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HORMONAL REGULATION OF UTERINE LUCINE-AMINOPEPTIDASE ACTIVITY IN RAT: EFFECT OF A TRIPHENYLETHYLENE ANTIESTROGEN, CENTCHROMAN

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ABSTRACT

Lysosomal enzyme-system has been well documented during pre-implantation uterine growth/proliferation and decidualization. Present study was conducted to determine the effects of a single antiimplantation dose (1.25mg/Kg, p.o., on day 1 *post-coitum*) of centchroman (CCN) on uterine localization of lucine-aminopeptidase (LAP) activity in Sprague-Dawley rats autopsied between 10:00-11:00h from day's 2-6 *post-coitum* (*p.c.*). In addition, localization of this enzyme activity was also carried out in ligated rat uterus (on days 4, 5 and 6 *p.c.*), and in immature ovariectomized (OVX) rats administered CCN (0.25 or 1.25 mg/kg, i.m. for 3 or 5 days). Parallel control groups of rats maintained were given vehicle only. Results revealed an increase in uterine LAP activity from days 2-4 *p.c.*, with its maximal staining intensity on day 4 (11:00 h) in control rats. Period of maximal endometrial sensitivity (day 5, 10:00 h *p.c.*) was marked by decreased LAP activity in antimesometrial stroma. In contrast, increased enzyme activity was observed in early deciduas immediate to post-sensitivity period (day 6, 10:00h *p.c.*). Post-coital CCN (1.25 mg/Kg) treatment caused marked inhibition in uterine LAP enzyme activity during pre- (day 4, 10:00h) - and post-sensitivity (day 6)-periods in CCN-treated or in ligated rats as compared to corresponding controls. But, it did not show any significant change in its staining reaction on day 2, except for increased mesometrial LAP activity on day 3 in treated-rats. Inhibition of endometrial sensitivity (day 5, 10:00h) by CCN caused localized increase in antimesometrial (stroma/epithelium) LAP activity. Uterine ligation too, caused an increase in uterine LAP activity on day 5. In immature OVX-rats, CCN treatment (0.25 and 1.25 mg/Kg) alone or in

conjunction with estradiol (1 or 10µg) caused an increase in uterine LAP activity as compared to estradiol treatment. Results of the study, (1) confirm the role of LAP enzyme during uterine growth and proliferation, and differentiation into deciduas as evidenced by its increased activity just prior (day 4) to- and post (day 6)–implantation periods. (2) Inhibition of uterine LAP enzyme activity during pre (day 4, 10:00 h) - and post (day 6; 10:00 h) - sensitivity periods by CCN treatment may be due to inhibition of estrogen action. While, increased LAP activity in antimesometrial endometrium on day 5(10:00 h) in CCN-treated or in ligated rats might be due to inhibition of blastocyst-implantation.

KEYWORDS: LAP – uterus – centchroman - rat.

INTRODUCTION

Lysosomes, the main source of lysosomal enzymes, function in the intracellular mechanism of action of steroid and protein hormones.[1,2] It has been shown to serve as a link between hormone receptor stimulation and in triggering of genic depression in target cell to activate cellular macromolecular synthesis.[3] The functional significance of lysosomal enzymes has been demonstrated in cellular atrophy/cell death under physiological and pathological conditions, in phagocytosis, exocytosis and disposal of insoluble material.[4-6] Amongst several aminopetidases concerned with the hydrolysis of variety of bioactive peptides, protein synthesis/maturation/ stability and in degradation processes[7], Lucine-aminopeptidase (LAP), one of the proteolytic lysosomal enzymes, shown to be predominantly involved in protein synthesis or its degradation processes.[8,9]

