EFFECT OF OVARIAN HORMONE ON THE GROWTH AND DIFFERENTIATION OF THE RAT BLASTOCYST DURING THE DELAYED IMPLANTATION I. PROGESTERONE

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By

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Introduction

In mammals, it is well known that implantation occurs generally at the late stage of blastocyst. It has been confirmed the implantation and normal pregnancy in the rabbit (1, 2) and hamster (3), which had been ovarictomized in a very early stage of pregnancy, can be induced by daily adminsitration of progresterone alone. However, the implantation does not occur in the rat recieving progesterone only after ovariectomy, rather in these animals a vaiable blastocysts remains free in the uterine lumen, e.g. delaying implantation (4–7).

Utilization of this phenomenon in the rat has revealed considerable information on the hormonal requirements and the mechanisms for implantation of ova in this species. Generally, the growth of blastocysts occurs rapidly in the phase just before implantation, though their growth rate differs among the species. In mouse, rat and gunia-pig, the size of blastocyst at implantation is approximately the same as that of the tubal ovum (8. 9). In the rat, there is little known about the growth and differentiation of blastocyst at the pre-implantation stage and during implantation and also no information on the embryonic development during the delayed implantation induced experimentally and during lactation. The experiment was attempted to analyse the effect of progesterone on the embryonic development during pre- and implantation stage, using the rat induced experimentally to delay implantation and the lactating rat.

Materials and Methods

Experimental animals; Adult virgin female rats of Wistar strain, weighing 150–250 g., were used. Vaginal smears were taken daily for at least two consecutive cycles and rats that had 4-day cycles were chosen for the experiments. The animals were devided into five groups, consisting of three or four sub-groups.

In all groups, the animals in proestrus were caged overnight with males and were checked for the presence of sperm the following morning. The day that spermatozoa were found in the vaginal semars, was designated as Day 1 of pregnancy. Group 1, as control, consisted of four sub-groups of intact and normal pregnant rats.

The treatment groups (group 2–4) consisted of pregnant rats in which artificial delayed nidation was induced by the method described by Cochrane & Meyer (1957).

Ovariectmy was performed on Day 2 of pregnancy, that is 36 hours after the estimated time of fertilization. The ovariectomized rats in the group 2, 3 and 4 were daily adminsitered 2,4 and 8mg of progesterone dissolved in sesame oil, respectively.

In group 5, rats were bred to fertile male and allowed to carry to term. A nest box was provided at 17 day post coitum and from 20 Day of pregnaney, the pregnant rat was coupled with a fertile male and allowed to mate in post partum estrus. The rats were observed daily for the occurence of parurition; and their smear was taken daily from the paturition to 48 hrs after the term and fertile mating was checked. In the lactating rat, the litter was adjusted to 8 young in order to obtain definite stimulus of suckling.

Collection of blastocyst: The animals of each group were killed at 102, 108, 126, 132 and 174 hours after the estimated time of fertilization, according to the experimental designs given in Fig 1 and 2.

Uterine horns were exposed by a mid-ventral operation excised and separated at the servical region. The uterine horns were flushed fluid with 0.85 percent

