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REACTIVITY OF OVARIECTOMISED FEMALE RATS AFTER ADMINISTRATION OF INJECTABLE OESTROGENS BY TEM MICROSCOPY

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ABSTRACT. The purpose of this electrone microscopy study was to identify and specify structural and ultrastructural changes occurring in the vulvar epithelium of ovariectomised female rats, as well as their reactivity to the administration of injectable oestrogens. We used 30 female Wistar white rats, distributed in four groups with 1 control group, to which oestrogenic treatment was administered. The hormone replacement therapy with injectable oestrogens (Estradiol, Estradurin, Sintofolin), at a dose of 0.2 mg/rat/day was administered for 14 days. Afterwards, all animals were sacrificed and vulvar biopsies were taken, which were then processed using optical microscopy (the semithin section technique) and transmission electron microscopy (TEM) techniques. This study showed that injectable oestrogen treatment over a period of 14 consecutive days enables the recovery of each tissue layer, with regard to the structural and ultrastructural modifications arising in ovariectomised female rats.

Keywords: *oestrogens, optical microscopy, transmission electron microscopy, structure, atrophy, vulvar hyperplasia*

INTRODUCTION

Variations in estrogen levels strongly affect cell growth and metabolism in a variety of tissues including the vulvo-vaginal epithelium, the ovary and uterus.

Estradiol is biosynthesized from progesterone, also produced from cholesterol, via intermediate pregnenolone. One principal pathway then converts progesterone to its 17-hydroxy derivative, 17-hydroxyprogesterone,

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and then to Δ4-androstenedione via sequential cytochrome P450-catalyzed oxidations. The action of an aromatase on Δ4 -androstenedione generates estrone, which is then metabolized by a dehydrogenase to the final compound, 17β-estradiol [1].

The reaction to injectable oestrogen therapy is quick, due to the presence of a large number of oestrogenic receptors, either ER-α or ER-β, in the genital tract, especially in the vulvar area [2,3].

The lack of of estrogen production characteristic to menopause is responsible for the various signs and symptoms, which frequently compromise the quality of life [4,5]. Of these, vulvar atrophy is highly prevalent and does not resolve spontaneously in time unlike other symptoms such as the vasomotor ones [6,7].

The vulvar epithelium reacts to various hormonal influences, much like the entire female genitive tract, i.e. the cells at this level have a specific type of receptivity to female sexual hormones and suffer structural and functional modifications due to the decrease and, even more, lack of such hormones [8,9].

During the metabolism of female hormones, many enzymes catalyse reactions such as aromatisation, oxidation, reduction, sulfonation, desulfonation, hydroxylation and methoxylation. These enzymes must all recognise and bind oestrogen but they have diverse structures [10].

Figure 1 shows the chemical structure of the injectable substances used in this study.

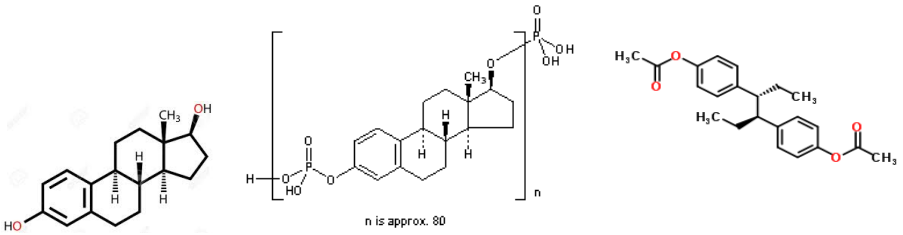

Figure 1. Structure for: a) Estradiol; b) Estradurin, (polyestradiol phosphate); c) Sintofolin (Hexestrol diacetate)

The novelty of this study is the possibility to investigate by new modern methods and a proprietary protocol the effects of estrogen injection on the vulvar structure.

In this study, we attempted to identify the structural and ultrastructural changes of the vulvar epithelium of ovariectomised female rats through optical microscopy and transmission electron microscopy, as well as their response to the administration of injectable oestrogens.

RESULTS AND DISCUSSION

Surgically induced menopause was demonstrated through postoperative estradiol level measurements, with statistically significant differences between the study groups. (Fig.2.)

