# Putative Effect of Wheat Germ Oil on Tongue Mucosa in Hypoestrogenic Rats

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**Original Article**

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## ABSTRACT

**Introduction:** Menopause is a physiological stage in the life of women. In the postmenopausal period, the oral cavity is susceptible to the endocrine disturbance caused by deficiency of oestrogen.

**Aim of Study:** This research aimed to investigate the wheat germ oil (WGO) putative effect on tongue mucosa in experimental postmenopausal hypoestrogenic rat model.

**Materials and Methods:** 40 virgin female rats, 6-month-old, were randomly classified into 4 equal groups; Group I (Sham group): Rats underwent sham operation of ovariectomy (OVX) and served as control for the experimental groups. Group II (OVX group): Rats underwent bilateral OVX operation. Group III (Sham + WGO group): Rats underwent sham operation similar to group I and received a daily oral dose of WGO for four weeks. Group IV (OVX + WGO group): Rats underwent bilateral OVX operation similar to group II and received a daily dose of WGO orally for 4 weeks similar to group III. At the end of experimental period, rats were euthanized and the tongue samples were processed for histological, immunohistochemical staining for PCNA and scanning electron microscopic study.

**Results:** Tongue mucosa of OVX group showed abnormal architecture with irregular arrangement of abnormally shaped papillae when compared with the tongue mucosa of sham and sham/WGO groups. Interestingly, OVX group treated with WGO exhibited enhancement in mucosa of the tongue with almost normal architecture. Immunohistochemical study revealed a significant decrease in PCNA expression in OVX group in comparison with those of other groups. However, OVX + WGO group simulated shame group in PCNA expression and displayed a significant increase in comparison with those of OVX group.

**Conclusion:** Estrogen deficiency caused deleterious effects on rats' tongue mucosa. However, WGO was able to abolish those effects. Hence, WGO is recommended as a prospective treatment for postmenopausal mucosal changes.

Forty, 6 month old, virgin female rats were randomly allocated into four equal groups; sham, ovariectomy (OVX), sham/ wheat germ oil (WGO) treated and OVX/ WGO treated groups. After four weeks, rats were euthanized and the tongue samples were processed for histological examination, immunohistochemical staining using proliferating cell nuclear antigen (PCNA) and scanning electron microscopic study.

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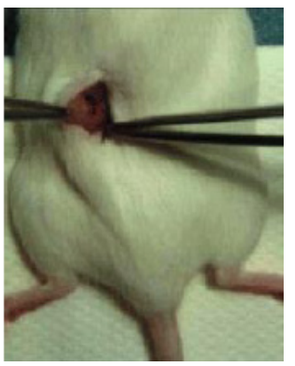

**Graphical Abstract**

Tongue mucosa of OVX group showed irregular arrangement of abnormally shaped papillae when compared with the tongue mucosa of sham and sham/ WGO groups. Interestingly, OVX/ WGO treated group revealed improvement in tongue mucosa with almost normal architecture. Immunohistochemical study revealed a significant decrease in PCNA expression in OVX group in comparison with those of other groups. However, OVX/ WGO treated group simulated sham group in PCNA expression and displayed a significant increase in comparison with those of OVX group

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**Key Words:** Estrogen deficiency, ovariectomy, tongue mucosa, wheat germ oil.

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## INTRODUCTION

Female menopause is a physiological condition that happens in the fifth decade of life. It refers to the time at which cyclic ovarian function terminates resulting in numerous characteristic changes affecting the whole body[1].

Endocrinological systemic hormonal alterations caused by estrogen deficiency during menopausal period may include mucosal atrophy of urogenital tissues, skeletal osteoporosis, cardiovascular diseases and neurological disorders[2].

