical test at the Ethics Commission of the Faculty of Dentistry Jember University no. 967/UN25.8/KEPK/DL/2020. Every two experimental animals kept in one cage. Every day the experimental animals were weighed and their health controlled and given standard food.

2.2 Fish scales

The fish scales were placed in an aluminum pan when the water temperature reaches 80°C. The scales were boiled for 60 minutes. Fish scales were oven-dried for 48 hours at 65°C. Fish scales were mashed, added 2L of 90% ethanol and soaked for 3x24 hours.

2.3 Material GCI (Glass Ionomer Restorative Cement)

The GCI filling material used was Gc Gold Label 9 Posterior Restorative GIC produced in the United States, consisting of 5g powder, 3g liquid (2.4 mL).

2.4 Research Groups

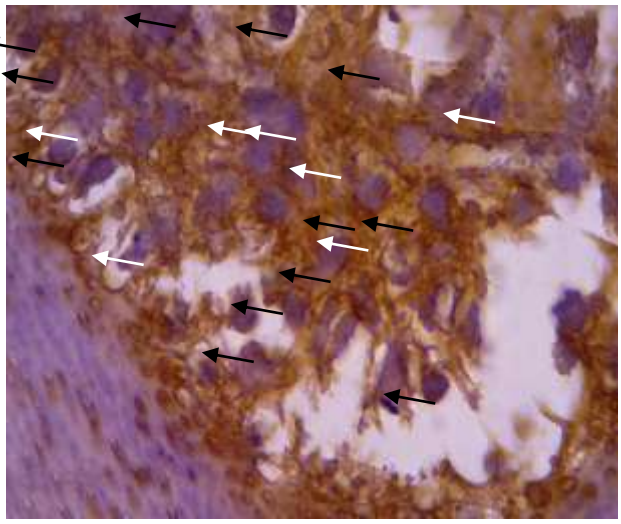
This study consisted of 3 research groups. C (Control): rats were made holes in their tooth. P1 (Treatment group 1): rats were made holes in their teeth and filled with GIC filling material. P2 (Treatment group 2): rats were made holes in their teeth and filled with GIC filling material with added 5% gouramy scales. The hole was made in the mandibular first molar using a fissure bur no.3 of the occlusal 0.5 mm deep. On day 3, 7, teeth were prepared for TLR2 and TLR4 analysis using immunohistochemical methods.

