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RESEARCH ARTICLE

Evaluation of the effect of Nicotine on Palatogenesis in Mice Pre and Post natally

El-SakhawyM.A.[1]; Sherif H.A.[2]; Abd El-Nasser A [2] and *Hussein SH[1]

1. Department of Cytology & Histology, Faculty of Vet.Medicine, Cairo University.
2. Department of Oral Biology, Faculty of Dental Medicine, Al-Azhar University

Manuscript Info *Abstract*

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Corresponding Author

Hussein SH

In this investigation fourty pregnant C57BL/6J mice were classified into two groups (I and II), each subdivided into two equal subgroups (Ia, Ib, IIa and IIb). Group I was the control group injected by sterile physiological saline daily from the 6ththrough the 15th gestation days. The Ia was sacrificed at 18th gestation days while; the Ib was sacrificed one day after delivery. Group II was the experimental group injected by 1.67 mg/kg N-Nitrosonornicotine (NNN) in saline of a final concentration 0.1% daily from the 6th - 15th gestation days. The IIa was sacrificed at 18th gestational days while the Ilb was sacrificed one day after delivery. The pregnant mice were sacrificed & their fetuses were measured and counted, decapitated, fixed in Bouin′s solution and prepared routinely for paraffin sectioning. For histological and morphometrical examination a routine H & E staining was performed, while for the immunohistochemical examination of Epidermal Growth Factor a serial sections were mounted on electrically charged slides. The cleft palate percentages of all groups were counted and statistically analyzed. Histological examination demonstrated that the palate of all control groups was normally developed, whereas the nicotine injected group demonstrated underdeveloped palatal shelves. The percentage of the cleft palate of nicotine injected group were 46.8% for IIa and 28.1% for IIb, also nicotine injected group showed complete closure but with persistence of epithelial seam in a percentage of 12.5% for IIa and 18.7% for IIb. Immunohistochemical examination demonstrated a moderate immune reaction to the EGF in the control group, while in the experimental group, the EGF expression in the palatal epithelium was negative and nearly absent in the mesenchymal tissue. Our investigation revealed that fetuses and offsprings were shorter and weighed less than those of the control group. Group II fetuses and offsprings had a smaller heads when compared to the control.

INTRODUCTION

Palatogenesis requires a complex series of events that result in growth of palatal shelves and differentiation of specific populations of palatal cells. All the events in palatogenesis are required to occur during a very narrow developmental time frame and in the correct sequence. Epidemiological studies have established a significant positive correlation between maternal smoking and an increased risk for orofacial cleft. The examination of the intraperitoneal injection of nicotine on mice daily from gestation days 6-15 during the organogenesis, reported that nicotine could inhibit the palatal fusion and linked consistently with the risk of cleft palate (Chung et al., 2000). Several growth factors have been reported to influence medial edge epithelium proliferation and apoptosis during palatogenesis. Supportive evidence indicated that teratogens altered these growth factor-related signaling pathways and induced cleft palate (Young et al., 2000). The present study aims to study and evaluate the effect of nicotine injection to pregnant C57BL/6J mice on the development of palate of their fetuses. This evaluation is carried out pre and post natally by histological, immunohistochemical and morphometrical studies.

Material and Methods:

Experimental animals:

Sixty adult C57BL/6J mice (40 females and 20 males) of 6-8 weeks age and 25-35 gm weights were utilized in this experiment. The animals were procured by the animal house, Faculty of Medicine, Assiut University. All mice were maintained in controlled environment of 22^{o}C with 16/8 light/dark periods, received food ad libitum with unconstructed access of water

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Animal breeding:

The animals (two females and one male) were caged together in polycarbonate cage covered by stainless steel wire and mated together from 5:00 p.m. to 9:00 a.m. of the next day when the male was separated from females. Detection of the vaginal plugs was indicated for the day zero of the pregnancy.