Estrogen may also play a biological role in the oral environment because of the presence of receptors of estrogen in salivary glands and oral mucosa similar to those found in reproductive tissues, bones, endothelial cells and ligaments rendering them as estrogen-responsive tissues[3,4]. Therefore, the decreased estrogen levels are usually associated with the development of numerous oral disturbances such as oral dryness or xerostomia due to decreased salivary secretion, gingivitis, diffuse gingival atrophy, periodontitis, oral candidiasis, dental caries, altered taste sensation, bleeding and oral mucosal ulceration[1,4]. Burning mouth syndrome is another potential significant complication which is associated with orofacial/dental pain, dysphagia and dysgeusia[5].

To mitigate the disturbing signs and symptoms of estrogen deprivation and to minimize the occurrence of its related chronic illnesses, both short-term and long-term hormone replacement therapy is frequently recommended in menopausal and postmenopausal females[6,7]. However, the use of such treatments is often controversial owing to numerous adverse effects, such as thromboembolism and cancer[8] which in turn rises the need for effective alternative therapy.

The extract of wheat kernel germ known as wheat germ oil (WGO) is accompanied by various health and nutritional benefits as improving physical endurance, decrease cholesterol levels of plasma and liver, and delaying aging effects[9]. This is properly related to its high content of health-beneficial bioactive compounds, as tocopherols, carotenoids, omega-3 fatty acids and phytosterols[10].

WGO is one of the most essential natural antioxidants, especially high in α-tocopherol, that is the most potent form of vitamin E.[11]. The unique cytoprotective properties of α-tocopherol can protect against oxidation of polyunsaturated fatty acids in biological membranes and in plasma lipoproteins. It has been reported as a powerful hepatoprotective agents that works efficiently against nicotine, immunosuppressive agents, chemotherapy, radiotherapy-induced oxidative stress and subsequent hepatic damage[12,13].

Moreover, WGO has been used as an ideal effective vaccine adjuvant that can elevate the efficacy of the immune response due to the presence of other steroids called squalene and biochemical precursor of cholesterol which was also reported to contribute either directly or indirectly to cancer treatment and prevention by the potentiation effect on co-administered anticancer agents[14].

Small amounts of phytosterols especially β-sitosterol and campesterol are found in WGO. Those phytosterols are involved in reduction of circulating plasma total LDL-cholesterol concentration through inhibition of intestinal cholesterol absorption. Also, WGO is a rich source of polyunsaturated fatty acids, specifically linoleic acid. , palmitic acid and lower amounts of oleic acid, linolenic acid, and stearic acid[15]. Thus, its use can improve several age-related health problems and is required for patients at risk of colon problems, diabetes, heart disease, and liver toxicity[16-18].

Accordingly, this research was performed to access the WGO capability to improve changes of tongue mucosa in ovariectomy- induced postmenopausal hypoestrogenic rat model.

## MATERIALS AND METHODS

### *Animals*

This research was performed according to the ARRIVE guidelines and was carried out after the approval of Research Ethical Committee, Faculty of Dentistry, October 6 University, Giza, Egypt (Number: RECO6U/ 15-2020).

The sample size was calculated depending on Charan and Biswas[19]. The estimated sample size was a total of 40 samples (n=40), 10 for each group.

40 virgin female albino rats (6 months old and weighing around 250-300 g) were utilized. They were placed in rat cages (5 rats / cage), numerically labelled, and kept in an animal house with adequate ventilation. in Faculty of Medicine, Zagazig University. Room humidity and temperature were kept at 60% and23 ˚C, respectively and normal photoperiod was kept (12 h light and 12 h dark). The rats were fed dry rat pellets and allowed drinking water adlibitum.

After one week acclimatization period, rats were classified randomly into four equal groups as follow:

**Group I** (Sham group): in which rats underwent sham operation of ovariectomy (OVX) and served as control for the experimental groups.

**Group II** (OVX group): Rats underwent OVX operation bilaterally.

**Group III** (Sham + WGO group): Rats underwent sham operation similar to group I and received WGO (El-Captain Company "Cap Pharm", Egypt) at a once daily dose of 1.5 ml/kg[20] by oro-gastric intubations. The first oral dose of WGO began at the day of operation and continued daily for 4 weeks post operatively.

