

## Effects of Interferon Alpha 2b (Inf-A2b) on the Development and Growth of Teeth in Mice (Histological and Electron Microscopic Study)

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**Abstract: Background:** Tooth development is a complex process results from epithelial-mesenchymal interaction, cellular proliferation, differentiation and apposition. Interferon is now widely used for the treatment of various malignant diseases, chronic viral hepatitis and have diverse effects as immune stimulation, tumor inhibition, reduced cell multiplication, and may be involved in the events leading directly to defective enamel formation. **Aim:** is to study the effects of interferon alpha 2b on the tooth germ of developing teeth in mice. **Material and method:** Twenty adult female mice and ten adult male mice (CD-1) were divided into two groups; control and experimental group, 10 females and 5 males mice for each group, the control group injected subcutaneously by 0.1 ml of sterile water and the experimental group injected subcutaneously by recombinant human interferon alpha 2b (Rhuifn- $\alpha$ 2b) 10.000 i.u in 0.1 ml sterile water, three times per week, two weeks before breeding and continued until the end of pregnancy, The Neonates from 5 pregnant mice were obtained immediately at birth (subgroup A) and 2 weeks after birth (subgroup B), samples were fixed in 10% neutral buffered formalin for routine H&E stain for histological interpretation and others were kept in gluteraldehyde for the electron microscopic study of the inner enamel epithelium cells. **Results:** by light microscope there were indistinct cell boundaries, degenerated ameloblast, destruction of the underlying basement membrane. Areas of cystic degeneration of the underlying dental papilla, increase intercellular spaces between the cells accompanied with areas of degeneration of the cytoplasm, and by electron microscope there were decreased number of mitochondria, and decreased rough endoplasmic reticulum, pychnotic nucleus showing high signs of degeneration, increased vacuoles within the cells even areas of empty cytoplasm. **Conclusion:** interferon - $\alpha$ 2b has adverse effects and interfere with tooth development and represents a threat that affects the developing tooth and should be avoided at least, during pregnancy.

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### 1. Introduction:

Cytokines include, Lymphokines-secreted by lymphocytes-, monokines- secreted by macrophages-, interleukins- produced mainly by T-cells, mononuclear phagocytes or by all tissue cells- and interferons (IFN-  $\alpha$  , IFN- $\beta$ ) which are produced by cells that have become virally infected, another type (IFN-  $\gamma$ ) is released by certain activated T-cells. Cytokines are important signaling proteins that are liberated during immune challenges and exhibit many modulatory activities (Alhashmi et al., 2000).

The interferons (IFN) are a group of proteins that inhibit viral replication and modulate a variety of immune responses. Jonasch and Haluska , (2001), classified IFNs into type I interferon - which is divided into (IFN- $\alpha$  ) that is secreted by leukocytes , (IFN-  $\beta$  ) that is produced by fibroblasts , (IFN-  $\delta$  ) that is secreted by leukocytes and (IFN-  $\iota$  ) that is secreted by ovine trophoblast. These types stimulate natural killer cells activation. – and type II interferons - which includes (IFN-  $\gamma$  ) that is secreted by T-

cells and natural killer cells - this type stimulates natural killer cells activation less than type I interferons.

Interferons have diverse effects as immune stimulation, tumor inhibition and reduced cell multiplication. Also Interferons improve lacrimation and salivation in S'jogren syndrome. On the other hand, IFN- $\alpha$  has no effect on fibroblast proliferation, while IFN - $\beta$  and IFN - $\gamma$  reduce the collagen synthesis of granulation fibroblast (Ferraccioli et al., 1996; Cornelissen et al., 1999).

Amelogenesis is a complex process which leads to the creation of a fully mineralized layer of enamel on the crowns of teeth. A stratified epithelium, the enamel organ, controls this process, it is widely accepted that cells of the inner most layer of the enamel organ, the ameloblasts, are mainly responsible for producing and secreting various proteins detected within the organic matrix of this hard tissue (reviewed in Boyde, 1989; Deutsch, 1989; Nanci and Smith, 1992). These cells also are responsible for controlling organization of enamel

into characteristic rod and interrod patterns (**Warszawsky et al., 1981**), and they likely influence movement of the large amounts of calcium and phosphate ions required for sustained growth of the unique apatite crystals which develop in this hard tissue (**Bawden, 1989; Takano et al., 1992**).

