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THE EFFECT OF PREGNANCY MILK ON THE EXPRESSION OF KALLIKREIN RELATED PEPTIDASE-4 (KLK-4) AND COLLAGEN TYPE 1 (Coll-1) IN AMELOGENESIS

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ABSTRACT
Background: *Tooth development during embryonic period is a complex process and requires adequate nutrients for the formation of healthy dental tissues. Kallikrein-related peptidase-4 (KLK-4) and collagen type 1 (Coll-1) are serine proteinases secreted by ameloblast during the transition and maturation stages of the amelogenesis processes, functioning to degrade the protein matrixes, so that the enamel can reach its final hardness. Pregnancy milk contains various nutrients expected to increase the KLK-4 expression of ameloblast cells in tooth development processes* ***Purpose:*** *This study aimed at determining the influence of pregnancy milk on the KLK-4 and collagen type 1 (Coll-1) expression of ameloblast cells in the tooth development processes.study* ***Method*** *The research subjects comprised of 10 pregnant female mice (Mus Musculus L.) that were divided into: control group (given sterile aquadest) and treatment group (given pregnancy milk + sterile aquadest) for 18 days followed by the collection of the tooth germ. The specimens were then stained using Imunnohistochemistry to see the KLK-4 and Coll-1 expressions. The data were analyzed using a pathway analysis.* ***Result:*** *The average KLK-4 and Coll-1 expression in the treatment group were higher than those in the control group. Based the pathway analysis, there were direct correlation of Pregnancy milk with Coll-1 expression and that with KLK-4 and Coll-1 expression as well as indirect correlation of pregnancy milk with KLK-4 expression.* ***Conclusion:*** *Pregnancy milk influences the Kallikrein-related peptidase-4 (KLK-4) and Coll-1 expression of ameloblast cells in the tooth development of the mice's fetuses*

Keywords: *Coll-1 pregnancy milk, Kallikrein-related peptidase-4 (KLK-4), Tooth development*
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INTRODUCTION

Tooth enamel is formed from a process called amelogenesis. Amelogenesis process is currently known to require a protein for tooth enamel density. Ameloblasts requires various proteins for tooth enamel maturation, such as amelogenin, ameloblastin, enamelin, kallikrein-4 and collagen type 1 [1]. Nutritional intake during pregnancy may prevent caries in child's teeth. Malnutrition during pregnancy may influence tooth eruption development and pattern and caries occurrence in the future [2,3].

Kalikrein-4 (KLK-4) was first found by a Japanese researcher characterizing it from pig teeth. Kalikrein-4 was isolated from pig teeth growth at average Ph (5.1-10.0), and was active at pH 6.1. The kalikrein-4 expression on tooth growth may be characterized using stage-specific northern blot hybridization, in situ hybridization and immunohistochemistry [4,5] .

Ameloblast lineage cell expresses collagen type I (Coll-1) [6]. Coll-1 is fibrous protein commonly found in remineralized tissue. COL1A1 serves as stimulator which influences proliferation and differentiation of ameloblast cells and adds to tissue hardness [7].

Pregnancy milk contains various nutrients necessary in the process of tooth formation and development, such as protein, calcium, phosphor, vitamin A, vitamin C and vitamin D [8]. Nutritional intake during pregnancy may prevent caries in child's teeth. Malnutrition during pregnancy may influence tooth eruption development and pattern and caries occurrence in the future [8–10]. Enamel formation requires nutritional intake and oxygen mediated by ameloblast cells called amelogenesis. The influence of pregnancy milk on the KLK-4 and Coll-1 expressions cannot be explained.

MATERIAL AND METHOD

This research was under approval of the Committee of Ethics, Faculty of Dentistry, Sultan Agung Islamic University, Semarang under No. 178/B.1-KEPK/SA-FKG/I/2020. The research was conducted at the Integrated Biomedical Laboratory, Faculty of Medicine, UNISSULA, Integrated Electron Microscope and Laboratory Unit Faculty of Medicine UNAIR, and Pathology-Anatomy Laboratory of Sultan Agung Islamic Hospital, Semarang.

This was a true experimental laboratory research with posttest only control group design. The research samples were 10 female mice (Mus Musculus L) which were divided into two groups, namely control group, in which 5 pregnant mice were given with sterile distilled water for 18 days, and treatment group, in which 5 pregnant mice were given with pregnancy milk at a dose of 117 mg dissolved in 0.5 ml sterile distilled water for 18 days.

