

## Tibolone Improves the Degenerative Changes of Tongue Mucosa in Ovariectomized Female Rats

Heba M. Eltokhey, Dalia H. Zahran\* and Amel M. Ezzat

Faculty of Dentistry, Tanta University, Tanta, Egypt

### Abstract

The aim of this study was to investigate the possible effect of sex steroid deficiency on the mucosa of rat tongue as well as the influence of Tibolone.

**Methods:** The tongue mucosa of ovariectomized rats (OVX) was compared to sham-operated rats by histology, immunohistochemistry and electron microscopy. The same evaluation was also performed after hormone replacement therapy.

**Results:** The OVX animals demonstrated significant degenerative changes of tongue mucosa such as reduced thickness of epithelium with an irregular, significantly thinner keratinized surface. Partial disappearance of lingual papilla with irregular epithelial ridges were also noted. At ultrastructural level there were wide intercellular spaces, swollen and/or degenerated mitochondria, irregular dense nuclei, dilated perinuclear cisternae, cytoplasmic vacuolization and decreased tonofillament aggregations. The results from immunohistochemistry showed the possibility that the turnover period was prolonged in OVX rats. The degenerative changes of tongue mucosa were inhibited after administration of Tibolone.

**Conclusions:** The overall results suggest that sex steroids have a specific role in the maintenance of normal tongue mucosa, and its deficiency deteriorates tongue mucosa ultrastructure and histology. Tibolone has a mild estrogenic action and has a good supportive effect on tongue structure.

**Keywords:** Ovariectomy; Tibolone; Tongue mucosa; Rat

**Abbreviations:** ER: Estrogen Receptor; HRT: Hormone Replacement Therapy; OVX: Ovariectomized; PCNA: Proliferating Cell Nuclear Antigen

### Introduction

In postmenopausal period the endogenous estrogen level decreases. This primary change provides for many characteristic alterations in almost all the body. The systemic aspects of menopause are well documented and include oral manifestations, such as changes in salivary secretion, burning mouth syndrome, oral dryness, gingivitis, bleeding, mucosal ulceration and altered taste sensation [1].

Estrogen acts through two intracellular receptor proteins ER $\alpha$  and ER $\beta$  which select, recognize, and bind the hormone to the cell cytoplasm or nucleus [2]. Leimola-Virtanen et al. (2000) have confirmed the presence of estrogen receptors (ERs) in oral buccal mucosa, gingiva, minor salivary glands, parotid and submandibular glands [3]. Changes caused by estrogen deficiency are also manifested in the vascular, urogenital and skeletal systems. In short, mucosal changes in postmenopausal period can be named atrophical [4,5].

Drugs used in the prevention of postmenopausal osteoporosis protect body against alterations caused by hypoestrogenism. Prevention of postmenopausal hypoestrogeny can be based not only on simple supplementation with missing hormone, but also drugs modulating ER can be used [6,7].

Tibolone is a synthetic steroid hormone drug that exhibits estrogenic, androgenic and progestogenic properties, but it has different effects than other hormone replacement therapy (HRT) preparations. Tibolone significantly increased the ER $\beta$  protein expression in bladder destrusor muscle of rats during low estrogen state, but had little effect on ER $\alpha$  expression [5]. It is used, as well as conventional HRT, for the treatment of hot flushes and for the prevention of postmenopausal

osteoporosis [8,9]. Tibolone also has favourable effects on vagina, climacteric symptoms, mood and sexual well-being in postmenopausal women [9].

The lingual dorsal epithelium of adult mammals is generally composed of regularly ordered columns of cells with different degrees of keratinization. The epithelium of the posterior cell columns of the filiform papillae shows hair-like, hard keratinization in most of the mammals, and the epithelium of the anterior cell columns of the filiform papillae shows the newborns skin – like soft keratinization. In rodents, the interpapillar epithelium shows very weak keratinization, which may be identical with parakeratinization; however, in many mammals other than rodents, this area shows no evidence of keratinization [10].

Several studies have showed the influence of the sex steroids on oral mucosa [4,11-13]. However, this is the first study to evaluate the possible supportive effect of Tibolone on the changes induced by ovariectomy model on structure of the tongue mucosa of rats.

