Histological and Immunohistochemical studies of the effect of vitamin C and *Nigella sativa* on the palate of albino mice's offspring after cadmium exposure.

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Abstract: Performance of the current study utilized a well elicted CD-1 albino mouse. 50 females divided into 5 groups, 10 females in each group separated from males for one month, all groups were get the drug doses twice a week for one month before gestation and during pregnancy until delivery. For breeding purposes each 2 females were mated with 1 male from 5 pm. until 9 am. at the following day. Female mice were classified into five groups. **Group I (**control group, female animals were injected intramuscularly with 0.5 ml of sterile normal saline); **group II** (dams were injected i.m. with 5 mg/kg body weight of cadmium chloride (Cd Cl_2) dissolved in 0.5 ml of sterile normal saline); **group III**; (treated with Cd Cl_2 as in group II, plus i.m. injection of 10 mg/ kg body weight of vitamin C. **group IV**(was injected i.m. with Cd Cl_2 as in group II and given 50 mg/kg body weight of *Nigella sativa* orally) and **group V**(animals were administrated cadmium and vitamin C as in group III and *Nigella sativa* as same as in group IV). After delivery, heads of pups were decapitated. The heads were fixed in Bouin`s fixative and prepared routinely for paraffin sectioning and staining for histological and immunohistochemical investigations**.** Morphological and histological observations showed that 44.77% of cadmium treated pups affected with cleft palate, 13.51% of them with secondary palatal cleft. All control group animals were normally developed, as well as the pups of the remaining groups which treated with vitamin C and / or *Nigella sativa* with cadmium. Immunohistochemical examination of transforming growth factor alpha revealed that, control pups expressed a strong immune reaction in the primary palatal region in the palatine epithelium, while it was moderately positive in the underlying mesenchymal tissue cells. The reaction was strongly expressed in the endothelium of the blood capillaries and in the ossifying centers of the secondary palatal region. Cadmium treated mice (group II) revealed negative immune TGF-α reaction. Cadmium and vitamin C treated animals expressed moderate TGF-α reaction in the palatine epithelium and faint reaction in the underlying connective tissue cells, endothelium of the blood capillaries and in the ossifying centers. Animals treated with cadmium and *Nigella sativa* expressed the same immune reaction as in group III. Cadmium, vitamin C and *Nigella sativa* treated pups expressed strong immune reaction of TGF-α similar to that of control group.

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1. Introduction

The cadmium is one of common environmental pollutants; it is an important metal and has several uses in industry such as electroplating, soldering, batteries, painting and as a plastic stabilizer. The cadmium toxicity considered as an environmental disease which resulted from the cumulative absorption of small amounts of cadmium until toxic levels are reached in the body which results in a toxic state which is linked with a number of health problems. Vitamin C as an antioxidant and immunoenhancer is one of the important water soluble vitamins and essential for collagen synthesis**(Naidu, 2003)**. The *Nigella sativa* (common food species) is immunoenhancer as it enhances T-cell mediated immunity through the improvement of T-helper to suppressor T-cell ratio. *Nigella sativa* seeds have an antioxidant actions and cytoprotective effect**(Smith and Reynard, 1992**and**Ali Blunden, 2003)**. The *Nigella sativa* has an antibacterial effect **(Thompson and Goldin, 1995)**, also it has anticancer effect**(Sadler**, **1985)**. The transforming growth factor alpha (TGF-α) is known to regulate cell proliferation and differentiation in the embryo. The induction of cleft palate was associated with altered expression of transforming growth factor alpha**(Luke**, **1988** and **Abbott and Best, 2005)**.The injurious effect of cadmium on the biological behavior of the body tissues have primary promoted the present investigation which will be investigated histologically and immunohistochemically to detect the effects of cadmium chloride administration on the palate

development of the albino mice's offspring subsequent to intramuscular injection of the drug to their mothers (dams) and to study the effect of vitamin C and Nigella sativa as an antioxidants and immunoenhancers on the effect of cadmium on mice offspring's palate development.

