1. Miale, J. B. "Laboratory Medicine: Hematology" 918 pp. Mosby, St. Louis, Missouri, 1962.

2. Rugh, R., "The Reproduction and Development of the Mouse." Burgess, Minneapolis, Minnesota, 1967, in press.

3. Russel, E. S., Neufeld, E. F., Higgins, C. T., Proc. Soc. Exptl. Biol. Med. 78, 761 (1951).

4. Halberg, F., Hamerston, O., Bittner, J. J., Science 125, 73 (1957).

5. Halberg, F., Zander, H. A., Houglum, M. W., and Muhlemann, H. R., Am. J. Physiol. 177, 361 (1954).

6. Urushiyama, Y., Nippon Igaku Hoshasen Gakkai Zasshi 19, 1333 (1959).

7. Pauly, J. E., Scheving, L. E., Anat. Record, 153, 349 (1965).

8.. Graser, F. M., Proc. Soc. Exptl. Biol. Med. 99, 407 (1958).

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Fertilization, Embryo and Fetal Survival Rates in Rabbits Isoimmunized with Semen, Testis, and Conceptus* (32927)

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Evidence in the mouse (1,2), guinea pig (3,4), rabbit (5), and cow (6) has indicated that immunization of females with homologous semen, spermatozoa, or testis will cause infertility. The infertility in mice was reported due to inhibition of fertilization (2). The exact nature of the infertility in other species, however, has not been clearly demonstrated to be fertilization failure, embryo mortality, fetal mortality, or a combination of these factors. The objective of the present study was to determine the effects of isoimmunization with semen, testis, 9-day-old conceptus, and seminal plasma on fertilization rate, embryo survival, and fetal survival in rabbits.

Materials and Methods. The rabbits used in the trials were sexually-mature estrous females of mixed breeding. The antigenic materials consisted of: semen from the pooled ejaculates of 2–4 male rabbits, seminal plasma from 2 vasectomized rabbits, testis material from mature rabbits that was homogenized with an equal volume of physiological saline, and homogenized conceptus material from rabbits 9 days pregnant. The antigenic materials were mixed with an equal volume of Freund's complete adjuvant and 0.5–0.75 ml of this mixture was given per injection. Rabbits injected with adjuvant and physiological saline served as controls.

In Trial 1, 25 rabbits were given 3 weekly intramuscular and subcutaneous injections of the antigen–adjuvant mixtures. One week after the last injection the females were artificially inseminated with 0.5 ml of diluted semen containing 15 to 25 million live sperm. Ovulation was induced by intravenous injection of 100 IU human chorionic gonadotropin (HCG) at the time of insemination. Embryo survival was determined 9 days after breeding by laparotomizing the rabbits and noting the number of embryos and corpora lutea present. Fetal survival was based on the number of viable fetuses at 28 days after breeding or the number of young born compared with the number of normal embryos at 9 days. Rabbits bred a second time were artificially inseminated from 4 to 6 weeks after an infertile breeding or from 3 to 4 weeks postpartum after removal of the litter.

In Trial 2, 30 rabbits were given 3 weekly injections of the antigen–adjuvant mixtures followed in 3 weeks by a fourth injection. One week after the last injection the rabbits were artificially inseminated and injected with

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HCG as described earlier. Fertilization rate was determined by flushing one oviduct *in situ* in each of the rabbits 26–28 hours after breeding. Embryo and fetal survival rates were determined as in Trial 1 and in addition the embryo survival rate was adjusted for fertilization rate in each group.

Trial 3 consisted of transferring ova recovered from bred-superovulated rabbits into 44 recipients that had been isoimmunized using the same procedures as in Trial 2. Five or six 2-cell ova were placed into the infundibular end of the oviducts of the recipients at 24–26 hours after HCG injection. The transfer occurred 5–7 days after the last immunization. The recipients were laparotomized at 9 days and killed at 28 days after HCG injection to determine embryo and fetal survival rates, respectively. The donors were superovulated by twice-daily subcutaneous injections of 0.5 mg of porcine FSH preparation for 3 days followed on the fourth day with an intravenous injection of 2 mg of equine LH. The rabbits were artificially inseminated at the time of LH injection and killed from 26 to 28 hours later. Ova were recovered by flushing the oviducts with a warm (37°C) solution of normal rabbit serum diluted 1:3 with modified Krebs solution.