2.5 Preparation Preparation and Immunohistochemistry methods

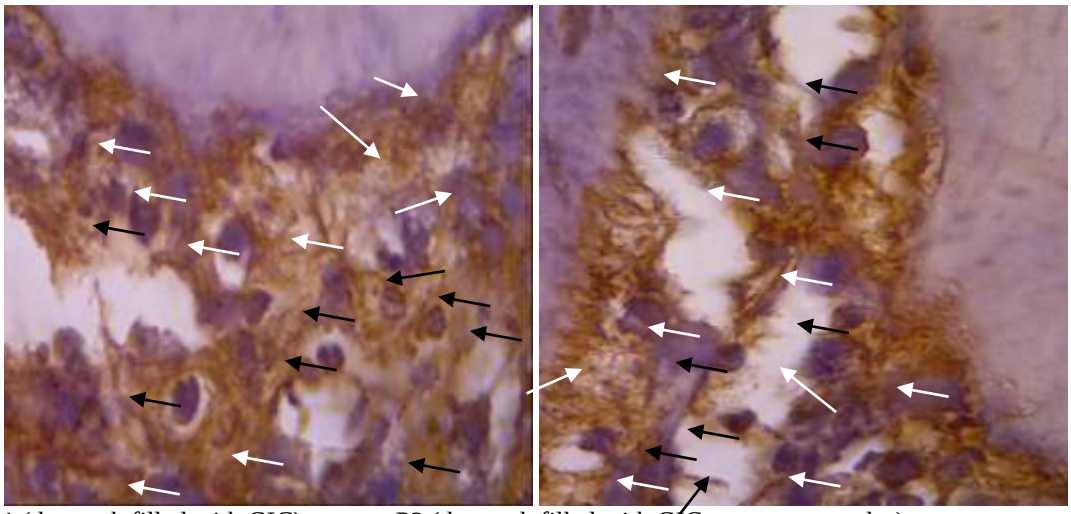
Dental and surrounding tissue were included in 10% formalin. After the decalcification was carried out, the tooth wase cut vertically to make preparations. The next step were deparaffinized, dried with tissue, marked with a pen, blocked paraffin, soaked in PBS (Phosphate Buffered Saline), dripped with 0.025% trypsin, washed with PBS 3 times each 2 - 3 minutes, dripped with H2O2 washed with PBS 3 times each. 2 - 3 minutes each, cutting with 5-6 microns microtome, the object were given polyolesin.
Immunohistochemical staining by: preparations incubated in Back-ground Sniper (protein blocking solution) 10 minutes at room temperature. The primary antibody was added 20 µl per preparation (adjusted until all parts were flooded) and then incised on a damp tray at room temperature (25ºC) for 60 minutes, washed with PBS for 2 x 2 minutes. Then, it Were given secondary antibody as much as 20 µl per preparation added and incubated at room temperature (25ºC) for 15 minutes then washed with PBS. The preparations were incubated with TrekAvidin-HRP (Horseradish Peroxidase) reagent for 10 minutes, then washed with PBS. DAB (Diamonobenzinidine) chromogen substrate was prepared: 1 µl Betazoid DAB Chromogen diluted with 600 µl Betazoid DAB Substarte Buffer immediately before use. The preparations were incubated in the above DAB chromogen substrate as much as 20 µl per preparation for 10 minutes, then the preparations were washed under running water. Mayer hematoxylin (counterstain) paint was added to the preparations, incubated for 1-3 minutes, then it washed under running water and dried. The preparation was dipped in alcohol, dried and cleaned. Then the entellan was poured on, covered with a cover glass. The cells analyzed were brown and counted under a light microscope with a magnification of 400 and 1000 times.

3. Results and Discussion

The expression of TLR2 and TLR4 could be observed in leukocyte cells, namely macrophages, neutrophils and lymphocytes using a light microscope with a magnification of 400 and 1000x (Figures 1 and 3). Cells expressing TLR2 and TLR4 will appear brownish cytoplasm, while cells that do not express TLR2 and TLR4 will appear cytoplasm in purple. Besides that, the brownish color around the cells also showed that the control group (just made holes) was darker and more visible.
Based on the results of the One Way ANOVA statistical test above, there was a significant difference with a significance value of 0.000 ($p < 0.05$). Followed by the LSD test to determine significant differences with a significance degree of 95% ($p < 0.05$). The results of this study indicated that the scales of gouramy were able to reduce the expression of TLR2 and TLR4, which means that there were fewer microorganisms infecting rat tooth pulp than controls.

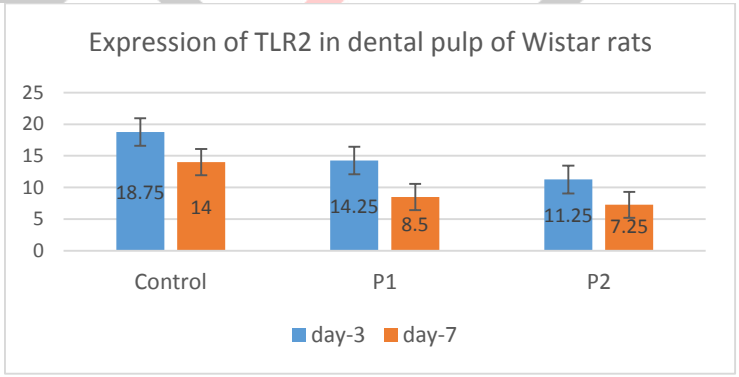


Control (the tooth were made a hole)