2. Material and methods

2.1. Experimental animals

The animals used in this study were the CD-1 albino mice. The experimental work has done in the pre-clinical farm of the Contractor Company for Biological Products and Vaccine, Cairo where the animals produced and live in the same place. The animals studied were 75, of five to six weeks of age, which were well elected as mature females weighed 260-280g., while the males weighed 280-300g.

2.2 Grouping of the experiment:

- **Group I:** The animals were injected intramuscularly in the thai with 0.5 ml of sterile normal saline twice a week and used as a control group.
- **Group II:** The animals were injected intramuscularly with 5mg/ kg body weight of cadmium chloride (El Nasr pharmaceutical chemicals Co. Abu Zaabal, Egypt) dissolved in 0.5 ml of sterile normal saline, twice a week. The experimental dose was determined by using dose of 10 mg/ kg body weight, all the treated animals were died. The sublethal dose which used in the current study was 5mg/kg body weight.
- **Group III:** The animals have been injected with cadmium chloride as same as group II and also were injected by intramuscular injection of 10mg/ kg body weight of vitamin C (Cevarol 1000 mg/5 ml ampoule, Memphis Co. for pharm.& chemical ind. Cairo, Egypt) two times per week.
- **Group IV:** The animals were injected with cadmium chloride as in group II and were also given 50 mg/ kg body weight of *Nigella sativa* oil orally twice a week. The *Nigella sativa* oil (Baraka 450 mg capsules, Pharco pharmaceuticals, Alexandria, Egypt) was given orally by injection through plastic syringe with blunt needle into the pharynx.
- **Group V:** The animals were treated as in group III and have been also given the *Nigella sativa*, as in group IV.

All groups of animals have been administrated the doses for one month before pregnancy, and during gestation period till delivery.

2.3 Investigations of pregnancy:

After one month of drug administration, each two females were housed together with one male in a single cage, for breeding purposes. Animals were mated from 5.00 pm. until 9.00 am. at the following day when the males were separated from females. The technique used for determination of pregnancy was based on the vaginal smears, this day was determined the day 0 of gestation. All groups were given the drugs doses as the regimen which has mentioned before for the whole pregnancy time until delivery. Each female was separated in a single cage near to delivery to conserve the offspring. All mice were maintained in a controlled environment and were provided with adequate laboratory diet and water.

2.4. Obtaining of specimens and tissue preparation:

After delivery, pups were collected, counted, and then sacrificed by decapitation using a sharp blade. The heads of each litter were fixed in Bouin's fixative, prepared routinely for paraffin embedding and sectioned serially parallel to the coronal plane for histological and immunohistochemical studies.

2.5. Histological examination

5-6 µm sections were stained with Harris haematoxylin and eosin **(Bancroft *et al.*, 1994).**

2.6. Immunohistochemical examination

For the demonstration of transforming growth factor alpha, tissue sections of 5 µm thick were cut from paraffin blocks and placed on positively charged (opti plus) slides for staining procedures. The avidin-biotin peroxidase complex method (ABC) outlined by **Ramos-Vara (2005)** was used. The transforming growth factor alpha receptors (TGF-α) were used in addition to a polyclonal rabbit anti-human p- catenin. All antibodies were in the form of prediluted antibody (conc. 1-50, Dako), which were ready to be used for the reaction procedures. Control slides were prepared using the same method omitting either primary or secondary antibodies.

3. Results

Group I (control group): Histological observations of different regions of secondary palate of the control group mice showed normal palatogenesis. Both palatal shelves attained a horizontal position, and fused completely with each other and with nasal septum and with the primary palate at the anterior plane(Fig. 1). The fused palatine shelves appeared as an extended continuous structure. No epithelial remnants were observed in the fusion area. The TGF-α in the primary palate of control pups demonstrated a strong staining reaction in the parabasal and superficial layers of the palatine epithelium, while in the underlying mesenchymal tissue cells the reaction was moderately positive (Fig. 2). High expression of TGF-α reaction was observed in the endothelium of the blood capillaries, the proliferating mesenchymal cells and in the ossification centers in the secondary palate.

