WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 8, Issue 12, 1264-1278. Research Article ISSN 2277– 7105

EFFECT OF CDRI - 85/287, AN ESTROGEN ANTAGONIST / ANTI-IMPLANTATION AGENT ON EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) DURING PRE-IMPLANTATION PERIOD IN RAT: AN IMMUNOCYTOCHEMICAL STUDY

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Article Received on
11 Sept. 2019,

Revised on 01 Oct. 2019,
Accepted on 21 Oct. 2019,

DOI: 10.20959/wjpr201912-16137

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ABSTRACT

Epidermal growth factor (EGF)/epidermal growth factor receptor (EGFR) have been shown to play a vital role in the uterine proliferation/endometrial decidualization. Present study was undertaken to determine the role of EGFR in rat uterus during pre-implantation period under the influence of an estrogen antagonist /anti-implantation agent, CDRI- 85/287. Results on immunocytochemical expression of EGFR showed strong staining intensity in uterine (luminal/glandular) epithelium, sub-epithelial endogenous stromal cells, exogenous leucocytes cells, blood vessel endothelium and in uterine muscularis (myometrium and serosa) region on day 3 *p.c.* On day 4 *p.c.*, staining intensity of EGFR was observed to be increased in uterine stroma and epithelium. Uterine muscularis region showed strong staining intensity of EGFR on day 4 *p.c.* similar to in day 3 *p.c.* Period of maximal endometrial sensitivity (day 5 *p.c.*) was characterized by marked an increase in endometrial EGFR staining intensity including stroma, epithelium and blood capillaries. Rats administered CDRI-85/287(2.5 mg/Kg, p.o., on day 1 *p.c.*) caused decrease in EGFR staining intensity in uterine peripheral stroma at antimesometrial side on day 3 as compared to control rats. On day 4, EGFR staining intensity decreased in antimesometrial stroma and circular muscle layer in treated rats, but uterine epithelial activity of EGFR was similar to controls. On day 5, a marked decrease in staining intensity of EGFR in entire endometrial stroma including blood capillaries was observed in treated rats. Results of the study indicate that the inhibition of EGFR staining pattern during pre-implantation days 3-5 by CDRI-85/287 may

be due to inhibition of estrogen dependent uterine proliferation and stromal differentiation into deciduas.

KEYWORDS: EGFR, Pre-implantation period, CDRI 85/287, Rat.

INTRODUCTION

Embryo-uterine interaction results in endometrial decidualization, triggers a programmed cell death, followed by endometrial proliferation and decidual cell reaction under the influence of steroid hormones viz. estrogen and progesterone.[1,2] The decidual stromal cells acquire specific functions related to recognition, selection, and acceptance of the allogeneic embryo, as well as to development of maternal immune tolerance.[3-6] Growth factors are known to play a pivotal role in reproductive physiology so as to regulate menstrual cycles, puberty, ovulation, implantation, decidualization and fetal growth via autocrine /paracrine system.[7,8] One of such factor, EGF and EGFR, a transmembrane glycoprotein with intrinsic protein-tyrosine kinase phosphotransferase activity[9], have been shown to play a vital role in uterine proliferation and stromal cell differentiation into deciduas as evidenced by its increased levels during decidualization *in vivo* and *in-vitro*.[10, 11] It has been also demonstrated that EGF and steroid hormones exerts similar effects and EGF may be the substitute for steroid hormones and vice versa[12] as evidenced by increased expression of EGFR with estrogen treatment in non-pregnant uterus as well as it exerts many actions.[13,14] It has been shown that a dose-dependent activation of transcription of the EGFR gene by ligand-bound estrogen receptor–alpha in Hela cells.[15] On the other hand, a possible correlation between progesterone and expression of EGFR system has been demonstrated which shows an increasing trend in the expression of EGFR from day 8 to its maximum on days 10 and 12 of pregnancy during progesterone dominant phase for decidualization.[16] One of the possible mechanism that estrogen and progesterone may exert mitogenic effects on uterus and oviduct by stimulating EGFR system which is a functional component of stromal cells membranes structure[17-19], and its expression was inhibited by antiprogestin RU 48622.[12] However, the mechanism that controls EGFR system mediated processes of uterine proliferation and decidualization, and modulations caused by antiestrogens is poorly understood.

