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BLASTOCYST PROTEASE AND IMPLANTATION: EFFECT OF OVARIECTOMY AND PROGESTERONE SUBSTITUTION IN THE RABBIT

By

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ABSTRACT

Protease activity in the rabbit trophoblast is demonstrated by a substrate film technique. The dissolution of egg coverings and the penetration of the trophoblast into the uterine epithelium are accompanied by a sharp maximum of proteolytic activity. After ovariectomy at 6 days post coitum, blastocysts are unable to dissolve egg coverings and to implant. These eggs do not have normal values of protease activity and a normal histochemical pattern of its distribution. Following progesterone application in ovariectomized does, enzyme activity and implantation processes are normal. The possible involvement of uterine protease inhibitors in the hormonal regulations is discussed.

Implatation processes in the mammal are well known to be regulated by maternal sex hormones (see Discussion). On the tissue involved in these processes (the blastocyst and the uterus), a lot of biochemical and histochemical data has already been compiled by many workers (for literature cf. *Denker* 1970*a,b*, 1971 *a,b,c*).

Many parameters have been shown to be regulated by maternal sex hormones (cf. *Adams & Lutwak-Mann* 1956; *Aldeen* 1970; *Beier* 1968, 1970; *Beier et al.* 1971; *Bever* 1959; *Bitman et al.* 1967; *Bo* 1961; *Böving* 1959; *Brody & Westman*

Dedicated to Prof. Dr. G. H. M. Gottschewski to his 65. birthday.

1958; *Brökelmann & Fawcett* 1969; *Conchie & Findlay* 1959; *Connell et al.* 1967; *Daniel & Krishnan* 1969; *Fishman & Fishman* 1944; *Gregoire et al.* 1961, 1967; *Gulyas & Daniel* 1969; *Hafez & White* 1967; *Hall* 1965; *Heap & Lammings* 1962; *Kirchner* 1971; *Lageron & Wegmann* 1964; *Lutwak-Mann & Adams* 1957; *Lutwak-Mann et al.* 1962; *Marcus & Shelesnyak* 1967; *Marley & Robson* 1971; *Mintz* 1971; *Mohla & Prasad* 1970; *Murdoch & White* 1969; *Prasad et al.* 1968; *Riggs et al.* 1968; *Rinard & Leonard* 1968; *Roskoski & Steiner* 1967; *Saldarini & Yochim* 1968; *Schmidt et al.* 1966; *Shelesnyak & Kraicer* 1960; *Shelesnyak & Marcus* 1969; *Sugawara & Hafez* 1967*a,b*; *Szego & Davis* 1967; *Vaes & van Ypersele* 1960; *Valadares et al.* 1968; *Weitlauf & Greenwald* 1968; *Wilson* 1969; *Zachariae* 1958). Nevertheless, it is very difficult to evaluate the significance of these biochemical changes for the success of implantation, for until now the mechanism of egg invasion is still unknown (for discussion see *Denker* 1970*b*, 1971*c*).

In studies of the histochemistry of implantation in the rabbit, we found in the invading trophoblast a very high activity of an enzyme which depolymerizes gelatin substrate films (*Denker* 1971*c*). The activity is mainly located in the abembryonal hemisphere of the blastocyst. It shows a sharp maximum at 7 $^1/_3$–7 $^1/_2$ days post coitum (d. p. c.), which is exactly the time when the egg coverings are dissolved in the abembryonal region and the trophoblast invades the uterine epithelium. Therefore we think this enzyme to be involved in the initiation of implantation events.

In our studies of hormonal regulation of the protease activity, we performed a series of ovariectomy experiments and hormone treatments. Animals were ovariectomized at exactly 6 d. p. c., for this treatment prevents egg implantation as described by *Lutwak-Mann et al.* (1962).

MATERIALS AND METHODS

27 nulliparous does, aged 11 to 23 months, were used, weighing from 2.6 to 5.1 kg. The animals were held in separate cages at a standard pellet diet and an illumination of 12 h per day. They were mated each to 2 fertile bucks. Laparotomy was performed at exactly 6 days 0 hours post coitum (6 d. p. c.) under thiobarbiturate anaesthesia (Thiogenal®) according to standardized general operative procedures (*Zimmermann* 1964), the details of the ovariectomy step being on principle as described by *Corner & Allen* (1929). The treatment of the controls consisted of laparotomy and counting of corpora lutea. Progesterone (Progesteron für biochemische Zwecke, Merck No. 8972) was dissolved in sesame oil (cf. *Wu & Allen* 1959) and injected subcutaneously at the following doses: 5 d. p. c.: 1 mg/1 ml; 6 d. p. c. (ovariectomy): 4 mg/2 ml; 7 d. p. c.: 4 mg/2 ml. For the demonstration of protease activity, the gelatin substrate film test (*Denker* 1971*d*) was used. For better localization it was modified in the following manner:

