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REPRODUCTIVE EFFICIENCY IN AGED FEMALE RABBITS GIVEN SUPPLEMENTAL PROGESTERONE AND OESTRADIOL

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Summary. The reproductive efficiency of twenty-eight aged does, 49 to 72 months old, was compared with that of eighteen young does, 6 to 13 months old. Fertilization rate and development *in vitro* of fertilized ova from rabbits induced to superovulate were not influenced by the doe's age. Ovulation rates following natural mating were only slightly reduced with age. However, the number of embryos per doe was much greater in young than in old does at 12 and 24 days *post coitum*. All young does had viable embryos, whereas the percentages of aged does with detectable implantation sites and viable embryos were 80 and 40, respectively, at 12 days *post coitum*, and 77 and 44 at 24 days *post coitum*.

Aged female rabbits were given supplemental exogenous progesterone and/or oestradiol benzoate in an effort to increase reproductive efficiency. Progesterone treatment had no effect on the total number of young kindled but did prolong the gestation period, increase the birth weight and result in fewer live young kindled/doe. When administered on Days 3 to 29 of pregnancy, 4 μg/day of oestradiol alone or in combination with 2 and 4 mg progesterone completely blocked pregnancy in all does. Starting on Day 5 of pregnancy, oestradiol levels of 1 μg/day, with or without progesterone, had no effect.

Chromosomal analysis of fourteen embryos revealed eleven normal females (44,XX), one normal male (44,XY), one abnormal embryo (45,XX) with an extra acrocentric chromosome and one embryo with a modal number of forty-two chromosomes in 35% of the metaphases. Since most of the embryonic wastage in aged rabbits occurred during the first 12 days *post coitum*, chromosome studies of embryos younger than 12 days *post coitum* are indicated.

Most of the embryonic wastage could not be attributed to ovulation rate, fertilization rate, ovum potential, CL function, circulating levels of progesterone and oestrogen, or to chromosomal anomalies of the fetuses. It was concluded that uterine factors apparently limit reproductive performance in aged rabbits.

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INTRODUCTION

Reproductive capacity is initiated at puberty, increases for a short time, and eventually decreases at a rate characteristic of the species (Krohn, 1964; Schultze, 1967; Talbert, 1968). It has been suggested that the decline in fecundity with age might be due to (1) an exhaustion of the oocyte population (Ingram, Mandl & Zuckerman, 1958), (2) an increase in the incidence of defective oocytes (Henderson & Edwards, 1968) and (3) a decline in the ability of the uterus to maintain pregnancy (Adams, 1964, 1970; Biggers, 1969). Embryo transfer studies in mice (Talbert & Krohn, 1966) and rabbits (Adams, 1964; Maurer & Foote, 1971) showed that aged females still produce an adequate number of fertilizable oocytes which are capable of normal development provided that they are transferred to a suitable environment, such as the uterus of a younger recipient. In hamsters, Thorneycroft & Soderwall (1969) reported that decreased litter size in senescent females was due not to a decreased ovulation rate, but to a marked increase in pre- and postimplantation deaths. By inference, these studies support the hypothesis that the major cause of reproductive failure is related to uterine factors.

Another factor could be a progesterone deficiency, since progesterone is required for pregnancy. Decreased progesterone output by the ovaries has been implicated (Maurer & Foote, 1972). The objective of the first two experiments reported in this paper was to determine if implantation and kindling rates could be improved in aged rabbits by supplying exogenous ovarian steroids. As reproductive efficiency was declining rapidly in the aged does, they were compared with young does with respect to ovulation rates, embryo development *in vivo* and *in vitro*, CL function and chromosomal abnormalities of the progeny.

MATERIALS AND METHODS

Experiment 1

Thirty-six aged rabbits, 30 to 53 months old, which had previously produced numerous litters (Maurer & Foote, 1971), were used to study exogenous hormone treatment during pregnancy. During a pre-experimental test of their reproductive capacity, these thirty-six does were mated naturally with fertile bucks and allowed to go to term. Subsequently, thirty-five of these does were randomly bred with fertile males (one would not mate) and assigned to a treatment group. The four treatment groups were: (1) untreated controls; (2) 2 mg progesterone on Days 3 to 9 and 4 mg on Days 10 to 29; (3) 4 μg oestradiol benzoate on Days 3 to 29; and (4) progesterone plus oestrogen at the same levels as in treatments 2 and 3. Day 1 was the day of mating. All steroids were dissolved in sesame oil so that 1 ml contained half of the daily dose. Injections were given subcutaneously every 12 hr. The gestation length of the doe was determined and the progeny were counted, weighed and sexed.