The mechanism of action of Interferon is explained by **Spitanly and Mave, (1984)**, who stated that binding of Interferon (IFN) to the intact cell membrane is the first step in establishing its effect. Interferon  $\gamma$  appears to have a different specific receptor from either (IFN- $\alpha$  and (IFN- $\beta$ ). As a result of this specificity, Interferon  $\alpha$  has an antiviral activity and induces macrophage tumoricidal activity *in vitro*. After binding, synthesis of a new cellular RNAs and proteins that mediate the biologic effect of Interferons. The correlation between Interferon and the ameloblast cell morphology and function until now is not clear whether interferon affects the ameloblast and enamel formation or not so we perform this research to show the effects of interferon on the tooth germ (ameloblast cells and enamel formation).

## 2. Materials and Methods

Twenty adult female mice and ten adult male mice (CD-1) of average weight 100-150 gm were used in this experiment. The animals were divided into two groups; control and experimental, 10 females and 5 males mice for each group, housed in separate cages and maintained on normal laboratory diet and water *ad libitum*.

### Group I: control group:

The 10 mature females that are ready for fertilization were injected subcutaneously by 0.1 ml of sterile water three times per week, two weeks before breeding and the injection continued until the end of pregnancy.

### Group II: Experimental group

The 10 mature females that are ready for fertilization were injected subcutaneously by recombinant human interferon alpha 2b (Rhuifn- $\alpha$ 2b, ABO Switzerland Co., Ltd) 10.000 i.u in 0.1 ml sterile water three times per week, two weeks before pregnancy and the injection continued until the end of pregnancy (**Denkers, 1999**).

For breeding purpose, each two mature injected females were housed together with one male in a separate cage and mated from 5:00 pm until 9:00am. Then pregnancy was inspected according to the examination of the vaginal plug. This day was determined to be day of pregnancy. After pregnancy, the control animals (10 pregnant females) and

experimental animals (10 pregnant females) were divided into two subgroups, five animals each:

Subgroup (A) (The Neonates) from 5 pregnant mice were obtained immediately at birth.

Subgroup (B) (The Off springs) were obtained two weeks after birth.

The heads of the control and experimental groups were obtained, fixed in 10% neutral buffered formalin for routine H&E stain for histological interpretation and others were kept in glutaraldehyde for the electron microscopical study.

### (I) Histological study : (Luna, 1968):

The sections were deparaffinized in xylene, 2 minutes each, to ensure complete removal of xylene from the sections. They were then dehydrated by passing the slide through descending grades of alcohol; 90%, 70%, and 50% for 2 minutes in each change, and finally, through distilled water. The serial sections specimens were then stained with the routine Hematoxyline and eosin stain.

### (II) Tissue preparation for transmission electron microscope (Sternberger, 1974):

Tissue preparation for ultrastructural study started in animal house as soon as the specimen was taken and were immediately fixed in 3% glutaraldehyde for 2 hour and buffered with 0.1 sodium phosphate at pH 7.4 for 2 hour at 4<sup>0</sup>C (2 rinses were used), post fixation in freshly prepared buffered 1% osmium tetroxide in veronal acetate buffer for 1-2 hours at 4C<sup>0</sup>, then washed twice in sodium phosphate buffer. Three changes each for 10 minutes were used to remove the excess of osmium tetroxide, dehydrating the specimens in ascending grades of ethyl alcohol, clearing were done by using undiluted propylene oxide as transitional solvent and infiltration with Araldite. First propylene oxide is applied in 2 changes, 20 minutes each. Araldite epoxy resin was used as an embedding material. The tissues were then embedded in prelabelled plastic capsules, and kept to polymerize at 60C<sup>0</sup> in an incubator for 48 hours.

The polymerized block was trimmed under a dissecting microscope into a smooth pyramid, by a sharp razor using LKB microtome for removal of excess embedding medium for the specimen surfaces.

Sectioning into ultra thin sections (900-1500A<sup>0</sup>) were carried out using LKB ultra microtome by using freshly prepared glass knives. The ultra thin sections were mounted on copper grids and were kept in a Petri dish. The grids were double stained with uranyl acetate and lead citrate. Lead citrate appears to give higher contrast level, while uranium gives a finer grain and thus better resolution. The stained sections on the copper grids were placed in the grid

holder 2x2 of a JEOL, JEM-100cx electron microscope and were examined at 60 Kv in the electron microscope unit of the high institute of public health, Alexandria University.

