

Cell and Organ Transplantation. 2022; 10(1): 54-60.
<https://doi.org/10.22494/cot.v10i1.138>

Morphometric analysis of skin in ovariectomized mice of different ages



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ABSTRACT

It is known that the deficiency of estrogens of any origin (physiological menopause, surgical removal of the ovaries, etc.) accelerates the aging processes of both the body in general and the skin in particular in women. Studies of the morphometric properties of skin caused by estrogen-deficient disorders may help in the development of more effective regenerative medicine approaches aimed at treating and preventing age-related skin changes.

THE PURPOSE of the study is to determine the morphological changes in the skin of young and old CBA/Ca mice with estrogen deficiency on the ovariectomy model.

MATERIALS AND METHODS. Skin samples from young (4 months) and old (16 months) mice two months after ovariectomy were compared with age-matched sham-operated mice. The thickness of the epidermis and dermis, the number of hair follicles, and sebaceous glands were determined on histological sections of the skin.

RESULTS. It was shown that the thickness of the epidermis was significantly smaller both in the group of young and in the group of old ovariectomized mice compared to the thickness of the epidermis of the control group of animals of the corresponding age. A significant increase in the thickness of the dermis was found in the group of young ovariectomized mice in comparison with the thickness of the dermis in the young control group. However, in the group of old ovariectomized mice, the thickness of the dermis did not differ significantly compared to the age-matched control group. No significant differences were found in the number of sebaceous glands and hair follicles in the studied groups.

CONCLUSION. Ovariectomy, as a model of artificial estrogen deficiency, is accompanied by significant changes in the epidermis in young and old animals and the dermis in young ovariectomized animals, without affecting the number of sebaceous glands and hair follicles.

KEY WORDS: skin; hair follicles; sebaceous glands; ovariectomy; morphometry

The skin is a unique organ that performs many functions, including protection against infection and ultraviolet light, temperature regulation, water balance control, and removal of harmful substances. Metabolic, genetic, hormonal, and environmental factors influence skin condition [19, 52].

At the same time, the skin is a hormone-dependent organ where receptors for many hormones, in particular estrogens, are located [6,37]. Sex hormones significantly affect the development of the skin and its derivatives, the chemical composition of the sebaceous glands and the water-lipid mantle, so their physiological levels are necessary to maintain the structural integrity and functional capabilities of the skin. Estrogens are the main hormones of the female body, which are formed from cholesterol in the ovaries [4].

Through binding to specific receptors (found in many dermal structures – keratinocytes, Langerhans cells, melanocytes, fibroblasts, seba-

ceous glands, hair follicles and blood vessels), estrogens are involved in the regulation of many functions of skin cells, including proliferation, morphogenesis, differentiation and apoptosis [5]. Estrogens affect the thickness, elasticity of the skin, its hygroscopic properties, blood supply, barrier function, hair growth [21].

Physiological removal of the ovaries (menopause) or surgical removal (ovariectomy) leads to the development of estrogen-deficient states of varying degrees of severity: from mild manifestations to severe functional disorders in the female body [3,4]. Levine et al. indicate that premature loss of ovarian function leads to increased DNA methylation, which is a well-known biological marker of accelerated aging [26].

Estrogen-deficient skin (EDS) is characterized by the appearance of dry skin, skin atrophy, a decrease in collagen content, water content, which is accompanied by a decrease in elasticity, barrier function, the appearance of wrinkles, poor wound healing. Most women begin to feel a

decrease in attractiveness and even psychological health [15, 35].

Slowing down of cell regeneration processes is one of the key factors of morphological changes in the epidermis and dermis during chronological aging. The proliferative potential of keratinocytes of the epidermis decreases, the number and functional activity of melanocytes and fibroblasts reduces with age [2, 44, 53].

Age-related decline in the functional activity of epidermal and hair follicle stem cells (SCs) is well known [16, 29]. Involutional skin changes are very closely related to the function of stem cells, so the use of these cells in antiaging strategies, as well as the treatment of patients using stem cells or their differentiated derivatives can be a universal technique in regenerative and aesthetic medicine [51].

The **PURPOSE** of the study is to determine the morphological changes in the skin of young and old CBA/Ca mice with estrogen deficiency on the ovariectomy model.

