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Full Length Research Paper

Effect of intra-uterine injection of H_2 receptor blockers on implantation in albino Wistar rats

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The role of histamine in various stages of female reproductive functions is now understood. The anti-implantation activity of various H_2 receptor blockers had been established earlier by our laboratory. The present studies were conducted to evaluate the local effects of direct intra-uterine injection of H_2 receptor blockers on implantation in albino Wistar rats. The individual administration of ranitidine, famotidine and roxatidine by direct intra-uterine injection on Day 4 of pregnancy in female albino Wistar rats produced 100% anti-fertility activity probably by acting on the H_2 receptors on the blastocyst. Control group animals delivered normal litters. Unilateral injection of roxatidine inhibited implantation in the treated and contralateral uterine horns of rats indicating its systemic as well as local anti-implantation activity while ranitidine and famotidine showed only the local effects on the treated uterine horns suggesting the blockade of locally derived uterine histamine in implantation. Histopathological examination of uterus revealed the absence of morphological damage to the endometrial epithelium. It is concluded that, the intra-uterine application of H_2 receptor blockers prevents conception in rats.

Key words: Anti-implantation activity H_2 receptor blockers, ranitidine, famotidine, roxatidine, intra-uterine injection, implantation.

INTRODUCTION

Recognizing that the currently available contraceptive options represent a limited choice for women, WHO initiated contraceptive research and development programmes had identified the process of implantation as a promising area for investigation (Griffin, 2005). Implantation is the most critical stage in the establish-ment of pregnancy. Blastocyst implantation involves a complex series of events occurring over time (Carson et al., 2000). In animals, an experimental evidence for local interactions between the blastocyst and endometrium at the time of implantation has been demonstrated [Kennedy, 1994]. Histamine appears to play a major role in all the following events viz . the follicular development and ovulatory process (Kathpalia and Prashad, 1990; Paczosca-Eliasiewicz and Rzasa, 1998), blastocyst implantation (Espey, 1980; Szego, 1965; Szego and Gitin, 1964), contractile activity of uterus (Szelag, 2002), lactation (Bealer and Crowley, 2001) and pregnancy maintenance (Kahlson, 1960; Cocchiara et al., 1988). The initial attachment reaction for implantation results from an intimate “cross–talk” between the trophectoderm of the active blastocyst and luminal epithelium of the receptive uterus, which occurs late on the Day 4 (22 00 to 23 00 h) of pregnancy (Das et al., 1994). A local inflammatory reaction, accompanied by accumulation of histamine in the uterus occurs around the time of implantation (Marcus and Shelesnyak, 1968). Uterine-derived histamine interacts with embryonic H_2 receptors in a paracrine fashion to initiate the process of implantation. The blastocyst H_2 receptors are the targets for uterine histamine in implantation (Zhao et al., 2000). Uterine epithelial cells have been described as the major source of histamine in the mouse uterus, which peaks on Day 4 of pregnancy (Paria et al., 1998). Histamine is also

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Abbreviations: u.h, Uterine horn; **µl,** microlitre; **g** , gram, **mg,** milligram; **kg,** kilogram; **ml,** milliliter; **h_2 receptor,** histamine 2 receptor.

responsible for the induction of decidualization (Barkai and Kraicer, 1996). In rabbit, inhibition of histamine release from mast cells by means of intra-luminal administration of disodium cromoglycate was reported to prevent implantation and the decidual cell reaction (Dey et al., 1978).

Considering the various roles played by histamine in various stages of gestation, considerably anti-implantation activity is expected for histaminergic antagonists. With a thorough knowledge of the role of histamine in various stages of reproduction, extensive on the anti-fertility studies of H_2 and H_1 receptor blockers have been carried out on the laboratory of DIPSAR. Although implantation was reduced in rats treated with a combination of H_1 and H_2 receptor blockers (Brandon and Wallis, 1977), the research conducted in our laboratory revealed that the oral administration of various H_2 recep-tor blockers namely cimetidine and ranitidine produced 80 to 84% anti-implantation activity in albino Wistar rats. Our findings on the anti-implantation activities of H_2 receptor blockers were also confirmed by Ahmed (1996), Zhao et al. (2000) who reported, uterine-derived histamine interacted with embryonic H_2 receptors in a paracrine manner to initiate the process of implantation and uterine epithelial cells were the major source of histamine which peaked on Day 4 of pregnancy. Hence, we have selected H_2 receptor blockers to evaluate their anti-implantation effects, which are non-steroidal and totally devoid of the side effects associated with hormonal contraceptives. Ranitidine, famotidine and roxatidine were administered by direct single intra-uterine injection on day 4 of pregnancy and their anti-implantation activity was compared with after oral administration. The present study is con-ducted to evaluate the local effects of direct intra-uterine injection of H_2 receptor blockers on implantation in albino Wistar rats.

