

Research Report

Effect of gestational diabetes mellitus on the expression of amelogenin in rat offspring tooth germ

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ABSTRACT

Background: Amelogenin is a major protein constituent of the developing enamel matrix that is critical for enamel formation. Mutations of amelogenin cause hypoplastic enamel phenotypes. Previous research found that infant of diabetic mother has higher risk for having enamel hypoplasia. **Purpose:** The aim of this study was to determine the effect of gestational diabetes mellitus on the expression of amelogenin in Wistar rats offspring tooth germ. **Methods:** Sixteen female Wistar rats, aged 2.5-3 months, body weight 150-200 g were used in this study, Wistar rats were mated and divided into two groups and treated on day 0 of pregnancy. Group A was DM group, consisting of 8 rats, induced by streptozotocin (STZ) injection 40 mg/kg BW. Group B was control group, consisting of 8 rats received citrate buffer injection. Thirty-two rat pups were decapitated on day 5. Immunohistochemical procedures were performed on molar tooth germ of the mandibular rat pups using antibody anti-AMELX to determine the expression of amelogenin. Examination carried out on the images using ImageJ software. All data were then statistically analyzed by Mann Whitney test. **Results:** There was no significant difference in the expression of amelogenin in the DM group and control group ($p > 0.05$). **Conclusion:** Gestational diabetes mellitus did not affect the expression of amelogenin in rat offspring tooth germ. Further study is needed to examine the pattern of amelogenin expression with measurement of glucose levels of rat pups.

Key words: Gestational diabetes mellitus, amelogenin expression, Wistar rats

ABSTRAK

Latar belakang: Amelogenin merupakan protein terbanyak pada matriks email yang berperan penting dalam pembentukan email. Mutasi pada amelogenin dapat menyebabkan email menjadi hipoplastik. Penelitian sebelumnya menunjukkan bahwa anak yang dilahirkan oleh ibu pengidap diabetes memiliki resiko lebih tinggi untuk mengalami hipoplasia email. **Tujuan:** Penelitian ini bertujuan untuk meneliti pengaruh diabetes mellitus gestasional terhadap ekspresi amelogenin pada benih gigi anak tikus Wistar. **Metode:** Enam belas ekor tikus Wistar betina, umur 2,5-3 bulan, berat badan 150-200 g digunakan dalam penelitian ini, dikawinkan kemudian dibagi menjadi dua kelompok dan diberi perlakuan pada kehamilan hari ke-0. Kelompok A merupakan kelompok diabetes mellitus, terdiri atas 8 ekor tikus diberi perlakuan diabetes mellitus dengan injeksi streptozotocin (STZ) 40 mg/kg BB. Kelompok B merupakan kelompok kontrol, terdiri atas 8 ekor tikus diberi injeksi buffer sitrat. Tiga puluh dua anak tikus yang lahir didekapitasi pada hari ke-5. Dilakukan prosedur imunohistokimia pada benih gigi molar rahang bawah anak tikus menggunakan antibodi anti-AMELX untuk mengetahui ekspresi amelogenin. Pemeriksaan dilakukan pada hasil foto menggunakan software ImageJ. Hasil kemudian dianalisa menggunakan uji Mann Whitney. **Hasil:** Tidak terdapat perbedaan yang bermakna pada ekspresi amelogenin kelompok kontrol dan kelompok diabetes mellitus ($p > 0,05$). **Simpulan:** Diabetes mellitus gestasional tidak mempengaruhi ekspresi amelogenin pada benih gigi anak tikus. Diperlukan penelitian lebih lanjut untuk mengetahui pola ekspresi amelogenin dengan pengukuran kadar glukosa darah anak tikus.

Kata kunci: Diabetes mellitus gestasional, ekspresi amelogenin, tikus Wistar

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder of carbohydrates, lipids and proteins, which occurs because the pancreas can not produce enough insulin or because the body can not use insulin effectively resulting in increased levels of glucose in blood (hyperglycemia).¹ Gestational diabetes mellitus (GDM) is diagnosed when DM begins or is first detected during pregnancy.² Hyperglycemia during pregnancy can cause complications to the mother and fetus. Maternal complications associated with GDM include hypertension and increased risk of developing diabetes after pregnancy. Fetal complications include macrosomia, hypoglycemia, hypocalcemia, polycythemia, and hyperbilirubinemia.^{3,4}

An infant of diabetic mothers has higher risk for having enamel hypoplasia.⁵ Another study also showed thinner enamel in pups born to diabetic mother because of decreased secretion of enamel matrix and ultrastructural changes in the secretory ameloblast. The secretory ameloblast were shorter and the ameloblast nuclei were smaller. There were intracellular metabolic disturbances in consequence to the lack of intracellular glucose.⁶

