Immunohistochemical expression of Ki- 67 in gingival tissues of Albino rats during pregnancy

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Abstract: Background: Different conditions of the oral cavity have been developed in association with pregnancy such as gingivitis and periodontitis. These conditions are mainly related to the elevated sex hormones, in addition to the presence of predisposing factors of the oral cavity such as plaque, calculus and bacteria. **Aim:** To investigate the alterations of the gingival tissue and the difference in the expression of Ki-67 between pregnant and non-pregnant rats as an indication of cell proliferation. **Material and Methods**: Rats were divided into two groups, the control (non-pregnant) and pregnancy groups. They were sacrificed at day 19 of gestation (late pregnancy) and the buccal gingival tissue at the mandibular molar regions was carefully dissected and processed for histological and immunohistochemical evaluation of Ki-67. ***Results:*** The gingival tissue of pregnant rats showed marked increase in thickness, elongation of epithelial ridges, and proliferation of cells of the basal layer. The stratum spinosum cells presented lightly stained cytoplasm, signs of hydropic degeneration and had pyknotic nuclei. Binucleated cells were present in the stratum spinosum and stratum granulosum. The blood vessels of the lamina propria were markedly dilated with extravasated RBCs. The number of Ki-67 positive cells were significantly increased in pregnancy group compared to the control. ***Conclusion***: The elevated sex hormones during pregnancy has an obvious impact on the gingival epithelium and increase the proliferative activity of epithelial cells even in absence of other initiating factors such as plaque and calculus.

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1. Introduction:

Pregnancy has been associated with various physiologic and psychological changes of the body that may also affect healthy women. Different conditions of the oral cavity have been developed in association with pregnancy such as gingivitis, periodontitis, xerostomia, enamel erosion or tooth mobility (1,2). Estrogen and progesterone are the main sex hormones that rise during pregnancy. The increased levels of these hormonesin blood and salivaresult in an increased gingival and periodontal disorders with concomitant increase in gingival exudates (3). Studies have also reported that increased sex hormones causes alteration of saliva composition and gingival immune response, changes of local blood supply and facilitation of proliferation of microorganisms (4).

It has been confirmed that estrogen and progesterone receptors are present in the gingiva, therefore the gingiva is a target organ for these hormones (5,6). Estrogen receptors have also been detected in PDL fibroblasts, periosteal fibroblasts, fibroblasts of the lamina propria and osteoblasts (7). Clinically, pregnancy gingivitis has been reported in 30-100% of all pregnant women(8), and the condition ranges from mild edemato sever hyperplasia, edema, erythema, pain and bleeding(9). Habashneh et al. showed that the incidence of gingival bleeding and inflammation in pregnant women is more than in the general population and that these effects are associated with microbial flora and the changes of hormonal levels(10).

Ki-67 is an important antigen specific antibody used as an indicative of increased cellular mitotic activity and proliferation in healthy and diseased tissues. It is a non-histone nuclear protein that is detected in proliferating cells throughout all G1, S, G2 and M phases of the cell cycle, while absent in inactive cells(11). Ki- 67 antibodies allow for immunohistochemical determination of tissue growth fractions. Increased expression of Ki-67 has been found in various inflammatory conditions such as rheumatoid arthritis (12), pancreatitis(13), atherosclerosis(14), diabetes (15)and chronic periodontitis(16).

Although the gingival epithelium undergoes remarkable changes as a result of increased levels of estrogen and progesterone during pregnancy (17), the consequent extension of gingival and periodontal changes is still controversial. Also pregnant women are prone to gingival overgrowth, even though the exact mechanism of the effect of sex hormones on the gingival tissues is unclear. Animal models of pregnancy are ideal for investigating the hormonal effects of pregnancy. Therefore, the aim of the current work is to investigate the alterations of the gingival

tissue and the difference in the expression of Ki-67 between pregnant and non-pregnant rats as an indication of cell proliferation.

