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MODULATIONS IN UTERINE LIPIDS AND GLYCOGEN UNDER THE INFLUENCE OF CDRI-85/287 DURING PRE-IMPLANTATION PERIOD IN RAT: A HISTOCHEMICAL STUDY

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ABSTRACT

In early pregnancy in rat, uterine lipids play a very vital role in the process of implantation especially for nutrition of blastocyst and in energy metabolism required for uterine proliferation. Thus present study was undertaken to study changes in histochemical localization of uterine lipids and glycogen under the influence of CDRI-85/287- a potent estrogen antagonist/antiimplantation agent in rat. Results revealed moderate staining intensity of uterine Red oil O-lipids and glycogen in uterine luminal epithelium on day 3 *post-coitum (p. c.*). While the stromal cells and glandular epithelium showed no lipid staining, intense staining of glycogen was noted in sub-epithelial stroma on day 3. On day 4 *p.c.* a marked increase in uterine (stroma/epithelium) lipids and glycogen staining reaction was observed. But, staining intensity for both lipids and glycogen on the antimesometrial side of uterus showed a decrease on day 5 *p.c.* as compared to day 4. Rats administered CDRI-85/287 at its contraceptive dose (2.5 mg/kg) on day 1 *p. c*. showed a significant increase in staining intensity of lipids in uterine stroma (particularly in leucocytic cells) and blood vessels on day 3 as compared to controls. But, exhibited decreased staining intensity in uterine luminal epithelium from days 3-5. However, uterine glycogen staining reaction showed a significant decrease in treated rats from days 3-5. Results of this study indicate inhibition in uterine lipids and glycogen content during pre-implantation period under the influence of CDRI 85/287, thus contributing to the potency of this novel molecule as an anti-implantation and anti-estrogenic agent. Findings are discussed in relation to role of glycogen and lipids during the process of implantation in rat.

KEYWORDS: Implantation-lipids-glycogen-85/287.

INTRODUCTION

The endometrial metabolites carbohydrates, lipids and proteins required for the nutrition of the implanting blastocyst and the production of high rate energy for rapid proliferation of the uterine tissues. The reactions depend on the enzymatic reactions necessary for glucose metabolism, the increasing demand for circulating blood through the network of spiral arteriols, the histological and cytological changes, which occur in the glandular epithelium and stroma, and, the stimulating action of the ovarian hormones.[1]

Uterine lipids probably serve as an energy source and are utilized in early pregnancy to meet the metabolic requirements of the blastocyst and nidation. The long chain unsaturated fatty acids are necessary for the growth and development of the trophoblast.[2] Moreover, rat embryo has been reported to utilize endometrial fatty acids esterified to triglycerides.[3] The histochemical techniques have shown that exogenous estradiol causes rapid disappearance of lipids[4,5] and progesterone on the other hand appears to facilitate the storage of lipids.[4, 6] Such hormones are specific, for stimulating the enzyme and coenzyme systems responsible for energy production. The cyclic variations in protein and carbohydrate metabolism are associated with biochemical processes such as nucleic acid synthesis, availability of glucose and glycogen reserve, which play an important role in cellular nutrition and fetal survival in vitro. Thus, gonadal hormones are important regulatory factors, where estrogen stimulates anaerobic glycolysis and glycogen deposition.[7] However, uterine glycogen has been reported to be the substrate used to sustain contractile activity, as its concentration was observed to be greater in implantation zone.[8, 9]

Hence, we have attempted to determine histochemically the effects of a potent estrogen antagonist and anti-implantation agent, CDRI-85/287[10-13] on cellular localization of some key parameters (lipids and glycogen), which play an important role in implantation and are regulated by gonadal hormones, during pre-implantation period in the rat uterus.

MATERIALS AND METHODS

Animals

Adult cycling female (150-180 gm) and male (200-250 gm) rats of proven fertility of Sprague Dawley strain were used in the present study. Rats were caged in the environmentally controlled rooms of the Institutes animal house. The temperature of the colony was

maintained at (24±1^{0}C) with 14 hours light and 10 hours darkness. Animals were fed with pelleted food (Hindustan Liver Ltd.) and water *ad libitum*.

