The Effect of Saltwater Fish Consumption by Female House Mice *(Mus Musculus*) on the Increasing Teeth Enamel Density of Their Pups: MicroCT Analysis

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Abstract

Dental caries represent a common chronic condition the four main contributory factors of which are hosts, agents, substrates and time. One factor where an increase in tooth enamel density could reduce damage caused by the demineralization process. One saltwater biota which contains various proteins having a function in supporting enamel maturation is saltwater fish. A Radiography Micro Computed Tomography Scanner, commonly known as a µ-CT Scanner, is a device used to quantify the increase in tooth enamel density. This study aims to examine the effect of saltwater fish powder on the ability of female mice to increase teeth enamel density in their pups. This study type was true experimental in character using the *Completely Randomized Design*. 24 impregnated mice (*Musmusculus*)used as samples which were then given saltwater fish powder derived from sardines (*Sardinellafimbriata*), splendid ponyfish (*Leiognathussplendens*) and cobs (*Euthynnusaffinis*). The mice were divided into three concentration groups: group 1 (1.07 mg/0.5 ml), group 2 (2.14 mg/0.5 ml) and the control group. Samples were observed with a SkyScan1173 Scanner, while the enamel density was measured using a GrayScale indicator and analyzed by means of a One Way Anova test. The results confirmed a significant difference between the enamel density in the control group with that of group 1 and group 2, whereas no significant difference existed between group 1 and group 2. There was an increase in tooth enamel density of those mice pups belonging to groups whose mothers had consumed saltwater fish.

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Introduction

Dental caries constitute a dental condition detrimental to tooth tissue, especially that of enamel due to bacterial activity, characterized by demineralization of an inorganic matrix which then impairs the organic matrix. The three main factors that play an important role in the development of caries are cariogenic microorganisms, cariogenic substrates and hosts (teeth). These factors interact when the ongoing demineralization and remineralization balance on the tooth surface is disrupted.[1]

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Tooth enamel is formed by a process called amelogenesis which necessitates protein to ensure its density. Ameloblast cells require various proteins such as amelogenin, ameloblasin andenamylein, essential for enamel maturation, to be present. High nutritional intake during pregnancy can prevent the occurrence of caries in a pup's teeth. Conversely, malnutrition during pregnancy can affect both the development and pattern of tooth eruption and caries in the future. During formation, the intake of nutrients and oxygen by enamel is mediated by ameloblast cells through a process called amelogenesis. The mechanism effect of saltwater fish consumption by pregnant mothers on the densityof the enamel of a pup's oldest teeth appears in explicable.[2,3]

Dental caries are rare in pups that consumesaltwater fish. Coastal inhabitants frequently consume saltwater fish which affects the pattern of daily consumption of food by mothers and children aged 7-12 years-old.[4] A study conducted by Abdulah (2009) stated that most fish catches consisted of *Osteichthyes*

class (boned fish), with *Ordo Perciformes* (Milkfish) constituting more than 50%.[5,6] The strongest saltwater fish consumption trends were among school-aged children living in coastal areas compared to those resident in non-coastal areas. According to a study conducted in Kelurahan Gempolsewu District Rowosari Kendal Regency, the incidence rate of saltwater fish consumption was 94.9%. A study conducted by Falah & Christiono (2013) argued that there is a significant link between the consumption of saltwater fish by women aged 20-40 years-old resident in coastal areas and their DMFT index.[7–9] An investigation conducted by Danifatis & Christiono (2016)[5] identified the significant effect of consuming various types of saltwater fish, crabs and squids during pregnancy on limited cases of Early Childhood Caries (ECC). The study was conducted by means of an observation of the oral cavity health of children aged from 24 to 71 months with 32 ECC samples, 16 positive and 16 negative, selected by consecutive sampling of the entire research population.