Grouping:

Fourty pregnant mice were divided into two equal groups of 20 mice each; Group I served as a "control group" and Group II is the "experimental one". Each group was subdivided into two equal subgroups a&b of 10 mice each. Animals of subgroups Ia (Control) and IIa (Experimental) were sacrificed at the 18th day of gestation, while animals of subgroups Ib (Control) and IIb (Experimental) were left until delivery.

Nicotine administration:

Experimental group **(**Group II) injected intraperitoneal with 1.67 mg/Kg N-Nitrosonornicotine (NNN) dissolved in saline of a final concentration 0.1% daily from the 6th through the 15th gestation days. Control group (group I) injected by saline instead of nicotine.

Sacrification:

Animals of subgroups Ia and IIa were removed by laparotomy, examined clinically for gross malformation and sacrificed by chloroform inhalation. Animals of subgroups Ib and IIb were left until delivery. One day after delivery, offsprings were examined clinically and sacrificed by chloroform inhalation

Tissue preparation for microscopic examination:

The heads of all fetuses and offsprings were fixed in Bouin′s solution, after proper time of fixation, the samples were dehydrated, cleared and embedded in paraplast. Coronal tissue sections of 5 µm thickness were cut. The deparaffinized tissue sections were then stained by routine Harris Haematoxylin and eosin stain (H&E) for general histologic examination and Periodic Acid Shiff (PAS) for demonstration of neutral mucopolysaccharides. The methods used were adopted as outlined by Drury &Wallington (1980). For immunohistochemical staining of Epidermal Growth Factor, coronal tissue sections of 5 µm thickness were mounted on electrically charged slides. Deparaffinization and rehydration of the tissue sections were performed in order to be ready for application of blocking of endogenous peroxidase using H_2O_2 methanol. Enzymatic treatment was performed by putting the slides in a solution to enhance the reaction in hot oven for 1 hour. Tissue sections, after that, were covered with non-immune protein blocking serum in humidity chamber for 30 minutes (protein blocking). Two- three drops of the monoclonal mice primary antibody were applied, following by incubation in a humidity chamber for 1 hour at room temperature. After washing the slides with phosphate puffer saline (PBS), they were incubated with biotylinated secondary antibody in humidity chamber for 30 minutes at room temperature. The Epidermal Growth Factor (EGFR) receptor in the cell membrane of MEE was evaluated by it′s monoclonal antibody (Golden Lab, USA) (clone TAB 346) using streptavidin biotin method. Diaminobenzidine (DAB) was applied to develop color. Tissue sections, after that, were counterstained with Mayer′s hematoxylin for 30-40 seconds in order to be ready for microscopic examination. The examination was performed by evaluating the concentration of antigen within tissue for estimating the relative intensity of a chromogen label of EGFR. The method used was outlined by Ramos-Vara (2005).

Statistical analysis:

Statistical analyses were performed using X2 test to determine the significant relationship between nicotine injection and the change in;

1- Weight and Length of the control and experimental fetuses and offsprings.

2- Width, Height and Circumferences of heads of control and experimental fetuses and offsprings.

Cleft palate frequency:
The frequencies of cleft palate in all of experimental and control groups were collected and statistically analyzed. Statistical analysis of recorded data of both groups was done using X2 test to determine the significant relationship (P=O) between nicotine injection and frequency of cleft palate induction.

Results:

Histological results:

Histological examination of group I:
Microscopic examination of coronal sections of secondary palate of control group either sacrificed at 18th gestational day or left until the one day after delivery revealed that, the palatine shelves of all fetuses and offsprings were in a horizontal position, well developed, and completely fused with each other (Fig. 1). The palatal shelves were also fused with the primary palate anteriorly and with nasal septum superiorly. At the area of fusion, the epithelial seam was completely degenerated with no epithelial remnant. The oral aspect of secondary palate was lined by stratified squamous epithelium. The nasal aspect of secondary palate was covered by pseudostratified columnar epithelium with goblet cells. The underlying mesenchymal tissue of the anterior and middle regions of the secondary palate, particularly, at the area of fusion showed an apparently condensed population of mesenchymal cells. The ossifying center showed increased mitosis of mesenchymal cells and formation of bony trabeculae (Fig. 2).

