

THE EFFECT OF DIABETES INDUCTION IN PREGNANT RATS ON MOLAR ROOT FORMATION OF OFFSPRINGS “HISTOLOGICAL AND IMMUNO-HISTOCHEMICAL STUDY”

Alaa Abdelkader Abdella*^{ID}, Dahlia Ghazy Mohamed Rateb**^{ID} and Nuha AbdulFattah Baraka***^{ID}

ABSTRACT

Objective: Diabetes mellitus (DM) as a chronic disease has multiple effects upon both general and oral health. This study aimed to evaluate the effect of DM in pregnant rats on root formation of their offspring.

Methodology: This investigation was carried out on forty-two Albino rats. Rats were divided into two groups (n=21): **group C (control):** offspring to mothers injected by intraperitoneal single dose of 1ml Citrate buffer, **group E (diabetic):** offspring born to mothers rendered diabetic through single intraperitoneal injection of Streptozotocin (40mg/kg body weight) dissolved in 1ml Citrate buffer. Their offspring were sacrificed one, two and four weeks after birth (E1, E2 and E3 subgroups respectively). The lower jaws of the offspring were excised and processed for histological and immunohistochemical investigation of osteopontin (OPN) antibody marker.

Results : Rats of E1 subgroup showed apparently thin epithelial diaphragm. E2 subgroup showed areas of degeneration between odontoblastic cells and periodontal fibers disorganization, while E3 subgroup showed increased cementum thickness, widening of cementocytes lacunae and massive congestion in pulpal blood vessels. Positive OPN immunoreactivity was observed in E1 subgroup in the epithelial diaphragm cells and primitive pulp tissue while E2 and E3 subgroups showed immunoreactivity in pulp, cementoblastic cells lining the cementum tissue and PDL fibers.

Conclusions: Diabetes mellitus induction in pregnant mothers adversely affected molars root formation of the offspring. Alterations in dental root forming cells, pulp, cementum and PDL in the offspring were associated with increased OPN immuno-expression.

KEYWORDS: Diabetes Mellitus, offspring, root formation, osteopontin

* Demonstrator of Oral Biology, Faculty of Dentistry, Al-Ahram Canadian University.

** Associate Professor of Oral Biology, Faculty of Dentistry, Ain Shams University.

*** Lecturer of Oral Biology, Faculty of Dentistry, Ain Shams University.

INTRODUCTION

Diabetes Mellitus (DM) is a chronic condition characterized by elevated blood glucose levels known as hyperglycemia. It is due to abnormality in insulin secretion, insulin function or both. This disease has many effects on various organs of the body, including eyes, bones, kidneys, blood vessels, liver and the nervous system as well (**Tao et al., 2015**).

A series of alterations in the oral mucosa of diabetic patients were observed by **Silva et al., (2015)**. Gingivitis, periodontitis, salivary gland dysfunction, altered taste and oral mucosal diseases that favor infections such as candidiasis were detected.

Experimentally induced DM in three week old male rats showed increase hypoplasia of enamel and reduction of dentin thickness with reduction in the rate of dentin mineral apposition and formation during early formation of dental crown (**Abbassy et al., 2015**).

El-Zainy (2018) postulated that experimentally induced diabetic rats showed uneven irregular cementum thickness with increased amount of cementoid matrix accompanied by areas of degeneration of periodontal ligament (PDL) fibers and increased inflammatory cells.

Yamunadevi (2020) reported that induced diabetic rat mother affected odontogenesis in the offsprings by suppression of cellular proliferation and enhanced apoptosis in molar tooth germ. Through activation of Toll like receptor 4/nuclear factor kappa B (TLR4/NF κ B) pathway.

Reviewing the literature, changes occurring during tooth root development of rats born to diabetic mothers were not thoroughly investigated. This study was directed to investigate the changes occurring during tooth root development in offsprings that were born to mothers with maternal-induced DM.