Cyclicity and mating

The vaginal smear of the rats was recorded daily for at least 15 days to assess the stages of the estrous cycle and confirm the regular cyclicity. The animals showing normal cyclicity were used in the experiment. They were cohabitated with adult male rats (200-250 gm.) of proven fertility in the ratio of 2 females to 1 male. Vaginal smears of co-habitated female rats were checked everyday in the morning for the presence of spermatozoa. The day vaginal smear was found to be sperm positive 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 and rats of group-2 were administered the compound 85/287(2.5 mg/kg) on day 1 *p.c.*, which served as treated group.

Collection of tissue

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 (5-8mm thick) from the middle portion of each uterine horn were fixed in different fixatives e.g. 10% Neutral formalin for histochemical localization of lipids; Bouin's and Cornoy fixation for localization of glycogen activity were used.[14]

Histochemical localization of lipids

Formalin fixed uterine frozen sections (8µm thick) were rinsed properly in triple distilled water and then in 60% isopropanol. Sections were stained with freshly filtered Oil Red O (0.5% in isopropyl alcohol, further diluted 6 ml stock solution with 4 ml of distilled water, filtered and used) solution for 30 minutes, differentiated briefly in 60% isopropanol, then washed in distilled water and counterstained with Mayer's haemalum for 5 minutes.[15] After washing in water at least 5 minutes sections were mounted in glycerin jelly on micro-glass slides for light microscopic examination.

Histochemical localization of Glycogen

Bouin's and Cornoys fixed paraffin embedded uterine sections of 5 µm thickness were rinsed and kept in xylene filled Coupling jars for 30 minutes to deparaffinize the uterine sections,

then transferred to absolute alcohol, hydrated up to 70% alcohol and stained with Ehrlich's haematoxylin for 5-10 minutes. The uterine sections were rinsed and rapidly differentiated in 1 % acid alcohol, hydrated and dehydrated with 70% alcohol. Then stained with Best's Carmine solution, differentiated, dehydrated and cleared in xylene and mounted in D.P.X. as per the method described previously.[15]

RESULTS

Lipids

Control rats killed on day 3 *p.c.* showed an intense staining of Red Oil O lipids in uterine luminal epithelium, comparatively weak staining intensity was observed in sub-epithelial stroma and blood vessels (Fig. 1A, B). No lipid reaction was noticed in uterine glandular epithelium and most of the endometrial peripheral stroma, as well as in muscularis layers (Fig. 1A). There was a further increase in the lipid staining intensity on day *4 p.c.* in endometrial stroma particularly in leucocytes compared to day 3 *p.c.* (Fig. 2A, B) rats. In this group of rats uterine luminal and glandular epithelium showed slight lipid activity (Fig. 2B). Serosa and myometrium showed no lipid activity except in blood vessels (Fig. 2A). On day 5 *p.c.* a marked decrease in lipid staining was observed in uterine stroma as well as in uterine epithelium as compared to day 4 *p.c*. rats (Fig. 3A, B).

CDRI-85/287 treatment caused a significant increase in the uterine lipid staining mostly in leucocytic cells and blood capillaries in peripheral stroma, while a decreased staining intensity was observed in uterine luminal epithelium and metrial glands on day 3 (Fig. 1 C, D). In uterine muscularis region strong lipid reaction was also seen in blood vessels located in between myometrium and serosa as well as in leucocytic cells (Fig. 1 C). A significant decrease in lipid staining intensity was observed in endometrial stroma particularly in leucocytic cells in antimesometrial site of uterus as well as in uterine luminal epithelium of treated rats as compared to corresponding control rats on day 4 (Fig. 2 C, D). On day 5, CDRI-85/287 treated rats showed decreased lipid staining reaction in endometrial stroma/uterine luminal epithelium comparable to controls of the same day (Fig. 3 C, D). Blood vessels, endometrial glands and muscularis showed similar activity as in controls (Fig. 3 C).