### Animals and Methods

#### Animals

Fifteen Wistar female albino rats (20 weeks old, 150–200 g) were

\*Corresponding author: Dalia H. Zahran, Faculty of Dentistry, Tanta University, Tanta, Egypt, E-mail: [dhzahran@gmail.com](mailto:dhzahran@gmail.com)

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obtained from physiology department in Alexandria University. The rats were housed under controlled laboratory conditions (room temperature  $23 \pm 2^\circ\text{C}$ , relative humidity  $60 \pm 5\%$ , with light-dark cycle of 12 h each).

### Surgical Procedure

After one week of acclimatization, the rats were anaesthetized with sodium pentobarbitone (35 mg/kg, *i.p.*), and bilateral ovariectomy (OVX) was done aseptically. Sham operation was done in the same manner but only exposing the ovaries. The animals were given prophylactic ampicillin (4000 IU/kg, *i.p.*) for 3 days and coloplast paste (Humlebaek, Denmark) was applied locally. The experiment was carried out in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication Number 85-23, Rev. 1985). The protocol was examined and approved by the local experimental animal ethics committee.

### Drug

Tibolone (Livial 2.5 mg tablets of Organon Comp.) was suspended in an aqueous solution of gelatin (5mg/ml). Drug was injected subcutaneously into the loose skin at the back of the neck of rats. A dose volume of 1mg/kg body weight was used [14].

### Groups

Group I (Sham, n=5) was sham-operated and received the vehicle only. Group II (OVX+P, n=5) was ovariectomized and received the vehicle only. Group III (OVX+T, n=5) was ovariectomized and received Tibolone. All treatments were given daily by subcutaneous injection. Twelfth week after the surgery, all rats were anaesthetized with ketamine and xylazine and euthanized by cervical dislocation. The rat was placed on a surface that it can be gripped and a rod was pressed firmly across the back of the neck. With the other hand, the base of the tail was quickly pulled causing separation of the cervical vertebrae from the skull. Tongue mucosa were carefully dissected for light and transmission electron microscopic examination at Faculty of medicine, Tanta University.

### Tissue Preparation for Light and Transmission Electron Microscopic Examination

Tongue mucosa was carefully dissected and immediately cut into two halves. One half was fixed in 10% formol saline, processed and paraffin sections were stained by hematoxylin and eosin stain. Immunohistochemistry for PCNA antibody was also made. The other half was cut into small pieces and fixed into 3% phosphate buffered gluteraldehyde, processed for ultra structure study and examined by Joel 100 CX transmission EM in EM Unit at Faculty of Medicine, Tanta University.

### PCNA (proliferating cell nuclear antigen) Antibody

To understand the possible underlying mechanisms of change in thickness of oral mucosa, we also investigated the difference in turnover cycle between the three groups using PCNA labeling. Tissue blocks were cut at  $3 \mu\text{m}$  and subjected to the biotin-streptavidin amplified system. To improve PCNA immunohistochemical expression, sections were submitted to a microwave in a citrate buffer pre-treatment (pH 6.0, 10 mM) for 15 min. The samples were then immersed in 3% methanolhydrogen peroxide solution for 10 min to block endogenous peroxidase activity, washed in tris-HCl buffer (pH 7.4) and incubated with anti-PCNA (Clone PC10, 1:3000 Sigma, St. Louis, MO, USA)

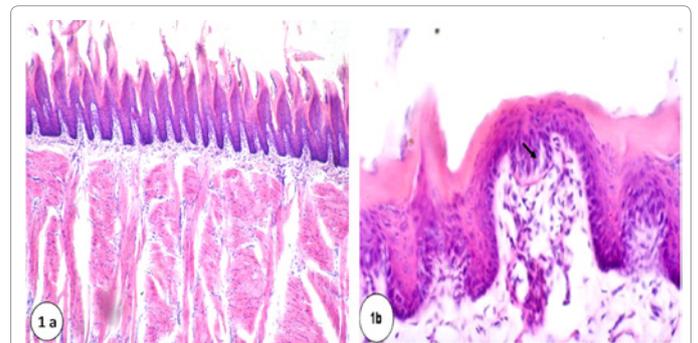
in 5 mM tris-HCl buffer for 18 h at  $4^\circ\text{C}$ . After washing in tris-HCl buffer (pH 7.4), the sections were incubated at room temperature with 1) biotinylated swine anti-goat, mouse and rabbit immunoglobulin (DAKO, Carpinteria, CA, USA) diluted 1:150 in tris-HCl for 30 minutes; 2) washed with tris-HCl twice for 10 min; 3) incubated for 30 min with horseradish peroxidase-conjugated streptavidin (DAKO) prepared according to the manufacturer instructions; 4) washed with tris-HCl; 5) incubated for 3 min with 0.01% diamine benzidine tetrahydrochloride (DAB) and 0.03%  $\text{H}_2\text{O}_2$  in 5 mM tris-HCl buffer (pH 7.4); 6) rinsed in distilled  $\text{H}_2\text{O}$  for 10 min and counterstained with Mayer's hematoxylin. To avoid false positive results, a series of tissue sections were stained with omission of the primary antibody or using adsorbed primary antibody with purified PCNA protein (25 ng PCNA per ml of anti-PCNA antibody diluted 1:3000 tris-HCl).