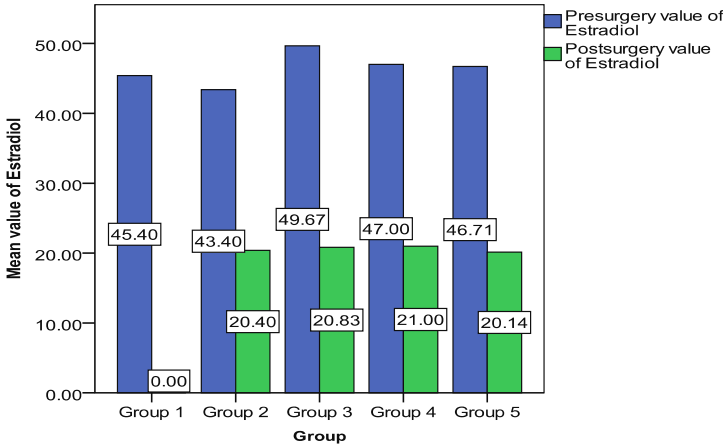

Figure 2. Comparing pre/postoperative estradiol values

The vulvar epithelium of group 1 had a normal structure on the semi thin section technique (Fig.3A).

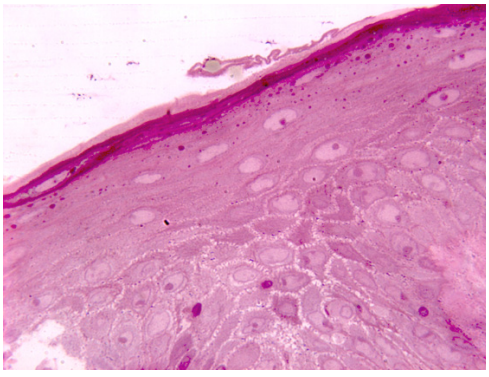

Figure 3A. Light microscopy micrograph showing a normal aspect of vulvar epithelium, containing partially keratinized cells of the basal, spinosum and superficial layers. Ob X 100.

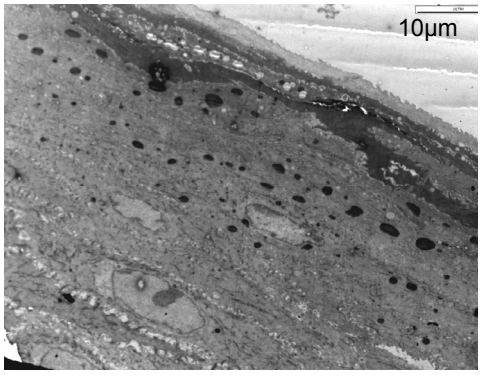

Figure 3B. Electron micrograph (TEM) image of vulvar epithelium showing normal ultrastructural aspects of cells components. Keratohyalin granules are apparent in superficial layer.

In group 2, however, we found both structural and functional changes of the vulvar epithelium due to ovariectomy. Cells in division are relatively scarce, or may even lack in some areas of the basal layer, suggesting the poor regenerative activity of the cells in this layer. In some areas we even found alterations of the entire epithelium, including the destruction of some cells of the basal layer. The superficial layer per se was absent. We even found some signs of destruction of the spinous layer. It is typical for the intercellular space of the spinous layer to be very dilated and include numerous damaged desmosomes, which leads to weak cohesion between cells and the potential disruption or destruction of the entire spinous layer. Of note is the presence of numerous blood vessels, especially veins, most of which are congested or overburdened with erythrocytes (Fig.4A).

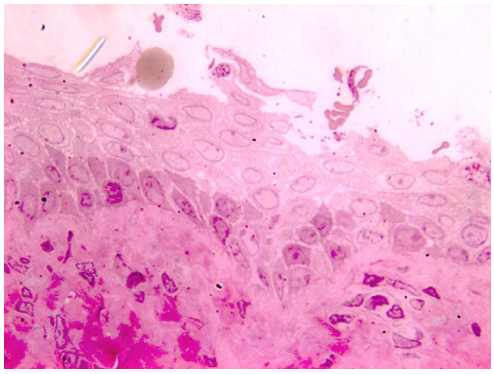

Figure 4A. Micrograph of vulvar (labia minor) epithelium from ovariectomized rats showing structural alterations with partially desquamated cells of the superficial layer and the disappearance of keratohyalin granules. Ob. X 100.