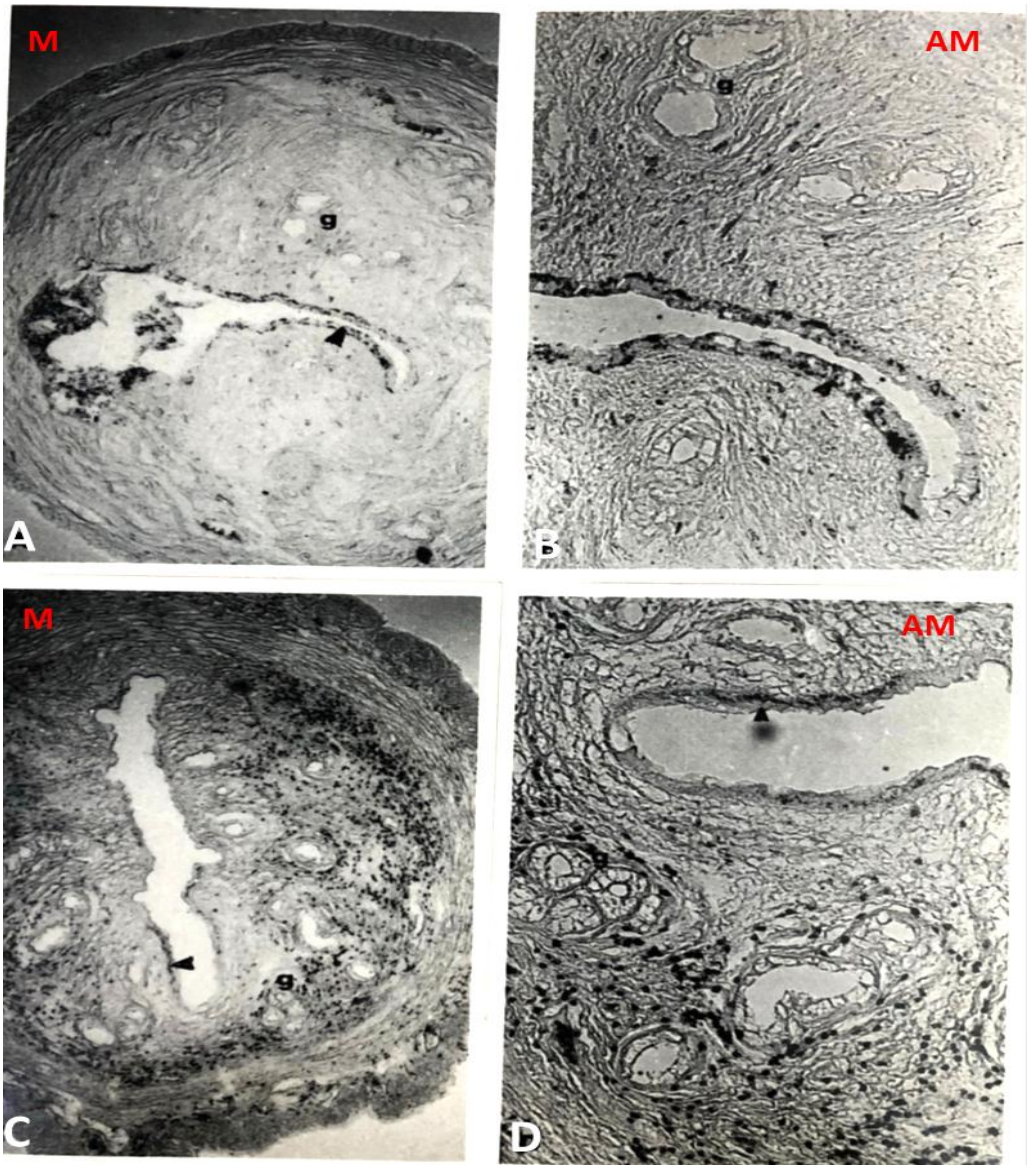

Figure 1: Oil red O lipid profile in rat uterus on day 3 p.c. showing staining reaction in uterine luminal epithelium (Arrow head) and in blood vessels/capillaries (A, B). CDRI-85/287 treatment (C, D) showing significant increase in lipid profile of stromal (leucocytic) cells but lipid activity in luminal (arrow head) and glandular (g) epithelium decreased as compared to control. AM-Antimesometrial side, M- Mesometrial side. Figures A, C: x40 & B, D: x100 magnification.