Compound CDRI-85/287, the non-steroidal estrogen antagonist (2-[4-2-N-piperidinoethoxy) phenyl]-3-phenyl (2H) benzo (b) pyran, has shown potential as an anti-estrogen and anti-implantation agent in rats.[20-23] It exerts competitive antagonism at ER level only in rats and monkeys.[24,25] The morphometric alterations caused by this anti-estrogen were the decrease

in uterine volume density, eosinophilic infiltration, mitotic cell number and uterine peroxidase activity in ovariectomized estrogen-stimulated uterus, mature normal cycling rat uterus during pre-implantation period and in pseudo pregnant rats.[22,26,27] Similarly, benzopyran derivative, 2-[piperidinoethoxyphenyl]-3-[4-hydroxyphenyl]-2H-benzo (b) pyran (K-1) has also been shown to induce apoptosis and inhibits estradiol-induced hyperplasia in rat uterus.[28-30] Previous study with CDRI-85/287 have been shown significant inhibition in the levels of ER, PR and decidual plaque cell formation in artificially induced decidual cell reaction in rhesus monkey.[25] Also, it showed inhibition in estradiol-17 beta-induced increase in uterine weight gain and nuclear/cytosolic estrogen receptors in ovriectomized hormone-primed immature rats[31-33] and mature pregnant rats.[33,34] In addition, progesterone hormone also interferes specifically with estrogen action via ER, PR, growth factors/enzymes, and depresses estrogen dependent growth.[25, 35-38]

The present study deals with immunocytochemical localization of EGFR in natural cyclic rat uterus during pre-implantation period under the influence of CDRI- 85/287, an estrogen antagonist and anti-implantation agent.

MATERIALS AND METHODS

Animals

Adult female cycling (170-180 gm) and male rats (200-225 gm) of proven fertility (Sprague Dawley strain) were used in the present study. Rats were caged in environmentally controlled conditions in the Institutes animal house. The temperature of the colony was maintained at (24 ± 1^0C) with 12 hours light and 12 hours darkness. Animals were fed with pelleted food (Hindustan Lever Ltd., Bombay) and water *ad libitum*. Animal studies were conducted according to the regulations of the Institutes Animal Ethics Committee (IAEC) and the protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India.

Cyclicity and mating

The vaginal smear of female rats was recorded daily for at least 15 days to assess the stages of the estrous cycle and confirm the regular cyclicity. Animals showing normal cyclicity were cohabitated with adult male rats (200-225 gm) of proven fertility in the ratio of 2 females: 1 male. Vaginal smears of co-habitated female rats were checked everyday in the morning for the presence of spermatozoa. The day of presence of sperm positive vaginal smear was considered as day 1 of pregnancy. Mated rats were assigned to different groups from days 3

to 5 of pregnancy. Animals were divided into two groups, group-1 consisting of normal pregnant rats, which served as control. Rats of group-2 were administered the compound CDRI-85/287 (2.5 mg/kg body weight, p.o., on day 1 *p.c.*) served as treated group.

Tissue collection, Fixation

Six animals from each group, control and treated, were sacrificed by cervical dislocation on days 3, 4 and 5 *p.c.* respectively. After removal under semi-sterile conditions uteri were freed from all attached fat, mesenteries, etc. Tissue pieces (5-8 mm thick) from the middle portion of each uterine horn were fixed in buffered formalin (3.7%) for 24h.