Since the blastocyst-uterus attachment reaction triggers cellular destruction called as ‘programmed cell death’ in uterine luminal epithelium and in surrounding antimesometrial endometrial stromal cells, an immediate response to trophoblast invasion into luminal epithelium, followed by endometrial decidualization, exerts the sequential morphological/histochemical/biochemical changes in uterus under the control of ovarian steroids (estrogen and progesterone), where estrogen predominantly plays an important role in pre-implantation uterine growth and proliferation, and progesterone in deciduogenesis.[10-16] A significant role of hydrolytic/proteolytic lysosomal enzymes has been postulated in cellular destruction during ovum implantation as well as in uterine modifications such as in cellular morphology/integrity, DNA/RNA and protein synthesis during pre-implantation uterine cell growth and proliferation and decidua formation in mammals including rodents.[17-21] LAP, one of the proteolytic lysosomal enzymes has been shown to play an important role

in uterine growth and proliferation, blastocyst-implantation, decidualization processes as well as in maintenance of pregnancy.[18,19,22-24]

Therefore, the present study was undertaken to determine the effect of a single antiimplantation dose (1.25 mg/Kg, p.o., on day 1 *p.c.*) of CCN, a triphenylethylene antiestrogen with weak inherent estrogen agonistic activity and shown to cause inhibition of estrogen action leading to inhibition in endometrial sensitivity/vascular permeability and blastocyst-implantation but, did not affect the secretion of nidatory estrogen[15], on uterine LAP enzyme activity during pre-sensitivity (days 2-4 *p.c.*), maximal endometrial sensitivity (day 5, 10:00 h) and post-sensitivity(day 6 *p.c.*) periods as well as during uterine ligation so as to explore more precisely the role of LAP enzyme in uterine preparation for its sensitization responsive to blastocyst-implantation followed by decidual transformation. Localization of uterine LAP activity was also carried out in OVX-immature rats administered CCN so as to determine the uterine enzymatic modifications under estrogenic/antiestrogenic mode of action of this novel nonsteroidal antiestrogen.

MATERIALS AND METHODS

Chemicals

Chemicals like L-lucyl--napthylamide, Garnet GBC (Fast Blue RR Salt), Triazma and steroid (estradiol-17β) were purchased from Sigma Chemical Co., St. Louis, MO, USA). All other indigenous chemicals used were of analytical grade.

Animals, treatment and tissue collection

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 (CPCSEA), New Delhi, India.

Adult (180-200 gm. body weight) Sprague-Dawley rats received from Institute's Breeding colony were maintained under standard laboratory conditions (12h Light: 12h Dark, 22±2 ^{0} C), free access to pallet diet (Lipton India Ltd, Bangalore) and tap water. Female rats were caged overnight with coeval males of proven fertility (3:1); vaginal smear examined microscopically on following morning; day of presence of spermatozoa in smears, considered as day 1 *post-coitum* (*p.c.*). Rats were randomized into 5 different groups containing 10 rats each group. Out of which 5 rats administered a single dose of CCN (1.25 mg/Kg, p.o.) on day 1 *p.c.*, whereas, the remaining ones (5 rats) were given oral suspension of gum acacia in

distilled water served as controls. Autopsy of rats was done by cervical dislocation between 10:00-11:00 h on days 2, 3, 4, 5 and 6 *p.c.*

In second experiment, bilateral uterine ligation was carried out at utero-tubalar-junction (UTJ) on day 1 *p.c.* so as to prevent entry of native embryos into uterine horns[25] under light anesthesia (solvent anesthetic ether) in total of 15 rats. And autopsy 5 rats/day (on days 4, 5 and 6 *p.c.* respectively) was done by cervical dislocation so as to explore the possible role of local embryonic steroid (estrogen) dependent LAP activity in endometrial sensitivity.

In third experiment, immature rats (at the age of 21 days) received from Institutes breeding colony were lightly anesthetized and ovariectomized(OVX) bilaterally under sterile conditions followed by 7 days rest period; divided into nine groups (Gr.) containing 6 animals in each. Gr. I rats injected intramuscularly with 0.1 ml of 4% EtOH in phosphate buffered saline (PBS) were served as control. Gr. II and III rats injected with estradiol-17β((1 and 10 µg/rat/day, i.m.). Rats in Gr. IV and VI were injected with CCN at the dose of (0.25 mg/Kg) and (1.25 mg/Kg, i.m.) repectively for 3 and 5 days alone or in combination with E2 (Gr. VII & VIII), so as to determine the effects of estrogenic/antiestrogenic mode of action of CCN on uterine lysosomal (LAP) enzymatic profile. Autopsy was done on the next day following the last treatment, uteri dissected out, immediately rinsed in chilled normal saline (0.9%), to free from blood clots and connective tissues, and the middle portion (5-7 mm) of both uterine horns were embedded in Kryoquick embedding medium(ICA, USA) for Cryosectioning at -20^0 C.

