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THE EFFECT OF THYMOQUINONE TOWARD TOOTH DISORDER PREVENTION IN POSTNATAL RAT OFFSPRING BORN FROM HYPERGLYCEMIA PREGNANT RATS

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Abstract

Background and aims. *Hyperglycemia in pregnancy was caused by reduced insulin production that led to tooth germ growth disorder. Thymoquinone could increase insulin production through pancreatic β-cells regeneration. The purpose of this study was to determine the effect of thymoquinone to prevent tooth disorder in rat offspring born from hyperglycemia pregnant rat.* ***Material and method.*** *Wistar rat offspring used in this research taken from pregnant rat induced hyperglycemia with streptozotocin 40 mg/kgBW divided into four groups. Rat offspring observation had been done on 3rd, 5th, and 7th day postnatal. The histological image of first maxillary molar tooth germ stained with Haematoxilin Eosin and Mallory's Trichrome. The parameters of rat offspring were body weight, blood glucose levels, tooth growth stages, tooth dimension width, and tooth germ enamel matrix.* ***Results.*** *Thymoquinone group had the widest tooth germ dimension compare to other groups. All rat offspring tooth germs were at the apposition-calcification stage in matrix enamel (pre-enamel). The analysis showed that no statistical differences between thymoquinone group and metformin group (p>0.05).* ***Conclusions.*** *Thymoquinone has same function with metformin to prevent tooth disorder in rat offspring born from pregnant rat induced hyperglycemia.*

key words: *hyperglycemic, tooth germ, rat offspring, thymoquinone.*

Background and aims

Hyperglycemia during pregnancy is glucose tolerance disorder occurred or known for the first-time during pregnancy. Physiological changes and hormonal factors such as cortisol, prolactin, progesterone, and human placental lactogen were thought to be the cause of insulin resistance because these hormones play a role homologically with insulin antagonist growth hormone [1]. Hyperglycemia occurs in 7% of pregnant population every year [2].

The increases of oxidative stress in the postnatal rat offspring and fetus will result in protein synthesis disorder, which could cause tooth growth and development disorder in dental matrix formation. Rat offspring born from hyperglycemic pregnant rat has enamel matrix

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formation inhibited than postnatal rat offspring born from pregnant rat who did not suffer from hyperglycemia [3].

The rat offspring born from hyperglycemic pregnant rat has risk of nutritional deficiencies, whereas fetus should adapt to hyperglycemic condition with body metabolism changes, hormone production, tissue sensitivity, blood flow distribution, and body growth delay. The existence of this adaptation would affect the body's structure and function permanently, including tooth formation, tooth development, and tooth mineralization. The lack of nutrition led to the decrease of tooth tissue thickness, enamel and dentine composition changes, and also teeth shape and size. Therefore, rat offspring born from hyperglycemic pregnant rats could have affect tooth growth by slowing down of cell differentiation, changing growth factors response in tooth hard tissue, and affecting the speed of dental matrix synthesis, such as collagen and non-collagen tissues. This process was causing enamel hypoplasia, delay in tooth eruption, and decrease in length of alveolar bone. Protein synthesis was known to be very importance on formation matrix and hard tissue calcification process on teeth [3,4].

According to the *World Health Organization* (WHO) report, traditional medicine was used as primary treatment for 70–80% of the human population [5]. *Thymoquinone* extract, one of traditional medicinal plants, is an active agent from Black Cumin (*Nigella sativa L.*) reported could reduce blood sugar levels [6-8]. *Thymoquinone* (Tq) was very effective to protect lipids, proteins, mitochondrial DNA, and nucleus DNA from damage caused by free radicals. Previous studies showed that anti-diabetic from *thymoquinone* was able to reduce blood glucose levels by increasing insulin production and regenerating pancreatic β-cells structure. Oxidative stress could be reduced through reduction of hepatic gluconeogenesis mechanism. Compounds found in *thymoquinone* such as *carvacol, p-cymene, t-anethol* and *4-terpinol* also had strong antioxidant activities that could inhibit the formation of free radicals [8,9,20].

