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VARIATIONS IN LYSOSOMAL ENZYMES OF UTERINE EPITHELIAL/STROMAL CELLS DURING PRE-IMPLANTATION PERIOD IN RAT UNDER CDRI-85/287

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ABSTRACT

Lysosomal enzymes play a pivotal role in uterine growth/proliferation, epithelial cellular destruction/programmed cell death during implantation and decidualization. However, exact role of uterine epithelial/stromal cells in triggering various biochemical and hormonal events during blastocyst-implantation is not yet clear. The present study was conducted to determine the lysosomal enzymes viz. Leucine aminopeptidase (LAP), cathepsin D and phospholipase A2 (PLA2) activity in uterine fractions viz. luminal washings(LW), epithelium(UE) and stroma(US) during pre-implantation days 3-5 *post-coitum* (*p.c.*) under the influence of CDRI 85/287 (2.5 mg/Kg, p.o.; on day 1 *p.c.*), an estrogen antagonist and antiimplantation agent. Results revealed the differential pattern of lysosomal enzymes activity in different uterine cells fractions. While the LAP activity showed a decrease on day 4 vs. day 3 *p.c.*, it increased on day 5 *p.c.* in all uterine fractions (LW, UE and US). The activity of cathepsin D increased to maximum on day 4 *p.c.* but showed decrease on day 5 in LW from control rats. Uterine epithelial activity showed an increase from day 3-5 p.c., but, there was a decline in stromal enzyme activity from days 3-5 *p.c.* Uterine PLA2 activity showed an increasing trend from day 3-5 *p.c.* Administration of CDRI 85/287 (2.5 mg/Kg) caused significant decline in lysosomal enzymes (LAP, Cathepsin D and PLA2) in LW, UE and US fractions on days 3, 4 and 5 as compared to corresponding controls. Results of the study show significant inhibition of lysosomal enzyme activity in all the uterine fractions under the influence of CDRI 85/287 indicating inhibition of implantation associated uterine growth and decidual cell transformation may be possibly due to antiestrogenic mode of action of the

compound. Results are discussed in relation to alterations caused in uterine pregnancy-specific 'key' lysosomal enzymes under the influence of this novel molecule.

KEYWORDS: Lysosomal enzymes, LAP, Cathepsin D, PLA2, Uterus, CDRI-85/287.

INTRODUCTION

In response to the ovarian secretion of progesterone and estrogen during early gestation period, the mammalian uterus, especially epithelial cells develop the capacity to perform several cellular functions vital to blastocyst implantation. Luminal epithelial cells control the uterine environment of the blastocyst by secretion and endocytosis and help to transmit information from the blastocyst to the underlying stromal cells to initiate decidualization. The endocytotic and secretary activity of the epithelial cells and their eventual self-destruction is controlled by the golgi-endoplasmic reticulum-lysosomal system within these cells.[1,2] Accumulation of various lysosomal enzymes helps in autolysis and cell death of the luminal epithelium in this species facilitating the process of implantation.[3-5]

Increase in vascular permeability of the uterine endometrium is one of the early features of blastocyst implantation, which involves participation of various deciduogenic substances like prostaglandin. Changes in the prostaglandin, synthetic capacity results from changes in the activity of enzymes of lysosomal origin necessary for its synthesis. Considerable evidences are available supporting the participation of various lysosomal enzymes, present in the uterine epithelium to the cell division and in the process of blastocyst implantation.[6] There are five main lysosomal enzymes, which are reported to take active part in the phenomenon of implantation.[7-9] Out of these phospholipase A_2, Leucine-aminopeptidase and Cathepsin D are the key enzymes of uterine lysosomal activity.

The exact role of uterine epithelial and stromal cells in triggering various biochemical and hormonal events during the implantation process is not yet clear. Methods to separate the individual cell types of the uterus can avoid the constraints placed on interpretation of experimentation due to complexity of the in vivo condition, which is unable to distinguish between the cell types and their individual hormonal sensitivities during various events of ovum implantation. Thus, epithelial and stromal cells were isolated and the effect of CDRI-85/287 on the levels of enzymes involved in the uterine lysosomal activity at different stages of implantation was investigated.

MATERIALS AND METHODS

Chemicals

L-leucine p-nitroanilide, p-nitroanline hemoglobin, tyrosine, triton-x-100, were purchased from Sigma Chemical Co., USA. Other chemicals were of analar grade quality available commercially.

