The Effect of Saltwater Fish Consumption by Mother Mice (*Mus Musculus*) on the Expressions of FABPs and Type 1 Collagen regarding Increase in Enamel Density

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Abstract

One marine biota containing various proteins supporting enamel maturation is saltwater fish. Protein is transferred from a mother to her child through the placenta, an organ that plays a critical role in fetal growth and development. The placenta with fatty acid-binding proteins (FABPs) expression serves as an interface for exchanging substances, producing proper hormones during pregnancy, and as a barrier. Early dental enamel formation is generally known as amelogenesis.

Type 1 collagen is one of the ameloblasts' proteins in amelogenesis.

This research aims to examine the effect of saltwater fish powder on the increase in the expression of FABPs in trophoblast cells and the increase in expression of type 1 collagen in ameloblasts.

This research belongs to true experimental with a completely randomized design. The sample consisted of 24 female house mice (Mus musculus) which were pregnant and received saltwater fish powder administration made of sardines (Sardinella Fimbriata), pony fish (Leiognathus splendens), and mackerel tuna (Euthynnus affinis). The mice were divided into three concentration groups: Group 1 with 1.07 mg/0.5 ml concentration, Group 2 with 2.14 mg/0.5 ml concentration, and Group 3 as the control group. The samples were stained to see FABP expression and the KLK-4 expression a. Data were analyzed using an independent sample t-test and pathway analysis.

The research results showed a significant difference in enamels between the Control Group and Group 1 and Group 2, and there was no significant difference between Group 1 and Group 2.

In mother mice that consumed saltwater fish powder, there was an increase in expression of FABPs and a decrease in expression of type 1 collagen.

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Introduction

Tooth enamel is produced by a process called amelogenesis. Amelogenesis requires specific proteins to build the density of tooth enamel. Ameloblasts require various proteins such as amelogenin, ameloblastin, and enamelin for tooth enamel maturation[1]. sufficient nutrition intake during pregnancy can prevent caries in children's teeth. Malnutrition in pregnancy can affect the growth and pattern of tooth eruption and may cause caries in the future[2]. During enamel formation, sufficient nutrition and oxygen intake are required and mediated by ameloblasts.

Study conducted by Yani & Dewanti (2019) mentioned the correlation between children's dental caries and 8-10 years old children's quality of life in Banyuputih Village, Wringing District, Bondowoso, the higher dental caries, the lower children's quality of life will be[3]. Study conducted by Christiono et al (2016) showed that 85.7% of children had ECC in Semarang, Central Java. The results also showed a significant relationship between caries status in children under five with maternal age, socioeconomic status and other incomes[4].

The saltwater fish powder is a food product made of either whole saltwater fish or fish processing waste. Overproduction causes fish accumulation on a large scale, decreasing fish prices. To solve the accumulation problem, fishermen often dry fish for poultry feed or to be

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further processed as fish powder. The fish powder is often made from sardines (*Sardinella Fimbriata*), pony fish (*Leiognathus splendens*), and mackerel tuna (*Euthynnus affinis*). Fish powder contains 5.56% b/b calcium (Ca), 3,34% omega-3, 0.12 linoleic acid, Eicosatrienoic acid <0,1%, 0,80% Arachidonic Acid, 1,44% EPA, and 0,98% DHA as measured by the inductively coupled plasma (ICP) and gas chromatography [5].

The main barrier which limits nutrition transfer across the human placenta is syncytiotrophoblast. Syncytiotrophoblast possesses two polarized plasma membranes, maternal-facing microvillus plasma (MVM) and fetal circulation oriented basal plasma (BM) membranes. Transportation characteristics of both plasma membranes will have a significant influence on the net placental nutrition transportation and fetal growth. Syncytiotrophoblast [6, 7] expresses many nutrient transporters that can be regulated by fetal, maternal, and placental signals [8]. Amino acid transfer through the placenta is a complex process critical for fetal growth and creates an intrauterine environment that predisposes health. In suboptimal fetal development, an intervention is required to increase amino acid transfer in the placenta, improving postnatal health field [6, 8].