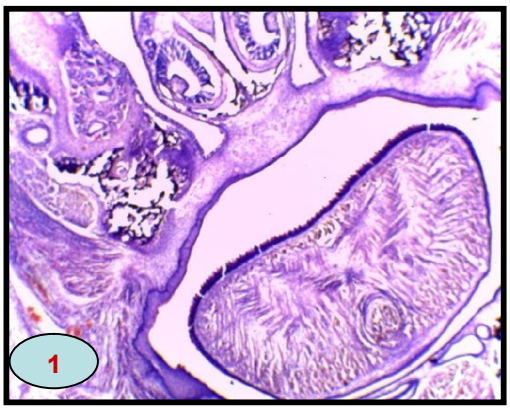
Group II (cadmium treated group): It was found that 30 of cadmium chloride treated pups of total 67 animals displayed cleft palate (Fig. 3),while the remaining 37 animals showed fused palatine shelves (Fig. 4) The percentage of cleft palate in group II was 44.77%. In cadmium treated animals with cleft palate, some of them revealed palatine shelves in vertical position on both sides of the tongue. The tongue was present in a

high position between these palatine shelves with complete communication between the oral and nasal cavities (Fig. 5). Some cases of cadmium treated animals with palatine shelves fusion showed persistent medial epithelial seam (MES) which was not completely degenerated (Fig. 6). Cadmium treated animals revealed negative immune reaction in the palatal epithelium and the underlying mesenchymal tissue cells and in the ossification centers (Fig.7).

Group III (cadmium and vitamin C treated group): In this group the palatine shelves attained a horizontal position, and fused with each other completely and with nasal septum at the primary palate (Fig. 8), and secondary palate. The palatal epithelial lining and the underlying connective tissue stroma appeared normal. Cadmium and vitamin C treated offsprings showed a moderate expression of TGF-α in the superficial palatine epithelium and faint reaction in the underlying connective tissue cells. Moderate staining reaction in the endothelium of the blood capillaries and mesenchymal cells of the ossification centers were observed (Fig. 9).

Group IV (cadmium and *Nigella Sativa* treated group): The histological study of this group showed, completely fused palatine shelves with the primary palate and with the nasal septum at the primary palate (Fig.10), and completely fused palatal shelves with each other and with the nasal septum at the secondary palate.The animals of this group revealed a moderate expression of TGF-α in the palatal epithelium, underlying mesenchymal tissue cells and the ossifying center cells.

Group V (cadmium, vitamin C and *Nigella Sativa* treated group): The animals in this group revealed normal palatogenesis. Both palatal shelves appeared in a horizontal position and fused completely together with the nasal septum at the primary palate(Fig.11), and the palatine shelves fused at the secondary palate**.** Cadmium, vitamin C and Nigella sativa treated pups revealed strong expression of TGF-α in the palatine epithelium, and in the underlying mesenchymal tissue cells. The reaction was highly expressed in the endothelium of the blood capillaries and underlying mesenchymal connective tissue cells and cells of the ossification centers (Fig. 12).