## Results

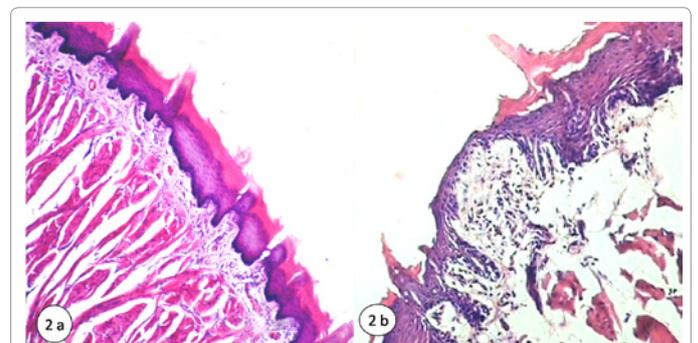
### Light Microscopic Examination

The dorsal tongue surface of the Sham group showed long finger like projections of filiform papillae with scattered fungiform papillae. The connective tissue papillae were normal (Figure 1).

In the OVX+P group the filliform of rat tongue appeared ill defined with loss of normal finger like projections. The tongue papillae showed thin detached keratin layer and thin epithelium. The epithelia ridges were few and shallow (Figure 2).



**Figure 1:** Photomicrographs of the dorsal tongue surface of Sham group showing: (a) Sharp conical projections of filiform papillae with thin smooth keratinized epithelial covering (H & E X 100 ). (b) Fungiform papilla and taste bud (arrow) (H & E X 200).



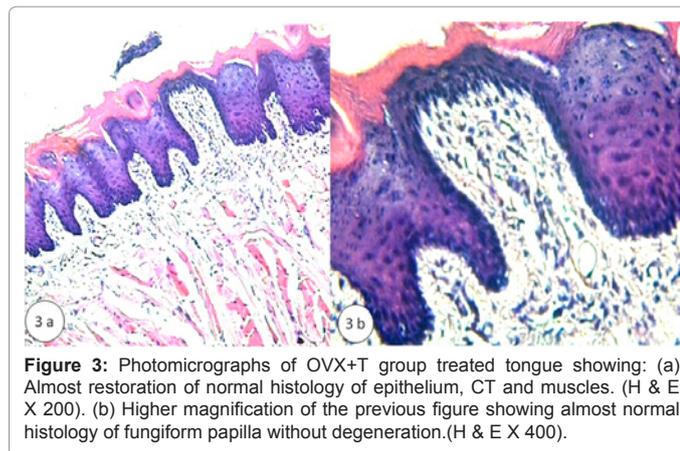
**Figure 2:** Photomicrographs of the dorsal tongue surface of the OVX+P group showing : (a) Ill defined filiform papillae, atrophy of epithelium and few and shallow epithelial ridges. (H & E X 200). (b) Ill defined, atrophied fungiform papilla with separation of keratin layer and disoriented muscle fibers . (H & E X 200).

In the OVX+T group there was almost restoration of normal tongue histology. The basal cell layer was strongly developed, and thick keratin layer was evident (Figure 3).