MATERIALS AND METHODS

The Institutional Animal Ethical Committee (IAEC) of DIPSAR approved this present studies. Female albino Wistar rats of proven fertility were used for the study. Animals were maintained under standard breeding conditions at 21.1°C and 50 - 60% relative humidity. Water and dry pellets were given *ad libitum.*

Drugs

Ranitidine was obtained as a gift sample from Torrent Pharma-ceuticals Ltd., Ahmedabad, Gujarat-382721. Famotidine was ob-tained as a gift sample from Nicholas Piramal India Ltd., Mumbai, Maharashtra-402302. Roxatidine was obtained as gift sample from Hoest Marion Roussel Ltd., Mumbai.

Vaginal smears

Vaginal smears were checked with 100 µl of saline using a Pasteur pipette and were observed under microscope. Vaginal smears were taken daily for monitoring reproductive cyclicity, sperm positivity and pregnancy. Stages of estrous cycle were identified based on vaginal cytology.

Preparation of drug solution

Ranitidine and roxatidine were dissolved in sterile saline to prepare a solution of the required concentration. Famotidine is sparingly soluble in water; it was suspended in 1% gum acacia suspension using sterile saline.

Anti-implantation studies of H_2 receptor blockers in pregnant female albino Wistar rats by oral route

Wistar female rats weighing 180 to 200 g were cohabited with adult male rats at a proportion of 3 females: 1 male. The presence of spermatozoa in the vaginal smear, taken the next morning documented as pregnancy day 1. The pregnant rats were divided into different groups such as experimental and the control. For oral administration H_2 receptor blockers were administered on different days of pregnancy that is, days 1 to 7 and day 4 at different doses. However the number of *C. lutea* and the implantation sites were counted after laparotomy on the 10th day. The animal was allowed to deliver and the number of litters delivered was counted. The percentage antifertility activity, pre-implantation and post-implantation losses were calculated. The results were statistically analyzed by student's 't' test.

Anti-implantation activity of H_2 receptor blockers after direct single intra-uterine injection (Bilateral intra-uterine administration)

The intra-uterine injection was done as per the method of Upadhyay et al. (1990). For intra-uterine administration, ranitidine (7.0 and 14 mg/0.1 ml/u.h), famotidine (1.0 and 2 mg/0.1 ml/u.h) and roxatidine (3.5 and 7.0 mg/0.1 ml/u.h) were directly injected into each uterine horn near the utero-cervical junction after midventral laprotomy. Sterile saline 0.1 ml was administered to the control groups for ranitidine and roxatidine whereas the control group of famotidine received 0.1 ml of sterile saline diluted 1% gum acacia. The uterine horn was held with forceps for 30 s after injection to prevent reflex. The abdominal wall was sutured, the skin was closed and the rats were returned to their cages. The number of *C. lutea* and the implantation sites were counted after laparotomy on the 10th day. The number of healthy embryos and resorbing fetuses were counted. On the other hand, the sum of the two figures was taken as the indication of the number of implantation sites. Embryos with bright red dish aspect and a clear margin were considered healthy; those with dull blue color, no clear margin and orientation and with some surrounding exudates were considered to be resorbing (Batta and Martini, 1975). The animal was allowed to deliver and the number of litters delivered was counted. The percentage antifertility activity, pre-implantation and post-implantation losses were calculated. The results were statistically ana-lyzed by One-way ANOVA test.

Anti-implantation activity of H_2 receptor blockers after direct single intra-uterine injection (Unilateral intra-uterine administration)

Pregnant female Wistar rats were taken and saline diluted Ranitidine; and Roxatidine and 1 % gum acacia diluted Famotidine were injected directly into right uterine horn while the 0.1mL of sterile saline was injected into the (left) contralateral horn of the same animal except for Famotidine, where 1 % gum acacia suspended in sterile saline was given to the control uterine horn on

Table 1. Effect of ranitidine on female rats when administered daily at the dose of 35, 70 and 140 mg/kg orally from 1-7 days of pregnancy.

S/No.	Groups	Mean no. of *Corpus luteum* (CL)	Mean no. of implants	Mean no. of litters	% Pre-implantation loss	% Post implantation loss	% Antifertility activity
1	Control	10.5 ± 0.71	9.16 ± 0.65	8.0 ± 0.68	12.69 ± 1.77	13.04 ± 2.0	26.26 ± 3.79
2	Ranitidine 35 mg/kg	15.0 ± 1.15	9.16 ± 1.92	6.5 ± 2.18	40.25 ± 9.74	42.34 ± 16.31	58.85 ± 12.58
3	Ranitidine 70 mg/kg	18.0 ± 0.51	9.83 ± 1.99	6.6 ± 2.33	45.4 ± 10.6 *	48.34 ± 17.11	63.46 ± 12.36
4	Ranitidine140 mg/kg	16.0 ± 0.81	10.6 ± 0.8	6.83 ± 1.6	32.94 ± 4.89	37.13 ± 12.24	46.61 ± 12.64
One way ANOVA	F				3.51	1.01	2.27
	df				3.20	3.20	3.20
	P				< 0.05	> 0.05	> 0.05

Values are expressed as mean ± SEM. * $p < 0.05$ when compared to control.

the 4th day of pregnancy. The abdominal wall was sutured and the rats were returned to their cages. Evidence of implants was ascertained under the observation by laprotomy on 10th day of pregnancy. The number of *C. lutea* and the number of implan-tation sites on the left and right uterine horns were counted. The results were statistically analyzed by student's 't' test.

