ANTIEMBRYONIC EFFECT OF BULL SEMINAL VESICLE FLUID IN MICE*

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Summary. Subcutaneous injection of the seminal vesicle fluid of bulls into female mice which were subsequently mated resulted in a reduction in the number of embryos. The action of a number of different enzymes on the seminal vesicle fluid did not remove its antifertilizing activity.

The supernatant resulting from precipitation of the seminal vesicle fluid with acetic acid and ammonium sulphate had considerable antifertilizing activity. Injection of this fraction before and after mating demonstrated the antiembryonic character of the antifertilizing substance. Neither the number of ovulated eggs nor their fertilization was affected by the antiembryonic substance and there was no adverse effect on lactation, but the body weight of young, and even of adult, animals declined.

The antiembryonic substance affects the embryo at an early stage and continues to affect the organism for a long time. Necrotic changes can be observed in embryos as early as the 3rd day after the first injection. The number of leucocytes decreases for a short time after injection of the antiembryonic substance.

INTRODUCTION

The aspermatogenic effect of bovine seminal vesicle fluid has been examined in a number of different animal species (Matoušek, 1969; Matoušek, Staněk & Veselský, 1972) and a preliminary investigation of the antifertilizing effect of this fluid has been carried out in mice and hens (Matoušek & Petrovská, 1969).

The antifertilizing effect of bovine seminal vesicle fluid is manifested by a reduction in the number of embryos in female mice injected with the fluid before mating. The present study elaborates and extends the findings of the preliminary studies.

MATERIALS AND METHODS

Collection of fluid

Bovine seminal vesicle fluid (SVFB) was collected from sexually mature

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healthy bulls at the slaughterhouse (Matoušek, 1969). Mixtures of SVFB from at least twenty bulls were kept frozen at -20°C until use.

Injection procedure and the determination of embryonic mortality

The antifertilizing and antiembryonic effect of the vesicular fluid was studied in female mice of the C57BL and CBA strains. The mice were injected subcutaneously with (1) a mixture of SVFB in four doses of 0.05 ml administered in the course of 8 days, 2 to 12 days before mating, (2) a mixture of SVFB treated with enzymes after precipitating the protein by means of alcohol-ether at a ratio of 1:5. The alcohol-ether induced precipitate was resuspended in 0.9% NaCl to a protein concentration of 3 to 4% (determined by the biuret procedure). Three doses of 0.1 ml of this fluid were administered in the course of 6 days, 2 to 12 days before mating and 2 to 7 days after mating, or (3) SVFB fractions were injected in two to five doses of 0.1 ml in the course of 2 to 5 days before and after mating. The fractions were administered by resuspending 10 mg of the dialysed and freeze-dried material in 1 ml 0.9% NaCl.

Determination of antifertility and antiembryonic effect

Mice injected before, and within 6 days after, mating were killed with ether 10 to 12 days after mating. Mice injected between the 7th and the 14th day of pregnancy were killed 4 days after the last injection and the numbers of live embryos and those which would eventually be aborted were determined at autopsy. Mice injected in the last quarter of pregnancy were not killed and their fertility was determined from the number of offspring born.

The time of mating was determined by the appearance of copulation plugs.

Sensitivity of the antiembryonic substance to the action of enzymes

Bull seminal vesicle fluid was treated with papain, ficin, trypsin, chymotrypsin, pronase, lysozyme, α -amylase, β -amylase and hyaluronidase (Koch-Light Laboratories Ltd, England) by the method described by Amano & Behrman (1968). The enzyme concentration used was 20 mg/ml phosphate buffer, pH 7.1. After the enzyme concerned had been allowed to act for 24 hr at 37°C , an alcohol-ether preparation was added to the mixture of SVFB and enzyme. The resulting precipitate was suspended in 0.9% NaCl and used for the study of the antiembryonic effect.

Methods of fractionation of vesicular fluid

Alcohol-ether. Vesicular fluid was dialysed against phosphate buffer, pH 7.2, and the proteins were precipitated by a 3:1 (v/v) ratio of ethanol:ether added to the dialysed vesicular fluid in the ratio of 1:5. The filtered precipitate was air-dried at room temperature and resuspended in 0.9% NaCl to a concentration of 3 to 4% protein.