Localization of LAP activity

Fresh frozen uterine sections cut (8µm thick) in Cryostat Chamber (at -20^0C) from different groups of rats, were processed further for histochemical localization of LAP activity as per method described by Culling.[26] Briefly, uterine sections adhered to glass slides were thawed and incubated in substrate medium containing 1% L-lucyl-(-napthylamide, Garnet GBC (Fast Blue RR Salt; 0.006 gm), 0.2M Tris buffer (pH 7.4) and 0.1N HCl for 3 hours. Control sections maintained were incubated in substrate deficient-medium. Uterine sections then rinsed thoroughly in triple distilled water and mounted in glycerine jelly. Enzyme activity was visually appraised, graded and microphotographed under Olympus Trinocular microscope (Olympus, Tokyo, Japan).

RESULTS

LAP enzyme activity in Control rats

In control rats, localization of uterine LAP activity showed moderate staining intensity in uterine (luminal and glandular) epithelium and in sub epithelial stroma on day 2 (10:00 h) *p.c.* which increased further on days 3 (in antimesometrial stroma) and 4 *p.c.*, with maximal enzyme activity in entire endometrium (including stroma, epithelium and blood capillaries) and in muscularis region on day 4 (11:00 h). Period of maximal endometrial sensitivity (day 5, 10:00h *p.c.*) was marked by decreased staining intensity of LAP activity in antimesometrial as compared to mesometrial side of uterus. In contrast, strong LAP activity was discerned in antimesometrial stroma (early deciduas) following post-sensitivity period (day 6, 10:00h *p.c.*) in control rats (Fig. 1A,C,E,G and Fig. 2 A,B).