The purpose of this study was to determine effects of *thymoquinone* to prevent tooth disorder in postnatal rat offspring born from pregnant rat induced hyperglycemia.

Material and method

Study design and patients

This study was an experimental laboratory with the post-test only control group design. This study was using 48 *Wistar Rattus norvegicus L* rat offspring born from hyperglycemic pregnant rat.

Laboratory, anthropometric and clinical data collection

This study was approved by the Research Ethic Committee Faculty of Dentistry, Jember University, Jember, East Java, Indonesia. This study used animal subjects, namely; pregnant white Wistar strain rats (*Rattus norvegicus L*). On 10^{th} day of pregnancy, rats were adapted and controlled health for 24 hours by observing the behavior, physical condition, and active movement.

The animal models were injected intraperitoneally with *streptozotocin* (STZ) 40 mg/kgBW. Blood sugar levels of pregnant rat were examined with glucometer before injected by STZ. STZ solution made by dissolved the STZ powder (Bioworld, USA) in 50 mg/ml of 0.1M *citric acid buffer* pH 4.5. STZ injection solution should prepare in less than 5 minutes before injected because the solution is unstable [10]. One day after receiving an injection of STZ, the blood glucose levels of pregnant rat were measured by glucometer (GCU, Indonesia).

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The presence of hyperglycemic was confirmed when the blood glucose levels of the subjects were above 120 mg/dl and showed signs of polydipsia, polyuria, polyphagia, and asthenia.

The animal subjects were divided into four groups, such as Group A (normal pregnant rat; blood glucose level <120 mg/dl and did not given any therapy during the pregnant period), Group B (negative control; pregnant rat with hyperglycemia and no therapy), Group C (treatment group 1; pregnant rat with hyperglycemia and Metformin therapy 100 mg/kgBW by intragastric 2 times a day in the morning and evening as positive control group), and Group D (treatment group 2; pregnant rat with hyperglycemia and *thymoquinone* therapy 80 mg/kgBW intragastric 1 time a day). The treatment was done from the 1st day after positive hyperglycemic until 7th postnatal. One sample rat offspring of each group (n=48) was taken after birth by simple random sampling and observed on 3rd, 5th and 7th postnatal days for body weight and blood glucose levels.

The animal subjects of rat offspring were sacrificed by the administering of overdose ketamine. Thereafter, their right maxillary was removed and fixed in 10% formalin for 24 hours to serve the tissue samples of first maxillary molar tooth germ. After fixation time had elapsed, the tissue samples were decalcified in 10% formic acid for paraffin-tissue embedding. Each paraffin block sliced 6 µm then stained with *Haematoxilin Eosin* (HE) to observe tooth development stage and measuring tooth dimension of right maxillary first molar such as mesio-distal of *inner enamel epithelium* (IEE), cervical-occlusal IEE, mesio-distal *outer enamel epithelium* (OEE), cervical-occlusal OEE, and cervical-occlusal *enamel organ* (EO). All samples slide also stain with *Mallory's trichrome* to observe tooth matrix formation. Observation had been done with 40x and 100x magnification using a light microscope that connected to Optilab 3.0 Raster Image application.

Statistical analysis

The data analysis carried out using *Shapiro Wilk normality test* and *Levene homogeneity test*. The result of statistical analysis showed data is not normally distributed and not homogeneous. Thereafter, the data carried out non parametric statistical tests using the *Kruskal Wallis test* ($p<0.05$) and followed by *Mann Whitney difference test*.

Results

The research showed that blood glucose levels in all pregnant rats who injected by STZ was increased above 120 mg/dl. These results indicated that pregnant rats had hyperglycemia also clinically all hyperglycemia samples seem polydipsia, polyuria, polyphagia, and asthenia. The amount of urine volume was not measured by a measuring device, but it seen from wet and easily dirty husks compared to the normal group. Weight measurements in postnatal rat offspring showed that group A (normal) had the highest body weight followed by group D (*thymoquinone*), while group B (negative control) and group C (positive control) had almost the same body weight on each observation day (Figure 1).

