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TNFα and IL-10 Expression in Dental Pulp after Administration of Gouramy Bone Paste (*Osphronemus gouramy*) and Calcium Hidroxide

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ABSTRACT

Mechanical trauma causes pulp exposure and make the invasion of pathogenic microorganisms into the dental tissue. This condition causes pulp inflammation characterized by the release of pro-inflammatory cytokine *tumor necrosis factor-α* (TNF-α) and anti-inflammatory cytokine interleukin-10 (IL-10). Gouramy bone paste (*Osphronemus gouramy*) contains amino acids, omega-3, omega-6, and flavonoids which is thought to accelerate healing of inflamed pulp tissue that triggers decreased TNF-α expression and increased IL-10 expression. This study aims to analyze the expression of TNF-α and IL-10 in dental pulp after administration of Gouramy bone paste (*Osphronemus gouramy)* and calcium hydroxide. The sample size consisted of 24 *Rattus novergicus* which were divided into 3 groups i.e (K1) without the application of direct pulp capping material, (K2) with the application of calcium hydroxide, and (K3) with the application of gouramy bone paste. Data measured are the number of inflammatory cells (neutrophils, macrophages, and lymphocytes) that express TNF-α and IL-10 on the 4th and 7th days histologically by immunohistochemical methods. Statistical test results showed that administration of gouramy bone paste in dental pulp decreased TNF-α expression and increased IL-10 expression more than those without gouramy bone paste with a significant difference ($p < 0.05$) . The administration of Gouramy fish paste can reduce TNF-α expression and increase IL-10 expression in dental pulp.

Keywords: cytokine; direct pulp capping; immunohistochemical; inflammation

INTRODUCTION

Mechanical trauma can cause pulp exposure, which will facilitate various pathogenic microorganisms to penetrate and spread to dental pulp tissue[(1)(2)]. Bacteria or products may be entered into the pulp through the cracks in the dentine, either from caries or pulp exposure because of trauma, through the expansion of the infection of the gums or via blood circulation[(3)]. Inflammation activates pro-inflammatory cytokines and anti-inflammatory cytokines through TLRs activation pathways as a sign of inflammation of the pulp. The main anti-inflammatory cytokine that can be an indicator of inflammation in the dental pulp is interleukin-10 (IL-10)[(4)]. IL-10 has the ability to limit the severity of the inflammatory response by reducing the production of proinflammatory cytokines such as TNF-α[(5)(6)]. TNF–α is a plays role as a mediator of the inflammatory response that plays an important role in the host's response. However, it also causes damage during chronic diseases and acute tissue injury[(7)].

Direct pulp capping is the placement procedure of biocompatible materials over exposed pulp to maintain vitality and enhance healing in the pulp[(8)]. The material commonly used for direct pulp capping is calcium hydroxide. Calcium hydroxide is a dental material that has strong antibacterial ability and can stimulate the formation of reparative dentin[(9)(10)]. Dental treatment using material such as calcium hydroxide has potential to cause side effects because it contain various active ingredients or chemical agents[(11)]. In addition, although calcium hydroxide has been clinically well received, this material has not been known to have an anti-inflammatory effect so it cannot reduce the inflammatory process in the pulp. Therefore, alternative material from natural ingredients are needed as a *direct pulp capping* material, which act as an anti-inflammatory, antibacterial, and can reduce the side effects of chemicals. An alternative ingredient which is thought to be used is gouramy fish bones.

Tridhar's research (2016) states that the protein in Gouramy bone is higher compared to other freshwater fish. Quality of protein is influenced by the composition of amino acids[(12)]. Research by Dewanti et al. (2019)

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showed that Gouramy fish bones contain omega 3, omega 6 and flavonoids which function as anti-inflammatory[13]. They works by inhibiting *cyclooxygenase* (COX) and *nuclear factor - kappa B (* NF-κB) transcription[14][15][16]. Inflammatory transcription factors that are inhibited by gourami fish are expected to reduce the expression of proinflammatory cytokines TNF-α and cause an increase in the expression of anti-inflammatory cytokines such as IL-10. TNF-α is the main cytokine in the acute inflammatory response to gram-negative bacteria and other microbes in inflammation secreted by various body cells[17]. Increased expression of TNF-α in tissues will cause acute inflammatory processes and stimulation of adaptive immunity . TNF-α secretion at excessive levels is a response to the high growth of microorganisms and can cause very heavy and fatal tissue damage[18][19]. According to Nirwana (2012), IL-10 is an anti-inflammatory cytokine that inhibit the inflammatory response in the pulp by inhibiting the production of proinflammatory cytokines (TNF-α)[20]. The decreased expression of TNF-α signifies the occurrence of inflammatory phases and repair response in the pulp tissue[20]. Based on these descriptions, researchers are interested in examining the expression of TNF-α and IL-10 after administration of Gouramy bone paste (*Osphronemus gouramy)* and calcium hydroxide on rats dental pulp.