Effect of H_2 receptor blockers on the functional morphology of reproductive organs

The effects of intra-uterine administration of H_2 blockers on the structure and function of reproductive organs was studied to assess its adverse effects, if any. Six groups of female rats were selected. The vaginal smear was also checked to monitor the reproductive cyclicity. Ranitidine (14 mg/0.1 ml/u.h), famotidine (2 mg/0.1 ml/u.h), roxatidine (7 mg/0.1 ml/u.h), sterile saline (0.1 ml/u.h) and 1% gum acacia suspended in sterile saline (0.1 ml/u.h) were injected directly to the non-pregnant uterus of the test groups, control groups respectively. The animals were killed, at specific time intervals viz. at the end of 1st day, at the end of 5th day and at the end of 10th day after the administration of test drugs individually along with control groups. The uterine horns were dissected, fixed in 10% buffered formalin and examined under a light microscope. Semi-thin sections were stained with hematoxylin and eosin and studied under a light microscope.

RESULTS

The percentage anti-fertility activity following daily oral administration of ranitidine for 1 to 7 days of pregnancy at the dose level of 35 mg/kg was 58.85%) . However, at the higher dose levels such as 70 and 140 mg/kg produced the percentage antifertility activity of 63.46 and 46.61 respectively. The control group for ranitidine, treated with saline produced the antifertility activity of 26.26% (Table 1). The percentage anti-fertility activities following daily oral administration of famotidine for 1 to 7 days of pregnancy at the dose level of 5 mg/kg was 70.55. Famotidine at the dose of 10 and 20 mg/kg orally produced percentage antifertility activity of 44.0 and 60.52%, respectively. The control group for famotidine produced the percentage antifertility activity of 28.54% (Table 2) . roxatidine at the dose of 17.5 mg/kg orally produced percentage antifertility activity of 61.85, while higher dose levels viz. 35 and 70 mg/kg orally produced percentage antifertility activities of 66.89 and 63.19 respectively. The control group for roxatidine, treated with saline produced the percentage antifertility activity of 25.76 (Table 3). When given orally only on Day 4 of pregnancy ranitidine, famotidine and roxatidine exhibited significant antifertility activities. Ranitidine at the dose of 140 mg/kg, produced percentage antifertility activity of 77.42, while that of control group produced 38.24 respectively (Figure 1). Famotidine at the dose of 10 mg/kg produced percentage antifertility activity 80.43, while that of control group produced 35.25, respectively (Figure 1). Roxatidine at the dose of 70 mg/kg produced percentage antifertility activity of 81.02 while that of control group was 28.77, respectively (Figure 1).

Bilateral intra-uterine administration of ranitidine at the dose of 7 mg/0.1 ml/u.h had produced the percentage pre-implantation and post-implantation losses of 68.75, 100.0 respectively whereas ranitidine at the dose of 14 mg/0.1 ml/u.h had produced percentage pre-implantation and post-implantation losses of 75.92, 100 respectively. The control group animals received sterile saline at the dose of 0.1 ml/u.h had produced percentage pre-implan-tation, post-implantation losses of 29.4 and 11.96, respectively (Figure 2). Famotidine at the dose of 1 mg/0.1 ml/u.h had shown the percentage pre-implan-tation loss, post-implantation loss of 83.93 and 83.33 respectively whereas famotidine at the dose of 2 mg/0.1 ml/u.h had produced percentage pre-implantation and post-implantation losses of 74.75 and 100.0, respectively. The control group animals received 1% gum acacia suspended in sterile saline at the dose of 0.1 ml/u.h had produced percentage pre-implantation and post-implantation losses of 29.28 and 12.38, respectively (Figure 3). Roxatidine at the dose of 3.5 mg/0.1 ml/u.h had produced the percentage pre-implantation and post-implantation losses of 80.16 and 83.33, respectively whereas roxatidine at the dose of 7.0 mg/0.1 ml/u.h had produced percentage pre-implantation and post-implantation losses of 82.23 and 100.0 respectively. The control group animals received sterile saline at the dose

Table 2. Effect of Famotidine on female rats when administered daily at the dose of 5, 10 and 20 mg/kg orally from 1 to 7 days of pregnancy.

