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Amelogenin and alkaline phosphatase expression in ameloblast after saltwater fish consumption in pregnant mice *(Mus musculus)*

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ABSTRACT

Background: *The intricate process of tooth formation during embryonic development ensures sufficient nutrition for the growth of healthy dental tissues. Amelogenin and alkaline phosphatase (ALP) are serine proteinases secreted by the ameloblast during the transition and maturation phases of the amelogenesis process. Consumption of saltwater fish is predicted to increase the expression of amelogenin and ALP in ameloblast cells during tooth formation. Only now have the function of each gene, tooth-forming cells, and the proteins they produce in the biomolecular amelogenesis of tooth enamel, which began during prenatal development, been clarified.* ***Purpose:*** *This study aims to determine how saltwater fish powder affects the ability of mother mice to increase the expression of amelogenin and ALP in cell ameloblast.* ***Methods:*** *Using a completely randomized design, this study was experimental and aimed to examine the effects of sardine (Sardinella fimbriata), splendid ponyfish (Leiognathus splendens), and tuna (Euthynnus affinis) powder. As samples, twenty-four female mice (Mus musculus) were used. Two groups of mice were created: group 1 (2.14 mg/0.5 ml) and the control group. The expression of amelogenin and ALP was determined using immunohistochemistry (IHC) and t-test ($p<0.05$).* ***Results:*** *Expression of ameloblast was significantly different between the treatment and control groups ($p<0.05$).* ***Conclusion:*** *The consumption of saltwater fish reduces the amelogenin and ALP expressions of mouse fetal ameloblast cells during tooth development in vivo.*

Keywords: *amelogenin; alkaline phosphatase; medicine; saltwater fish consumption; tooth development*
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INTRODUCTION

The growth and development of children's teeth must be considered at an early age, specifically during the embryonic stage of tooth development. At the stage of tooth growth and development, abnormal teeth are found in many children. The development of teeth begins approximately 28 days after conception.[1] The mineralization phase of primary teeth begins during the fourteenth intrauterine week, and all primary teeth will be fully mineralized after birth.[2,3]

Calcium and phosphate comprise around 95% of enamel, making it the most complicated tissue in the human body. One of the proteins that become engaged in the enamel development stage is called amelogenin. Ameloblasts, responsible for 80–90% of the total protein, are responsible for the production of this hydrophobic protein.[4] This protein is expressed in secretions until the post-secretory ameloblast stage. Amelogenin is also essential for the formation of hydroxyapatite prisms and the production of standard enamel thickness.[5] The high alkaline phosphatase (ALP) levels produced by differentiated cells in the stratum intermedium during the last stages of tooth formation are responsible for this. The epithelial cells that make up enamel are diverse, each differentiating to perform

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a somewhat distinct set of tasks. These cells include the stratum intermedium cells, which have a role in ameloblast development and promote enamel mineralization via strong ALP activity. ALP expression is elevated in the stratum intermedium layer.[6,7]

Tooth formation in the prenatal period occurs in the fourth intrauterine month, when the enamel and dentin deposit structures will begin to form. Teratogens and poor nutrition during pregnancy significantly affect the development of the primary and young permanent teeth. Nutritional deficiency during pregnancy impacts tooth size, time of tooth eruption, defects in tooth mineralization that can increase the risk of caries, and impaired salivary gland formation.[8] The process of tooth development and growth, which is divided into several stages, allows the occurrence of abnormalities or growth abnormalities starting at the initiation stage of tooth bud formation, which, if disturbed, can cause hypoplasia, anodontia, oligodontia, or odontogenic tumors.[1,9,10]

Disruption in the histodifferentiation stage can lead to amelogenesis imperfecta or dentinogenesis imperfecta. Disturbances in the morph differentiation stage cause disturbances in the shape of the teeth.[11] The hypoplastic/hypomineralization effect can decrease tooth strength and delay tooth eruption time. Exogenous and endogenous variables lead to the factors that cause the above disturbances in growth and development. Endogenous factors include genetics. The role of each tooth-forming gene and cell, as well as the protein they produce in the biomolecular calcification of tooth enamel that begins in prenatal time, has not yet been elucidated.[12] Numerous studies have documented the impact of daily nutrition on the development of teeth. According to reports, providing proteins such as saltwater fish powder containing calcium and omega-3 optimizes enamel density.[9] Based on the preceding information, the purpose of this study is to determine how saltwater fish powder affects the ability of mother mice to increase the expression of amelogenin and ALP in cell ameloblast.