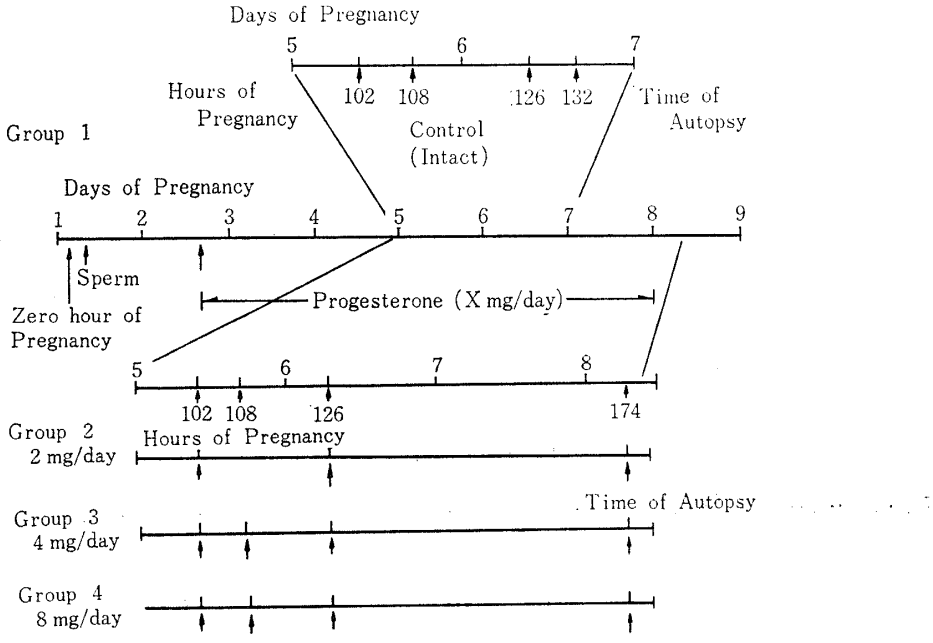

Fig. 1. Experimental designs for intact rats and treated rats.

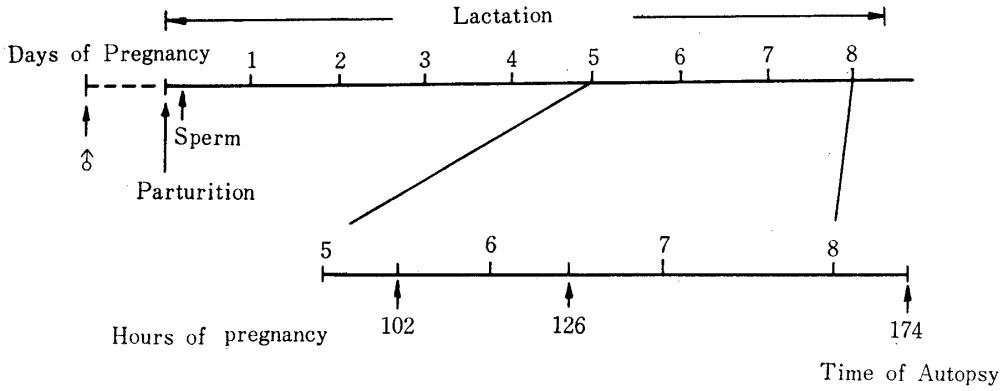

Fig 2. Experimental design for the lactating rats.

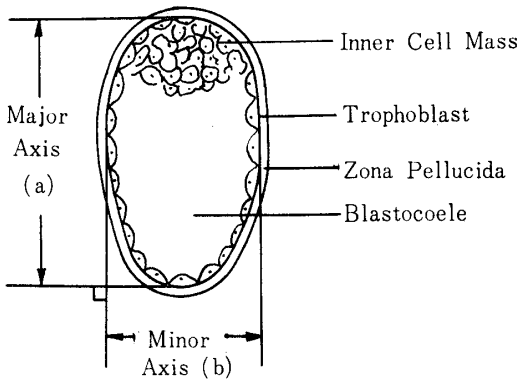

Fig. 3. Method used to measure axes of blastocyst.
Major axis (a), Minor axis (b).

saline solution to obtatin the live blastocyst. The flushed fluid was collected in a petri dish and with the aid of microscope, the blastocysts were grouped, drawn into a micropipette and transfered to the centre of a small depression slide. The lengths of the major and minor axes of blastocyst was an imaginary line which bisected diameter and perpendicular to the major axis. (Fig. 3). The area of blastocyst was estimated by the following formula:

$$S = \pi (a/2) (b/2)$$

Where S, is area of blastocyst; a and b are lengths of the major and minor axes, respectively.

The area of blastocoele was estimated as well as the case of the entire blastocyst.

Statistical analyses were performed for the studies on area of the entire blastocyst and blastocoele.

The presence or disappearance of zona pellucida was noted by direct observation of the live blastocyst. The time of disappearance of zona pellucida was checked in each group and the results discussed among each group.

Results

The results obtained from this experiment were summarized in Table 1.

A total of 436 of the fertilized ova were recovered and 371 of 436 (85%) developed in normal balstocyst. 15% of ova recovered were in the molura stage. In the intact rat control group, implantation was observed at 132 hours after fertilization. However, the rats ovariectmized following injection of progesterone and lactating rats resulted in complete failure of implantation.

The change in the major and minor axes of the entire blastocyst:

As shown in Fig. 4, the length of the major and minor axes increased with the progress of the time after the fertilization, although the rate of increase differed in each group. In the control group, the remarkable increase in the length of the major axis occurred between 108 and 126 hours after fertilization, while the minor axis increased gradually until initiation of implantation.