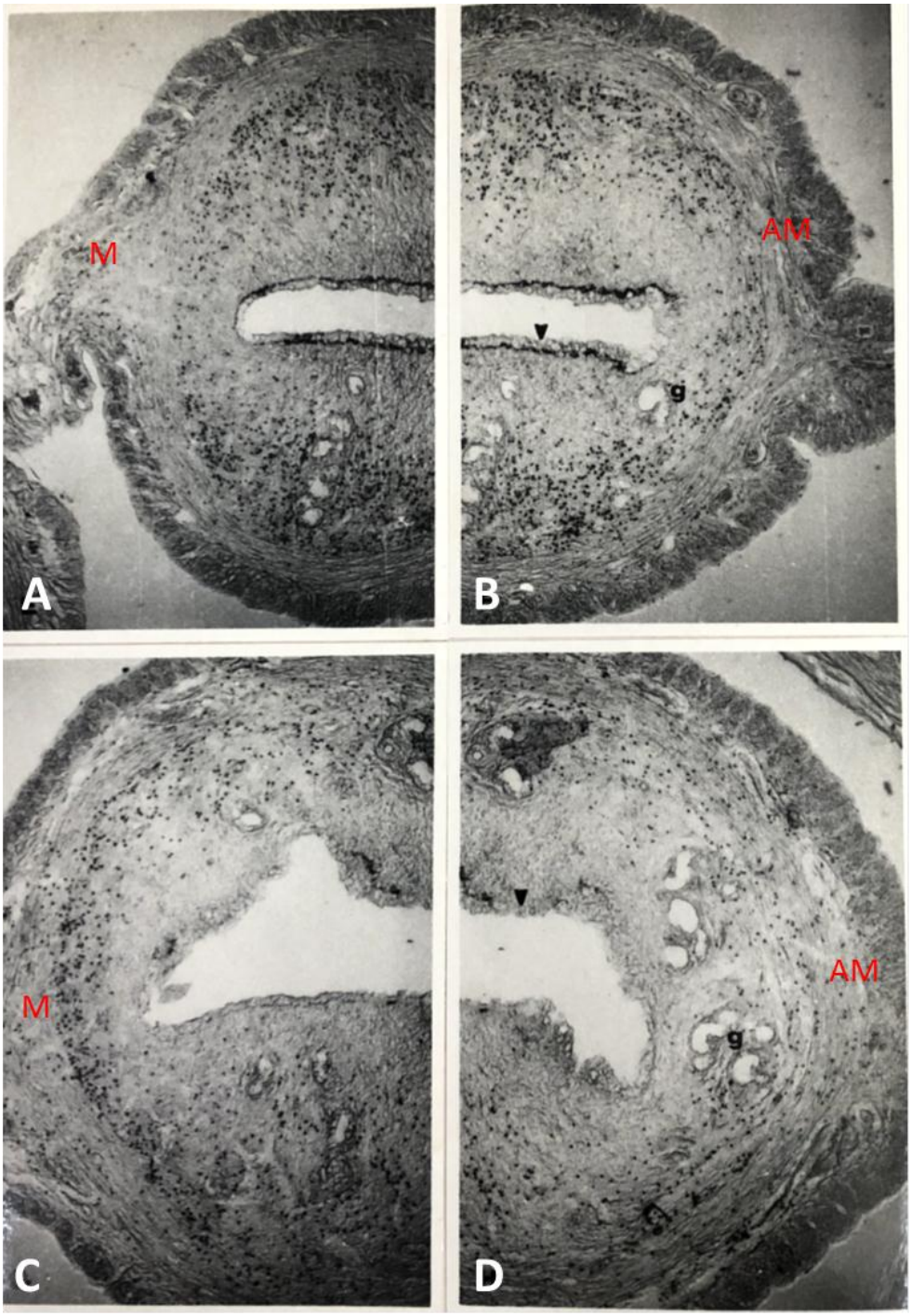

Figure 2: Showing increased lipid staining on day 4 p.c.(A,B) vs. day 3(Figure 1 A,B) in entire stroma. Uterine luminal epithelium (Arrow head) showing lipid staining at the basal region. In rats administered CDRI-85/287 exhibit a decline in lipid staining of luminal epithelium(Arrow head) but, similar or slight decrease in activity can be seen in endometrial stroma(C,D). Figures A-D: x40 magnification.