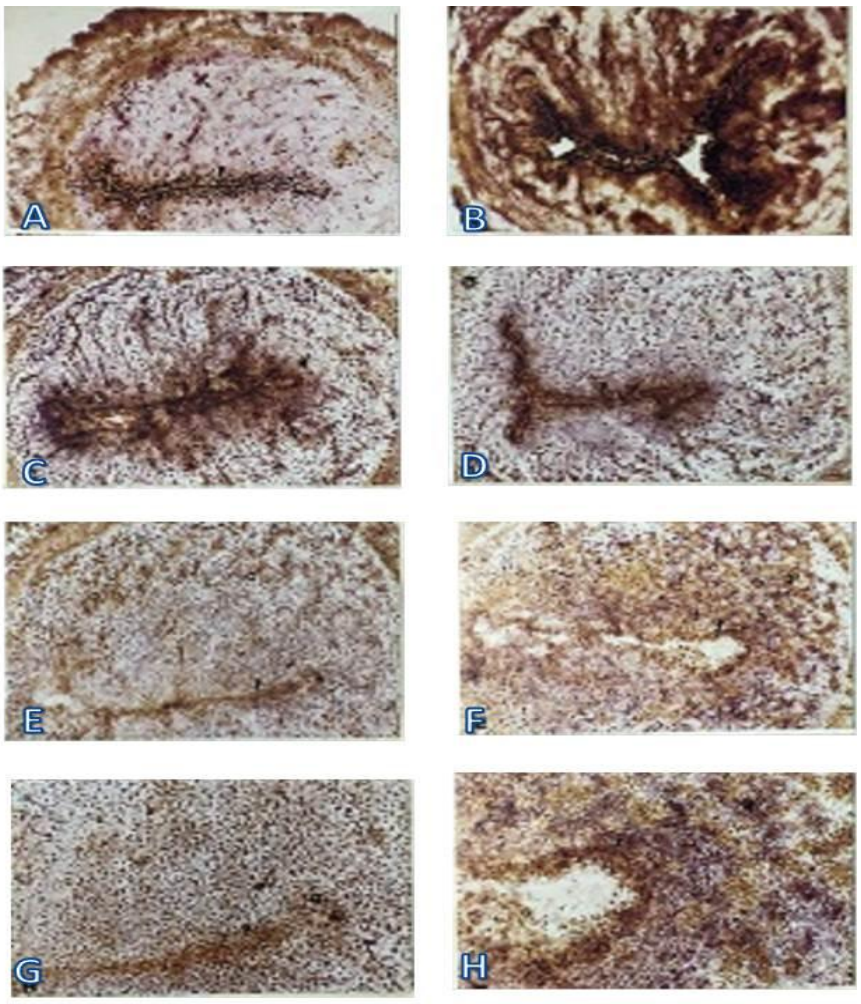

Figure 1: Uterine cross sections showing LAP enzyme activity in control and CCN treated rats from days 3-5 *p.c.* (A) Showing strong staining intensity of LAP activity in uterine luminal (arrow) and glandular (arrow head) epithelium, and in sub-epithelial

stromal cells on day 3 *p.c.* Note the higher staining reaction in uterine epithelium than in stroma. (B) Uterine LAP activity in CCN-treated rat on day 3. Note the increased endometrial activity in treated rat uterus. (C) On day 4 *p.c.* increased LAP activity in entire endometrium including muscularis (serosa/myometrium) can be seen as compared to day 3 *p.c.* control (A). In contrast, enzyme activity showed marked decrease in uterine stroma in CCN-treated (D) rat. (E, G) Showing decreased LAP enzyme activity in antimesometrial and mesometrial stroma on day 5(10:00h) *p.c.* In CCN-treated rat (F, H) increased LAP activity can be seen in endometrial antimesometrial stroma. Magnification for all microphotographs (A-F): X40; (G-H): X100; G-Endometrial glands; Arrow head- Luminal epithelium.

CCN treatment

Post-coital treatment of CCN (1.25 mg/kg; p.o., on day 1 *p.c.*) did not show any significant change in uterine (stromal/epithelial) LAP activity on day 2, but, exhibited elevated enzymatic profile in mesometrial endometrium on day 3 as compared to corresponding control rats. On day 4 (10:00 h), a marked decrease in uterine LAP activity was observed in treated rats. On day 5 (10:00 h) enzyme activity was found to be increased in antimesometrial stroma/epithelium in CCN treated rats. In contrast, LAP enzyme activity decreased in endometrial stroma and in glandular epithelium except strong intensity in uterine luminal epithelium on day 6 (Fig. 1B, D, F,H and Fig. 2 C).

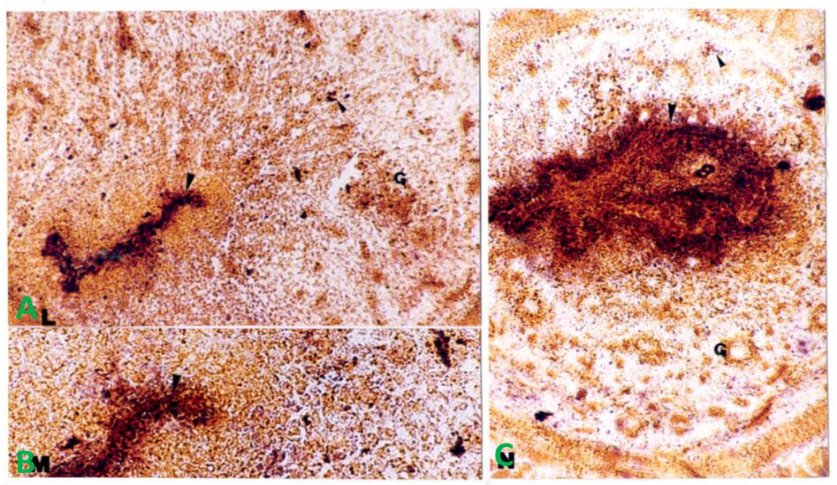

Figure 2: On day 6 *p.c.*, note the LAP positive endometrial decidual cells in control rats (A, B). Luminal epithelium show strong reaction of LAP and luminal closer. CCN-treated (C) rat also showed strong enzyme reaction in luminal epithelium similar to control. But, showed decreased enzyme reaction in uterine stroma in treated rats.

