DMPA- Induced Changes in Estrogen and Progesterone Receptors of Ampulla of Rat-oviducts: An Immunohistochemical Study

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Abstract To evaluate the changes in estrogen (ER) and progesterone (PR) receptors in oviducts, their immunohistochemical expression was investigated using albino rats in estrous cycle and after administration of depot-medroxy progesterone acetate (DMPA). Materials and methods: Twenty rats underwent this study were classified into groups, five rats were sacrificed in the morning of first day (follicular stage); another five rats were sacrificed in the morning of the second day (luteal stage); and ten rats were injected with 12.5 mg subcutaneous DMPA in the first day of cycle, then sacrificed in the next day to show changes in ER and PR. Paraffin-embedded blocks were prepared from the ampullae of oviducts. Then, immunohistochemical staining was performed for each block. Results: ER immunoreactivity mainly nuclear was expressed in the glands, pseudoglands, stromal cells and vascular endothelia in the follicular phase; becoming cytoplasmic in the luteal phase, expressed mainly in the cytoplasm of luminal epithelial cells, and nearly the same picture after DMPA injection. PR immunoreactivity mainly nuclear was expressed in the glands, pseudoglands and stromal cells in the follicular phase; becoming moderately expressed in the luteal phase, but reached a maximum after DMPA injection. Conclusions: DMPA injection caused increased expression of PR but not affecting ER expression.

Keywords Oviduct, Estrogen Receptors, Progesterone Receptors, Immunohistochemical Expression, DMPA

role in reproduction, as it is the site of fertilization [2]. Moreover, it provides an environment that enhances and supports fertilization and early embryonic development, along the travel of embryo toward the uterine cavity [3,4]. In spite of having little knowledge about the mechanism of the embryo transport within the tube, both tubal peristalsis and ciliary activity are thought to be important for successful embryo transport[5]. These reproductive events are regulated mainly by the plasma and/or peritoneal fluid ovarian steroid hormones, estrogen and progesterone, which influence the ciliary activity, the composition of tubal fluid and tubal peristalsis [5-7]. The influence of ovarian steroids is likely mediated through their corresponding receptors which vary in concentration according to menstrual cycle [8]. Despite extensive research on expression of sex steroid hormone receptors in female reproductive tissues of humans and laboratory rodents, expression, localization and function of these receptors in the oviduct remain unclear [9]. At the same time, the precise effect of hormonal contraceptives such as DMPA "a long-acting progestin" on the Fallopian tubes remains unclear. Regarding this aspect, the short estrous cycle in the rat, lasting four to five days, makes it an ideal animal for such research [10].

Aim of the study

To highlight the immunohistochemical localization, and distribution of estrogen and progesterone receptors in the normal oviducts in rats in follicular and luteal phases, and after administration of exogenous progesterone "DMPA".

1. Background

The oviduct (synonyms Fallopian tube, salpinx, uterine tube) firstly described by Gabriel Fallopius in 1561, was considered a simple connection between the ovary and the uterus for a long time. Today, it is regarded as one of the most dynamic reproductive organs, whose functional aspects are not yet fully understood [1]. It plays a pivotal functional

2. Materials and Methods

2.1. Animals and Experimental Design

Twenty sexually mature female albino rats, 6-8 weeks old and weighting 230-380 gm were housed in stainless-steel cages. The animals were housed cage at the animal house of Zagazig Faculty of Medicine under standard conditions of

temperature (22 to 24°C), luminosity (12:12 h light/dark cycles) and humidity (60%). Food and water were available ad libitum. Experimental procedures were approved by the *Scientific and medical research center 'ZSMRC'* of Zagazig university. The animals were divided into two groups; each one was formed of ten rats. The animals of the two groups were separated in individual cages under good healthy conditions. Daily vaginal smears were done to determine the phase of estrous cycle for each animal [11]. Five animals from the first group were anesthetized with an overdose of ether, and sacrificed in the morning of the first day of cycle (follicular phase); the other five animals of this group were sacrificed in the morning of the second day (luteal phase). The second female group (ten animals) was injected in the first day of cycle, subcutaneously with 12.5 mg DMPA "Depo-Provera" [12]. One day after treatment, the treated animals were sacrificed. The ventral abdominal wall of each animal was opened through a longitudinal section.