### 3. Results

#### Histological Results

Tooth germs of the control group at one day postnatal animals were found to be in the late bell stage of odontogenesis with predentin formation in all specimens. Tooth germs of the control group animals composed of well developed outer enamel epithelium, stellate reticulum, stratum intermedium, inner enamel epithelium and odontoblastic layer arranged in palisading pattern with predentin deposition. The dental papilla and bony crypt were normally developed (Fig.1).

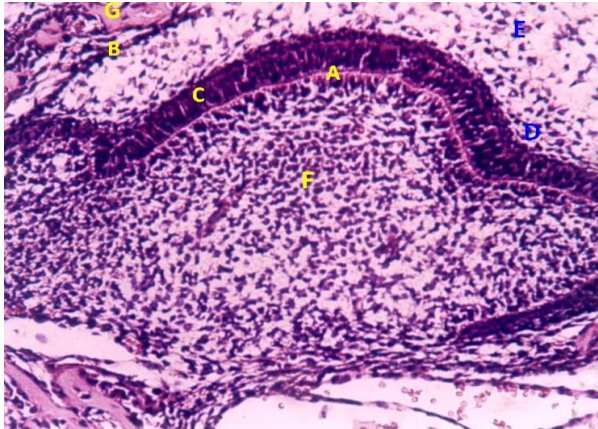


Fig . (1): Dentin matrix formation (A), outer enamel epithelium (B), inner enamel epithelium (C), stratum intermedium (D) and stellate reticulum (E). Note also the dental papilla (F) and the bony crypt (G) (H& E Stain, X 200).

Tooth germs of the experimental animals at one day postnatal were found to be in the early bell stage of odontogenesis. The enamel organs appeared poorly organized with less differentiated cell layers. Dental papillae were affected by cystic degeneration as well as the dental sacs were poorly developed. The cystic degeneration of dental papillae and the absence of odontoblastic layers in all specimens of experimental animals, were the characteristic features of this group (Fig.2).

The histological examination of the control animals 2 weeks after birth revealed, normal thickness and formation of the enamel, dentin and predentin layers. The odontoblastic layer appeared in normal palisading pattern with the presence of cell free – zone and normal architecture of the pulp tissue. Normal development of epithelial root sheath of

Hertwig's, epithelial diaphragm, ectomesenchymal tissue and bone trabeculae or bony crypt (Fig.3).

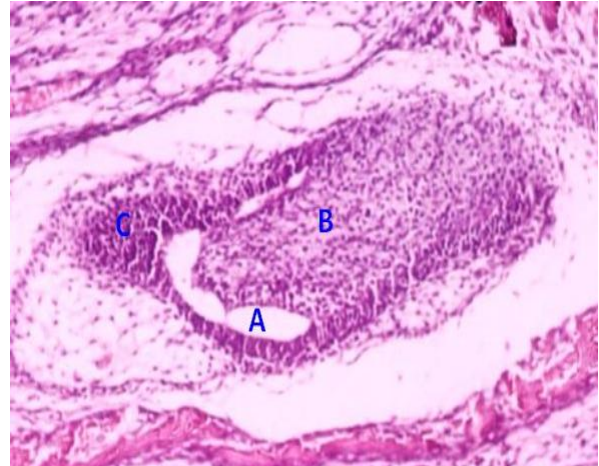


Fig . (2): The first molar tooth germ in experimental group showing less developed tooth germ with the absence of odontoblastic layer (A) Note also the dental papilla (B) with the proliferative inner enamel epithelium and stratum intermedium (C) (H& E Stain, X 200) .

