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AN IMMUNOHISTOCHEMICAL STUDY ON ESTROGEN RECEPTOR-ALPHA (ER-A) EXPRESSION IN THE RABBIT UTERUS DURING EARLY PREGNANCY AND PSEUDOPREGNANCY

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ABSTRACT

Estrogen is essential factor for establishment of normal pregnancy in mammals. Estrogenic action in uterus was found to be mediated by intracellular, primarily nuclear receptor called estrogen receptor-alpha (ER-α). In the current study; immunohistochemistry was used to localize ER-α in the uterus of pregnant and psudopregnant rabbits. ER-α was strongly expressed in non pregnant uteri in nearly all cellular compartments as luminal and glandular epithelium, stromal cells and myometrial smooth muscle cells. Marked down regulation of the ER-α in luminal but not in glandular epithelium was observed during early periods of pregnancy and pseudopregnancy. Weaker glandular staining was only observed during postimplantation periods. Stromal cells were always immunopositive even after implantation till day twelve of pregnancy. Myometrium was expressing the receptor till after implantation. ER-α was always nuclear in immunopositive cells. Our results revealed that, ER-α was involved in the process of progestational proliferation in the rabbit uterus; in addition, the receptor expression is tissue specific and maternally controlled.

INTRODUCTION

Estrogen hormone is important for normal preimplantation uterine changes and embryonic development in the reproductive tract ***(Weitlauf, 1994)***. It is thought that estrogen regulates the female reproductive functions primarily through the nuclear estrogen receptor alpha (ER-α). After hormone binding become the ER-α activated and serve as a transcription factor that modulates the expression of target genes ***(Beato, 1989; Das et al., 1997; Rissman et al., 1997)***. During early pregnancy, ER-α was immunohistochemically localized in smooth muscles of uterine arteries in both human and rabbit uteri ***(Perrot-Applant et al., 1988)***. ER-α was observed in the decidual cells of primates (baboon) ***(Hild-Petito et al., 1992)*** and human ***(Wang et al., 1992)***. On the other hand, the ER-α was shown to be down regulated in uterine luminal and glandular epithelium after implantation and during late pregnancy in human ***(Wu et al., 1993)*** in cat ***(Li et al., 1992)*** and in pig ***(Geisert et al., 1993)***. During Preimplantation period, the endometrium in rabbit undergo progestational proliferation in correlation with a high serum level of estrogen ***(Davies and Hoffman, 1973)*** which reach a peak value during the first week of gestation in rabbit ***(Challis et al., 1973 ; Kriesten and Murawski, 1988)***. As the rabbit is an induced ovulator animal; the uterine changes could be exactly recorded in relation to days of pregnancy considering day 0 (estrous stage) is the day of mating or treatment with hCG (human Chorionic Gonadotropin).

The rabbit is a laboratory animal model and has economic value as well; studying of rabbit reproduction is of great importance. Moreover a detailed study on the expression of ER-α during the preimplantation period in rabbit uterus was not carried out before. The current study demonstrate immunohistochemically, the expression of ER-α in the uteri during preimplantation period of both pregnant and pseudopregnant rabbits.

MATERIALS AND METHODS

Animals:

For this experiment, 40 sexually mature virgin female Zika-hybrid rabbits (Oryctolagus cuniculus) were used and divided into three groups. The experiments were started after a three weeks resting period. In group I, Rabbits were mated twice with fertile males and then injected with 75 i.u. hCG (human chorionic gonadotropin) in the vena auricularis lateralis to ensure ovulation. The day 0 of pregnancy is the day of mating. In group II, the pseudopregnant rabbits received only 75 i.u. hCG i.v. and day 0 of pseudopregnancy was the day of hCG injection. In group III, The non-pregnant rabbits were neither mated nor injected with hCG.

Rabbits were divided as following:

Items	Group I	Group II	Group III
Number	24	9	7
Physiological status	Pregnant days 1,3,4,6 and12	Pseudopregnant days 3,4 and 6	Non pregnant

Sampling:

Rabbits were narcotized with 50mg/kg pentobarbital in 5ml physiological saline through i.v. injection then bleded by opening the carotid arteries. The abdominal cavity then opened and the genital tract was exposed for material collection. specimens from uteri were fixed in Bouin's solution for immunohistochemistry. In mated rabbits, pregnancy was confirmed by the presence of embryos in the uterine tube and/or uterine flushings in early pregnancy.