**Group IV** (OVX + WGO group): Rats underwent bilateral OVX operation similar to group II and received a daily WGO dose for 4 weeks similar to group III.

### *Surgical procedure of ovariectomy*

Bilateral OVX was carried out with a minimally invasive surgical technique and under sterile conditions. As described previously by Kalu *et al*.[21], intraperitoneal injection of pentobarbital sodium (15 mg/kg) was used to induce anaesthesia in the rats then bilateral OVX was conducted. In sham operation of OVX, rats underwent a similar surgical procedure by exposing the ovaries and replacing them in the same position. The success of OVX was confirmed by the serum analysis of estradiol level (E2) consecutive to OVX[22] in El-Borg laboratory, Zagazig branch.

### *Euthanasia and samples collection*

Four weeks postoperatively, with an overdose of pentobarbital sodium, animals were sacrificed confirmed with cervical dislocation. The tongue was entirely removed and dissected from the midline into two halves. The right halves were prepared for histological examination and immunohistochemical study while the left ones were prepared for scanning electron microscopic examination.

### *Histological analysis*

Specimens were fixed immediately after collection of right halves of tongues with 10% buffered formalin solution for 48 h then dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin. Each block was sectioned with a microtome, and five serial slices (5µm thick) were produced and stained with hematoxylin and eosin (H&E) for histological evaluation of any potential structural changes. The slides were examined and a digital color CCD camera (Olympus, DP73, Tokyo, Japan) mounted on a light microscope (Olympus BX53, Tokyo, Japan) used to capture the images at Faculty of Dentistry, Zagazig University.

### *Immunohistochemical analysis*

Proliferating cell nuclear antigen (PCNA) expression was used as a well-known marker for cell proliferation to detect any possible changes in its index[23]. Using the Avidin–Biotin–Complex (ABC) method, immunohistochemical staining for PCNA expression was performed. For negative control, phosphate buffered saline (PBS) was used instead of the primary antibody. A positive PCNA reaction was determined by the presence of brown colored reaction localized to the nucleus while the negative control tissue demonstrated absence of specific staining. Using blinded analysis by two experienced research associates, the number of positive immunoreactions was identified which was then followed by image analysis using the Image J software (version 4.10.03, Nikon, Tokyo, Japan).

### *Scanning electron microscope analysis*

The left halves of the tongues were immediately fixed (2.5% glutaraldehyde for 72 hours and post-fixation in 1% OsO4 for 24 hours), dehydrated in graded ethanol, dried, mounted on stubs and coated with gold using a sputter coater that converted electrically non-conductive samples into conductive ones hence enabled a tightly focused electron beam to be scanned across the sample surface by scanning electron microscope (JEOL JSM-636 OLA at an accelerating voltage of 15kv) in Electron Microscopy Unit, Mansoura University.

### *Statistical analysis*

Data were collected from serum E2 level and PCNA immunohistochemical analysis then were tabulated and statistically analysed by statistical package for social sciences (SPSS) software version 11.0 (SPSS Inc, Chicago, IL, USA) using t- test for serum E2 level analysis and one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for PCNA immunohistochemical analysis to reveal statistical difference among groups. Results were considered statistically significant at $P < 0.05$.

## RESULTS

### *Serum analysis of estradiol level (E2)*

Statistical analysis of serum E2 level confirmed the success of OVX in rats through significant decrease in their serum E2 level consecutive to OVX in comparison to that of sham operated rats (Table 1).

### *Histological results*

Examination of the dorsal surface of the tongue of the sham group revealed normal architecture of tongue papillae. The filiform papillae long finger like projections composed of a lamina propria core covered by a keratinized stratified squamous epithelium and had properly formed epithelial rides. The fungiform papillae had typical mushroom shaped appearance with taste buds found on their superior surfaces (Figure 1A). In contrast, the OVX group displayed short, ill-defined filiform papillae with a thinner epithelium, an uneven thinly keratinized surface and atypically formed epithelial ridges (Figure 1B). In sham + WGO group, tongue papillae simulate those of sham group. The filiform papillae appeared well defined with regular shape and typical epithelial stratification and keratinization with normally shaped fungiform papillae (Figure 1C). Interestingly, OVX + WGO group exhibited marked improvement and reconstruction of the long projections of filliform papillae with almost normal epithelial thickness and keratinization (Figure 1D).