In the treatment groups, the length of the major and minor axes increased in proportion to the dose of progesterone and it was observed that the rate in the increase of the major axis was larger in comparison with that of the minor axis.

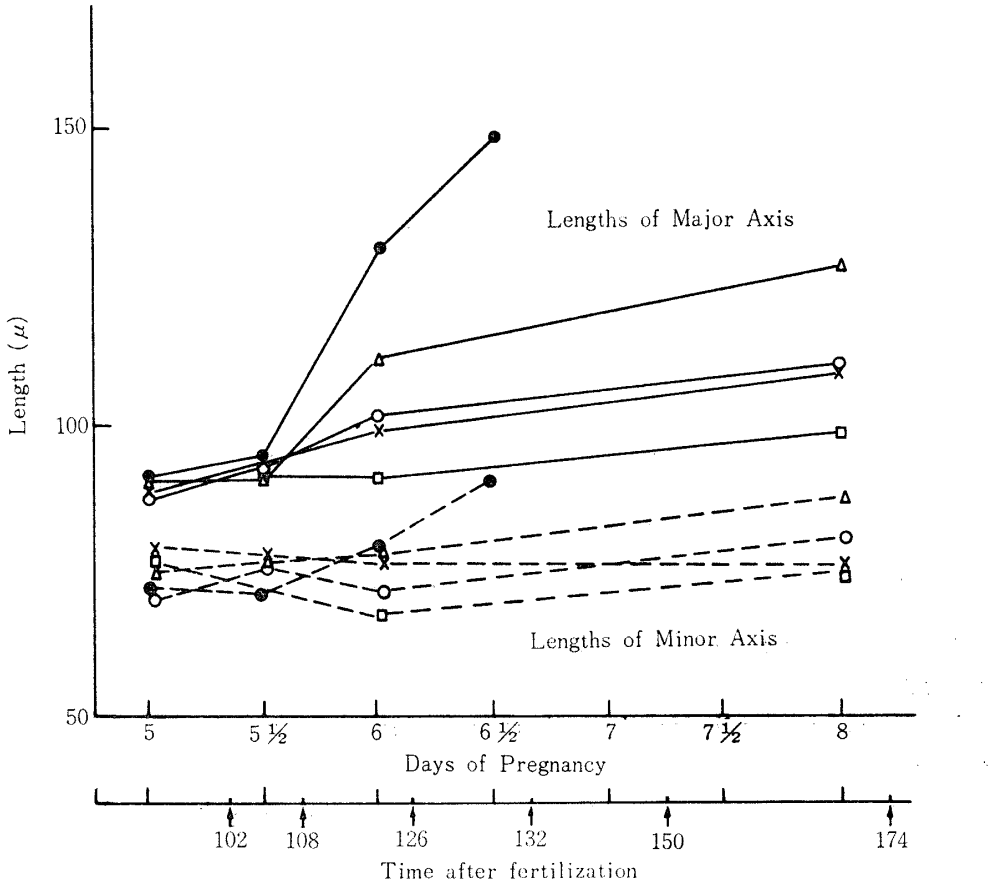

Fig. 4. Changes in major (—) and minor (- -) axis lengths of rat blastocysts. •, Intact animals; □, ○ and △ are 2,4 and 8 mg of progesterone treated animals, respectiveely; ×, Lactating rats.

Table 1. Changes in lengths and area of entire blastocyst and blastocoele from each groups.