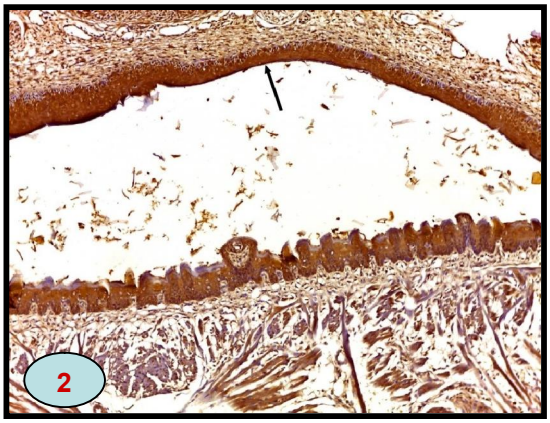
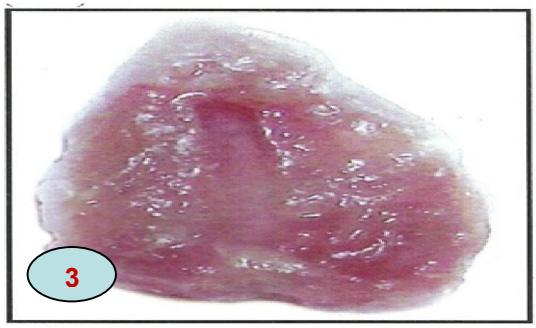


Fig. 1: Photomicrograph of coronal section of control animal, primary palate, showing complete fusion of palatine shelves with the primary palate. H&E X 40.

Fig. 2: Photomicrograph of coronal section of complete palatal shelves fusion in control animal (G. I), showing, strong transforming growth factor alpha expression in the palatine epithelium (arrow), moderate staining reaction in the underlying mesenchymal tissue. TGF-α x 100.



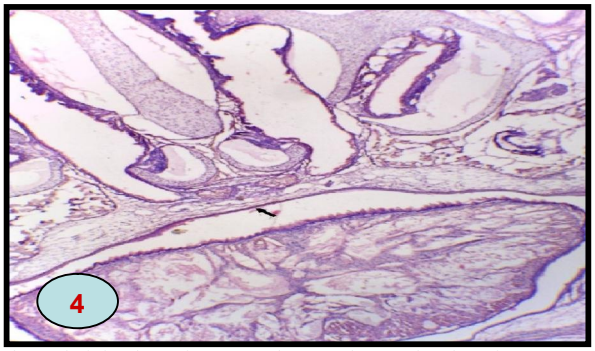


Fig. 3: Gross picture of cadmium treated mice head, showing complete cleft in the primary and secondary palatal region X 10.

Fig. 4: Photomicrograph of coronal section of cadmium treated pup, showing complete palatine fusion with thin epithelial lining (arrows). H&E X 100.