2.2. Tissue Sampling

Oviducts were identified and removed. Both ampullae from the right and left oviducts of each animal were dissected. Immediately after excision, the ampulla was carefully cleaned from the surrounding tissues, and then cut into small pieces of equal length. Tissue specimens were fixed in 10% neutral buffered formalin for 12 hours before dehydration in ascending grades of alcohol then paraffin embedding.

2.3. Immunohistochemistry

According to Santiago et al., [13] with modifications: Serial sections from the paraffin embedded blocks were cut at 5µm thickness and mounted on slides coated with poly-l-lysine (Sigma). Slides were deparaffinized, rehydrated through graded concentrations of alcohol to distilled water, transferred to sodium citrate buffer (pH 6.0), and heated two times for 10 min in a microwave oven, set at 800 W. Slides were cooled between microwave irradiations for 5min. After this procedure, slides were washed twice with 10mM PBS, pH 7.4, and incubated successively in: 3% hydrogen peroxide in PBS for 30 min (at room temperature), 1% normal goat serum plus 1% hydrogen peroxide in PBS for 30 min; 0.5% Triton X-100 in PBS for 30 min; PR, or ER mouse monoclonal antibody, ready to use (DAKO, USA) for 12 hours at 4°C in a humid chamber. Slides were incubated with a biotinylated secondary antibody for 2 hours at room temperature and later with conjugated streptavidin-peroxidase for 1 hour. Sections were washed twice with PBS among incubations. Peroxidase activity was evidenced by using 3, 3'-diaminobenzidine chromogen solution in the presence of hydrogen peroxide for 10 min. After washing, sections were counterstained with Mayer's hematoxylin, then sections were dehydrated and a cover slip was applied with mounting medium (DPX). Negative

Control sections were incubated with pre-immune goat serum (1:2000) in place of the primary antibody. Positive control sections were taken from endometrial tissue of the same rat.

2.4. Morphometric Analysis

Scoring of nuclear or cytoplasmic immunoreactivity of ER or PR in cells was done by the two authors (RH, AH) blindly and independently through manually counting the number of stained cells. Five hundred cells in the most strongly positive section were counted by light microscopy (400_× magnification). Overexpression or positivity was defined as more than 20% of the examined cells displaying staining.

2.5. Statistical Analysis

For statistical analysis, the categories were divided into these two categories; positive and negative, or strong and weak reaction. Data were represented as numbers and percentages. The differences were compared for statistical significance by chi-square test. Difference was considered significant at $P < 0.05$. The statistical analysis was performed using (SPSS 16.0 for Windows; SPSS Inc. Chicago, Illinois, USA).

3. Results

In the follicular phase, ER was immunoreactive (i.e. more than 20% of the examined cells). There was strongly nuclear positivity in the glands, pseudoglands, the luminal epithelium and the stromal cells in four rats (80%) associated with positivity occurred in the luminal epithelial cells in only one rat (20%) ($P = 0.001$). Nuclear immunoreaction was strong in the endothelial cells of vasculature of three rats (60%) associated with strong nuclear reactivity in the stromal and muscle cells in 4 rats (80%) ($P = 0.001$), (Table 1, Figures 1-6).

Figure 1. ER positive reaction in the glands, pseudoglands, and negative in luminal epithelial cells (nuclear staining), rat oviduct, follicular phase, x 100.

