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Original article

# Comparison of rat tooth eruption in rats born from diabetic mothers

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## ABSTRACT

**Background:** *Tooth eruption begins after crown and root formation and may be delayed by gestational diabetes mellitus. Metformin can control blood glucose levels through gluconeogenesis inhibition, and consuming thymoquinone for diabetic treatment will regenerate pancreatic β cells and reduce oxidative stress.* **Purpose:** *The objective of this study is to compare the tooth eruption in rats that were born with diabetes and are being treated with either metformin or thymoquinone.* **Methods:** *This study used 48 Wistar rats (Rattus norvegicus L.), and the rat sample was divided into four groups, including rats who were born from healthy mothers, rats who were born from untreated diabetic mothers, rats who were born from diabetic mothers that were treated with metformin and rats who were born from diabetic mothers that were treated with thymoquinone. Diabetes was induced by intraperitoneal injection of a single dose of streptozotocin (40 mg/kg BB). Each rat sample was taken with simple random sampling from different mothers, and body weight, blood glucose levels and levels of tooth eruption were recorded. Eruptions of the maxillary right first molar were measured from the cusp of the tooth to the alveolar epithelial lining.* **Results:** *Based on the measurements of tooth eruption, it was found that groups A, C and D were closer to mucosa on day 1, 7 and 14 than group B. Based on statistical analysis, there were significant differences ($p = 0.03$) between group B and groups C and D.* **Conclusions:** *Rats born from untreated diabetic mothers have more delays in tooth eruption than those born from diabetic mothers who are treated with metformin and thymoquinone. Thymoquinone has the potential to be an alternative to metformin because it has been shown to be similarly effective.*

**Keywords:** *diabetes; eruption; thymoquinone; tooth*

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## INTRODUCTION

Tooth eruption is a normal physiological process, but it is considered to be abnormal if there is interference or delay. As teeth reach their functional position in the jaw arch, the tooth germs move through three distinct phases, including the pre-eruptive phase, the eruption phase and the post-eruptive phase. In the post-eruptive phase, the teeth are in the functional position.[1]

As in humans, a rat's teeth develop through interactions between dental epithelium and neural crest cells. A rat's first molar develops through multiple stages, including dental sheet proliferation, the bud stage, the cap stage, the bell stage and finally, the eruption phase.[2] There are various factors that affect tooth eruption, such as root development, alveolar bone remodelling and periodontal ligaments and other predisposing factors, such as endocrine hormones, vascular changes and enzymatic degradation.[3] In humans, the onset of diabetes during pregnancy is known as gestational diabetes mellitus (GDM), and it can lead to negative effects on the child's tooth eruption. A women with GDM has insulin deficiencies that can cause metabolic abnormalities and lead to malnutrition in the fetus.[4,5] Severe and prolonged malnutrition in the early life of a fetus can later cause delays in the tooth eruption phase.[1] Diabetes is usually treated by controlling glucose levels in the blood with healthy diet and exercise, but prescription drugs, such as metformin, can also be used to control glucose levels by absorbing glucose through glucose transporters (GLUTs).[6] Metformin has gastrointestinal side effects, such as diarrhoea, nausea and vomiting, and these side effects occur in approximately 50% of patients.[7] To reduce

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the side effects of metformin, this researcher tried using thymoquinone (Nigella sativa L.; derived from black cumin seeds). It is bioactive, and it can control blood glucose levels and regenerate pancreatic β cells.[8]

Thymoquinone is a terpenoid compound (essential oil) and has volatile properties that are difficult to dissolve in water, therefore, it is recommended that thymoquinone be dissolved in an oil solvent (herbal or olive oil). Many functions of thymoquinone offer health benefits,[9] but its effectiveness in preventing tooth eruption disruption has not yet been reported. Other side effects have also have been reported, such as allergic rash, stomach problems and, with long-term use, hyperlipidaemia,[10] but these need further research. The purpose of this study is to examine tooth eruption in rats born from untreated diabetic mothers and in rats born from mothers treated with either metformin or thymoquinone.