Immunohistochemistry (IHC):

After fixation for 18 hours in Bouin's solution, tissues were processed using routine histological techniques to obtain 5µm sections. Tissue sections were dewaxed in xylol and rehydrated by descending dilutions of ethyl alcohol, these sections were pretreated by boiling in 10mM citrate puffer solution (pH 6.0) in microwave oven for 30 minutes. The citrate buffer solution was prepared as following:

- Solution A 0.1 M citric acid (21.01g citric acid in 100 ml dist. water)
- Solution B 0.1 M sodium citrate (29.41g sodium citrate in 100ml dist. water)
- 9ml solution A + 41ml solution B + 450ml dist. water = 500ml citrate puffer

After pretreatment, sections were washed in distilled water then subjected to indirect method of immunohistochemistry by using peroxidase enzyme activity. The endogenous peroxidases were blocked by incubation of the sections in 3% (v/v) H2O2 in methanol at room temperature for 30 min followed by washing (3 times x 5 minutes) in PBST(phosphate buffered solution with Tween20). Antibodies and

serum were diluted in 3% BSA (Bovine serum albumen) in PBS. in Unspecific reactions were blocked by normal goat serum in 10% for 30 min at room temperature. The primary antibody anti ER-α (Mouse monoclonal [1D5] to Estrogen Receptor alpha) was applied at dilution of 1:50 and incubated at 4ºC overnight. Sections were carefully washed three times with PBST for 15 min (3x5) and incubated with the secondary antibody, a peroxidase conjugated polyclonal goat anti-mouse antibody at a dilution of 1:250 for 1 hour at room temperature. After three times of washing with PBST for 15min (3x5), immunostaining was visualized by 10% (v/v) 3,3-diaminobezidine (chromogen substrate) in stable hydrogen peroxide for 2 minutes. Afterwards the background was stained light blue with haematoxylin for 10 sec.. Finally, sections were covered in entellan and examined under light microscope. Negative controls omitting the primary antibody were included in each experiment and were always blank.

RESULTS

In the uterus, the estrogen receptor-alpha (ER-α) was expressed in all uterine compartments including luminal and glandular epithelium, endometrial stroma, Myometrium and perimetrium showing a specific pattern of expression in the form of nuclear staining in the immunopositive cells.

In non pregnant rabbits, luminal and glandular epithelium was immunopositive in all cells (Fig.1A,B,C) the reaction was similar intensity in both luminal and glandular epithelium. Numerous stromal cells but not all them were immunopositive specially those aggregated beneath the luminal epithelium (Fig. 1C). Both inner circular and outer longitudinal smooth muscle layers were immunopositive (Fig. 1B) while

connective tissue elements between them were immunonegative. The perimetrial epithelium (serosa) was immunopositive while the subserosal connective tissue cells were negative.

In pregnant rabbits, at day one of pregnancy, the receptor expression was similar to that found with the non pregnant ones but there was increase in uterine glands and more folded endometrium (Fig 2A,B).

At day three of pregnancy, luminal epithelium which looks like pseudostratified (characteristic for day3 – ***Davis and Hoffman 1973***) was lighter stained than the uterine glands which clearly darker stained (Fig. 3A,C,D). Stromal cells were immunopositive (Fig. 3D). Myometrium was positive (Fig.3A). Day four of pregnancy (Fig.4A) showed staining pattern similar to day three of pregnancy.

At day six of pregnancy, the luminal epithelium which show symplasm formation (characteristic for day 6 - Fischer et al., 1986) was weakly stained or negative (Fig. 5A,C) while the uterine glands were immunopositive showing nuclear staining (Fig. 5C). Both myometrium (Fig.5A) and stromal cells were immunopositive.

At day twelve of pregnancy, At the site of implantation, the decidual stromal cells were darkly stained while both luminal epithelium and uterine glands were immunonegative (Fig. 5D). on the other hand, embryonic tissues were negative.

In pseudopregnant rabbits, the expression of ER-α at days 3,4 and 6 was similar to that found in the pregnant rabbits at the corresponding days 3, 4 and 6 (see Figures: 3B,4B and 5B for days 3,4 and 6 of pseudopregnancy respectively).

