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Relationship of *in Vivo* Gamete Aging and Exogenous Hormones to Early Embryo Development in Rabbits* (34001)

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Noyes and Thibault (1) concluded that many factors influence the functional life of spermatozoa in the female genital tract. In the rabbit, capacitation of spermatozoa must take place before fertilization can occur (2). Rates of capacitation and sperm survival vary, depending on the location of sperm in the female genital tract (3, 4) and upon various hormonal states (1, 3, 5, 6). Fertilizing capacity of spermatozoa in the mated doe diminishes after 22 hr *in utero* (7).

Adams (8) obtained a high fertilization rate following administration of hormones to induce superovulation. Kennelly and Foote (9) found no differences in the fertilization rate of superovulated and nonsuperovulated does inseminated immediately after giving ovulating hormone. Tesh (10) reported a decrease in the fertilization rate in superovulated does with *in vivo* aged spermatozoa, and he suggested that oocytes produced by superovulation are in some way deficient.

It has been reported that the rabbit secondary oocyte has a fertilizable life span of 3.5–8 hr (11–13). Ovulation is delayed in does treated with follicle-stimulating preparations (9, 15). This delay explains the apparent lag in development of embryos obtained from FSH-primed does compared with nonprimed does examined at the same time interval after LH injection (15). The present investigation was conducted to study the critical parameters of aging spermatozoa and secondary oocytes in FSH-primed and control (nonprimed) does using initial cleavage *in vivo* and development during culture as test criteria.

Materials and Methods. The experimental design was a 2 × 4 arrangement with control and FSH-primed groups each subdivided into four subgroups inseminated either 10 hr before LH injection, at the time of LH injection, or 5 or 10 hr after LH injection. These times are designated as +10, 0, —5, and —10 Thus, the sperm were allowed to age in the female as much as 10 hr longer than normal to 10 hr less than normal. Since the late inseminations were expected to coincide with many of the ovulations (14), ovulated oocytes were expected to age at least 4–6 hr (2) during the period required for sperm capacitation before possible fertilization.

Forty-eight uniparous does were assigned to the eight groups, giving six does per group. The FSH-primed does received FSH subcutaneously 2 times daily for 3 days (0.5 mg of FSH/injection) followed by an intravenous injection with LH the fourth morning (2.5 mg of Armour PLH). Controls received only the LH. The time of LH injection was controlled over a 20-hr period so that semen collected from one fertile male could be used promptly and simultaneously to inseminate all eight does comprising one complete 2 × 4 replicate. The same semen donors were used throughout the experiment. Semen was diluted with physiological saline, containing penicillin G and streptomycin sulfate, so as

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TABLE I. Percentage of Oocytes and Embryos at the Time of Collection.

Interval (hr)	Total embryos or oocytes	Stage at collection (%)		
		1-cell	2-cell	4-cell
Control group				
+10	42	55	45	0
0	46	17	11	72
−5	40	13	2	85
−10	37	22	78	0
FSH group				
+10	223	43	37	20
0	214	33	13	54
−5	211	19	18	63
−10	242	67	30	3

to give 100×10^6 motile sperm/ml, and 0.1 ml was inseminated/doe.

Twenty-nine to 30 hr after LH injection each doe was killed and the embryos and/or oocytes were recovered. The stage of development was recorded. Most of the embryos and oocytes were cultured in bovine serum (16). They were observed *in vitro* daily for 6 days. All ova recovered in the 1-cell stage are referred to as oocytes. Although many were zygotes not yet cleaved, it was not possible to distinguish them at this stage because the experimental design required that they be placed promptly and undamaged in culture.

Results. The stage and percentage of oocytes cleaved are presented in Table I and Fig. 1. A chi-square analysis on total cleaved ova in each group showed that the control group had a higher percentage of cleaved oocytes $\chi^2 = 13.3$, 1 *df*, $p<0.005$). However, the analysis of variance, taking into account groups, subgroups and their interaction, resulted in no difference ($p>0.10$) between groups or among subgroups.

The percentage of ruputre points accounted for by embryos and/or oocytes was 85 and 83% in the control and FSH groups. Corresponding values among subgroups ranged from 74 to 98%.

Development in culture is presented in Table II and Fig. 1. Blastocyst development differed between groups ($p<0.10$) and among intervals ($p<0.05$). Over all subgroups a higher proportion of cleaved ova placed in culture from FSH-primed does (58%) developed into blastocysts than cleaved ova from control does (28%) ($\chi^2 = 33.1$, 1 *df*, $p<0.005$). Both groups had 1-celled ova develop into normal blastocysts, indicating that these were zygotes at the time of collection. In the subgroups, particularly striking is the consistently higher proportion of blastocysts which developed from embryos and embryos plus zygotes recovered from the FSH-treated does, as compared to the controls (Fig. 1).

The percentages of cleaved ova when placed in culture for the control +10, 0, −5, and −10 subgroups were 48, 85, 88, and 77%, and after culture were 65, 96, 88, and 94%, respectively. Corresponding values for the FSH-primed groups were 56, 68, 79, and 37%, 89, 96, 93 and 81%, respectively. The overall percentages of cleaved ova before and after culture for the control group were 75 and 86%. Similar values for the FSH-primed group were 61 and 90%.

Discussion. The result of aging spermatozoa (+10 subgroups) is shown by the lower rate of cleavage observed both before and after culture. This is particularly evident in the control group in which no embryos had reached the 4-cell stage at the time of collection (Table I), and only 65% had cleaved after culture. Spermatozoa would have been in the female tract for 20 or more hours by the time of ovulation, so this decrease in fertility is in agreement with studies on naturally mated animals (7).