Preparation of substrate films. – Spread 0.3 ml of gelatin solution (5 % (w/v))

(Gelatine Merck No. 4070) on a slide over 6 × 2.5 cm. Let cool and dry in a horizontal position. Fix in a pre-cooled mixture of 10 ml commercial formaldehyde solution (Formaldehydlösung min. 37 Gew. % säurefrei, Merck No. 3999) plus 90 ml 50 % ethanol at 4°C for 6 hours. Wash 20 min in running tap water, ≦ 20°C, rinse in distilled water. Air drying. Dry substrate films can be stored at room temperature up to one week. For use, pre-incubate films in wet chambers (Petri dishes, sealed with vaseline) in an atmosphere of volatile buffer pH 7.0 (ethylene diamine – acetic acid buffer, *Fasella et al.* 1957) for several hours at 37°C.

Protease tests. – Uterine swellings are excised and quenched with liquid N_2. Unfixed 14 μm cryostat sections of the blastocyst in the uterus are mounted on warm pre-incubated substrate films and incubated in the wet chambers in the atmosphere of the volatile buffer at 37°C (as for pre-incubation) for 16 h. Transfer rapidly to a fixative (formol 30 ml plus distilled water 70 ml, 4°C, ≧ 20 min). Wash in running tap water and distilled water, stain in 0.5 % toluidine blue in borate buffer pH 10.0 at 4°C for 30 min. Wash in running tap water and distilled water. Air drying. For mounting, dehydrate air dried films in graded alcohols (beginning from 80 %), clear in xylene, mount in a synthetic resin.

At least 2 sets of sections were prepared from different parts of each blastocyst tested. Each set was mounted on at least 2 substrate films, ≧ 4 sections per slide.

RESULTS

Control laparotomy at 6 d. p. c. does not alter egg development, implantation and trophoblastic protease activity, as compared with the results described earlier (*Denker* 1971*c*). (Fig. 1).

After *ovariectomy* at 6 d. p. c. there is a marked *loss of embryos* from 7 to 8 d. p. c. (Table 1). 8 d. p. c. only 8 % of the eggs could be recovered at all, only 3 % appearing to be in a viable state. In contrast to the controls, blastocysts are *unevenly spaced* in the uteri of spayed animals. The size of the blastocysts appeares to be normal at 7 d. p. c. but reduced at 7 1/3 to 8 d. p. c. The diameters could not be measured exactly for the eggs had to be let in the uterus for quenching. On the other hand, representative data are obtainable from the literature (*Lutwak-Mann et al.* 1962). *Blastocyst coverings* are not dissolved after ovariectomy until at least 7 1/3 d. p. c. (cf. Table 2). Unfortunately no sections could be taken from the 2 expanded blastocysts found at 8 d. p. c. The 4 collapsed eggs flushed from the uteri at 8 d. p. c. still had coverings which of course were disrupted (artifact?). The trophoblastic *protease activity* begins to rise in spite of ovariectomy at 6 d. p. c. On the other hand it does not reach the same values as in the controls. Differences are considerable at 7 1/3 d. p. c. (Figs. 3–6). The normal histochemical *pattern* of enzyme distribution is different at 7 d. p. c. and 7 1/3 d. p. c. At 7 d. p. c. all parts of the blastocyst show a marked activity except of the inner layer of the embryonic disc. This pattern is seen in the controls and after ovariectomy as well. At 7 1/3 d. p. c. the normal pattern is characterized by the contrast

between an even broader lysis zone at the site of the abembryonic (invading) trophoblast and a negative reaction of the embryonic third of the blastocyst (embryonic disc and surrounding trophoblast). The pattern is typically developed in the controls (Fig. 1). After ovariectomy at 6 d. p. c., the pattern does not develop beyond the 7 d stage (Figs. 3 and 4). In these eggs, on the other hand, regions differ markedly in activity. Those parts which are adjacent to the uterine epithelium show only insignificant activity. Parts of the egg, which are distant from the uterine tissues (e. g. by bridgeing the furrows of the mucosa) shown a high activity. They are often covered by an unusually great lot of mucus (Figs 4–6).