Group	Day of pregnancy	No. of blastocyst	Lengths of Axes (μ)				Area ($\times 10^{-3}$ mm^2)	
			Blastocyst		Blastocoele			
			Major	Minor	Major	Minor	Blastocyst	Blastocoele
Intact (Control)	5	27	90.3 ± 7.2	71.3 +10.8	44.0± 5.2	52.2+ 9.6	5.1 ±1.0	2.0 ±1.0
	$5^1/_2$	29	93.7 ± 8.3	70.7 ±11.5	37.6± 7.1	45.6±16.1	5.2 ±0.9	1.4 ±0.9
	6	28	129.7 ± 8.7	78.9 ± 7.6	66.3± 8.4	52.8± 9.6	8.1 +2.2	3.0 ±1.9
	$6^1/_2$	12	148.2 ±10.6	90.5 ± 8.9			10.7 ±1.6	
Progesterone 2mg/day	5	17	90.0 ± 5.3	76.6 ± 6.5	31.1±11.0	50.8±14.1	5.4 ±0.7	1.6 ±0.9
	6	25	90.9 ± 7.6	67.3 ± 4.7	33.3± 9.3	37.9±12.1	4.8 ±0.8	1.2 ±0.9
	8	12	99.8 ±10.4	76.0 ±10.4	50.1±12.3	33.8±11.8	6.0 ±1.5	3.3 ±1.1
Progesterone 4mg/day	5	24	87.6 ± 7.0	70.1 ± 6.9	28.8±11.4	41.8±10.3	4.8 ±1.1	1.0 ±0.8
	$5^1/_2$	32	92.5 ± 6.6	75.6 ± 7.5	40.7± 9.7	54.8± 8.1	5.6 ±1.3	1.9 ±1.1
	6	22	101.3 ± 9.3	71.3 ± 8.4	44.7±11.2	45.6± 9.9	5.7 ±1.5	1.8 ±1.3
	8	17	110.9 ±12.4	80.7 ± 7.2	60.9±12.4	57.2±11.6	7.0 ±1.3	3.0 ±1.6
Progesterone 8mg/day	5	20	89.9 ± 6.2	75.9 ± 6.5	31.1±12.4	46.4±11.2	5.3 ±0.9	1.4 ±0.9
	$5^1/_2$	31	90.3 ± 6.4	76.2 ± 8.1	42.8± 9.7	55.1±10.4	5.4 ±0.7	1.9 ±0.7
	6	18	111.3 ± 7.9	78.0 ± 6.0	57.5±11.2	57.5± 9.8	6.9 ±1.3	2.9 ±1.9
	8	26	127.7 ± 6.8	87.0 ± 7.3	78.3± 9.4	65.6±10.0	8.7 ±1.6	4.4 ±1.3
Lactating	5	13	88.4 ± 8.5	79.6 ± 5.9	33.2± 9.4	50.8± 9.0	5.6 ±1.2	1.5 ±0.8
	6	5	99.1 ± 8.3	76.2 ± 9.8	38.1±10.4	50.8±12.4	6.0 ±0.9	1.9 ±1.0
	8	23	109.6 ±10.6	75.9 + 8.4	68.8±10.1	59.1± 9.3	6.7 ±0.8	3.4 ±1.6