The enamel layer of teeth is formed by ameloblast cells which produce mineralized enamel with a large number of highly mineralized prism.[10,11] These cells also regulate the precipitation of hydroxyapatite crystals in the maturation stage. The accuracy of micro-computed tomography (µCT) technology when employed to estimate the mineral content (weight and/or density) and volume is comparable with that for directly weighed and sectioned enamel. Different sets of mandibular incisors taken from mice were used for dissections and µCT reconstructions. Microdissection technique is destructive, time-consuming and requires considerable manual dexterity. Micro-computed x-ray tomography (µCT) is a non-destructive method that perceives mineral content and density at different x-ray intensity before and after passing through an object.[12]

According to Kraus & Jordan, intra uterine teeth development begins at 11 weeks.[13] Genetic disorders could cause mutations which impede protein function or result in the failure of protein formation. A study conducted on house mice demonstrated that the presence of mutations in extracellular enamel is caused by the misfolding of amelogenin proteins. Such proteins are required by ameloblasts during the process of amelogenesis and, consequently, cases of misfolding can lead to susceptibility to Amelogenesis Imperfecta.[14] Tooth enamel is the hardest tissue in mammals and enamel maturation contains less than 1% of all the organic matter controlled by ameloblast cells.[15] Tooth development in subjects began with embryogenesis on the 11th day, continued during the cap stage on the 15th day and the bell stage on the 18th day.[16]

The distribution of saltwater fish powder that contains calcium and Omega 3 and other proteins is believed capable of optimizing the formation of inorganic and collagen (organic) materials at the prenatal period resulting in the development of enamel density. Based on the background outlined above, the author aims to prove the effect of saltwater fish powder on the ability of female mice to increase the enamel density of their pups.

Methods

This study which incorporated the use of experimental subjects was approved by an ethical review conducted by the Ethics Commission, Faculty of Dental Medicine, Universitas Airlangga, with certificate number 010/HRECCFODM/II/2018. The study type was true experimental incorporating a Completely Randomized Design. The sample consisted of 10-week old impregnated mice (*Mus musculus*) with a body weight of ± 20-30 gram and in a healthy condition (nimble, energetic, possessing clean, injury-free skin and bright eyes). The samples consisted of 24 mice, namely: a control group, group 1- administered with 1.07 mg/0.5 ml 3 times a day every 6-8 hours and group 2 - administered with 2.14 mg/0.5 ml 3 times a day every 6-8 hours. The control group, dose group 1 and dose group 2 were sacrificed on the 18th day of pregnancy.

Mice (*Mus musculus*) that had undergone a 7-day environmental adaptation were kept in plastic enclosures fitted with wire lids. The pedestal of the cage was covered with wood shavings that were replaced every three days, while limitless (*ad-libitum*) supplies of food and drink were provided. A pregnancy test was conducted on day 3. If vaginal plugs could be seen in the vulva of the mice, it was declared the 0th day of pregnancy. The subjects were then divided into groups of five for each cage.

The materials used in this study consisted of dehydrated saltwater fish derived

from sardines (*Sardinellafimbriata*), splendid ponyfish (*Leiognathussplendens*) and cobs (*Euthynnusaffinis*), an extraction of saltwater fish in powder form and emulsifier CMC (*Carboxy Methyl Cellulose*) (Faculty of Chemistry UGM, Indonesia). The procedure for making fish powder comprised the following steps: weighing the fish according to dosage and dissolving it in hot water at 70°C. Saltwater fish powder was homogenized, being crushed and softened using an ultraturax device (IKA, Germany). 1% CMC material was stirred for about 15 minutes until homogenous. The calcium value of saltwater fish powder was one of 5.56% b/b usingan ICP (Inductive Coupled Plasma) method and the omega 3 level was 3.34% as measured by means of gas chromatography.

10-20 ml of chloroform (Henan Haofei Chemical Co., Ltd, Indonesia) were used to euthanise the subjects. The chemicals used for dental histology preparations were 10% formalin and alcohol atrespective concentrations of 70%, 80%, 95% and 96% of alcohol absolute, xylol, paraffin and Hematoxilin Eosin (HE).

A surgical procedure was performed on the subjects following the adminstering of an anesthestic in the form of chloroform. The subjects were locked in a jar before 10-20 ml of chloroform was poured over cotton balls which were subsequently placed in the confined space. After two and five minutes, observation of respiration and heart beat was conducted to check whether these had ceased in order that the jar could be opened. Prior to surgery, the subjects were killed by dislocating the collarbone to ensure they had actually died before being secured to a surgical board by means of pins. An initial incision was made in the abdomen or uterus using curved scissors. After the right and left corpus luteum could be observed, each of which was still covered by the amniotic membrane, the fetus and placenta were removed from the uterus. The tissue was then fixed using formalin in order that an observation of placental and prenatal tooth tissue could be performed.