After *progesterone treatment,* abembryonal blastocyst coverings are dissolved and the eggs implant quite normally. The protease activity is developed to the normal values and the normal pattern of 7 1/3 d. p. c. (Fig. 2). At 8 d. p. c. it shows the same regression as in the controls.

DISCUSSION

Lutwak-Mann et al. (1962) demonstrated that after ovariectomy of rabbits at 6 d. p. c. eggs do not implant, although morphological differentiation and expansion proceed for some time. The blastocysts were found to be unevenly spaced and to have a lower content of lactate. The implantation failure and

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All eggs figured have been obtained after sacrifice at 7 1/3 d. p. c. In each figure, the mesometrial site of the uterus is at the top.

Fig. 1.

Control. 10 ×. Note the broad lysis zone at the abembryonal two thirds of the blastocyst tissue in contrast to the nearly negative reaction of the embryonic third.

Fig. 2.

Ovariectomy + Progesterone. 10 ×. The proteolytic activity and the pattern of its distribution are comparable to those found in the control (Fig. 1).

Fig. 3.

Ovariectomy. 13 ×. Blastocyst coverings not yet dissolved. The activity and the histochemical pattern are comparable to the 7 d 0 h stage (cf. Denker 1971*c* Fig. 7 a). (In this section, the embryonic disc is not to be seen).

Fig. 4.

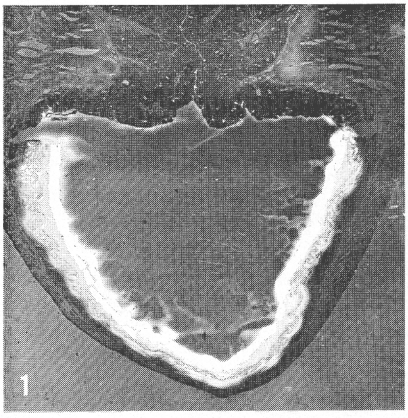
Ovariectomy. 11 ×. Two blastocysts adjoining each other. Any remarkable activity is found only in those parts of the eggs which are remote from the uterine tissues.

Fig. 5.

Ovariectomy. 23 ×. Strong activity only in a region where the blastocyst tissue bridges a depression of the mucosa and where a lot of mucus is deposited.

Fig. 6.

Ovariectomy. 60 ×. Blastocyst coverings not yet dissolved. The activity is confined to the wall of the blastocyst. Endometrium negative.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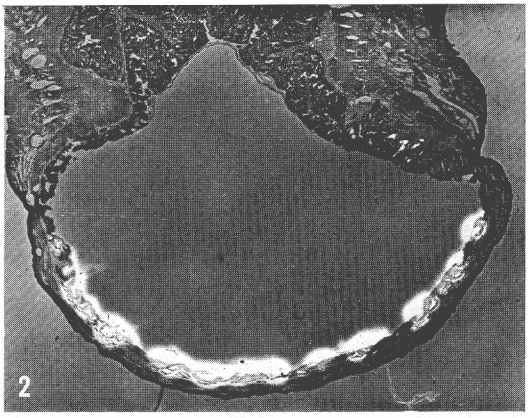
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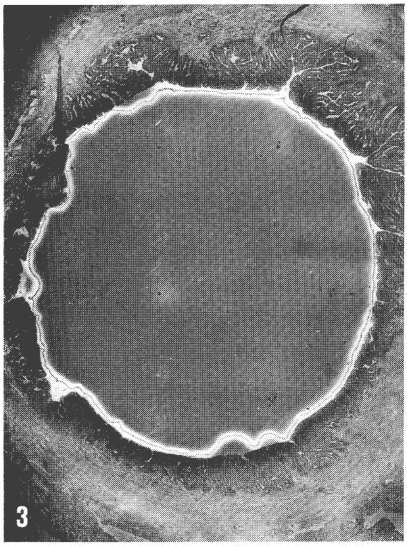
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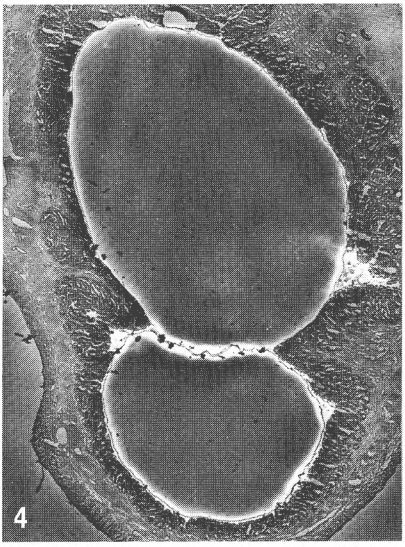
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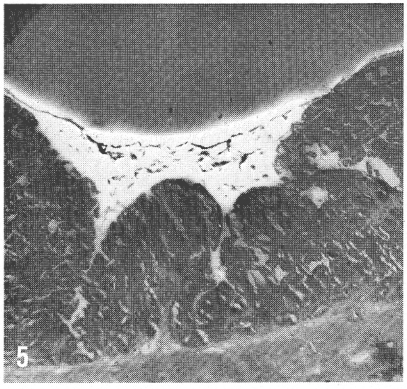
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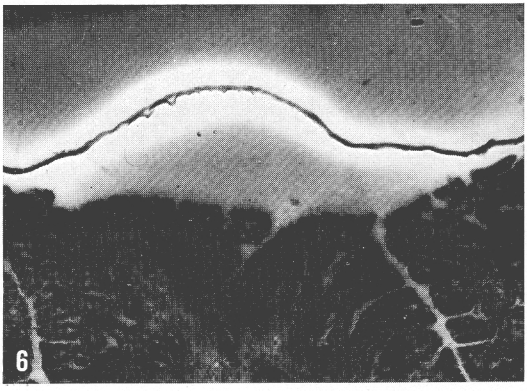
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Table 1.
General observations. All animals were laparotomized at 6 d 0 h p. c.