### *Immunohistochemical results*

Examination of PCNA immunohistochemical expression revealed strong nuclear staining of the basal cells of tongue epithelium with anti- PCNA antibody in sham and sham + WGO groups. On the other hand, OVX group exhibited weak nuclear staining of basal cells. Whereas, OVX + WGO group showed improved PCNA immunoreactivity than OVX group and depicted moderate to strong nuclear staining of basal cells (Fig. 2 A-D).

Statistical analysis of the number of positively immunostained cells for PCNA demonstrated that sham group simulated those of sham + WGO and OVX + WGO

groups with no significant difference between them. Conversely, OVX group revealed a significant decrease in the number of PCNA positively immunostained cells in comparison with those of other groups. Moreover, sham + WGO and OVX + WGO groups exhibited a significant difference between them regarding the number of their positive PCNA immunostained cells (Table 2).

### *Scanning electron microscope analysis*

Topographical examination of the dorsal surface of the tongue of the sham group showed regular arrangement of conical shape fine pointed filliform papillae. Also, fungiform papillae with their classical appearance as dome shape eminences on the tongue surface were obvious (Figure 3A). On the other hand, the dorsal surface of the tongue in OVX group exposed cracked mucosa and appeared craggy. The filiform papillae were short and exhibited irregular arrangement while the fungiform papillae appeared ill- defined and lost their normal architecture (Figure 3B). In sham + WGO group, the filiform papillae appeared well defined conical shape and exhibited regular distribution on the dorsal surface of tongue besides normally shaped fungiform papillae similar to sham group (Figure 3C). Moreover, OVX + WGO group exhibited restoration of almost regular shape and arrangement of filiform papillae in addition to reestablishment of fungiform papillae of almost normal architecture (Figure 3D).