Animals

Adult virgin cycling female (150-180 gm) and male (200-250 gm) rats of proven fertility (Sprague-Dawley strain) were used in the present study.

Cyclicity and mating

The vaginal smears of female rats were recorded daily for 3 consecutive cycles to check the cyclicity. The females showing regular cyclicity were cohabited with males and mated females showing sperm positive vaginal smears were divided into two groups each comprised of 8 rats i.e. control/85/287 treated at its contraceptive dose (2.5 mg/kg on day 1 *post-coitum*).

Collection of tissues

On days 3, 4 and 5 *post-coitum (p.c.)* animals were killed under aseptic conditions using anesthesia. For each study 8 rats were used. Uterine tissue from both control and treated rats was removed and transferred to an ice-cold petridish. Adherent fat and mesentery were stripped off from the uteri and tissue weighed in a torsion balance. All the subsequent manipulations of the uterine tissue were carried out at 4^0 C. the uteri of 2 rats were pooled together for isolating epithelial and stromal cells, as it was essential to get sufficient quantity of different cell types to be used in different enzyme bioassays.

Removal of the uterine epithelium

The uterine epithelium was removed from the stroma and myometrium following the method of Fagg et al.,[10] In short each uterine horn was flushed with 0.1 ml of ice-cold isotonic-saline (pH 7.4) to collect the luminal fluid. The uterine horn was slit open, cut transversely into pieces, placed in a 15 ml test-tube containing 1.3 ml of ice cold isotonic saline and 5 smooth glass balls of approximately 5 mm diameter. The contents of the tube were mixed for 2-3 minutes using a laboratory vortex mixture operated at its highest speed. The residual fluid so formed consisted of a suspension of intact nuclei together with the remaining epithelial

cell constituents and was termed as "epithelial-fraction". The residual uterine horn was removed for preparation of stromal fraction.

Preparation of luminal washing from the epithelial fraction

The "epithelial fraction' was centrifuged at 800 rpm for 10 minutes at temperature (4^0 C) to get the nuclear pellet. The supernatant obtained following centrifugation was pooled with the flushed out luminal fluid and was termed as "luminal-washing" (LW). The nuclear-pellet was finally suspended in 0.5 ml of distilled water and was termed a "uterine epithelium" (UE).

Preparation of uterine stroma

The residual uterine horns, containing the stromal cells and the myometrium were homogenized using a glass homogenizer by adding 1 ml of ice-cold isotonic-saline at 4^0C and the resultant supernatant was termed as 'uterine-stroma"(US) which also consists of the myometrial cells.

Following enzymes (Cathepsin D, Leucine-aminopetidase and Phospholipase A_2) were estimated in various uterine cells fractions. All the fractions were in duplicates or triplicates in all the assays. Measurements of LAP levels involved two steps. Activation of the enzyme[11] and estimation of enzyme activity by continuous method of Walter and Appel.[12] Activity of Cathepsin D was measured by the method of Barrett and Heath[13] with minor modifications and PLA2 by the method of Neiuwenhuizen et al.[14]

Protein contents from the same samples, which were used for enzyme estimation of each group of rats was estimated by the method described by Lowry et al.,[15] using students "t" test.

RESULTS

Leucine-aminopeptidase (LAP)

This enzyme (Figure 1) in general showed a decline in its levels in luminal washing, epithelial as well as stromal cells of control rat uterus on day 4 of pregnancy (vs. day 3, $p < 0.001$). However, the values again showed an increase on day 5 in all the three fractions (viz. luminal washings, epithelium and stroma). From these results it is evident in normal pregnant animals that there is a decline on day 4 p.c. only. After the administration of compound CDRI 85/287 on day 1 p.c. a significant decline was noticed in the enzyme levels in luminal washings on day 3 p.c.(vs. control, $p < 0.005$), which further declined on day 4 (vs. control

p< 0.002) of presumed pregnancy. Although these values showed an elevation on day 5 (vs. day4) in treated rats (p < 0.001), but the decline was evident when compared to controls of the same day. In the uterine epithelium a gradual decrease in the enzyme levels was noticed on day 4 (vs. day 3 controls), which again showed an increase on day 5). However, on the whole in treated animals the uterine epithelium showed low levels of the enzyme activity on all the days of pre-implantation period and the day of implantation, viz. 3, 4 & 5 p.c. In the stroma as well CDRI 85/287 administration affected enzyme levels on days 3, 4 & 5 p.c.(control vs. treated, day 3 p <0.001, day 4 p < 0.001, day 5 p < 0.001).