Ammonium sulphate. Bull seminal vesicle fluid was diluted with an equal part of water and the pH was adjusted to 7.0 with 1 M-NaOH. Ammonium sulphate was added to a concentration of 3 M. After 24 hr, the mixture was centrifuged and the precipitate and supernatant were separated. The precipitate was

dissolved in 0.9% NaCl, dialysed against water and freeze-dried. The supernatant was also subjected to dialysis and freeze-drying.

Acetic acid and ammonium sulphate. One part of SVFB was diluted by 2½ parts of 2% acetic acid and the mixture was allowed to stand for 30 min at room temperature. After centrifugation, ammonium sulphate was added to the supernatant, to a final concentration of 3 M. After 1 hr at room temperature, the precipitate formed was centrifuged and the supernatant was dialysed and freeze-dried, yielding a basic fraction which was used in subsequent injection procedures.

Electrophoretic analysis of fractions of vesicular fluid

(1) Starch gel electrophoresis was carried out in a glacial acetic acid + formic acid buffer (pH 1.7) according to the method described by Aschaffenburg (1966). Urea (47 g/120 ml buffer) was added to prevent formation of protein aggregates. Electrophoresis was run at 300 to 350 V and 42 to 47 mA for about 16 hr. The gels were cooled with running water.

(2) Disc electrophoresis carried out according to the method described by Ornstein (1964) was used for the investigation of the character of proteins. It was carried out in an apparatus devised by Davis (1964). Standard conditions, i.e. a 7.5% gel for electrophoresis at pH 9.4 and a 15% gel for electrophoresis at pH 4.3, were utilized, according to instructions provided by the firm Canalco. After dialysis, 0.3 mg dry protein was applied to acrylamide gels which were 0.5 × 8 cm in size.

Injection procedures

(A) Basic fraction, in three doses of 0.1 ml daily (10 mg protein in 1 ml), was injected into twenty-nine female mice 2 to 12 days before mating and into 318 females at different periods after mating. In the days following, the females were killed and the number of embryos was determined.

(B) Sixteen female mice were injected in the same way and mated after 3 to 10 days. The females were killed 2 days after mating. The eggs were washed from the oviducts and those at the two-cell stage with a second polar body were regarded as fertilized (Pavlok, 1968).

(C) Basic fraction in five doses of 0.1 ml daily was injected into eight lactating female mice (1 to 10 days after the delivery) and into four mice during the last quarter of pregnancy. The offspring were weighed at weekly intervals after the 1st week.

(D) Basic fraction in five doses of 0.1 ml daily was injected into twenty female mice aged 14 to 20 days. Young females were weighed weekly and after achieving a weight of 21 g, they were mated and their fertility was investigated.

(E) Twelve mice were injected daily for 3 days with 0.1 ml basic fraction, beginning on the 4th day after mating. The mice were killed in groups of three on Days 7, 8, 9 and 10 of pregnancy. Twenty-four mice received a similar series of injections starting 8 days after mating. These mice were killed on Days 10, 11, 12 and 13 of pregnancy.

(F) Investigation of the dose of basic fraction necessary to produce 100%

embryonic mortality was carried out on ninety-six mice. These were injected with 0.2 to 0.5 ml as well as the normal 0.1-ml dose.

(G) Those mice which aborted after the injection of basic fraction were mated again after 20 to 100 days and then killed 10 to 12 days after mating. The number of embryos was recorded.

(H) Sixteen mice were tested 4 days after mating by the addition of 10 mg of basic fraction to the drinking water, and the number of embryos was recorded 12 days after mating.

(I) Basic fraction in five daily doses (1 mg/day) was injected into twenty-four mice 3 days after mating. Eight control mice received a similar series of injections of 0.9% NaCl. The mice were decapitated 3 and 10 days after the last injection and blood samples were taken for the determination of the number of

Table 1. Fertility of mice injected with bull seminal vesicle fluid following exposure to the action of various enzymes

<i>Injected fluid</i>	<i>Injections before or after mating</i>	<i>No. of mice injected</i>	<i>Average no. of embryos/female</i>
0.9% NaCl (Control)	Before	27	6.44 ± 2.38
SVFB + papain	Before	17	3.06 ± 3.58 **
	After	30	2.76 ± 3.91 **
SVFB + ficin	After	14	3.92 ± 4.20 *
SVFB + trypsin	Before	36	3.91 ± 3.72 **
SVFB + chymotrypsin	Before	13	3.15 ± 3.38 **
	After	20	3.00 ± 3.50 **
SVFB + pronase	Before	16	2.75 ± 3.50 **
	After	51	1.78 ± 2.72 **
SVFB + lysozyme	After	38	2.21 ± 3.00 **
SVFB + α -amylase	After	12	4.08 ± 4.08
SVFB + β -amylase	After	12	2.33 ± 3.10 **
SVFB + hyaluronidase	After	13	2.15 ± 2.92 **

SVFB = Bull seminal vesicle fluid.