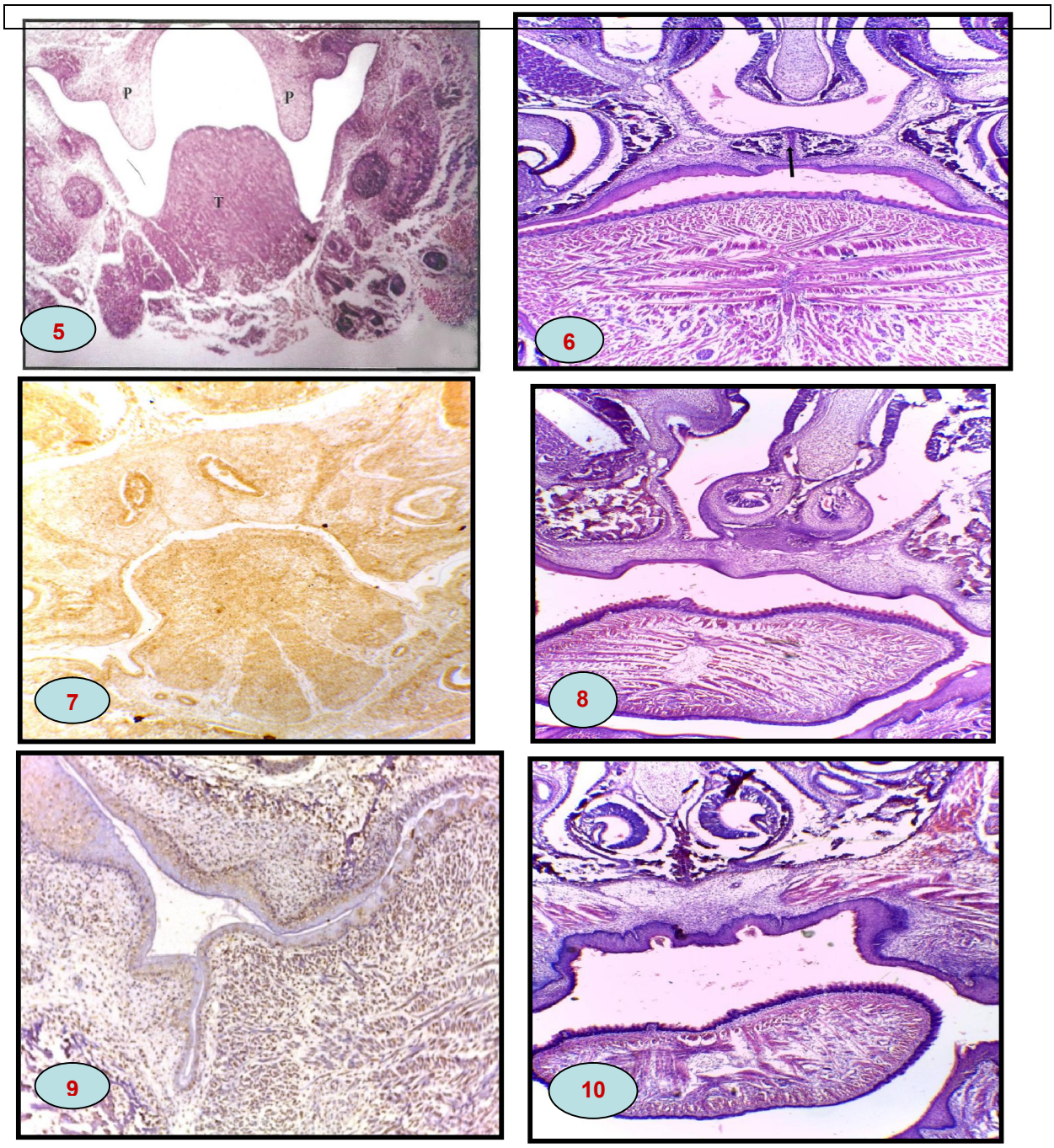


Fig. 5: Photomicrograph of coronal section of G II pup, secondary palatal region, showing cleft palate, vertical palatine shelves (p) beside the tongue (T). H&E X 100.

Fig. 6: Photomicrograph of coronal section of G II pup, showing median epithelial seam (MES) (arrow). H&E X 100.

Fig. 7: Photomicrograph of coronal section in G II mice, showing negative TGF-α Staining reaction in the palatine epithelium, underlying mesenchymal tissue cells and ossification centers (arrows). TGF-α. X 100

Fig. 8: Photomicrograph of coronal section of cadmium and vitamin C treated pup (G III), showing complete palatine shelves fusion with the primary palate. H&E X 100.

Fig. 9: Photomicrograph of coronal section of G III animal, showing moderate TGF alpha staining reaction in the palatine epithelium and underlying mesenchymal tissue cells. TGF-α. X 100

Fig. 10: Photomicrograph of coronal section of cadmium and *Nigella sativa* treated mouse (G IV), primary palatal region, showing complete palatine shelves fusion with the primary palate and with the nasal septum. H&E X 100.