Amelogenin is a hydrophobic protein that is expressed by ameloblast. This is the most abundant protein of the enamel extracellular matrix, compose 80-90% of total protein, and is expressed in the secretory until post-secretory stage of ameloblast.⁷ Amelogenin is essential for well-organized hydroxyapatite prism formation and for producing normal enamel thickness. In vivo studies of amelogenin null mice showed the occurrence of enamel hypoplasia, seen chalky-white staining on incisivus. Enamel thickness was less than 10% of normal enamel.⁸ Amelogenin expression can be influenced by several factors, such as blood glucose and calcium levels.^{9,10} The result of this experiment is then expected to give information about the effect of Gestational Diabetes Mellitus on the expression of amelogenin in rat offspring tooth germ.

MATERIALS AND METHODS

Sixteen female Wistar rats, aged 2.5-3 months, with 150-200 g body weight were adapted to the metal cages for 1 week, given the standard feed and drink *ad libitum*. The rats were kept on a 12-h light-dark cycle at 22–24° C. The day that spermatozooids appeared in vaginal smears (day 0 of pregnancy), 8 rats were intraperitoneally treated with 40 mg STZ (Sigma, St. Louis, MO, USA)/kg BW, dissolved in 50 mM citrate buffer, pH 4.5. Eight control rats were run in parallel, and received the medium. The experimental procedure was approved by the Ethics and Advocacy Unit of the Faculty of Dentistry Gadjah Mada University. Animals were weighed and blood glucose levels were measured with Accu-Check Active (Roche, Germany) on day 0, 7, 14, and 19 of pregnancy. Rats with fasting blood glucose levels above 120 mg/dL and showed

the sign polydipsia, polyuria, poliphagia, and asthenia were considered as having diabetes.^{4,11} Two pups from each litter were selected at random and decapitated on day 5 after birth.

Mandibular molar tooth germ of rat pups were taken and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS formalin) for 24 hours, decalcified using 10% EDTA at 4° C for 14 days and embedded in paraffin. 3 µm thick cross-sectional sections were stained with immunohistochemistry. Samples were deparaffinized with xylol and rehydrated with serial alcohol.

After deparaffinization and hydration, the sections were treated with 0.3% H₂O₂ in methanol for 15 minutes to reduce endogenous peroxidase activity, then washed with distilled water and Tris EDTA followed by administration of antigen retrieval application (in citric buffer pH 6) by heating for 15 minutes in a microwave to open antigen-covered and washed with Tris EDTA. They were then blocked with different normal serum (background snipper) at room temperature for 10 minutes followed by hatching the primary antibody and incubated at 4° C for 18 hours; primary antibodies anti-AMELX diluted with PBS (1:1000). After washing with Tris EDTA, the sections were incubated with secondary antibody (Trekki universal link) for 10 minutes at room temperature, washed with Tris EDTA and treated with Trekkavidin-HRP label for 10 minutes. Sections were then washed with Tris EDTA and staining for peroxidase was performed with DAB chromogen (1:200 in substrate) in a dark room for 3 minutes then washed with distilled water. For maximum staining, counterstain with haematoxylin meyers performed for 2 minutes and terminated by washing with water tap for 2 minutes. The section were then dehydrated with serial alcohol followed by xylol. Next stage was mounting the slide. Normal rat tooth germ was used as positive control.

Images of the cross-sectional section of immunohistochemistry-stained molars were captured using a light microscope connected to camera (Optilab). Amelogenin expression was identified as brownish yellow spots in the cytoplasm. Amelogenin expression in ameloblast was observed by measuring the density of amelogenin using ImageJ software. Greater value stated on the ImageJ software showed greater density of amelogenin, and greater density of amelogenin means that amelogenin expression getting weaker.

RESULTS

The means of fasting blood glucose level and body weight of female rat are presented in Figure 1. Fasting blood glucose levels in diabetic group increased after injection of STZ. The highest fasting blood glucose level was in diabetic group day 14 of pregnancy (329.00 ± 97.33 mg/dL), and the lowest was in control group day 19 of pregnancy (81.39 ± 7.05 mg/dL) (Figure 1A). Fasting blood glucose levels in diabetic group decreased on day 19, but