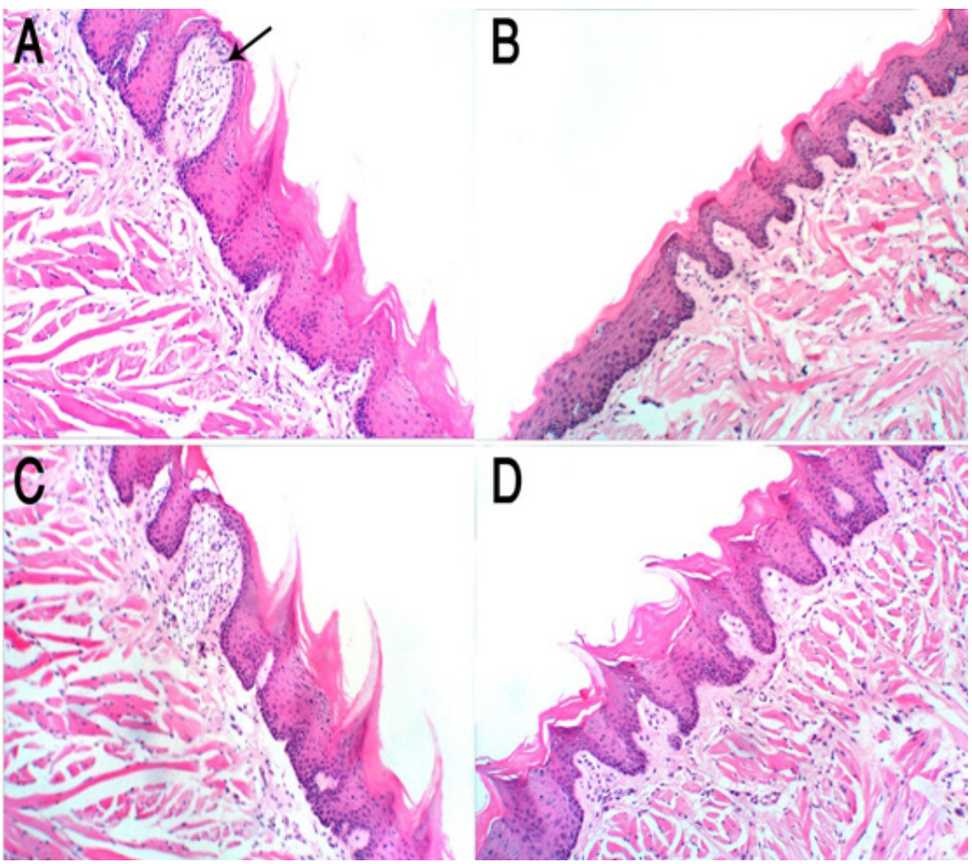


**Fig. 1:** Histological photomicrographs showing rat tongue dorsal mucosa of different groups stained with H&E stain: (A) Sham group shows numerous long finger- like projections of filiform papillae and a mushroom shaped fungiform papilla (arrow). (B) Ovx group shows ill- defined filiform papillae with irregular keratinized surface and abnormally shaped epithelial ridges. (C) Sham + WGO group shows regular filiform papillae with normal keratinization. (D) Ovx + WGO group shows regular filiform papillae with almost normal architecture. (H& E, A- D X 200).

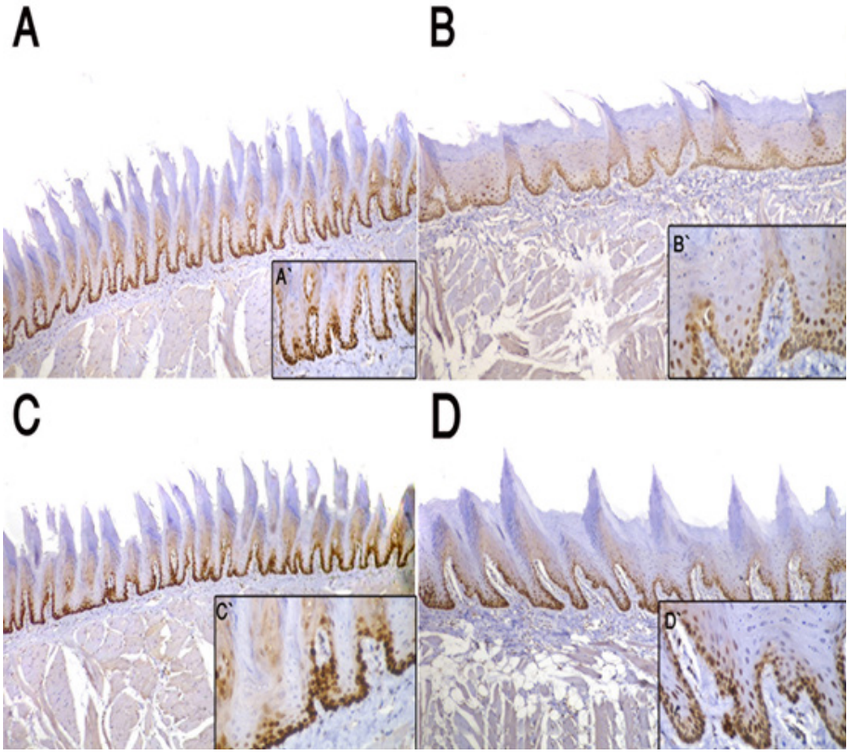


**Fig. 2:** Photomicrographs of PCNA immunohistochemical-stained sections of the rat tongue dorsal mucosa of different groups: (A, A`) Sham group shows strong PCNA immunoreactivity. (B, B`) Ovx group shows weak staining immunoreactivity to PCNA. (C, C`) Sham + WGO group shows strong PCNA immunoreactivity. (D, D`) Ovx + WGO group shows moderate to strong immunoreaction. (PCNA immunostain, A-D X 200 and A`-D`X400)

