Potential Effect of Bovine Colostrum on Tongue Mucosa in Ovariectomized Rats

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Abstract: Background: Menopause is a physiologic stage in women life. Oral cavity is subjective to the endocrine disturbance linked with menopause particularly in sex steroid hormone deficiency. Aim of study: To investigate the possible effect of bovine colostrum consumption on rat tongue mucosa during estrogen deficiency. Material and Methods: Twenty four, 6 month old, virgin female Sprague–Dawley rats were randomly allocated into four equal groups; Sham, Sham/Colost, Ovx and Ovx/Colost groups. Colostrum was given by oro-gastric intubations for one month after confirmation of estrogen deficiency. Then animals were euthanized by overdose of anesthesia. Samples were processed for routine histological Hematoxyline and Eosin (H&E) and immunohistochemical staining using proliferating cell nuclear antigen (PCNA) and P53. Statistical analysis was used to compare PCNA and P53 expression between all groups. Results: Histologically, Ovx group tongue mucosa showed abnormal shaped filiform papillae when compared with the tongue mucosa of Sham and Sham/Colost groups. Interestingly, Ovx/Colost group revealed improvement in tongue mucosa with almost normal architecture. Immunohistochemically, Ovx group had a significant PCNA expression ($p<0.05$), extended to prickle cell layer. However, other groups PCNA expression was limited to the basal cell layer. Unexpectedly, P53 showed no expression in all groups. Conclusion: Estrogen deficiency affected the tongue mucosa in different ways resulting in altered mucosal turnover and architecture. However, bovine colostrum was able to abolish those effects.


Key words: Colostrum, Ovariectomy, Estrogen deficiency, PCNA, P53, Tongue mucosa

1. Introduction

Menopause in women is a physiological state that gives rise to adaptive changes at both systemic and oral level. Estrogen deficiency arising as a result of menopause along with physiological age-related factors are responsible to the alterations detected within the oral cavity in postmenopausal women[1]. Along with osteoporosis, hormonal changes during menopause lead to a much higher risk for gingivitis and advanced periodontitis [2,3]. Additionally, many significant adverse changes in the oro-facial complex are associated with menopause. One of the major oral problems in these women is burning mouth syndrome that is accompanied by dysgeusia, dysphagia, and oro-facial/dental pain. Besides, hyposalivation associated subjective oral dryness or xerostomia is another common manifestation in post-menopausal women which in turn leads to potential complications as mandibular dysfunction, diffuse gingival atrophy or oral ulcerations, oral candidiasis and dental caries [4-7].

Estrogen might have a biological role in the oral environment since the oral mucosa and salivary glands express estrogen receptor (ER) and might, therefore, be estrogen-responsive tissues. This, in turn, suggests that mucosal disorders and oral discomfort are responsive to hormonal treatment [8].

To improve the unpleasant symptoms associated with estrogen deficiency and to prevent some of the chronic illnesses common to post menopausal women, prevention of postmenopausal hypooestrogen is recommended. This can be based not only on simple supplementation with hormone, hormone replacement therapy (HRT) of either estrogen or estrogen and progestin, but also drugs modulating ER can be used [9-10]. However, each of them is associated with numerous side effects.

Colostrum is the first specific diet of mammalian neonates that is secreted by mammary gland during the first two to four days of lactation. It is considered as a unique natural secretion given to the neonate since all the mother’s lifetime achievements pass to the baby through colostrum [11]. The nutritional composition of colostrum including proteins, carbohydrates, fat, vitamins and minerals favors its use as an ancient food for modern times and hence can be considered as a perfect nutraceutical [12]. Interestingly, colostrum is not only a source of nutrients but it also rich in immunoglobulins, antimicrobial peptides and other bioactive molecules including growth factors that could lay good foundation for the immune system and exert mineralization promoting function beside their essential contribution in cell proliferation, differentiation, growth and regeneration [13-15]. Cytokine profile of human colostrum declared the presence of several novel biologically active proteins or cytokines include chemotactic factors (B-
lymphocyte chemo-attractant, macrophage inflammatory proteins, pulmonary and activation-related chemokine, leukemia inhibitory factor, oncostatin-M) and anti-inflammatory factors (tissue inhibitor of metallo-proteinases-1, -2, macrophage-derived chemokine)\textsuperscript{[16]} . Noteworthy, previous studies revealed the efficiency of using different colostrum-containing hygiene products in the form of dentifrice, gel and mouthwash in alleviating symptoms and treating oral mucosal lesions in patients with primary Sjögren’s syndrome and those with oral lichen planus. Similarly, purification of antimicrobial factors proteins as lactoperoxidase, lysozyme and lactoferrin from bovine colostrum then using them in oral health care products improves xerostomia\textsuperscript{[17, 18]} . It is remarkable that studies on neonatal porcine revealed the role of colostrum in estrogen receptor-\( \text{ER} \) expression in neonatal reproductive tissues. Relaxin (RLX), a lactocrine-active peptide found in porcine colostrum, stimulates estrogen receptor-\( \alpha \) (ESR1) expression required for normal developmental programming of neonatal reproductive tissues\textsuperscript{[19]}.

