Effects of Ovarian Hormones on the Growth and the Differentiation of the Mouse Blastocyst During Delayed Implantation

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Introduction

In mammals egg division continues from the fertilization to the uterine arrival. In the uterus, the eggs develop into blastocysts and are implanted after a certain period depending on each species. However, the implantation does not occur in the lactating or ovariectomized mouse and rat at early pregnancy. In these animals, viable blastocysts remain free in the uterine lumen, i. e., delayed implantation. It is known that implantation is induced by the administration of progesterone and estrogen to ovariectomized delayed-implanting mouse and rat. Utilization of this phenomenon in the mouse and rat has offered considerable informations on the hormonal requirements and the mechanisms for implantation in these species.

In ovariectomized experimentally delayed-implanting rat, the growth and the differentiation of the delayed blastocyst were observed at beginning 12 hrs. after the simultaneous administration of estrone and progesterone. Implantation occurred within the subsequent 24 hrs.[1,2] It is known that delayed-implanting mouse blastocyst increases CO_2 production,[3] amino acid incorporation[4,5] and protein content[6] with its growth and differentiation after estrogen administration. However, in the mouse, there is little known about the growth and differentiation of the blastocyst during the pre-implantation period. Accordingly this experiment examines the effect of progesterone and estrogen on the blastocyst growth and differentiation by the measuring axis length of the blastocyst and blastocoele.

Materials and Methods

Mature virgin female mice of ICR strain, 6–8 weeks old, were maintained in light-controlled (12 hrs. light/12 hrs. dark), air conditioned rooms (20±1°C) and fed

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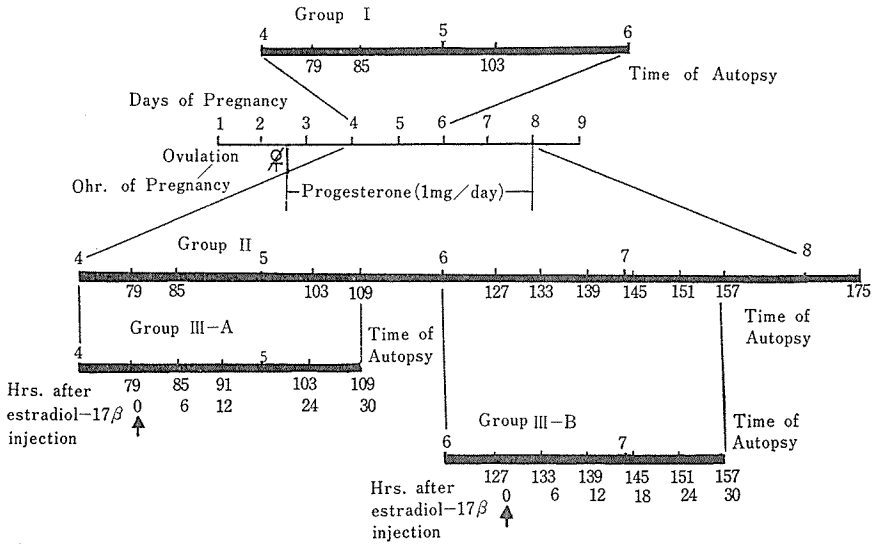

Fig. 1. Experimental design for intact mice and treated mice.

a standard diet. These experimental animals in proestrus or estrus were caged overnight with males and were checked for vaginal plug in the following morning. The day that the vaginal plug was found was designated as Day 1 of pregnancy. According to the result of the time of ovulation in ICR mice in our laboratory, the ovulation was considered to occur on Day 1 at 2 A. M. (unpublished data) and this time was designated as 0 hr. of pregnancy.

The experimental designs are shown in Fig. 1. The pregnant mice were divided into three groups, Group I, II and III. Group III was subdivided into Group III-A and III-B. Group I was the intact control. Group II, III-A and III-B were ovariectomized on Day 2 of pregnancy and were daily administered 1 mg progesterone dissolved in 0.1 ml olive oil. Groups III-A and III-B were administered 0.5 μg estradiol-17β dissolved in 0.1 ml olive oil on Day 4 and Day 6 of pregnancy, respectively. The time of autopsy in each group is shown in Fig. 1.

The uterine horns were exposed by a mid-ventral incision, excised and separated at the cervical region. The uterine horns were flushed with KRB solution. The flushed fluid was collected in a watch glass.