METHODS

Animal Model

This research was approved by the Ethical Committee Faculty of Dentistry, University of Jember Indonesia (580/UN25.8/KEPK/DL/2019). Samples used male rats (*Rattus novergicus*), aged 2-3 months, body weight 150-200 grams. The rats were kept in separate cages in a well-ventilated room at standard experimental conditions.

Gouramy Bone Powder

Gouramy bone powder was made by separating the fish bones with meat then boiled (70˚C-100˚C for 30 minutes) and dried using an oven (65˚C for 48 hours). The fish bones that have been dried subsequently comminuted using a blender to become powder.

Gouramy Bone Paste

Gouramy bone paste was made by mixing the basic paste extract (magnesium carbonat, calcium carbonat, glycerine, TEA (triethanolamine), propylene glycol, and aquadest) with Gouramy bone powder.

Research Group

The sample size consisted of 24 *Rattus norvegicus* which were divided into 3 groups. The number of each sub group consisted of 4 rats (4th day and 7th day); in the treatment groups, perforations were made in the teeth to direct pulp capping using Gouramy bone paste.

- K1 (Control): Cavity prepared + perforated + without direct pulp capping material applied + temporary fillings (*orafil*)
- K2 (Calcium hydoxide): Cavity prepared + perforated + applied with calcium hydroxide + temporary fillings (*orafil*)
- K3 (Gouramy bone paste): Cavity prepared + perforated + applied with Gouramy bone paste + temporary fillings (*orafil*)

The 4th and 7th day rats were sacrificed serially after which their teeth were prepared for analysis of the number of TNF-α and IL-10 expression by immunohistochemistry.

Immunohistochemistry Methods

Mixing was done 3 times for deparaffinization using xylol. The xylol was eliminated with rehydration by soaking the stratified alcohol in a row for 3 minutes. The mixture was flooded with 3% H2O2 solution for 10 minutes and then washed 4 times with PBS for 5 minutes, after that it was dropped with *Super Block* and incubated for 8 minutes at room temperature. Washed with PBS for 5 minutes. Reacted with primary antibody against TNF-α (1:250)/IL-10 (1: 300), then incubated for 24 hours. Washed with PBS 4 times for 5 minutes.

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Gived 1 drop of *UltraTek Anti-Polyvalent* (yellow lid), then incubated for 10 minutes at room temperature. Washed again with PBS, then gived 1 drop of *UltraTek HRP* (red lid) and incubated for 10 minutes at room temperature. Washed using PBS, after that gived 4 drops of *chromogen* DAB to the DAB *substrate* and incubated for 5-15 minutes. The next step, washed with PBS and aquadest for 5 minutes. Washed with distilled water and added with Meyer-HE for 10 minutes. Washed with tap water and then with distilled water. The preparations were dried, added with entelan, and covered with a coverglass. Microscopic analysis was done under a light microscope with 1000x magnification, which was analyzed over 4 fields of view. The parameter was the amount of inflammatory cells (neutrophils, macrophages, and lymphocytes) that express TNF-α and IL-10. The data were analyzed descriptively and by One Way ANOVA, followed by an LSD test.

RESULTS

The expression of TNF-α and IL-10 can be observed in inflammatory cells leukocytes i.e macrophages, neutrophils and lymphocytes using a light microscope with a magnification of 1000x. Inflammatory cells that express TNF-α and IL-10 will appear as brown cell membranes (cytoplasm), whereas inflammatory cells that do not express TNF-α and IL-10 will appear purple/pale cytoplasm. The results of the study can be seen in figure 1 and figure 2. ANOVA (P <0.05) showed that a significant difference between groups can be seen in table 1 and table 2.