Collagen is the most abundant protein in the human body. It covers 30% of the protein composition in the human body. Collagen is a type of organic structure that builds bones, teeth, joints, muscles, and skin. Collagen fibres have strong resistance against pressure. The word collagen came from Greek which means glue or to produce adhesive. Collagen is an extracellular protein that covers most of the connective tissues. The collagen structure is characterised by a triple-helical domain formed by three polypeptide chains bound by hydrogen bonds and hydrophobic interactions field [9]. Chemically, collagen consists of two amino acids, hydroxyproline and hydroxylysine.

Meanwhile, cells responsible for collagen synthesis are fibroblasts, chondroblasts, osteoblasts, and odontoblasts. Type 1 collagen is the most common form found in skin, tendons, bones, dentin, and pulp tissues. The amount of collagen deposited by fibroblasts is continuously regulated by collagen metabolism synthesis. The turnover of collagen and other ECM proteins is controlled by MMP and TIMP, produced by granulocytes, macrophages, epidermal cells, and myofibroblasts[10].

Materials and methods

This research used experimental animals that were previously reviewed ethically by the ethics committee of the Faculty of Dentistry, Airlangga University, with certificate number 010/HRECCFODM/II/2018. Therefore, its type was true experimental and employed a completely randomised design. The units of analysis used in this research were adult female pregnant house mice (*Mus musculus*) aged 100 weeks with ± 20-30 grams in body weight, looked healthy, agile, and energetic, and possessed bright eyes and spotless skin. The samples were 24 mice divided into three groups. Group 1 received 1.07 mg/0.5 ml saltwater fish powder administration three times a day every 6-8 hours. Group 2 received 2.14 mg/0.5 ml saltwater fish powder administration thrice a day every 6-8 hours. The final group is the Control Group. The mice in all groups were killed on the 18th prenatal day.

The female house mice, which had undergone environmental adaptation for seven days, were kept in plastic cages equipped with wired covers. The bottom part of each cage was covered with wooden shavings. The wooden shavings were replaced with fresh ones per three days. The mice were given food and water indefinitely (ad-libitum). A pregnancy test was conducted on day three. The day when the female mice had a vaginal plug on their vulva was established as day zero of pregnancy. The pregnant mice were then grouped into each cage containing five mice.

The materials used in this research were dried products of saltwater fish such as sardines (*Sardinella Fimbriata*), pony fish (*Leiognathus splendens*), and mackerel tuna (*Euthynnus affinis*). The extractions of saltwater fish in the form of powder were treated with a carboxymethyl cellulose (CMC) emulsifier. The active agents in the saltwater fish were calcium and omega-3 (The Faculty of Chemistry, Gajah Mada University, Indonesia). The procedure for making saltwater fish powder as a material test was as follows: The powder was weighted according to the given dosage and dissolved in hot water at 70°C temperature. Next, the powder was homogenised in order to be crushed and grinded using Ultra Turrax Homogenizer (IKA, Germany). 1% CMC emulsifier was added to it. It

was then stirred until homogenous for about fifteen minutes. The content level of calcium in the saltwater fish powder was 5.56% b/b, identified by the Inductive Coupled Plasma (ICP) method. In comparison, the level of omega-3 was 3.34%, as identified by the gas chromatography method.

Chloroform (Henan Haofei Kimia Co, Ltd, Indonesia) of 10-20 ml was used to euthanize the mice. The chemical materials used for dental histology preparation were 10% formalin and alcohols with 70% absolute alcohol concentrations, 80% xylol, 95% paraffin, and 96% Hematoxylin and Eosin (H&E).

The procedure for house mice surgery was started with euthanasia using chloroform. The house mice were put into jars, and the jars were closed tightly. 10-20 ml chloroform was poured into cotton, which was later put into the jars where the house mice were. After two-five minutes, the mice's breath and heart rate were observed. Once the house mice were breathless, the jar lids were removed. Before surgery, collarbone dislocation was performed on all house mice to ensure their deaths. The lifeless mice were then placed on a surgery board using pins. The surgery was started from the abdomen or uterus using bent scissors. After observing the right and left sides of the corpus luteum, which were still wrapped by the amniotic membrane, the fetus and placenta were removed from the uterus and then fixated using formalin. After that, the placenta and prenatal dental tissues were observed.