2. Material and Methods:

Animals and Experimental design:

Twenty adult female albino rats (200-250g) were used in this study. The rats were kept under controlled temperature and lighting conditions. Animals were fed standard laboratory diet and tap water for at least one week acclimatization period prior to experiment. At the start of the experiment, rats were divided equally into two groups. The control group were housed in a separate cage until the end of the experimental period. Animals of the pregnancy group were housed with male rats and vaginal smears were recorded daily in the morning. Successful mating was determined according to the presence of vaginal plug or sperms in the vaginal smear. This is considered as day one of pregnancy. Since the gestation period in rats is 21 days, pregnant rats were sacrificed at day 19 of gestation (late pregnancy). The research protocol was reviewed and approved by the Research Ethics Committee, Faculty of Dentistry, Tanta University.

Histological and Histochemical investigations:

After anesthesia, the buccal gingival tissue at the mandibular molar regions was carefully dissected and immediately fixed in 10% neutral buffer formol for histological techniques. After histological processing techniques, specimens were embedded in paraffin blocks, stained with hematoxylin and eosin stain, and examined by light microscope. For immunohistochemical staining, sections were prepared in silanized slides by the Avidin Biotin Peroxidase method. Ki-67 antibodies were used at a dilution of 1:50 in buffer specific for the primary antibody. Slides were examined under light microscope at a magnification of x400 (Olympus, Japan), and the evaluation was carried out by two observers to ensure objectivity. Positive Ki- 67 expression was identified as brown staining of cell nuclei. Five random sections for each animal were examined and digitalized under a magnification of x400. Computerized image analysis software was used to count the number of positive Ki-67 cells regardless of the intensity of the staining. Statistical analysis was done using SPSS software (23 Version). Data was expresses as mean value ± SD. Unpaired t-test was used to compare the mean number of Ki-67 positive cells between both groups, P value < 0.05 to control for alpha error.

3. Results

Histological results:

Examination of H & E stained sections from the gingival mucosa of non-pregnant rats (group I) showed the masticatory mucosa of the gingiva formed of stratified squamous ortho-keratinized and lamina propria. The laminapropria consisted of a papillary layer formed of loose vascular connective tissue and a reticular layer formed of denser connective tissue. Gingival epithelium interdigitated with connective tissue forming interconnected rete ridges that were seen separated byconnective tissue papillae (Figure 1A). The gingival epithelium was formed of stratum basale which appeared as a layer of small cuboidalcells resting on the basement membrane, and their nuclei were closely packed. Stratum spinosum appeared as several layers of polygonal cells, with central rounded vesicular nuclei. Stratum superficiale was seen as flattened superficial cells with flattened nuclei and finally the outer most layer was the stratum corium (Figure 1B). In group II (pregnant rats), an apparently increased thickness of the epithelium and elongation of epithelial ridges were noticed in some areas (Figure 2A). Proliferation of cells of basal layer was present. Several layers of the stratum spinosum cells presented signs of hydropic degeneration and had pyknotic nuclei (Figure 2A). Stratum spinosum cells presented lightly stained cytoplasm. Also, nuclear changes of stratum spinosum cell layers were present such as binucleation. The papillary layer showed inflammatory cell infiltrate (Figure 2B). In other areas of the gingival epithelium binucleated cells are detected in stratum spinosum and stratum granulosum cell layers (Figure 3A). The blood vessels of the lamina propria were markedly dilated with extravasated RBCs (Figure 3B).

Immunohistochemical results

In the healthy control gingiva of group I, Ki-67 positive cells were observed only in the basal and parabasal layers of epithelium (Figure 4), while the gingival samples from pregnant rats of group II showed widely distributed Ki-67 positive cells throughout all layers of gingival epithelium (Figure 5). The number of positive cells were calculated and unpaired t-test was used to compare the difference in Ki-67 expression of the epithelium of both groups (Figure 6). The mean value of Ki-67 positive cells in the epithelium of non-pregnant rats= 99± 14 (n=50), while the mean value of Ki-67 positive cells in the epithelium among pregnant rats= 188±22 (n=50). Unpaired t-test value = 24.133, the two-tailed P value is less than 0.0001. By conventional criteria, this difference is considered to be extremely statistically significant.