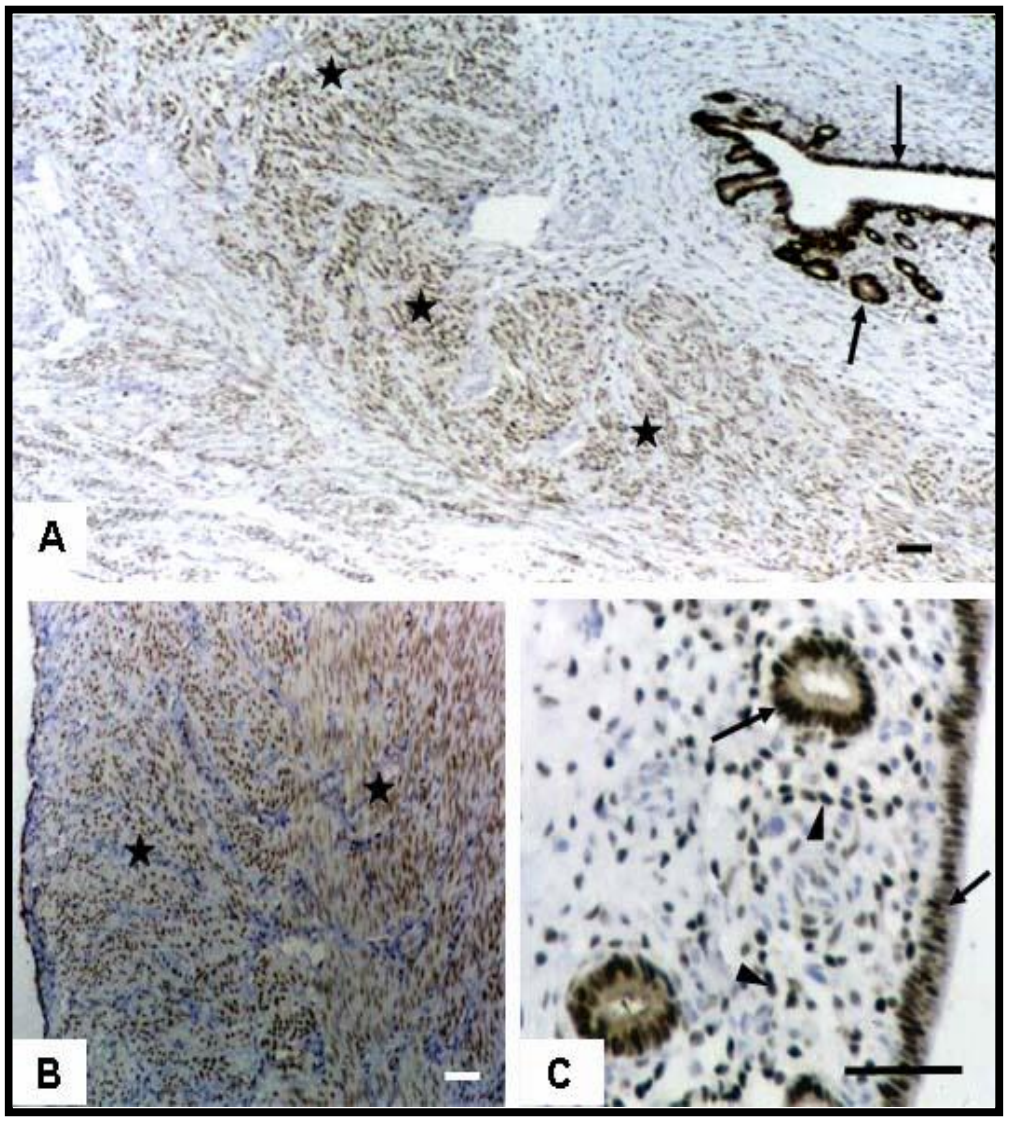

Fig. (1): In non pregnant rabbit estrogen receptor-alpha (ER-α) was expressed in luminal epithelium and uterine glands (A,C arrows) showing nuclear localization (C-arrows). Myometrial muscle cells were immunopositive (A,B stars) showing nuclear staining. Stromal cells were immunopositive (C, arrow heads). Bars (50um).