MATERIALS AND METHODS

The experimental study was performed on female CBA/Ca mice: young 2 months old (average body weight 19.8 g) and old 14 months old (average body weight 22.5 g) from the vivarium of the D. F. Chebotarev State Institute of Gerontology of the National Academy of Sciences of Ukraine. Mice were kept under standard conditions with a fixed light regime of 12:12 hours and received a standard diet and water *ad libitum*. The animals were divided into 4 groups of 4 mice each: young (ovariectomized and control) and old (ovariectomized and control).

Animals were anesthetized by intraperitoneal injection of a 2.5 % solution of 2,2,2-tribromethanol (*Sigma*, USA) at a dose of 400 mg/kg, and bilateral ovariectomy was performed in compliance with all rules of aseptic and antiseptics. Animals of the appropriate age, which underwent a sham operation – a same surgery without removing the ovaries served as control groups. The wounds were sutured in layers; the animals were kept under a heater until coming out of anesthesia. 60 days after ovariectomy, the animals were euthanized by cervical dislocation under anesthesia with a 2.5 % solution of 2,2,2-tribromethanol 400 mg/kg. After euthanasia, skin from the mice back was excised for morphological examination.

Histological processing of the skin was performed according to the standard procedure [1]. Skin samples were fixed in a 10 % solution of neutral formalin for 24 hours. Formalin-fixed tissues were dehydrated by placed in ethyl alcohol, gradually increasing the concentration from 70 % to 96 %, and then embedded in paraffin blocks (*Leica Biosystems Richmond, Inc.*, USA). Histological sections with a thickness of 5-6 μm were prepared on a rotary microtome HN 325 (*Thermo Scientific*, USA). Hematoxylin-eosin staining was used and sections were analyzed on a BX-41 microscope (*Olympus*, Japan), photographed with a SP-500 UZ digital camera (*Olympus*, Japan) with subsequent image processing using the software DP-soft ver. 3.1 (*Olympus*, Japan).

Morphometric analysis was performed using Image J software (*Wayne Rasband, NIH*, USA). The thickness of the epidermis (μm), dermis (μm), the number of hair follicles and the number of sebaceous glands on the section area (units/mm^2) were determined.

The Statistica v 7.0 (*Statsoft Inc.*, USA) software was used for statistical analysis of the obtained results. Arithmetic mean values of parameters and their standard errors in groups ($M \pm m$) were calculated. Comparison of parameters between groups was carried out using the non-parametric Mann-Whitney test. Differences were considered reliable when reaching the level of significance $p < 0.05$.

RESULTS AND DISCUSSION

The epidermis in the experimental groups is represented by 2-4 layers of epithelial cells. On the surface of the epidermis, areas of delamination of keratinized cells are observed in old ovariectomized and old animals

of the control group (Figs. 1, 2). The dermis in all groups of animals is represented by numerous collagen fibers, fibroblasts, and single lymphocytes. Histological analysis of skin sections shows the presence of multiple swellings between collagen fibers in the group of old ovariectomized, old control, and young ovariectomized animals (Figs. 1, 2, 3). Sebaceous glands have a multilobular structure and unequal size. In many lobes of the sebaceous glands, there is an absence of secretion in the group of old ovariectomized and old control groups (Figs. 1, 2). Hair follicles in the experimental groups did not change.

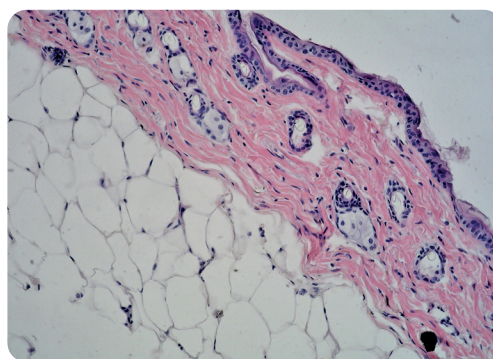


Fig. 1. Photomicrograph of a histological section of the skin of ovariectomized old mouse. Light microscopy, hematoxylin and eosin staining; ob. x20, oc. x10.

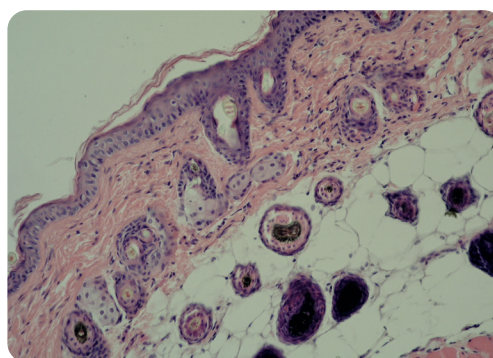


Fig. 2. Photomicrograph of a histological section of the skin of an old mouse (control group). Light microscopy, hematoxylin and eosin staining; ob. x20, oc. x10.