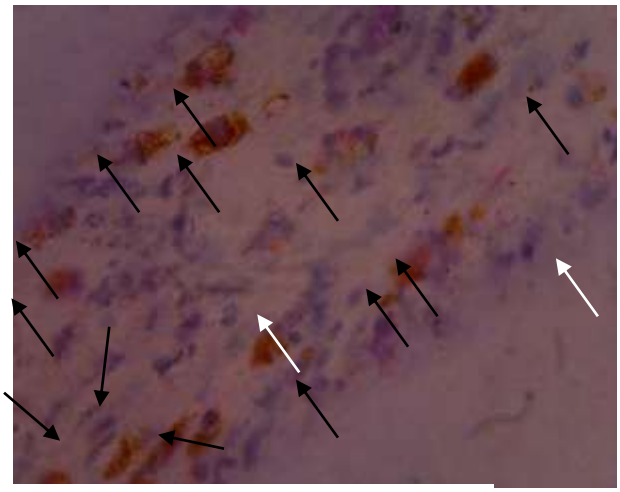


P1 (the tooth filled with GIC) P2 (the tooth filled with GIC + gouramy scales)

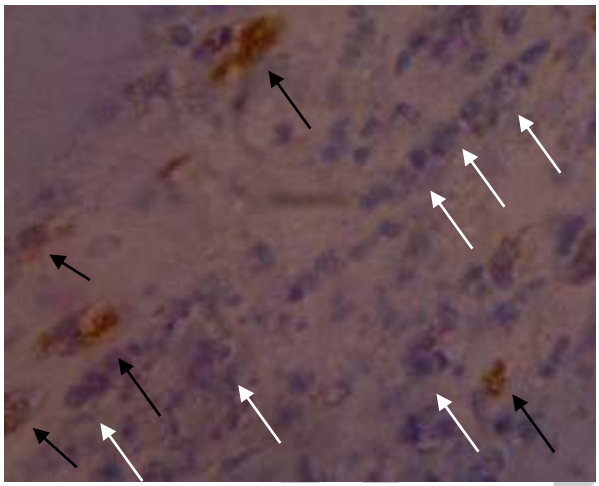
Figure 1. Immunohistochemical results of TLR2 expression. Cells expressed TLR2 were shown black arrows, while yellow arrows were cells that did not express TLR2. Analysis using a light microscope at 1000x magnification.



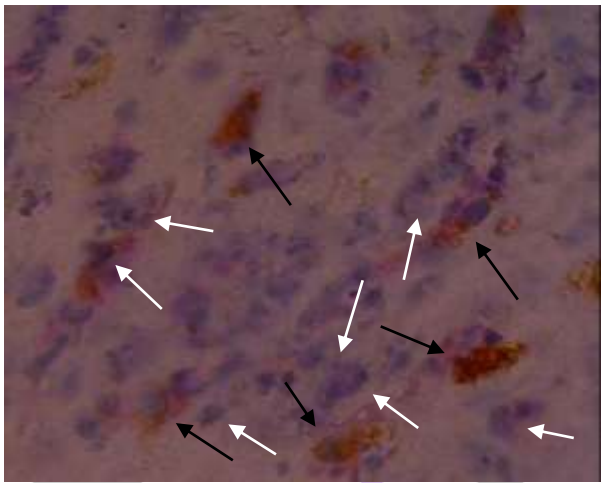
Figurer 2. The figure was Bar chart Expression of TLR2 in dental pulp of Wistar rats



Control (the tooth were made made a hole)



P1 (the tooth filled with GIC)



P2 (the tooth filled with GIC + gouramy scales)

Figure 3. Immunohistochemical results of TLR4 expression. Cells expressed TLR4 were shown black arrows, while yellow arrows were cells that did not express TLR4. Analysis using a light microscope at 400x magnification.

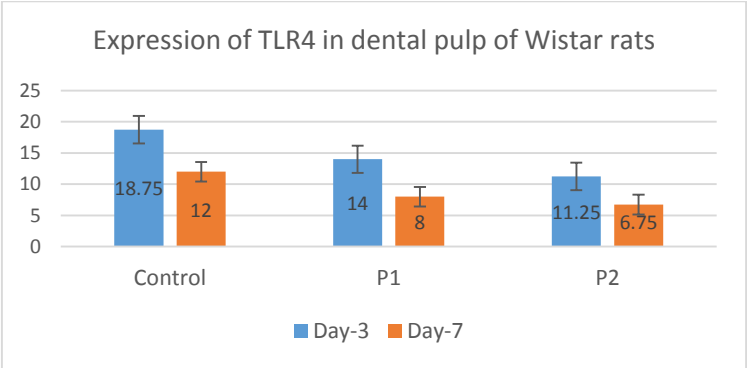


Figure 4. Bart chart Expression of TLR4 in dental pulp of Wistar rats

Physiologically, the homeostatic mechanism will continue so that inflammatory cells will remain in the network for immune system defense, which begins with receptor activation [13]. That the receptors on the cell surface are not constant because they can be influenced by the situation and condition of a tissue. Inflammatory cells will trigger an increase in the expression of TLR2 (up-regulation) and TLR4 if there are pathogenic microorganisms that cause infection to enter the tissue. The introduction of PAMPs from these microorganisms will induce an immune response to eliminate pathogens [14,15].