MATERIALS AND METHODS

Following seven days of environmental adaption, female mice (*Mus musculus*) were housed in plastic cages with wire mesh. The hardwood shavings at the bottom of the cages were changed every three days, and the animals had free access to food and water (ad libitum). On the third day, a pregnancy examination was performed. If the female mice's vulva developed a visible vaginal plug on a given day, that day was recognized as day zero of pregnancy. The pregnant mice were subsequently housed in five-cage groups and provided with a regular diet.

The Ethics Committee of the Faculty of Dental Medicine at Airlangga University had already assessed the ethical treatment of animals in this research. Therefore, a certificate with the number 010/HRECCFODM/II/2018 was granted. This research used a completely random experimental design. As the unit of analysis, 10-week-old, 20–30 gram, healthy-appearing adult female *Mus musculus* mice were employed in this investigation (agile, not lethargic, clean skin without wounds, bright eyes). Both the treatment and control groups were comprised of 24 mice. The average intake of saltwater fish powder was converted into a dosage of 2.14 mg/0.5 ml.

The dose is delivered thrice daily, every 6 to 8 hours. Both experimental and control groups were slaughtered on the eighteenth day of gestation. Material for this research was prepared at the Faculty of Chemistry, Universitas Gadjah Mada, Indonesia, by drying sardines (*Sardinella fimbriata*), splendid pony fish (*Leiognathus splendens*), and tuna (*Euthynnus affinis*), reducing them to a powder, and adding the emulsifier carboxymethylcellulose (CMC). In the first process of making saltwater fish powder, the test material was dose-weighed and dissolved in hot water heated to 70 degrees Celsius. Using an ultra-turrax (IKA, Germany), sea fish powder was homogenized to crush and grind, and 1% CMC material was agitated for 15 minutes until homogeneous. After the fish powder and CMC had been combined, the mixture was deposited in the appropriate containers. The inductively coupled plasma technique revealed the calcium content of saltwater fish powder to be 5.56% w/w. At the same time, the gas chromatography method determined the omega-3 content to be 3.34% w/w. Mice were euthanized with 10 to 20 cc of chloroform (Henan Haofei Kimia Co., Ltd, Indonesia). For dental histology preparations, formalin 10% alcohol was employed in concentrations of 70%, 80%, 95%, and 96% absolute alcohol, xylol, paraffin, and hematoxylin-eosin.[9]

The surgical operation was done on mice while sedated with chloroform. Mice were put in hermetically sealed jars, then 10 to 20 cc of chloroform was poured onto cotton and placed in the jars with the mice. After two to five minutes, breath and heart rate measurements were taken. If the mice were not breathing, the cover of the jar was removed. Before surgery, the mice's cervical spines were dislocated to ensure their demise. The mice were put on the surgical board using pins. Surgical incisions were made in the abdomen or uterus using curved scissors. The embryo was removed from the uterus after examination of the right and left corpus luteum. To analyze prenatal dental tissue, they were simultaneously coated by an amniotic membrane and preserved in formalin. Since the detected substance interacts with enzyme-labeled antibodies, immunohistochemical responses are specific. A color indication shows the existence of an enzymatic process (chromogen). Among the chromogens available are naphthol (blue) and DAB (brown). Using mice from each housing arrangement, immunohistochemical preparations are produced in the same way as histopathological preparations up to the stage of tissue sectioning. [13] The preparations were examined using a Leica DM 750 Germany light microscope at 100x magnification to observe all fields of view, and then at

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400x magnification for a more thorough study. Under a microscope with a magnification of 400x, the edges of the peripherally organized ameloblast cells (amelogenin sc-33121 Santa Cruz Biotechnologi, INC, Europe, ALP bs-1535R-HRP Bioss, Boston, Massachusetts) may look brown. Two researchers and analysts conducted computations with 400x magnification in particular visual fields, specifically on ameloblast cells, with 95% clinical agreement.