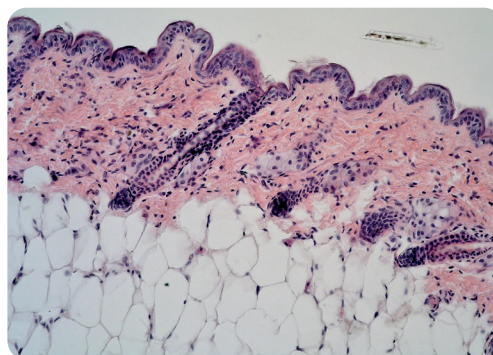


Fig. 3. Photomicrograph of a histological section of the skin of ovariectomized young mouse. Light microscopy, hematoxylin and eosin staining; ob. x20, oc. x10.

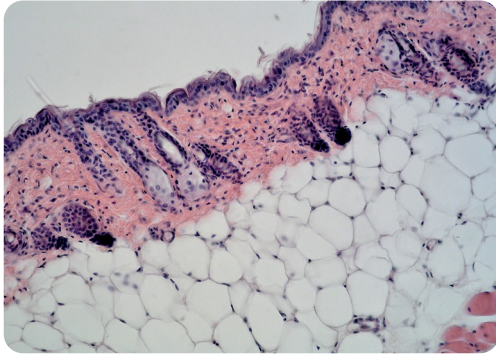


Fig. 4. Photomicrograph of a histological section of the skin of a young mouse (control group). Light microscopy, hematoxylin and eosin staining; ob. x20, oc. x10.

The thickness of the epidermis differed depending on the experimental group. In young ovariectomized mice, the thickness of the epidermis ($11.5 \pm 0.6 \mu\text{m}$) was significantly less ($p < 0.05$) compared to the control group without ovariectomy ($15.2 \pm 1.0 \mu\text{m}$) (Fig. 5). The thickness of the epidermis in the group of old ovariectomized animals ($12.1 \pm 2.0 \mu\text{m}$) was significantly less ($p < 0.05$) compared to the control group ($19.8 \pm 2.4 \mu\text{m}$).

This confirmed the data of other researchers. In particular, Yue Chen et al. showed that the thickness of the epidermis decreases in ovariectomized mice compared to controls [13]. Zaki's study shows that the thickness of the epidermis is significantly reduced in old rats [56].

According to the obtained data, the thickness of the dermis also differed in the experimental groups (Fig. 6). Thus, in young ovariectomized mice, the thickness of the dermis ($98.5 \pm 5.9 \mu\text{m}$) was significantly higher ($p < 0.05$) compared to the control group ($81.6 \pm 2.3 \mu\text{m}$). In the group of old ovariectomized animals, the thickness of the dermis ($83.4 \pm 8.1 \mu\text{m}$) was not significantly different from the control group ($95.9 \pm 7.7 \mu\text{m}$), but we observed a tendency of the dermis thickness to decrease.

The data obtained by us do not coincide with the data of other researchers. Yue Chen et al. demonstrated that the thickness of the dermis in ovariectomized mice was comparable to that in control mice [13]. According to Zaki, the thickness of the dermis decreased in adult and old groups of rats [56]. In postmenopausal women, there is a decrease in collagen content in the skin of the thighs by 2 % per year during the 15 years of menopause [10]. The gradual decrease of collagen in the skin is accompanied by a decrease in the thickness of the dermis, a decrease in the resistance of the skin to mechanical damage in response to the action of traumatic factors.