## MATERIALS AND METHODS

This study was conducted in vivo with a post-test as the only control group, it was approved by the Medical Research Ethics Committee at the Faculty of Dentistry, University of Jember, No.586/UN25.8/KEPK/DL/2019, and it was carried out in a biomedical and animal science laboratory.

Sixteen pregnant Wistar rats were divided into four groups with each group consisting of four samples. Group A consisted of normal pregnant rats with blood glucose levels < 126 mg/dl, group B consisted of pregnant diabetic rats who received no treatment, group C consisted of pregnant diabetic rats who received metformin treatment, and group D consisted of pregnant diabetic rats who received thymoquinone treatment. The pregnant Wistar rats from groups B, C and D were induced with diabetes using streptozotocin (STZ) obtained from Bioworld (GeneLinx International inc., Dublin, Ohio, USA) on day 10 of gestation by dissolving 40 mg/Kg/bw STZ powder in 50 mg/ml 0.1 M citric acid buffer solution with a pH of 4.5.[11,12] One day after STZ injection, blood glucose levels were measured at > 126 mg/dl, and the rats were categorized as diabetic. After inducing diabetes in groups B, C, and D, group C was given metformin obtained from Hexapharm Jaya Laboratories (Kalbe Company, Bekasi, Indonesia) at a dose of 100 mg/kg/bw dissolved in 1.5 ml of distilled water and administered intragastrically using an intragastric tube twice a day (every morning and evening). Group D was given thymoquinone obtained from Merck (Sigma Aldrich Inc., St. Louis, Missouri, USA) at a dose of 80 mg/kg/bw[13] dissolved in 1.5 ml of olive oil and administered intragastrically once a day.[14] Rats in groups C and D received their respective doses everyday up until 14 days postnatal.

After each of the rats gave birth, a research sample was obtained by taking one baby rat from each of the mother rats in all four groups on days 1,7 and 14 postnatal using simple random sampling. This provided 48 baby Wistar rats whose teeth were observed (Figure 1).