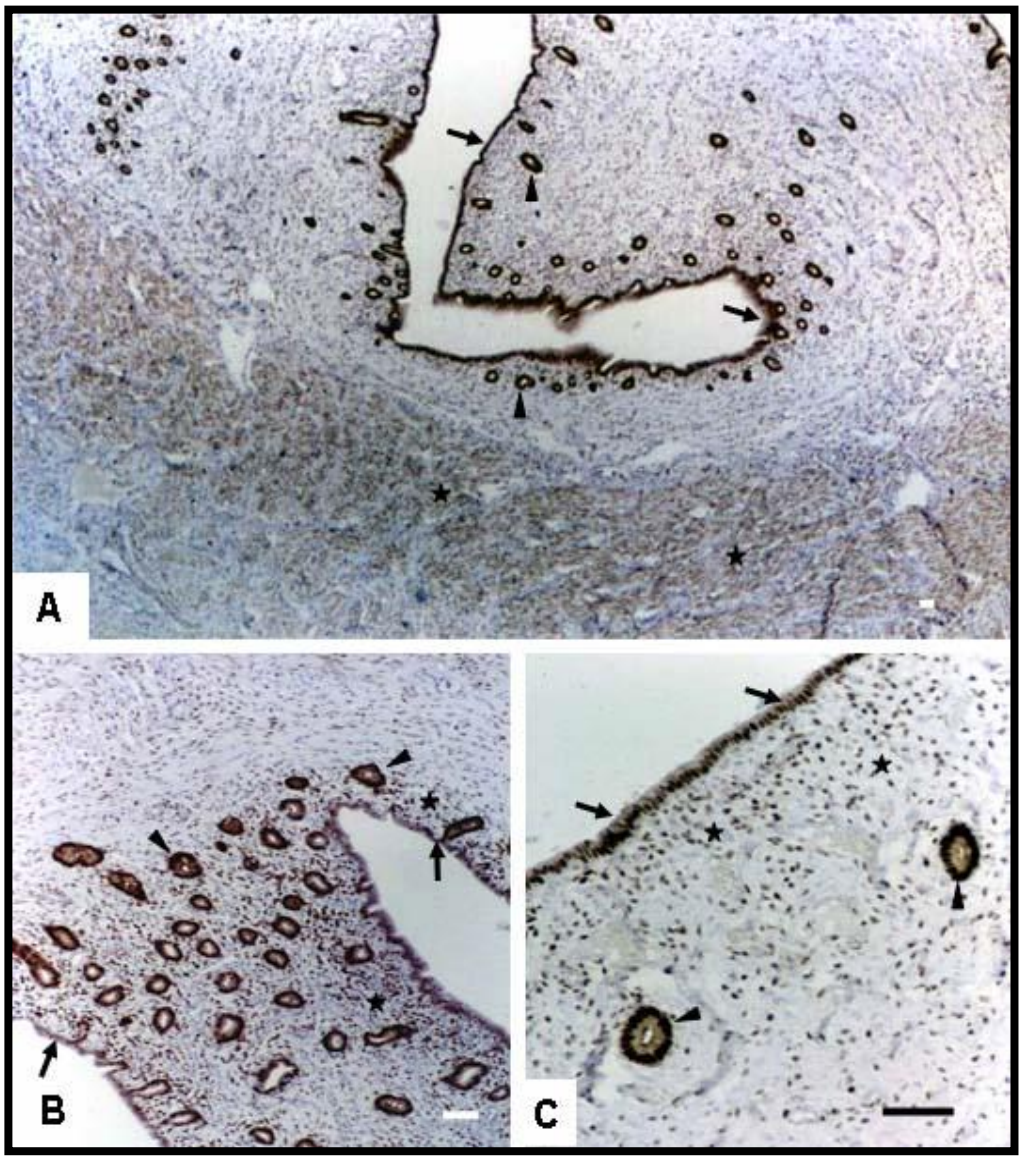

Fig. (2): At day 1 of pregnancy, ER-α was strongly expressed in luminal epithelium (A,B,C arrows) and uterine glands (A,B,C arrow heads). Myometrium and stromal cells were immunopositive (A,B,C stars) Notice the increased number of uterine glands. Bars (50um).