The lengths of the major and minor axes of the blastocysts and blastocoeles were measured with a micrometer under a dissecting microscope. The major and minor axes of the blastocysts and blastocoeles are shown in Fig. 2. The areas of blastocysts (S) and blastocoeles (S') were estimated by the following formulae:

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

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

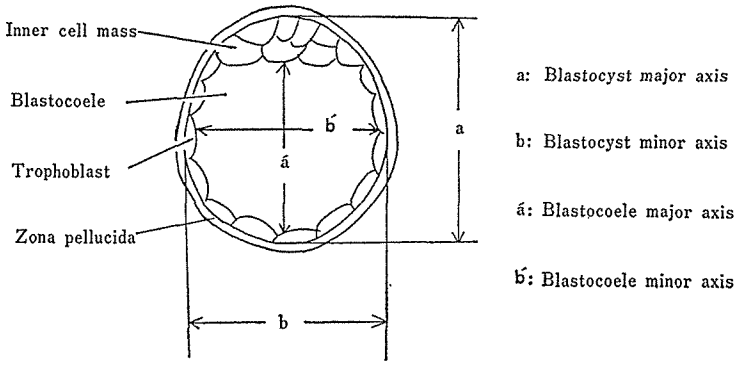

Fig. 2. Method used to measure axes of blastocyst.

The implantation sites were examined by direct observation after the intravenous injection of pontamine sky blue dissolved in physiological saline solution. The presence or absence of zona pellucida was noted by direct observation of the blastocyst.

Statistical analysis was performed by analysis of variance and Student's t-test.

Results

Changes in the major and minor axes of the blastocyst and blastocoele.

The results are summarized in Table 1. A rapid increase in the major axis length of the blastocyst and blastocoele were observed in Group I between 79 and 103 hrs. ($F^2_{124}=79.3^{***}$, $F^2_{122}=38.6^{***}$). The major axis length of blastocyst at 103 hrs. increased 1.8 fold as compared with that at 79 hrs. The minor axis length of the blastocyst and blastocoele in Group I increased between 79 and 85 hrs.

In Group II, the major axis length of the blastocyst and blastocoele increased from 85 to 127 hrs. and the significant increases were observed between 103 and 109 hrs. and between 157 and 175 hrs. (blastocyst). The minor axis length of the blastocyst and blastocoele decreased from 79 to 103 hrs. and the significant decrease was observed in the blastocoele between 79 and 85 hrs.

In Group III–A, a rapid increase in the major axis length of the blastocyst and blastocoele observed between 85 and 91 hrs. but no difference in the minor axis length of the blastocyst and blastocoele was observed among these times.

In Group III–B, the major and minor axis length of the blastocyst and blastocoele increased gradually from 133 to 157 hrs. and a significant increase was observed in the minor axis length of the blastocyst between 133 and 139 hrs. The significant differences between Group II and III–B were observed in the major and

Table. 1. Changes in lengths of blastocyst and blastocoele in each group.

Group	Hours of Pregnancy( )*	Lengths of Axis (μ)※			
		Blastocyst		Blastocoele	
		Major	Minor	Major	Minor
I	79	67.5±1.3[a]	60.8±1.5[p]	46.1±1.8[y]	57.4±1.7[m']
	85	86.2±2.3[b]	75.8±2.2[q]	59.6±2.1[z]	72.2±2.9[n']
	103	124.9±7.1[c]	75.2±3.8	81.5±4.7[a']	74.7±3.8
II	79	92.1±2.9	85.9±3.2	51.9±7.0	85.2±3.3[o']
	85	88.9±5.1	74.8±2.9	58.5±3.0	70.3±4.1[p']
	103	93.9±3.3[d]	66.9±4.7	60.2±4.6[b']	59.8±4.4
	109	106.6±3.6[e]	73.9±3.6	73.4±3.3[c']	67.7±3.7
	127	115.8±6.3	75.0±4.2	83.4±5.5	72.0±4.3
	133	113.1±4.7	69.9±5.9	81.0±4.2	73.1±3.9
	139	114.4±4.6	79.7±2.8	76.5±4.6[d']	79.1±2.7
	145	111.0±4.9[f]	75.8±3.0	78.6±3.9[e']	76.1±2.9
	151	115.8±3.6[g]	78.1±3.3[r]	82.7±4.0	76.9±3.3
	157	106.9±4.9[h]	72.0±2.9[s]	75.9±4.9[f']	68.1±3.9[q']
	175	119.1±3.6[i]	75.7±3.2	85.2±4.1	72.5±3.1
III-A	85 (6)	87.8±4.7[j]	82.1±5.8	63.0±4.5[g']	82.1±5.8
	91 (12)	113.6±5.4[k]	74.3±5.3	85.5±6.4[h']	74.3±5.3
	103 (24)	110.0±3.6[l]	78.3±2.5[t]	74.6±4.0[i']	75.5±2.7
	109 (30)	—	—	—	—
III-B	133 (6)	122.1±4.6	71.4±3.5[u]	82.9±3.8	71.4±3.5
	139 (12)	121.3±4.4	87.6±3.3[v]	88.1±4.2[j']	87.2±3.5
	145 (18)	138.0±6.1[m]	87.0±4.7	99.8±5.3[k']	87.0±4.7
	151 (24)	131.6±6.4[n]	90.9±4.2[w]	90.0±8.0	87.4±4.3
	157 (30)	133.5±8.6[o]	96.8±4.6[x]	93.6±6.0[l']	94.5±5.4[r']