Histological examination of group IIa:
The frequency of cleft palate in this group was 15 fetuses from total 32 fetuses (46.8%). The histological findings of group IIa sacrificed at 18th gestational day showed variety of histological appearance classified as follows:

Fetuses with cleft palate (Group IIa):
The majority of clefts were bilateral cleft; the palatal shelves were underdeveloped and vertically directed lied lateral to the highly positioned tongue. The tongue was reached to the nasal septum occupying both the nasal and oral cavities (Fig. 3). There is an increase in the thickness of the epithelium covering the palatine shelves. On the other hand, in unilateral clefts the palatal shelves were poorly developed and vertically positioned. The tongue was in the high position if compared with those of control group but in lower position than in cases of bilateral clefts (Fig. 4). The ossifying centers showed a decreased mitotic figures and decrease in bony trabeculae formation.

Fetuses with complete palatine closure (Group IIa):
The palatine shelves were correctly directed in horizontal position, well developed, and fused together and with primary palate anteriorly and with nasal septum posteriorly. At the fusion area, the epithelium seam was persisted with the signs of apoptosis (Fig. 5). The underlying mesenchymal tissue showed a little signs of condensation. The tongue was in normal position but under- developed.

Histological examination of group IIb:
The frequency of cleft palate in this group was 6 fetuses from total 32 fetuses (28.1%). This group demonstrated a variety of histological appearance classified as follows:

Offsprings with cleft palate (Group IIb):
This group show both bilateral and unilateral cleft. In bilateral clefts the palatal shelves were under developed with the tip of the palate shelf pointed vertically toward the tongue which was located in higher position reaching the nasal septum. The tongue was poorly developed (Fig. 6). In the unilateral clefts the palatine shelves were directed horizontally, one of them fused with the primary palate and nasal septum, while the other fails to reach the midline (Fig. 7). The underlying mesenchymal tissue of shelves was not condensed by mesenchymal cells. The ossifying centers showed little bony trabeculae and little mitotic figures in the mesenchymal cells of the ossifying centers.

Offsprings with palatine closure (Group IIb):
The palatal shelves were completely fused with complete degeneration of medline epithelial seam. The underlying connective tissue appeared condensed particularly at the area of fusion. The tongue was well developed and correctly positioned occupying the oral cavity (Fig. 8). Some fetuses showed a well developed and horizontally directed palate but with persistence of epithelium seams at the line of fusion, in spite of increased thickness of epithelium covering the palatal shelves.

Histochemical results

The examination of the acid mucopolysaccharides revealed a negative result for both control and experimental groups. On the other hand, the use of Periodic Acid Schiff technique for demonstration of neutral mucopolysaccharides revealed a strong PAS positive reaction in the palate epithelium and moderate reaction in the mesenchymal cells and bony trabeculae for the control group (Fig. 9). The PAS reaction of the experimental group revealed moderate amount of PAS positive substance in the palatal epithelium and faint reaction in the mesenchymal tissue.

Immunohistochemical results

The saline injected group (control group) showed moderate immune reaction for the Epidermal Growth Factor in the palatal epithelium. Also the mesenchymal cells and the ossifying centers expressed the same immune reaction (Fig. 10). On the other hand, examination of immunohistochemically stained slides of experimental group (nicotine injected group) revealed that immune expression of Epidermal Growth Factor was lacking in the palatal epithelium and nearly absent in the mesenchymal cells, ossifying centers and epithelial seam cells (Fig. 11).

Morphometrical measurements:

The intraperitoneal injection of 0.1 % nicotine sulfate daily from the 6th through the 15th gestational days affects the fetuses and offsprings. These fetuses and offsprings were shorter and weighed less than those of the control group. Group II fetuses and offsprings also had a smaller heads when compared to the control. Data was summarized in tables 1-5.