I Current No.	II Time of sacrifice (d. p. c.)	III Kind of treatment	IV No. of corpora lutea	V No. of viable eggs recovered		VI Size of blastocysts		VII Blastocysts evenly distributed (+) or unevenly distributed (Φ)
				n	%[1]	normal (No. of eggs)	undersized (No. of eggs)	
1	7	control	10	9	90	9		+
2	"	"	9	9	100	9		+
3	7	ovariectomy	11	3	27	3		Φ
4	"	"	8	8	100	8		Φ
5	"	"	14	0	0			
6	7 1/3	control	9	8	89	8		+
7	"	"	10	8	80	8		+
8	7 1/3	ovariectomy	12	4	33		4	Φ
9	"	"	11	7[2]	64		7	Φ
10	"	"	7	0	0			
11	7 1/3	ovariectomy +	16	6	38	6		+
12	"	progesterone	10	8	80	8		+
13	"	"	10	10	100	9	1	+

Table 1 (cont.).

I Current No.	II Time of sacrifice (d. p. c.)	III Kind of treatment	IV No. of corpora lutea	V No. of viable eggs recovered n	V No. of viable eggs recovered %[1]	VI Size of blastocysts normal (No. of eggs)	VI Size of blastocysts undersized (No. of eggs)	VII Blastocysts evenly distributed (+) or unevenly distributed (ϕ)
14	8	control	16	15	94	14	1	+
15	"	"	10	9	90	9		+
16	"	"	9	8	89	8		+
17	8	ovariectomy	12	0	0			
18	"	"	10	2	20		2	ϕ
19	"	"	13	0	0			
20	"	"	11	0[3]	0			
21	"	"	10	0	0			
22	"	"	13	0	0			
23	"	"	8	0[3]	0			
24	8	ovariectomy +	8	8	100	8		+
25	"	progesterone	15	14	93	14		+
26	"	"	11	7	64	4	3	+
27	"	"	12	12	100	10	2	+

1) Corpora lutea = 100 %.
2) 3 collapsed blastocysts found in the uterine washings.
3) 2 collapsed blastocysts found in the uterine washings.

Table 2.
Observations on protease activity. All animals were laparotomized at 6 d 0 h p. c.

I Current No. (cf. Table 1)	II Time of sacrifice (d. p. c.)	III Kind of treatment	IV No. of eggs tested	V Protease activity (No. of eggs)			VI Pattern of activity (No. of eggs)			VII Abembryonal blastocyst coverings	
				normal	slightly diminished	considerably diminished	≙ 7 d	≙ 7 1/3 d	≙ 8 d	not dissolved (No. of eggs)	dissolved (No. of eggs)
1	7	control	4	3	1		4			4	
2	"	"	2	2			2			2	
4	7	ovariectomy	6	1	5		6			6	
5	"	"	3		3		3			3	
6	7 1/3	control	5	5				5			5
7	"	"	7	7			1	6		1[1]	6
8	7 1/3	ovariectomy	4			4	4			4	
9	"	"	4			4	4			4[2]	
11	7 1/3	ovariectomy + progesterone	4	3	1			4			4
13	"	"	4	4				4			4
15	8	control	5	5					5		5
16	"	"	4	4					4		4
–	8	ovariectomy	–								
24	8	ovariectomy + progesterone	5	4	1				5		5
27	'	"	5	5					5		5