( )* Hours after estradiol-17β injection.
※ Values are expressed as Means±S.E.
Statistical differences were evaluated by Student's t-test ($P<0.05$) as follow ; a-b, b-c, b-j, d-k, e-l, f-g, f-m, n-o, p-t, q-u, r-s, v-w w-x, w-d', y-e', z-f', a'-g', b'-c', l'-m', h'-i' and j'-k'.

the minor axis length of the blastocyst and the blastocoele on and after 139 hrs. (12 hrs. after estradiol-17β injection).

The area of blastocyst and blastocoele.

In Group II, the mean area of the blastocyst between 127 and 157 hrs. (6.8± 0.2, $\times 10^{-3}$mm^2) were significantly larger than that between 79 and 109 hrs. (5.7± 0.2, $\times 10^{-3}$mm^2). The mean area of the blastocoele between 127 and 157 hrs. (4.8± 0.2, $\times 10^{-3}$mm^2) were also significantly larger than that between 79 and 109 hrs. (3.6±0.2, $\times 10^{-3}$mm^2). However, a significant increase was not observed in compar-

Table 2. Changes in area of blastocyst and blastocoele in each group.

Group	Hours of Pregnancy( )*	Area ($\times 10^{-3}$mm^2)※	
		Blastocyst	Blastocoele
I	79	3.3±0.1[a]	2.2±0.1[l]
	85	5.3±0.2[b]	3.6±0.2[m]
	103	7.6±0.7[c]	5.0±0.5[n]
II	79	6.3±0.4	4.1±0.4
	85	5.3±0.4	3.3±0.3
	103	5.1±0.5[d]	3.0±0.4[o]
	109	6.3±0.5	4.0±0.4
	127	7.1±0.8	4.9±0.6
	133	6.7±0.6	4.8±0.5
	139	7.2±0.5	4.8±0.4[p]
	145	6.7±0.5[e]	4.7±0.4[q]
	151	7.1±0.5[f]	5.1±0.5
	157	6.1±0.4[g]	4.2±0.4[r]
	175	7.2±0.4	4.9±0.4
III-A	85 (6)	5.7±0.7	4.2±0.5
	91 (12)	6.7±0.8	5.1±0.9
	103 (24)	6.8±0.4[h]	4.5±0.3[s]
	109 (30)	—	—
III-B	133 (6)	7.0±0.4	4.7±0.4
	139 (12)	8.5±0.6	6.2±0.5[t]
	145 (18)	9.6±0.9[i]	7.0±0.7[u]
	151 (24)	9.7±0.9[j]	6.6±0.8
	157 (30)	10.3±0.4[k]	7.1±0.7[v]

( )* Hours after estradiol-17β injection.
※ Values are expressed as Means ± S.E.
Statistical differences were evaluated by Student's t-test ($P<0.05$) as follows ; a-b, b-f, c-g, d-h, e-j, k-l, l-p, m-n, m-q, n-r and o-s.

ison with each area in autoptic sequence (Table 2, Figs. 3 and 4).

In Group III-A, significant differences were not observed in the area of blastocyst and blastocoele. The differences of the area of blastocyst and blastocoele in Group I, II and III-A at 85 hrs. were not significant and the area of blastocyst and blastocoele at 103 hrs. in Group I and III-A were significantly larger than that in Group II. This result was similar to those observed in the major axis length of the blastocyst and blastocoele (Table 1).