The aim of this study was to examine the capability of bovine colostrum to alleviate changes in rats tongue mucosa in experimental postmenopausal hypoestrogenic model.

2. Material and methods

Animals

Twenty-four virgin female Sprague–Dawley rats (6 months in age and approximately 250-300 g in weight) were used. Animals were housed - at faculty of Medicine-Zagazig University- in individual cages and received a standard diet for rodents and tap water \textit{ad libitum}. Room temperature and humidity were maintained at 23 °C and 60%, respectively. The light cycle was fixed at 12 h. All animal experiments were carried out in accordance with the guidelines of the National Institutes of Health (NIH) for the care and use of laboratory animals (NIH Publication, Number 85-23, Revised 1985).

Surgical procedure

Animals were assigned randomly into four equal groups; Sham operated group (Sham; n=6), Sham operated group received colostrum (Sham/Colost; n=6). Ovariectomized group with colostrum treatment (Ovx/Colost; 1.7g/Kg, n=6)\textsuperscript{[20]}.

Ovariectomized group with no treatment (Ovx; n=6).

After one week acclimatization in an animal house, each group underwent an operation. The animals were anesthetized with intraperitoneal injection of pentobarbital sodium (15 mg/kg body weight) for the surgical procedure. In the ovariectomized group, bilateral ovariectomies were performed by the dorsal approach as described previously\textsuperscript{[21]}.

The sham-operated groups underwent a similar surgical procedure, exposing the ovaries and replacing them in the same position.

Evaluation of ovariectomy

The results of ovariectomy were confirmed by two procedures. First, an autopsy was performed in all animals at the time of sacrifice. In a successful case of ovariectomy, the uterus becomes atrophic and the ovaries no longer remain\textsuperscript{[21]}. Second, the serum estradiol level (E2) was analyzed in El-Borg laboratory, Zagazig branch.

Drug administration

Daily dose of colostrum (Lovely Health Ltd, Auckland, New Zealand) was dissolved in 0.3 ml of physiological saline solution, and applied by oro-gastric intubations for one month after confirmation of ovariectomy.

Euthanasia and sample collections

One month after the onset of treatment, animals were sacrificed with an overdose of pentobarbital sodium, confirmed with cervical dislocation. The whole tongue was removed, cut at midline, and immediately fixed with 4% buffered formalin solution.

Tissue preparation

After fixation, the specimens were dehydrated by a graded ethanol series and embedded in paraffin with the midline contacting the bottom. 5\( \mu \) sections were cut from midline, and the cutting directions were adjusted perpendicular to the surface of the tongue. Representative sections were stained with H&E for conventional histological assessment using light microscope (Leica ICC50 HD).

Immunohistochemistry

Immunohistochemical labeling was performed using the Avidin–Biotin–Complex (ABC) method. Representative sections were deparaffinized in xylene and re-hydrated through a descending series of ethanol concentrations. The sections were washed with TBS (20 mM Tris- HCl, 150 mM NaCl, pH 7.4). Then they were incubated in 0.3% \textit{H}2\textit{O}2 in \textit{dH}2\textit{O} at room temperature (30 min) to inhibit endogenous peroxidase. Antigen retrieval was performed according to the manufacture instructions. Slides were placed in 100 \( \mu \) blocking solution (Abcam), for 30 minutes at room temperature. Then incubated with primary monoclonal antibodies; ( anti- PCNA primary antibody, Clone PC10, Santa Cruz, Se 56, USA at dilution 1:300/ anti P53 primary antibody, PAb 240, Abcam, ab26, UK at dilution 1:250) at 4°C overnight. Sections were washed in 1X Phosphate buffered saline (PBS) and then incubated with secondary biotinylated antibody (anti-mouse) (in blocking buffer for 1 hour at room temperature in a humidified chamber. To perform peroxidise visualization; sections were incubated in ABC.
solution for 1 hour at room temperature. Color reaction was then developed by adding DAB solution (0.5 mg/ml DAB and 0.1% H2O) onto the sections. When color reaction was satisfactory, it was stopped by rinsing with H2O for 5-10 minutes, and then sections were counterstained with hematoxylin for 2 minutes. Sections were gradually dehydrated and mounted with coverslips. Immunohistochemical staining was assessed using light microscope.