MATERIAL AND METHODS

A) Animals

Six female Albino rats (250g weight) were housed in the Animal House Unit, Faculty of Medicine, Ain Shams University under controlled temperature, humidity and dark-light cycle with free access to bread, vegetables and water. They were kept there for one week for adaptation and observation in wire mesh cages. The females in estrus were mated overnight with males (two females per male). A vaginal smear was used to ensure presence of sperms indicating the first sign of pregnancy. The pregnant rats were divided into two groups (n=3): control & experimental groups.

All animal experimental procedures used were approved by institution guidelines of Ain Shams Ethical committee (approval n= FDASU/RecIM121719).

Diabetes induction in pregnant rats

Female pregnant rats were fasted for 14 hours and diabetes was induced at the 10th day of pregnancy (**Damasceno et al., 2014**) by an intraperitoneal single injection of 40 mg/kg body weight Streptozotocin (STZ) freshly dissolved in 1 ml Citrate buffer (0.01 M; pH 4.5) (**El-Zainy et al., 2018**). After the injection, animals were given free access to water and food. Control pregnant animals were injected by 1ml Citrate buffer.

Blood samples were obtained via vein puncture of the tail under ether anesthesia. A plasma glucose level greater than 300mg/dl confirmed the presence of diabetes. The glucose level was measured three days after drug injection using a glucometer (**El-Zainy et al., 2018**) and monitored during pregnancy (22 days).

After delivery, forty-two Albino rats (offsprings of both healthy and diabetic mother rats) were housed in wire mesh cages, each cage contained seven rats. Rats of one week old were lactated by

mother while those two weeks and four weeks old were separated to be fed bread and milk.

B) Animal grouping

Control group (C): Offspring of non-diabetic mother

Constituted of 21 pups born to pregnant rats injected by 1ml Citrate buffer at 10th day of pregnancy. Pups were subdivided into three subgroups (C1, C2, and C3) (n=7) that were sacrificed one week, two weeks, four weeks after birth respectively.

Experimental group (E): Offspring of diabetic mother

Constituted of the offspring born to pregnant rats injected by STZ and was subdivided into three subgroups (E1, E2 and E3) (n=7) sacrificed at dates corresponding to those of control subgroups.

C) Sample collection and preparation

The offspring of each subgroup was sacrificed by an intracardiac anesthetic overdose of sodium thiopental (80 mg/kg) at the determined dates. Their mandibles were dissected, divided into halves and washed thoroughly under tap water to remove blood and adhering tissues then the samples were fixed immediately in 10% buffered formalin solution.

The molar areas were dissected and immersed in daily refreshed 14% ethylene diamine tetra-acetic acid (EDTA) solution 10-14 days for decalcification (Novack, 2013). The embedded specimens were sectioned buccolingually by microtome to a thickness of four to six microns to be stained with H&E staining and immunohistochemical staining for OPN.

Immunohistochemical method

Rabbit polyclonal antibody was used (purchased from Dako). All sections were incubated in hydrogen peroxide block for ten minutes to reduce nonspecific background staining due to endogenous peroxidase then washed four times in Tris-Buffer-Saline (TBS)

(pH 7.6, 0.1M) and the primary antibody was applied and incubated for 15 minutes. Sections were washed back four times in fresh TBS buffer. Horseradish peroxidase polymer were applied and incubated for 15 minutes at room temperature and the sections were washed again four times in buffer. One drop of DAB Plus Chromogen were added to 2ml of DAB Plus substrate and mixed by swirling and applied to the tissues then incubated for five minutes. Finally, sections were washed four times in distilled water and mounting media was used. Brown coloration is an indicator for OPN reactivity. OPN immunoreaction is cytoplasmic.

Histomorphometric analysis

The space area % between odontoblasts was examined in ten separate fields of H&E stained sections from the different subgroups of the study under x20 magnification. Surface area spaces between odontoblast cells field was calculated (in pixels) as the average of the pooled readings from these fields in each specimen.

Statistical analysis

Data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 26.0. Descriptive statistics were calculated in the form of mean \pm standard deviation (SD). In the statistical comparison between the different groups, the significance of difference was tested using one of the following tests:

- 1- Student's *t*-test (Unpaired) was Used to compare between mean of two different groups of numerical (parametric) data.
- 2- One way ANOVA (Analysis of variance) was used to compare between more than two different groups of numerical (parametric) data followed by post-hoc tukey. P value <0.05 was considered statistically significant and highly significant if <0.001.