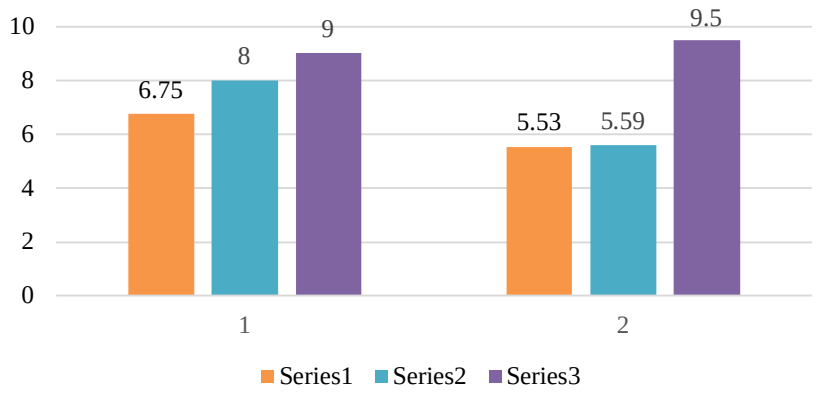

Figure 1. Histogram average number of inflammatory cells expressing IL-10

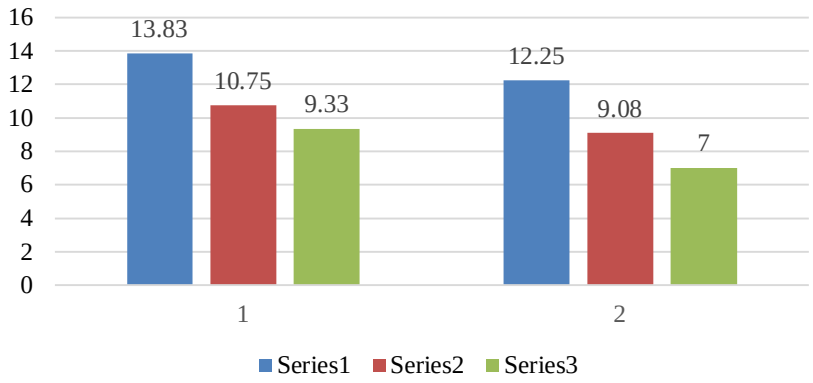

Figure 2. Histogram average number of inflammatory cells expressing TNF-α

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Table 1. Summary of ANOVA number of inflammatory cells expressing IL-10

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	59.875	5	11.975	7.245	.001
Within Groups	29.750	18	1.653		
Total	89.625	23			

Table 2. Summary of ANOVA number of inflammatory cells expressing TNF-α

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	631.407	7	87.630	146.897	.000
Within Groups	14.317	24	.597		
Total	627.724	31			

Histological observations can be seen in figure 3. (IL-10 4th day expression), 4. (TNF-α 4th day expression), 5. (IL-10 7th day expression), 6. (TNF-α day expression 7th). The picture was taken at the same place that is on the roof of the pulp and observed in 4 different field of view.

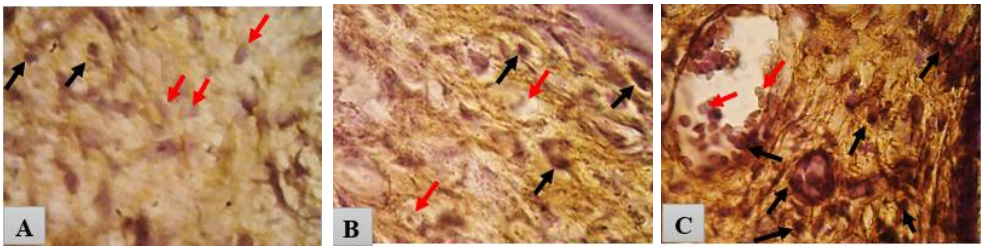

Figure 3. Histological picture of pulp tissue of left mandibular first molar rats, inflammatory cell that express IL-10 designated with black arrow, A) Control group on the 4th day, B) Calcium hydroxide group on the 4th day, C) Gouramy bone paste group on the 4th day.