Magnification for microphotographs (A,C): X40; B: X100. G -Endometrial glands; Arrow head- luminal epithelium; Thin arrow head-Blood vessels.

Uterine ligation

In ligated rat, decreased staining intensity of LAP enzyme activity was observed in uterine stroma and epithelium on day 4 p.c. but, showed elevated profile of this enzyme in on day 5 (10:00 h) as compared to appropriate controls. By day 6, stromal LAP enzyme activity decreased but, showed strong enzyme reaction in uterine epithelium (Fig. 3A-F).

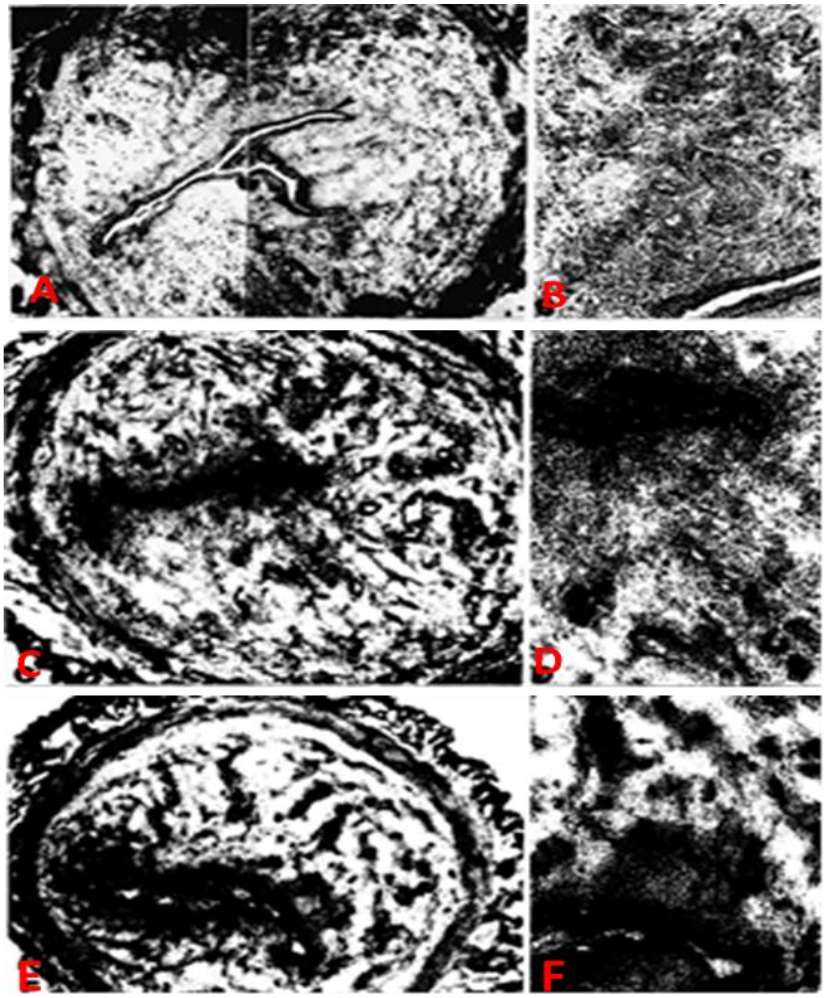

Figure 3: Show uterine LAP activity in ligated rats. (A) Showing decreased uterine LAP activity except positive staining in stromal leucocytic cells and uterine epithelium on day 4 as compared to control rat (Figure1C). (B) A magnified view of (A) showing strong intensity in stromal leucocytes. In ligated rats autopsied on day 5 (C, D), exhibit increased endometrial LAP activity compared to in control (Figure 1E) rat. On day 6 (E, F) uterine cross section of ligated rat showing decreased stromal enzyme activity

except strong staining intensity in uterine epithelium. All photographs (Figures A, C, E) were taken at X 40 magnification. Figures B, D, F are at x100 magnification.