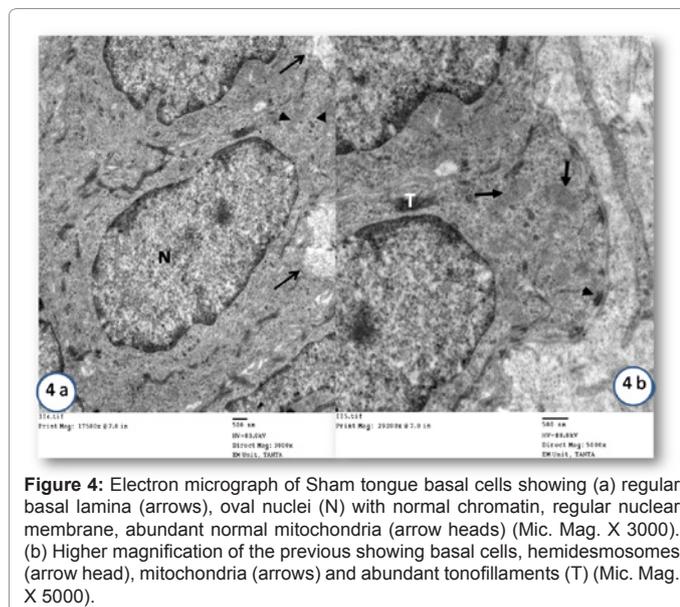
### Transmission Electron Microscopic Examination

Electron microscopic examination of the Sham group revealed normal basal, prickle, granular and cornified layers. The basal cells showed euchromatic nuclei, abundant free ribosomes, well developed rough endoplasmic reticulum and numerous mitochondria (Figure 4a). Desmosomal junctions were seen in between the adjacent cells with normal intercellular spaces and normal tonofillament aggregations (Figure 4b).

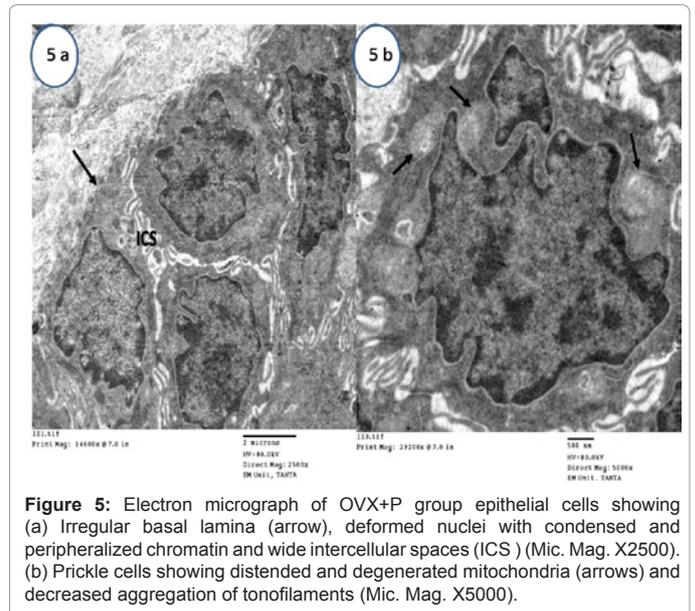
The OVX+P group revealed wide intercellular spaces (Figure 5a), numerous mitochondria that sometimes appeared swollen and/or degenerated (Figure 5b). Some epithelial cells showed irregular dense nuclei and dilated perinuclear cisternae. Their cytoplasm revealed marked vacuoles, decreased tonofillament aggregations (Figure 5b). There was several mitosis in the upper prickle cell layers (Figure 6a). The cornified layer was thinner than that of Sham animals with fewer keratohyaline granules (Figure 6b).



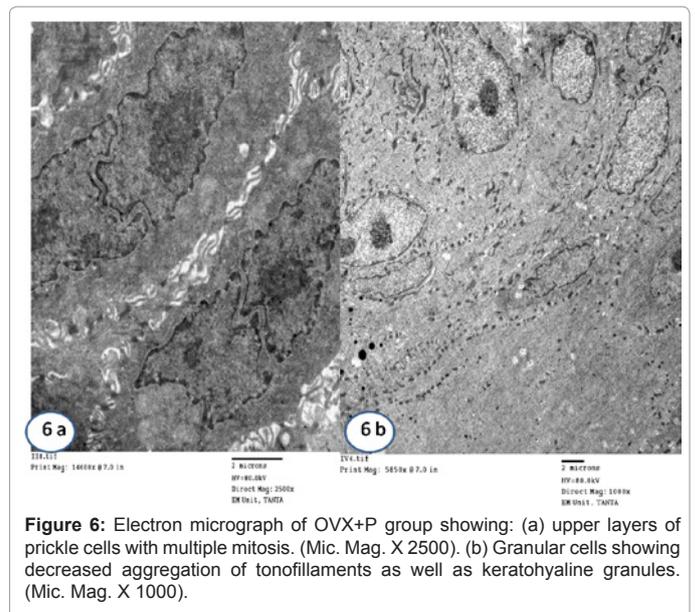
**Figure 3:** Photomicrographs of OVX+T group treated tongue showing: (a) Almost restoration of normal histology of epithelium, CT and muscles. (H & E X 200). (b) Higher magnification of the previous figure showing almost normal histology of fungiform papilla without degeneration.(H & E X 400).