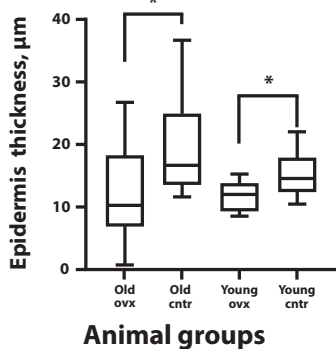


Fig. 5. The average thickness of the epidermis on histological sections of the skin in animals of experimental groups, μm . Notes: ovx – ovariectomized; * – $p < 0.05$

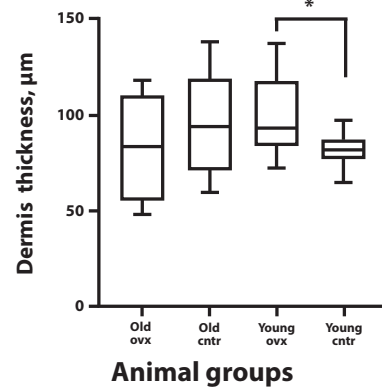


Fig. 6. The average thickness of the dermis on histological sections of the skin in animals of experimental groups, μm . Notes: ovx – ovariectomized; * – $p < 0.05$

It is known that estrogen deficiency is accompanied by structural changes in the dermis, such as: a decrease in sebum production, collagen content, skin thickness and elastin fibers [55]. The decrease in skin thickness in conditions of estrogen deficiency is mostly due to the loss of skin collagen [10], as well as a decrease in the rate of collagen deposition [8]. According to Affinito et al., the level of skin collagen of types I and III decreases by 30 % in the first five years after menopause [7]. Levels of collagen type I (80 %) and type III (15 %), which are most abundant in the skin, correlate with estrogen levels [45]. Moreover, systemic and local hormone replacement therapy with estrogens increases collagen synthesis in conditions of estrogen deficiency [12, 14, 54]. According to researchers, the peak of collagen content in women's skin is observed between the ages of 25 and 34, followed by a gradual decline. According to a study by Sibilla et al, collagen content was reduced in postmenopausal women compared to premenopausal women [46].

It is known that estrogen-deficient skin is accompanied by dryness due to a decrease in the activity of sebaceous glands, skin hydration, and a violation of the barrier properties [15]. Morphometric analysis of sebaceous glands (Fig. 7) showed no significant changes in their number in the group of old ovariectomized animals ($28.2 \pm 5.3 \text{ units/mm}^2$) in comparison with the control group ($26.0 \pm 1.8 \text{ units/mm}^2$) and in the group of young ovariectomized animals ($27.4 \pm 6.1 \text{ units/mm}^2$) in comparison with the young control group ($24.0 \pm 6.6 \text{ units/mm}^2$).

Individual sections of the sebaceous glands do not contain their secretion and appear optically empty in a histological skin sample. The counting of empty sebaceous glands shows (Fig. 8) similarly the absence of significant changes in their number in the group of old ovariectomized animals ($2.8 \pm 0.6 \text{ units/mm}^2$) compared to the control group ($8.1 \pm 3.3 \text{ units/mm}^2$), and in the group of young ovariectomized animals ($1.2 \pm 1.2 \text{ units/mm}^2$), compared to the young control group ($1.4 \pm 0.9 \text{ units/mm}^2$).

It is known that after menopause, the activity of sebaceous glands in women gradually decreases [52]. According to Zaki et al., the number of sebaceous glands decreased with age in rats, but there were no obvious morphological changes in the glands in adult and old rats [56].

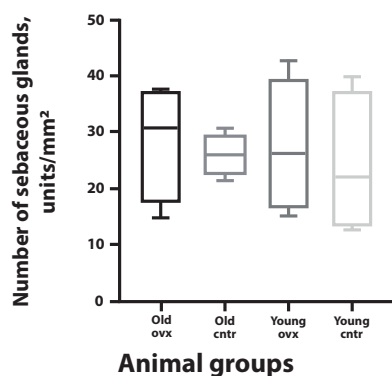


Fig. 7. Average number of sebaceous glands on histological sections of skin in animals of experimental groups, unit/mm². Note: ovx – ovariectomized.

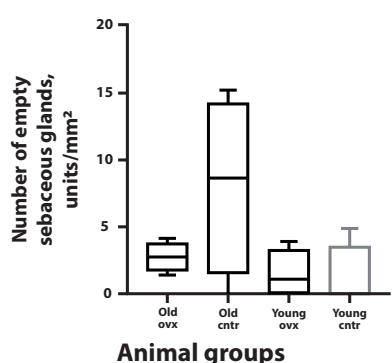


Fig. 8. The average number of empty sebaceous glands on histological sections of the skin in animals of experimental groups, units/mm². Note: ovx – ovariectomized.

The number of hair follicles did not significantly differ (**Fig. 9**) in the group of young ovariectomized patients (61.1 ± 9.3 units/mm²) relative to the parameter (57.8 ± 18.5 units/mm²) of the young control group, and in the group of old ovariectomized animals (31.6 ± 8.1 units/mm²) versus (33.5 ± 4.1 units/mm²) in the group of old control animals.