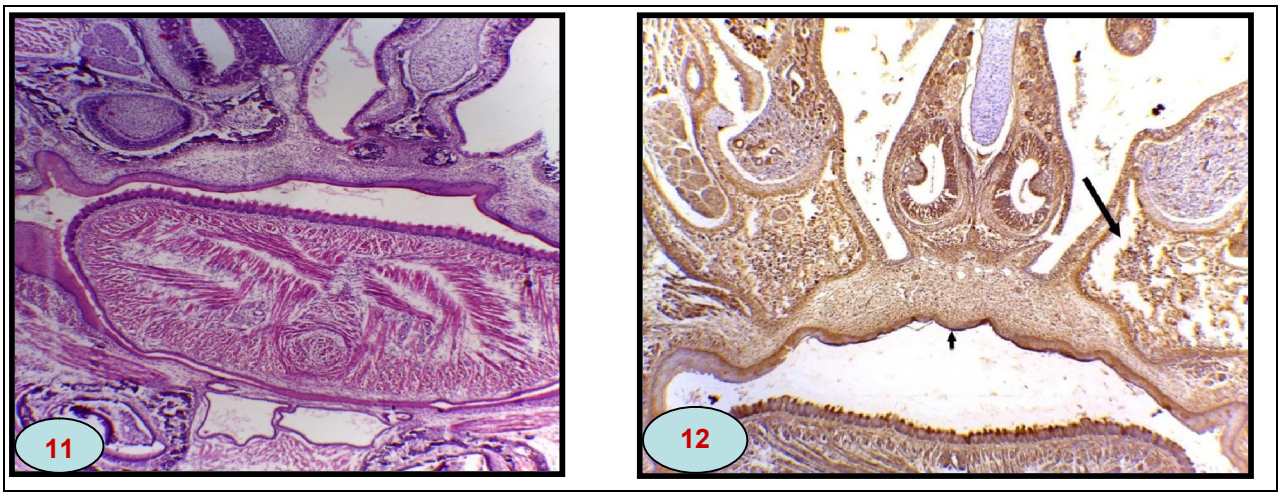


Fig. 11: Photomicrograph of coronal section of G V animal, secondary palatal region, showing complete palatal shelves fusion. H&E X100.

Fig. 12: Photomicrograph of coronal section of G V pup, showing strong stainingTGF-α reaction in the palatine epithelium (small arrow) and underlying mesenchymal tissue cells and ossifying center (big arrow). TGF-α x 100.

4. Discussion

Cadmium is one of teratogenic agents which produce cleft palate in rodents **(Salvatori *et al.*, 2004**). Most teratogenic agents interfere with or modify or inhibit proliferation of palatal shelves mesenchyme, normal shelves growth, lingual movement, palatal shelves elevation and horizontalization and apoptosis of the median edge epithelium, all these factors caused cleft palate formation.The toxicity of cadmium had a cumulative effect**(Kangsen Mai *et al.*, 2006)**. In the current study CD-1 dams injected intramuscularly with 5mg/kg body weight of cadmium chloride twice a week, one month before and during pregnancy until delivery, obtained 44.77% pups with cleft palate. These results revealed that, cadmium at the present dose, period and route of administration obviously, interfere with the normal organogenesis and growth of the palatine shelves, retarded the downward movement of the embryonic tongue in between the palatine shelves, and consequently inhibited midline palatal shelves movement and fusion.These findings were coincidental with the findings reported by other investigators utilized cadmium and rodent models **(Walker and Fraser, 1965; Naya *et al.*, 1997** and**Sultan *et al.*, 2000)**. In the control group pups the palatal shelves appeared normal, fused with other and nasal septum and had normal lining epithelium, no epithelial remnants noticed in the midline region, as same as the finding was reviewed by numerous investigators**Fraser (1971); Fitchett and Hay (1989)** and **Gibbins *et al.* (1999)**.In the remaining groups, III, IV and V which were treated with cadmium, vitamin C and | or *Nigella sativa*, the palatine shelves were normally fused, with normal epithelial lining and without epithelial remnants in the middle region. These findings revealed the protecting effect of vitamin C against Cd cytotoxicity. These results were parallel with the findings stated by many authors **Fox (1983); Pharikal *et al.* (1988)** and **Akhere *et al.* (2008)**.Regarding the *Nigella sativa*, up to available literatures, there was no study investigated the effect of Nigella sativa on the palate of mice exposed to cadmium during embryogenesis, the *Nigella sativa* possessed modulating effect on the embryotoxicity of Cd on the palate of the mice, which is going with the findings of investigations negotiated the protective effect of *Nigella sativa* against cadmium toxicity on rat and mice**(Kantre *et al.*, 2005** and **Massadeh *et al.*, 2007)**.Immunohistochemical observations of transforming growth factor alpha in the current study revealed that, the expression of immune reaction was strong in group I pups (control group) in the lining epithelium and the proliferating mesenchymal tissue cells and the endothelium of the blood capillaries and the ossifying centers. These findings simulate those that showed TGF-α and its receptor was detected in all the human palates regardless of the stage of fusion. They were more highly expressed in the epithelial cells than in the mesenchymal cells of the palatal shelves**(Helene and Domique, 1994)**. In the cadmium treated mice, the reaction was faint to negative, that means the adverse effect of cadmium on the proliferation of the epithelial, endothelial and mesenchymal cells of the developing animals. These results were comparable with investigations which studied the relation between TGF-α and cleft palate formation **(Shiang *et al.*, 1993; Shaw *et al.*, 1996; Bryant *et al.*, 2001; Abbott and Best, 2005** and **Alexander, 2006)**. **Alexander (2006)** concluded that cleft palate was associated with altered expression of TGF alpha.. Biologic support for a role of transforming growth factor arises from its presence at