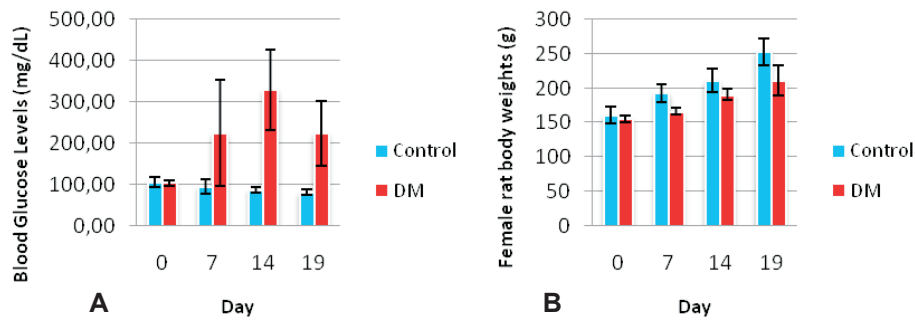


Figure 1. Means of fasting blood glucose level (A) and body weight (B) of female rat in control and DM group. Fasting blood glucose and body weight were measured on pregnancy day-0, 7, 14 and 19.

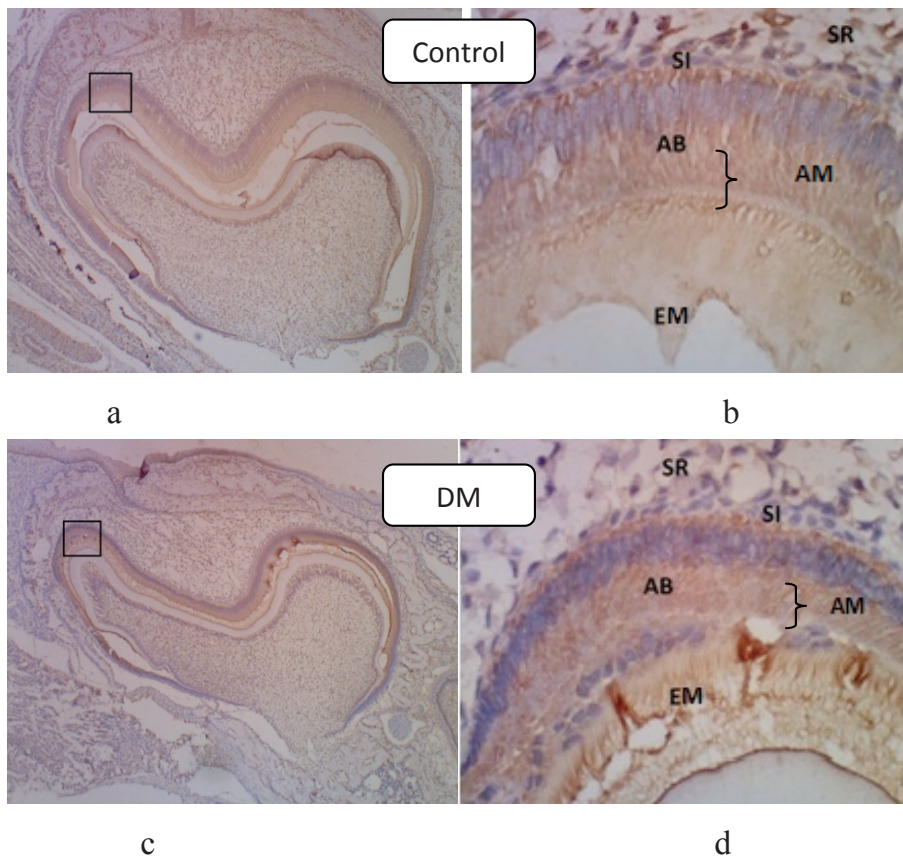


Figure 2. Sections showing localization of amelogenin protein in the cross-sectional mandibular molar tooth germ using an immunohistochemical technique. Amelogenin expression in ameloblast were marked with brownish yellow granules in the cytoplasm. a, c. Control/DM group was observed at 40x magnification, b, d. Control/DM group was observed at 400x magnification. EM, Enamel; AB, Ameloblast; SI, Stratum Intermedium; SR, Stellate Reticulum; AM, Amelogenin.

still above 120 mg/dL. There was no increase of fasting blood glucose levels in the control group. Rats body weight were increased in each observation either in the control and DM group. Control rats had body weight means greater than diabetic rats, with the greatest body weight mean was on control group day 19 of pregnancy (252.80 ± 19.91 g) and the lowest was in DM group day 0 of pregnancy (155.79 ± 4.64 g) (Figure 1B).