Immunohistochemistry

Epidermal growth factor

Serial transverse sections (thickness 5µm) were processed for immunohistochemistry employing internal domain F4 of EGFR as per method described previously.[39] Briefly, deparaffinized endometrial tissue sections were hydrated and trypsinized (in 0.1 % trypsin in $CaCl_2$, 9.9 mM at pH 7.6) for 30 min at 37^0 C. After washing with Tris buffered saline (TBS) the endometrial sections were treated with streptavidin-biotin blocking reagent (Sigma, USA) and then covered with NRS (1:5 dilution) for 20 min to block non-specific binding. Then sections were incubated in EGFR- F4 antibody in a dilution (1:5) for 18 h at 4^0C. After washing with TBS, endometrial sections were treated with biotinylated sheep antimouse immunoglobulin (1:200) for 1 h and washed with TBS again for treatment with alkaline phosphatase labeled – streptavidin for 30 min. Lastly, sections were treated with alkaline phosphatase substrate solution (Sigma, USA) for 20 min, washed with triple distilled water, then counterstained with haematoxylin and mounted in glycerine jelly. Immunostaining for EGFR in rat uterus was narrated under Olympus Trinocular microscope (Olympus, Japan) and microphotograhed.

RESULTS

EGFR staining intensity was observed to be strong in uterine (luminal/glandular) epithelium, sub-epithelial endogenous stromal cells, exogenous leucocytes cells, blood vessel endothelium and in uterine muscularis (myometrium and serosa) region on day 3 *p.c.* Whereas, in rats autopsied on day 4 *p.c.*, an increase in staining intensity of EGFR activity was observed in endometrial stroma and in uterine glandular and luminal epithelium. Staining intensity for EGFR in uterine muscularis region was very strong on day 4 *p.c.* similar to in

day 3. On day 5 *p.c.* a marked increase in endometrial EGFR staining intensity was observed in entire stroma, glandular and luminal epithelium and in blood capillaries (Figures 1-3 A, B).

In rats treated with CDRI-85/287(2.5 mg/Kg, on day 1 *p.c.*), EGFR staining intensity showed a decrease in uterine peripheral stroma in antimesometrial side of uterus than in mesometrium on day 3. But showed strong activity in sub epithelial stromal cells and in uterine luminal and glandular epithelium. Uterine muscularis region (myometrium and serosa) showed strong activity similar to in control rats on day 3 (Figure 1C, D).