Mean measurements of weight and length of control and experimental fetuses and offfsprings:

Table 1 Table 2

Length (mm)	
Control	Experimental
Fetuses (t=192.378)**	
21.33	15.02
Offsprings (t=153.22)**	
21.61	15.19

Weight (gm)	
Control	Experimental
Fetuses (t=17.79)**	
0.9	0.32
Offsprings (t=13.78)**	
1.12	0.54

** Means the value showed highly significant relationship.

Mean measurements of head size of control and experimental fetuses and offspring (Table 3, 4 & 5)

Table 3

Head circumferences (mm)	
Control	Experimental
Fetuses (t=63.487)**	
20.23	17.9
Offsprings(t=53.50)**	
20.41	18.12

Table 4

Head height (mm)	
Control	Experimental
Fetuses (t=31.42)**	
5.86	4.9
Offsprings(t=20.915)**	
6.07	5.11

Table 5

Head width (mm)	
Control	Experimental
Fetuses (t=27.42)**	
5.98	5.02
Offsprings (t=1.25 n.s)	
6.2	5.21

** Means the value showed highly significant relationship.
n.s Means the value showed non- significant relationship.

Fig.1: Photomicrograph of coronal section of control group Ia sacrificed at 18th day of gestation at the middle third of the palate, showing: complete palatine closure with complete degeneration of the epithelial seam (arrows) and advanced calcification of hard palate. (H&E x 100)

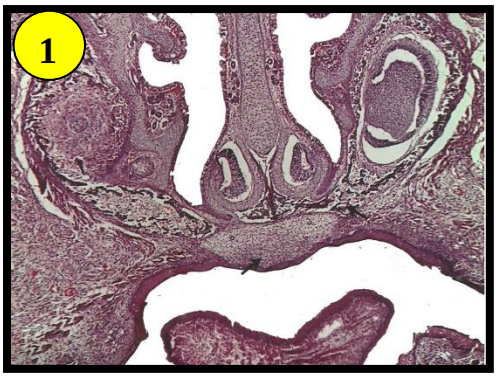


Fig. 2: Photomicrograph of the ossifying center at coronal section of control group Ib, at the middle third of the palate showing: increased mitotic figures of the mesenchymal cells increased deposition of bone trabeculae. (PAS x 400)

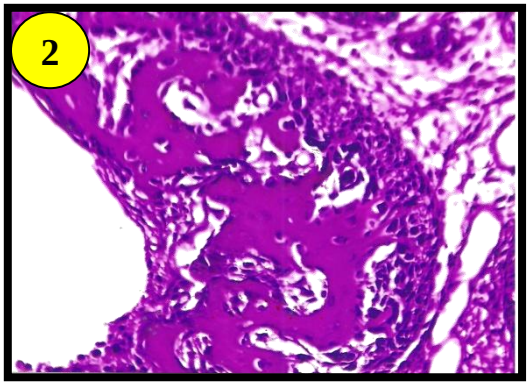


Fig. 3: Photomicrograph of coronal section of experimental group IIa, at the middle third of the palate, showing: bilateral cleft palate (arrows). (H&E. x 200)

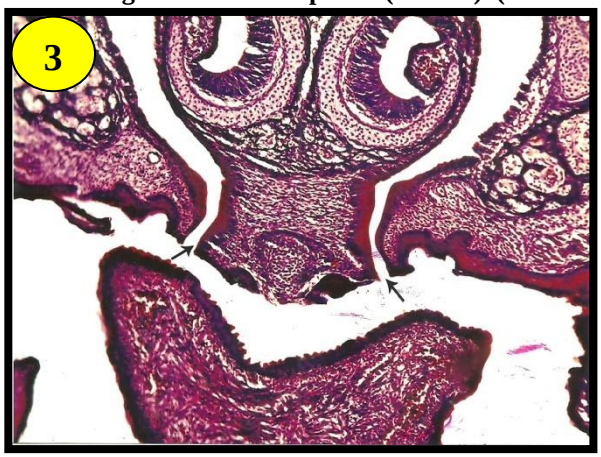


Fig. 4: Photomicrograph of coronal section of experimental group IIa, at the middle third of the palate, showing: unilateral cleft palate (arrow) with under developed and vertically positioned palatal shelf. (H&E. x 40)