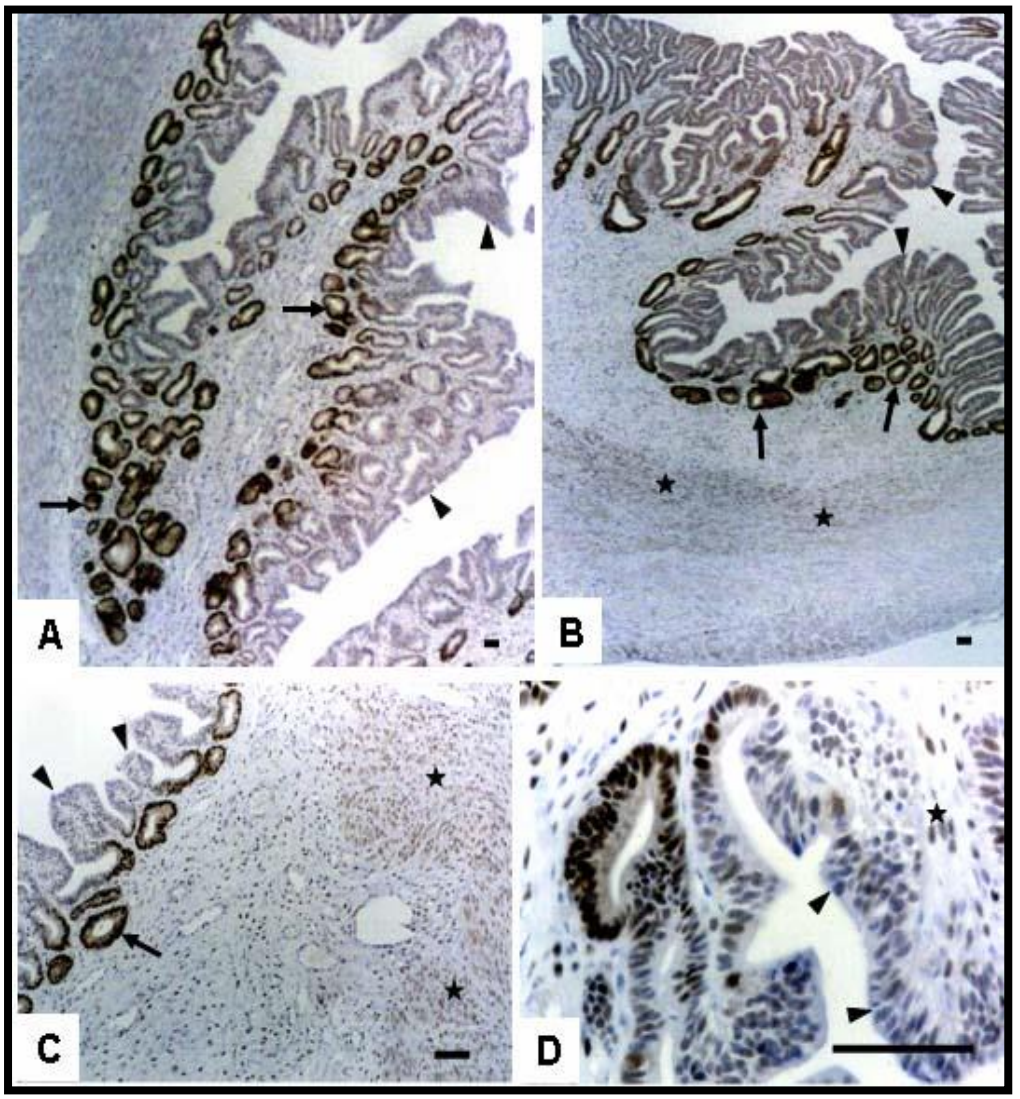

Fig. (3): At day 3 of pregnancy (A,C,D) and pseudopregnancy (B); ER-α expression was prominent in uterine glands (A,B,C arrows) showing nuclear staining (D, arrow). Weaker stain was observed in luminal epithelium (A,B,C,D arrowheads). Both myometrium and stromal cells were immunopositive (B,C,D stars). Bars (50um).