* Statistically significant, $0.05 > P > 0.01$.

** Statistically highly significant, $P < 0.01$.

red and white blood cells and for differential white blood cell determination. Differential counts of 100 to 200 cells were made on smears stained with May-Grünwald's and Giemsa's stain.

RESULTS

Antifertilizing effect of the seminal vesicle fluid of bulls

The subcutaneous injections of SVFB into female mice mated 2 to 10 days after the last injection resulted in a reduction in the number of embryos. In twenty mice injected with SVFB, seventy-one embryos were detected on the 12th day of the pregnancy while, in twenty mice injected with 0.9% NaCl, 144 embryos were found (3.55 ± 3.20 and 7.02 ± 1.40 /female). In eight mice, the

SVFB of sexually immature bulls did not evoke any reduction in the number of embryos ($6.25 \pm 1.95/\text{female}$).

None of the enzymes used succeeded in abolishing the antifertilizing activity of SVFB, and only α -amylase effected a reduction in activity (Table 1).

After dialysis and precipitation of the protein fraction by alcohol-ether, the antifertilizing substance was found in the precipitate. Ten mice injected with the precipitate had forty-three embryos at 12 days of pregnancy, while females injected with the supernatant had seventy-seven embryos.

The active substance occurred in both the ammonium sulphate supernatant and the precipitate. The mean number of embryos in thirty-one mice injected with freeze-dried precipitate amounted to $3.22 \pm 3.16/\text{female}$. Injections of the supernatant material reduced the number of embryos in sixteen mice to $2.50 \pm 3.43/\text{mouse}$. Acetic acid (2%) precipitated some non-reactive proteins but in combination with ammonium sulphate, the very active (Table 2)

Table 2. Antiembryonic activity of the basic fraction in the supernatant of bull vesicular fluid following precipitation by acetic acid and ammonium sulphate

<i>Time of first injection</i>	<i>No. of mice injected</i>	<i>Average no. of embryos/female</i>
Control 0.9% NaCl	12	5.75 ± 1.90
2 to 10 days before mating	29	$2.65 \pm 3.17^{**}$
On the day of mating	12	$1.16 \pm 1.90^{**}$
1 day after mating	13	$2.23 \pm 3.01^{**}$
2 days after mating	12	$3.08 \pm 3.58^{*}$
3 days after mating	17	$0.17 \pm 0.32^{**}$
4 days after mating	54	$1.37 \pm 2.10^{**}$
5 days after mating	43	$1.30 \pm 2.05^{**}$
6 days after mating	13	$1.00 \pm 1.90^{**}$
7 days after mating	26	$2.57 \pm 2.80^{**}$
8 days after mating	12	$2.66 \pm 2.08^{*}$
9 days after mating	13	$1.92 \pm 2.70^{**}$
10 days after mating	11	$3.09 \pm 3.36^{*}$
11 days after mating	11	$2.36 \pm 2.75^{*}$
12 days after mating	15	$2.20 \pm 2.91^{**}$
13 days after mating	11	$1.72 \pm 2.50^{**}$
14 days after mating	26	4.72 ± 2.61
15 days after mating	13	4.77 ± 2.96
16 days after mating	7	5.57 ± 1.92
17 days after mating	9	6.11 ± 1.67

* Statistically significant, $0.05 > P > 0.01$.

** Statistically highly significant, $P < 0.01$.

supernatant basic fraction was obtained. Its partial purification and concentration in the active substance A can be seen in Plate 1 and Text-fig. 1.

Mode of action of the antifertilizing substance in bull seminal vesicle fluid

Even after treatment with enzymes, injections of SVFB before and after mating (Table 1) demonstrated the probable antiembryonic character of the antifertilizing substance. Injections of the partially purified basic fraction supported this observation. Females receiving the basic fraction in three doses of 0.1 ml daily from the day of the mating till the 13th day of the pregnancy showed a significant increase in embryonic mortality (Table 2), while injections

TEXT-FIG. 1. Disc electrophoresis of the seminal vesicle fluid of bulls (SVFB) and the basic fraction of this fluid (BF) at pH 9.4 and pH 4.3. A = antiembryonic active fraction.