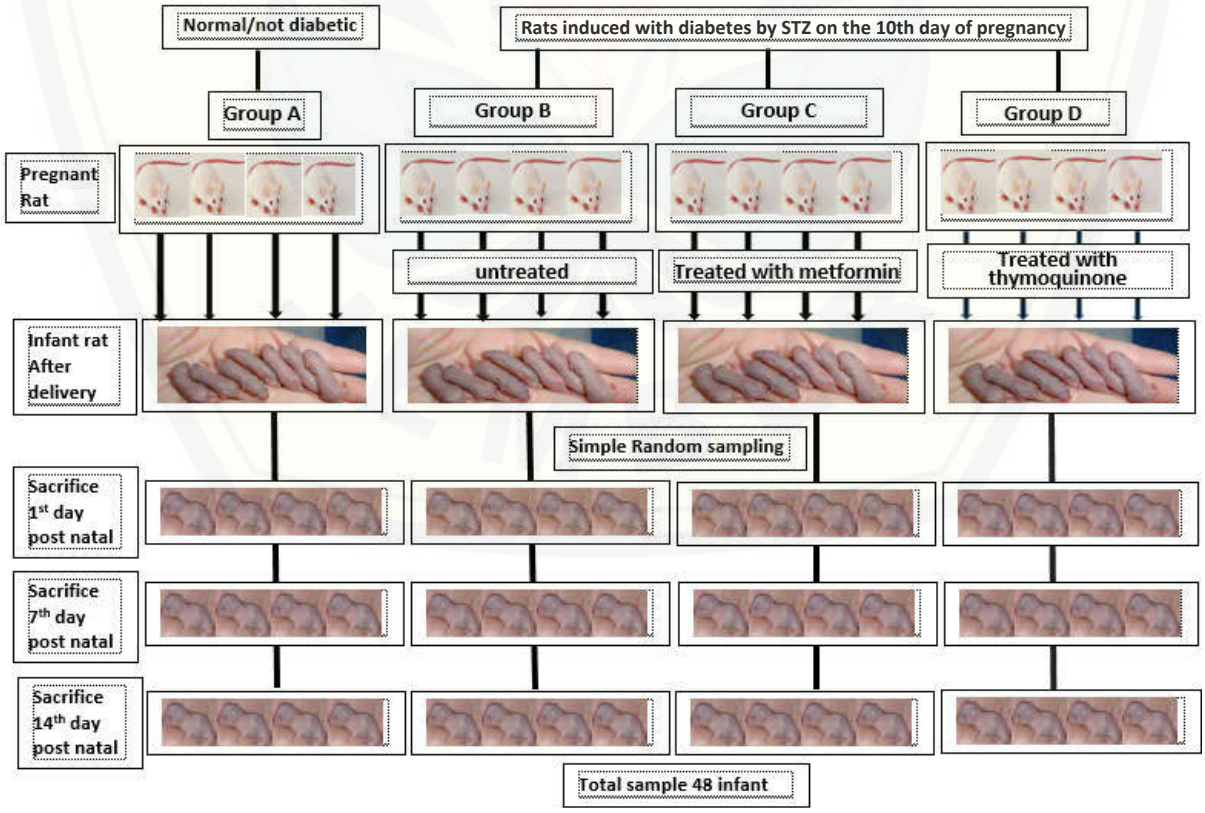


**Figure 1.** Grouping and sampling diagram.

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Postnatal rats from each group were euthanized on days 1, 7 and 14 with ketamine obtained from Kepro BV (Maagdenburgstraat, ZA, Deventer, Netherlands) using the overdose method. In each of the rats, the molar region of the right maxillary was removed and placed in a pot with 10% formalin solution for 24 hours and then decalcified using a 10% formic acid solution for 7 days to remove inorganic material from the bones. The tissue was then processed using the paraffin-embedded tissue technique. The paraffin block was cut using microtome with a thickness of 6 µm, and Hematoxillin & Eosin was used for staining. Histopathological appearance was observed under a microscope (Olympus CX-21) at 40x magnification that was connected to an OptiLab advanced V2 camera and computer obtained from Miconos (Yogyakarta, Indonesia) to view histological images. The distance from the outer enamel epithelium of the tooth germ to the surface of the epithelial of the alveolar epithelial lining on the maxillary right first molar was measured using Raster Image Processor software (Miconos, Yogyakarta, Indonesia), and pre-dentin/pre-enamel formations, Hertwig's epithelial root sheath (HERS) formations, tooth root formations, and bifurcation formations were also observed.[2,15]

Statistical package for the social sciences (SPSS) software (version 26, IBM, New York, USA) was used. The normality test of the data used Shapiro Wilk and the homogeneity test used the Levene test ($p \leq 0.05$). If the statistical test results were not normally distributed and not homogeneous, then the data was tested using the Mann-Whitney and Kruskal-Wallis tests (nonparametric; $p \leq 0.05$).

## RESULTS

The results showed increases in blood glucose levels in groups B, C, and D after STZinjections (blood glucose ≥ 126 mg/dl; Table 1). Based on the Mann-Whitney test, there were significant differences ($p \leq 0.05$) between all the groups across all observation days (Table 2). Average bodyweights of the rats 1 day postnatal showed that group B had the lowest average bodyweight, and based on statistical tests between all groups, only group A and B were significant at 1 day postnatal ($p = 0.01$). In groups C and group D at 14 days postnatal, the average bodyweights differed but not significantly ($p = 0.04$; Table 3).

**Table 1.** Pregnant diabetic rat average blood glucose levels (mg/dl)

| Group | STZ post injection | 7th Day | 14th Day |
|---|---|---|---|
| A | 96±5.4 (without STZ injection) | 66.75±13.5 | 66.75±13.5 |
| B | 411.5±77.9 | 250.75±233.6 | 169.75±99.6 |
| C | 144.6±31.7 | 299.00±260.8 | 149.00±12.2 |
| D | 23.3±172.2 | 141.00±18.3 | 97.50±1.2 |