high levels in the epithelial tissues of the medial edge of the palatal shelves at the time of shelf fusion in mice **(Barrandon and Green, 1987; Shaw *et al.,* 1996** and**Machida *et al.,* 1999)**. Genetic support for the role of transforming growth factor alpha in clefting comes from the close association of TGF alpha alleles with human nonsyndromic cleft palate only and nonsyndromic cleft lip and palate**(Shiang *et al.,* 1993; Machida *et al.,* 1999** and **Alexander, 2006)**. Cadmium and vitamin C treated offspring, TGF-α showed a moderate immune reaction of in the palatine epithelium and moderate staining reaction was showed in the underlying connective tissue cells and endothelial cells of the blood capillaries and the ossifying centers, which revealed the protective effect of vitamin C as an antioxidant against cytotoxic effect of cadmium **(Akhere *et al.,* 2008** and **Sema *et al.,* 2008).**In group IV animals which treated with cadmium and *Nigella sativa* the TGF alpha expressed a moderate reaction in the palatal epithelium, underlying mesenchymal tissue cells and the endothelium of the blood capillaries and the ossification centers, which revealed the repairing effect of *Nigella sativa* on the adverse effect of cadmium on the palate of the mice**(Kantre et al., 2005** and **Massadeh *et al.,* 2007)**.The treated pups with cadmium, vitamin C and *Nigella sativa* showed a strong TGF alpha staining reaction as the same in control group. These result were comparable with the results of the studies that reported the protective effect of vitamin C and *Nigella sativa* against cytotoxicity and embryotoxicity of cadmium on the mice **(Barrandon and Green;1987,Kantre et al,2005;Massadeh et al,2007and Sema et al,2008). Miettinen et al(1999)**established that abnormalities in the gene for transforming growth factor Alpha were linked to cleft palate syndrome. TGF alpha is a growth factor with many known functions, yet how it related to cleft palate was a mystery until now.

Conclusion:

Cadmium chloride prevents palatine shelves fusion and interferes with normal palatogenesis. Furthermore, the drug adversely affected transforming growth factor alpha formation. Cadmium might cause abnormalities in the gene responsible for TGF alpha.Vitamin C has an antagonizing effect of the cytotoxic effect of cadmium, also Nigella sativa have a protective effect against the embryotoxic effect of cadmium, while vitamin C and *Nigella* sativa together had overcome the adverse effect of cadmium on the palate formation of the mice`s offspring.

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