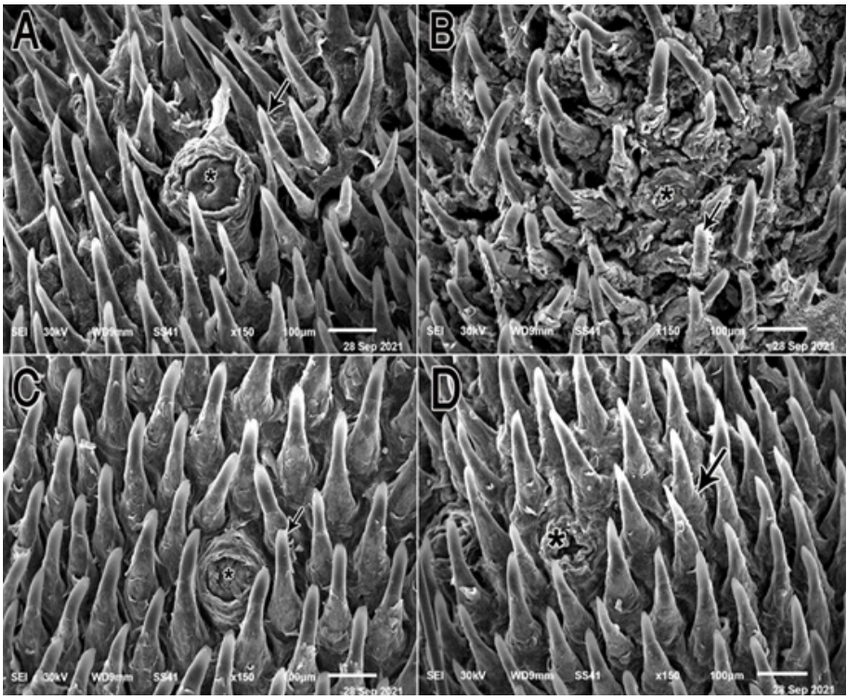


**Fig. 3:** Scanning electron micrographs of the tongue dorsal surfaces of different groups: (A) Sham group shows typical conical shape filliform papillae with regular arrangement (arrow) and normal appearance of fungiform papilla (*). (B) Ovx group shows cracked tongue surface with irregular arrangement of short filliform papillae (arrow) and ill- defined fungiform papilla (*). (C) Sham + WGO group shows regular shape of filliform (arrow) and fungiform (*) papillae. (D) Ovx + WGO group shows almost normal filliform (arrow) and fungiform (*) papillae. (Mic. Mag. A- D X 150).

**Table 1:** Statistical analysis of serum E2 level in OVX and sham operated rats

| | Sham operated rats | OVX rats |
|---|---|---|
| Range | 65 – 94 | 14 – 28 |
| Mean ± SD | 78.60 ± 12.70 | 20.20 ± 5.45 |
| T. test | 9.449 | |
| *P. value* | 0.001* | |

* means significant difference

**Table 2:** Statistical analysis of PCNA expression among groups

| | Group I Sham group) | Group II Ovx group) | Group III (Sham + WGO group) | Group IV (Ovx + WGO group) | |
|---|---|---|---|---|---|
| Range | 173 – 202 | 94 – 139 | 172 – 211 | 135 – 186 | |
| Mean ± SD | 188.20±12.28 | 117.40±17.30 | 192.40±16.41 | 167.00±19.53 | |
| F test | 21.515 | | | | |
| *P value* | 0.001* | | | | |
| I & II | I & III | I & IV | II & III | II & IV | III & IV |
| 0.001* | 0.649 NT | 0.060 NT | 0.001* | 0.001* | 0.028* |

## DISCUSSION

The current research aimed to investigate the possible changes in tongue mucosa in OVX rats and evaluate the ability of WGO in alleviating those apparent changes. To accomplish the current work, rats were the experimental model of choice. In accordance with Lelovas *et al*.[24], rats were considered a valuable model in postmenopausal research because of similarities between the human and rat in the pathophysiologic responses combined with the husbandry and financial advantages. Virgin females were utilized in this research to eliminate probable effects of lactation and pregnancy. Moreover, using them allowed eliciting an estrogen-deprived state by OVX, to serve the aim of tackling post-menopausal effects[25].