**Statistical analysis**

Immunostained sections were analyzed for PCNA and P53 positive (+ve) cells by Image J analysis system (V 1.48S) using light microscope. For statistical analysis, all measurement data were presented as mean ± standard deviation. All statistical analyses were performed using one-way analysis of variance followed by Dunnett’s post hoc test to compare all groups with the Ovx one. Values of \( p < 0.05 \) indicated a statistically significant difference. PrismPad Graph (V 5.01) was used for data analysis.

3. Results

**Histological findings**

In Sham group, the dorsal surface of the tongue was normal. It revealed numerous, thin and long finger- like projections of filiform papillae with a lamina propria core covered by a keratinized epithelium; possessing the classical appearance of the basal, spinous, granular and cornified epithelial cells beside the normal shape of epithelial ridges (Figure 1, A and A’). In Sham/Colost group, no remarkable histological changes were observed. The filiform papillae appeared well defined with regular shape and typical epithelial stratification and keratinization (Figure 1, B). At higher magnification, the basal cell layer appeared well developed and the normal mitotic activity was evident in the suprabasal layers (Figure 1, B’). On the other hand, Ovx group exhibited ill-defined filiform papillae with thin and irregular corneal (keratinized) surface and abnormally shaped epithelial ridges (Figure 1, C). Higher magnification of their tongue mucosa revealed abnormal shape of the basal and suprabasal cell layers. They were harboring some prickle-like cells (White arrowheads in figure 1, C’). Moreover, some cells exhibited hyperchromatic nuclei (Red arrowheads in figure 1, C’) while others appeared to be binucleated (Black arrowheads in figure 1, C’). Interestingly, in Ovx/Colost group, the filiform papillae appeared almost regular with almost normal epithelial stratification and keratinization (Figure 1, D and D’).

**PCNA and P53 immunohistochemical findings**

In both Sham and Sham/Colost groups, PCNA immunoreactivity was evident exclusively in the basal cell layer (Figure 2, A and B). However, in Ovx group, PCNA immunoreactivity was evident in some prickle cells together with the basal cell layer (White arrowheads in figure 2, C). Interestingly Ovx/Colost group revealed almost normal PCNA immunoreactivity that was localized in basal cell layer (Figure 2, D). Surprisingly, no obvious P53 immunoreactivity was detected in any of these groups (Data not shown).

**PCNA - positive cells counting**

Statistical analysis using One – way ANOVA test revealed a significant difference between all groups, (\( p \) value =0.0134). Dunnett’s post hoc test revealed a significant PCNA expression difference between ovariectomized rats and other groups.

4. Discussion

The present study attempted to address two research points. The first, the possible changes in tongue mucosa in ovx rats, in particular, those in the proliferative and apoptotic activity. The second, the capability of bovine colostrum to alleviate those changes. Similarities in pathophysiologic responses between the human and rat skeleton, combined with the husbandry and financial advantages, have made the rat a valuable model in postmenopausal research\[^{22}\]. In the present study, 6-months old, never pregnant, female rats were used in this study to avoid possible pregnancy and lactation related effects.