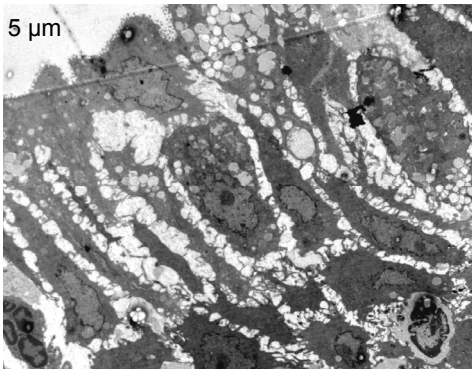

Figure 4B. Electron micrograph picture of labium minor (vulvar) epithelium from ovariectomized rats. Ultrastructural alterations of desmosomes between cells leading to larger intercellular spaces and loss of cohesion between epithelial cells.

In groups 3, 4 and 5, contrary to group 2, the semithin sections reveal hyperplasia of all cellular layers of the vulvar epithelium. Such findings indicate hyperplasia occurring in the vulvar tissues, with the regeneration and, respectively, preservation of structures in similar conditions to those of animals from group 1. We found relatively numerous cells in division, suggesting that the administration of injectable oestrogens had the ability to stimulate cell regeneration in the basal layer. We also noted the presence of Merkel cells, indicating the fact that this layer of cells not only had a normal structure, but that their metabolic activity had actually been stimulated. The granular layer was poorly represented in the normal epithelium and lacked completely in group 2, of ovariectomised rats. On the other hand, it appears that in this case, the administration of oestrogens

stimulated the synthesis of keratin granules and their accumulation in the form of a distinctive granular layer, and then as a keratinised superficial layer, even slightly horned in the external, apical area of the vulva. In the upper part of the chorion, towards the basal membrane of the epithelium, we found several elastic and collagen connective fibres, as well as a relatively numerous cell population, including fibroblasts, fibrocytes, eosinophils and mast cells (Fig.5A).

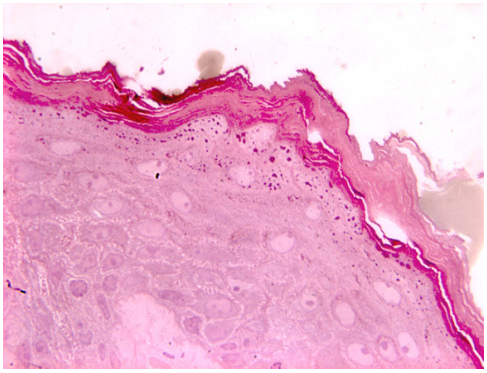

Figure 5A. Micrograph of vulvar epithelium from ovariectomized rats treated with oestrogen showing the protective effects of oestrogens. All cells in the epithelium appear to have a normal structure. Keratohyaline granules and the keratin layer are evident. Ob. X 100.

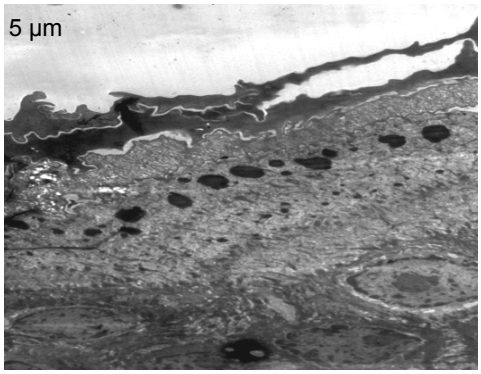

Figure 5B. Electron micrograph of labia minor (vulvar) epithelium from ovariectomized rats treated with oestrogen showing the protective effects of oestrogens. All cells have almost normal ultrastructure and the keratohyaline granules and keratin layer are present.

The ultrastructural studies using transmission electron microscopy (TEM) in group 1 revealed a normal ultrastructure of all layers of the vulvar epithelium (Fig.3B).