Table 3. Development of zygotes in mice injected with the basic fraction in the supernatant of bull vesicular fluid following precipitation by acetic acid and ammonium sulphate

Day of mating after last injection	No. of mice injected	No. of eggs		
		Total	Two blastomeres	Uncleaved
3	1	11	11	0
4	3	23	23	0
5	5	43	40	3
6	2	19	19	0
7	1	10	10	0
8	1	7	7	0
9	1	8	8	0
10	2	14	12	2
Total	16	135	130	5
Average/female		8.43 ± 1.11	8.12 ± 1.03	0.31 ± 0.27

Starch-gel electrophoretic separation of the seminal vesicle fluid of bulls (SVFB) and the basic fraction of this fluid (BF). A = antiembryonic active fraction.

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which were not begun until the 14th, and especially 16th and 17th days, were ineffective.

Neither the number of eggs ovulated nor their fertilization was affected by the active substance. The mean numbers of two-blastomere embryos in sixteen mice injected 3 to 10 days before mating were 8.12 ± 1.03 (Table 3). The onset of oestrus in the injected females was not changed when compared to that in control animals.

The antiembryonic substance did not appear to have any effect on the lactation of suckling females. There was no difference in the weight of fifty-eight offspring of eight females, which were injected with increased doses of the basic fraction at the beginning of lactation, compared with that of the offspring of control mice. (The average body weight for experimental mice at the age of 6 weeks was 20.60 ± 1.70 g, and that for control mice was 20.21 ± 1.71 g). Neither the body weight (20.32 ± 0.88 g) nor the fertility (8.23 ± 2.31 offspring/mouse) was affected.

Injections of the basic fraction given to twenty females at the age of 14 to 20 days significantly decreased their weight increase. The average body weight for the injected mice was 17.50 ± 1.80 g. The difference compared with the control group (20.21 ± 1.71 g) is statistically very significant ($P < 0.001$). A similar weight decrease (about 10%) was noticed after injections of the antiembryonic substance into adult mice. Even those mice which were injected with the basic fraction at the age of 14 to 20 days or in maturity showed no essential health disturbances or increased mortality. Twenty mice which were injected in youth showed no increase of embryonic mortality when sexual maturity was reached 25 to 35 days after the end of the injections. The average number of embryos on the 12th day of pregnancy was 7.16 ± 0.80 .

The active antiembryonic substance affects the embryo very early. It is possible to observe necrotic changes in embryos on the 3rd day after the first injection. When the basic fraction is administered up to the 5th day after mating, the death of the blastocysts probably occurs immediately after, or even before, nidation since, from the 7th day after mating, no blastocyst traces can be seen and the uteri have a normal non-pregnant appearance. In twenty-four mice which received their first injection 8 days after mating, 134 embryos were found on the 10th to 13th days of pregnancy and, of these, 112 were aborted and only twenty-two were normal.

In an investigation of the effects of various doses of the basic fraction on embryonic mortality, it was found that injection of 2 mg per female resulted in an average of only 1.17 ± 1.79 live embryos/mouse for twenty-three mice, 3-mg resulted in an average of 1.21 ± 1.89 /mouse for thirty-two mice, 4 mg resulted in the death of all the embryos in twenty-two mice and 5 mg produced sterility in eighteen out of nineteen mice.

From the results of the study of embryonic mortality in mice injected 10 or more days before mating and from the fertility results in 155 mice which aborted after basic fraction injections and which, at different periods after abortion, were mated again, the antiembryonic substance appears to be effective in mice for a relatively long time. Repeated and highly significant embryonic mortality occurs for the second time in mice injected between 20

and 25 days after the end of the injections. A significant ($P=0.05$ to 0.01) decrease of fertility is apparent up to 50 days from the last injection (Table 4).

By contrast, the addition of basic fraction to the drinking water in a double dose (10 mg/mouse) was ineffective. In sixteen mice treated in this way, the average number of embryos per mouse was 5.87 ± 3.03 .