This research employed some instruments, namely mice cage in size of 40cm x 30cm, food and drink containers, plastic pot, cotton bud, feeding tube, analytical balance, anatomical tweezers, surgical scissors, syringe volume 1ml, blade and scalpel, container in capacity of 20cc, microtome, object glass, cover glass, staining jar, clearing xylol, water bath, and light microscope. The materials used were Prenagen® mommy moka, marker KLK-4 from HRP-Linked Polyclonal Antibody anti KLK-4: MBS2042557-HRP, MyBioSource, Inc. USA, marker (COL1A1) from Antibodi Monoclonal (MoAb) anti (COL1A1) ab88147 methylene blue 1%, sterile distilled water, cotton, alcohol 70%, formalin solution 10%, and chloroform,

The laboratory mice were mated by combining three female mice with two male mice in a cage. The combination of male and female mice was conducted when the female mice were having estrus phase. The estrus phase was determined by observing the result of vaginal swab under microscope at 400x magnification as marked with disappearance of leukocyte and nucleated epithelium, replaced with horned epithelial cell or cornification. Vaginal plug was observed the morning after and in case vaginal plug was found, this means the mouse has been copulated and enters day-0 of pregnancy.

Administration of dose of pregnancy milk was calculated based on conversion of (Laurense & Bacharach 1964), conversion of dose of human (70 kg) to mouse (20 gr) = 0.0026. The suggested dose of pregnancy milk was 45000 mg per day. The dose of pregnancy milk for pregnant mice was (20 gr) = (45000 x 0.0026) mg = 117 mg. In this research, it used dose of pregnancy milk of 117 mg dissolved in 0.5 ml sterile distilled water [11].

All pregnant female mice were divided into two groups, namely control group in which 6 pregnant mice were given with sterile distilled water and treatment group in which 6 pregnant mice were given with pregnancy milk at a dose of 117 mg dissolved in 0.5 ml sterile distilled water. The pregnancy milk was administered in the morning (at ±09.00 WIB) and afternoon (at ±16.00WIB) orally using feeding tube. In pregnancy on day-18, all pregnant mice were dissected and taken for fetal tooth germ. In the next phase, a histopathologic preparation (IHC preparation) using tissue microtechnique method. microscopic observation was then made to calculate the count of expression of KLK-4 and Coll-1 using light microscope equipped with Sigma camera at 100x & 400x magnifications. The KLK-4 and Coll-1 expressions were calculated using the Allred Score in combination with Hotspot method.

The data were analyzed using multivariate statistical test with path analysis.

RESULTS

After observation on the KLK-4 expression was conducted through histological preparation on mouse fetal tooth germ dyed using Immunohistochemistry (IHC) dyeing. The of KLK-4 and Coll-1 expressions were observed under light microscope equipped with Sigma camera at 100x and 400x magnifications, and calculated using Allred score in combination with hotspot methods that gives positive reaction of brownish cytoplasm on polyclonal anti KLK-4 antibody as follows

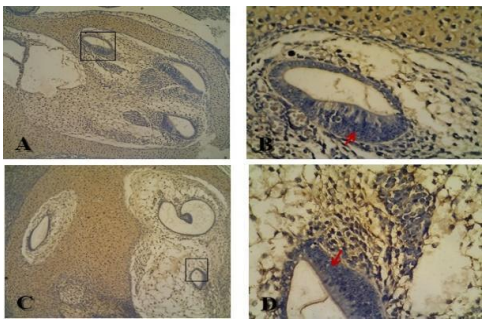

Figure 1. Immunohistochemical dyeing KLK-4. A,B with control group. C,D with treatment group. A,C at 100x magnification, B,D at 400x magnification. Arrow shows depiction of KLK-4 expression in pregnancy on day-18.

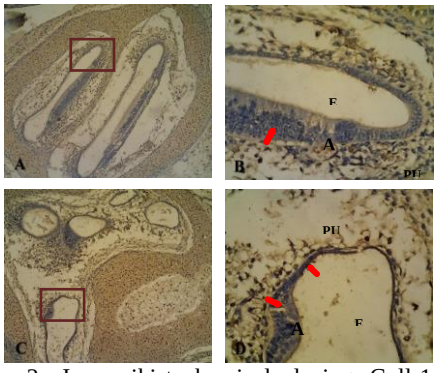

Figure 2. Immunihistochemical dyeing Coll-1. A,B with control group. C,D with treatment group. A,C at 100x magnification, B,D at 400x magnification. Arrow shows depiction of expression of Coll-1 in pregnancy on day-18.