**Figure 4:** Electron micrograph of Sham tongue basal cells showing (a) regular basal lamina (arrows), oval nuclei (N) with normal chromatin, regular nuclear membrane, abundant normal mitochondria (arrow heads) (Mic. Mag. X 3000). (b) Higher magnification of the previous showing basal cells, hemidesmosomes (arrow head), mitochondria (arrows) and abundant tonofillaments (T) (Mic. Mag. X 5000).



**Figure 5:** Electron micrograph of OVX+P group epithelial cells showing (a) Irregular basal lamina (arrow), deformed nuclei with condensed and peripheralized chromatin and wide intercellular spaces (ICS) (Mic. Mag. X2500). (b) Prickle cells showing distended and degenerated mitochondria (arrows) and decreased aggregation of tonofillaments (Mic. Mag. X5000).



**Figure 6:** Electron micrograph of OVX+P group showing: (a) upper layers of prickle cells with multiple mitosis. (Mic. Mag. X 2500). (b) Granular cells showing decreased aggregation of tonofillaments as well as keratohyaline granules. (Mic. Mag. X 1000).

Tongue mucosa of the OVX+T group revealed obvious improvement marked by nearly normal intercellular spaces, mitochondria, rough endoplasmic reticulum, nuclear shape and chromatin distribution (Figure 7a,7b). In addition there were numerous keratohyaline granules in granular cell layer as compared to the OVX+P group (Figure 7c).

### PCNA Immunohistochemical Observations

In the Sham group, PCNA immunoreactivity was evident in cells of the basal layer (Figure 8). Meanwhile, OVX+P sections showed that PCNA immunoreactivity was evident in the upper layers of prickle cells (Figure 9). OVX+T sections revealed that PCNA immunoreactivity was almost normal and located in basal layer (Figure 10).