RESULTS

H&E results

Examination of H&E stained sections of control subgroups showed that one week old C1 subgroup revealed horizontally directed epithelial diaphragm at the apical end of the completed crown surrounding coronal dental pulp (Fig.1A). C2 subgroup showed progressive root formation with plump, closely arranged odontoblasts bordering dental pulp. Plump cementoblasts bordering acellular cementum of developing root were seen interspaced with PDL attachment and initial PDL fibers orientation was detected (Fig.1B). C3 subgroup revealed advanced tooth root development with pulp of normal architecture and primitive arrangement of PDL fibers (Fig. 2 E). Higher magnification revealed cellular cementum at the apical part of developing root with cementocytes embedded in calcified cementum tissue. Some flattened cementoblasts were seen bor-

dering cellular cementum (Fig. 2F).

On examination of histological sections of experimental subgroups, E1 subgroup revealed apparently thin epithelial diaphragm. C.T proliferation zone was detected in loosely arranged connective tissue of dental pulp (Fig. 1 C). E2 subgroup revealed apparently thin dentin layer. Odontoblastic cells showed absence of the physiologic cell-to-cell contact and areas of degeneration in between cells. Haphazardly arranged PDL fibers were seen with scarce fibers inserted into cementum that also lacked bordering cementoblasts (Fig. 1D). E3 subgroup revealed large, degenerated areas and disorganized fibers in the PDL region close to the cementum surface (Fig. 2H). Extensive areas of degeneration were detected in cellular cementum as well as widened cementocytes associated with marked pyknotic nuclei (Fig. 2I). Prominently widened and congested blood vessels were seen in pulp connective tissue (Fig. 2G).

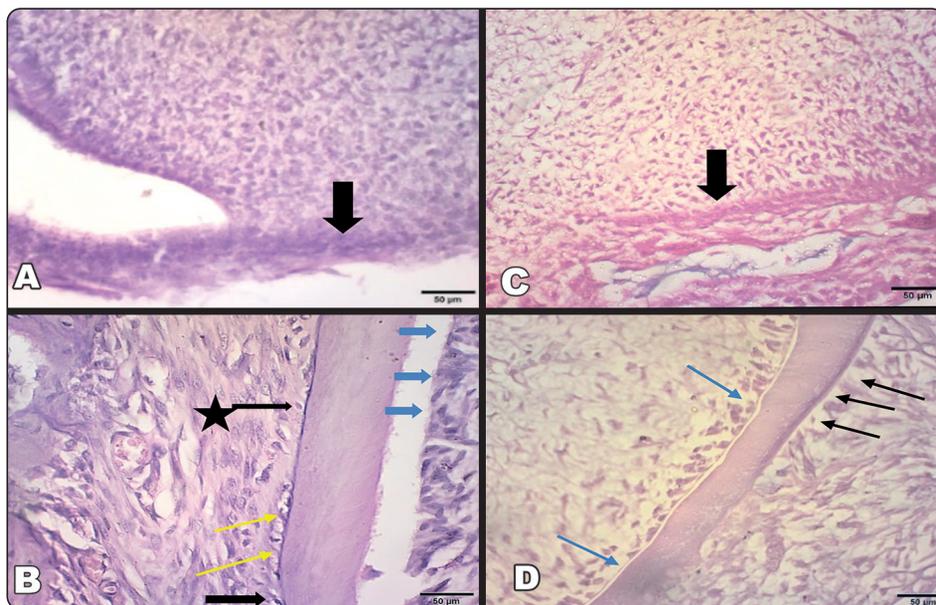


Fig. (1) Photomicrographs of first molar of subgroups:

- A. Subgroup C1 showing horizontally directed epithelial diaphragm at the apical end of fully developed crown (black arrow) [H&E. org.mag. x400].
- B. Subgroup C2 showing closely vertically arranged, plump odontoblasts and cementoblasts bordering root dentin and acellular cementum tissues respectively (blue and black arrows). Well-organized PDL fibers (black star) with fibers inserted into cementum are seen (yellow arrows) [H&E. org.mag. x400].
- C. Subgroup E1 showing apparently thin epithelial diaphragm with odontoblastic cells in the inner pulp zone (black arrow) [H&E. org.mag. x400].
- D. Subgroup E2 showing odontoblastic cells with absence of the physiologic cell-to-cell contact and areas of degeneration (blue arrows). Haphazardly arranged PDL fibers are seen with scarce fibers insertion into cementum and lack of cementoblasts bordering cementum (black arrows) [H&E. org.mag. x400].

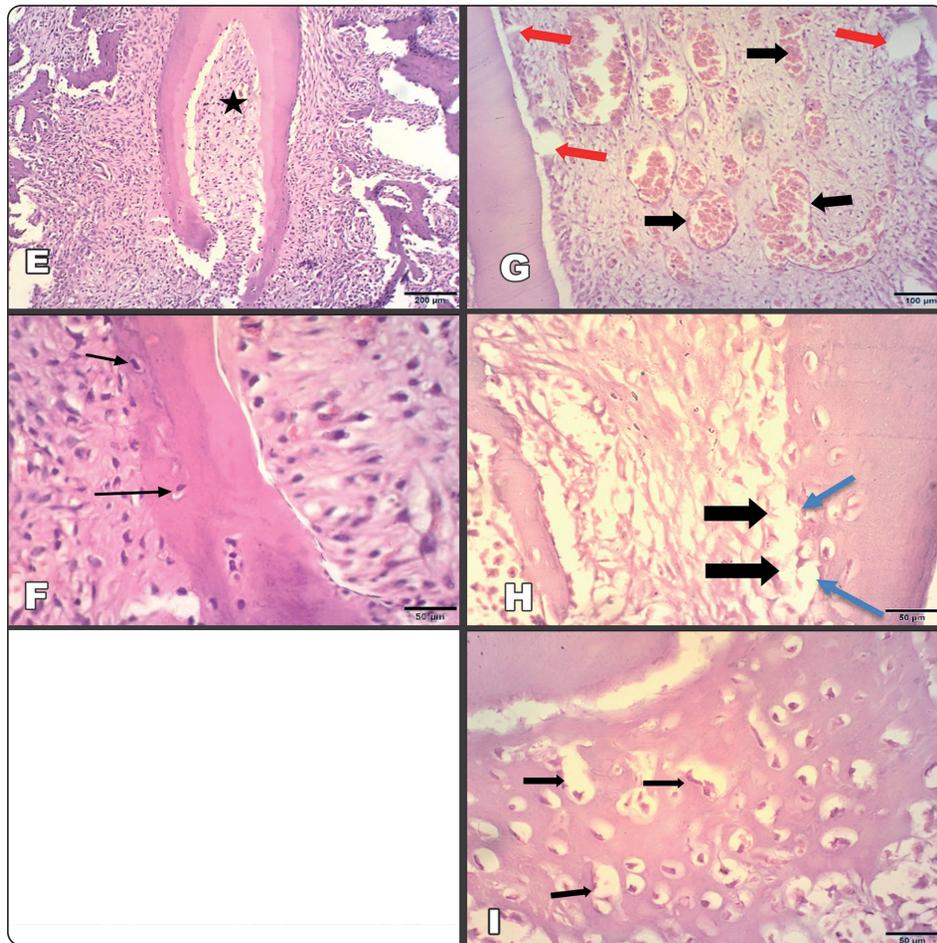


Fig. (2) Photomicrographs of first molar of subgroups:

- E. Subgroup C3 showing incompletely formed root surrounding pulp of normal architecture (black star) [H&E. org.mag. x100].
- F. Higher magnification of photomicrograph E of subgroup C3 showing cellular cementum containing cementocytes embeded in cementum (black arrow) bordered by few flattened cementoblasts. [H&E. org.mag. x400].
- G. Subgroup E3 showing multiple areas of degeneration in the pulp and interruption in odontoblastic cell layer (red arrows). Multiple dilated and congested blood vessels are seen (black arrows). [H&E. org.mag. x200]
- H. Subgroup E3 showing disorganized periodontal ligament fibers with areas of degeneration (black arrows), irregular cemental surface (blue arrow) with absence of cementoblasts and some widened cementocyte lacunae [H&E. org.mag. x400].
- I. Subgroup E3 showing cementum at the apical end of developed root showing numerous widened cementocytes lacunae sometimes associated with pyknotic nuclei (black arrows) [H&E. org.mag. x400].