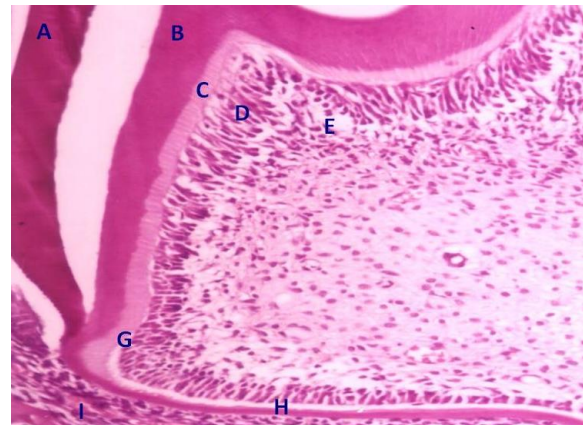


Fig . (3): Photomicrograph of tooth development of control group with higher magnification of the previous figure showing enamel matrix(A), dentin (B), predentin(C), odontoblastic layer(D), cell free-zone(E), pulp tissue(F). Note epithelial root sheath of Hertwig's(G), epithelial diaphragm (H) and surrounding ectomesenchymal tissue (I) (H& E StainX 200).

The histological examination of tooth development in experimental animals at 2 weeks postnatal revealed the following findings. The most characteristic feature is the disturbance in the pulp tissue, there were cystic degeneration, indistinct or absence of odontoblastic layer with the presence of some inflammatory cells and in some specimens complete absence of the pulp tissue. In regard to the hard dental tissue formation, there were small amount



of enamel matrix, uneven thickness of dentin and predentin layers. Also, there were defect or absence of epithelial root sheath of Hertwig's and epithelial diaphragm in all examined sections of experimental animals (Fig.4).

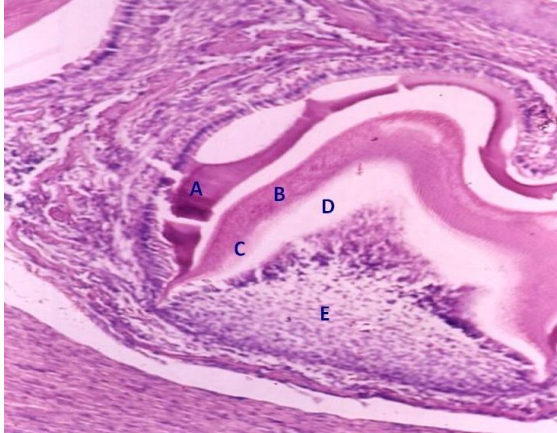


Fig.(4): Photomicrograph of first molar odontogenesis of experimental animals showing, enamel matrix covered by degenerated ameloblasts (A) abnormal thickness of dentin (B), thin layer of predentin (C), absence of odontoblastic layer (D), and the remaining pulp tissue appeared abnormal (E) in shape with the presence of some inflammatory cells. Note incomplete formation of epithelial diaphragm (H&E Stain, X 100).

#### Electron Microscopic Results:

Cells of the inner enamel epithelium are low columnar with centrally placed nucleus, Golgi apparatus and centrioles located at the proximal end while mitochondria and ribosomes are scattered all over cytoplasm. Cells of IEE interact and arrange themselves determining the shape of DEJ and the crown shape. IEE is separated from CT by basement membrane and cell free zone which contain fine argyrophilic fiber and cytoplasmic processes. There are increases in number of cell organelles in the control group, subgroup A (at birth). while in subgroup B (2 weeks after birth) the inner enamel epithelium of this group after the first layer of dentine is formed will be induced to be converted into ameloblast of tall columnar secretory cells forming the enamel and have increase in the number of mitochondria, rough endoplasmic reticulum and cell organelles (Fig. 5, 6).

In group II: Experimental group, The inner enamel epithelium showed decrease in the nuclear size, increase intercellular spaces between the cells accompanied with areas of degeneration of the cytoplasm, decreased number of mitochondria,

decreased rough endoplasmic reticulum in group II subgroup A (at birth) Fig. (7). While in subgroup B (2 weeks after birth) the inner enamel epithelium of this group showed more pyknotic nucleus with signs of degeneration, increased intercellular spaces between the cells more than subgroup (A), increased vacuoles within the cells even areas of empty cytoplasm can be seen, more decreased number of mitochondria, decreased and dilated rough endoplasmic reticulum (Fig. 8).