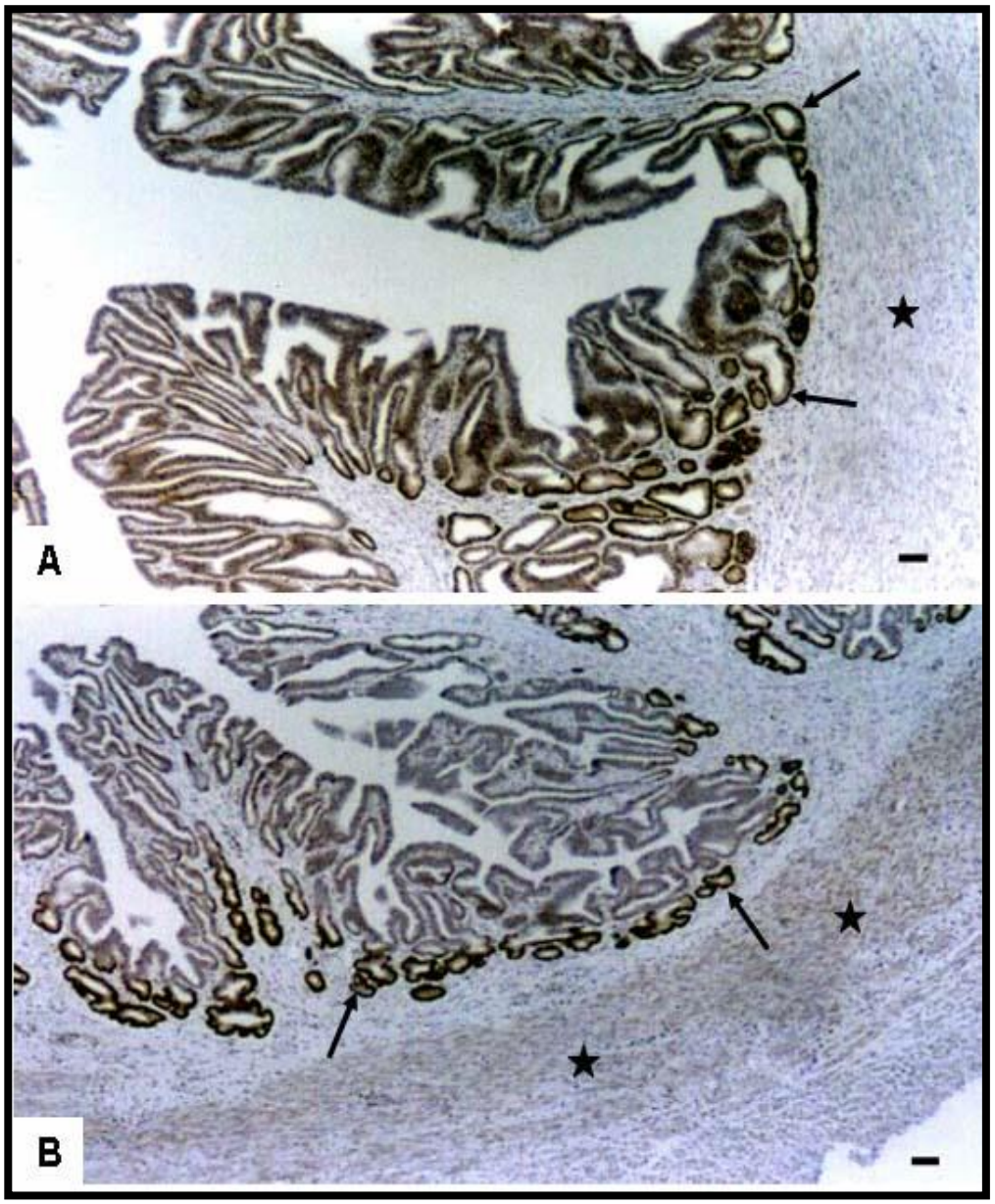

Fig. (4): At day 4 of pregnancy (A) and pseudoprenancy (B); ER-α showed strong expression in uterine glands (arrows) and Myometrium (stars). Luminal epithelium attained weaker staining. Bars (50um).