Experiment 2

Thirty-three does remaining from Exp. 1, 39 to 62 months old at this stage, were used to study lower dosages of progesterone and oestrogen. Does were

randomly mated and assigned to four treatment groups as follows: (1) controls receiving only 2 ml sesame oil on Days 5 to 27; (2) 2 mg progesterone on Days 5 to 9 and 4 mg on Days 10 to 27; (3) 1 μg oestradiol benzoate on Days 5 to 9 and 2 μg on Days 10 to 27; and (4) progesterone plus oestrogen at the same dosages as in treatments 2 and 3. Half of the daily dose was given every 12 hr in 1 ml sesame oil. The day of mating was taken as Day 1.

All does were subjected to laparotomy on Day 12 and the numbers of CL and implantations were recorded. At the same time, uterine blood-flow rate was estimated in four randomly selected does, two pregnant and two non-pregnant, from each treatment group (L. L. Larson & R. H. Foote, unpublished observations). All does were allowed to kindle and the number, weight and sex of the young, as well as the gestation length were recorded.

Experiment 3

Before this experiment, the thirty-one living aged does, 42 to 65 months old at this stage, were exposed to fertile bucks and allowed to kindle. Subsequently, twenty-eight aged does, 49 to 72 months old and eighteen young does, 6 to 13 months old were randomized by age groups and previous breeding performance into three treatment groups. Does were mated naturally, and if they did not mate twice they were artificially inseminated. Does in Group 1 were treated with FSH, and superovulated ova were collected 28 to 30 hr after the injection of pituitary LH, as described by Maurer, Hunt & Foote (1968). The young embryos were cultured to test the effect of maternal age on developmental potential. Does in Groups 2 and 3 were killed 12 and 24 days *post coitum*, respectively, to examine pre- and postimplantation losses.

Does were started on the experiment so that replicates from each age and treatment group were killed on the same days. At autopsy, the number of follicles, implantations and viable embryos in Groups 2 and 3 were recorded. The ovaries, CL, adrenals and pituitaries were weighed.

A random sample of embryos was taken from Groups 2 and 3 for cytogenetic studies. Cells from entire embryos were grown in tissue culture and were harvested after arrest in metaphase (Dunn, McEntee & Hansel, 1971). Metaphases were photographed for karyotyping and analysis.

RESULTS

Control matings were conducted annually for 4 years when the does were 7 to 30, 18 to 41, 30 to 53 and 42 to 65 months of age. The third and fourth control matings took place before Exps 1 and 3. The respective data for the criteria of reproductive performance at the four control matings were: does mated—100%, 100%, 100%, 77%; does kindled—81%, 94%, 97%, 46%; number of young kindled/doe—5·9, 5·7, 4·3, 2·9. Thus, reproductive performance was severely impaired after 4 years.

Experiment 1

All nine does receiving supplemental progesterone during pregnancy and seven of ten control does kindled. None of the does receiving exogenous oestro-

gen alone or with progesterone kindled. The total number of young kindled/doe was 4·8 for control does and 4·7 for does treated with progesterone. Progesterone treatment in comparison with the controls resulted in a prolonged gestation period (34 versus 32 days, $P<0{\cdot}005$), heavier young kindled (61 versus 51 g, $P<0{\cdot}10$) and fewer young kindled alive (2·6 versus 4·6, $P<0{\cdot}005$). The sex ratio was not affected by treatment and was normal (55% male:45% female).