4. Discussion:

Previous studies have confirmed the impact of pregnancy on the gingival tissues even in healthy individuals (18). The elevated levels of sex steroid hormones that occur in late stage of pregnancy has an

effect on the physiology of gingival epithelium inducing cellular proliferation. Animal models have been used to study the periodontal conditions and rats have been used in several experimental studies as an animal model to study the gingiva and periodontium.

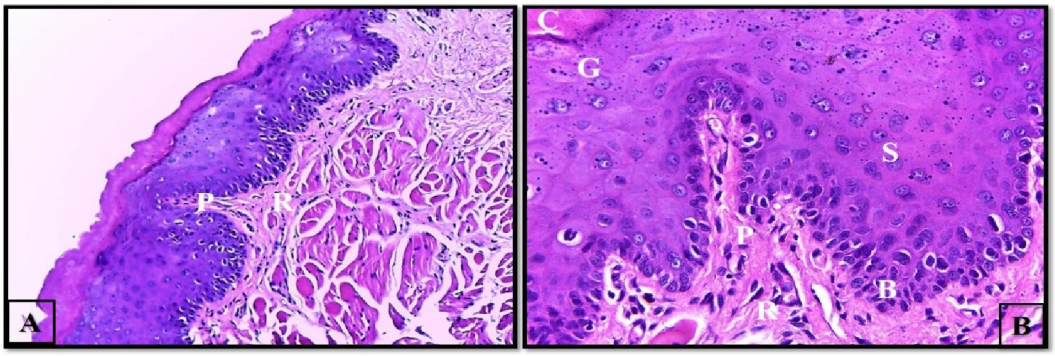

Figure (1): Photomicrograph of the group I (control group) showing; A: The gingival mucosa is formed of stratified squamous keratinized epithelium. The lamina propria consists of papillary (P) and reticular (R) layers. Connective tissue papillae interdigitate with epithelial ridges. B: The gingival epithelium is formed of four distinct layers, the stratum basale (B), Stratum spinosum (S), Stratum granulosum (G) and Cornified layer (C). Notice many inflammatory cells in the lamina propria. (H & E staining. Mic. Mag. A:10x, B 40x)

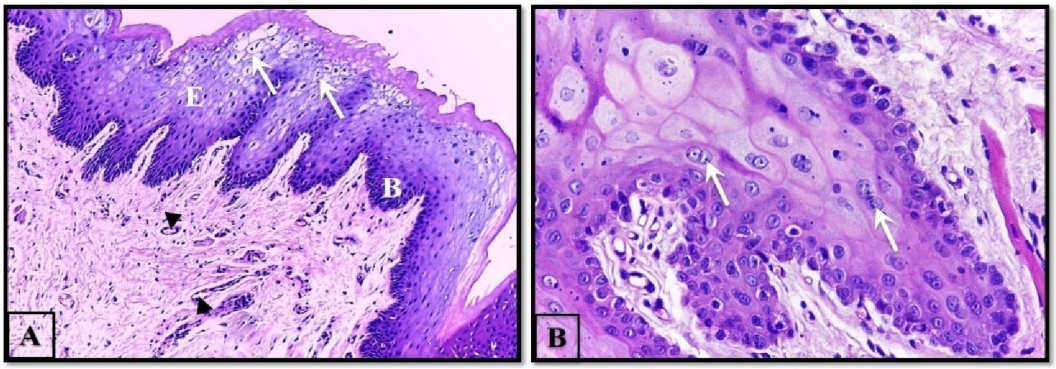

Figure (2): Photomicrograph of group II (pregnancy group) showing; A: Area of apparent increased thickness of the epithelium (E), elongation of epithelial ridges and increased fibrous tissue and blood vessels in the lamina propria (arrow heads). Area of proliferation of basal layer (B) is noticed. Several layers of the stratum spinosum cells have signs of hydropic degeneration and pyknotic nuclei (arrows). B: Cells of the stratum spinosum appeared lightly stained and numerous binucleated cells are evident (arrows). (H & E staining. Mic. Mag. A 10x, B 40x)