The Shapiro-Wilk test from IBM Statistical Package for Social Sciences Statistics 26 was used to assess the normality of distribution, continuing descriptive analysis to acquire mean and standard deviation, homogeneity, and significance of independent t-test at $p<0.05$.

RESULTS

The Shapiro-Wilk test was performed to determine the normality of the distribution between all research data, with a total of eight replications for each treatment group and seven replications in normal mice because one mouse died. The results of the Shapiro-Wilk test showed a normal distribution of amelogenin and ALP expression data ($p>0.05$). For amelogenin and ALP data, descriptive analysis was continued to get the independent t-test mean and standard deviation homogeneity (Table 1). HPA of amelogenin in the treatment groups is shown in Figure 1A-C, and control groups are shown in Figure 1D-F.

In ameloblast cells of the oral tissue treated with saltwater fish powder, amelogenin expression was reduced. The decrease in amelogenin in the dental tissue fed with saltwater fish powder appeared to be significant compared to the control group. According to the analysis's findings, the control group had lower levels of Alkaline Phosphatase (ALP) expression in dental cells than the group that received saltwater fish powder ($p<0.05$) (Table 2). HPA of ALP in the treatment groups is shown in Figure 2A-C, and control groups are shown in Figure 2D-F.

There was an increase in the expression of ALP in ameloblast cells in the dental tissue treated with saltwater fish powder. This increase was significant compared to the control group.

Table 1. Mean expression of amelogenin from ameloblast cells in fetal tooth tissue of mice

Group	n	Amelogenin Expression					p
		Mean	SD	Median	Minimum	Maximum	
Control	7	4.64	0.66	4.40	4.10	5.80	0.004*
Treatment	8	3.13	0.77	2.95	2.30	4.90	

Description: * significant level $p<0.05$

Table 2. Mean number of ameloblast cells expressing alkaline phosphatase in fetal tooth tissue of mice

Group	n	Alkaline Phosphatase Expressions				p
		Mean	SD	Minimum	Maximum	
Control	7	3.00	0.54	2.50	3.90	0.000*
Treatment	8	5.98	0.63	4.80	6.70	