The enamel density was measured using a Micro Computed Tomography Scanner (Skyscan, Kontich, Belgium), commonly known as a µ-CT Scanner, which is a device used to map and project the structure of a material in three-dimensional space (3D projection) with the help of x-rays. Fetal mice samples were dehydrated for 24 hours to remove tissue moisture. Specification of the irradiation applied is as follows: Scanner, SkyScan1173, *Source of Voltage* (kV) = 20, *Source of Current* (uA) = 60, *Number of Rows* = 2240, *Number of Columns* = 2240. The Enamel Density result was measured using a *GrayScale* indicator (Figure 1).

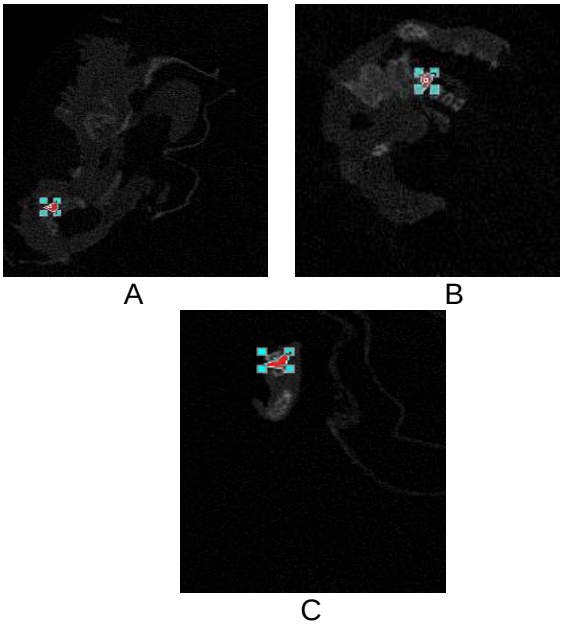

Figure 1. Enamel Density Measurement of House Mice. (A) Molarsof Subjects in the Control Group. (B) Molars of Subjects in Group 1. (C) Molars of Subjects in Group 2.

Results

The mean value of the tooth enamel density of group 2 was the highest at 124.5 ± 2.03, while the lowest was that of the control group at a value of 89.50 ± 1.41 (Table 1).

Group	N	Median	SD
Control	8	89,50	1.41
Group 1	8	124,5	2.03
Group 2	8	116,5	1.18

Table 1. Median and Standard deviation of enamel Density.

The results of the enamel density analysis with a Grayscale assessment indicator were processed using a One Way Anova statistical test. The result of the control group and group 1 was sig. 0.002 ($p < 0.05$) with a significant

difference; the value of the control group and group 2 was sig. 0.009 ($p < 0.05$) also with a significant difference, while the result of group 1 and group 2 was sig. 0.477 ($p > 0.05$) with no significant difference.

Discussion

The development of enamel (amelogenesis) can be divided into several stages: presecretory, secretory, transition and maturation. Each stage is described according to morphology and ameloblast function. Ameloblasts are single cell layers that cover the development of enamel and are responsible for its composition. Ameloblasts constitute that part of the enamel consisting of an outerepithelial layer, reticulum stellate, intermedium stratum and inner enamel epithelium. The prececretory stage is initiated when the mineralization process in an area later referred to as the dentinoenamel junction (DEJ) commences.[17,18,19]

Optimum maternal nutrition is important for both the formation of the matrix and mineralization of primary teeth, also being requiredin infancy and childhood for permanent teeth calcification. The presence of nutritional deficiencies during pregnancy might interfere with ameloblast activity at the calcification stage, resulting in a potential lack of protein matrix production and hypoplasia.[20,21]

In this study, a higher mean of Grayscalevalue in the treatment group for density enamel.This study aims to discover the degree of remineralization occurring in primary tooth enamel, while building on that conducted by Rusu (2014)[22] which proved the MicroCT method to be an effective tool for evaluating new bone growth in rats. This method enables both the numerical quantification of new bone regeneration and a determination of the bone healing process. In the tooth calcification process, calcium is an inorganic material present in the highest concentration and is a major component of enamel and dentin formation. The process begins after four months of intrauterine development at which point the calcification of teeth is inseparable from the process of calcium metabolism. Intercellular fluid and blood should contain calcium ions at sufficient precipitation levels. A factor that influences the addition of these ions is calcium intake during the calcification process.[9]