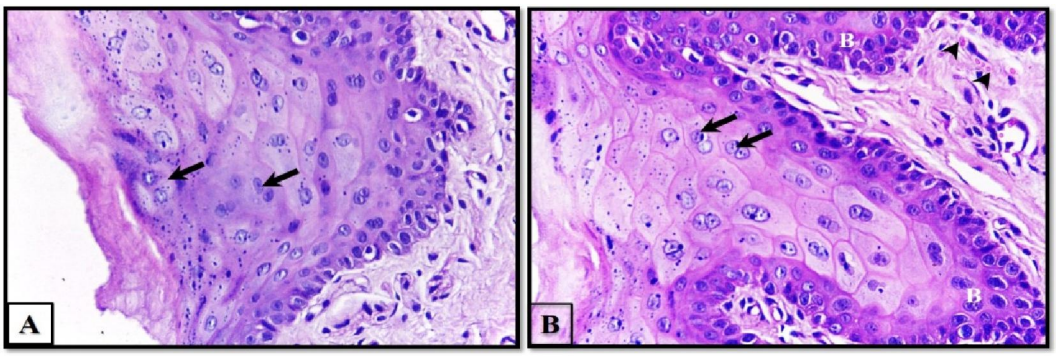

Figure (3): Photomicrograph of group II (pregnancy group) showing; A: Binucleated cells of the stratum spinosum and stratum granulosum (arrow). B: Areas of proliferation of the stratum basale is evident (B). Notice, the dilated blood vessels of the papillary layer with extravasated RBCs (arrow head). (H & E staining. Mic. Mag. A & B 40x)

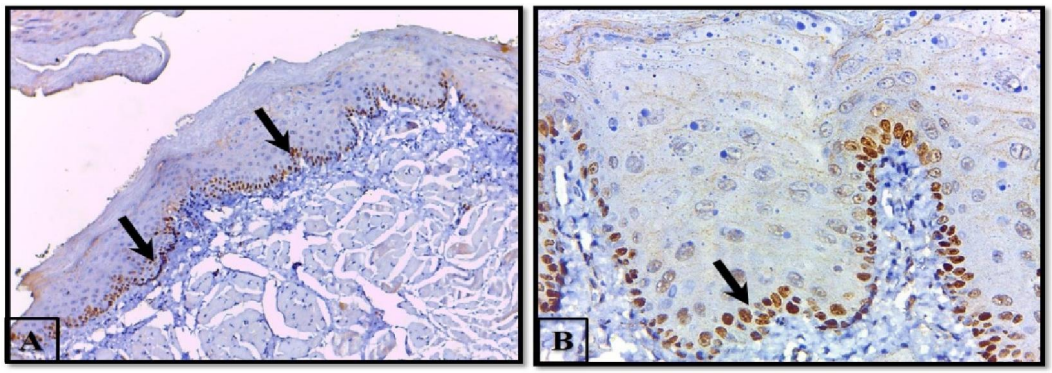

Figure (4): Immunohistochemical staining for Ki-67 of group I (control group) showing Ki-67 nuclear expression evident in basal, parabasal layers (black arrows). (Immunohistochemical staining. Mic. Mag. A 10x, B 40x)