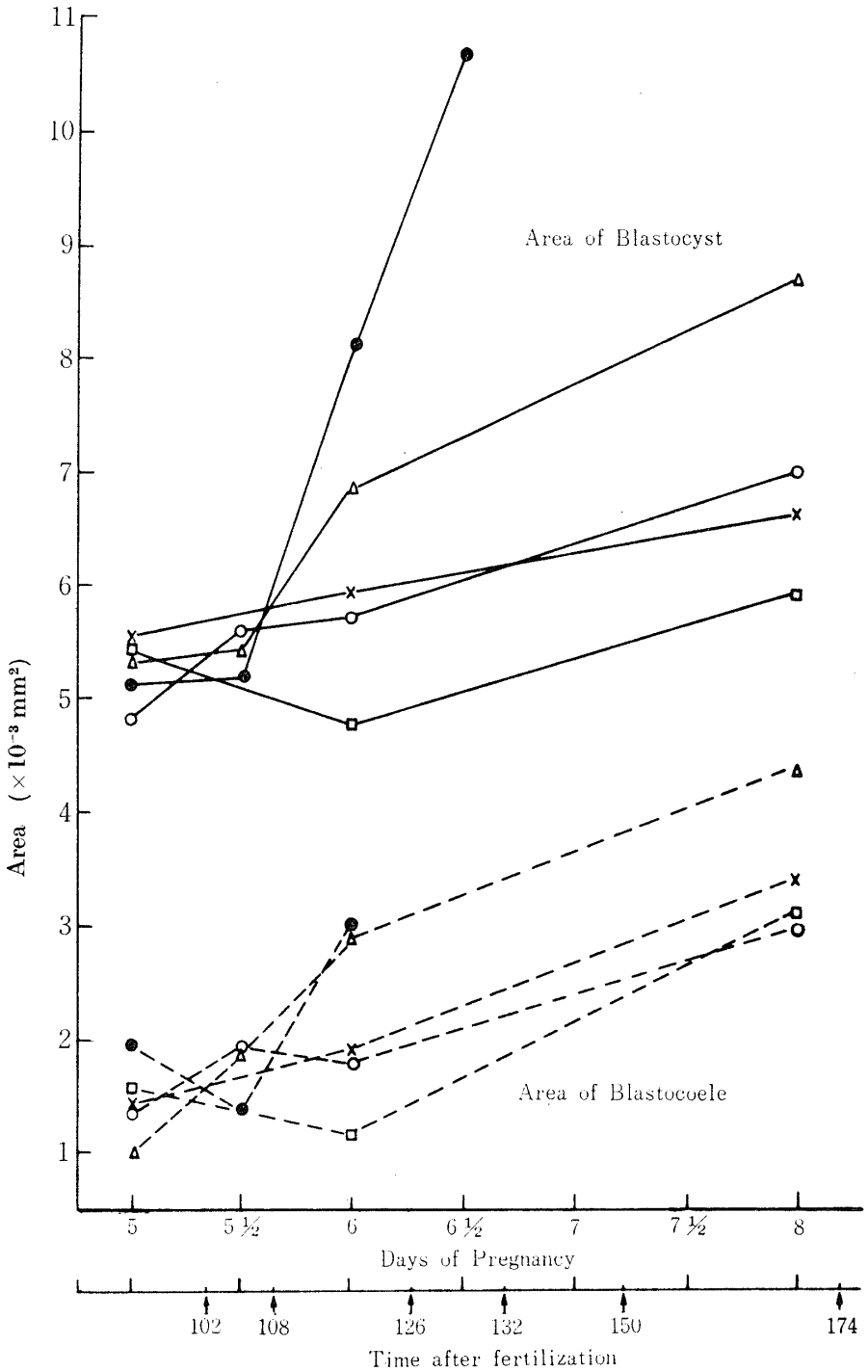

Fig. 5. Changes in area ($10^{-3}mm^2$) of blastocyst (—) and blastocoele (- -) from the rats in each groups. •, Intact animals; □, ○, and △, are 2,4 and 8mg of progesterone treated animals, respectively; ×, lactating animals.

In group 4 in which rats were injected with 8 mg of progesterone, the increase in the major axis at 126 hours occurred in the same mode observed on the blastocyst in the control group, but the former increase was less than that of the latter.

After 126 hours of fertilization, a gradual increase in the major and minor axes continued during the delayed implantation.

In the lactating rat, the increase of the major and minor axes was the same as the rate observed in group 3 which was injected with 4 mg of progesterone. The remarkable increase, observed in control and 8 mg groups, did not occur at 126 hours after fertilization.

The area of blastocyst and blastocoele:

At 102 and 108 hours, there was no difference among all groups in area of the entire blastocyst and blastocoele. (Fig. 5)

In the blastocysts at 126 hours, difference in the rate of increase of their areas was observed among all groups ($P<0.05$). The blastocyst of the intact rat was significantly larger than any of the others ($P<0.01$). In the intact group, the area of blyastocyst at 132 hr. increased in 2-folds in comparison with that of blastocyst at 102 hours.

In group 2, received 2 mg of progesterone daily, it was noted that a reduction in area of blastocyst at 126 hrs. was signicatly different as compared to that at 102 hrs. ($P<0.05$) and that the area of blastocyst increased slightly thereafter. In the progestrone 4 mg/day-group, there was a slight and linear increase in the area of blastycst until 174 hours after fertilization. In the progesterone 8 mg/day group, the rapid increase in area of blastocyst and blastocoele occurred at 126 hours after fertilization and their area in this group was significantly larger than that of progesterone 4 mg/day group at 126 hrs ($P<0.01$).

The area of blastocyst from lactating rats was observed to have a nearly similar change as that obtained from the rats in 4 mg/day progesterone group. The observed increases in the area of pre-implantation blastocysts were associated with the increases in minor and major axes and also these increases in the area corresponded to the increase in area of blastocoele.

Disappearacens of zona pellucida:

About disappearances of zona pellucida, there was a difference between the control group and any the other groups. It was showed that the time in

Table 2. Disappearance of zona pellucida: Percentage of zonae-free blastocyst from each groups.

Day of pregnancy	Intact (Control)	Progesterone(mg)			Lactating group
		2	4	8	
5	29.6	0	0	0	0
5½	55.2	—	0	0	0
6	100	76.0	72.8	66.6	100
6½	100	—	—	—	—
8	—	100	100	100	100

disappearance of zona pellucida did not differ among the treatment groups with progesterone or the lactating group.