• Immunohistochemical results:

Examination of IHC stained section with OPN antibody showed that C1 subgroup revealed positive cytoplasmic immunostaining for OPN in cells of epithelial diaphragm (Fig.3A). C2 subgroup revealed positive immunostaining for OPN in the cementoblastic cell layer bordering the developing root surface and also in the surrounding periodontal ligament fibers (Fig.3B). C3 subgroup revealed positive immunostaining for OPN in cellular cementum and in

the PDL fibers area (Fig.3C).

E1 subgroup revealed positive immunostaining for OPN in the epithelial diaphragm area in addition to pulp C.T cells (Fig.3D). E2 subgroup revealed marked immunoreactivity in pulp, cementoblasts bordering the formed cementum and in PDL fibers (Fig.3E). E3 subgroup revealed prominent immun-expression for OPN in cementoblasts, PDL fibers, endothelial lining of pulp blood vessels and as well as pulp tissue (Fig.3F).

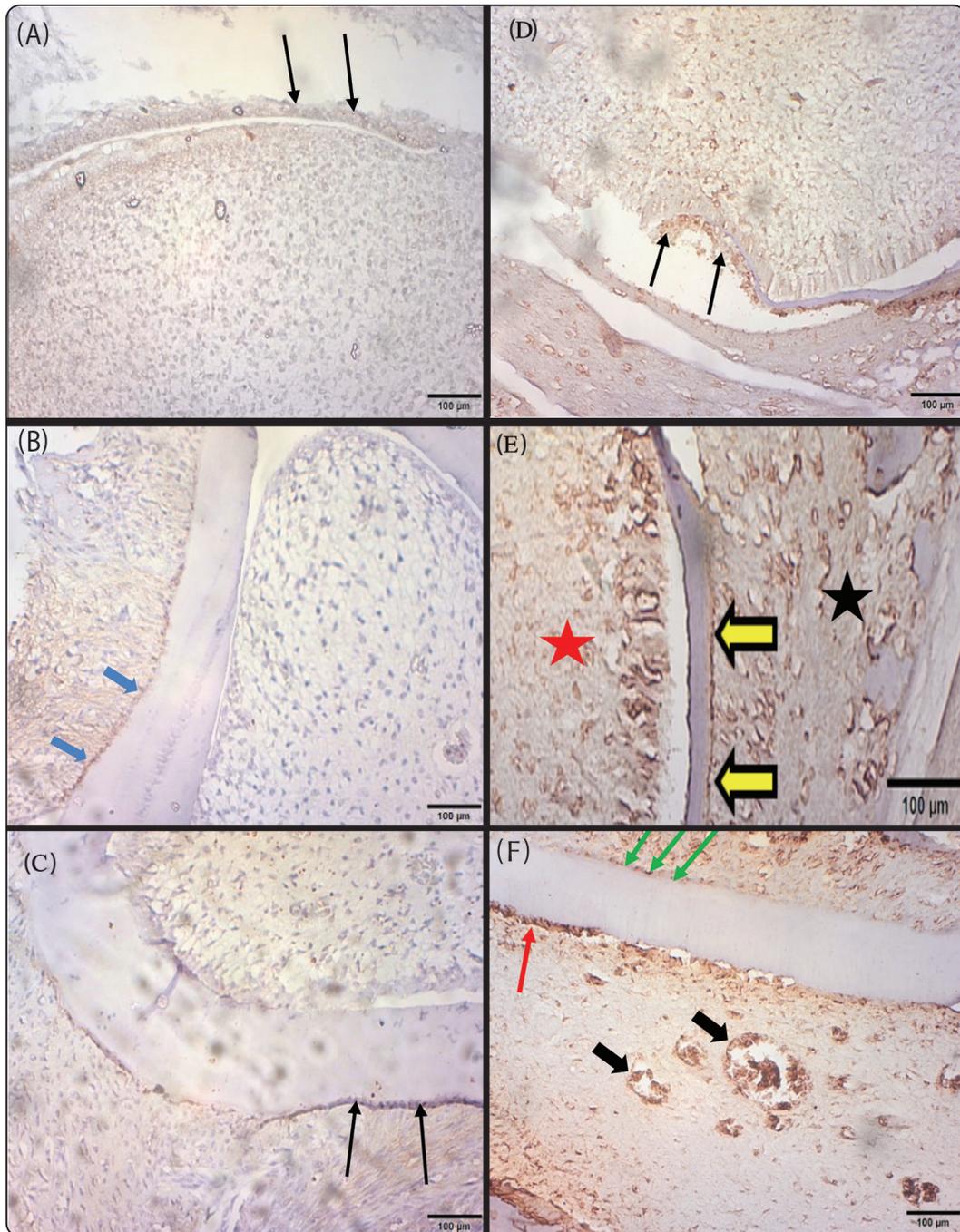


Fig. (3) Photomicrographs of OPN labelling (Anti-OPN.org.mag.x200):

- A. Subgroup C1 showing positive cytoplasmic immunostaining for OPN in the epithelial diaphragm (black arrows).
- B. Subgroup C2 showing positive immunostaining for OPN in the cementoblastic cell layer facing the developing root surface (blue arrows).
- C. Subgroup C3 showing positive immunostaining for OPN in cellular cementum (black arrows).
- D. Subgroup E1 showing positive immunostaining for OPN in the epithelial diaphragm area in addition to pulp C.T. tissue (black arrows).
- E. Subgroup E2 showing marked immunoreactivity in cementoblasts bordering cementum (yellow arrows). Pulp (red star) and PDL tissues (black star) are also positive immuno-stained.