**Table 2.** Statistical test results (Mann-Whitney) for blood glucose levels of pregnant diabetic rats

| Group/day | | A | | | B | | | C | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 7 | 14 | 1 | 7 | 14 | 1 | 7 | 14 | 1 | 7 | 14 |
| A | 1 | | | | .021 | | | .034 | | | .050 | | |
| | 7 | | | | | .020 | | | .032 | | | .032 | |
| | 14 | | | | | | .042 | | | .020 | | | .060 |
| B | 1 | .021 | | | | | | .289 | | | .480 | | |
| | 7 | | .020 | | | | | | .372 | | | 1.000 | |
| | 14 | | | .042 | | | | | | 1.000 | | | .355 |
| C | 1 | .034 | | | .289 | | | | | | .827 | | |
| | 7 | | .032 | | | .372 | | | | | | .275 | |
| | 14 | | | .020 | | | 1.000 | | | | | | .240 |
| D | 1 | .050 | | | .480 | | | .827 | | | | | |
| | 7 | | .032 | | | 1.000 | | | .275 | | | | |
| | 14 | | | .060 | | | .355 | | | .240 | | | |

A: Normal group; B: Negative control group; C: Metformin group; D: Thymoquinone group

**Table 3.** The average bodyweight of postnatal rats

| Group | Postnatal Weights (g) | | | |
|---|---|---|---|---|
| | 1st Day | | 7th Day | 14th Day |
| A | 6.50±0.6 | p=0.01 | 10.25±2.9 | 18.00±4.3 |
| B | 4.75±0.5 | | 8.25±2.1 | 18.00±5.0 |
| C | 6.33±1.2 | | 8.67±1.2 | 15.00±1.0 |
| D | 6.00±1.0 | | 9.33±2.5 | 18.00±0.0 |

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At 1 day postnatal, the development of the maxillary right first molar of each of the rats was in the bell stage and pre-dentin/pre-enamel was detected, except for one sample in group B that had growth retardation and was still in the cap stage. On day 7 postnatal, observations showed that all samples had entered the stage of apposition and calcification with the formation of HERS, except for one sample in group B that was still in the bell stage. The growth and development of teeth on day 14 postnatal in groups A and C showed that all samples had entered the eruption stage, marked by the formation of roots and root bifurcation. In group B, 50% of the samples were in the eruption stage, and in group D, 66% of the samples were in the eruption stage (Figure 2).

Based on measurements of the distance from the cusp of the maxillary right first molar to the alveolar epithelial lining, it was observed that the distances decreased from day 7 to day 14 (Table 4). Statistical analyses showed that there were significant differences between group B and group C and significant differences between group B and group D (significance of 0.03; $p \leq 0.05$; Table 5).

**Table 4.** Measurements of eruption distance of right maxillary first molars (μm)

| Group | 1st Day | 7th Day | 14th Day |
|---|---|---|---|
| A | 348.00±69.9 | 322.00±88.5 | 196.77±69.7 |
| B | 339.58±81.9 | 397.00±95.1 | 259.23±32.3 |
| C | 323.58±45.3 | 368.16±175.4 | 153.61±74.3 |
| D | 267.98±8.4 | 344.00±129.3 | 152.03±47.4 |

**Table 5.** The statistical test results (Mann-Whitney) for the measurements from maxillary right first molar cusps to the alveolars epithelial lining

| Group/Day | | A | | | B | | | C | | | D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 7 | 14 | 1 | 7 | 14 | 1 | 7 | 14 | 1 | 7 | 14 |
| A | 1 | | | | .773 | | | .480 | | | .289 | | |
| | 7 | | | | | .248 | | | 1.000 | | | .480 | |
| | 14 | | | | | | .248 | | | .480 | | | .480 |
| B | 1 | .773 | | | | | | 1.000 | | | .157 | | |
| | 7 | | .248 | | | | | | .724 | | | .289 | |
| | 14 | | | .248 | | | | | | .034 | | | .034 |
| C | 1 | .480 | | | 1.000 | | | | | | .827 | | |
| | 7 | | 1.000 | | | .724 | | | | | | .593 | |
| | 14 | | | .480 | | | .034 | | | | | | .513 |
| D | 1 | .289 | | | .157 | | | .827 | | | | | |
| | 7 | | .480 | | | .289 | | | .593 | | | | |
| | 14 | | | .480 | | | .034 | | | .513 | | | |