In this study, the highest protein and mineral content of saltwater fish powder was found to be that of calcium and omega 3. An increase in enamel density showed that calcium and omega 3 could penetrate the placenta barrier. Calcium will bind to CaT1 in order to penetrate the placenta membrane before being transported by Calmodulin to the fetus. A study conducted by Turnbull et al. states that calmodulin examined with a calbindin 28 kDa biomarker acts as an active means of calcium transport.[23] Other field studies have suggested that omega-3 polyunsaturated fatty acids (ω-3 PUFAs) increase bone strength. The ω-3 PUFA in bones affect changes in calcium absorption, osteoblast differentiation, lipid oxidation, eicosanoid production and inflammation.[24,25]

Omega 3 polyunsaturated fatty acid transport tends to act as anautocrine which can self-regulate uptake, transport and metabolism in the placenta. A study states that omega 3 polyunsaturated fatty acids can increase Placental FATP1 and FATP4 mRNA expression in humans and mice. Polyunsaturated fatty acids such as DHA, EPA, AA and LA represent a natural ligand against peroxisome Proliferator-Activated Receptors (PPARs), which possess anti-inflammatory properties that could suppress the expression of proinflammatory cytokine in the placenta at the end of pregnancy.[26] Factors such as proinflammatory and hormonal cytokines are capable of regulating the transportation of proteins in the placental system. Insulin, interleukin-1 (IL-1), and tumor necrosis factor (TNF) could stimulate the activity of the amino acid transporter.[27]

Polyunsaturated fatty acids taken by the placenta and transported to the fetus originated from two sources in the maternal circulation: nonesterified fatty acids (NEFAs) and esterified fatty acidsin triglycerides (TGs). Fatty acid transport proteins (FATs) are important integral membrane proteins for the absorption of cells from long-chained fatty acids. The study of trophoblast cell cultures on the placenta has explored a mechanism that regulates placental FATs, especially at mRNA expression levels. Peroxisome proliferator-activated receptor (PPAR)γ regulates FATP1, FATP2 and FABP4 mRNA expression. Fatty acids are esterified, beta-oxidized or transferred to the fetus via transport by Fatty Acid Binding Proteins (FABPs) in the cytosol in syncytiotrophoblast.[27]

Polyunsaturated fatty acid is reputed to be capable of increasing tooth density strength against caries. Other field studies have proven that Omega 3 fatty acids, especially DHA, positively affect bone minerals in BMD (Bone Mineral Density) in adult males.[28] A study by Luke (2011) mentions that the consumption of Omega 3 could change the absorption of Ca^{2+} and osteoblast differentiation. There was an increase in Ca^{2+} absorption, decreased levels of Ca^{2+} in urine and elevated levels of Ca^{2+}in bones in a 5-week study on Spraque-Dawley rats that were fed Omega 3.

The density of intrauterine teeth is below the average of those already classified. In this study, the mean Grayscale value of the control group was 89.50, while those of the two treatment groups were 124.5 and 116.5 respectively. A Grayscale image is a digital image that possesses only one channel value on each pixel, therefore appearing as a gradation of black and white which produces a gray effect. In this type of image, color is expressed intensity ranging from 0 to 255. A value of 0 signifies black, while a value of 255 denotes white. There are various degrees of gray from black to near white which have a depth of 8-bit color.[29]

This study could not use a Hounsfield Unit (HU) form of measurement because Source Voltage (kV)=20 was employed. This study differs from that which Metscher (2009)[30] conducted by using microCT in mice embryos with Source Voltage (kV)=60 at an average energy of 41 kev. The differentiation between hard and soft tissue did not appear detectable. Osmium tetroxide has become the most common contrast agent for microCT imaging on soft tissue. Osmium possesses advantageous energies capable of binding electrons for strong x-ray absorption, while it is also known to bind cell membranes and other structures, including nerves.[31,32]

Conclusion

From this study, it can be concluded that there is an increase in tooth enamel density in mouse pups born to females that consume saltwater fish powder.

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