S/No.	Groups	Mean no. of *Corpus luteum* (CL)	Mean no. of implants	Mean no. of litters	% Pre-implantation loss	% Post implantation loss	% Antifertility activity
1	Control	10.83 ± 0.60	8.5 ± 0.42	7.66 ± 0.21	21.13 ± 3.09	9.12± 3.18	28.54 ± 2.81
2	Famotidine 5 mg/kg	13.83 ± 0.79	9.0 ± 1.96	3.83 ± 1.60	35.8 ± 13.17	44.1 ± 16.82	70.5 ± 12.5*
3	Famotidine10 mg/kg	13.66 ±0.33	10.66 ± 0.55	7.66 ± 1.11	21.80 ± 3.97	28.4± 9.39	44.0 ± 7.91
4	Famotidine 20 mg/kg	12.33 ± 0.42	6.33 ± 1.72	4.83 ± 1.57	48.72 ± 14.16	29.53± 15.03	60.52 ± 33.11
One way ANOVA	F				1.72	1.35	3.33
	df				3.20	3.20	3.20
	P				> 0.05	> 0.05	< 0.05

Values are expressed as mean ± SEM. * p < 0.05 when compared to control.

Table 3. Effect of roxatidine on female rats when administered daily at the dose of 17.5, 35 and 70 mg/kg orally from 1 to 7 days of pregnancy.

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Values are expressed as mean ± SEM. * p < 0.05, **p < 0.01 when compared to control.

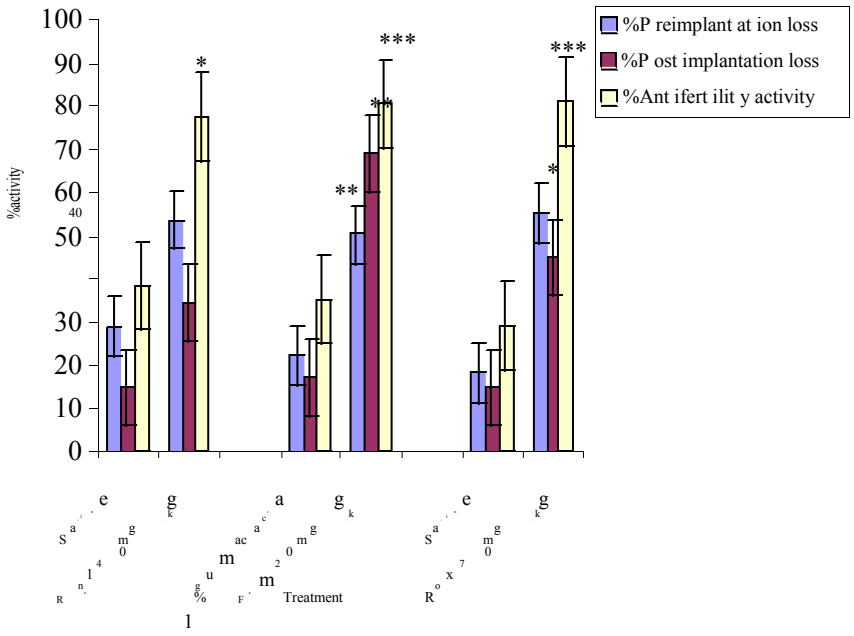

Figure 1. Effect of ranitidine, famotidine and roxatidine in female rats when administered orally at the dose of 140, 20 and 70 mg/kg, respectively on 4th day of pregnancy. The mean values are indicated, while the vertical line indicates ± SEM.

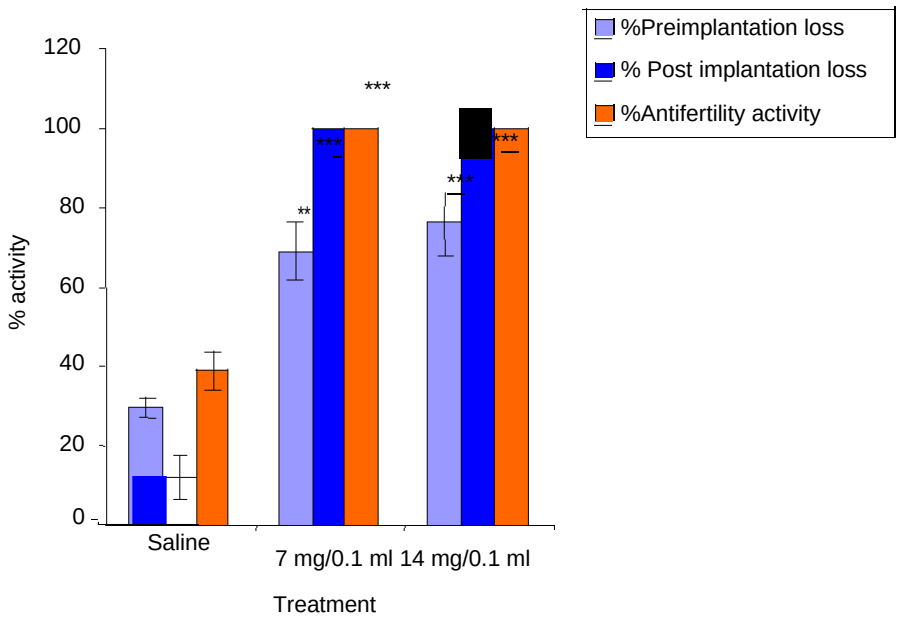

Figure 2. Effect of Ranitidine on female rats when administered at the dose of 7.0 and 14.0 mg/0.1 ml/u.h by bilateral intra-uterine injection on 4th day of pregnancy. The mean values are indicated, while the vertical line indicates ± SEM.