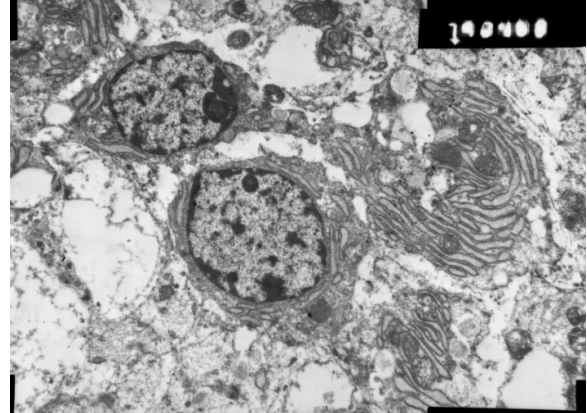


Fig. (5): Electron micrograph of the cells of the inner enamel epithelium of the control group showed increase in number of cell organelles. (X3000).

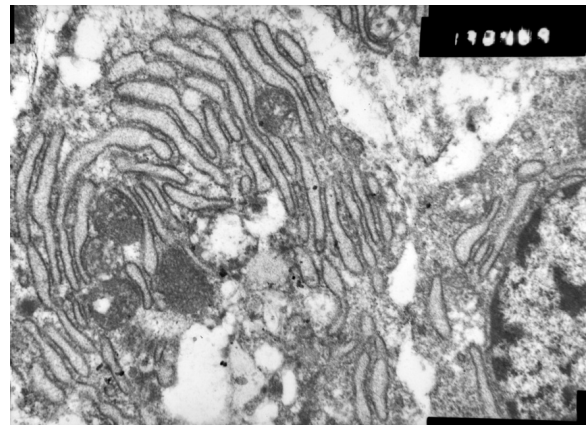


Fig. (6): Higher magnification of the previous electron micrograph of the cells of the inner enamel epithelium of the control group showed the increase in number of cell organelles. (X7500).

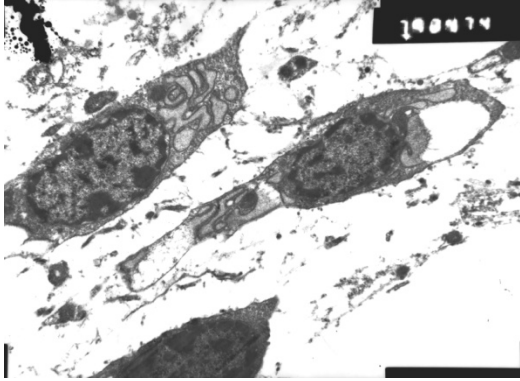


Fig. (7): Electron micrograph of the cells of the inner enamel epithelium of the experimental group subgroup (A) showed decrease in the nuclear size, increase intercellular spaces between the cells accompanied with areas of degeneration of the cytoplasm, decreased number of mitochondria, decreased and dilated rough endoplasmic reticulum. (X3000).

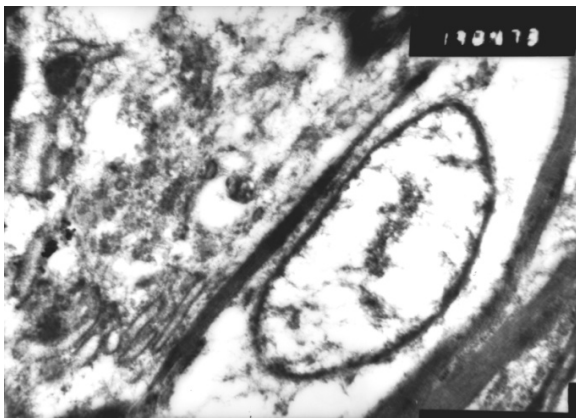


Fig. (8): Electron micrograph of the cells of the inner enamel epithelium of the experimental group subgroup (B) showed marked decreased RER, increased vacuolation with very little cell organelles, and pyknotic nucleus showing high signs of degeneration. (X7500).

#### 4. Discussion

The antiviral activity of IFNs and their biological effects, had been carried out, there is still lack of reports on the deleterious effects of IFN- $\alpha$  2b on the oral tissues. Our results demonstrated that interferon  $\alpha$ -2b most definitely interfered with normal odontogenesis of tooth germs. Although it is not possible to suggest the exact mechanism of action of interferon based on the present histological findings, it is obvious that the present dose of interferon may interfere with cytodifferentiation of the inner enamel epithelium and subsequently inhibited normal amelogenesis (Cohn, 1957; Khan *et al.*, 1979; Saad, 1987; Ali, 1988).