In this study, rats verified the success of OVX by significant decline in their serum E2 level consecutive to OVX which were compatible with numerous histological, immunohistochemical and topographical changes in the tongue mucosa. The histological changes included ill-defined tongue papillae, reduced epithelium thickness together with thinner keratinized surface and irregular epithelial ridges compared with other groups. These findings are in accordance with previous researches that reported increased tongue discomfort in OVX rats and detected the ability of ovarian estrogens deficiency in OVX rats to alter cells and surface histology including reduction in thickness of tongue epithelium and irregular keratinized surface[26,27].

Noteworthy, the proliferative activity of the tongue mucosa was investigated using PCNA immunohistochemical staining. The choice of PCNA in the current study was based on the evidence that PCNA is a ubiquitous cell cycle marker protein that is crucial for DNA repair, DNA replication, cell proliferation and cell cycle progression,[28]. The tongue mucosa of OVX rats exhibited significant decrease in the number of PCNA positively immunostained cells in comparison with those of other groups. This finding could be attributed to the degenerative changes caused by OVX on tongue mucosal cells. Similar result was reported by Seko *et al*. who observed significant reduction in the PCNA positive cells percentage in OVX rats indicating possible epithelial turnover periods delay which could induce oral mucosa thinning[27].

Moreover, in this study, topographical examination of tongue surface by scanning electron microscope revealed cracked tongue mucosa with irregular shape and arrangement of tongue papillae. These findings matched the previous researches reported that reduced salivary flow rate appear to be estrogen-dependent, often leaving menopausal and postmenopausal women with a chronic sense of dry mouth and suffering from atrophic tongue[29,30].

Estrogen deficiency effect on tongue mucosa is due to the presence of estrogen receptors in the oral mucosa which are affected directly by estrogen levels variations[4]. The impact of estrogen on mucosa was proved by the ability of estrogen to stimulate proliferation and differentiation of epithelial cells leading to up-regulated epithelial keratinization[31]. Multiple target genes that control cell proliferation, including c-Myc gene, may be regulated by estrogen[32]. Also, estrogen could regulate telomerase, the enzyme that regulates the lifespan of cell proliferation by preserving telomeres; so, estrogen insufficiency results in telomerase inhibition, and shortening, and decreased cell proliferation[33]. Worth mentioning, estrogen deficiency could induce oxidative stress as a result of increasing the accumulation of mitochondrial reactive oxygen species (ROS) and decreasing the effectiveness of antioxidant systems which in turn can lead to cellular damage and dysfunction[34].

Interestingly in the present work, OVX rats treated with WGO showed recovery of hypoestrogenism effect on tongue mucosa and restoration of almost normal mucosal architecture and thickness. Moreover, the number of PCNA positively immunostained cells in the OVX + WGO group simulated that of shame group and displayed significant increase in comparison with those of OVX group. This improvement in tongue mucosa could be explained by the beneficial antioxidant activity of WGO which could be attributed to its high content of α-tocopherol, the most effective form of vitamin E, which protects DNA and cell membranes from oxidative damage[11]. α-tocopherol functions not only as the most potent naturally occurring scavenger of ROS, but also it has cell signaling and gene regulatory functions[35]. As well, WGO is rich in fat-soluble carotenoids especially; beta-carotene, lutein, and zeaxanthin which have antioxidant activity and are known to prevent health problems associated with oxidative stress[11]. Moreover, WGO is rich in spermidine which is a naturally occurring polyamine exhibiting anti-aging properties through regulation of cell growth, proliferation and death[36]. Besides, WGO contains phytosterols which

are non-steroidal compounds that are functionally or structurally resembling estrogens and may attach to estrogen receptors, mimicking the conformational structure of estradiol and work as partial agonists, agonists, or antagonists inducing estrogen-responsive gene products[37].