- F. Subgroup E3 showing OPN immunopositivity in pulp tissue and blood vessels endothelium (black arrows). Odontoblasts (red arrows) and cementoblasts (green arrows) are also immunostained.

Statistical Results

Area% of spaces between odontoblasts:

As shown in table (1) and figure (4), there was statistical difference between all subgroups, subgroup E3 showed highest mean of area % of spaces between odontoblastic cells followed by subgroup E2 and the lowest mean value was recorded by subgroup E1.

As shown in table (2) and figure (5), C1, C2 and C3 subgroups showed non significant difference (P=0.18). Within experimental subgroups, E2 subgroup showed non significant difference compared to E1 subgroup while E3 subgroup showed significant difference compared to E1 subgroup. E3 subgroup showed significant increase compared to E2 subgroup.

TABLE (1): Showing mean and standard deviation of area % of spaces between odontoblasts in control and experimental subgroups.

	Control group	Experimental group	P value
1 week of birth	113.62±18.94	147.70±24.62	0.01*
2 weeks of birth	102.68±14.67	172.11±24.59	<0.001**
4 weeks of birth	98.15±11.39	4175.64±521.95	<0.001**

SD: standard deviation* : significance <0.01 ** : highly significance <0.001

TABLE (2): Comparison of area % of spaces between odontoblast cells.

	1 week postnatally	Two weeks PN	Four weeks PN	P value
Control group (C)	113.62±18.94	102.68±14.67	98.15±11.39	0.18
Experimental group (E)	147.70±24.62	172.11±24.59	4175.64±521.95	<0.001**
		P1=0.98 NS	P2=<0.001** P3=<0.001**	