Creating immunohistochemistry preparation is similar to making histopathology preparation until the tissue cutting stage field. The preparation examination was initially started with an observation with a LEICA DM 750 light microscope with 100x magnification to observe all fields of view. Then the magnification was increased by 400 times. The areas to be observed were determined in advance; the edges of the ameloblasts were arranged in the periphery. For HPA: the histopathological images of tissues undergoing the enamel hardness process were tooth enamel and transfer proteins in the placenta. For IHC: expression FABPs, type 1 collagen with FABP monoclonal antibody and type 1 collagen looked brownish with 400x magnification in the microscope examination. Calculations were carried out with 400x magnification on specific visual fields, namely ameloblasts and trophoblasts. The study was carried out by two people, the researcher and the analyser, with 95% clinical agreement. The research reagent used FABP-3 bs-11283R-HRP and typed one collagen sc-59772.

Results

All data of research results, with eight replications per treatment group and seven replications in the control group because one mouse died, were tested with the Shapiro-Wilk test to check their normal distribution. The test showed a normal distribution of the expressions of FABPs and typed 1 collagen ($p>0,05$). For FABPs data, the type 1 collagen data was further analysed descriptively in order to acquire the values of mean and standard deviation as well as homogeneity using the independent T-Test. The analysis test was used to identify the strength of relationships among variables under study.

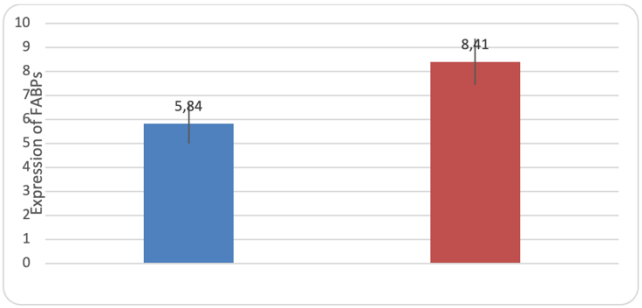

Figure 1. The mean value of expression of FABPs in the control group (blue) and treatment groups (red).

Cells that express FABPs can be seen in Figure 1. There is an increase in the expression of FAPBs of trophoblasts in the placenta tissues with saltwater fish powder treatment. The growth is significantly higher than the one in the control group.

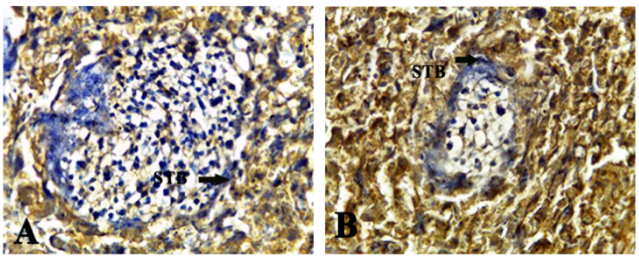

Figure 2. HPA and FABP images of treatment groups (A) and control group (B) with 400x magnification. In image A, the expression of

FABPs (as indicated by arrow) has strong brown staining in the cytoplasm. In image B, the expression of FABPs (as indicated by arrow) has weak brown staining in the cytoplasm. Note: STB: Syncytiotrophoblast, Ss: Stem Cells.

Groups	N	Expression of FABPs				$p_{1\text{-tailed}}$
		$\bar{x}$	SD	Minimum	Maximum	
Control	7	5.84	0.83	4.50	6.75	0.000*
Treatment	8	8.41	0.96	7.20	10.25	

Table 1. The mean values of expression of FABPs of trophoblasts in mice's fetal placenta tissues.
Note: * significant at α=0,05.

The analysis result showed that the expression of FABPs in the trophoblasts in the treatment groups which received fish powder was higher than the one in the control group (p<0,05).

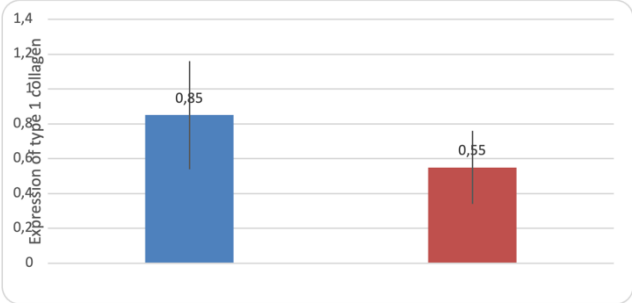

Figure 3. The mean value of the expression result of type 1 collagen between the control group (blue) and the treatment groups (red).