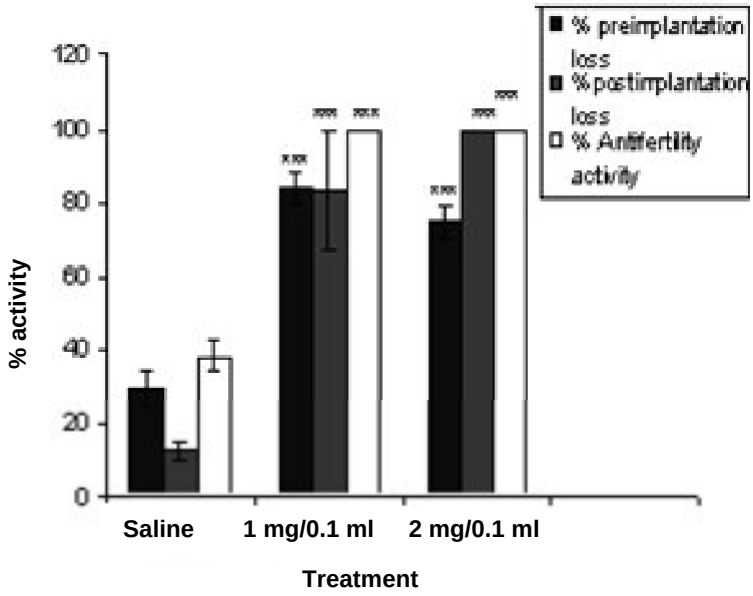

Figure 3. Effect of Famotidine on female rats when administered at the dose of 1.0 and 2.0 mg/0.1 ml/u.h by bilateral intra-uterine injection on 4th day of pregnancy. The mean values are indicated, while the vertical line indicates ± SEM.

of 0.1 ml/u.h had produced percentage pre-implantation and post-implantation losses of 21.37 and 7.45 respectively (Figure 4). Ranitidine, famotidine and roxatidine produced 100% anti-fertility activity probably by acting on the H_2 receptors on the blastocyst (Figures 2 - 4) while the control group animals delivered normal litters.

Unilateral administration of H_2 blockers reduced the mean number of implants in the treated uterine horn compared to the contralateral horn (Table 4 and Figure 6). The control uterine horn had shown normal implantation.

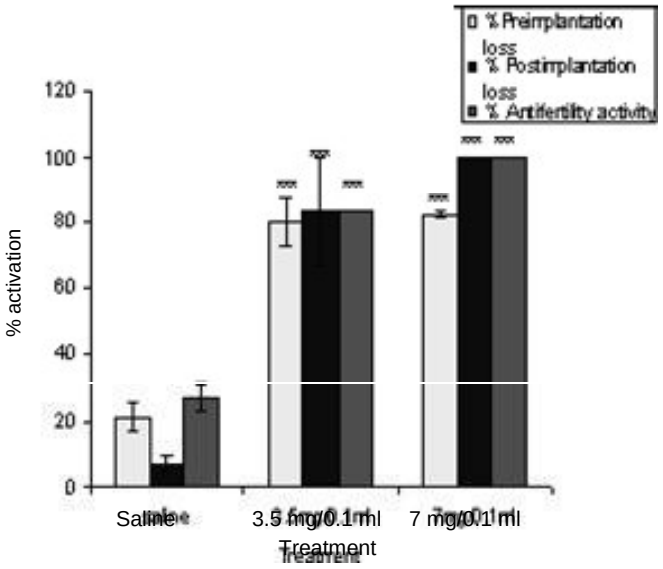

Figure 4. Effect of Roxatidine on female rats when administered at the dose of 3.5 and 7.0 mg/0.1 ml/u.h by bilateral intra-uterine injection on 4th day of pregnancy. The mean values are indicated, while the vertical line indicates ± SEM.