The measurement of average blood glucose levels in postnatal rat offspring on all observation days mostly showed up to 120 mg/dl (Figure 2). Statistical analysis showed group A (normal) had no difference with group C (positive control) and group D (*thymoquinone*) on all observation days ($p>0.05$), while the statistical analysis in group A (normal) had a significant difference with group B (negative control) of 0.029 ($p<0.05$).

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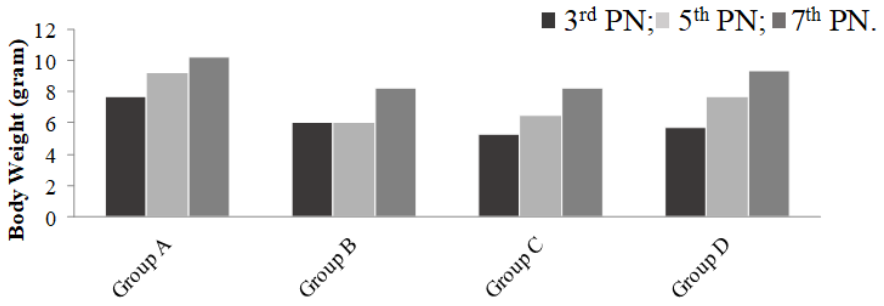

Figure 1. *The average body weight of postnatal rats offspring.*

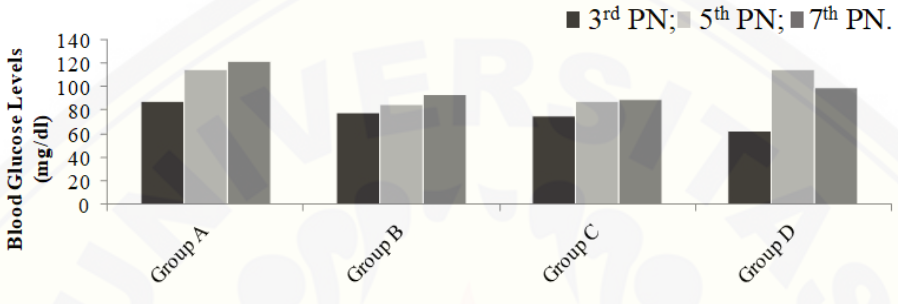

Figure 2. *The average blood glucose levels of postnatal rats offspring.*

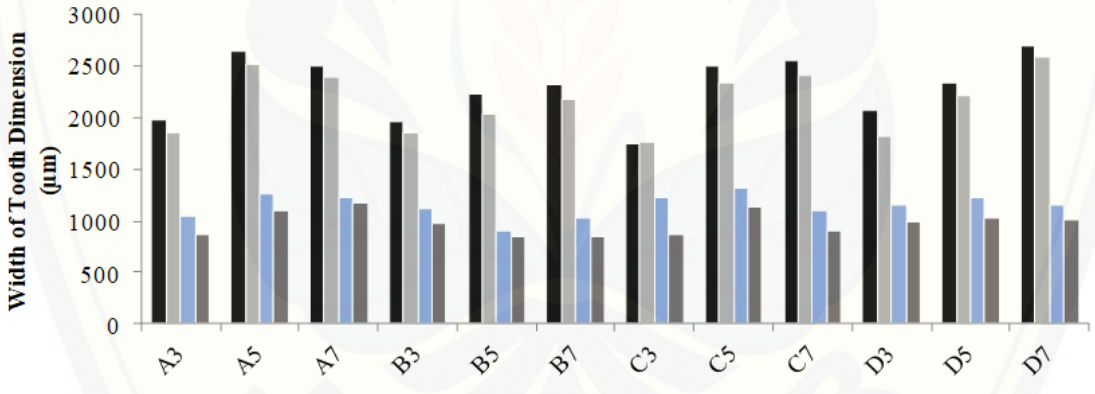

Figure 3. *The average width of tooth dimensions in postnatal rats offspring. Description: OEE: outer enamel epithelium; IEE: inner enamel epithelium; PN: postnatal; Panel A3, 5, 7 are Group A day 3rd, 5th and 7th; Panel B3, 5, 7 are Group B day 3rd, 5th and 7th; Panel C3, 5, 7 are Group C day 3rd, 5th and 7th; Panel D3, 5, 7 are Group D day 3rd, 5th and 7th;*