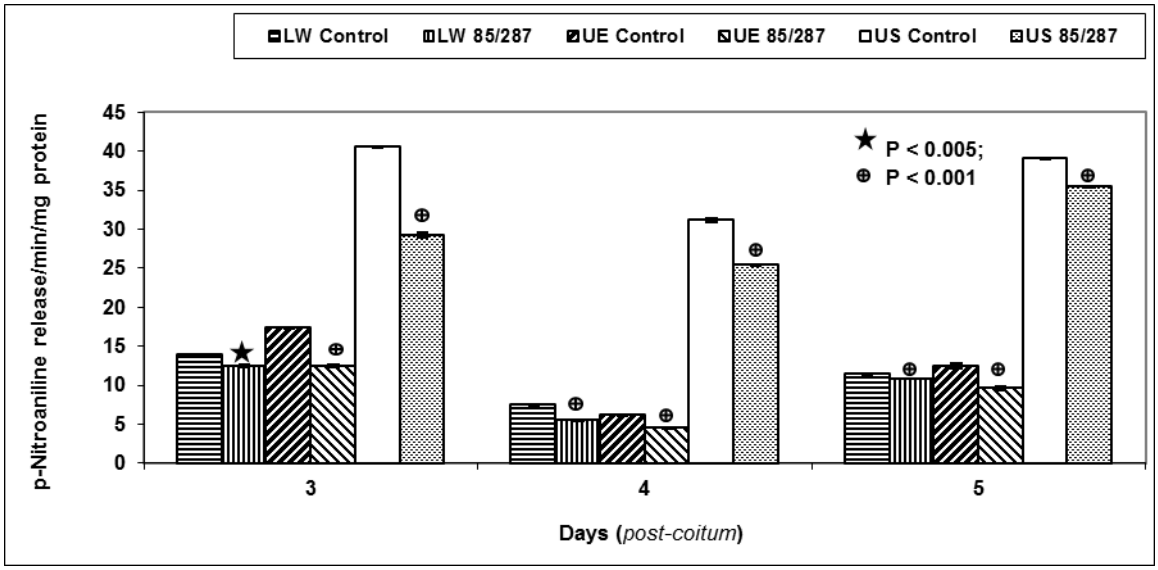

Figure 1: Showing the effect of 85/287 on Leucine amino-peptidase levels in uterine luminal washings (LW), epithelium (UE) and stroma (US) during pre-implantation days 3-5.

Cathepsin-D

In normal pregnant rats the enzyme activity (Fig. 1b) showed an increase in luminal washing on day 4 compared to day 3 (p<0.001) but a significant decrease was observed on day 5, when compared to day 4 p.c. (p < 0.001). In the uterine epithelial fraction a gradual, but significant increase was noticed in the activity if the enzyme from day 3 to 5 (p <0.001). In the uterine stroma a declining trend in Cathepsin-D levels was noticed form day 3 to 5. The activity decreased significantly (p <0.01) on day 4 as compared to day 3 p.c., which decreased further on day 5 (p <0.001, vs. day 4). It is evident from results presented in Figure 2, that administration of the compound (CDRI 85/287) on day 1 p.c. affected the enzyme levels in all the cell fractions on days 3, 4 and 5 *p.c*. there was a significant decrease in the activity of the enzyme in the luminal washings both on day 3 and 4 (p <0.001) as compared

to corresponding controls. Similarly, the decline in the uterine luminal washings of treated rats on days 5 p.c. was also significantly lowered ($p < 0.01$).

In the uterine epithelium the enzyme activity decreased significantly on all the days ($p < 0.001$) viz. 3, 4 7 5 in treated rats as compared to controls. On the other hand the enzyme activity declined significantly on day4 than day 3 ($p < 0.01$) and day 5 ($p < 0.02$) in treated rats.