In group 2, the electron microscopy images showed multiple complex changes brought on by ovariectomy, visible in all cells of the vulvar epithelium, as well as in all components of the chorion. In the spinous layer, it was apparent that intercellular spaces were expanded due to the depletion of desmosomes, leading to a weakened intercellular cohesion. There were no granular or keratinised superficial layers, and the last row of cells in the spinous layer reached the surface of the epithelium. The entire chorion was congested, to the extent that all its cellular and fibre components had a dense aspect, with several alterations. It is interesting, however, that some of the eosinophils and neutrophils were in a process of alteration, with a ruptured cell membrane and granules scattered in their vicinity. Smooth muscle cells were rare and separated by large spaces, occupied chiefly by eosinophils and neutrophils, but also by mast cells (Fig .5B).

In all groups treated with oestrogens, i.e. groups 3, 4 and 5, we found that the injectable oestrogen treatment lead to the protection and regeneration of the vulvar structures affected by ovariectomy, but not in all areas. Hyperplasia was present more evidently in the chorion than in the epithelium. It is certain that in the epithelium, the oestrogen treatment led to an increase in keratin synthesis, suggested by the presence of an evident granular layer. The cytoplasm of cells appeared dense because of the abundance of ribosomes, small granules of keratin, mitochondria, tonofilaments and even the presence of lipid droplets. In the chorion, there were abundant elastic and collagen fibres, along with a large number of fibroblasts, fibrocytes, mast cells and eosinophils. Furthermore, we found blood vessels and myelinated and non-myelinated nerve fibres of sensitive formations, probably Meissner corpuscles. Such nerve fibres were also present in areas of lipid accumulation, either in large deposits or in small droplets, belonging to sebaceous glands and probably located on the external and basal side of the vulva (Fig.4B).

Optical microscopy and transmission electron microscopy are particularly useful methods in the study of the microanatomy of the genital tract, facilitating the in-depth understanding of the structural and ultrastructural modifications occurring at this level during menopause [11,12].

This experimental study showed that bilateral ovariectomy triggers important and complex changes in the vulvar epithelium [13,14]. The experimental model of hormone replacement therapy with surgically induced menopause through bilateral ovariectomy in female rats is well known [15,16].

Our results on the study of semithin sections from a normal vulvar epithelium, found multiple and complex changes induced by ovariectomy in group 2 rats. These changes, both structural and functional, occurred in every layer of the vulvar epithelium: relatively scarce cells in division, sometimes completely absent in some areas of the basal layer, suggesting a poor regenerative activity of the cells of this layer. In certain areas we even found alterations of the entire epithelium, including the destruction of some cells of the basal layer. The superficial layer per se was absent. The rats in groups 3, 4 and 5, when compared to group 2, had hyperplasia of all cell layers of the vulvar epithelium on the semithin sections.

The ultrastructural investigations confirm the results of the structural study based on semithin sections, showing that the injectable oestrogen treatment helped protect and rebuild the structure of the vulvar epithelium, the chorion and the muscle layers affected by ovariectomy.

Based on these results, we concluded that all three types of injectable oestrogens used helped to treat vulvar atrophy [17,18].

This study showed that injectable oestrogen treatment over a period of 14 consecutive days enables the recovery of structural and ultrastructural modifications occurring in each layer in ovariectomised female rats, similar to other results published in literature [19,20].

CONCLUSIONS

Experimentally-induced menopause causes important and polymorphic changes in the entire vulvar epithelium, with various degrees of ultrastructural alterations.

The injectable oestrogen treatment had a stimulating effect on the structural and functional regeneration of the vulvar epithelium.

This study showed that Optical Microscopy and Transmission Electron Microscopy can be used as complex complementary diagnostic methods, useful in the understanding and clinical assessment of vulvar changes occurring during menopause, as well as in their adjuvant therapy.

EXPERIMENTAL SECTION

This study used a total of 30 female Wistar white rats, with an average weight of 200 g, obtained from the laboratory animal facility of the "Iuliu Hatieganu" University of Medicine and Pharmacy of Cluj-Napoca. Throughout the experiment, the rats were given standard food and water ad libitum, thus observing the standard conditions required by the current legislation on the protection of laboratory animals.