Injections of the basic fraction did not affect the average number of erythrocytes either in a group of twelve mice killed 3 days after the end of the injections ($5,796,000 \pm 775,000$) or in twelve mice decapitated 10 days after receiving the injections ($5,994,000 \pm 516,000$) when these levels were compared with those of controls ($5,517,000 \pm 775,000$). The number of leucocytes was decreased ($P = 0.05$ to 0.02) in twelve experimental mice killed 3 days after the final

Table 4. Embryonic mortality of mice rendered temporarily sterile after injections of the anti-embryonic active fraction and secondarily mated again after various time intervals

<i>No. of days from the last injection to the second mating</i>	<i>No. of mice mated</i>	<i>Average no. of embryos/female</i>
Control	14	6.07 ± 2.14
20 to 25	15	$1.46 \pm 2.12^{**}$
26 to 30	27	$3.40 \pm 3.50^{*}$
31 to 35	32	$3.87 \pm 3.16^{*}$
36 to 40	14	$3.14 \pm 2.18^{*}$
41 to 50	13	3.61 ± 3.55
51 to 60	11	5.54 ± 2.68
61 to 70	11	5.90 ± 2.64
71 to 80	15	5.20 ± 2.45
80 to 100	17	5.64 ± 2.65

* Statistically significant, $0.05 > P > 0.01$.

** Statistically highly significant, $P < 0.01$.

basic fraction injections (2962 ± 675 compared to the control level of 4118 ± 1087), but not in mice killed 10 days after the injections (4223 ± 816). Differential white blood cell values were not changed significantly ($77 \pm 8\%$ of lymphocytes and $23 \pm 8\%$ of heterophils in the group of mice killed 3 days after the last injections, $84 \pm 7\%$ lymphocytes and $16 \pm 7\%$ heterophils in the mice killed 10 days after the injections, and $81 \pm 7\%$ lymphocytes and $19 \pm 7\%$ heterophils in the control group).

DISCUSSION

The results presented in this paper indicate that the so-called antifertilizing substance of the seminal vesicle fluid of bulls (Matoušek & Petrovská, 1969) has such an effect only after fertilization of the egg. It does not disrupt the oestrous cycle, ovulation or the actual fertilization process, as may be inferred from the undisturbed development of the zygote at the two-blastomere stage in mice injected 3 to 10 days before mating. The embryonic mortality observed in mice injected after mating is further proof of the specific teratogenic effect aimed at the developing embryo.

An interesting problem is the relative ineffectiveness of the antiembryonic substance on embryos in the last quarter of their development. It is possible that the antiembryonic substance does not have enough time to evoke the damage (even when in younger embryos, the damage occurs several hours after injection of the active substance) or that, in this developmental stage of the embryo, some barriers are present which do not allow the substance to act effectively. The possibility cannot be excluded that the antiembryonic substance attaches itself to a component of the embryo which is present only during a certain period of embryonic development.

There appears to be no disturbance of the oestrous cycle, ovulation or lactation and it may be presumed that the antiembryonic substance does not interfere with the hormonal activity of the individual.

The embryonic mortality is unlikely to have an immunological origin. Embryonic damage occurs very early after the injection of the active substance when antibodies cannot have been fully formed. In experiments with rabbits, no difference in the quantity and quality of antibodies was found between injected females whose embryos had been aborted and those whose embryos developed normally (J. Matoušek, unpublished results). The immunological situation seems to be the same as in males receiving injections of the aspermatogenic factor (Matoušek *et al.*, 1972).

The antiembryonic substance affects the mouse organism for a relatively long time. This suggests the possibility of a specific teratogen circulating in the maternal blood or bound to maternal tissues and released to become effective when the embryonic cells undergo development.

The effective factor in the antiembryonic substance is unknown. In general, cell cleavage or the metabolic processes specific to the embryonic tissues can be disturbed in the blastocyst either before or after nidation. However, it seems unlikely that this substance would be effective as a common cytostatic agent. It is true that the number of leucocytes after injections of the antiembryonic substance was significantly decreased, but only for a few days and the differential white blood cell count of heterophils and lymphocytes was practically not affected at all. The accompanying digestive disorders typical for cytostatic agents were not observed.

The antiembryonic substance may be identical with the aspermatogenic factor of the seminal vesicle fluids of bulls (Matoušek, 1969; Matoušek *et al.*, 1972). All fractions which have an active aspermatogenic effect also appear to be active from the antiembryonic aspect (J. Matoušek, unpublished results). The position and character of the presumed aspermatogenic and antiembryonic fractions on electrophoretograms are identical.

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