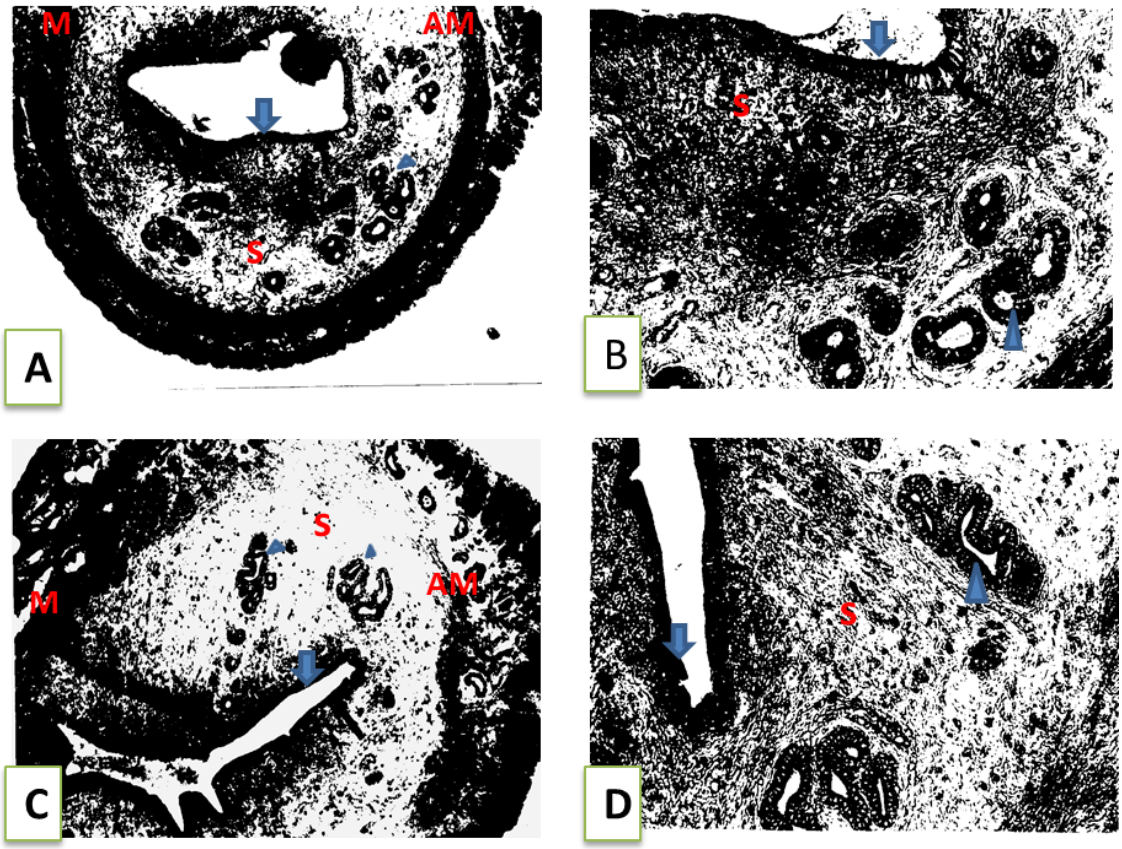

Figure 1: Showing immunocytochemical localization of EGFR activity on day 3 *p.c.* in control (A, B) and CDRI 85/287-treated (C, D) rats. Note the strong staining intensity of EGFR activity in uterine luminal (Arrow)/ glandular (Arrow head) epithelium, sub-epithelial stroma (S), leucocytes, blood vessels and in uterine muscularis (myometrium and serosa) region on day 3 *p.c.*(Figures A, B). In CDRI-85/287(2.5 mg/Kg)-treated rats, EGFR staining intensity showed a decrease in peripheral antimesometrial stroma (S) but, strong intensity can be seen in uterine glandular/luminal epithelium, sub-epithelial mesometrial stroma and in uterine muscularis region (Figures C, D). M –

Mesometrium, AM - Antimesometrium side of uterus. Figures A, C: x40 & B, D: x100 magnification.

On day 4, staining intensity of EGFR activity was markedly decreased in antimesometrial stroma except uterine glands which showed strong activity in treated rats as compared to controls. In mesometrial side of uterus, there was a strong activity in uterine luminal/glandular epithelium and sub epithelial stroma similar to in control rats (Figure 2C & D).