Since the epithelial-mesenchymal inductive interaction is important to occur permitting normal development, as demonstrated by some investigators<sup>(19)</sup> and because interferon appeared to interfere with such normal development, retardation of odontogenesis resulted. Such retardation in development was obvious in experimental fetuses at birth as well as at two weeks after birth.

Several investigations have been performed to explain its mechanism on embryonic development and differential growth. Such studies believed the mechanism to be related to cell injuries that might lead to interfering with cell division and cell death will be the end result (Goldfeder 1963; Storer 1966). The cellular injury has been postulated to be due to a number of possible factors, among which are: Toxic effect of protein breakdown products, inactivation of enzyme system, coagulation or flocculation of protoplasmic colloids and denaturation of nucleus proteins. Moreover Lengyel, (1982), reported that Interferons exert widely overlapping pleiotropic effects including inhibition of cell growth and modulation of certain immune reactions.

The revealed degenerative changes in the present study in the form of cystic degeneration of dental papilla, disturbance or absence of odontoblastic layer, defect or absence of both epithelial root sheath of Hertwig's and epithelial diaphragm as well as in some specimens complete absence of dental pulp were in accordance with the observation reported by Selzer *et al.*, (1994).

The cause of these degenerative changes was explained by Kamachi *et al.*, (2002), who were working on human salivary gland (HSG), they found that IFN- $\gamma$  produces apoptosis in the HSG cells. This apoptosis was assessed by DNA fragmentation and disruption of mitochondrial transmembrane potential. They also cleared that DNA fragmentation and apoptosis were determined in HSG cells cultured with IFN- $\gamma$ , and mitochondrial dysfunction also appeared to be involved in this process because disruption of mitochondrial transmembrane potential was reported.

Mahamed *et al.*, (2005), reported that activated T cell produce predominantly Th1 cytokines such as interferon  $\gamma$  and TNF- $\alpha$ , which can exacerbate immunity mediated alveolar bone loss. Moreover Alayan *et al.*, (2007), added that both Th1 and Th2 cytokines play an important role in maintaining alveolar bone homeostasis. The kinetics of alveolar bone loss seen in cytokine gene knockout mice indicates that bone loss is age dependent and late in onset.

The biologic activities also include inhibition of cell proliferation, enhancement of the cytotoxic

activities of lymphocytes, the expression of cell surface antigens and stimulation of the phagocytic and tumoricidal activities of macrophages. These properties may play an important role in vivo antiviral and antitumor effects of IFNs (Cornelissen et al., 1999).

However, Interferons have diverse effects as immune stimulation, tumor inhibition and reduced cell multiplication. Also Interferons improve lacrimation and salivation in Sjogren syndrome (Nanci & Smith 1992). Otsuji et al., 1992, investigated the localization of interferon gamma and granulocyte colony – stimulin factor receptors during early amelogenesis in rat molars. In the newborn rats, interferon gamma –receptor was localized in the preameloblasts, in the cervical loop, in the proximal and distal ends of presecretory ameloblasts, the outer enamel epithelium, the dental lamina, and in bone. In 5 day – old rats, it was confined to the proximal ends of the presecretory and secretory ameloblasts. These findings indicate that the interferon gamma receptor is upregulated in the cells of the enamel organ and may be involved in the events leading directly to defective enamel formation.

Musch et al., 2005, their clinical findings suggest that IFN  $\alpha$  could interfere with the physiological barrier function of endothelial and possibly also epithelial cells and this is agreed with our ultramicroscopic results in the increase in the intercellular spaces between the cells in the experimental groups.

Lin et al., 2008, investigated that Interferon  $\alpha$  has direct effects on LLC-PK<sub>1</sub> cells, affecting parameters such as morphology, viability, and transport, including dome formation and sodium-dependent glucose transport. DNA synthesis was decreased, and IFN $\alpha$  caused a blockade in the G2/M phase of the cell cycle in proliferating, non differentiated cells and this is agreed with our results in degeneration of the nuclei in the experimental groups.

From the obtained results of the current study, it is evident that the interferon -  $\alpha$  2b therapy is a matter of double edged weapons. Its deleterious effect should not be neglected and a careful follow up should be undertaken especially in patients with prolonged treatment.

In general, interferon -  $\alpha$  2b has adverse effects on tooth development and represents a threat that affects the welfare of developing fetus and should be avoided at least, during pregnancy.

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