Table 1. Immunohistochemical expression of ER and PR in the tissue elements of ampulla of oviduct in follicular and luteal phases and after DMPA injection

Phase	Rat No.	Tissue elements	ER				PR			
			+ve	-ve	N, or C	P value	+ve	-ve	N, or C	P value
Follicular	5	Luminal epithelial cells	1(20%)	4(80%)	N N	0.001	1(20%)	4(80%)	N N	0.001
		Glands, pseudoglands	4(80%)	1(20%)			4(80%)	1(20%)		
		Stromal cells, muscle cells	4(80%)	1(20%)	N N	0.002	5(100%)	0	N -	0.001
		Vascular endothelial cells	3(60%)	2(40%)			0	5(100%)		
Luteal	5	Luminal epithelial cells	4(80%)	1(20%)	C C	0.001	3(60%)	2(40%)	N N	1.000 (NS)
		Glands, pseudoglands	1(20%)	4(80%)			3(60%)	2(40%)		
		Stromal cells, muscle cells	0	5(100%)	- N	0.001	3(60%)	2(40%)	N -	0.001
		Vascular endothelial cells	1(20%)	4(80%)			0	5(100%)		
After DMPA injection	10	Luminal epithelial cells	8(80%)	2(20%)	C C	0.001	8(80%)	2(20%)	N N	1.000 (NS)
		Glands, pseudoglands	2(20%)	8(80%)			8(80%)	2(20%)		
		Stromal cells, muscle cells	0	10(100%)	- N	0.001	8(80%)	2(20%)	N -	0.001
		Vascular endothelial cells	2(20%)	8(80%)			0	10(100%)

Figure 2. ER positive reaction in the glands, pseudoglands and stromal cells, and negative in luminal epithelial cells (nuclear staining), rat oviduct, follicular phase, x 150. **Figure 3.** ER positive reaction in the glands, pseudoglands and stromal cells, rat oviduct, follicular phase, x 200. **Figure 4.** ER positive reaction in the glands, pseudoglands, stromal cells, and negative in the luminal epithelial cells, rat oviduct, follicular phase, x 200.

Figure 5. ER positive reaction in the glands, pseudoglands and stromal cells, rat oviduct, follicular phase, x 200.

Figure 8. ER positive reaction in the endothelial cells of vascular channels (nuclear staining), negative in the stromal cells, rat oviduct, follicular phase, x 400.

Figure 6. ER positive reaction in the glands, pseudoglands, and stromal cells (nuclear staining), rat oviduct, follicular phase, x 400.

Figure 9. ER positive reaction in the glands (cytoplasmic, or membranous), rat oviduct, luteal phase, x 400.

In the luteal stage, we noticed a strong cytoplasmic reactivity for ER in the luminal epithelial cells in four rats (80%) associated with low reactivity in the glands, pseudoglands in one rat (20%) ($P=0.001$). Low nuclear reactivity was noticed in the endothelia in one rat only (20%), associated with absence of reactivity in the stromal or muscle cells ($P=0.001$), (Figures 7, 8).

Figure 7. ER positive reaction in the endothelial cells of vascular channels, negative reaction in the stromal and luminal epithelial cells, rat oviduct, follicular phase, x 100.

Figure 10. PR positive reaction in the luminal epithelial cells and stromal cells, rat oviduct, luteal stage, x 100.

For PR immunoreactivity in the follicular phase, there was a strong immunoreaction in the glands, pseudoglands in 4

rats (80%) associated with strong reaction in luminal epithelial cells in only one rat (20%) ($P=0.001$). The stromal and muscle cells were positive in all examined five rats (100%), associated with negativity in the endothelial cells ($P=0.001$), (Figure 9). In the luteal phase, we also found a strong nuclear reaction for PR in the luminal epithelial cells and glands or pseudoglands in three rats (60%) (non-significant "NS" association), and in stromal and muscle cells in three rats (60%), associated with negativity in the endothelial cells in all examined rats ($P=0.001$), (Figure 10).