## CONCLUSION

Estrogen deficiency has adverse effects that could harm tongue dorsal surface. Due to its considerable beneficial ingredients, WGO displayed its ability to alleviate the degenerative effects caused by estrogen deficiency and restore the normal tongue mucosal architecture, which consider it a prospective treatment for postmenopausal mucosal changes.

## CONFLICT OF INTERESTS

There are no conflicts of interest.

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**الملخص العربى**

# التأثير المحتمل لزيت جنين القمح على الغشاء المخاطي للِّسان في الفئران منخفضة الاستروجين

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**خلفية الدراسة:** يُعد سن انقطاع الطمث مرحلة فسيولوجية طبيعية في حياة المرأة. قد يتأثر تجويف الفم باضطرابات الغدد الصماء المرتبطة بسن انقطاع الطمث؛ خصوصًا نقص الهرمون الستيرويدي الجنسي.

**الهدف من الدراسة:** فحص التأثير المحتمل لزيت جنين القمح على الغشاء المخاطي في ألسنة الفئران أثناء نقص هرمون الإستروجين التجريبى بعد سن اليأس.

**المواد ومنهج الدراسة:** تم استخدام أربعون من إناث الفئران العذارى تبلغ من العمر ستة أشهر. قُسمت الفئران عشوائيًّا إلى أربع مجموعات: مجموعة أُجريت لها جراحة وهمية و هى المجموعة الضابطة ، ومجموعة أُجريت لها جراحة وهمية وتلقت المعالجة بزيت جنين القمح لمدة اربع اسابيع، ومجموعة مُستأصَلة المبايض لم تتلقَّ اى علاج، ومجموعة مُستأصَلة المبايض تلقت المعالجة بزيت جنين القمح لمدة اربع اسابيع. وبعد انتهاء التجربة تم التضحية بالفئران وتم أخذ عينات من ألسنة الفئران وتجهيزها لإجراء صبغات الهيماتوكسيلين والأيوسين، والتلوين الكيميائي النسيجي المناعي باستخدام المستضد النووي للخلايا المتكاثرة, والفحص بالميكروسكوب الإلكترونى الماسح.

**النتائج:** من الناحية النسيجية، أظهرت النتائج أن الغشاء المخاطي للِّسان في مجموعة الفئران مُستأصَلة المبايض التي لم تتلقَّ المعالجة قد ظهرت عليه حُليمات خيطية شاذة الشكل بالمقارنة بالغشاء المخاطي للِّسان في كلٍ من مجموعة الفئران التي أُجريت لها جراحة وهمية ولم تتلقَّ المعالجة ومثيلتها التي تلقت المعالجة بزيت جنين القمح. ومن المثير للاهتمام أن مجموعة الفئران مُستأصَلة المبايض التي تلقت المعالجة بزيت جنين القمح أظهرت تحسنًا في الغشاء المخاطي للِّسان حيث بدا ذو بِنية طبيعية إلى حدٍ كبير. وفيما يتعلق بالتعبير الكيميائي النسيجي المناعي، أظهرت النتائج أن تعبير المستضد النووي للخلايا المتكاثرة في مجموعة الفئران مُستأصَلة المبايض التي لم تتلقَّ المعالجة بزيت جنين القمح كان أقل من باقى المجموعات, بينما مجموعة الفئران مُستأصَلة المبايض التي تلقت المعالجة بزيت جنين القمح أظهرت زيادة فى تعبير المستضد النووي للخلايا المتكاثرة بالمقارنة بمثيلتها التى لم تتلقَّ المعالجة بزيت جنين القمح .

**الإستنتاجات:** أثَّر نقص هرمون الإستروجين على الغشاء المخاطي للِّسان بصورة كبيرة محدثًا تغييرات ضارة فى انسجته, ومع ذلك، كان زيت جنين القمح قادرًا على إبطال تلك الآثار. ومن هنا تظهر التوصية باستخدام زيت جنين القمح للسيدات بعد انقطاع الطمث كعلاج متوقع لتغيرات الانسجة.