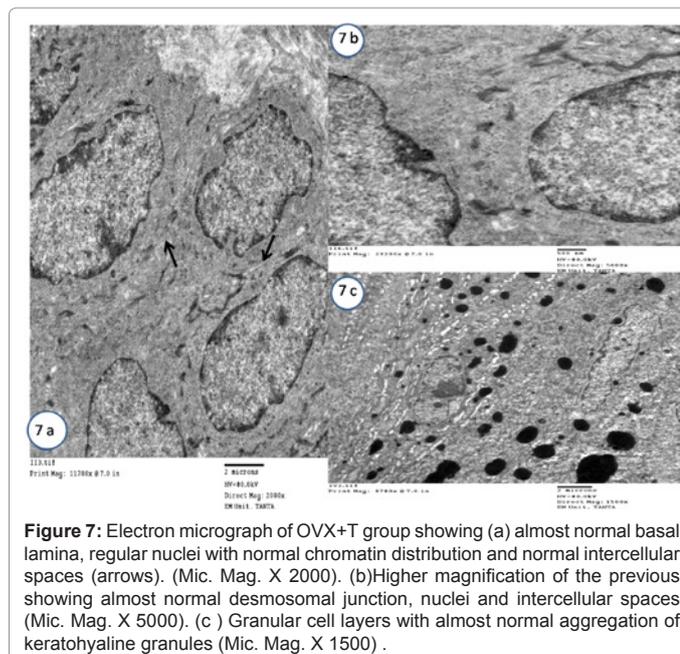
### Discussion

HRT has been used extensively to relieve systemic alterations

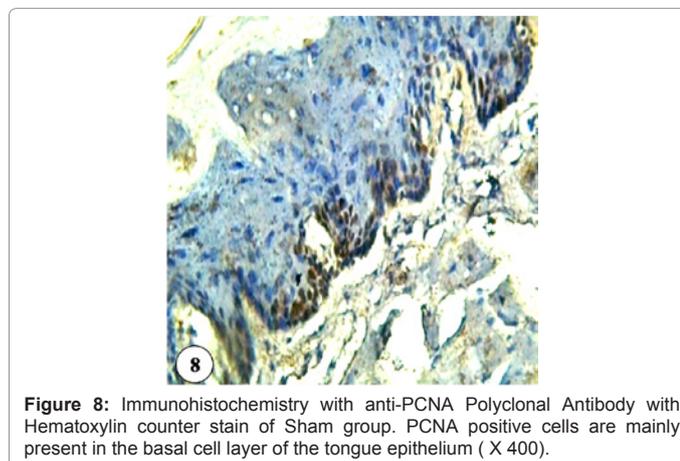
occurred in postmenopausal women. Unfortunately, however conventional HRT seems to have serious side-effects such as an increased risk of malignancies, in particular, breast and gynecologic cancers [15]. For this reason, new drugs, such as Tibolone, a synthetic steroid that is distinct from currently available HRT because of its mode of action, without having an estrogen-like stimulating effect on the endometrium or breast, may be more attractive [16].

The present study attempted to clarify the effect of a sex steroid hormone deficiency as well as the effect of its replacement on the tongue epithelium of OVX rats. 20 weeks-old female rats (never pregnant) were chosen in this study to simplify factors such as pregnancy and delivery.

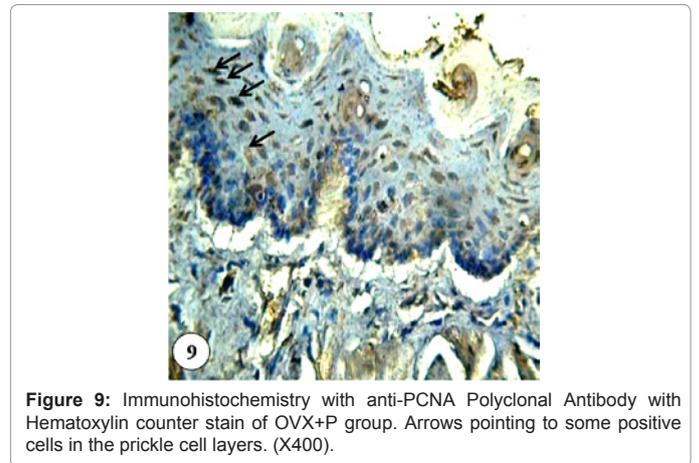
Based on structural changes between the investigated groups, it is concluded that lack of endogenic estrogen and HRT supplementation cause changes in tongue mucosa at tissue level. In our study, the epithelial ridges of tongue mucosa were few and short after OVX. This observation was reported by Rahnama et al. (2004) in buccal mucosa of OVX rats [11]. Also, in accordance with the histological findings



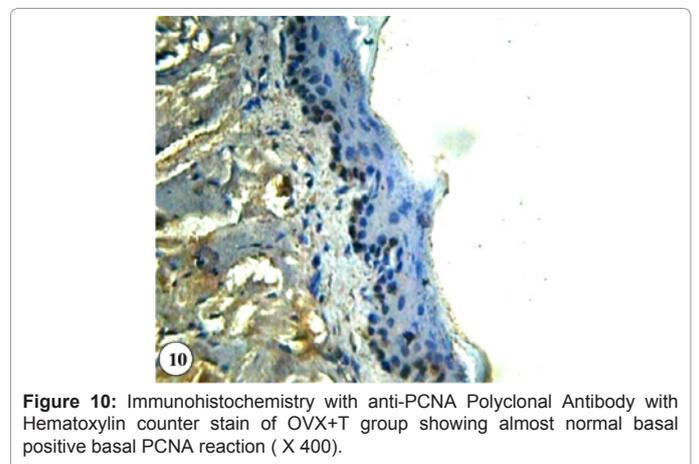
**Figure 7:** Electron micrograph of OVX+T group showing (a) almost normal basal lamina, regular nuclei with normal chromatin distribution and normal intercellular spaces (arrows). (Mic. Mag. X 2000). (b) Higher magnification of the previous showing almost normal desmosomal junction, nuclei and intercellular spaces (Mic. Mag. X 5000). (c) Granular cell layers with almost normal aggregation of keratohyaline granules (Mic. Mag. X 1500) .