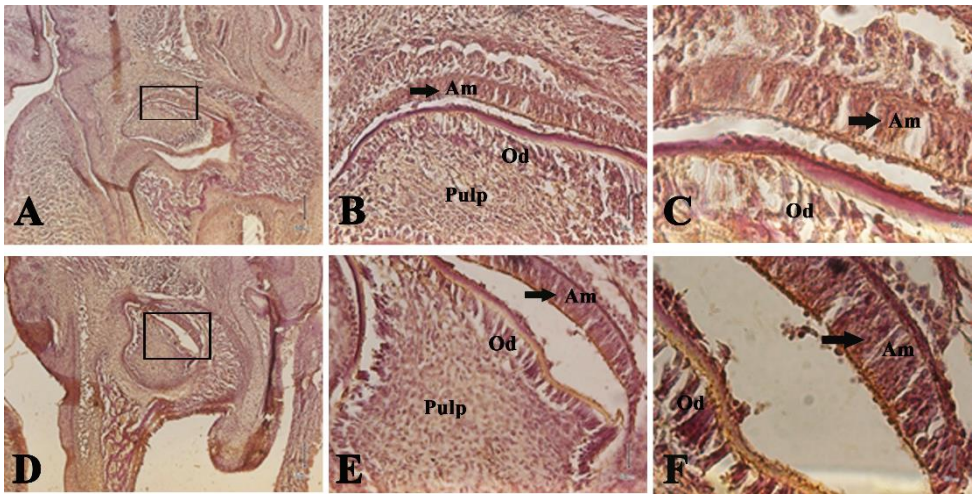

Figure 1. HPA of amelogenin. A, B, and C in the treatment group. D, E, and F in the control group. A, D 100x magnification, B, E 400x magnification, C, F 1000x magnification. B and C showed amelogenin expression with weak brown staining of the cytoplasm. Figures E and F show the expression of amelogenin with solid brown staining in the cytoplasm. Information: Am: Ameloblast, Od: Odontoblast, Pulp: Dental pulp.

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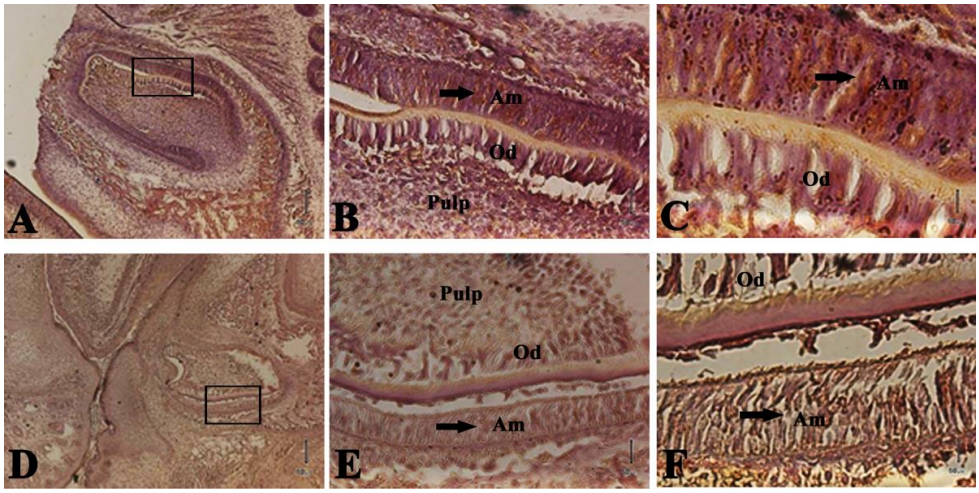

Figure 2. HPA of alkaline phosphatase. A, B, and C in the treatment group. D, E, and F in the control group. A, D 100x magnification, B, E 400x magnification, C, F 1000x magnification. B and C showed alkaline phosphatase expression in the cytoplasm with a solid brown color. E and F showed weak brown alkaline phosphatase expression in the cytoplasm. Am: Ameloblast, Od: Odontoblast, Pulp: Dental pulp.

DISCUSSION

Ameloblast cells are responsible for forming dental enamel, a dense limb of the tooth. Ameloblasts are a unique kind of tooth cell responsible for the secretion of three major enamel-specific matrix proteins. These enamel-specific matrix proteins include amelogenin, ameloblastin, and enamelin. This matrix protein is expressed by ameloblasts in the secretory stage, and its presence is necessary for enamel biomineralization.[14] For mice that become pregnant when they eat fish powder with calcium and omega-3 polyunsaturated fatty acids, the calcium will bind to transient receptor potential cation channel subfamily V member 6 (TRPV6) to get through the placental membrane. It will then be carried to the mouse fetus by a Ca2+-binding protein with the help of a Ca2+ pump. Omega-3 polyunsaturated fatty acids in non-esterified fatty acids are natural ligands that signal PPARs to tell FATPs to break through the placental membrane. Fatty acid binding protein will then transport the FATPs. Ca2+ will be absorbed by ameloblast cells through Ca2+ sensing receptors. FATPs make the insulin growth factor (IGF) send out more signals. IGF will send a transduction signal into the cell nucleus. This will turn on P38 mitogen-activated protein kinase (MAP) and p44/p42 MAP, which will increase heat shock protein 27 as a chaperone molecule to keep the cell's structure intact. IGF will help move the amelogenin protein secreted by ameloblast cells during the secretory stage.[3,9,16]