After DMPA injection, we noticed cytoplasmic immunoreactivity of ER in luminal epithelial cells in eight rats (80%), associated with cytoplasmic reactivity in two rats (20%) in the glands, pseudoglands ($P=0.001$), and reactive in the endothelial cells in two rats (20%) associated with negativity in the stromal cells in all examined rats ($P=0.001$).

After DMPA injection; the nuclear immunoreactivity reached the maximum for PR in the glands, pseudoglands and luminal epithelial cells in eight rats (80%) (NS association), and reactive in stromal and muscle cells in eight rats (80%), associated with negativity in the endothelial cells of vasculature of all ten rats (100%) ($P=0.001$), (Figure 11).

Figure 11. PR positive reaction in the luminal epithelial cells, and stromal cells (nuclear staining), rat oviduct, DMPA-treated animal, x 100.

4. Discussion

The estrous cycle in the rat consists of four stages known as proestrus, estrus, metestrus and diestrus [11]. During proestrus, estrogen level increases and ovarian follicles grow fast [14]. This phase lasts about 12 h [11]; and corresponds to follicular phase in humans. Ovulation occurs during the night of estrus 10-12 h after the luteinizing hormone (LH) surge. In the absence of mating at the time of ovulation, the corpora lutea are transiently functional and secrete a small amount of progesterone [15]. This phase called metestrus lasts approximately 21 h [11], corresponding to luteal "secretory" stage.

The mammalian oviduct is not just a conducting bridge

between the ovary and uterus, but it is also a sophisticated secretory organ that maintains and modulates the dynamic fluid-filled environment, necessary for fertilization and early embryonic development [16]. From the point of view of chemical composition, the tubal fluid contains ions, amino acids, proteins, growth factors, enzymes, hormones, the gamete- and embryo-protective and immunosuppressive components – taurine and hypotaurine [17] and source of energy – lactate, pyruvate and glucose [18]. The fluid originates from transudation of blood plasma and the active secretion of the epithelial cells [19]. Lauschová [20] mentioned that tubal fluid originating from blood plasma passes into the lumen via transudation from the blood vessels. However, it seems another source contributing to the tubal fluid that comes out from the glands noticed at the bottoms of crypts (Figures 1,2). The current finding is in contrary to previous results suggesting that the histoarchitecture of the Fallopian tube is simple, with a non-glandular mucosa [21]. The glands might produce a lubricating substance which facilitates the passage of the oocyte and blastocyst in the narrow isthmus [22].

The cilia of the oviduct epithelium together with muscular contractions and the follicular fluid play a major role in the transport of sperms and oocytes and are important for fertilization [23]. Different hormones have been tested for their effects on ciliary activity. Estrogen receptors (ERs) are members of the nuclear receptor superfamily and are involved in regulation of fallopian tube functions (i.e., enhancement of protein secretion, formation of tubal fluid, and regulation of gamete transport) [17]. In this study; in the follicular phase, we found increase immunoreactivity for estrogen receptors in the glands, pseudoglands, associated with negativity in the luminal epithelial cells with high significance ($P=0.001$), this indicating proliferative activity that start in these glands, which may represent the reserve of luminal epithelial cells. We noticed also strong reactivity in the stromal cells, muscle cells that was associated with increased nuclear reactivity in the endothelial cells of vasculature ($P=0.001$), indicating their proliferative activity, and participation in activity of the fallopian tubes. In the luteal phase, there was increase cytoplasmic reactivity of ER, in the luminal epithelial cells, associated with decrease reactivity in the glands or pseudoglands ($P=0.001$), a sign for increased transcriptional activity, hence the secretory reactivity of the luminal epithelial cells, and decrease proliferative activity. In the same direction, we found decreased nuclear immunoreactivity of the endothelial cells associated with no reactivity in the stromal cells ($P=0.001$). Other authors stated that estrogen receptor (ER) is immunolocalized to epithelial cells in the ampullary and fimbrial sections of the Fallopian tube and is reputed to increase throughout the follicular phase before reaching a plateau in the luteal phase [25]. Progesterone receptor (PR) in the Fallopian tube was first characterized in the early 1990's [8,26]. Some studies showed that immunoexpression of epithelial PR expression was most intense in the follicular phase but it decreased along the