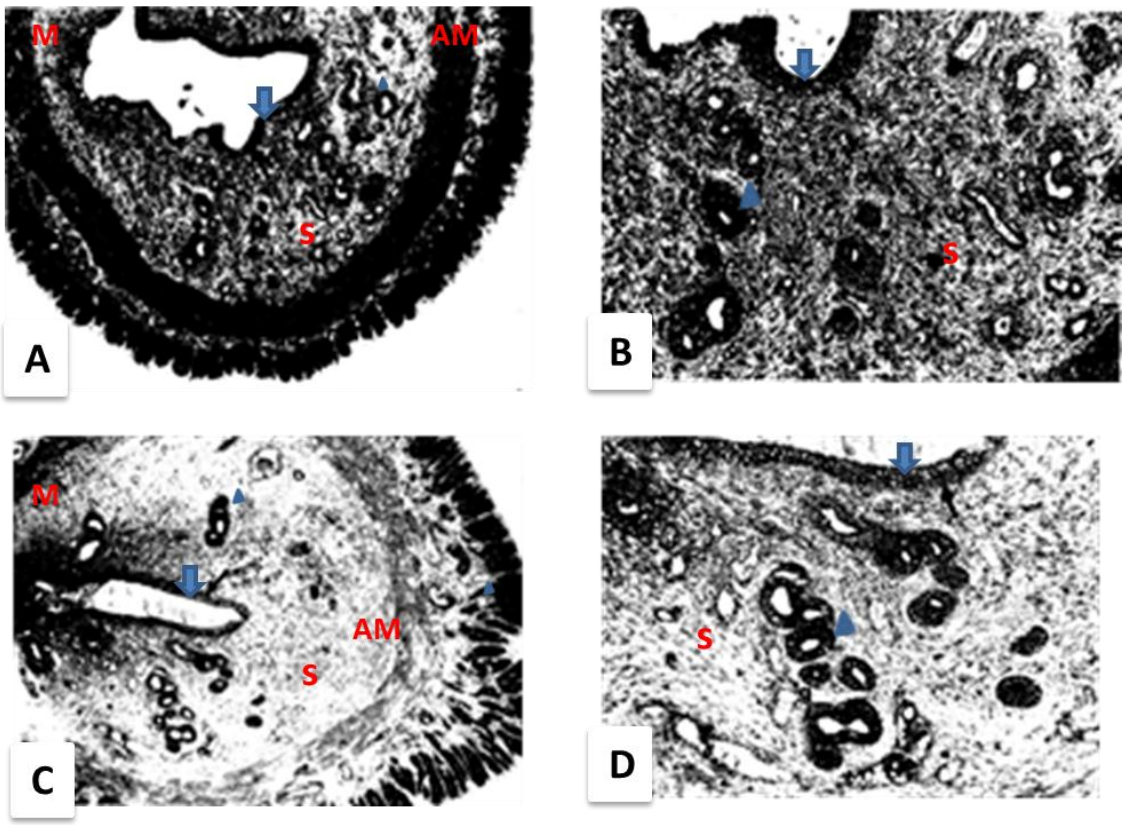

Figure 2: Showing increased staining intensity of EGFR in uterine endometrial stroma, epithelium and in muscularis region on day 4 *p.c.* than in day 3 *p.c.* in control rats (Figures A, B). In CDRI 85/287- treated rat uterus, decreased staining intensity in antimesometrial stroma (S) can be seen except positive activity in glandular (Arrow head) and luminal (Arrow) epithelium, mestrometrial stroma and muscularis region as compared to controls (Figures C, D). M – Mesometrium, AM- Antimesometrium side of uterus. Figures A, C: x40 & B, D: x100 magnification.

On day 5, there was a marked decrease in EGFR staining intensity in entire endometrial stroma and blood capillaries in CDRI 85/287-treated as compared to control rats. But,

exhibited strong staining intensity in uterine (luminal/glandular) epithelium somewhat similar to in controls (Figures 3C, D).