Mechanical trauma that causes the dentin to open could facilitate the invasion of pathogenic microorganisms into the pulp tissue. These microorganisms will enter the pulp tissue through the dentinal tubules to bind to the extracellular receptors of TLR2 and TLR4. TLR2 and 4 in pulp tissue are found in various cells, namely macrophages, neutrophils, lymphocytes, odontoblasts, fibroblasts, pulp stem cells, dendritic cells, and mast cells. TLR2 and 4 found on the cell surface will hold attachments with PAMPs from microorganisms to trigger intracellular transduction signals [13, 14, 16,17]. Furthermore, activating the transcription factor, namely NF-κβ, can stimulate the production of various proinflammatory cytokines such as TNFα, IL-1, IL-8 and so on [18]. The

increase in inflammatory cells would trigger an increased in TLR2 and TLR4 expression that occurs from day 3 to activate the immune response and provide a signal to produce proinflammatory cytokines. Enggardipta research explained that inflammatory cell infiltration reaches a peak on day 4 to day 5 [19]. It was understandable why on day 7 the number of cells expressing TLR2 and 4 also decreased.

This was thought to be due to the anti-inflammatory and antibacterial properties of the gouramy scales. The content contained in gouramy scaless were amino acids, omega-3, omega-6, and flavonoids [6, 20]. Flavonoids act as an immunomodulator. Natural ingredients that contain flavonoids have the ability to boost the immune system [21]. The biological activity of flavonoid compounds against bacteria is carried out by damaging the cytoplasmic membrane of bacteria consisting of lipids and amino acids by reacting them with alcohol groups in flavonoid compounds. This process will cause the cell wall to be damaged and these compounds can enter the bacterial cell nucleus. The difference in polarity between the lipids that make up DNA and the alcohol groups in flavonoid compounds will cause a reaction that damages the lipid structure of the bacterial DNA so that the bacteria will experience lysis and die [22].

Amino acids will accelerate the work of macrophages, resulting in TGF (Tumor Growth Factor) -β1 to stimulate fibroblast production. TGF-β1 will diffuse through the dentinal tubules, resulting in an increase in collagen synthesis and can inhibit TLR signaling activity. The TLR signaling inhibitory pathway can be regulated in several ways, namely the receptors are internalized by the endocytosis process, the receptors are destroyed or stored in vesicles, and the receptor activity is modified so that they cannot be ligand-bound, or bound, but form ligand-receptor complexes that do not induce cellular responses. The mechanism of action of these amino acids will affect the decrease in TLR2 and TLR4 expression (down-regulation) so that it is thought to accelerate the tissue healing process [14, 17, 23, 24, 25].

Omega-3 works as a trigger for fibroblasts to accelerate collagen synthesis so that pulp healing can occur [26]. Healing in the pulp will suppress the expression of proinflammatory cytokines secreted by NF-κβ (nuclear factor-kappaB). Inhibition of NF-κβ activation can be through the molecular signaling pathway in several ways, namely blocking stimulating signals at an early stage, disrupting the stage in the cytoplasm in the NF-κβ pathway by inhibiting IkK activation (IkB kinase), and inhibiting the translocation activity of NF-κβ into the nucleus [24]. Resolvins E1 is a derivative of EPA while protectin D1 is a derivative of DHA, it are as a reducer of inflammation and anti-inflammation which works to mobilize macrophages to eat neutrophil cells and clear the remains of the phagocytosis process [25,26]. This mechanism is a sign that the inflammation will end, then it enters the tissue healing stage which is marked by a decrease in TLR2 and TLR4 expression.

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