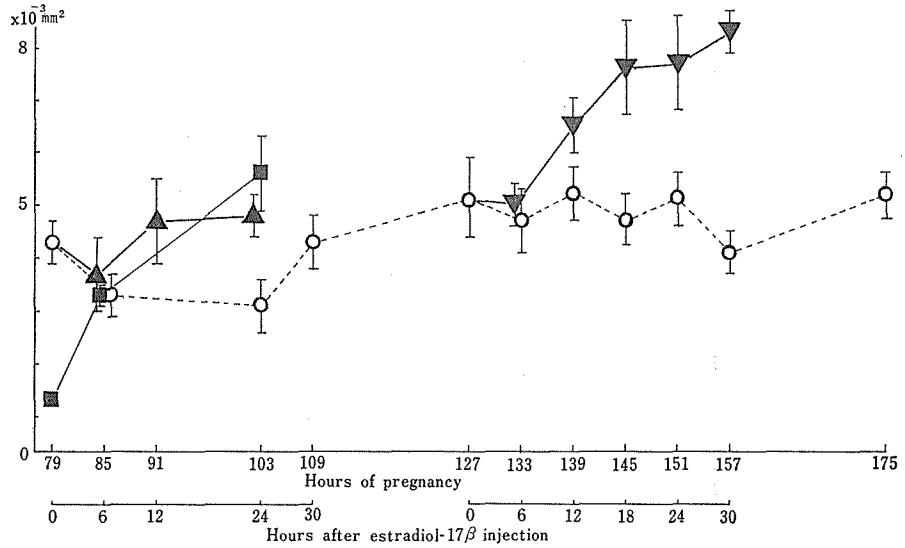

Fig. 3. Changes in the area of blastocyst. ■ Group I : intact control ; ○ Group II : progesterone (1mg/day) ; ▲ Group III–A : progesterone (1mg/day) + estradiol–17β (Day 4 injection) ; ▼ Group III–B : progesterone (1mg/day) + estradiol–17β (Day 6 injection). S.E. calculated.

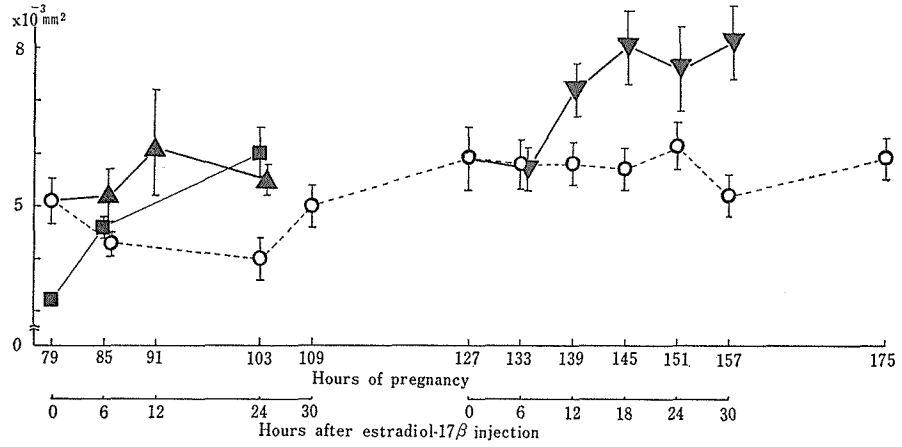

Fig. 4. Changes in the area of blastocoele. ■ Group I : intact control ; ○ Group II : progesterone (1mg/day) ; ▲ Group III–A : progesterone (1mg/day) + estradiol–17β (Day 4 injection) ; ▼ Group III–B : progesterone (1mg/day) + estradiol–17β (Day 6 injection). S.E. calculated.

In Group III–B, the area of blastocyst and blastocoele began to increase gradually at 133 hrs. (6 hrs. after estradiol–17β injection). The area of blastocyst and

blastocoele in Group III-B were significantly larger than those in Group II from 139 to 157 hrs. except for 139 hrs. (blastocyst) and 151 hrs. (blastocoele). The area of blastocyst at 157 hrs. increased 1.4 fold as compared with that at estradiol-17β injection. In Group III-A, the area of the blastocyst at 103 hrs. increased 1.1 fold as compared with that of the blastocyst at the estradiol-17β injection.

Development in the recovered egg.

A total of 572 eggs were recovered; 420 had developed into normal blastocyst, 57 were in the morula stage and 95 were abnormal.

All blastocysts recovered at 103 hrs. in Group I and at 109 hrs. in Group II

Table 3. Percentage of Zona-free blastocyst and number of pontamine sky blue reaction site in each group.