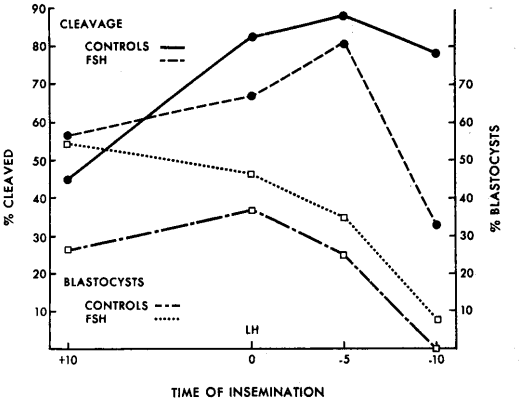

FIG. 1. Percentage of cleaved ova at the time of collection and the percentage which became blastocysts in culture.

TABLE II. Development of Oocytes and Embryos in Culture.

Interval (hr)	No. of blastomeres prior to culture	No. of oocytes or embryos cultured	Stage of development in culture (%)				Percentage of cleaved ova that became blastocysts
			No change	< morula	Morula	Blastocyst	
Control group							
+10	1	21	67	23	0	10	29
	2–4	19	5	5	43	47	50
	Total	40	38	15	20	27	44
0	1	7	29	71	0	0	0
	2–4	39	23	23	10	44	57
	Total	46	24	30	9	37	49
−5	1	5	0	60	0	40	40
	2–4	35	0	63	14	23	23
	Total	40	0	62	13	25	25
−10	1	8	25	25	50	0	0
	2–4	27	11	70	19	0	0
	Total	35	14	60	26	0	0
Group total	1	41	44	37	9	10	17
	2–4	120	11	43	18	28	32
	Total	161	19	41	16	24	29
FSH-primed group							
+10	1	90	26	54	11	9	12
	2–4	115	0	5	5	90	90
	Total	205	11	26	8	55	62
0	1	66	12	82	0	6	7
	2–4	139	2	14	17	67	68
	Total	205	5	36	12	47	48
−5	1	42	33	67	0	0	0
	2–4	161	4	27	26	43	45
	Total	203	10	35	20	35	38
−10	1	116	31	58	9	2	3
	2–4	69	0	30	51	19	19
	Total	185	20	48	24	8	10
Group total	1	314	26	63	7	4	6
	2–4	484	2	18	22	58	59
	Total	798	11	36	16	37	42

Aging of oocytes (−10 subgroups) had a pronounced effect, especially in the control group. The fact that no 4-celled embryos were recovered at the time of collection is explainable by the late insemination. Sperm would not have capacitated until 4–6 hr after the beginning of ovulation (2); therefore, fertilization would be delayed and embryos produced would not have had time to develop to the 4-cell stage when collected. Of particular importance is the finding that, while 78% of the aged oocytes were in the 2-cell stage at collection, none developed to the blastocyst stage in culture. This result *in vitro* is in agreement with the finding of reduced blastocyst development *in vivo* when mating was delayed for 10 hr (17). In the corresponding FSH-primed subgroup only 30% of the cleaved ova had reached the 2-cell stage, but 19% of these developed in culture into blastocysts. This is consistent with the hypothesis that the oocytes were not aged as long prior to fertilization due to the reported prolonged and later time of ovulation in FSH-primed does (9, 15). Consequently, this delay in ovulation would lead to less de-

velopment than in the controls at the time of collection, but the probability of subsequent development was increased by fertilization of more recently ovulated oocytes. Apparently, aged oocytes in the control group were able to cleave following penetration by the capacitated sperm, but had lost their capacity to form a blastocoele.

The results in Table II and Fig. 1 suggest that spermatozoa aged in a FSH-primed environment (+10 subgroup) retain their ability to fertilize oocytes and initiate normal development longer than spermatozoa in control does. This is indicated by the higher proportion cleaved initially, 57 vs. 45%, higher proportion cleaved after culture, 89 vs. 65%, and higher proportion of blastocysts formed in culture, 55 vs. 27%. The 1-cell zygotes that formed blastocysts (Table II) must have resulted from oocytes ovulated at least 17–18 hr after LH administration, or they should have undergone cleavage at least once prior to recovery (9, 18). Thus, sperm were capable of initiating development, at least to the blastocyst stage, after more than 27 hr in the FSH-primed does. This longer fertilizable life of the spermatozoa placed in FSH-primed does could have resulted from changes in the uterine environment induced by the FSH, which also may affect the capacitation rate of spermatozoa (2, 5, 6).

Overall the proportion of oocytes from FSH-primed does which cleaved and the proportion which developed into blastocysts were equal or higher than in the controls. In no way did the embryos from the FSH group show any deficiencies, contrary to the report by Tesh (10). The lower percentage of cleaved zygotes at any comparable standardized time of collection merely reflects the shorter interval between ovulation and collection in superovulated does. Thus, the superovulation technique appears to be well suited to studies of this type, particularly as it provides large numbers of embryos for study.

Summary. Oocytes aged before fertilization by delaying insemination for 10 hr resulted in embryonic failure in culture prior to the blastocyst stage, similar to previous studies *in vivo* (17). Aging of spermatozoa for 10 hr before inducing ovulation with LH administration reduced fertilization and cleavage rates, and reduced the proportion of embryos that developed into blastocysts. The fertilizable life span of spermatozoa was prolonged by the environment of FSH-primed does. Overall the development in culture of embryos recovered from FSH-primed does was equal to or better than the controls. Some one-celled zygotes along with 2- and 4-celled embryos recovered from both groups developed into blastocysts in culture. Thus, the combined techniques of superovulation and embryo culture appear to be well suited to studies of this kind.

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