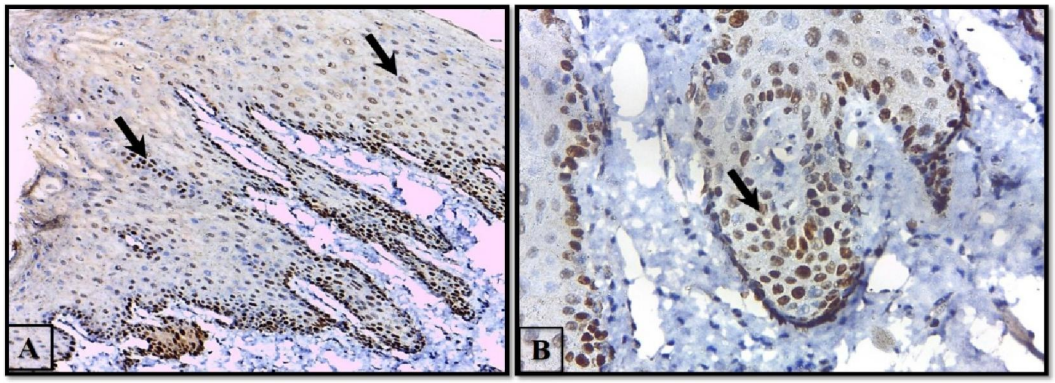

Figure (5): Immunohistochemical staining for Ki-67 of the group II (pregnancy group) showing Ki-67 nuclear expression is evident in basal, parabasal and stratum spinosum layers (arrows). (Immunohistochemical staining. Mic. Mag. A 10x, B 40x)

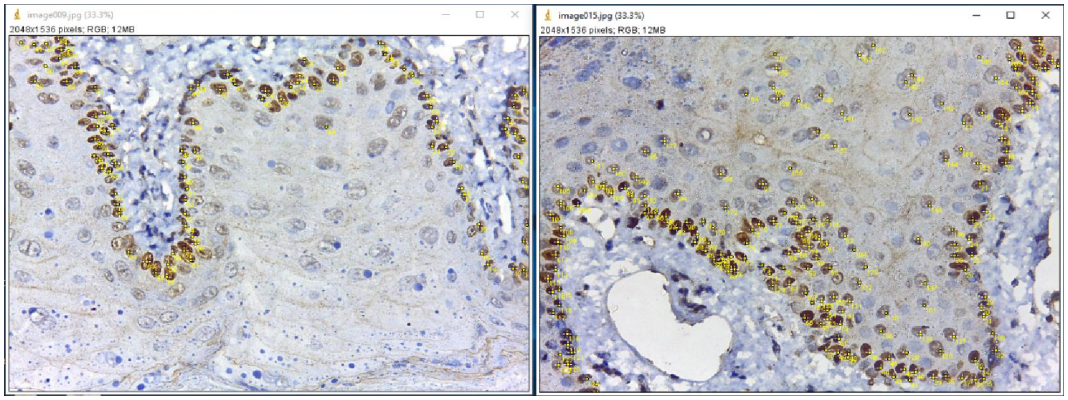

Figure (6): Digital images of Immunohistochemical staining for Ki-67 of both groups (A: control group; B pregnancy group) under a magnification 40x showing counting of Ki-67 positive cells in both sections.

This is because the anatomy of the periodontium at the molar region in rats share many similarities with humans. In addition, rats as experimental animal model are easy to care and handle(19). In spite of this, it is uncertain that the periodontal findings in rats can be applied totally on human periodontal abnormalities because the rodents have distinct oral microbiome that are quite different from those in humans (19). Accordingly, in our study, by excluding the predisposing factors of gingival alterations that might potentiates the effect of pregnancy on gingiva, it can be claimed that our findings are the sole result of

hormonal changes of the gingival tissues associated with pregnancy.