Mesio-Distal OEE; Mesio-Distal IEE; Servico-Oklusal OEE; Servico-Oklusal IEE.

The measuring of width germ tooth of right maxillary 1st molar from 3th, 5th and 7th postnatal rat offspring showed that group B (negative control) had smaller dimensions than group C (positive control), while group D (*thymoquinone*) and group C (positive control) had same dimensions with group A (normal) (Figure 3).

Histological appearance by H &E staining showed that all dental samples of postnatal rat offspring had been range advanced bell stage such as from pre-enamel/pre-dentin formation undergoes to apposition and calcification process. Stage of apposition and calcification could be observed as thinning eosinophilic of rest enamel matrix or dark colour (basophilic

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stain) after decalcified process whereas a pre-dentin formation who detected by light eosinophilic color above dental papilla (Figure 4). *Mallory's trichrome* staining showed formation of dentine collagen matrix that stained as blue and pre-dentine seen as yellow stain, whereas pre-enamel stained as red (Figure 5 and 6C). However one sample Group A, B, C and two sample Group D on 3rd day showed slightly late in apposition, they have been still in matrix collagen formation that stained blue (Figure 6B), meanwhile there is one sample of Group B, the group negative control more lately until on 7th day did not any formation of matrix collagen. It could be occurred by malnutrition of rat offspring (Figure 6A).