**Figure 8:** Immunohistochemistry with anti-PCNA Polyclonal Antibody with Hematoxylin counter stain of Sham group. PCNA positive cells are mainly present in the basal cell layer of the tongue epithelium ( X 400).



**Figure 9:** Immunohistochemistry with anti-PCNA Polyclonal Antibody with Hematoxylin counter stain of OVX+P group. Arrows pointing to some positive cells in the prickle cell layers. (X400).



**Figure 10:** Immunohistochemistry with anti-PCNA Polyclonal Antibody with Hematoxylin counter stain of OVX+T group showing almost normal basal positive basal PCNA reaction ( X 400).

Seko et al. (2005) and Saruhan and Ketani (2006) have suggested that, the tongue epithelium in OVX animals presented an irregular corneal (keratinized) surface and apparently smaller epithelial thickness than in the control animals [12,13].

The underlying mechanism of these histological changes is not known. One possible explanation could be the proliferative activity of epithelial cells. The changes in the oral mucosa as a result of estrogen deficiency occur possibly at the tissue level, because estrogen influences the proliferation, differentiation, and keratinization of the oral epithelium and stimulates the proliferation of fibroblasts [12].

PCNA is a known marker for cell proliferation. It acts as an auxiliary to DNA delta polymerase. The synthesis of PCNA begins to increase in the late G1 phase of the cell cycle. After reaching a peak in the middle S-phase, it returns to its initial condition in the late S phase. PCNA is widely used as a marker of proliferation in both experimental and clinical pathology [17,18].

The PCNA immunoreactivity was seen in basal cell layer of the Sham group while, OVX+P sections showed PCNA immunoreactivity in the upper prickle cell layers. Biagiotti et al. (2000) studied the proliferation rate in different epithelial layers of both rat and rabbit tongue [19]. They reported that activity was two to three times higher in upper cell layers of epithelium whereas PCNA localization revealed that the basal cell layers of tongue epithelium are the only cell layers that contain proliferating cells. Seko et al. (2005) found that the percentage

of PCNA positive cells was significantly reduced in tongue of the OVX rat's and suggested a possible delay in epithelial turnover which could induce thinning of oral mucosa in OVX rats [12].

On the other hand Lai et al. (2000) detected a sustained, high level of PCNA expression together with apoptosis in luminal epithelium of rat uterus for 7 days after OVX and suggested an association between PCNA expression and epithelial atrophy [20]. The authors stated that growth of tissues depends not only on the rate of cell proliferation, but also on the rate of cell death. The imbalance of either one would cause abnormal growth or atrophy of the tissues. Therefore, they concluded that PCNA expression does not necessarily reflect cell proliferation but functions other than cell proliferation should be taken into consideration.

The results obtained from transmission electron microscopic examination of tongue epithelial cells of OVX+P rats were comparable to those observed by Yang et al (2009) who confirmed that estrogen deficiency deteriorated bladder ultra structure and histology in OVX rats [5]. The authors reported wide intercellular spaces, dilated rough endoplasmic reticulum, swollen mitochondria as well as derangement and decrease of myofilaments in smooth muscle cells in OVX bladder of rats.

Some authors have reported the benefits of Tibolone administration in several tissues in OVX animals [5,21]. The same benefits were also seen in this work in the tongue of rats treated with Tibolone. Histologically, the basal layer of epithelium improved in the OVX+T group than in OVX+P group. The PCNA immunostaining appeared almost normal in OVX+T sections and was localized to basal cells. Ultrastructural examination of the same group revealed obvious improvement of epithelial tongue cells. Similar improvement in vaginal epithelium of OVX rats following Tibolone administration was also reported by Henriques et al. (2011) who concluded that Tibolone administered in high dosages and for a long period of time has proliferative and trophic effects, regenerative hyperplasia, reversing vaginal atrophy, and does not have dysplastic or neoplastic effects in the vagina of OVX rats [21].

It is to be concluded that estrogen deficiency secondary to OVX leads to an increase tongue epithelium disorders as well as unique ability to alter cell histology. Tibolone reduces these pathological effects and helps approaching mucosal structure to normal.

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