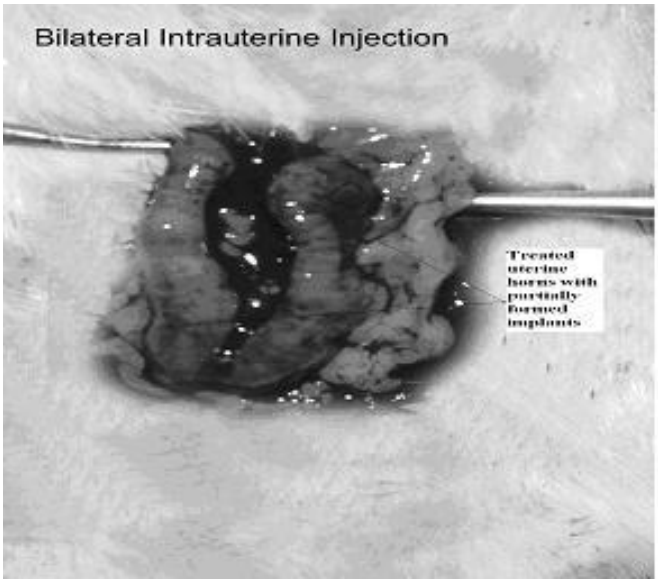

Figure 5. Uterine horns following the bilateral intra-uterine injection of H_2 receptor blockers. The implants were partially formed and were dark bluish–red in color and not following normal spacing.

Most of the embryos in the treated uterine horn as observed on the 10th day of pregnancy had abnormal macroscopic characteristics (Figure 6). Histopathological examination following H_2 blockers treatment revealed that it did not produce any tissue necrosis or ulceration of the endo-metrial epithelium and were devoid of any morphological damage when administered directly to the uterus. The treatment with H_2 receptor blockers did not interfere with normal reproductive cyclicity of the female rats as evidenced by the vaginal smear cytology (Table 5).

DISCUSSION

The role of histamine in implantation has long been considered important. However, its site of formation and mode of action in this process was not clearly understood. Paria et al. (1998) had shown that epithelial cells are the primary source of histamine in mouse uterus. Zhao et al. (2000) reported that the effects of H_2 receptor antagonists viz. ranitidine and famotidine on inhibition of implantation were specific and not due to non-specific toxic effects. Hence, H_2 receptors on the blastocyst but not the uterus are the primary target for uterine histamine for implantation. The absence of H_1 and negligible presence of H_3 in blastocyst as well as non-responsiveness of blastocyst to H_1 agonists or antagonists place H_2 as the primary target for histamine action during im-plantation. The above histamine assumption is consistent with the report that H_1 receptor deficient mice have the normal embryo development and implantation (Inoue et al., 1996). Thus, it appears that the expression of H_2 receptor in the blastocyst is important for further blastocyst growth and implantation.

The anti-implantation activities of H_2 receptor blockers were compared to their respective control groups. When the dose of H_2 receptor blockers were increased from lower dose to higher dose, there was no further increase in activity, in fact there was slight reduction in activity, which was statistically insignificant (Tables 1 - 3). It was found that the anti-implantation activity following oral ad-ministration was not dose-dependent and also, the anti-implantation activity was more pronounced when given on the day 4 of pregnancy by single oral administration (Figure 1) rather than 1 to 7 days of pregnancy (Tables 1 -3). In the present studies, H_2 receptor blockers viz. ranitidine, famotidine and roxatidine were administered by direct single bilateral intra-uterine injection on the Day 4 of pregnancy. Control group animals received same volume (0.1 ml) of 0.9% sterile saline solution and 1% gum acacia suspended in sterile saline. All the control group animals became pregnant and delivered normal litters, while animal treated with H_2 receptor blockers had shown 100% antifertility activity (Figures 2 - 4). In ranitidine treated groups, the implants formed were less in number and were small. Some of the implants were fused together and were possessing dark bluish color (Figure 5). It was observed that those implants were not following normal spacing, color and orientation as that in control. In roxatidine treated group, the implants were less and were partially formed. Famotidine produced less implants and the implants were fused together and were

Table 4. Effect of ranitidine, famotidine and roxatidine on female rats when administered by unilateral intra-uterine injection on 4th day of pregnancy.

S/NO.	Groups	Mean no. implants in control uterine horn	Mean no. implants in treated uterine horn
1	Ranitidine 7.0 mg/0.1 ml	4.16 ± 0.70	1.83 ± 0.40 *
2	Ranitidine 14.0 mg/0.1 ml	3.16 ± 0.54	0.83 ± 0.30 **
3	Famotidine1.0 mg/0.1 ml	4.66 ± 0.95	0.83 ± 0.53**
4	Famotidine2.0 mg/0.1 ml	5.0 ± 0.36	1.0 ± 0.51 ***
5	Roxatidine 3.5 mg/0.1 ml	4.5 ± 1.22	2.16 ± 0.90
6	Roxatidine 7.0 mg/0.1 ml	1.33 ± 0.61	0.83 ± 0.47

Values are expressed in mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to control uterine horn.