This study found a significant decrease in the expression of amelogenin with the provision of saltwater fish powder (Figure 1). This is done by Wu et al.,[17] and C-terminal brushite-interaction amelogenin with AFM at low concentrations is stated (1-10 nM). Several clinical states cause ameloblasts to express varying amounts of amelogenin, and the amelogenin found in ameloblasts during enamel development may be a reaction to hypomineralization. Consequently, in the hypomineralized state, amelogenin is up-regulated in ameloblasts.[18,19] Amelogenesis and the formation of the tooth structure are connected to the function of the tooth tissue. The mineralization of calcium phosphate is an essential component of this process. The modulation of calcium levels inside the cell will cause an increase in acidity, which in turn will cause amelogenin levels to decrease.[20,21]

During the secretory phase, the protein amelogenin is secreted and degraded by kallikrein-4. After 18 days of gestation, when the enamel is fully formed and matrix development has begun, amelogenin expression was assessed in these fetuses. Mice begin developing dental enamel between 14.5 and 16.5 days when immunohistochemistry reveals a transition from the cap to the bell stage; at the bell stage, ameloblast cells have started to differentiate.[22] According to Wahluyo et al.,[23] in the examination of the effect of sodium fluoride on the development of ameloblasts and kidney proximal tubular cells, the expression of amelogenin was shown to be significantly lower in the control group than in the treatment group.

In addition to the liver and osteoblasts (new bone-forming cells), the intestines, proximal renal tubules, placenta, and milk-producing mammary glands provide ALP. A buildup of blockage in the bile ducts causes a rise in serum ALP levels (cholestasis).[24] Liver (hepatobiliary) and bone (bone resorption) illnesses are two common conditions that can be diagnosed with the help of the ALP test. In bone diseases like Paget's disease, an aberrant increase in osteoblastic activity (bone cell production) leads to high ALP levels. In youngsters, elevated levels of a protein called alanine aminotransferase can be observed before and after puberty (physiological). Bone remodeling can be accelerated in vitro by increased synthesis of bone

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alkaline phosphatase from gingival mesenchymal stem cells cultured in platelet-rich fibrin.[25–27]

This study showed a significant increase in the treatment group given saltwater fish powder (Figure 2). Indeed, research conducted by Lacruz et al.[28] claimed that ALP is crucial to the calcification process. A study on experimental mice increased the development of dental enamel and mineralization of the stratum intermedium by ameloblast cells showed decreased ALP expression in tissue hypoxia after 24 and 48 hours.[29] Research by Zhang et al.[30] found that ALP was lower in hypoxia, the mitochondrial count was lower, the endoplasmic reticulum was smaller, and mineralization was slowed when mitochondria were damaged. An elevation of ALP in the treatment group did not correlate with decreased tissue oxygen saturation. As the number of osteoblasts involved in bone formation increases, so will the amount of the ALP enzyme being expressed.[31,32]

During the histodifferentiation phase of the tooth creation process, the animals used in this research were put to sleep on day 18. As a result, the ALP level was elevated in tooth enamel tissue, particularly in the stratum intermedium. Because ALP plays an important role in the mineralization process, the intermediate layer of tooth enamel contains a high concentration of the enzyme's expression. One important disclaimer is that in vivo research can only provide an incomplete representation of the amelogenesis process. Despite this, it was discovered that the expression of amelogenin and ALP increased on day 18 of prenatal consumption of saltwater fish powder. This study did not look at MMP-20, known to be one of the progenitors of tooth enamel, nor did it look at any other proteins that can be tested.[6,22] In conclusion, the consumption of saltwater fish powder can reduce the number of ameloblast cells expressing amelogenin and ALP in mice.

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