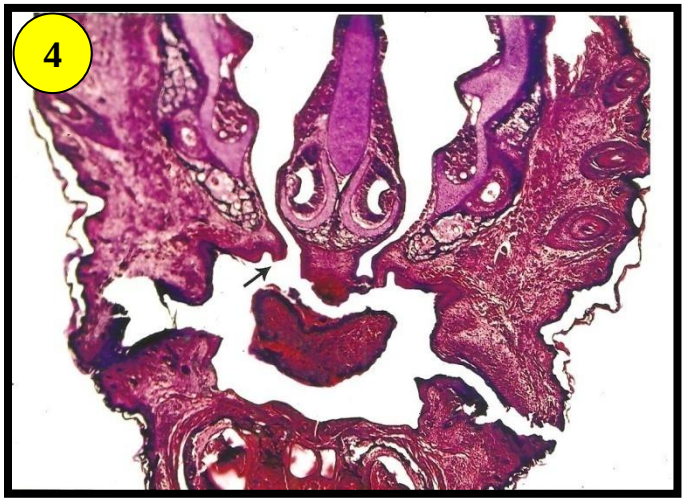


Fig. 5: Photomicrograph of coronal section of experimental group IIa at the middle third of the palate, showing: horizontally directed and well developed palatine shelves with persistence of epithelium seam at the fusion area (arrow). (H&E. x 200)

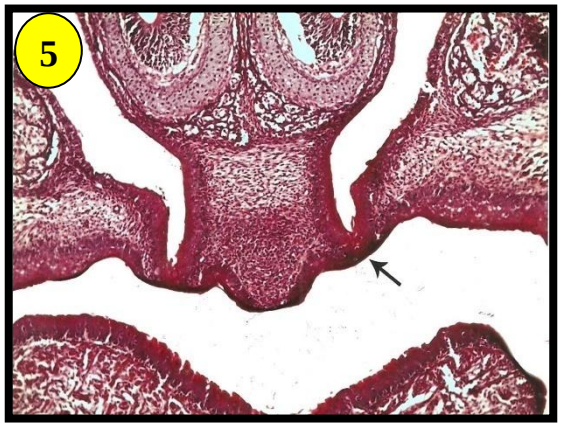


Fig. 6: Photomicrograph of coronal section of experimental group IIb at the middle third of the palate, showing: bilateral cleft palate (arrows) with vertically positioned palatal shelves. The tongue was under developed and highly positioned. (H&E. x 40)

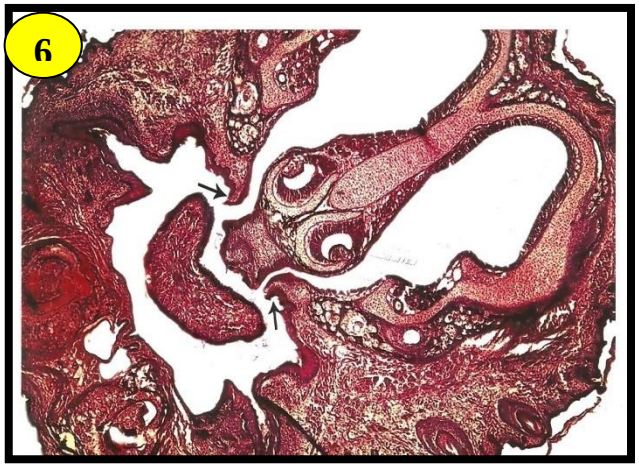


Fig. 7: Photomicrograph of coronal section of experimental group IIb at the middle third of the palate, showing: unilateral cleft palate (arrow) with under developed palatal shelves. (H&E. x 40)