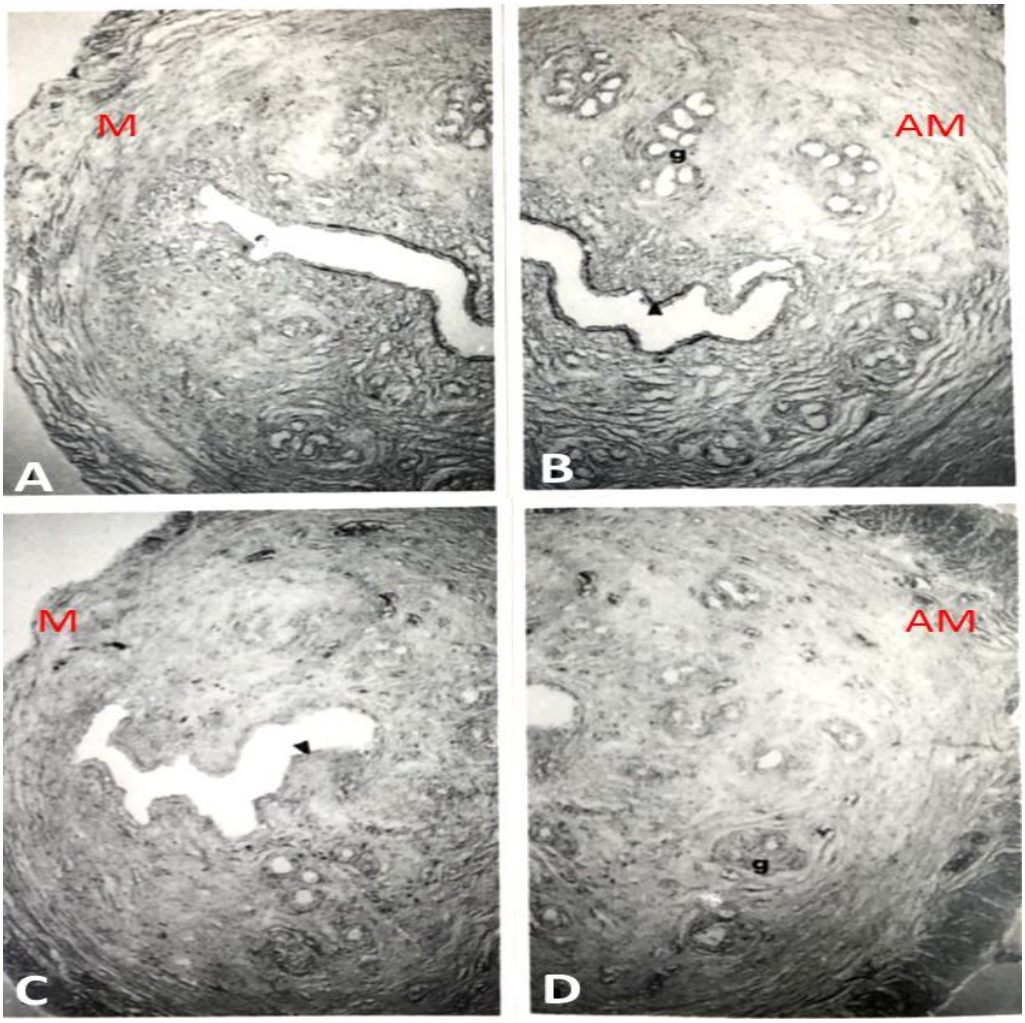

Figure 3: Uterine sections showing decreased lipid staining reaction in stroma and epithelium (Arrow head) in control (A, B) as compared to day 4 p.c. rats. Compound CDRI 85/287-treated (C, D) rats showing decreased activity of lipid in luminal (Arrow head) and glandular (g) epithelium as compared to control rats. All figures were micro photographed at x40 magnification.