Cells that expressed the type 1 collagen can be seen in Figure 3. There was a decrease in the expression of type 1 collagen of the ameloblasts in the dental tissues with saltwater fish powder treatment. The decrease in type 1 collagen in dental tissues with saltwater fish powder treatment was significantly lower than in the control group.

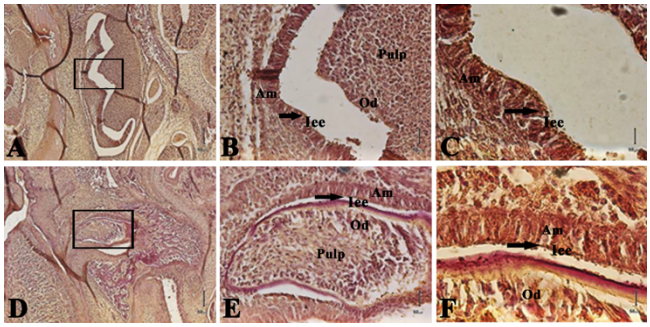

Figure 4. Images A, B, and C show the HPAs in type 1 collagen in the treatment groups, while images D, E, and F show HPAs in the control group. Images A and D received 100x magnification, images B and E 400x magnification, and images C and F 1000x magnification. In images B and C, brown staining expressions in the inner enamel epithelium are observable. In images C and F, brown staining expressions in the inner enamel epithelium are observable. Note: Am: Ameloblasts, Od: Odontoblasts, Iee: Inner Enamel Epithelium, and Pulp: Dental pulp.

Groups	n	Type 1 Collagen				$p_{1\text{-tailed}}$
		$\bar{x}$	SD	Minimum	Maximum	
Control	7	0.85	0.31	0.50	1.28	0.022*
Treatment	8	0.55	0.21	0.35	0.93	

Table 2. The mean values of ameloblasts that excreted type 1 collagen in the mice's fetal tooth tissues.
Note: * significant at α=0,05.

The analysis showed that the type 1 collagen in the groups with powder fish treatment was higher than in the control group (p<0,05).

The path analysis results in the treatment groups with powder fish administration, after a path significance coefficient test, was conducted (p<0,05), followed by trimming and building, showed a mechanism of the power path of tooth enamel as displayed in Figure 5. The powder fish administration has a strong relationship with FABP (B=-0,841) and type 1 collagen (B=-0,536),

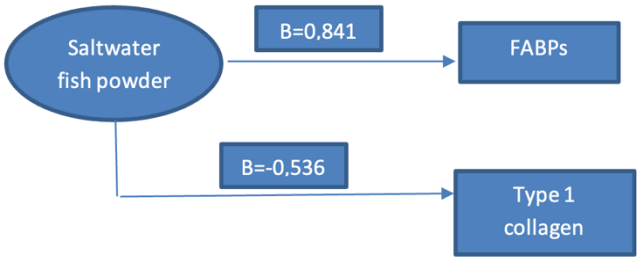

Figure 5. The analysis of density mechanism path in the amelogenesis model with powder fish administration (bold blue arrows).

Discussion

The saltwater fish powder, which contained calcium and omega-3, increased enamel density which was tested with micro-CT with the density in the intrauterine teeth below the average teeth that were calcified. In his studies, Christiono et al. acquired the mean grayscale value of 89.50 in the control group and

124.5 and 116.5 in the treatment groups [11, 12].

FABPs are proteins that can transport fatty acids in the placenta tissue. Another protein that has a similar ability is fatty acid translocase (FAT) [8]. The powder fish administration, which contained fatty acid, was transported by FABPs. An omega-3 fatty acid is essential in expressing a protein in the placenta. It determines the mRNA and levels of fatty acid proteins in protein transport during pregnancy yield [13, 14].

This research found an increase in the expression of FABPs in the trophoblasts after being administered with saltwater powder fish containing omega-3 and calcium. The field study states that fatty acid stimulates expressions of the intracellular fatty acid-binding proteins FABP-4 and FABP-3. FABPs, in general, are depicted as intracellular proteins which can influence lipid fluxes, metabolism, and signalling in cells. These proteins are essential mediators for metabolism and inflammation processes locally and systematically. Therefore, they become potential therapeutic targets for the immunometabolic disorders field [15, 16].