Histological amelogenin expression can be seen in Figure 2a, b, c, d. Means of amelogenin density in control group greater than DM group (Figure 3). The greater amelogenin density means the amelogenin expression getting weaker. This suggests that amelogenin in DM group were expressed stronger compared with control group. Normality test results were 0.033 for control group and 0.102 for DM group. These results indicate that data

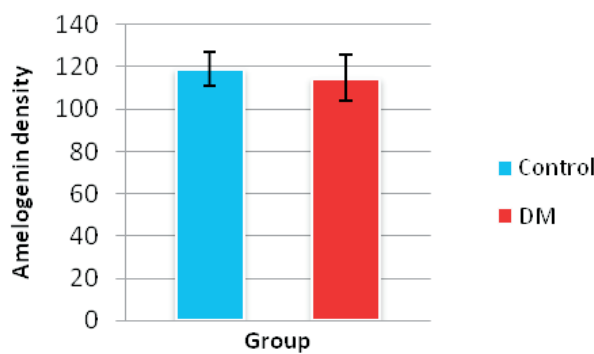


Figure 3. Means and standard deviations of amelogenin density in rat offspring tooth germ. Amelogenin density was measured using ImageJ software.

has a significance $p < 0.05$ for control group and $p > 0.05$ for DM group which means the control group data were not normally distributed, so it could not proceed with the parametric test. Mann Whitney test results showed p -value = 0.224 ($p > 0.05$). These results indicate that there was no significant difference in the amelogenin density between control and DM group, which means maternal diabetic condition had no significant effect on the amelogenin expression.

DISCUSSION

Results of this study indicate that there was an increase in fasting blood glucose levels of diabetic group compared with control group. Diabetic group had glucose level above 120 mg/dL. Increasing of fasting blood sugar levels in diabetic rats probably caused by necrosis of pancreas beta cells. Streptozotocin selectively induce necrosis in pancreatic beta cells via DNA methylation. DNA damage is caused by free radicals that are released by STZ. Nitrosurea in STZ causes cellular toxicity through decreased levels of NAD^+ and production of free radicals. Streptozotocin is also able to act as a donor of nitric oxide (NO) and generate reactive oxygen species (ROS). Necrosis of beta cells causes a decrease in the biosynthesis and secretion of insulin and blood glucose levels.¹² Rat also showed the signs of DM, i.e polydipsia (abnormal thirst), polyuria (increased urine volume), polyphagia (excessive hunger) and asthenia (weakness due to the inability to use glucose as a source of energy). This finding agrees with previous studied.¹¹ In this research, the weight of rat had increased either in control and diabetic group. Diabetic group weight was lower than control group, although diabetic group consumed more food and beverages. This was probably caused by metabolic disorders due to diabetic conditions.¹³ There were disturbances in the metabolism of carbohydrates, proteins and lipids in diabetic rats.¹ Low weight gain during pregnancy could be a cause of the low

number of LPA foetuses in this group.¹³

Weight gain as well as the results of abdominal palpation during pregnancy showed that the rat had been pregnant. Pregnant rats was determined by palpation on the abdomen on day 13 of pregnancy. Enlargement in the abdomen suggests there were multifoetuses in uterus.¹⁴

The results showed that the diabetic condition did not have a significant effect on amelogenin density. It means that diabetic condition had no effect on the expression of amelogenin significantly. The absence of significant effect is likely due to normal fasting blood glucose levels and normal serum calcium levels in rat pups. Examination of blood glucose levels in rat pups was not done in this research. However, based on the results of previous studied, blood glucose levels of rat pups born to diabetic rats parent could be significantly high compared to control.¹⁵ Another study showed that STZ-offspring were initially hypoglycemic but became normoglycemic by weaning and remained normal up to at least 15 wk of age.¹⁶

Other possible causes of the absence of significant differences in the expression of amelogenin in this study was due to the normal serum calcium levels in rat pups. Serum calcium levels remained relatively constant since each cell has basic requirements for calcium. Low serum calcium levels will stimulate production of parathyroid hormone. Parathyroid hormone then increase resorption of bone matrix stimulate osteoblasts to release factors that increase the number and activity of osteoclasts. Increased bone resorption would increase serum concentrations of calcium and phosphate. Parathyroid hormone increases the absorption of calcium and decreases the absorption of phosphate in the kidneys causing fosfaturia. Increased calcium reabsorption in the renal tubules by transport proteins (epithelial calcium channel, calbindin-D28K and plasma membrane Ca^{2+} -ATPase) in children born to mothers with diabetes will normalize serum calcium levels.¹⁷ Parathyroid hormone also increase the activity of $1-\alpha$ -hydroxylase, resulting in increased synthesis of 1.25 -dihydroxyvitamin D which causes an increase in calcium absorption in the small intestine.¹⁸

It can be concluded that Gestational Diabetes Mellitus does not affect the expression of amelogenin in Wistar rat offspring tooth germ. Further research is needed to examine the expression patterns of amelogenin with measurement of blood glucose and serum calcium levels in diabetic offspring.

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