In the control group, disappearance of zona pellucida at 102, 108, 126 and 132 hr. was 29.6, 55.2, and 100 percent, respectively. However, there was no disappearance of zona pellucida in the treated groups until 108 hr. after fertilization. At 126 hours, about 75 percent of the recovered blastocysts had loss of zona pellucida and none of blastcyst had zona pellucida at 174 hr. In lactating rats, by 108 hr. all of the recovered blastocysts were zonae-encased and by 126 hr. all of them were zonae-free.

Discussion

Changes in the lengths of axes and area of blastocysts:

In the previous works conducted by many workers, it was confirmed that implantation in the intact rat occurs at fifth day of pregnancy.

In this experiment, it was found that ova implantation initiate at 132 hr. after fertilization e.g. at 6½ day of pregnancy, while the blastocysts in the progesterone-treated and lactating rats remains free in the lumen of uterus. Blastocysts from intact rats, studied succeedingly after fertilization, demonstrate morphological changes before implantation. At a accurate stage of development, 108 hr. after fertilization, the area of the blastocyst remains unaltered. During the subsequent 24 hrs, the rapid expansion of blastocyst and blastocoele occur until implantation is accomplsihed by 132 hrs, after fertilization. Under the proper hormonal condition, delayed blastocysts from the ovariectomized animals treated with progestrone do not demonstrate the morphological change, which had been observed in the blastocysts from intact rats, except group 4 injected with 8 mg of progesterone daily. It is frequently stated that the development of blastocyst in the delayed implantation remains during the period of delay. (10)

It is clearly shown, from the results of these experiments, that the blastocyst from the ovariectomized rat treated with progesterone continues the gradual development during delays and also that their development during the period of delayed nidation induced by ovariectmy followed progesterone treatment occurs in proportion to the dosage of the hormone. In lactating rats, the rate of increase in the area of blastocyst is observed, as nearly similar to that of the rats treated with 4 mg of progesterone daily. This observation seems to suggest that the lactating rat with 8 pups secretes the amount of hormone, which corespond to the biological signfiicance of 4 mg of progesterone, from their ovary daily. But, this assumption must be supported by evidence which can be obtained from other experiments.

Disappearace of zona pellucidae:

The blastocysts from intact rats lose their zona pellucidae in the 5th Day of

pregnancy. This result substantiate those of Dickmann & Noyes (1961) and Yasukawa & Meyer (1966), they established 112 hr. after fertilization, as the time when blastocysts from intact rats are zonae free. From the present experiment, it is demonstrated that about 75 percent of delayed blastocysts from progesterone treated animal is their zonae-free on the 6th Day of pregnancy and that all of them are free of their zonae on the 8th Day.

Our results suggest that the disappearance of zonae is not dependent on the age of blastocyst.

The literature, reviewed by Böving (1963), contains the published results of a large volume of work on the mechanism of the disappearance of the zonae, but, there is a paucity of information on the mechanism.

It is notable that the time of shedding of the zonae is closely associated with the time of the appearance of the morphological changes.

The disappearance of the zonae cannot be atributed to the increase of pressure caused by the expansion of blastocyst, but must be due to another factor, for example, the alteration in the function of trophoblastic cells. The morphological alterations in 24 hrs before implantation may be indicative of changes in properties and functions of trophoblast which synthesize and release the substance acts to invasion of trophoblast into uterine epithelium.

Summary

The effect of progesterone on the embryonic development during pre-implantaion and implantation stages was investigated in the rat induced experimentally to delay implantation and in the lactating rat. The results obtained are summarised as follows:

1) In intact rat control group, implantation was observed at 132 hours after fertilization, while the rats ovariectomized followed progesterone treatment and lactating rats resulted in complete failure of implantation.

2) The remarkable increase in the length of major axis of blastocysts from intact rats between 108 and 126 hours after fertilization. The growth of blastocysts from these animals at 132 hr. increased in 2-folds as compared with that of them at 102 hours after fertilization.

3) In treatment groups, the growth of blastocysts continued gradualy during the period of progesterone treatment.

4) In lactating rats, the growth of blastocysts was nearly similar to that obtained from group 3, in which rats were treated daily with 4 mg of progesterone.

5) The disappearance of zona pellucida of blastocysts from the intact rat was markedly ealier (1 or half day) than any of the others. But, there was no difference among the treatment or lactating groups.

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