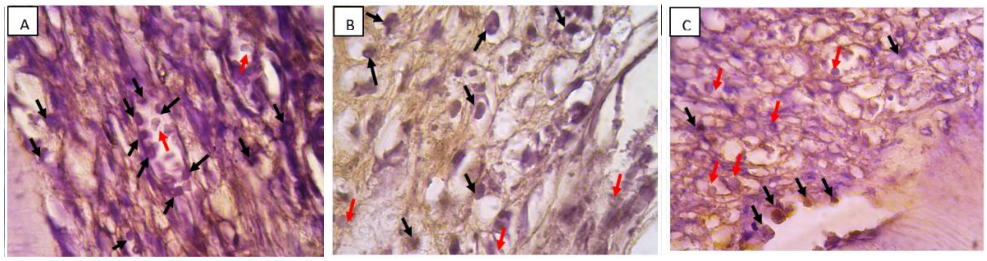

Figure 4. Histological picture of pulp tissue of left mandibular first molar rats, inflammatory cell that express TNF-α designated with black arrow, A) Control group on the 4th day, B) Calcium hydroxide group on the 4th day, C) Gouramy bone paste group on the 4th day.

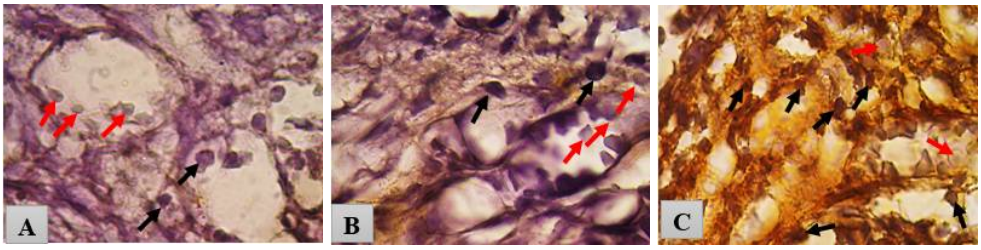

Figure 5. Histological picture of pulp tissue of left mandibular first molar rats, inflammatory cell that express IL-10 designated with black arrow, A) Control group on the 4th day, B) Calcium hydroxide group on the 7th day, C) Gouramy bone paste group on the 4th day.

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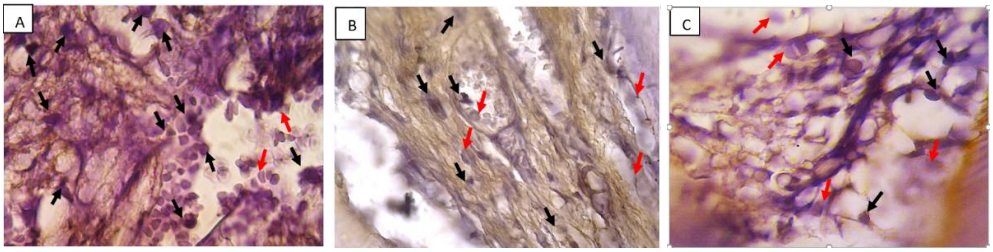

Figure 6. Histological picture of pulp tissue of left mandibular first molar rats, inflammatory cell that express TNF-α designated with black arrow, A) Control group on the 4th day, B) Calcium hydroxide group on the 7th day, C) Gouramy bone paste group on the 4th day.

DISCUSSION

Physiologically, the existence of mechanical trauma and invasion of microorganisms on the pulp will trigger an immune response. Pulp immune responses occur from day 0 after trauma. Trauma and microorganisms will be recognized by *Toll-Like-Receptors* (TLRs). TLRs will activate transcription factors, NF-κB (*Nuclear Factor Kappa Beta)* which work to release various pro-inflammatory cytokines such as TNF α[21][22][1]. IL-10 is a major anti-inflammatory cytokine that inhibits the production of pro-inflammatory cytokines such as TNF-α[5]. Inhibition of pro-inflammatory cytokines will reduce inflammation in the pulp so that the healing process occurs in the pulp.

The results obtained in the control group had higher TNF-α expression and lower IL-10 expression than the calcium hydroxide group or Gouramy bone paste group. This is thought to be due to the control group inflammation continues. Pro-inflammatory cytokines (TNF-α) in this group experienced a continuous increase in production, while anti-inflammatory cytokines (IL-10) in the production control group would decrease. The administration of direct pulp capping material helps reduce the inflammatory process by increasing the expression of IL-10 to inhibit pro-inflammatory cytokines TNF-α, while the control group is not treated with the addition of direct pulp capping material so that the inflammatory process will only rely on the immune response of experimental animals. In accordance with research conducted by Sumarna (2017), that the control group was not treated with pulp capping material and was only given a temporary fillings *orafil,* the inflammatory process and wound healing relied solely on the immune response of experimental animals without the aid of antiinflammatory and antibacterial treatments. Meanwhile, the provision of treatment materials that are anti-inflammatory and antibacterial can accelerate the body's immune response in the fight against an injury[23].