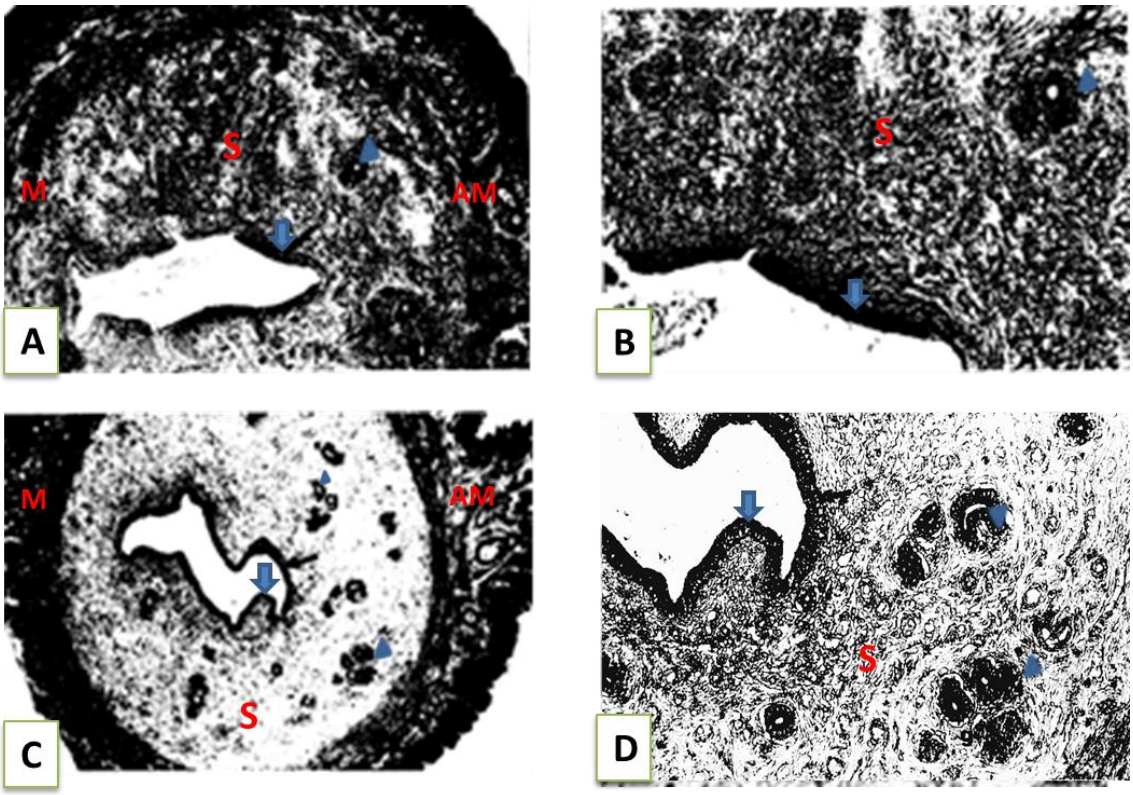

Figure 3: On day 5 p.c. a marked increase in endometrial EGFR staining intensity in uterine endometrial stroma (S), luminal epithelium (Arrow) and glandular epithelium (Arrow head), and in muscularis region can be seen than in day 4 *p.c.*(Figures A, B). In CDRI 85/287-treated (Figures C & D) rats, a marked decrease in EGFR staining intensity in entire stroma (S) except positive staining in uterine luminal(Arrow) / glandular (Arrow head) epithelium similar to in control. M – Mesometrium, AM-Antimesometrium side of uterus. Figures A, C: x40 & B, D: x100 magnification.

DISCUSSION

Endometrial decidualization involves embryo-uterine interaction, triggers programmed changes in epithelial/stromal cells transformation and plaque cell formation under the influence of steroid (estrogen and progesterone) hormones. A critical evaluation of the immunocytochemical expression of EGFR shows strong staining intensity in uterine (luminal/glandular) epithelium, sub-epithelial stromal cells, exogenous leucocytes cells, and blood vessel endothelium on days 3 and 4 *p.c.* Period of maximal endometrial sensitivity (day 5 *p.c.*) was characterized by marked increase in endometrial EGFR activity in entire

endometrium. Treatment of CDRI-85/287 (2.5 mg/Kg, p.o., on day 1 *p.c.*) caused a decrease in EGFR staining intensity in uterine stroma at antimesometrial side on day 3 and 4 as well as a marked decrease on day 5 in entire stroma as compared to control rats. However, strong EGFR intensity in uterine circular muscle layer in treated as well as in control rats may be indicative of stored energy. The expression of EGFR in human normal endometrium, decidua and trophoblast cells of early pregnancy has been demonstrated earlier to explore the effect of EGFR on cell proliferation and development. EGFR expression was present in the proliferative and secretory endometrium, decidua and trophoblasts of early pregnancy, and it was significantly higher in the decidua and trophoblasts than that during the menstrual cycle.[40] Our previous studies in ovariectomized hormone-primed rhesus monkeys showed maximal immunostaining of EGFR during decidualization (Unpublished data), similar to study of Yue et al.,[41] who suggested maximal localization for EGF, TGFα, HB-EGF, AR and EGFR mainly in glandular and luminal epithelium on days 16, 20 and 25 of menstrual cycle. Estrogen treatment from 0-10 days has also been reported to enhance mitotic proliferation in luminal in OVX-rhesus monkeys. The existing evidences have shown that the steroid hormones (estrogen and progesterone) may exert their mitogenic effects on uterus through stimulation of EGFR system.[18,19,42,43] In addition, progesterone stimulates the expression of 17 beta-HSD type 2, which catalyzes the conversion of the potent estrogen into its inactive form, estrone, in epithelial cells and various effects of progesterone on uterine epithelium have shown to be mediated by stromal PRs.[19,44] It has been also shown that antimesometrial implantation site, blastocyst attachment and uterine epithelium enhances EGFR genes as well as there was an up regulation in expression of EGFR genes during peri-implantation decidualization in rabbit.[45]