An additional 4 rabbits were immunized by injections of washed epididymal sperm (250×10^6) mixed with an equal volume of Freund's complete adjuvant using the procedure outlined for Trial 2. The epididymal sperm were collected by flushing the epididymides of 3 mature rabbits. The sperm were washed 10 times with phosphate buffered saline (pH 7.2) by centrifugation.

Serum samples were collected from rabbits before injection and at the time of breeding. The samples were heated at 56°C for 30 min and the titers of sperm agglutinins were determined by the gelatin agglutination method. (7).

The data in Trials 1 and 2 were analyzed by chi-square, and the data of Trial 3 by Duncan's new multiple range tests (8).

Results. In Trial 1 (Table I) the percentages of corpora lutea represented by normal embryos at 9 days after first breeding were reduced highly significantly to 36, 28, and 41% in rabbits immunized with semen, testis, and conceptus, respectively, in comparison with 89.7% in the saline controls. No viable embryos were found in 2 of 5, 3 of 5, and 2 of 7 rabbits in the 3 respective immunized groups. The average embryo rates in the remaining rabbits of the 3 groups were 54.5, 58.3, and 58.2%, respectively, which were still significantly less than the controls. Fetal survival rate was significantly lower in the rabbits immunized with semen (55.6%) and testis (44.2%) and tended to be lower in the conceptus group (71.9%) than the saline-control rabbits (85.7%). Rabbits immunized with seminal plasma had embryo and fetal survival rates (88.6 and 83.9%) similar to those of the controls. Following the second breeding the embryo survival rates increased to 65.4, 51.2, and 71.8% and the fetal survival rates increased to 76.5, 77.3, and 86.3% in rabbits immunized with semen, testis, and conceptus, respectively. All the rabbits bred for a second time had conceived. The embryo survival rate tended to be less ($p<.10$) for the semen group and was significantly less

TABLE I. Embryo and Fetal Survival Rates in Rabbits after Isoimmunization with Semen, Testis, Conceptus, and Seminal Plasma.

	First breeding				Second breeding			
	No. of:		Survival (%)		No. of:		Survival (%)	
Immunizing treatment	Rabbits	CL[a]	Embryo	Fetal	Rabbits	CL[a]	Embryo	Fetal
Semen	5	50	36.0	55.6	5	52	65.4	76.5
Testis	5	50	28.0	44.2	4	43	51.2	77.3
Conceptus	7	78	41.0	71.9	7	71	71.8	86.3
Seminal plasma	4	35	88.6	83.9	2	19	82.6	94.7
Saline control	4	39	89.7	85.7	4	41	87.8	83.3

[a] Corpora lutea

TABLE II. Fertilization, Embryo, and Fetal Survival Rates in Isoimmunized Rabbits.

Immunizing treatment	Breeding no.	No. of rabbits[a]		Ova[b] fertilized (%)	Survival[b] (%)		
		Bred	Fertile		Emb.	Adj. emb.[c]	Fetal
Semen	1	8 (75)	3 (32)	55.6	28.6	51.3	50.0
	2	5 (49)	4 (37)	87.5	60.0	68.6	58.3
Testis	1	6 (58)	2 (21)	30.0	0.0	0.0	—
	2	5 (59)	4 (49)	71.4	26.9	37.6	28.6
Conceptus	1	6 (51)	6	100.0	33.3	33.3	50.0
	2	5 (54)	5	95.0	56.7	59.6	70.6
Seminal plasma	1	5 (40)	5	100.0	84.2	84.2	87.5
	2	3 (32)	3	91.7	70.6	76.9	75.0
Saline control	1	5 (45)	5	95.2	91.3	95.2	85.7
	2	4 (45)	4	88.9	79.2	86.4	84.2

[a] Values in parentheses represent total number of corpora lutea per group.
[b] Values are for fertilized females only.
[c] Adjusted embryo survival is based on fertilization rate within treatment group.

for the rabbits injected with testis than those injected with saline (87.8%).