SD: standard deviation

NS= non-significant

P1: significance between 1wk & 2wks,

P3: significance between 2wks & 4wks

*:significant. <0.05

**highly significant <0.001

Test used: One way ANOVA followed by post-hoc tukey

P2: significance between 1wk & 4wks,

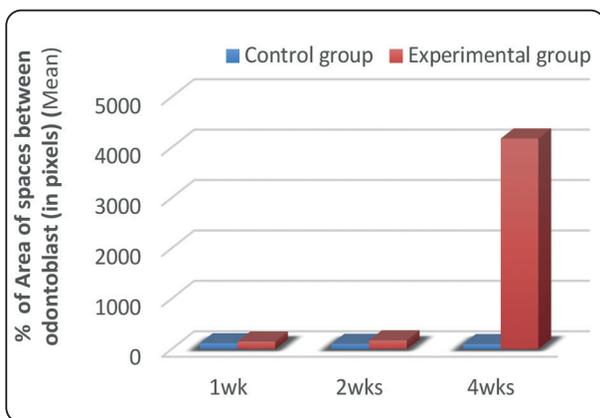


Fig. (4): Bar chart of means of area % of spaces between odontoblasts in control and corresponding experimental subgroups.

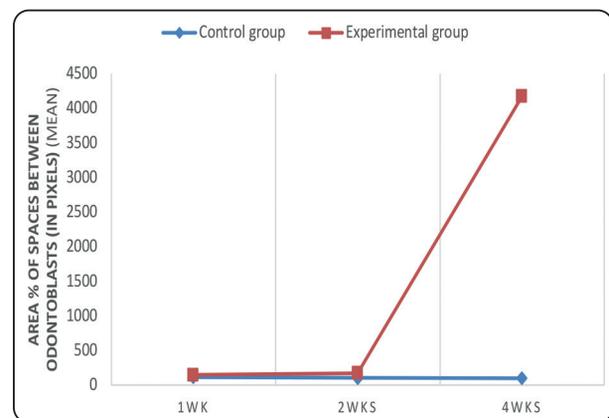


Fig. (5): Mean of area % of spaces between odontoblasts in control & experimental subgroups.

DISCUSSION

The dental root as a portion of dento-alveolar complex was monitored in this study to detect the alterations caused by maternal DM. Studies establishing relationships between cementum and diabetes were found to be few in number (**Gokhan et al., 2004**).

In the present work, one week aged rats of offspring were chosen for beginning of examination because root development of mandibular first molar starts at 5th postnatal day (**Sohn et al., 2014**). Molars of Albino rat are morphologically distinct from human molars, but their enamel, dentin and cementum are nearly histologically the same. In addition, this model is helpful to study teeth formation, since tooth-related cells can be observed sequentially in all stages of development (**Park et al., 2017**).

In H&E stained sections of the present work, the epithelial diaphragm of the experimental subgroup E1 appeared thinner when compared to the corresponding control subgroup C1. **Chen et al. (2017)** suggested that maternal gestational diabetes in rat model resulted in many effects on tooth development in offspring. The same investigators declared that in the tooth germ of offspring of diabetic rats, increased apoptosis level of epithelial cells was observed.

In subgroup E2, dentin of apparently thinner thickness when compared to the corresponding control was detected. This result come along with those of **Abbassy et al. (2015)** that experimentally induced DM significantly reduced rate of dentin mineral apposition and formation. The same investigators explained that the metabolic functions of odontoblasts could be hindered by the elevated blood glucose level associated with DM. Further investigations to explore the effect of induced maternal diabetes on rate of dentin formation are required.

In this study, examination of odontoblasts in subgroups E2 showed an interruption in the continuity of odontoblastic layer in the developing root with absence of physiologic cell-to-cell

adhesion, in addition to areas of degeneration that were detected between the odontoblasts.

These results could be explained based on the conclusion of **Chen et al. (2017)** reporting that hyperglycemia significantly affected cell proliferation and apoptosis in offspring tooth germs by suppression of odontoblast cell proliferation and enhancement of apoptosis through (TLR4/NF- κ B) signaling pathway.

TLR4/NF- κ B signaling pathway is reported to be one of the important pathways activated by maternal diabetes, also shown to be crucially important in impairing embryo development. (**Devaraj et al., 2011**). It is also closely associated with the dental tissue-derived stem cells. However, the exact role of this signaling pathway activated by maternal diabetes during the offspring tooth development remains largely unknown (**Lin et al., 2012**).