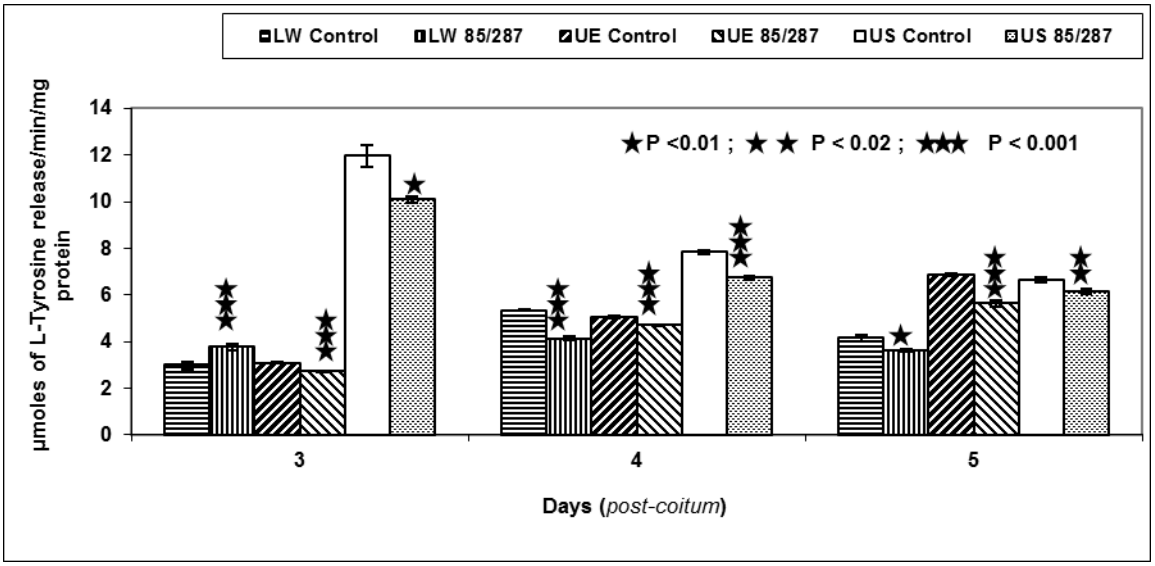

Figure 2: Showing the effect of 85/287 on Cathepsin D levels in uterine luminal washings (LW), epithelium (UE) and stroma (US) during pre-implantation days 3-5.

Phospholipase-A2

The activity of phospholipase in general (Figure 3) showed an increase from day 3 to day 5 p.c. in all uterine cell fractions viz. luminal washings, uterine epithelium and stroma (Fig. 3), which increased further on day 5 (vs day 4, $p < 0.005$) in the luminal washings of control rats. After the administration of the compound 85/287 on day 1 p.c. the activity of the enzyme was inhibited in the luminal washings on all the days (day 3, $p < 0.001$; day 4, $p < 0.001$ and day 5, $p < 0.005$ vs respective controls).

In the uterine epithelium the enzyme didn't show much variation in its activity from day 3 to day 4 but an increase was noticed on day 5 ($p < 0.005$, when compare to day 4 p.c.) in normal pregnant rats. After the administration of compound 85/287 on day1 p.c. the activity of the enzyme was inhibited significantly on days 3 ($p < 0.005$) and 5 ($p < 0.001$) as compare to day 4 p.c. ($p < 0.01$) in treated rats.

The activity of the enzyme was noticed to increase on day 4 vs. day3 ($p< 0.05$), which increased further on day 5 vs. day 4 ($p < 0.01$) in the uterine stroma of normal pregnant rats. However, on the whole in treated rats the uterine stroma showed low levels of the enzyme activity o all the days of pre-implantation period (day 3, $p <0.005$; day 4 ($p <0.005$; and day 5 ($p < 0.001$) as compared to controls of the same day.