The fertilization check in Trial 2 (Table II) revealed that, compared with the saline controls, isoimmunization with seminal plasma had no effect on fertility, whereas immunization with semen and testis tended to decrease the incidence of fertile rabbits and significantly lowered the rate of fertilization and embryo survival in the fertile rabbits. The fertilization and adjusted embryo survival rates following first breeding were 55.6 and 51.3% in semen-immunized rabbits, 30.0 and 0.0% in the testis group, 100 and 84.2% in the seminal plasma group and 95.2 and 95.2% in the saline controls. Rabbits immunized with conceptus had a fertilization rate (100%) within the normal range but a significantly decreased rate of embryo survival (33.3%) compared with the controls. Fetal survival tended to be lower, but was not significant because of the small numbers involved, in rabbits immunized with semen (50.0%) and conceptus (50.0%) in comparison to animals immunized with seminal plasma (87.5%) and saline (85.7%). The lack of embryo development in the testis group precluded any determination of fetal survival. Following the second breeding there was an improvement in the fertilization, embryo, and fetal survival rates to 87.5, 68.6, and 58.3%, respectively, in semen-immunized rabbits and to 71.4, 37.6, and 28.6%, respectively, in the testis-immunized rabbits. Rabbits immunized with conceptus had embryo and fetal survival rates of 59.6 and 70.6% which represented increases over the results of the first breeding. These values, except for the embryo survival rate in the testis group, were not significantly different from the values for the control rabbits. There was no difference noted between first and second breeding in the fertility of the seminal plasma and saline groups.

In Trials 1 and 2 the serum titers of sperm agglutinins were from 1:32 to 1.128 for rabbits that were infertile after immunization with semen and testis, respectively. The fertile rabbits of these groups had respective titers of 1:2 to 1:32 and 1:2 and 1:16. Isoimmunization with seminal plasma caused agglutinin titers of 1:4 to 1:64 but with no relationship to fertility. Normal sera and sera from rabbits immunized with conceptus and saline had a titer of 1:2 or less. In Trial 3 (Table III), compared with the embryo survival rate of transferred ova in the saline control rabbits (71.6%), survival was comparable in rabbits immunized with seminal plasma (65.5%) and significantly lower in rabbits immunized with semen (38.4%), conceptus (45.4%), and testis (21.8%). Fetal survival was significantly reduced (16.7%) in the testis group, whereas in the other groups it was similar (81.1–83.6%) to that of the controls

TABLE III. Survival Rates of Fertilized Ova Transferred into Isoimmunized Recipient Rabbits.

Recipient treatment	No. Recipients	No. Ova tran.	Survival (%) Embryo	Survival (%) Fetal
Semen	10	112	38.4	81.4
Testis	10	110	21.8	16.7
Conceptus	8	86	45.4	81.1
Seminal plasma	8	84	65.5	83.6
Saline control	8	88	71.6	88.9

(88.9%). One rabbit each in the semen and testis groups failed to have any viable embryos present at 9 days. By 28 days after ova transfer there was 1 additional rabbit in the semen group and 6 rabbits in the testis group that had lost all their embryos. There tended to be an association of embryo loss with an increased titer of sperm agglutinins in the sera of rabbits immunized with semen and testis (Table IV).

The four rabbits immunized with epididymal sperm were killed 26 hours after insemination. None of 27 ova recovered from 3 rabbits and 3 of 8 ova from the fourth rabbit were fertilized compared with 14 of 15 ova recovered from 2 untreated rabbits. The 3 infertile rabbits had serum titers of agglutinins of 1:128, 1:64, and 1:64 and the partially fertile rabbit had a serum titer of 1:32.

Discussion. The results indicate that isoantibodies to the antigens of semen and testis can reduce fertility by three means; (a) prevention of fertilization, (b) induction of early embryo mortality, and (c) induction of fetal mortality. The embryo and fetal loss induced in the rabbit differs from the results reported for the mouse in which only fertilization inhibition was noted (2). This loss, however, is in agreement with the work in cattle which indicated that isoimmunization with semen increased the incidence of early embryo loss (6).