In this study, the gingival epithelium in pregnant rats was characterized by marked increase in thickness compared to non-pregnant rats. Also, proliferation of the basal cell layer was noticed in pregnant group. These observations matched the results of Fahey et al. who confirmed that sex steroid hormones can induce proliferation of epithelial cells in different tissues such as the mammary glands (20). The proliferation of the basal cells might be considered as protective response of the epithelium to the weak epithelial barrier that resulted from the effect of estrogen on the epithelium leading to cellular proliferation. Estrogen has a well-known regulatory effect on cellular proliferation, differentiation and keratinization in target tissues including keratinocytes and gingival fibroblasts (21,22) and estrogen and progesterone receptors have been located in the gingiva. Estrogen receptor has been classified into alpha and beta receptors that belong to a superfamily of nuclear receptors. They function as transcriptional factors or associated with other transcriptional factors that regulate the expression of many genes. Also, in addition to the nuclear receptors, a third estrogen receptor, GPR30, is located in the plasma membrane and endoplasmic reticulum and is associated mainly with non-genomic responses (23). In addition estrogen has an important role in the regulation and modulation of epithelial permeability through the regulation of epithelial tight junction resistance (24). It also decreased the capillary resistance and enhance the blood flow. It was reported that progesterone has a low metabolism in the gingival tissue during pregnancy suggesting active functions of this hormone (25). Also the elevated levels of progesterone could down regulate the production of IL-6, thus making the gingiva less resistant to the inflammatory mediators produced by the bacteria (26). Progesterone has also been confirmed to affect the collagen production through decreasing glycosaminoglycan production, which, in addition to collagen are principle components of the gingival matrix (27). However, it is worth mentioning that the hormonal changes that occurred during pregnancy significantly potentiate the response of gingival tissue to local factors leading to the hyperplastic response but doesn't alter healthy gingiva (28). These facts could represent an explanation for the observed thickening of the gingival epithelium in pregnant rats.

In the current study, it was observed that the epithelial ridges in the epithelium of pregnant rats were markedly elongated. This elongation of epithelial ridges was suggested to be directly related to the increased number of basal cells that can undergo mitosis (29). Additionally, an obvious feature of the cells of the stratum spinosum of pregnant rats in this study is the increased number of binucleated cells as well as the cells with signs of hydropic generation, pyknotic nuclei and lightly stained cytoplasm. A report published by D"Agostini et al. has confirmed that periodontitis has been associated with gingival cells with micronuclei and binucleated cells (30). Examination of the lamina propria in pregnant rats showed that the blood vessels of the lamina propria were increased and has an eosinophilic substance. In agreement with this finding, it was concluded that steroid hormones might have a specific role on gingival vasculature, and the main cause of pregnancy related gingival changes was suggested to be the associated increase in vascular flow of the gingival tissue(31). Progesterone was also able to increase the permeability of blood capillaries and venules through the formation of gaps in the normally intact endothelial lining(32). In addition, Cetinkaya et al. suggested that the increase number of blood vessels in the gingival tissue was due to upregulation in the number of macrophage phenotypes expressing growth factors(33). Estrogen and progesterone have been reported to affect the gingival vasculature, and this effect might explain the associated increased edema, erythema, hemorrhage and gingival crevicular exudate that occurred during pregnancy and other stages of the reproductive cycle(28).

To assess the proliferative activity of the epithelial cells, Ki-67 immuoexpression was evaluated. The immunohistochemical localization of Ki-67 in this study showed that Ki-67 positive cells of the control group were seen in all specimens mainly in the basal and parabasal layers of the gingival epithelium in agreement with other studies (34). Ki-67 is one of the best markers that can be used to assess the proliferative rate of the cells. Control gingival tissue is characterized by normal rate of cell turnover as compared to other tissues, and the physiologic proliferative activity are located at the basal and parabasal cells. In the pregnancy group, Ki-67 positive cells were detected not only in basal and parabasal cells but also in stratum spinosum and granulosum throughout all epithelial cell layers. The increased expression of Ki-67 positive cells in all layers of epithelium in pregnant rats indicated increased proliferating activity of all epithelial cells which could be attributed to the significant effect of sex hormones on cellular proliferation.

In conclusion, the current study showed that elevated sex hormones during pregnancy has an obvious impact on the gingival epithelium increase the proliferative activity of epithelial cells even in absence of other initiating factors for gingival and periodontal alterations such as plaque and calculus. Although local factors are responsible for initiating gingival enlargement or inflammation during

pregnancy, the hormonal effects also have an obvious role in changing the gingival physiology and inducing various alterations. In general, maintaining a good oral hygiene is very important to minimize the hormonal effects on the gingiva during pregnancy.

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