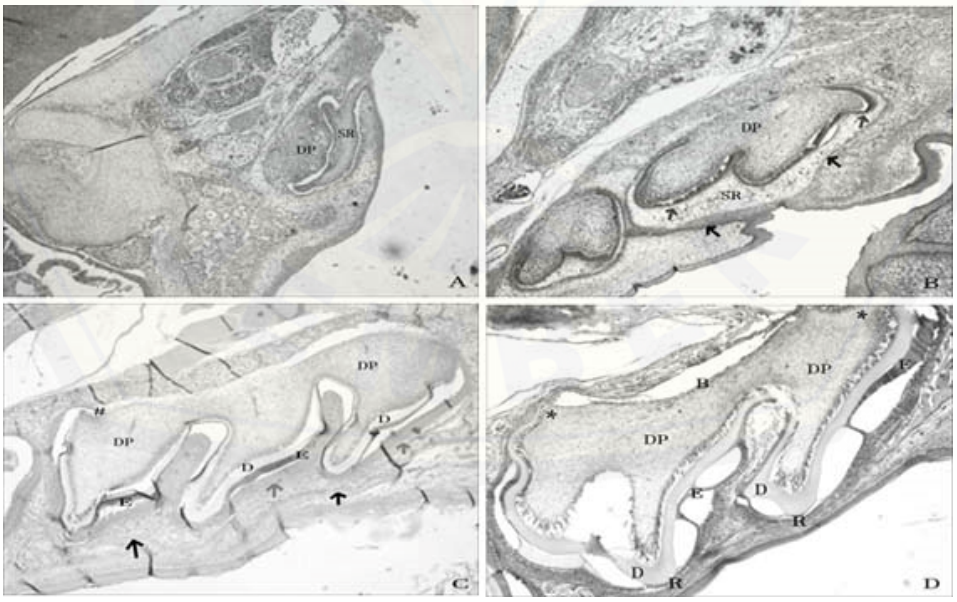


**Figure 2.** Histological features of a right maxillary M1 in a postnatal rat. A: Delayed tooth development shows the cap stage (sample from group B; day 1 postnatal); B: A histological picture of the bell stage (groups A, C and D; day 1 postnatal); C: The apposition and calcification stage on day 7 postnatal; D: Shows eruption (grey arrows indicate inner enamel epithelium, black arrows indicate outer enamel epithelium, hashtags indicate HERS and stars indicate root formation). Notes: DP: Dental Papilla; SR: Stellate Reticulum; D: Dentine; E: Enamel; R: Reduce enamel epithelium; B: Bifurcation. (A-C: HE Staining 40x magnification; D: HE Staining 5x magnification)

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## DISCUSSION

Diabetes induced by STZ injection can affect glucose oxidation and decrease biosynthesis and insulin secretion through the GLUT-2 glucose transporter. This leads to decreased sensitivity of peripheral insulin receptors and can increase insulin resistance and blood glucose levels.[10,11]

There were decreases in blood glucose levels in the negative control (group B) who had not received treatment. A study showed that this may be due to spontaneous self-repair mechanisms because, although streptozotocin is an alkylating agent and can damage DNA and pancreatic β-cells, it is dose-dependent, and the study showed that after eight hours of exposure to STZ, 55% of mitochondrial DNA cells repaired themselves and rose to 70% in 24 hours. [12] Meanwhile , the increase in blood glucose levels on day 7 in the rats treated with metformin was thought to be due to damage of pancreatic β cells from the STZ injection. The effects of metformin were seen after day 14 when there were decreases in blood sugar levels, but normal levels were not reached due to metformin's ability to reduce glucose absorption through the GLUT and inhibit gluconeogenesis.[13] Decreased blood glucose levels were found in group D because thymoquinone can stimulate insulin release by inhibiting oxidative stress, and it stimulates the regeneration of pancreatic cells.[14]