Group	Hours of Pregnancy( )*	No. of animal	No. of blastocyst	Percentage of Zona-free Blastocyst	No. of Pontamine sky blue reaction site
I	79	6	58	1.7	0
	85	8	52	25.0	0
	103	10	17	100.0	124
II	79	4	13	15.3	0
	85	6	16	25.0	0
	103	6	16	50.0	0
	109	9	19	100.0	0
	127	3	15	100.0	0
	133	7	16	100.0	0
	139	5	14	100.0	0
	145	6	12	100.0	0
	151	6	22	100.0	0
	157	6	16	100.0	0
	175	10	17	100.0	0
III-A	85 (6)	4	8	0.0	0
	91 (12)	3	8	100.0	0
	103 (24)	11	23	100.0	11
	109 (30)	3	0	0.0	9
III-B	133 (6)	5	14	100.0	0
	139 (12)	5	19	100.0	0
	145 (18)	4	12	100.0	0
	151 (24)	10	21	100.0	46
	157 (30)	6	12	100.0	36
Total		143	420		226

( )* Hours after estradiol-17β injection.

lost their zona pellucida, but in Group I and II, 25% of the blastocysts were zona-free at 85 hrs. (Table 3). In Group III-A, all the blastocysts retained zona pellucida at 85 hrs. but had lost them all at 91 hrs. All blastocysts recovered from Group III-B were zona-free.

Time of implantation.

In Group I at 103 hrs., all animals had pontamine sky blue reaction and the number of reaction sites were 124 (Table 3). In Group II, the implantation was not observed and delayed implantation was induced. In Group III-A and III-B, pontamine sky blue reaction was observed between 24 and 30 hrs. after estradiol-17β injection.

Discussion

The area of the blastocyst and blastocoele from Group III-A and III-B treated with progesterone plus estradiol-17β were larger than that from the progesterone-treated group on and after 12 hrs. estradiol-17β injection. The area of blastocyst increased about 1.1 and 1.4 fold, in Group III-A and III-B respectively at 24 hrs. after estradiol-17β injection. The blastocyst morphology is affected by ovarian hormones, but little information is available concerning the morphology of the delayed blastocyst in the mouse.

In the ovariectomized rat treated with progesterone, the axis length and the area of the blastocyst continued to develop gradually during the delayed implantation[1,2]. In this experiment, the blastocyst treated with progesterone did not develop continuously. As for the changes in the major axis length and the area of blastocyst and blastocoele, the means between 127 and 157 hrs. were significantly larger than those between 79 and 109 hrs. The increases in the axis length and the area of blastocyst and blastocoele within a short term observed in Group I, III-A and III-B were not observed in the progesterone treated group (Group II), but this would suggest that the growth of the blastocyst did not completely stop during delayed implantation. During this period of delayed implantation, the unattached blastocyst, free-living in the uterus has been shown to enter a state of arrested development or dormancy characterized by a cessation of mitosis and DNA synthesis[7] and by a dramatic depression in the levels of RNA synthesis[8], protein synthesis[9] and carbon dioxide production.[10]

In this experiment, the size of the blastocyst increased after estradiol-17β injection. Many studies have been performed on embryonic and uterine metabolism after estrogen administration. As demonstrated by the incorporation experiments of labelled nucleotides and amino acids, the synthesis of RNA, DNA and protein by the mouse blastocyst and the uterus during delayed implantation increases considerably after estrogen administration[11-14]. Delayed-implanting blastocysts from

animals treated with progesterone show a significant increase in CO_2 production after injection of estrogen. Dickson *et al*[15,16]. reported that estrogen induced the activation of metabolism and implantation of dormant mouse blastocyst, associated with trophoblast changes which spread from the abembryonic pole to the embryonic pole. Also it is suggested that estrogen brings about the changes of the cell membrane permeability with the consequent changes in the intracellular pool radioactivity[17]. These results may be that estrogen induces both the activation and the cell membrane permeability of diapausing mouse blastocyst in order to accelerate the growth and the differentiation of the blastocyst.

It takes a certain time interval until estrogen activates the blastocyst metabolism after the injection. Carbohydrate metabolism[18] and protein synthesis[12] of the mouse blastocyst *in vivo* accelerated by estrogen requires 12 to 18 hrs. after the estrogen injection. RNA, protein and DNA synthesis were investigated autoradiographically in the mouse blastocyst. RNA synthesis increased between 15 and 30 mins. after estrogen injection, protein synthesis did between 30 and 45 mins., and DNA synthesis did not increase until 24 hrs.[19]. In Group III–B of this experiment, the increase in the area of the blastocyst began at 6 hrs. after estradiol–17β injection, so it may be seen that the activation of metabolism by estradiol–17β operated between 0 and 6 hrs. after the injection. Furthermore, pontamine sky blue reaction showed that implantation occurred from 24 to 30 hrs. after estradiol–17β injection both in Group III–A and III–B. As the above-mentioned, we think that estrogen induces both the activation of metabolism and the increase of the area of dormant mouse blastocyst and these active blastocysts begin to implant after estrogen injection.