In current study, the histological findings were compatible with the significant decline in serum level of E2 consecutive to ovariectomy. Tongue mucosa in Ovx group showed numerous histological changes such as ill - defined filiform papillae with thin and irregular corneal surface and abnormally shaped epithelial ridges. Higher magnification revealed abnormal shape of the basal cell layer with some prickle-like cells accommodated within. Also, few cells exhibited hyperchromatic nuclei while others appeared to be binucleated. These observations are in agreement with Seko *et al.* and Saruhan and Ketani who stated that ovariectomy led to an increased tongue epithelium disorders as well as its unique ability to alter cell and surface histology. Also, they reported reduced thickness of the tongue epithelium with an irregular keratinized surface and irregular rete-peg in ovx rats\[^{23, 24}\]. Similarly, Rahman *et al.* declared that low estrogen level in ovx rats led to atrophic changes in oral mucosa including shortening of epithelial ridges with reduction in the thickness of basal and granular cell layers of epithelium\[^{25}\].
Figure 1: H&E stained sections showing rat tongue dorsal mucosa. (A) Tongue mucosa of Sham group shows numerous, thin and long finger-like projections of filiform papillae with a lamina propria core covered by a keratinized epithelium beside the normal shape of epithelial ridges. (A’) Higher magnification of boxed area in (A) showing the classical appearance of the basal, spinous, granular and cornified epithelial cells. (B) Tongue mucosa of Sham/Colost group showing well defined, regular shaped filiform papillae with typical epithelial stratification and keratinization. (B’) Higher magnification of the boxed area in (B) showing well developed basal cell layer with normal mitotic activity in the suprabasal layer. (C) Ovx group shows ill-defined filiform papillae with thin and irregular corneal surface and abnormally shaped epithelial ridges. (C’) Higher magnification of boxed area in (C) shows abnormal shape of the basal and suprabasal cell layers with some prickle-like cells within (White arrowheads). Also, some cells exhibit hyperchromatic nuclei (Red arrowheads) while others appear to be binucleated (Black arrowheads). (D) Tongue mucosa of Ovx/Colost group shows regular filiform papillae with normal keratinization. (D’) Higher magnification of boxed area in (D) showing normal epithelial stratification. (Original Magnification; A-D X200 and A’-D’X1000).
Figure 2: Immunodetection of PCNA in rat tongue dorsal mucosa. (A) Shows PCNA immunoreactivity in tongue mucosa of Sham group. Its expression is exclusive to the basal cell layer. (B) Tongue mucosa of Sham/Colost group shows PCNA expression limited to the basal cell layer. (C) In Ovx group, PCNA immunoreactivity is evident in some prickle cells together with the basal cell layer (White arrowheads). (D) Ovx/Colost group shows almost normal PCNA immunoreactivity that is localized to the basal cell layer. (DAB, Original Magnification A-D X400).

Figure 3: PCNA mean expression in tongue mucosa. Statistical analysis using One-way ANOVA test revealed a significant difference between all groups, (p =0.0134). Dunnett’s post hoc test revealed a significant PCNA expression difference between ovariectomized rats and other groups. Error bar=Standard deviation.

The effect of estrogen deficiency on tongue mucosa is attributed to the existence of estrogen receptors in oral mucosa, thus any variations in hormone levels directly affect it\(^9\). The underlying mechanism contributed to this mucosal change is not well known. Many investigations had reported the role of estrogen in maintaining normal cell proliferation and differentiation. Vittek et al., reported estrogen effect on cellular proliferation, differentiation and keratinization of the rat gingival epithelium\(^{26}\). Also, Van der Burg et al., and Greenberg et al., supposed that estrogen could regulate several target genes that control cell proliferation, including the proto-oncogene c-Myc \(^{27, 28}\). In addition, Sharyn et al., suggested that estrogen might regulate telomerase, the enzyme that controls the proliferation lifespan of cells by maintaining telomeres\(^{26}\). Interestingly, other investigations on rats uterus and prostate demonstrated the capability of estrogen to accelerate the synthesis of epidermal growth factor which could result in cellular proliferation, differentiation, and survival \(^{30-32}\).
Accordingly, in our study, the reported mucosal changes could be due to disturbance of proliferation resulted from hypoestrogenism.

PCNA, also known as cyclin, is an intra nuclear polypeptide of 36 kDa that shows peak synthesis during the S phase of the cell cycle. It plays an important role in DNA synthesis, DNA repair, cell cycle, progression and cell proliferation[33]. In the present study, PCNA expression was limited to basal cell layer in all groups except the Ovx rats. Surprisingly, PCNA expression in Ovx rats was significantly higher than other groups (p=0.0134). Its expression was extended to the prickle cell layer beside its expression in the basal cell layer. This finding coincides with Eltokhy et al., who observed extended PCNA immunoreactivity in the upper layers of prickle cells of tongue in ovx rats[34]. Similarly, Lucan et al., reported uneven distribution of PCNA positive cells in basal and intermediate cell layers of urothelium in ovx rats[35]. In contrast, Seko et al., reported significant decrease in the percentage of PCNA positive cells in ovx rats suggesting a possible delay in epithelial turnover periods which could induce thinning of oral mucosa[23].