luteal phase, to be undetected in the late luteal phase [25]. In our study; in the follicular phase, PR reactivity occurred in the glands, pseudoglands associated with negativity in the luminal epithelial cells ($P = 0.001$). There was marked reactivity in the stromal cells associated with negativity in the endothelial cells ($p = 0.001$). However, in the luteal phase, the immunoreactivity becomes moderate in the luminal epithelial cells, glands, pseudoglands (NS association), stromal cells, and muscle cells but still negative in the endothelial cells ($P = 0.001$), this indicating moderate secretory activity and ciliray beats along through the luteal stage. In comparison with other animals, Tienthai et al., [27] found that: ER α and PR in Thai swamp buffalo oviduct vary according to the phase of ovarian activity and the regional function of the oviduct; the intensity and proportion of ER α and PR staining was very intense in uterine tubal junction (UTJ) and isthmus of swamp buffalo oviduct during follicular phase and less intense during luteal phase. These findings correspond to the earlier reports in heifers and cows [28,29]. We found association between immunoreactivity of stromal cells, muscle cells and the epithelial cells (luminal, glandular or pseudoglandular). The same result was found by Okada et al. [9] and stated that: There's interaction between epithelial and stromal cells via intermediate molecules which are produced by ER- and PRs-positive stromal cells, that control ciliogenesis regulation. After DMPA injection, we found high cytoplasmic immunoreactivity for ER in the luminal epithelial cells, associated with low reactivity in the glands, pseudoglands ($P = 0.001$) and low nuclear reactivity in the stromal cells, associated with negativity in the endothelial cells, this indicates low proliferation in the glands, stromal cells, endothelium, still some proliferative activity in the luminal epithelial cells. PR reactivity was found to be high in all epithelial cells, glands association, stromal cells, and negative in the endothelial cells ($P = 0.001$), indicating the stimulatory effect of exogenous progesterone on the PR release, thus more inhibition on the ciliary beats. Progesterone has proven to cause reduced ciliary activity and may at a high level be a cause of ectopic pregnancy [30]. Similarly, Zhao et al. [31] found that levonorgestrel, a derivative of progesterone, reduces the ciliary beat frequency without damaging ciliated cells. Ciliary dysfunction caused by endometriosis or smoking may be the reason for reduced fertility in some women [5,32]. Comparable results were obtained by Vereide et al. [33]. They stated that in patients with endometrial hyperplasia after intermittent treatment with oral medroxyprogesterone acetate for three months, there was a reduction in immunohistochemical expression of two isoforms of PR (A and B) in the epithelial and stromal cells of the endometrium. From these notices, it might be concluded that DMPA can enhance PR in the fallopian tubes but reduces them in the uterine epithelial and stromal cells. In comparison to other drugs, mifepristone (progesterone antagonist) increases PR in fallopian tube while levonoprogesterone (progesterone agonist) does not change PR concentration in fallopian tubes [34].

5. Conclusions

In conclusion, the administration of exogenous progesterone modulates PR, consequently affecting the function of the fallopian tube and providing a possible mechanism of contraception. Further studies are needed to understand the mechanisms by which progestins mediate stromal-epithelial interactions in female reproductive tissues. The understanding of such mechanisms is essential for the development of new contraceptive methods or the improvement of those already available [35]. Moreover, the glands or pseudoglands appearing different in immunoreactivity from the surface epithelium need more clarification in the future studies.

Abbreviations

DMPA: Depot-medroxy progesterone acetate
ER: Estrogen receptors
PR: Progesterone receptors
N: Nuclear expression
C: Cytoplasmic expression
NS: Non significant

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contributions

All authors contributed equally to this work. All authors read and approved the final manuscript.

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