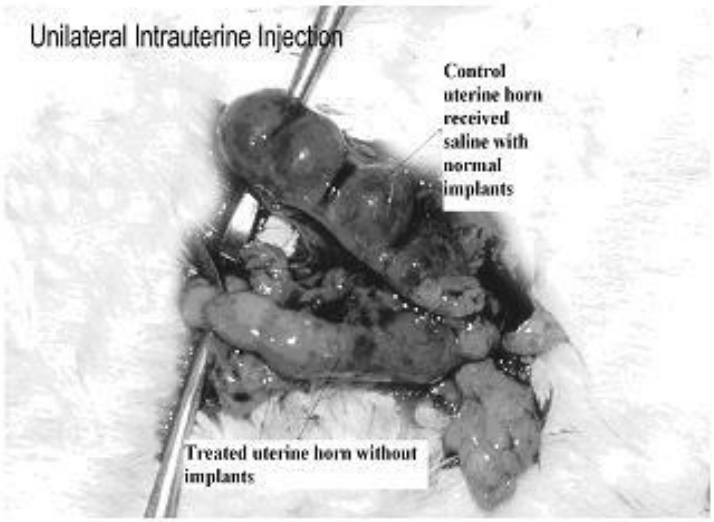

Figure 6. Right Uterine horn following the unilateral intra-uterine injection of H_2 receptor blockers and left uterine horn following the intra-uterine injection of sterile saline. The implants in the left uterine horn were normal and there was complete absence of implants in the right horn.

possessing bluish color. Those morphological signs were considered compatible with resorption of embryos (Pedron et al., 1985). The partially formed implants were resorbed in the uterine horn; whereas, all uterine horns of control groups had evidence of normal implantation and delivered the litters (Figure 7) . Our observations are in accordance with those of Batta and Martini (1975).

Unilateral injection of roxatidine produced less number of implants not only in the drug treated uterine horn but also in the contralateral uterine horn suggesting its systemic effects in addition to the local actions while rani-tidine and famotidine produced partially formed implants in the treated horns and were resorbed. The implants formed in the contralateral horn were normal, healthy and were delivered after the gestation period (Figure 6). In many cases, the treated uterine horns were inflamed without the formation of implants. The intra-uterine administration of H_2 receptor blockers did not alter the cyclical changes to the uterus and it showed that the ability of the uterus to respond to the ovarian hormones (Table 5). Histopathological findings revealed that it did not produce any tissue necrosis or ulceration of the endometrial epithelium and revealed that those that were not producing any morphological damage when given directly to the uterus, however the moderate acute inflammatory reaction was produced in not only the ranitidine and roxa-tidine treated groups (Figures 8 and 9).

Statistical analysis

Results are shown as mean ± SEM. Statistical analysis was performed using graph Pad instat, statistical software, version 3.01. $P < 0.05$ was considered statistically

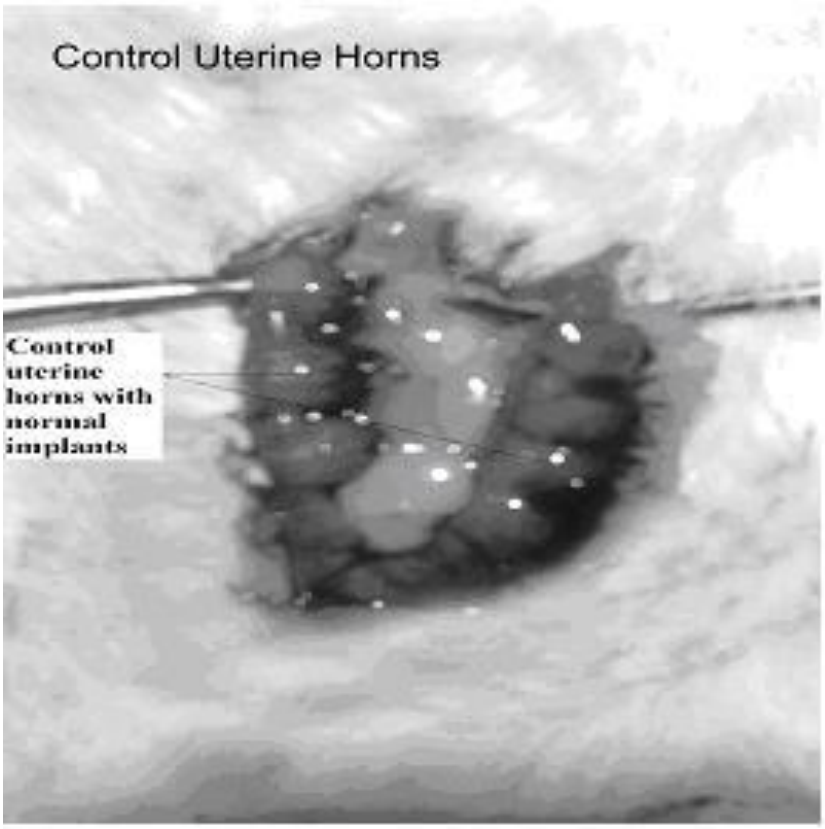

Figure 7. Control uterine horns following the bilateral intra-uterine injection of sterile saline. The implants formed were following normal spacing and orientation.

Table 5. Histo-morphological effects of intra-uterine injection of H_2 receptor blockers.