The following study groups were created:

- Group 1 - control group (no surgical intervention, no oestrogenic treatment, premenopausal), including 5 subjects.
- Group 2 - operated, menopausal, without treatment, including 5 subjects.
- Group 3 - operated and treated with Estradiol, i.e. a natural oestrogen, at a dosage of 0.2 mg/day/rat, for a period of 14 days, including 6 subjects.
- Group 4 – operated and treated with Estradurin, i.e. a synthetic oestrogen, at a dosage of 0.2 mg/rat every 7 days, for a period of 14 days, including 7 subjects.
- Group 5 – operated and treated with Sintofolin treatment, i.e. a synthetic oestrogen, at a dosage of 0.2mg/day/rat, for a period of 14 days, including 7 subjects.

Estradiol (Biofarm, Bucharest, Romania, Zip code: 031212) was used for group 3; each 1 ml-vial of injectable oily liquid contained 2.5 mg estradiol, which was diluted in 9 ml neutralised and sterilised sunflower oil, so that for a dose 0.2 mg of estradiol/rat/day, we administered 0.8 ml of oily solution. Estradurin, (Pharmacia & Upjohn Company LLC (a subsidiary of Pfizer Inc.)), 7000 Portage Road Kalamazoo, MI 49001 United States, is a synthetic oestrogen which was used for group 4; each 2 ml-vial contained 80 mg of powdered polyestradiol phosphate, diluted in 38 ml of distilled water, so that for 0.2 mg of estradiol/rat/day, we administered 0.1 ml solution. Estradurin was administered at 7-day intervals since it is a powerful phosphatase inhibitor, with a particularly slow release, ensuring considerable

oestrogenic activity for a prolonged period of time, even weeks after the injection. Sintofolin (Terapia S.A, 400632, Cluj-Napoca, jud. Cluj, Romania) a synthetic oestrogen, was used for group 5; each 2 ml vial of injectable oily liquid contained 5 mg of hexestrol diacetate, which was diluted with 8 ml of neutralised and sterilised sunflower oil, so that 0.4 ml of oily solution was administered for 0.2 mg/rat/day.

Bilateral ovariectomy was performed in 25 of the 30 female rats included in the study. We considered menopause installed 15 days after surgery. For confirmation, the estradiol level was tested 15 days after surgery and compared with pre-surgery hormonal levels.

The bilateral ovariectomy in female Wistar rats was performed in accordance with the technique described by Waynforth et al [21]. The animals were anesthetized by intramuscular injection of a mixture of Xylasine (10 mg/kg, Xylocontact) and ketamine (100 mg/kg).

Once menopause was confirmed in all groups, we began administering various injectable oestrogen hormonal formulas, over a 14-day period. In the final stage of the study, 15 days after treatment, all animals were sacrificed, using the cervical dislocation method. Biopsies from the vulva and vagina were taken from all groups, and the samples were then processed using optical microscopy (the semithin section technique) and transmission electron microscopy (TEM) methodologies and techniques [22,23,24].

The study protocol was approved by the Ethics Committee of the “Iuliu Hatieganu” University of Medicine and Pharmacy of Cluj-Napoca (approval number no. 116/06.03.2015).

The tissue samples were immediately immersed into a 2.7% glutaraldehyde solution, in phosphate buffer 0.1 M. 7.2, so as to prefix them for 90 minutes at a temperature of +4°C. In the first stage, 500 nm-thick semithin sections were taken from the blocs obtained, and properly modelled for optical microscopy studies, followed by ultrafine 40-60 nm-thick sections from the selected areas of the same blocs, for electronic microscopy studies. The sections were made with a Leica UC 6 ultramicrotome, using DDK diamond knives, and then collected onto electrolytic grids and double-contrasted with uranyl acetate and lead citrate, prior to their examination under a Jeol JEM 1010 transmission electron microscope (TEM). We used a Megaview III camera for the capture of images which were then stored in a database using the Soft Imaging Analysis application.

The semithin section technique was used for optical microscopy studies, on sections 500 nm-thick obtained from the same blocs processed for electronic microscopy research with the same ultramicrotome. These sections were stained with Epoxy tissue stain, specific for epoxy synthetic resins. We used an Olympus BX 51 microscope to examine the sections and a CCD Media Cibernetics camera to capture the images, using the Image Pro Plus software.

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