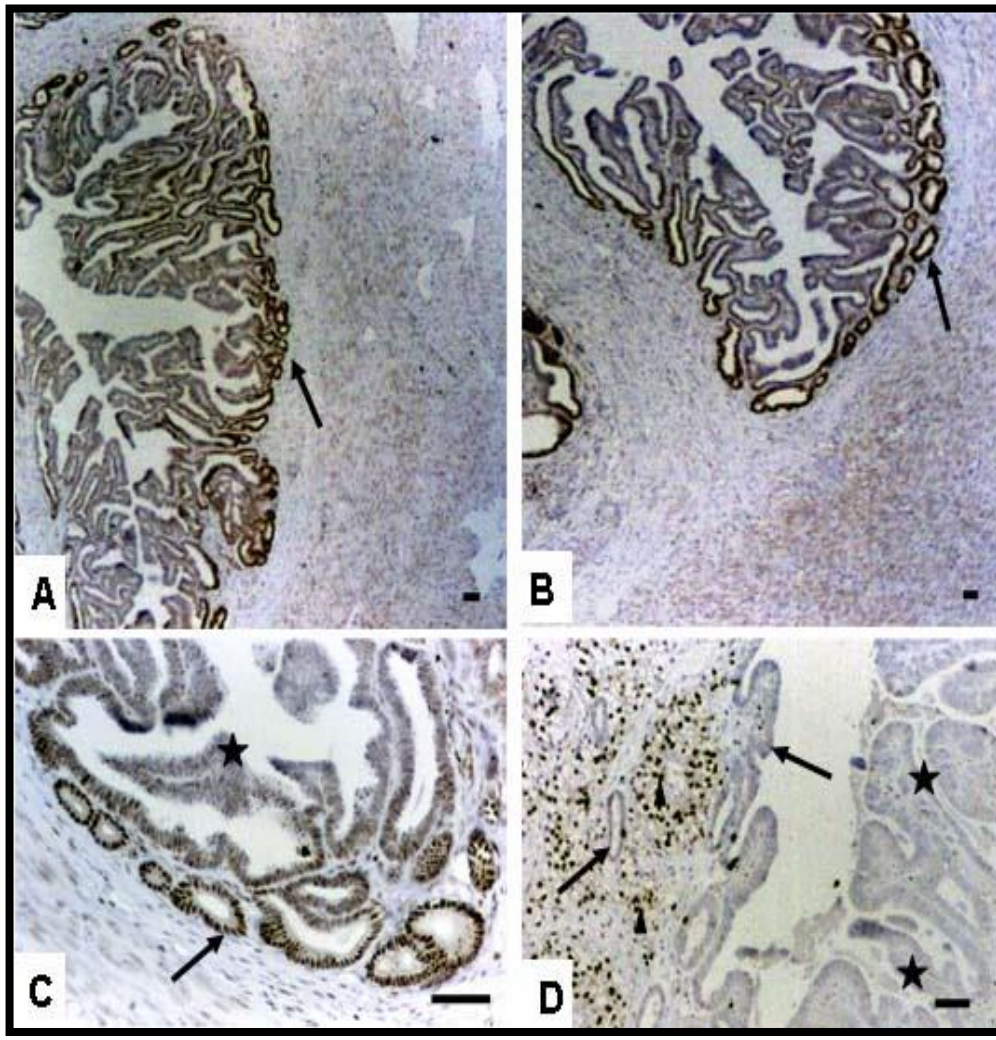

Fig. (5): At day 6 of pregnancy (A,C) and pseudopregnancy (B). ER-α expression was still stronger in uterine glands (A,B,C, arrows) than luminal epithelium which shows symplasm formation (C, stars). At day12 of pregnancy (5 days post implantation), ER-α was expressed in stromal cells (D-arrows heads) while luminal and glandular epithelium were immunonegative (arrows). The embryonic tissues (stars) were also immunonegative. Bars (50um).

DISCUSSION

It is known that, estrogen hormone is necessary for normal progestational proliferation and preimplantation uterine changes ***(Weitlauf, 1994)***. Estrogen hormone perform its action through the nuclear estrogen receptor- alpha (ER-α). After binding the receptor become active and serve as a transcription factor that modulate the expression of target genes ***(Beato, 1989; Das et al., 1997; Rissman et al., 1997)***. Moreover, ER-α is essential for normal reproductive tract, it was found that receptor deficient mice showed abnormal reproductive tract ***(Gorski and Hou, 1995; Moffatt et al., 1998)***.