There were differences in the rate of increase between the blastocyst areas of Group III–A and those of Group III–B. It suggests that the effect of estrogen on the blastocysts on Day 4 differed from that on Day 6. Same events were noted that in delayed-implanting rat, there was the difference in the rate of increase of the blastocyst area when estrone was injected on Day 5 or Day 8[2]. On the other hand, SMITH[20] showed that the number of animals where implantation sites existed and the number of implantation sites per animal following estradiol–17β injection on Day 4 were fewer than those on Day 6. It may be that the difference in the estrogen sensitivity existed in the uterus and the blastocyst during delayed implantation. Therefore it is concluded from this experiment that the sensitivity of the blastocyst to estrogen was different between Day 4 and Day 6.

Furthermore, it is known that adenosine 3′: 5′–monophosphate (c–AMP) activates the metabolism in the uterus and the blastocyst. The dormant trophoblast cells changed the morphology into the active state[21] and the implantation was induced[22], when c–AMP was administered to the delayed implanting mice. These reports sug-

gest that c-AMP mimics the early action of estrogen in the delayed blastocyst and the uterus. Further experiments on the relationship between the action of c-AMP on the blastocyst and the growth and differentiation of the blastocyst are necessary.

Summary

Effects of progesterone and estradiol-17β on the growth and the differentiation of the mouse blastocyst during the pre-implantation and implantation stages was investigated inducing to delayed implantation by ovariectomy. When the ovariectomy was followed by daily injection of 1 mg progesterone (Group II), the area of blastocyst on the 6th day of pregnancy was significantly larger than that on the 4th day of pregnancy. When a single dose of estradiol-17β (0.5μg) was injected to ovariectomized and progesterone treated mice on the 4th or 6th day of pregnancy (Group III-A and III-B), the implantation was observed between 24 and 30 hrs. after the injection. In addition the area of blastocyst in Group III-A was significantly larger than that in Group II after 24 hrs. estradiol-17β injection and was as large as those in intact group (Group I) and Group II after 6 hrs. estradiol-17β injection. The area of blastocyst in Group III-B was also significantly larger than that in Group II on and after 18 hrs. estradiol-17β injection. These results indicate that progesterone and estradiol-17β are important for the growth and the differentiation of delayed-implanting mouse blastocyst and they induce the implantation.

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摘　　要

卵巣ホルモンが着床遅延マウス胚盤胞の成長と分化に及ぼす影響

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マウス・ラットの受精卵の着床は，泌乳，または，卵巣除去により遅延する。着床遅延中の胚盤胞は，子宮腔内でその成長や物質代謝が抑制され，休眠状態にあり，プロジェステロンとエストロジェンを投与することにより，胚盤胞は大きさを増し，やがて着床することが知られている。ラット胚盤胞の着床前の大きさの変化については，詳細に研究されているが，マウスでは，殆ど研究されていない。本実験は，マウスにおいて卵巣除去により着床遅延を誘起し，卵巣ホルモン投与によって着床するまでの間の，胚盤胞の大きさの変化を胚盤胞および胚盤胞腔の長軸，短軸，面積について調べた。その結果，妊娠2日目卵巣除去後，プロジェステロン1mgを連日投与した群(GII)で，着床は全くみられず，胚盤胞面積の変化は，妊娠6日目の平均値が，妊娠4日目の平均値より有意に大きかった。GIIと同様，プロジェステロン投与に加え，エストラジオール—17β を妊娠4日または，妊娠6日に投与した2群 (GIII-A, GIII-B) で，着床は，エストラジオール—17β投与後24～30時間にみられた。また，GIII-A の胚盤胞面積は，エストラジオール17β 投与後6時間に，無処置群 (GI) およびGII と同じであったが，投与24時間後では，GII より有意に大きかった。さらに，GIII-B胚胞盤面積も，エストラジオール—17β投与18時間以後に，GIIより有意に大きかった。これらのことから，プロジェステロンとエストラジオール—17βは，着床遅延中の胚盤胞の大きさを増大させ，また，受精卵の着床に重要な役割を果たすことが示唆された。