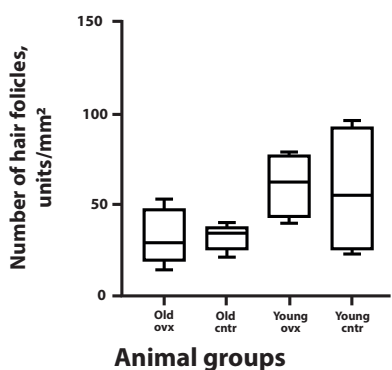


Fig. 9. The average number of hair follicles on histological sections of the skin in animals of experimental groups, units/mm². Note: ovx – ovariectomized.

The data we obtained do not coincide with other studies. Thus, according to a study by Zaki et al., the number of hair follicles in rats decreased in adults and old animals compared to young animals [56].

Premature ovarian failure is a relatively common condition affecting

1-3 % of adult women under the age of 40 [22]. The ovaries of patients with premature ovarian failure are characterized by the loss of secondary follicles and the cessation of folliculogenesis, which leads to a decrease or cessation of estrogen production and infertility. The mechanism of depletion of ovarian follicles is associated with accelerated atresia of follicles, but the specific pathogenesis is not fully understood yet [18].

Estrogen-deficient conditions cause acceleration of the biological aging of the body, in particular the skin [38]. Hormonal changes in women change the external condition of the skin, its structure and functions, which manifests in the form of dryness, the appearance of wrinkles, and atrophy [41]. Aging of the skin against the background of low levels of estrogen in the blood also adds to the negative impact on the psycho-emotional state of women and behavior in modern society.

According to scientists' forecasts, women will live about one third of their lives in a state of potential estrogen deficiency [34]. Therefore, it is extremely important to study the changes in the female body that occur against the background of estrogen deficiency and choose therapy aimed at compensating for estrogen deficiency, for example, the prescription of hormone replacement therapy with estrogens [57].

The ovariectomy model is widely used to study the effect of estrogen hormone deficiency on the female body [20]. Artificially induced estrogen deficiency causes various changes both at the level of the body and at the level of the skin in women (the changes are described above). Systemic and local treatment programs are used to eliminate the clinical manifestations of estrogen deficiency and improve the quality of life.

The following types of therapy are used to correct the changes occurring in women's bodies:

- systemic hormone replacement therapy with estrogens (HRT),
- topical hormone therapy with estrogens (creams, ointments, patches),
- phytoestrogen therapy,
- bioidentical hormones,
- cosmeceuticals,
- regenerative cellular products.

The prescription of menopausal hormone therapy requires a thorough examination of the patient (clinical blood test, coagulogram, examination by a gynecologist, breast specialist, ultrasound examination of the vessels of the lower extremities), and is associated with an increase in the risk of oncological diseases (endometrium, ovaries, breast), stroke, thromboembolic diseases [33, 40, 43].

Locally applied fat-soluble molecules of estradiol are well absorbed through the epidermis and have a positive effect on the condition of the skin [28, 40]. However, long-term local use of estrogen in the form of a cream or gel in combination with systemic estrogen therapy can lead to an overdose of hormones [48, 50].

The term "bioidentical hormone" refers to a compound with the same molecular structure as a hormone of endogenous origin. However, pills, gels, and suppositories, which consist of several hormones – estradiol, estrone, estriol, progesterone, testosterone, and dehydroepiandrosterone (DHEA) – are gaining practical application. Such complex hormonal systems require careful monitoring and prescription only by a medical professional [39, 42].

Cosmetic products contain a number of antioxidants of plant origin, and they also meet the FDA safety criteria for substances that can be introduced into products for topical use without a prescription [24, 25].

Many studies have examined the effects of polyphenols, which are also known as phytoestrogens, on skin conditions in menopausal women [27]. Topical application of 4 % Genistein in the gel form to the facial skin of postmenopausal women contributed to an increase in the thickness of the epidermis by 20 % according to morphological research [32]. According to the study of Silva et al. topical use of estradiol and Genistein increased facial collagen type I and III in postmenopausal women in both groups. The increase in collagen concentration was significantly higher in the group treated with topical estradiol [47].