In our study, ER-α was strongly expressed at day 0 (estrous state) in all uterine compartments including epithelium (luminal and glandular), endometrial stromal cells and smooth muscle cells of myometrium. At day 3 till day 6 of early pregnancy (before implantation in rabbit) there were a marked down regulation of ER-α in luminal epithelium which showed proliferation and symplasm formation; it is known that estrogen hormone serum level forms a peak value during the first week of pregnancy in rabbit ***(Kriesten and Maurawski, 1988)***. In pregnant sheep, the ER-α level was inversely related to the rate of cell proliferation in most of uterine compartments ***(Zheng et al., 1996)***. In rabbit, the animal under investigation, the down regulation of ER-α in rabbit uterus was clear in the luminal and glandular epithelium which become immunonegative after implantation at day 12; it is worthy to mention that the ER-α was shown to be down regulated in uterine luminal and glandular epithelium after implantation and during late pregnancy in human ***(Wu et al., 1993)*** in cat ***(Li et al., 1992)*** and in pig

(Geisert et al., 1993). On the other hand, ***Brenner et al., (1990)*** hypothesized that, progesterone suppress the ER-α expression in all uterine compartments in primates. In human uterus, ER-α persists in human uterus during early pregnancy in particular cell compartments as walls of blood vessels and myometrium despite of continual high levels of circulating progesterone ***(Hild-Petito et al., 1992)***, however, in our study the glandular epithelium was immunopositive at the time of implantation.

Stromal cells were immunopositive till day 12 (5 days post implantation); these cells are descendents of the decidual cells which share in rabbit placenta formation; similar results were observed in decidual cells of human uterus ***(Wang et al., 1992)***. In the rabbit uterus, myometrium was always immunopositive during early pregnancy, but weaker staining obtained after implantation (toward day 12 of pregnancy); however, ER-α was present in myometrial smooth muscle cells as early as day 18 postovulation in Baboon ***(Hild-Petito et al., 1992)*** indicating the effect of species difference on the receptor expression.

In the current study, there were no difference in ER-α expression during the preimplantation period in both pregnant and pseudopregnant rabbits indicating that the embryo do not modulate the receptor expression, comparable results where no differences in expression between implantation and non implantation sites of the uterus suggests that the direct contact with the developing embryo does not regulate the ER-α ***(Hild-Petito et al., 1992; Geisert et al., 1994)***.

It seems that, different uterine cell types were responding to estrogen and expressing ER-α according to its physiological and biological status.

In the rabbit uterus, ER-α was predominantly nuclear in all stained compartments; this was compatible with findings recorded in human uterus ***(Press et al., 1995)***, ovine uterus ***(Zheng et al., 1996; Spencer and Bazer, 1995)***, Baboon uterus ***(Hild-Petito et al., 1992)***.

In conclusion**,** during early pregnancy, ER-α was expressed in different uterine tissues in a tissue specific manner according to its physiological state as proliferation in luminal epithelium, secretion in uterine glands and placenta formation in stromal cells. The similarity in the receptor expression in both pregnant and pseudopregnant uteri indicates that the expression of ER-α is primarily maternally controlled.

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تعبير مستقبل الاستروجين ألفا في رحم الأرنب أثناء بداية الحمل و الحمل الكاذب: دراسة نسجوكيمياء مناعية

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يعتبر الاستروجين عامل أساسي في حدوث حمل طبيعي في الثدييات. تأثير الاستروجين يحدث عن طريق مستقبل داخل الخلية تحديدا في نواتها يسمى مستقبل الأستروجين ألفا.

في الدراسة الحالية، النسجوكيمياء المناعية قد استخدمت لتحديد موقع مستقبل الاستروجين ألفا في رحم الأرانب أثناء بداية الحمل و الحمل الكاذب. وجد أن مستقبل الاستروجين ألفا كان موجودا بقوة في أنسجة الرحم في الأرانب غير الحوامل. مع بداية الحمل و الحمل الكاذب وجد أن تعبير هذا المستقبل قد قل بشكل ملحوظ في الخلايا الطلائية للطبقة الداخلية لكنه ظل قويا في غدد الرحم. أما فترة ما بعد ثبوت الجنين في الرحم كان المستقبل ضعيفا في غدد الرحم. الخلايا الضامة كانت دائما ايجابية في كل العينات حتى بعد اليوم الثاني عشر من الحمل.

مستقبل الاستروجين ألفا كان دائما في الأنوية في الخلايا الموجبة.

نتائج البحث توضح أن مستقبل الأستروجين ألفا مشترك في أنشطة الرحم للاعداد للحمل في رحم الأرنب بالإضافة إلي أنه موجود في خلايا محددة و تتحكم فيه الهرمونات الجنسية للأم.