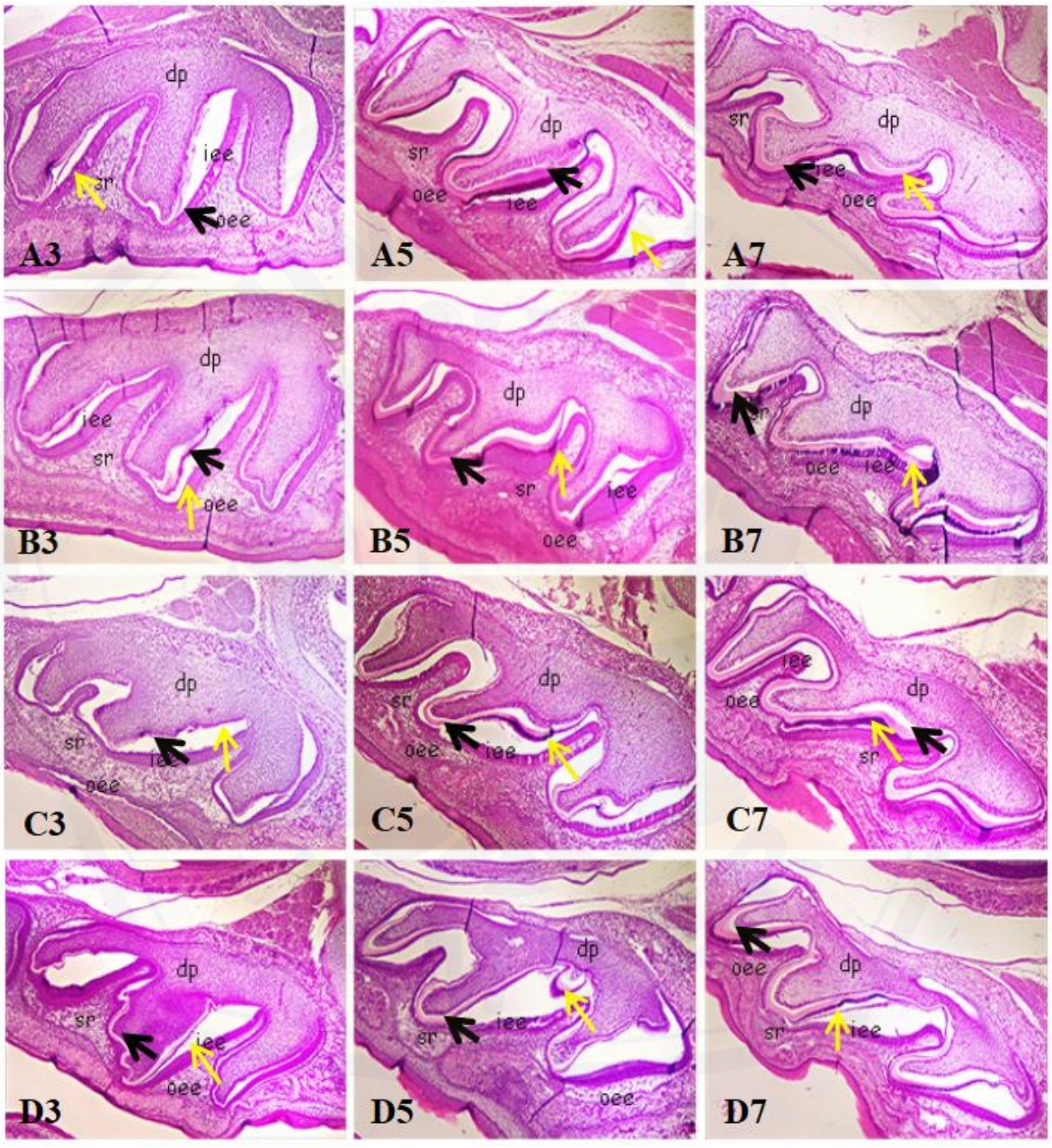

Figure 4. *Histological observation of 1st molar tooth germ in postnatal rat offspring with 40x magnification in normal group (A), negative control group (B), positive control group (C), and thymoquinone treatment group (D) using Haematoxilin Eosin staining showed tooth germ in the stage of apposition and calcification. Description: DP: Dental papilla; IEE: Inner enamel epithelium; OEE: Outer enamel epithelium; SR: Stellate reticulum; black arrow: dentine matrix; yellow arrow: pre-enamel. Panel A3, 5, 7 are Group A day 3rd, 5th and 7th; Panel B3, 5, 7 are Group B day 3rd, 5th and 7th; Panel C3, 5, 7 are Group C day 3rd, 5th and 7th; Panel D3, 5, 7 are Group D day 3rd, 5th and 7th.*