Glycogen

Histochemical localization of glycogen activity on day 3 *p.c.* in control rats, was observed to be moderate in uterine epithelium whereas in endometrial sub epithelial stroma and leucocytic cells exhibited intense staining of glycogen activity. Serosa and myometrium showed higher activity and blood vessels embedded in endometrial stroma and uterine muscularis revealed moderate activity. On day 4 *p.c.*, a marked increase in glycogen contents was seen in entire endometrium as compared to day 3 control rats, where uterine stromal and epithelial cells showed very intense staining of glycogen activity. However, significant decrease in glycogen staining reaction was observed in stroma and epithelium on day 5 *p.c.*

compared to day 4 rats, but higher glycogen activity was noticed in sub-epithelial stromal region than in peripheral stroma. Higher staining intensity was observed in mesometrial side compared to weak staining of anti-mesometrial side of uterus (Figure 4).

Rats treated with CDRI 85/287 (2.5 mg/kg, on day 1 *p.c.*) autopsied on day 3 and 4, exhibited decreased glycogen content in uterine stroma particularly in leucocytes as compared to controls, while glycogen staining intensity was similar in uterine epithelium. On day 5, a marked decrease in glycogen staining in entire endometrium (stroma/epithelium) was observed as compared to corresponding control rats. In myometrium and serosa there was a slight change (decrease) in glycogen contents in treated rats as compared to controls during pre-implantation days viz. days 3, 4 & 5 *p.c.*(Figure 4).

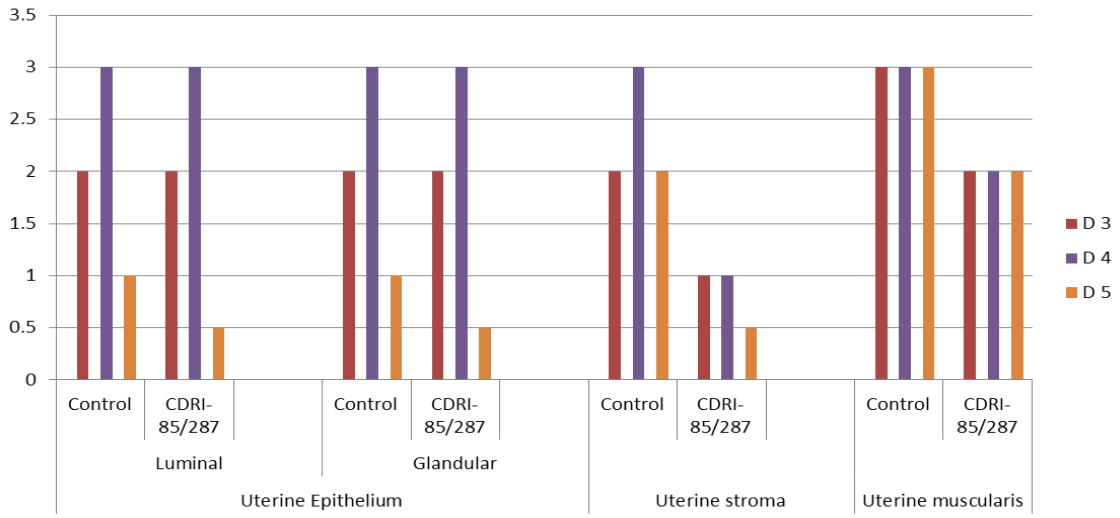

Figure 4: Histogram of glycogen staining intensity in uterine epithelium (luminal and glandular), stroma and muscularis region in control and CDRI 85/287- treated rats during pre-implantation days 3-5 *p.c.* (D3-Day 3 *p.c.*; D4- Day 4 *p.c.*; D5- Day 5 *p.c.*), 3- Strong intensity, 2- Moderate intensity, 1- Weak intensity. 0.5-Very weak intensity, 0- negative.