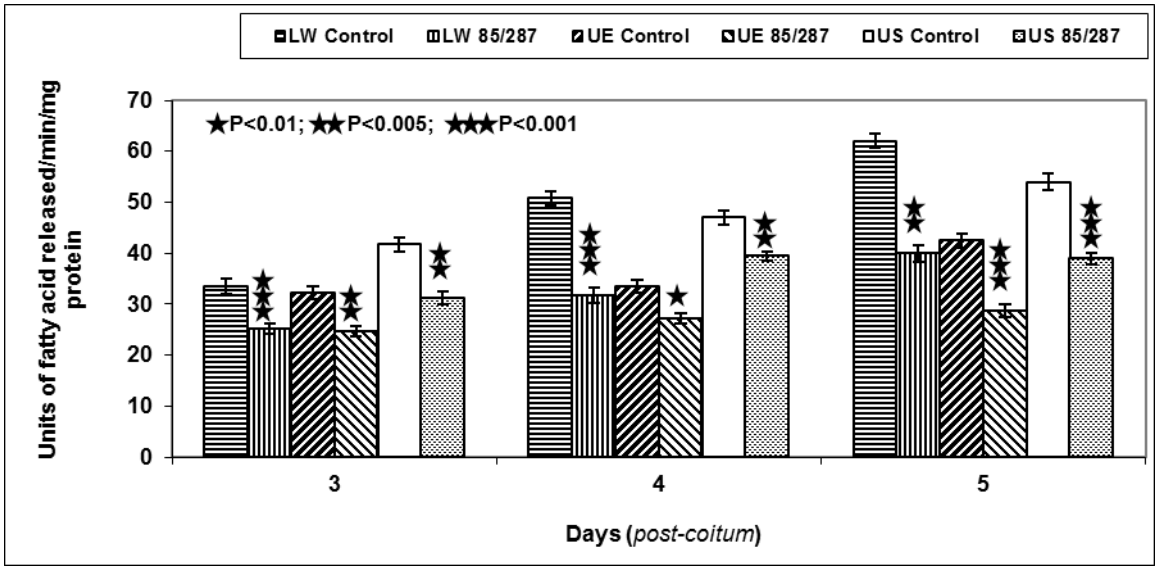

Figure 3: Showing the effect of 85/287 on Phospholipase A_2 levels in uterine luminal washings (LW), epithelium (UE) and stroma (US) during pre-implantation days 3-5.

DISCUSSION

The control of cellular degradative process during implantation is poorly understood. Histochemical studies have identified typical lysosomes in the luminal/glandular epithelial cells[16] and various lysosomal enzymes in the endometrial epithelium. Considerable evidence has been accumulated related to the levels of various lysosomal enzymes during implantation and early decidualization process.[7,8,17] It has therefore, been suggested that in rodents, there is active lysosomal involvement in blastocyst survival, formation of the implantation chamber in the embryo/uterus and attachment reaction during early gestation period.

The activity of LAP, a membrane bound exopeptidase is reported be an indicator enzyme, involved in endometrial protein release and in supplying nutrition to the blastocyst.[18] The enzyme showed peak activity in the luminal washing on day 3, the time when the embryos were present in the uterus and physiologically most active. The activity of the enzyme dropped significantly on day 4 when compared to day 3 p.c. in control rats and peak level activity was detected on day 5 only. The increase in LAP activity corresponds to the period of

blastocyst expansion, when the secretion of the progestational uterus only is available to the embryo as a source of nutrition and energy.[18] Studies by others show that the LAP activity in the endometrial tissue is progesterone dependent.[19] The rise in the activity of the enzyme on day 3 p.c. in our assay system, when the progesterone dominance prevails in the uterus and sudden decline on day 4 p.c., when estrogen started its effect to induce implantation of ovum, support the results reported by others.[19] The biological and physiological events leading to implantation of the blastocyst in the uterus normally depend upon a precise balance between estrogen and progesterone.[20] Thus, the altered ratio of estradiol and progesterone in the peripheral plasma following the anti-estrogen treatment seems to be responsible for the diminished activity of the enzyme.

The various cell populations of the uterus have been reported to respond differentially to the treatment of antiestrogens[21-23] similar to their differential responses to the steroid hormones.[24] CDRI 85/287 has been shown to prevent pregnancy in rats when administered in a single oral dose on days 1, 2 or 3 of pregnancy[25] and also inhibits decidualization in rhesus monkey[26] and rat.[27] Thus, abolition of the pre-implantation peak of the LAP activity in luminal washings, uterine epithelium and uterine stroma may be attributed to inhibitory effect of compound.