Untreated diabetes in pregnant rats has significant effects on the bodyweight of their offspring. The rats in the negative control (group B) had lower bodyweights on day 1 when compared to the other groups. This was perhaps due to the stress of endoplasmic reticulum placenta (ERP) in utero. It is known that the function of ERP is related to nutrient transportation, and stress to the ERP usually results in phosphorylation from translational initiation factor 2 and eukaryotic initiation factor 2 α (eiF2α) that are targets for several serine kinases that phosphorylate serine and lead to the inhibition of protein translation and the signalling of mammalian target of rapamycin (mTOR) that functions as a protein kinase serine/threonine that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy and transcription. It also functions as a protein kinasetyrosine that activates insulin receptors and insulin-like growth factor 1 (IGF-1) receptors, and this can inhibit transportation of nutrients to the fetus.[16,18] Disruption in placental function can cause intrauterine growth restriction (IGR) that can result in a baby being born with low birthweight.[19] From day 7 to day 14, the bodyweight of each rat in the four groups increased in line with their ability to eat. This might be the rat born from diabetic mother rat without treatment also able to achieve the same weight as rat born from a normal rat.

The rats in group D appeared healthy, had normal bodyweights and showed normal growth and development when compared to the rats in group A who were born from healthy mothers. It can be concluded that thymoquinone, when given to diabetic mothers during pregnancy, can function as an anti-hyperglycaemic medication.[20] This was shown at the end of the study on day 14 when the mothers' glucose reached normal levels and breastfeeding was sufficient for normal growth and development. Nevertheless, on the day 14, it was found that, although the rats in group C gained weight consistently up to day 14, they still gained weight at a slower rate than the rats in other groups. This may be due to the fact that their mothers were consuming metformin. It has been reported that metformin works on the central nervous system and can reduce appetite. [21] Loss of appetite in the mother rat can affect nutritional adequacy during breastfeeding and may reduce their offspring's bodyweight.[21]

Rats born from diabetic mothers may experience growth retardation and delays in the growth and development of their teeth. Severe diabetes in pregnant rats can also cause metabolic disorders and protein-energy malnutrition in the fetus due to oxidative stress that is known to disturb cell signalling during growth and development. In the absence of antioxidant activity, oxidative stress continues to increase and can cause extensive cell damage to formed protein, DNA, and lipids.[9] It was reported that a mother's nutrition during pregnancy is directly related to the primary teeth eruption of her offspring.[22] Through thymoquinone therapy, delayed eruption can be prevented because thymoquinone is an antioxidant that can protect pancreatic β cells by decreasing oxidative stress and increasing the production of endogenous antioxidants in the body, such as glutathione peroxidase, superoxide dismutase and catalase, and improve metabolic disorders that cause malnutrition of protein-energy in the fetus.[22,23] In diabetic pregnant rats, metformin therapy can improve insulin sensitivity because it improves blood glucose levels, and developmental tooth disorders can be prevented.

The teeth development process continues into the eruption stage. Tooth eruption is a process that begins immediately after the crown is formed, followed by the formation of roots that are regulated by HERS.[1] The process of eruption could be seen in each rat on day 7 through day 14 by observing the shortening of the distance between the tooth cusp (outer enamel epithelium) and the alveolar epithelial lining. In general, a rat tooth will start to erupt when a crown reaches two thirds the length of its root.[3] On the day 14 of observation, the negative control group was seen to have delayed eruption which was indicated by the distance of the tooth cusp to the alveolar epithelial lining which was deeper than the other groups. Statistical tests showed that there were significant differences between the negative control and group C and significant differences between the negative control and group D ($p = 0.03$; $p \leq 0.05$). The rates of tooth eruption in groups C and D (treated groups) were not significantly different. It was reported that root development takes place once HERS has formed. [24] HERS is an epithelial bilayer that extends to the apical below the cervical margin. The formed HERS proliferates and enters the cervical margin area to form a barrier between the dental papilla and the dental follicle (periodontium).[24]

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The formation of HERS was signalled by the secreted protein Sonic hedgehog (Ssh/Msx2;Msh-like gene invertebrates) and IGF-1 that have significant roles in tooth growth and development.[24] It was also reported that a lack of IGF-1 can result in stunted tooth growth and development, [25] and this is directly related to the tooth germs of rats born from diabetic mothers who have delayed eruption phases caused by a reduction of IGF-1. From this study, it can be concluded that rats born from untreated diabetic mothers have more delays in tooth eruption when compared to those born from mothers treated with metformin or thymoquinone. Thymoquinone has the potential to be an alternative to metformin because is similarly effective.

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