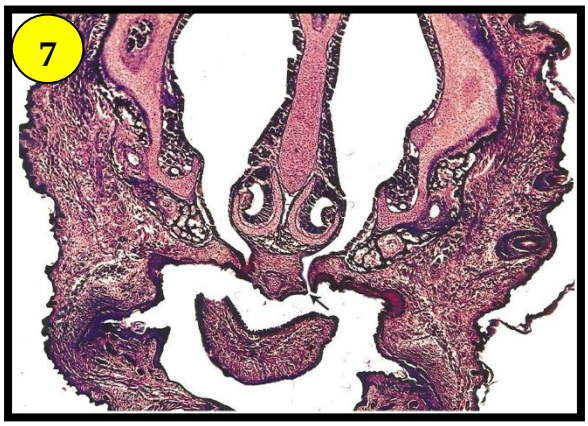


Fig. 8: Photomicrograph of coronal section of experimental group IIb, at the middle third of the palate, showing: complete fusion of palatine shelves and well developed tongue. (H&E. x 40)

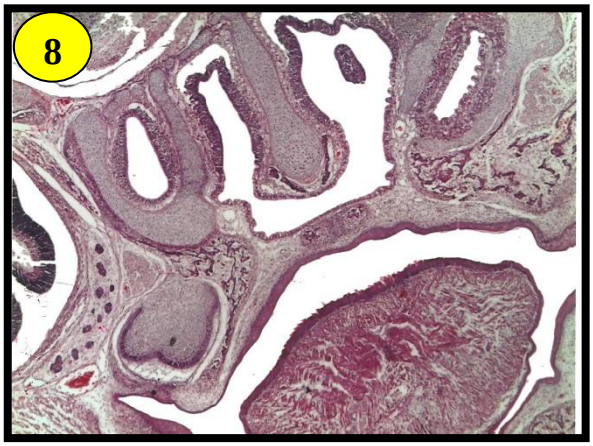


Fig. 9: Photomicrograph of coronal section of control group, at the middle third of the palate, showing: strong PAS positive reaction in the palatal epithelium (arrow) and moderate reaction in the mesenchymal tissue and bone trabeculae. (PAS x 400)

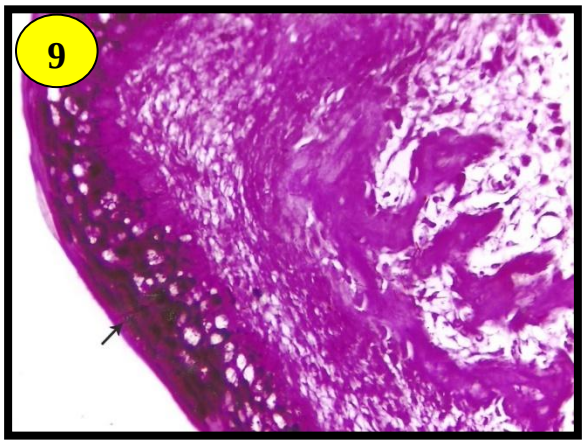


Fig. 10: Photomicrograph of coronal section of control group at the middle third of the palate, showing: Expression of Epidermal Growth Factor in the superficial layer of the palatal epithelium (arrow). (EGF x 100)