DISCUSSION

Studies in the past[6,16] have confirmed the findings that ovarian hormones affect uterine lipids and major fractions of lipids in the uterine epithelium are present as neutral fat (i.e. triglycerides)/ fatty acids.[6,16,17] Most of the triglycerides are used for phospholipid synthesis and as energy sources for the cell multiplication/developmental process.[3] Epithelial growth and secretion of fluid have a high-energy demand and the presence of neutral lipids adjacent to the synthetic and secretory mechanisms would help to satisfy such a demand.[16]

From the staining reactions obtained in our experiment strong lipid staining in the uterine luminal epithelium and weak lipid staining in sub-epithelial stroma and blood capillaries was discernible on day 3 *p.c.* in rats. Lipid staining intensity increased in the endometrial stroma particularly in leucocytes and in the base of uterine luminal epithelium of day 4 *p.c.* It is known that this is the time when very intense mitotic replication is dying down in these cells. Thus fatty acids which are no longer needed for phospholipid synthesis or as an energy source are probably stored as triglyceride droplets. It may be pointed out that increased staining intensity on day 4 may also be as a result of estrogen surge.[4,6,18] Therefore strong lipid staining especially in luminal epithelium, leucocytes and endometrial stroma on days 3 and 4 *p.c.* was noticed. A decrease in the staining intensity in uterine stroma as well as uterine luminal epithelium on antimesometrial side on day 5 is probably associated with initiation of the decidualization and implantation. Under the influence of the Compound 85/287 a general decrease was noticed in the staining intensity on all the days (3, 4 and 5) *p. c.* due to potent antiestrogenic activity.[19]

The accumulation of glycogen in the deciduas (antimesometrial side) and laterally to the embryo extends laterally and enlarges with further development. In our studies, the staining intensity of glycogen was observed to increase from day 3 to 4 *p.c.* On day 3 *p.c.* the activity was noticed to be intense in the sub-epithelial stromal cells and leucocytic cells in the endometrial stroma, myometrium and serosa. But on day 4 *p.c.* the glycogen staining was observed to be intense in the entire endometrium. This increased staining intensity of uterine glycogen has been attributed to synergistic action of rising levels of estrogen on these days.[20] Carrington and Bailey[21], Shinkarenko et al[22] and, Malini and Vanithakumari et al[23] showed increase in glycogen content in ovariectomized rats after beta-sitosterol and estradiol treatment. It is also considered that glycogen metabolism may provide a nutritional element to the early conceptus at a time, when it is dependent on histotrophic activity.[19,24] Greenstreen and Fotherby[25] have also shown that there is increased glycogen turnover during early pregnancy. The uterine glycogen level was observed to be decreased in the stroma and epithelium on day 5 as compared to days 3 and 4 *p.c.* in normal pregnant rats. These results are comparable to those of Rajalakshmi et al[26], which coincides with the presence of blastocyst in utero.[27] Thus, suggesting that glycogen mobilization is necessary to meet the energy requirements of the nidation response.[28]

In our results after the administration of CDRI 85/287 at its contraceptive dose (2.5mg/kg) on day 1 *p.c.*, we observed a general decline in the glycogen staining intensity from days 3-5 *p.c.* mainly in the stromal portion as compared to controls. This inhibition in glycogen content during pre-implantation and on implantation day is due to antiestrogenic activity as it is well established that estrogen causes increase in glycogen content.[29] Moreover, estrogen is reported to be necessary for implantation of the blastocyst in the rat.[30, 31] Mohla and Prasad[32] have reported an interference with normal glycogen metabolism with certain triphenylene antiestrogens. Srenivasulu et al[19] observed variation in glycogen content after treatment with CDRI 85/287 and suggested non-utilization of this energy substrate in the presence of this antiestrogenic compound.

CONCLUSION

It is evident that this study that CDRI 85/287 interferes with certain important biochemical constituents in different cellular compartments as studied histochemically in the rat uterus. Thereby leading to interference with the sequence of the events involved in the preparation of uterine sensitivity for implantation of the blastocyst. Thus, contributing to anti-implantation effect of CDRI –85/287, this in turn may be attributing to its potent anti-estrogenic activity.

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