Enzyme activity in immature rats

In immature rats, estradiol-17β (E2, i.m.) treatment at 1 or 10 µg administration for 3 or 5 days induced stimulation in uterine epithelial LAP activity but, showed decreased stromal enzyme activity as compared to OVX control. CCN treatment (0.25 mg/Kg or 1.25 mg/Kg) alone or in combination with E2 caused an increase in uterine stromal LAP activity as compared to E2-treated rats, which was comparable to that in OVX- rat (Fig. 4 and 5).

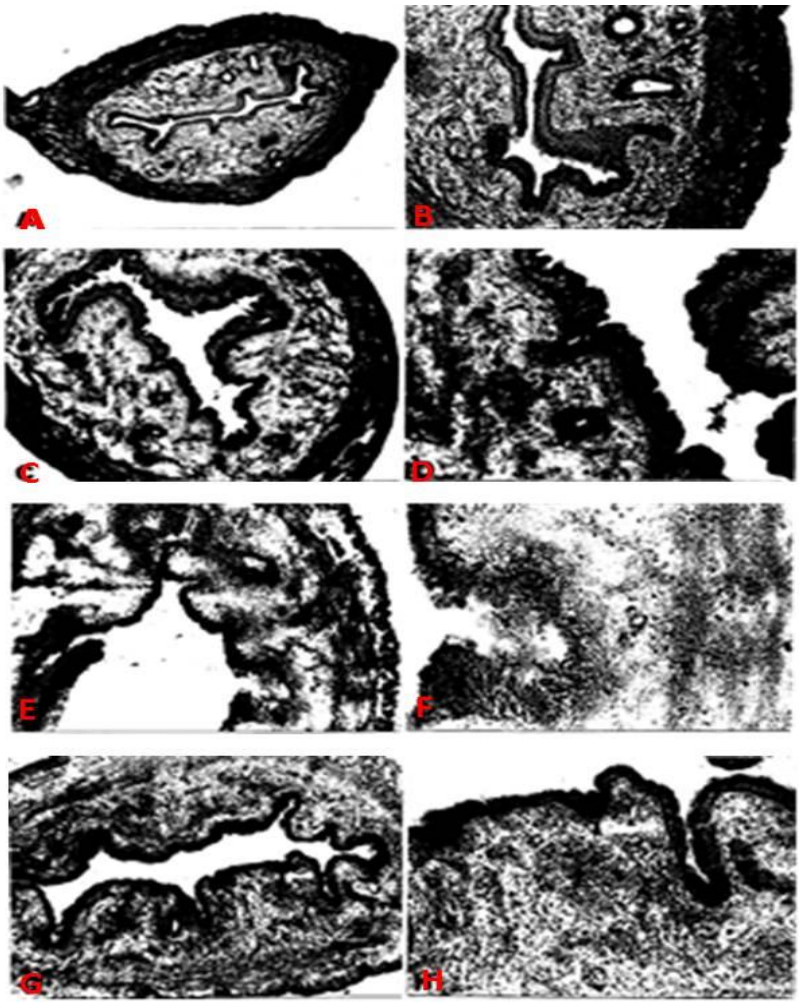

Figure 4: Showing uterine LAP activity in immature rats. Enzyme activity in OVX-Control (A, B), CCN (1.25 mg/Kg) (C, D), E2 (10µg) (E, F) and CCN + E2 (G, H) treated groups. Note the strong staining intensity of LAP in uterine epithelium but, decreased intensity in stroma/epithelium in E2-treated as compared to in OVX-C rats. Increased stromal LAP reaction can be visualized in CCN(C, D) or E2+CCN (G, H)-treated rat as

compared to E2 treatment. (Magnifications for Figures A, C, E, and G: X 40 and B, D, F: X 200).