S/No.	Groups	Days of sacrifice	Vaginal cytology	Dating	Acute inflammatory reaction
1		1	Proestrous	Early proliferatory	+ +
2	1% Gum acacia	5	Proestrous	Late proliferatory	+ +
3		10	Metestrous	Proliferatory	+/-
1		1	Proestrous	Proliferatory	+
2	Sterile saline	5	Diestrous	-	-
3		10	Metestrous	-	-
1		1	Proestrous	Secretary	+ +
2	Ranitidine	5	Estrous	Secretary	+ + +
3		10	Diestrous	Secretary	+ +
1		1	Proestrous	Proliferatory	+
2	Famotidine	5	Proestrous	Secretary	+ +
3		10	Diestrous	Proliferatory	+
1		1	Proestrous	Proliferatory	+ +
2	Roxatidine	5	Proestrous	Atrophy	++++
3		10	Diestrous	Secretary	+ + +

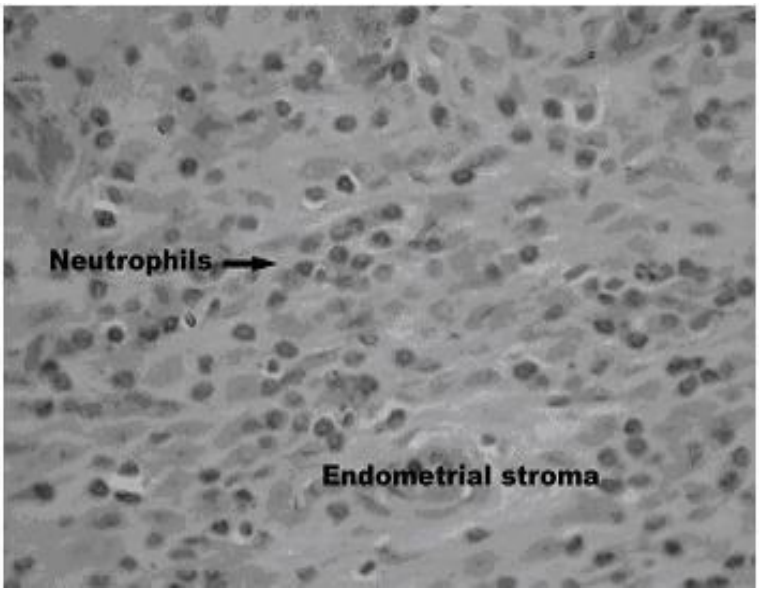

Figure 8. High power photomicrograph from T.S of uterine horn of rat collected at the end of 10th day after intra-uterine injection of Ranitidine (HE × 400 ×).

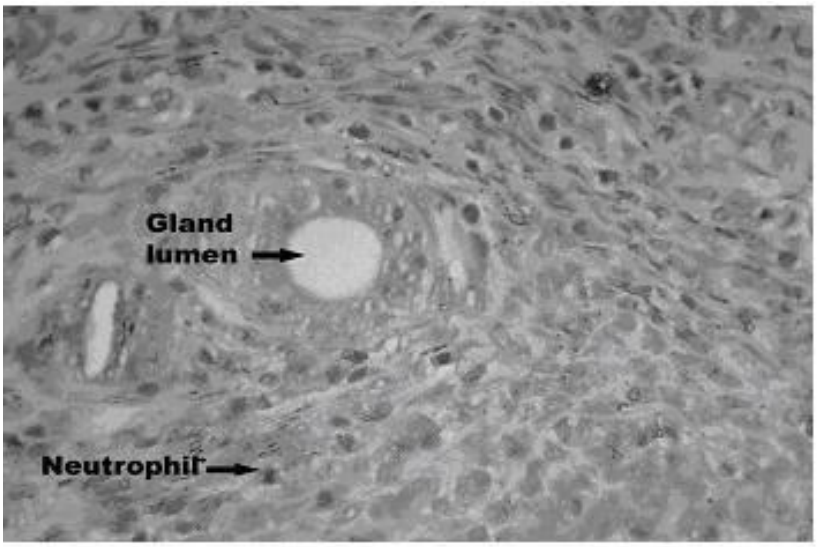

Figure 9. High power photomicrograph from T.S of uterine horn of rat collected at the end of 10th day after intra-uterine injection of Roxatidine (HE × 400×).

significant. Statistical comparisons were done using one-way analysis of variance (ANOVA) and student's 't' test.

Conclusion

The direct intra-uterine administration of H_2 receptor blockers on Day 4 of pregnancy in female albino Wistar rats produced 100% anti- fertility activity, which further confirms the role of uterine histamine in implantation. H_2 receptor blockers treated rats had normal reproductive cycles as indicated by the vaginal smears and did not produce any adverse effects to the uterus of female rats. The intra-uterine injection of H_2 receptor blockers adversely affects conception, exploring the possibility of one more non-steroidal molecule for intra-uterine contraceptive

device.

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