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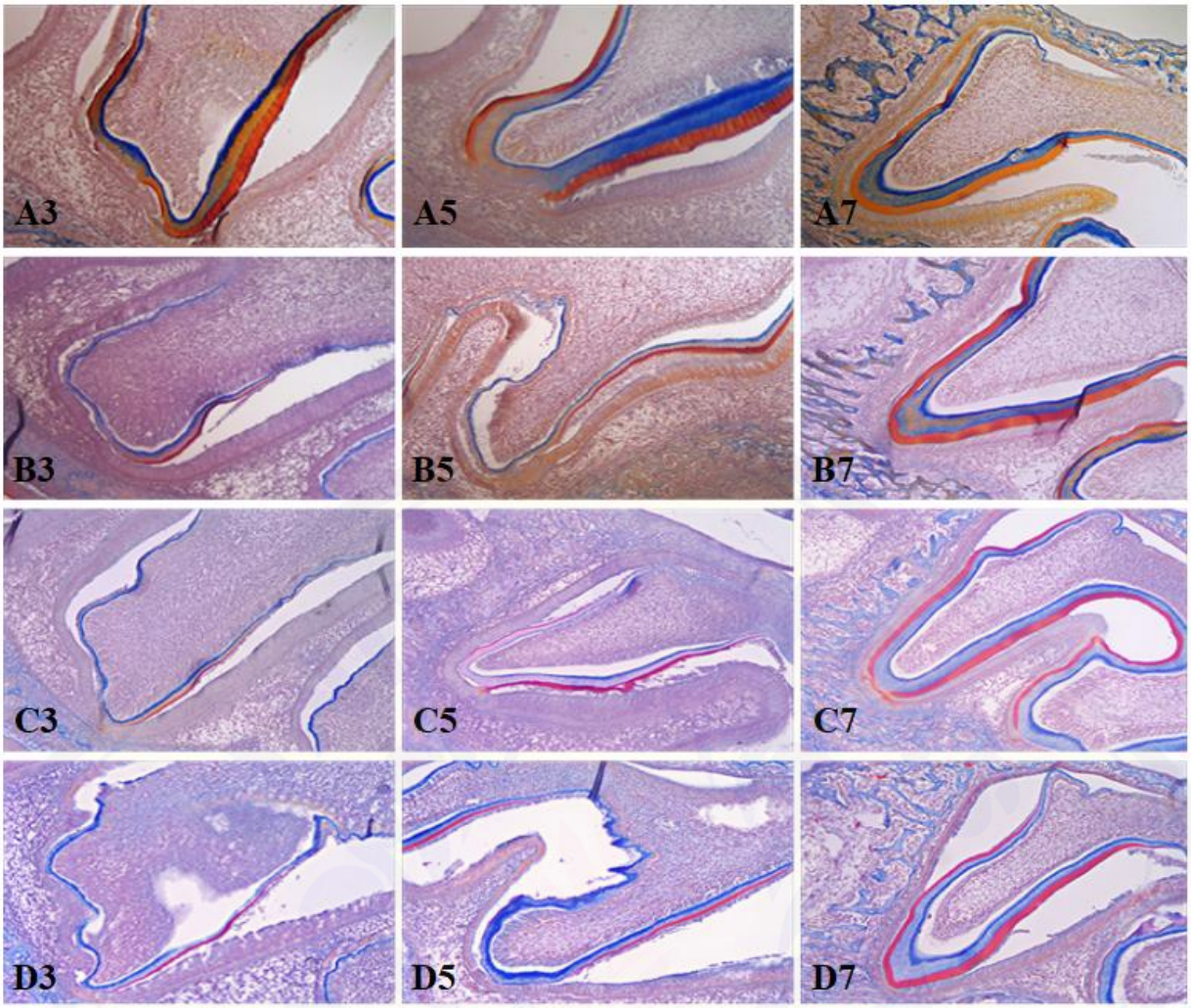

Figure 5. *Histological observation of 1st molar tooth germ in postnatal rat offspring with 100x magnification in normal group (A), negative control group (B), positive control group (C), and thymoquinone treatment group (D) using Mallory's Trichrome staining showed dentine collagen matrix stained as blue, pre-dentine stained as yellow, pre-enamel stained as red, mature enamel matrix that had been calcified and lost due to the decalcification process would appear as an empty gap between IEE (Inner Enamel Epithelium) and dental papilla, alveolar bone matrix also clearly showed in 7th day. Panel A3, 5, 7 are Group A day 3rd, 5th and 7th; Panel B3, 5, 7 are Group B day 3rd, 5th and 7th; Panel C3, 5, 7 are Group C day 3rd, 5th and 7th; Panel D3, 5, 7 are Group D day 3rd, 5th and 7th.*