During post partum involution of the uterus for regression of the deciduoma, complex proteins and polysaccharides are degraded, as tissues are destroyed and reabsorbed. During these physiologic and degradative processes, there is increase in activities of the lysosomal enzymes like cathepsin-D.[28] In this study the Cathepsin D activity was observed to increase in luminal washings on day 4 as compared to day3 and then decrease on day 5 p.c. in the uterus of rats. In the uterine epithelium the enzyme level shows an increase from day 3 to day 5 reactions.[29]

Previous studies shown that lysosomal cathepsin D mostly concentrated in the luminal and glandular epithelium.[30-32] Moreover, all the hormone dependent changes are also best revealed in luminal washings and in epithelial cells rather than in uterine stroma. In this context, it may be noted that Cathepsin D prior to implantation i.e. on day 4 has been reported to accumulate within the epithelium possibly because of the development of the ability of endometrium to undergo decidualization[33], which contributes in secretion finally to the uterine lumen and hence results in the deterioration and eventual removal of uterine epithelial cells facilitating the cellular autophagic attachment and subsequent implantation of the

blastocyst. During the pre-implantation period lysosomes of the luminal epithelial cells appear to degrade material taken into the epithelial cell by endocytosis.[34] Thus, our results of decrease in Cathepsin D activity in luminal washings from day 4 to day 5 and increasing trend from day 3-5 in the uterine epithelium is suggestive of its relation to the development of the ability of the endometrium to undergo a decidual reaction.

The anti-implantation effect of CDRI 85/287 has also been reflected in the activity pattern of this uterine lysozyme i.e. Cathepsin D.[35] A significant fall in Cathepsin D activity prior to implantation following the anti-estrogen treatment can possibly be due to decreased level of circulating steroidal hormones, particularly estradiol which has been reported to regulate the lysosomal structure.[8,36] Moreover, the antiproliferative effect of the antiestrogens on the luminal and glandular epithelial cells as evidenced by Martin[23,37] may also result in decreased secretion of the lysosomal protease. Although Cathepsin D has generally been measured in the myometrium, but according to Slone and Bird[38] the activity in the endometrium was twice the specific activity in the myometrium. Previously demonstrated by immunohistochemical staining of the uterus of pregnant rat[30] and human endometrium[39], the luminal and glandular epithelium contained the greatest concentrations of Cathepsin D, which in our results is inhibited significantly during early pregnancy period in mated rats administered CDRI 85/287 on day 1 p.c. Thus, these results may suggest that the ability of CDRI 85/287 to inhibit cathepsin D may be related to the inhibition of development of the activity, a period of achieving the maximum sensitivity to the endometrium for the implantation of blastocyst.

The activity of lysosomal PLA2 provides free arachidonic acid by activating a membrane receptor system and is widely accepted as the rate limiting enzyme in the biosynthesis of prostaglandins (PGs). The increased level of PLA2 activity on day 5 of pregnancy corresponds to the peak production of PGE2 6 OXO-PGF1 on day 5[40] and the PGE2 peak on day 4 of pregnancy corresponds to the higher level of PLA2 on the same day. However, different views are expressed in this context, according to some authors, the increase in prostaglandin production as evidenced by Phillips and Poyser[40] and Pakrasi et al.,[41] increased activity of PLA2[42] is not the only factor responsible for increased vascular permeability[43] proceeding implantation, but leukotrienes of various kinds are also believed to be involved in the induction of decidualization reaction. The PLA2 activity exhibited a

significant increase on day 4 of pregnancy in uterine stroma which may also help to facilitate the induction of decidualization leading to implantation of blastocyst as also been reported.[42]

The increase in vascular permeability has been reported[44-46] to be interrupted by the action of the antiestrogen at sub-cellular level and thereby interfere with the estrogen induced implantation and decidual response in rat. Since, PLA2 is considered to be the rate-limiting enzyme in generating free arachidonic acid leading to prostaglandin synthesis[41,47] and hence interference with prostaglandin synthesis due to alteration in PLA2 secretion leads to inhibition of decidualization process under the influence of CDRI 85/287 probably due to the altered ratio of estradiol to progesterone in the peripheral plasma.

CONCLUSION

The results of the study highlight the effect of CDRI-85/287 on the levels of uterine lysosomal enzymes in rats during early days of presumed pregnancy. The diversity in the changes in lysosomal enzyme activities suggest that in rodents there is active lysosomal involvement in the formation of the implantation chamber in the uterus and embryo attachment reaction during early gestation. Thus, it may be plausible to mention that changes in the activities of these enzymes levels accompanied by changing pattern of steroidal hormones and receptors may contribute to the anti-implantation potential of this novel molecule in rat.

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