The unexpected expression of PCNA in the current study could represent abnormal behavior of tongue mucosal cells in response to hypoestrogenism. Moreover, the PCNA expression does not necessarily reflect cell proliferation and functions other than cell proliferation could be considered[30]. Basal cell layer in oral mucosa harbor the stem cells required to maintain the epithelium[37]. Estrogen deficiency was found to decrease the proliferation ability and differentiation potential of mesenchymal stem cells of ovx rats [38]. Together, estrogen deficiency may also affect epithelial stem cells in oral mucosa in the same way.

P53 is a transcriptional activator, regulating the expression of genes involved in growth arrest, DNA repair and apoptosis hence it plays an important role in cell cycle control and apoptosis[39]. Surprisingly in the present study, no obvious P53 immunoreactivity was detected in any of our groups. Ogden et al., found that p53 protein was not expressed in normal, benign, or premalignant oral mucosa. They proposed that its identification would appear to correlate with oral malignancy[40]. Normal p53 protein has a very short half life therefore can be hard to detect in normal tissues that are free of stress. Thus, p53 protein was extremely hard to detect by immunohistochemistry in noncancerous tissues [41].

On the other hand, Win et al., found positive p53 expression at the basal layer in human oral mucosa [42]. They referred that expression to genotoxic stress, caused by physical, chemical or microbiological agents that commonly act in the oral cavity. This may lead to p53 accumulation in these epithelial cells for physiological response. Also, Nylander et al., reported that P53 protein could remain in the tissues longer either due to mutations or a defect in the degradation pathway or by binding to other proteins such as certain DNA virus-encoded proteins[43].

In the current study, Ovx rats treated with colostrum showed almost normal mucosal architecture and thickness. This matches with Vaillati et al., who reported that intravaginal gel, containing purified bovine colostrum improved vaginal hemodynamics and thickness of vaginal epithelium in rats with ovx-induced vaginal atrophy[44]. In accordance with the depicted recovery of hypoestrogenism effect on tongue epithelial cells, PCNA expression in Ovx/Colos group was limited to the basal cell layer similar to Sham and Sham/Colos groups. These results could be related to the constructive effects of colostrum on mucosa, since it contains several important factors with multiple regenerative effects that extend all over the body such as fibroblast growth factors that had shown to be a powerful stimulator of angiogenesis and a regulator of cellular migration and proliferation. Also, it has platelet-derived growth factor (PDGF) that could stimulate the production of other growth factors, including insulin-like growth factor-1 (IGF-1)[41].

Interestingly, colostrum is the only natural source of two major growth factors namely, transforming growth factors (TGF-α and TGF-β), which shown to regulate cellular migration and proliferation, and insulin-like growth factor-1 (IGF-I) that provide remarkable repair and growth capabilities. As well, colostrum expresses growth hormone that has shown to accelerate regeneration and contains high concentrations of three major growth factors namely; vascular endothelial growth factor (VEGF), hepatic growth factor (HGF) and epidermal growth factor (EGF) that have growth-promoting and protective effects[45]. Moreover, colostrum contains superoxide dismutase (SOD), amylase (AM) and alkaline phosphatase (ALP) enzymes which are indispensable for normal growth and development[48].

In addition, colostrum contains several non peptide constituents such as glutamine, polyamines, and nucleotides that cause increased proliferation of cells [49]. Jouan et al., declared that bovine milk and colostrum contain a large number of hormones from either steroid or peptic origin. The main categories to which these molecules belong are gonadal (estrogens, progesterone, androgens), adrenal (glucocorticoids), pituitary (prolactin, growth hormone) and hypothalamic hormones (growth hormone releasing hormone (GRH), luteinizing hormone releasing hormone (LH-RH), thyrotropin-releasing hormone (TRH))[50]. Moreover, colostrum
has an antagonistic effect to hypoestrogenism, Torre et al., found that colostrum could increase the proliferation of canine skin fibroblasts [45]. Additionally, in a study performed on mouse and human, it was found that alpha lipid colostrum/natural extract combination could stimulate an increase in the frequency of bone marrow stem cells, support self-renewal and promote release into the peripheral circulation. They suggested that inclusion of certain classes of functional foods in the diet could influence the cells that support healing and regenerative cellular pathways [51].

To conclude, estrogen deficiency affects the proliferation and the regeneration of the tongue dorsal surface mucosa, leading to abnormal papillae form. Due to its massive beneficial constituents, bovine colostrum demonstrated its ability to alleviate the hypoestrogenism effect resulting in restoring regular epithelial turnover and normal mucosal architecture.

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