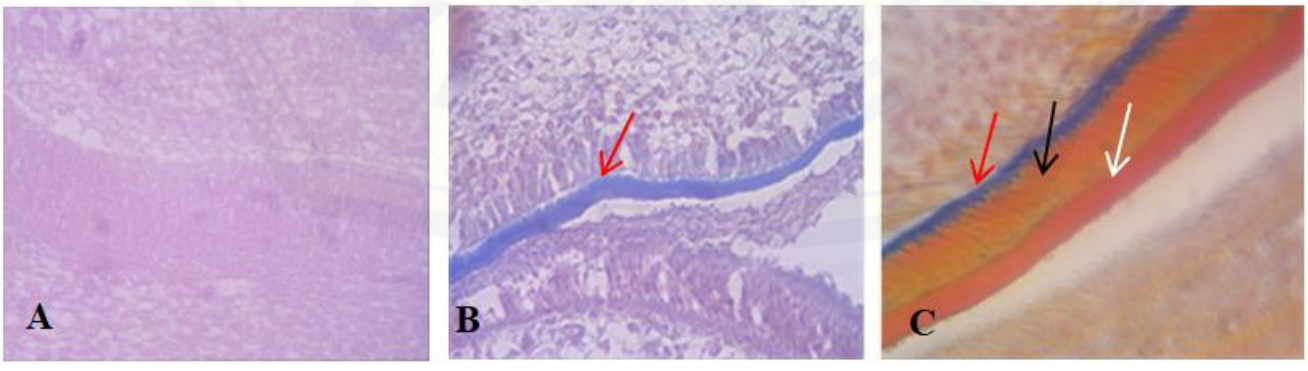

Figure 6. *Histological observation of 1st molar tooth germ in postnatal rat offspring with 400x magnification by Mallory's Trichrome staining (A) no formation of matrix collagen (B) formation of dentine collagen matrix that appears as blue (red arrow). (C) pre-dentin that appears as yellow/orange (black arrow), pre-enamel appears as red (white arrow), and the mature enamel matrix looks like an empty gap between IEE (Inner Enamel Epithelium) and dental papilla who indicated calcified enamel had been formed.*

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Discussion

The induction of hyperglycemia by injection of a single dose STZ 40 mg/kgBW in this research might cause increased blood glucose levels until above 120 mg/dl of pregnant rat, in the other hand also seen the hyperglycemia pregnant rat had increasing of feed consumption (polyphagia), water consumption (polydipsia), and urine production which characterized by easily wet in husks (polyuria) [3,4].

STZ is a diabetogenic agent that caused pancreatic beta cell necrosis and inhibits insulin secretion [12]. STZ enter pancreatic β cells through the GLUT2 glucose transporter resulted decreasing the sensitivity of peripheral tissue receptors causes increasing insulin resistance and blood glucose levels [14]. Rat offspring born from hyperglycemic pregnant rat had smaller body weights and tooth size, and enamel matrix formation was inhibited compare to rat offspring born from pregnant rat who did not suffer from hyperglycemia.

Weight gain of rat offspring along observation day in all samples has been due to growth and development process. In this study, negative control group had low body weight compare to normal group and treatment group. Disturbing of nutrient metabolism of hyperglycemic pregnant rat has resulted in insufficient nutrition to offspring growth, in this case that matter occur not only during pregnancy but also during breastfeeding. There is research reported that long-term effects of hyperglycemia along pregnant rats could reduce the amount and change the composition of milk during lactation in neonatal and postnatal breastfeeding rat offspring. This condition may cause why rat offspring of negative control group who had lower body weight compared to rat offspring who born from normal pregnant rat. The body weight difference between these groups was statistically significant on day 7. This result also supported by Touger, *et al.*, (2011) report that offspring body weight who born from mother hyperglycemic during pregnancy have lower body weight than control group (normal) on day 7 until day 21 postnatal [13,14]. Meanwhile, the average body weight of rat offspring in Group D closely with the average of rat offspring body weight group A who born from normal pregnant rat.

Due to tooth growth and development in this study showed there is one sample of negative control group on 3rd day had slightly late in tooth apposition, histologically it seem in matrix collagen formation phase (Figure 6B) also there is one sample in this more severe because on 7th day postnatal did not any seem tooth matrix collagen formation (Figure 6A). Based on the measurements of 1st right molar tooth width of rat offspring, it was found that that the largest dimensions of tooth germ were found in the *thymoquinone* group. Meanwhile, the negative control group had smaller tooth germ width average compare to other groups. This result supported by Dewi (2014) who said that the tooth germ width of rats offspring in hyperglycemia group was smaller than control group, the researcher considered that matter due to increase of blood glucose levels which correlated with occurrence of malnutrition. As known that increasing blood glucose levels of pregnancy rat in this research associated with pancreatic β-cells damage by the presence of DNA methylation reducing the amount of NAD^+ and increasing free radical production through the action of cell toxicity [3]. Free radicals would cause DNA damage and reduced the ability of pancreas to produce insulin and then cause disruption metabolism of carbohydrates, fats, and proteins that are useful as a source of nutrition [4].