It was further reported that hyperglycemia inhibited the differentiation and proliferation of human dental pulp stem cells **Yan et al. (2017)** and **Horsophonphong et al. (2020)** which could account for absence of odontoblasts.

In subgroup E2 and E3 of the present study, PDL fibers were haphazardly arranged and showed areas of degeneration. Very few PDL fibers were inserted into cementum which displayed irregular surface especially in E3 subgroup. In a parallel study, **El-Zainy et al., (2018)** reported disorganization and degeneration of PDL fibers with partly or complete attachment loss of Sharpey's fibers to bone and cementum in induced diabetic rats. The same investigators explained that high glucose level inhibited PDL cell differentiation, decreased the proliferative capacity of PDL cells and induced resistance to growth factors with partial or complete destruction of Sharpey's fibers attachment to cementum.

Examination of H&E sections of subgroup E3 showed that cementum thickness was markedly increased at the apical part of the root. These results were coincident with **Gokhan et al. (2004)** and **El-Zainy et al. (2018)** who clarified that an increase in the thickness of cementum layer could arise from

systemic conditions like diabetes.

In the present study, areas of degeneration between odontoblastic cells were observed in H&E stained sections. The statistical analysis showed that the area percentage of spaces between odontoblasts was significantly higher in experimental groups which is in parallel with conclusion of **Chen et al., (2017)** reporting that maternal diabetes affected the offspring odontoblasts by suppression of differentiation capacity and enhancement of apoptosis.

Statistical results of area % spaces between odontoblasts revealed that E3 subgroup showed the highest mean of spaces which was highly significant compared to E1 and E2 subgroups. This could indicate the prolonged effect of maternal diabetes on root dentin of offspring.

Osteopontin (OPN) is the first extracellular matrix protein identified in bone (**Karpinsky et al., 2017**). In streptozotocin-induced diabetic rats, it was found to play a role in the development of diabetic vascular complications such as atherosclerosis (**Inagaki et al., 2010**).

In our study C2 and C3 subgroups showed immuno-expression of OPN in cementum and the bordering cementoblasts. **Bosshardt (1998)** postulated that in normal conditions root-lining cells in rodent molar expressed mRNA for OPN and this non-collagenous matrix protein accumulated on the root surface. **Hirata et al. (2009)** also observed in two weeks old rats an intense labelling of OPN in cementum and cementoblasts of developing mandibular first molar where it was concluded that mature cementoblasts secrete OPN.

In the current study, the increase OPN immuno-expression detected in subgroups E2 and E3 is parallel to results of **Inagaki et al (2010)**, on radicular odontoblastic cells of diabetic rat. In addition, the same investigators reported weak OPN immuno-expression in nondiabetic rats which go along with control results of our study. It was also concluded that hyperglycemia significantly stimulated OPN production and alkaline phosphatase activity

indicating that high glucose level increased hard tissue-forming production of dental pulp cells.

Immunohistochemical results of experimental sections of E2 and E3 subgroups of the present study revealed marked increase in immuno-expression of OPN in cementoblasts compared to their corresponding control groups indicating the increased activity of cementoblasts that could be the reason for increased thickness of cementum under diabetic condition (**Gokhan et al., 2004**).

In the present study, OPN expression in PDL was well detected in experimental subgroups E2 and E3 compared to their corresponding control subgroups. These results were in agreement with **Yilmaz et al. (2018)** who stated that the expression of OPN in bone and PDL of STZ-induced diabetic rats showed a significant increase in reactivity compared to their control subgroups as hyperglycemia increased the fibroblastic immuno-expression for OPN.

In E3 subgroup of the present study, a marked immuno-expression of OPN in BV endothelium was detected. This is parallel with results of (**Inagaki et al., 2010**) who correlate the role of OPN with development of diabetic vascular complications and calcifications.

CONCLUSION

From the result of the present study, it could be concluded that diabetes induction in rat mothers adversely affected dental root forming cells, pulp, cementum and PDL fibers of developing molar roots in the offsprings. This was associated with increased OPN immuno-expression.

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