Peroxisome proliferator-activated receptors (PPARs) are important natural ligands in intrauterine fetal development. They are essential in fatty acid oxidation, especially in regulating transcription factors. Long-chain polyunsaturated fatty acids (LC-PUFAs), such as arachidonic acid, linoleic acid, docosahexaenoic acid and eicosapentaenoic acid, are PPAR agonists. Research in mice's placenta examined the increase in PPARs in metabolism, catabolism regulation and fat synthesis[18]. An increase in PPARs expression was observed during the luteal phase, and a decrease was found during the follicular phase in steroidogenesis. These receptors participate in the function of steroidogenesis, cytokine production, and angiogenesis during the estrus cycle [17-19].

Polyunsaturated fatty acids taken by the placenta and transported to the fetus are from two sources in the mother's circulation: non-esterified fatty acids (NEFAs) and esterified fatty acids in triglycerides (TGs). Fatty acid transport proteins (FATs) are integral membrane proteins critical to absorbing cells from long-chained fatty acids. Research in the cell culture of trophoblasts in the placenta has explored a mechanism that regulates FATs in the placenta, especially in mRNA the level of expression of mRNA. The Peroxisome proliferator-activated receptor (PPAR)γ regulates FATP1, FATP2, and the expression of FABP4 in mRNA. Fatty acids undergo esterification, beta-oxidation, or transportation to the fetus by fatty acid-binding proteins (FABPs) in the cytosol in syncytiotrofoblasts[8]. Polyunsaturated fatty acids are assumed to increase tooth density against caries. Another study in a different field shows that omega-3 fatty acids, especially DHA, positively influence bone mineral density (BMD) in male adults [20 14, 21]. Field Lukas et al. (2011) study mentions that omega-3 consumption can change Ca^{2+} absorption and osteoblast differentiation. The study was conducted on male rats (*Spraque-Dawley*) aged five weeks. In the rats given omega-3, there was an increase in Ca^{2+} absorption, reducing Ca^{2+} levels in urine and increasing Ca^{2+} levels in the bones [22].

Collagens are fibrous proteins located primarily in the connective tissues of the body. They produce and maintain cartilage, bones, tendons, ligaments, blood vessels, cornea, dentin, and other body tissues. Type 1 collagen can be found in the skin, tendons, ligaments, bones, teeth, intervertebral discs, and scar tissues. In addition, several studies have shown that collagen is detected in tooth enamel and ameloblasts [23]. Collagen acts as a chemical signal that can bind to the extracellular matrix of osteoprogenitor cells (integrin α2ß1). The interaction of type I collagen with integrin α2ß1 causes the activation of the extra-cellular regulated kinase (ERK) pathway, thereby causing phosphorylation and the potential for RUNX2 transcription. Then the RUNX2 bond called OSE2 (osteoblast-specific cis-acting element 2) stimulates the expression of osteoblast-specific genes, including BMP-2, VEGF, TGF-ß, ALP, osteocalcin, osteopontin, and type I collagen, so that it can be summarized that type 1 collagen from gurami scales induces osteoblast cell proliferation [24, 25].

This research showed a decrease in the expression of type 1 collagen in the treatment groups compared to the control group. Tooth enamels are formed with few proteins (less than 1% of organic materials) [26]. During amelogenesis, collagen expression decreased from the secretory to mineralisation and maturation phases, followed by another decrease in the enamelled matrix until the post-metamorphic juvenile teeth phase-field [27]. Studies conducted on Atlantic salmon and zebrafish found a

reduction in the expression of ameloblasts during the amyloid deposition and then another decrease during the maturation phase-field [28, 29]. Type I collagen application derived from gouramy fish extract (Osphronemus gouramy) in male Wistar rats showed increased osteogenesis and angiogenesis in the process of bone regeneration [30].

Conclusions

Based on the research that had been conducted, it was concluded that the administration of saltwater fish powder to mother mice 1) could increase the number of trophoblasts expressing FABPs in the mice and 2) reduce the number of ameloblasts expressing type 1 collagen in the mice's fetuses.

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Declaration of Interest

The authors report no conflict of interest.

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