Calcium hydroxide group has the expression of TNF-α were lower and the expression of IL-10 were higher than the control group allegedly due to calcium hydroxide will be ionized into ions of Ca^{2+} and OH^-. The presence of Ca^{2+} ions can bind with carbon dioxide to calcium bicarbonate so that bacteria lack CO2 supply[24]. Ion Ca^{2+} will also activate TGF-β which plays a role in the activation of odontoblast differentiation so that it can stimulate hard dental tissue mineralization[25]. OH^- is a strong base, pH 11-13. OH^- will interfere with the permeability of the cell membranes of bacteria that come into contact with calcium hydroxide is by damaging lipopolysaccharide bacterial cell membrane, causing bacteria and their products become lysis, the raising pH of the environment that inhibits the activity of bacterial enzymes for the oxidation of other enzymes and proteins of cells, also interfere with DNA bacteria and inhibits DNA replication [24] [25].

The gouramy bone paste group showed the result of a decrease in TNF-α expression and an increase in IL-10 expression. This is thought to have occurred because of the increased process of tissue repair in the pulp. The reduced expression of TNF-α indicates a decrease in the inflammatory phase and the response of tissue repair[20]. According to research by Nirwana (2012), increased IL-10 expression functions to regulate inflammation so that the healing process of the pulp is accompanied by an increase in TGFβ-1 that will cause proliferation and differentiation of pulp *stem cells* to odontoblasts. Odontoblasts will function as *secreting cells to* produce collagen type I. The collagen matrix is very important for the mineralization of hard dental tissue (dentin) [1].

The Gouramy paste group had lower TNF-α expression and higher IL-10 expression than the control group and calcium hydroxide group. This is thought to be due to the anti-inflammatory content in the form of amino acids, flavonoids, omega 3 and omega 6 in the bones of Gouramy fish. Amino acids and flavonoids can inhibit the cyclooxygenase (COX) enzyme so that it will suppress the amount of prostaglandins, prostacyclin,

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endoperoxide, thromboxin, hydroperoxide acids, and leukotrienes[26]. This can reduce inflammatory activity by decreasing the process of phagocytosis and neutrophil lysis[23].

Other ingredients that are omega 3 and omega 6 also function as anti-inflammatory. Omega 3 derivatives are *Eicosapentaenoic Acid* (*EPA*) and *Docosahexaenoic Acid* (*DHA),* while omega 6 derivatives are *Arachidonic acid* (AA). DHA and AA will provide strong anti-inflammatory effects under physiological conditions by reducing the production of TNF-α, while EPA works by inhibiting the activation of NF-κB[15]. The inhibition of NF-κB transcription can inhibit the production of pro-inflammatory cytokines TNF-α, with inhibition of pro-inflammatory cytokines will increase production of anti-inflammatory cytokines such as IL-10. Increased expression of IL-10 will accelerate the inflammatory process so that an improvement process occurs in the pulp tissue[1].

In addition, the flavonoid content in Gouramy fish bones also has antioxidant and antibacterial effects[23][27][28]. Antioxidant effects of flavonoids can improve the body's defense system against infectious agents that enter the body and prevent damage to body cells[23][27]. Anti-bacterial effects of flavonoids work by preventing bacterial DNA gyrase using the ability to replicate and change inhibited bacteria. The biological activity of flavonoid compounds against bacteria is carried out by destroying the cytoplasmic membrane of bacteria consisting of lipids and amino acids by reacting them with alcohol groups on flavonoid compounds. This process will cause cell membranes damage and these compounds can enter the bacterial cell nucleus. The difference in polarity between the constituent DNA lipids and alcohol groups in flavonoid compounds will cause reactions that damage the lipid structure of bacterial DNA so that bacteria will undergo lysis and death[28].

CONCLUSION

The administration of gouramy bone paste to the dental pulp can decrease TNF-α expression and increase IL-10 expression more than the control group or calcium hydroxide group.

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