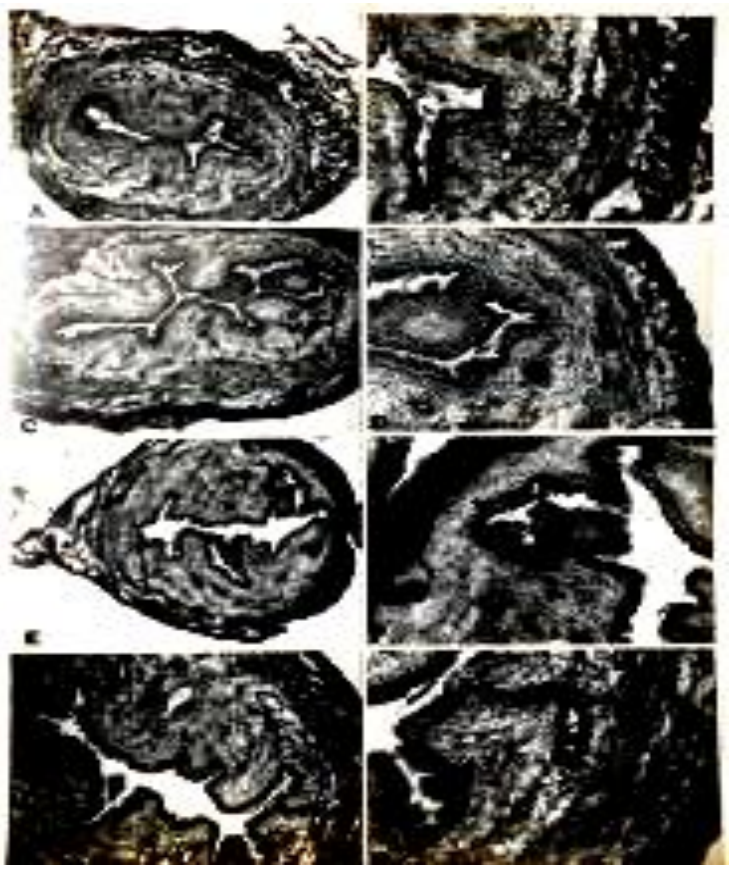

Figure 5: Uterine LAP activity in immature OVX-Control (A, B), E2 (1 µg) (C, D), CCN (0.25mg/Kg) (E, F) and in CCN (0.25 mg/Kg) + E2 (1 µg)-treated (G, H) rats for 3 days. Note the strong epithelial LAP activity and decreased stromal intensity in E2-treated(C, D) compared to control (A, B) rat. CCN (E, F) alone or in conjunction with E2 (G, H) showing increased stroma enzyme activity as compared to E2 (E, F). Arrow - Glandular epithelium; Arrow head–Luminal epithelium. (Figures A,C,E,G:X40; B,D,F,H:X100 magnifications).

DISCUSSION

The lysosomal enzyme, LAP has been shown to play an important role in pre-implantation growth and proliferation as well as in 'programmed cell death' of uterine luminal epithelium/stroma during blastocyst-implantation and in early decidualization.[20,27] The pre-implantational uterine events have been demonstrated to be dependent predominantly on pre-nidatory luteal phase estrogen secretion.[11,14,15] Results of the study show an increasing trend in uterine LAP activity from days 2-4, with its maximal enzyme activity in entire

endometrium (stroma/epithelium) just prior (day 4, 10:00h p.c.) to implantation, have been demonstrated to be due the influence of circulating estrogen level as reported to its maximum on day 4 p.c.[22,27-29]