1) Dissolution of blastocyst coverings just beginning.
2) 2 eggs showing small gaps in the blastocyst coverings (artifact?) but no protease activity in these regions.

the loss of eggs were thought to result at least in part from changed *myometrial tonus.*

Our results on morphological features are in good agreement with these findings. In addition, our observations on the proteolytic enzymes of the blastocyst suggest some further failure in the development of normal biochemical activities of the blastocyst occurring after ovariectomy. No doubt spaying at 6 d. p. c. does not prevent the rising of *trophoblastic protease* activity up to 7 d. p. c. But on the other hand the maximum values normally found at 7 1/3 d. p. c. are not reached, so that in this stage eggs of ovariectomized does differ markedly in activity from the controls (Figs. 1 and 3). This is correlated to the failure or dissolution of the blastocyst coverings and invasion into the endometrium in spayed does. We think this observation to be a further argument for the hypothesis that the trophoblastic protease is one of the factors which cause the dissolution of egg coverings and the penetration of the trophoblast into the uterine epithelium.

In *other species* a comparable protease activity of the trophoblast has been described only in the guinea pig (*Blandau* 1949; *Owers & Blandau* 1968, 1971). Up to now no activity has been found in the blastocyst of the rat (*Blandau* 1949) and the mouse (*Bergström* 1970). *Mintz* (1971) stressed the view that uterine enzymes may be involved in the mouse.

The arrest of implantation events induced by ovariectomy could be completely compensated, when appropriate daily doses of *progesterone* were given in a large volume of sesame oil. The protease activity is normal in these eggs (Fig. 2). This is contrary to the observations of *Lutwak-Mann et al.* (1962), who did not succeed in hormone substitution in this critical period of implantation. Most of the successful hormone replacement experiments in the rabbit were performed after ovariectomy at earlier stages (*Allen & Corner* 1929; *Hafez* 1963; *Pickworth & Lamming* 1967; *Rennie & Davies* 1965; *Wu & Allen* 1959). The choice of the solvent (sesame oil) and the great volume of injected fluid are certainly important (cf. *Wu & Allen* 1959). The fact that progesterone alone is capable to replace the ovarian function at implantation confirms the view that there is no special need for oestrogens in this phase in the rabbit, in contrast to some rodents. (Effects of oestrogens in the mouse and rat: cf. *Buchanan* 1969; *Dickmann* 1967; *Hedlund & Nilsson* 1971; *Humphrey* 1969; *Mayer* 1963; *Nutting & Meyer* 1963; *Orsini & McLaren* 1967; *Potts & Psychoyos* 1967; *Psychoyos* 1962, 1967; *Yasukawa & Meyer* 1966. The effects of progesterone on the differentiation of trophoblastic giant cells in the mouse are demonstrated by *Dickson* (1967) and *Eaton* (1968). The effects of progesterone on implantation in the hamster are shown by *Orsini & Psychoyos* (1965), and in the sheep by *Bindon* (1971).

The effect of progesterone on the rabbit trophoblastic protease activity does not necessarily mean that the hormone acts directly on the blastocyst. *Protease*

inhibitors in the uterine secretion might play an important role in the hormonal regulation of the trophoblastic protease activity. Such inhibitors have been described by *Beier* (1970). Evidence for the occurrence of protease inhibitors in the stroma tissue was shown by *Gräfenberg* (1910) and *Schmidt-Matthiesen* (1968). We observed that the rabbit trophoblast shows reduced protease activity when it is experimentally brought into close contact with the endometrium (*Denker* 1971*c*). There are also significant differences in activity between parts of the trophoblast adjoining the uterine tissue and more remote parts. These differences are especially remarkable in ovariectomized does at 7 1/3 d. p. c. (Figs. 4–6). So after ovariectomy at 6 d. p. c. an abnormally high level of uterine protease inhibitors might persist or might be produced. Before the answer to this question can be obtained reliable quantitative data on the activities of trophoblastic and uterine proteases and of protease inhibitors must be available. The quantitative interpretation of the results of histochemical substrate film tests is always difficult (cf. *Denker* 1971*a*). Biochemical studies are now in progress.

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