Nutritional deficiencies during pregnancy due to diabetes have direct impact on the

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development of enamel and dentine in the primary and permanent teeth during fetal growth [16]. That impact such as dentin and enamel maturation disturbance, decrease tooth composition, tooth shape and size disorder and late tooth eruption, also have influence to soft tissue, and saliva composition [4,15]. One of nutrients that needed during growth and development is calcium. Calcium plays a role in the process of mineralization and calcification of tooth, calcium deficiency results in dental hypocalcification and delay in eruption. Enamel contains inorganic ingredients up to 96% and dentin 70%, and around 37% of these inorganic ingredients is calcium. Nutrition also influences the speed of tooth collagen matrix and non-collagen tissue synthesis that the place for calcium salts deposition [3,14,16].

As known that normal tooth growth and development still have any several variation that related to the presence of recessive genes obtained from the parent [17], in this study also we found any slightly variation of tooth germ growth and development of 1st molar in normal group on 3rd day observation a number 25% of sample which still in matrix formation while most samples were inorganic apposition stage. The tooth developing process occurs under genetic control including stimulation of interactions between epithelial cells and mesenchyme regulation. The interaction between epithelial-mesenchymal cells will trigger mitosis process, preventing apoptosis near the surface of the tissue, affecting cell shape, cell differentiation, and stimulating the release of a number of organic and inorganic molecules [17] that will occurs through a series of stages that begin from bud phase, cap phase, bell phase, apposition and calcification intrauterine and postnatal [18]. Those process could disturb by any factors such as hypoinsulinemia [12].

Hyperglycemia during pregnancy could cause deficiency nutrition in rat offspring that is led to hypoinsulinemia and effect to abnormal glucose metabolism resulted tooth malformations and neonatal hypoglycemia of rat offspring [12]. This matter likely seem in this research group B, C and D on begin of delivery, however the body weight and tooth development of rat offspring is getting better due to decrease in mother rat blood glucose level after therapy metformin and thymoquinone (Group C and D) close to normal group (group A). Meanwhile in this study we found one sample rat offspring of group D who treated with *thymoquinone* had body weight only 3 grams, this body weight was the lowest compared to other samples and had low blood glucose levels were less than 20 mg/dl. Based on statistical analysis by *Mann Whitney Test* there were no significant differences between group A (normal) and group D (*thymoquinone*) on all observation days ($p>0.05$).

The ability of *thymoquinone* to inhibit *glucose-6-phosoatase* enzyme could reduce blood glucose levels by increasing insulin production and regenerating pancreatic β-cell structure with a mechanism decreasing hepatic gluconeogenesis and increasing their antioxidant effects through ROS modulation [19]. *Thymoquinone* could improve glucose homeostasis through modification of the main enzyme activity of carbohydrates through increased insulin secretion in STZ-induced hyperglycemic rat models [20]. The ability of *thymoquinone* to repair pancreatic β-cells can restore nutritional metabolism to normal [15].

Conclusions

Based on this research we considered that thymoquinone had similar function with metformin to prevent tooth disorder in rat offspring born from pregnant rat induced

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hyperglycemia with blood glucose levels, body weight, tooth growth stage, tooth dimensions width, and tooth germ enamel matrix is same with postnatal rat offspring who born from normal postnatal rat.

Further research is needed more study to know molecularly role play of thymoquinone using other parameters and methods to prevent fetal tooth disorders that cause hyperglycemia in pregnancy periods.

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