Period of maximal endometrial sensitivity (day 5; 10:00 h p.c.) was marked by decreased LAP enzyme activity in antimesometrial endometrium compared to its maximal activity in mesometrial stroma. This shows similarity with the previous investigations demonstrating that this decreased enzyme activity of lysosomal enzymes may be due to cell autolysis/programmed cell death during blastocyst-implantation.[20,27,30,31] Presence of LAP activity in trophoblast cells and inner cell mass of blastocyst as well as its localization in antimesometrial stroma surrounded to the implanted-blastocyst[19], indicated the functional significance of blastocyst-originated proteolytic lysosomal enzymes in induction of blastocyst adhesion of with uterine epithelium and in initiation of proteolysis in uterine epithelium/sub epithelial stroma.[20,24,32] It results in increased uterine events, such as cellular permeability, protein synthesis and decidual cells transformation and its proliferation, linked with nutrition of embryo via proteases and peptides.[20,33] Increased endometrial LAP activity following post-sensitivity period (day 6 p.c.) in the present study confirm the functional significance of this enzyme in early decidualization of stromal cells as also been reported previously in rat, mice and rabbits.[19,20, 34]

Inhibition of endometrial sensitivity[15] on day 5 (10:00h) by a single oral administration (1.25 mg/kg, p.o., on day 1 p.c.) of CCN caused a characteristic increase in LAP activity at antimesometrial side of uterus as compared to its decrease in corresponding control rats. Uterine ligation too, exhibited increased antimesometrial enzyme activity on this day. There are also reported evidences that have been shown an increase in uterine LAP activity by antiestrogens, like CI-628 citrate, tamoxifen and CCN itself in hamster.[27,35] Ultra-structural studies have been shown increased lysosomal characteristic in uterine epithelium in experimentally delayed implantation model.[36] Moreover, increased LAP activity in inter-implantation side of uterus as compared to implantation side has been also reported.[27] These studies are in agreement with the results obtained in the present study which displayed an elevated enzymatic profile in uterine epithelium/stroma not only in CCN-treated but also in ligated rats on days 5 and 6 (only in luminal epithelium; indicate increased hydrolysis/proteolysis in relation to inhibition of endometrial sensitivity leading to inhibitory effects on blastocyst-attachment reaction and related uterine transport and permeability

processes.[25,37] On the other hand, increased uterine (stromal/epithelial) LAP activity in OVX immature rats administered CCN (0.25mg/Kg or 1.25mg/Kg) alone was similar to as in ovariectomized rats which have been demonstrated to be due to hydrolytic changes related to uterine atrophy.[19] CCN treatment in combination with E2 too caused an increase in stromal LAP activity as compared to decreased staining pattern of enzyme activity in E2 treatment.

However, results of the study showed that CCN treatment or uterine ligation (on day 1p.c.), cause marked decrease in uterine LAP activity just prior (day 4) to- and post (day 6) - sensitivity periods, which is in agreement with the previous investigations[22,30] indicating its decreased enzymatic profile with tamoxifen or CCN just prior to implantation in hamsters, and correlated with the decreased plasma level of estradiol. In contrast, in rat, CCN treatment did not cause any alteration in circulating estrogen and progesterone hormones level[15] but, shown to cause an inhibition in uterine cytosolic estrogen receptors and an increase in nuclear estrogen receptors[38-40] in relation to inhibition of endometrial sensitivity.[15] This inhibitory effect on the uterine LAP activity on the day of estrogen 'surge' (day 4) and immediate to post-sensitivity period (day 6) in CCN treated rats, indicate an inhibition in uterine growth and proliferation, and early decidualization events, may be due to inhibition of estrogen action. In addition, uterine LAP activity although did not show any significant change on day 2, its increased localization on day 3 in treated rats, might be due to uterotrophic response of CCN.[41]

CONCLUSION

Results of the study confirm the role of proteolytic LAP enzyme in uterine growth and proliferation and early decidualization processes as evidenced by its increased activity just prior (day 4) to- and post (day 6)–sensitivity period. Inhibition of uterine LAP activity during pre (day 4, 10:00 h) - and post (day 6; 10:00 h) – sensitivity periods may be due to inhibition of estrogen action thereby leading to interference in pre-implantational uterine growth and proliferation. But, its increase in antimesometrial side of uterus on day (days 5 (10:00 h) in CCN-treated or in ligated rats in relation to inhibition of endometrial sensitivity responsive to decidualization, may be due to inhibition of blastocyst-implantation.

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