The antigens in semen responsible for inducing the antifertility isoantibodies originate in the testis and are probably sperm bound, as antibodies to washed epididymal sperm inhibited fertility, whereas antibodies to the antigens of seminal plasma had no effect on fertility. The exact nature of the action of the antifertility isoantibodies is still to be elucidated. Treatment of semen before insemination with an antiserum to semen was reported to inhibit fertilization and increase embryo loss in rabbits (9). Thus it appears that antibodies to sperm can act directly on the sperm cell to cause fertilization failure, embryo mortality and fetal loss and also directly on the developing conceptus to induce embryo and fetal loss. There is at least one antigen common to semen and conceptus that is not found in the serum of rabbits (10).

Isoantibodies to conceptus antigens appear to be detrimental to fertility after fertilization has occurred and primarily before implantation. The lack of conceptus antibodies to interfere with fertilization was observed after treatment of semen in the rabbit (10). A high incidence of fetal mortality has also been seen in pregnant rats following passive immuniza-

TABLE IV. Survival Rates of Transferred Ova in Relation to Sperm Agglutination Titer in the Sera of Recipients.

	Isoimmunized recipients								
	Semen			Testis			Seminal plasma		
Sperm agglut. titer	No. of rabbits	Survival (%) Embryo	Survival (%) Fetal	No. of rabbits	Survival (%) Embryo	Survival (%) Fetal	No. of rabbits	Survival (%) Embryo	Survival (%) Fetal
1:128	3	8.8	33.3	0	—	—	0	—	—
1:64	2	63.6	100.0	3	6.2	0.0	1	58.3	85.7
1:32	3	41.2	71.4	6	25.8	23.5	2	72.7	87.5
1:16	1	40.0	50.0	1	41.7	0.0	2	70.0	85.7
1:8	1	75.0	100.0	0	—	—	3	60.0	77.8

tion with rabbit antiserum to rat placenta (11) and kidney (12).

Summary. Isoimmunization of rabbits with semen, testis, and conceptus resulted in lowered embryo and fetal survival rates compared with immunization with seminal plasma and physiological saline. *In situ* recovery of ova from one oviduct of rabbits after insemination revealed a depression of fertilization rates in rabbits immunized with semen and testis and normal rates in rabbits immunized with conceptus, seminal plasma, and saline. Embryo survival rate adjusted for fertilization rate and the fetal survival rate were lowest in the testis group, intermediate in the semen and conceptus groups, and normal in the seminal plasma and saline groups. There was an increase in fertility of the 3 low-fertility groups from first to second breeding. Embryo survival rates of transferred ova were similar to those described above for the immunized groups. Fetal survival, however, was decreased only in rabbits immunized with testis. Isoimmunization with washed epididymal sperm induced antibodies and infertility in rabbits. In general, there was negative association between fertility and the serum titer of sperm agglutinins in rabbits immunized with semen and testis.

1. Edwards, R. G., Nature **203**, 50 (1964).

2. McLaren, A., Nature **201**, 582 (1964).

3. Katsh, S. Am. J. Obstet. Gynecol. **78**, 276 (1959).

4. Otani, Y., Behrman, S. J., Porter, C. W., and Nakayama, M., Intern. J. Fertility **8**, 835 (1963).

5. Behrman, S. J. and Nakayama, M., Fertility Sterility **16**, 37 (1965).

6. Menge, A. C., J. Reprod. Fertility **13**, 445 (1967).

7. Kibrick, S., Balding, D. L., and Merrill, B., Fertility Sterility **3**, 419 (1952).

8. Steel, R. G. D. and Torrie, J. H., "Principles and Procedures of Statistics." McGraw-Hill, New York, 1960.

9. Kiddy, C. A., Stone, W. H. and Casida, L. E., J. Immunol. **82**, 125 (1959).

10. Menge, A. C. and Protzman, W. P., J. Reprod. Fertility **13**, 31 (1967).

11. Brent, R. L., Proc. Soc. Exptl. Biol. Med. **125**, 1024 (1967).

12. Brent, R. L., Am. J. Anat. **115**, 525 (1964).

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