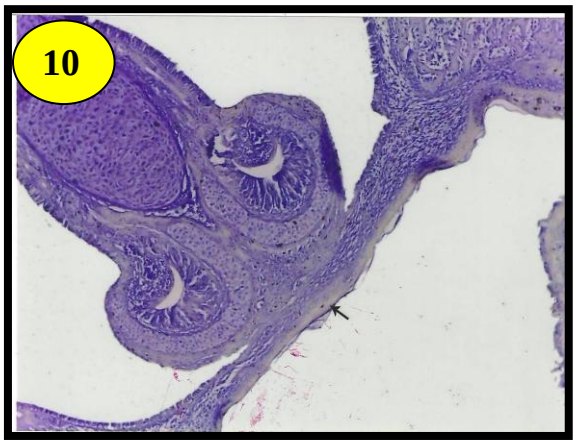
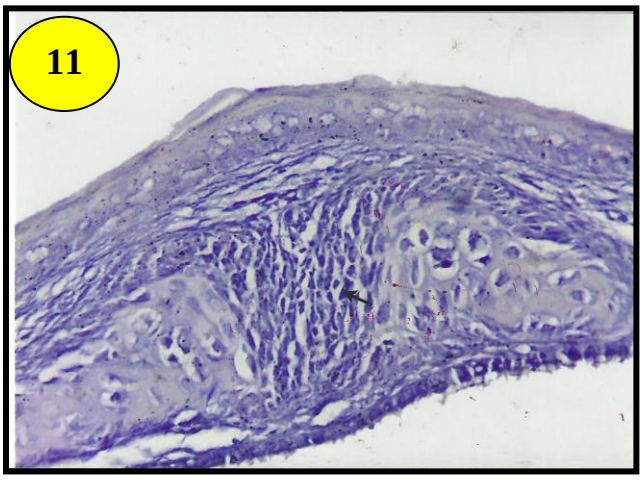


Fig. 11: Photomicrograph of coronal section of experimental group at the middle third of the palate, showing: negative reaction to EGF in the palatal epithelium and medline epithelial seam. (EGF x 400)



Discussion:

The results of the present study observed that the spontaneous palatal clefts in the control group were (0%). The epithelial seam of the control group was completely degenerated and no epithelial remnant observed at the site of fusion of palatal shelves, which was similar to that reported bySaad et al. (1990) & Takashi et al. (2005).

Nicotine injected group of our study showed 15 fetuses with cleft palate (46.8%) and 9 offsprings with cleft palate (28.1%), this in contrast to that reported by Saad et al.(1990). The percentage difference of cleft palate might be related to the different administration dose and/or administration route to the pregnant mice. Also, these differences might be due to the different species of animal used by different authors.

Histological results of cleft palate mice in our study showed that, nicotine affect the entire steps of normal palate development. Maternal injection of nicotine affects the growth of palatal shelves, elevation of the palatal shelves into horizontal position and adhesion and fusion of the opposing palatal shelves leading to cleft palate. These results were in agreement with the observations of many authors (Taya et al., 1999, Ito et al., 2003, Yamamoto et al., 2003 &Gritli-Linde, 2007).

Most of cleft palate fetuses or offsprings showed under developed palatal shelves and this related to the inhibition of proliferation and growth of both epithelial and mesenchymal tissues. This finding was parallel to that reported by Ito et al. (2003) who reported that nicotine inhibits the palatal shelves initiation and vertical growth due to its effect on the molecular control of palatal shelves initiation and growth. Nicotine thought to entail the complex singling cascades with transcription factors and growth factors including the Epidermal Growth Factors and Transforming Growth Factors and their receptors (Calzolari et al., 2004 & Rice et al., 2004).

Many fetuses and offsprings showed vertically directed palatine shelves instead of horizontally positioned which might be related to failure of tongue drooping during mandibular development. This finding was coincidentally with Kaartinen et al. (1997) &Taya et al. (1999). Additionally, failure of drooping of the tongue indicate that nicotine not only affect the palatal development but also might interfere with normal tongue and mandible development and growth.

Kaartinen et al. (1997), Taya et al. (1999), Martinez-Alvares et al. (2000), Gato et al. (2002) &Tudela et al. (2002) reported that nicotine also affect the adherence and fusion between the palatal shelves by affecting the cell adhesion molecules and desmosomal components and growth factors essential for initial adhesion of palatal shelves, increasing the surface area of medial edge epithelium through induction of cellular bulges and filopodia and promoting degeneration of medial edge epithelium.