Blood vessels/blood venules and decidual cell reaction in vascularized stroma also showed maximal staining intensity for EGFR in control rats on day 5, the day of implantation in rats. Previous studies have shown that progesterone stimulates estradiol-primed human endometrial stromal cells to decidualize around blood vessels, which are positioned to prevent peri-implantation hemorrhage during endovascular trophoblastic invasion by expressing tissue factor, the primary cellular mediator of hemostasis.[6,46] It has been also demonstrated that endometrial stromal cells from luteal phase and pregnant endometrium enhance the expression of tissue factor mRNA and protein[47-49] and progesterone controls the decidualization process via increased expression of TF mRNA and protein levels[48] which were more in estrogen + progesterone than in progesterone alone in *In-vitro* conditions in

case of human endometrial stromal cells[48], there by triggering complex pathway of intracellular gene-activating phosphorylation.[11,50,51] Whereas, in *In-Vivo* conditions, progesterone alone is reported to be able to maintain normal expression of EGFR and progesterone dependent protein in OVX- rats[12] and mare[16] so as to maintain uterine proliferation and decidualization of stromal cells.[12,52] In normal pregnancy, in pigs, uterine EGFR concentration increases from days 1-6 of pregnancy as reported by Wollenhaupt et al.[53] In human endometrium estrogen stimulates the synthesis of EGFR and that progesterone does not appear to modulate this effect.[54] CDRI-85/287 has been shown to cause inhibition in the progesterone receptor concentration (both cytosolic and nuclear) on days 24 and 30 and in estrogen receptor on day 30 of cycle in rhesus monkeys[25] and rats[24,55] probably via inhibition in estradiol induced transcription activation leading to inhibition of timed histometric and morphometric events. Moreover, the inflammatory cells have been demonstrated to be the main source of cytokines and growth factors, having many diverse functions, and play an important role in facilitation of endometrial remodeling as well as regression and in pre-menstrual events.[8,42,56,57] There are also reports that these migratory cells synthesize and secrete a variety of collagenolytic peptides/superoxide radicals/lysosomal hydrolytic enzymes indicating their role in cell lysis/spontaneous rupture of cell membranes.[58,59] *In-Vitro* studies have shown that EGF and bFGF increase levels of proteolytic enzymes produced by stromal cells undergoing decidualization.[60, 61]

CONCLUSION

Present study on immunocytochemical localization of EGFR in rat uterus during pre-implantation period show an increasing trend in EGFR activity from days 3-5 *p.c.* with maximal activity on day 5 *p.c.* Inhibition of EGFR staining intensity by CDRI-85/287 during pre-implantation days 3-5, with maximal decrease on day 5, indicate that this decrease may be due to inhibition of estrogen-dependent uterine proliferation and stromal differentiation into decidua. Findings may be useful to study the mechanism of action of uterine proliferation/decidalization.

ACKNOWLEDGEMENT

This work was supported by grants from Ministry of Health and Family Welfare, Govt. of India. CDRI communication number: 10001.

Authors' declaration: Author has no declaration of interest.

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