Genistein belongs to the group of isoflavones. They have a structural

similarity to the 17-beta-estradiol, can interact with the estrogen receptor and are therefore classified as phytoestrogens [36]. Isoflavones have a high affinity for estrogen receptor β , which is more common in the skin, bones, and cardiovascular system, and a low affinity for estrogen receptor α , which is found in the mammary glands and uterus [11, 17]. Therefore, due to the ability of tissue selectivity, isoflavones are also considered to be selective modulators of estrogen receptors [30].

Human stem cells play a key role and have important potential applications in cell therapy, which is a possible alternative for various diseases, including reproductive dysfunction. The use of bone marrow mesenchymal stem cells (BM-MSCs) can restore the structure and function of damaged ovarian tissues [9, 16, 31]. According to a study by Igboeli P. et al, injection of autologous BM-MSCs into the ovary of women resulted

in an approximately 50 % increase in ovarian volume compared to contralateral control ovaries that persisted until the end of the study (1 year). Serum estrogen levels increased by approximately 150 % compared to preoperative levels. The patients experienced menstrual episodes and also reported a marked improvement in menopausal symptoms, which also persisted until the end of the study (1 year). The procedure of BM-MSCs transplantation was very well tolerated; no side effects were reported [22].

The development of strategies for both systemic treatment and topical agents for age-related skin changes is extremely important for improving the quality of women's life in the perimenopausal and menopausal periods, as well as for the implementation of achievements in clinical aesthetic medicine [23].

CONCLUSION

Ovariectomy is accompanied by significant morphological changes of the dermis and epidermis in female CBA/Ca mice. A probable decrease in the thickness of the epidermis was established both in the group of young and in the group of old ovariectomized mice in comparison with control animals of the corresponding age. A significant increase in the thickness of the dermis was found in the group of young ovariectomized mice compared to the thickness of the dermis in the young control group, however, in the group of old ovariectomized mice, the thickness of the dermis did not differ significantly compared to the age-matched control group. It was found that there were no differences in the number of sebaceous glands and hair follicles in both young and old ovariectomized mice compared to age-matched control groups. There were foci of edema between collagen fibers in a group of old ovariectomized, old control and young ovariectomized mice.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

УДК 611.77:618.11-089.87+612.67

Морфометричний аналіз шкіри у оваріоектомованих мишей різного віку

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РЕЗЮМЕ

Відомо, що дефіцит естрогенів будь-якого походження (фізіологічна менопауза, хірургічне видалення яєчників тощо) прискорює у жінок процеси старіння як організму загалом, так і шкіри зокрема. Дослідження морфометричних властивостей шкіри, викликаних естрогендефіцитними станами, можуть допомогти у розробці більш ефективних підходів регенеративної медицини, спрямованих на лікування та попередження вікових змін шкіри.

МЕТА ДОСЛІДЖЕННЯ: визначення морфологічних змін у шкірі мишей лінії CBA/Ca молодого та старого віку з дефіцитом естрогенів на моделі оваріоектомії.

МАТЕРІАЛИ ТА МЕТОДИ: Досліджували зразки шкіри молодих (4 міс.) та старих (16 міс.) мишей через два місяці після оваріоектомії у порівнянні зі зразками від тварин відповідного віку з псевдооперацією. На гістологічних препаратах шкіри визначали товщину епідермісу та дерми, кількість волосяних цибулин, сальних залоз.

РЕЗУЛЬТАТИ. Показано, що товщина епідермісу була достовірно меншою як у групі молодих, так і у групі старих оваріоектомованих мишей порівняно з товщиною епідермісу контрольної групи тварин відповідного віку. Встановлено достовірне збільшення товщини дерми у групі молодих оваріоектомованих мишей в порівнянні з товщиною дерми у молодій контрольній групі, проте у групі старих оваріоектомованих мишей товщина дерми достовірно не відрізнялася щодо контрольної групи відповідного віку. Не було знайдено достовірних відмінностей у кількості сальних залоз та волосяних цибулин у досліджуваних групах.

ВИСНОВКИ. Оваріоектомія, як модель штучного дефіциту естрогенів, супроводжується суттєвими змінами епідермісу у молодих та старих тварин та дерми у молодих оваріоектомованих тварин, не впливаючи на кількість сальних залоз та волосяних цибулин.

КЛЮЧОВІ СЛОВА: шкіра, волосяні цибулини, сальні залози, оваріоектомія; морфометрія.