The histological examination of fetuses and offsprings with complete palatine closure of nicotine injection group revealed that the epithelial seam at the site of fusion was persisted with no signs of degeneration. This indicates that maternal nicotine administration led to continued medial edge epithelium cells proliferation and decreased medial edge epithelium cells apoptosis and decreased epithelial mesenchymal transformation. This finding might also indicate that, nicotine not only induced cleft palate malformation but also interfere with the apoptotic process of the epithelial seam. These results might indicate that, nicotine could act to prevent the formation of lysosomes needed for apoptosis by interfering with synthesis of lysosomes (Shuler et al., 1991).

Takashi et al. (2005) reported that the persistence of the epithelial seam in complete palatine closure was related to the effect of nicotine on the molecular mechanisms critical to DNA synthesis and cell proliferation of medial edge epithelium.

Additionally, Apoptosis reduction by binding of nicotine to nicotine acetylcholine receptors found in the palatal epithelium also reported by Minna (2003) & West (2003, 2004). This hyposis was further postulated by the observations of Kang & Svoboda (2003) who reported that nicotine can inhibit the palatal fusion by binding to neuronal nicotine acetylcholine receptors. Others revealed that the apoptosis reduction was due to the direct inhibitory effect of nicotine on the apoptosis protease-activating factor1 which is essential for medial epithelial seam degeneration (Cecconi et al., 1998 &Honarpour et al., 2000).

The present investigation noticed that there was a marked reduction in ossification of the palatal shelves of nicotine injected group compared with saline injected group even with degeneration of medial epithelial seam. This might be due to the marked decrease in mesenchymal condensation with subsequent decrease of differentiation of mesenchymal cells into osteoblasts. These observations simulate that recorded by Hartridge et al. (1999).

Periodic Acid Schiff (PAS) reaction used in the current study revealed that, the nicotine treated animals showed moderate PAS positive substance in the superficial layer of palatine epithelium and faint reaction in the palatine mesenchymal tissue. While the reaction was strongly positive in the control group, that revealed the effect of nicotine on the role of mucopolysaccharides in the development of palate. Ferguson (1981) demonstrated that depressed neutral mucopolysaccharides synthesis in fetuses and offsprings was associated with cleft palate. He also postulated that normal shelf elevation is brought about rapidly by an intrinsic turger shelf force generated by binding of water to mucopolysaccharides. Moreover, Lu et al. (2008) revealed that animals with cleft palate were associated with greatly decreased mucopolysaccharides synthesis.

Immunohistochemical staining of the Epidermal Growth Factor used in the present study showed a negative reaction in the palatal epithelium and medial epithelial seam of the experimental group. While the EGF was expressed in the palatal epithelium of the control group. Our result point to a tight regulatory relationship between the EGF distributions and the normal medial edge epithelium apoptosis and differentiation with consequence inhibition of palatal fusion.

Our results were similar to Nakayama et al. (2002) who revealed that, the cleft palate induction was related to the inhibitory effect of nicotine to the singling pathways between the EGF and their receptors. Also John (2004) reported that the disturbance in EGF induces the epithelial cells to keratinize, and as a consequence the normal sequence of steps of palate fusion was inhibited.

Morphometrical analysis of nicotine injected mice indicates that there was a significant decrease in all over the measurements of the fetuses and offsprings. This indicated that the maternal administration of nicotine affect the general development and growth of the fetuses and offsprings. Our results were also similar to the morphological findings reported by Schuller et al. (2000) who noticed that there were a general retarded growth of the fetuses and offsprings and delay in their normal pattern of maturation. The decrease in the circumference of the head reported in our result may indicate the parallel decrease of the palate size even with complete palatine closure.

Nicotine injected group of our study showed 15 fetuses with cleft palate (46.8%) and 9 offsprings with cleft palate (28.1 %), this in contrast to that reported by Saad et al. (1990). The percentage difference of cleft palate might be related to the different administration dose and/or administration route to the pregnant mice. Also, these differences might be due to the different species of animals used by different authors.

Conclusion:

Our study revealed a significant positive correlation between nicotine administration and palate formation. Nicotine inhibited the palatal fusion and linked consistently with the risk of cleft palate.

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