The data for average value of expressions of KLK-4 are presented in table 1. below.

Table 1. Average and standard deviation of control group for KLK-4 and Coll-1 expressions.

Group	Number of Samples	Average	Standard Deviation
KLK-4 Expression	5	0.5914	0.2214708
Coll-1 Expression	5	23.6	10.85357

The control group that was given only sterile distilled water only has the average value of KLK-4 0.5914 with a standard deviation of 0.2214708 and Coll-1 23.6 with a standard deviation of 10.85357

Table 2. Average and standard deviation of KLK-4 and Coll-1 expressions in the treatment group

Group	Number of samples	Average	Standard Deviation
KLK-4 Expression	5	2.6674	1.373329
Coll-1 Expression	5	49.2	23.33881

The treatment group that was only given with pregnancy milk has the average value of KLK-4 2.6674 with a standard deviation 1.373329 and Coll-1 49.2 with a standard deviation of 23.33881

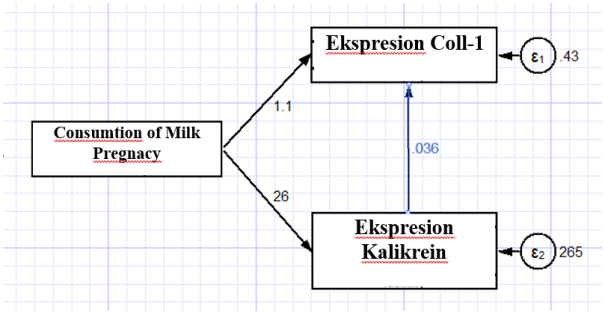

Figure 3. Path analysis

The result of path analysis shows direct influence of consumption of pregnancy milk with Coll-1 and KLK-4 expressions with Coll-1. Indirect influence of consumption of pregnancy milk is with KLK-4 expression.

DISCUSSION

Kallikrein is believed to play an important role in tooth enamel biomenieralization, and any disturbance in protein kallikrein may cause amelogenesis imperfect [12,13]. Amelogenesis imperfecta is a disorder caused by genetic factor as marked with defect on enamel, namely hypomineralization or hypoplasia. Amelogenesis imperfecta is influenced by mutated specific gen, which may lead to disturbance to the level of protein [12,14]. Kallikrein gen 4 (KLK4 known as prostase / KLK-L1), located at chromosome 19q13.4, is one member of newly found KLK gen family [10,15].

Amelogenesis process occurs in 3 phases, in secretory phase there is secretion of dental enamel protein followed with detached secretory organelles, getting shorter and loss of Tomes processes will become tooth enamel. In maturation phase, there is formation of enamel matrix by activating protein kalikrein-4 [16].

This research finds significant improvement in the of kallikrein-4 expression after administration of pregnancy milk. Research on Kalikrein-4 associated with formation of tooth enamel by evaluating enamel formation expects the importance of kallikrein-4 activity in tooth enamel development [1]. Protein Kalikrein-4 is derived from gen cluster kallikrein which serves in protein maturation process that will become enamel matrix. Kallikrein-4 and MMP-20 are two proteins secreted by ameloblast cells into enamel matrix. Kalikrein-4 cannot be activated without assistance of MMP-20 in the research conducted by Ryu et al. in vitro [1,4,11,17] .

Collagen is fibrous protein mainly located in the connective tissue in the body. This serves to produce and preserve cartilage, bone, tendon, ligament, blood vessel, cornea, dentin, and other body tissues. Collagen type I may be found in skin, tendon, ligament, bone, tooth, discus intervertebralis, and scar tissue. Some researches state that collagen is detected in tooth enamel in ameloblast cells [18].

This research discovers the increase of collagen type 1 expression in treatment group compared to the control group. Tooth enamel is formed only by a small amount of protein (less than 1% organic matters) [19]. During amelogenesis process, collagen expression decreases from secretory phase to mineralization and maturation phases, followed with a decrease in enameloid matrix through post-metamorphic juvenile teeth [7]. Researches on Atlantic salmon and zebrafish result in decreased expression of ameloblast during ameloid deposition, and decrease in maturation phase

[20]. Based on the research result, it is concluded that administration of pregnancy milk presents significant influence in increasing the Kallikrein-related peptidase-4 (KLK-4) and Collagen type 1 (Coll-1) expressions with ameloblast cells during mouse fetal teeth growth and development.

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