Experiment 2

Exogenous hormone treatment was not started until Day 5 of pregnancy and the oestrogen was reduced from Exp. 1. The latter no longer blocked pregnancy. However, supplying exogenous progesterone and/or oestrogen did not affect ($P>0{\cdot}05$) the number of implantations on Day 12 (Table 1). The percentages of CL represented as implantation sites for the control, progesterone, oestrogen and progesterone-plus-oestrogen treatment groups, respectively,

Table 1. Experiment 2. Implantation rate in aged does given supplemental exogenous steroids

Criteria	*Control*	*Progesterone*	*Oestrogen*	*Progesterone + oestrogen*
No. of does subjected to laparotomy on Day 12	8	9	8	8
No. of does pregnant	6	5	6	5
No. of corpora lutea*	5·4	5·0	7·0	6·3
No. of implantations*	4·4	1·8	3·9	3·8

No criterion varied significantly ($P>0{\cdot}05$) due to treatment. Dose of progesterone: 2 to 4 mg; dose of oestrogen: 1 to 2 μg.

* Group means based on the total number of does in the treatment group.

were 81, 36, 55 and 60%. The low percentage of implantations in the progesterone-treated group resulted partly from four does not being pregnant. Due to the death of three pregnant does before kindling and to the high fetal death loss in does used to study uterine blood-flow rates, kindling data are not given, although there was no apparent treatment effect. For does which were only subjected to laparotomy, the embryonic wastage from ovulation to Day 12 was 28% and the fetal loss from Day 13 to kindling was 21%. The sex ratio was normal.

Experiment 3

At the time of this study, the old does averaged 53 months of age and their reproductive performance in control matings was reduced. When treated with FSH, the mean superovulatory response of twelve ovulations was much less ($P<0{\cdot}005$) than the fifty-eight ovulations observed in does induced to superovulate at 9 months of age. However, there was no difference between young and aged does in fertilization rate (83% versus 81%) or in embryo development during culture *in vitro* ($P>0{\cdot}05$). The embryo development in culture averaged 3·7 for both groups on a scoring system where 0 = no cleavage and 6 = ex-

panded hatching blastocyst. Aged does had larger ($P<0.005$) pituitaries (49·8 mg versus 30·8 mg) than young does.

In the animals mated naturally, ovulation rate was significantly ($P<0.05$) greater in young does than in aged does (Table 2). However, this difference is insufficient to account for the much larger number of implantations in the young does. All the young does ovulated and had viable embryos, but two of the aged does failed to ovulate. Of the aged does that ovulated, 89% and 88% had detectable implantations but only 50% and 57% of those with implantation sites had viable embryos at 12 and 24 days *post coitum*, respectively.

Young does had more follicles ($P<0.005$) and heavier CL ($P<0.05$) at 24 days *post coitum*. Other characteristics of the young and aged does at 12 days compared with young and aged does 24 days *post coitum*, respectively, were: body weight, 2·1 versus 2·3 kg and 2·2 versus 2·5 kg; pituitary weight, 25·1 versus 49·3 mg and 30·8 versus 45·8 mg; adrenal weight, 226 versus 312 mg and

Table 2. Experiment 3. Age effect on reproductive efficiency* of rabbits

Criteria	12 *days* post coitum		24 *days* post coitum	
	Young	*Aged*	*Young*	*Aged*
Age (months)	9	53	8	58
No. of does	6	10	6	9
No. of does ovulating	6	9	6	8
No. of corpora lutea	8·0[a]	5·7[b]	7·3[a]	5·6[b]
No. of follicles	16·2[a]	9·0[b]	47·3[c]	16·3[a]
Total luteal tissue (mg)	102·0[a]	79·5[b]	120·7[a]	70·8[b]
Average CL wt (mg)	13·0[a]	12·4[a]	16·5[a]	10·7[b]
No. of does pregnant	6	8	6	7
Total no. of implantations/doe	6·2[a]	3·0[b]	6·8[a]	3·1[b]
No. of viable embryos/doe	5·7[a]	0·8[b]	6·0[a]	1·2[b]

* Mean response by group based on the total number of does in the treatment group.
[a,b,c] Means bearing different superscripts are significantly different ($P<0.05$).

237 versus 340 mg; and ovarian weight, 566 versus 953 mg and 652 versus 1025 mg.

In the cytogenetic studies, good growth with plentiful metaphases was obtained with tissue from fourteen embryos. Four embryos were from two young does and ten were from four aged does. Five embryos were obtained 12 days *post coitum* and nine were obtained 24 days *post coitum*. Cells from two degenerating embryos failed to grow. Twenty photographed metaphases were analysed for each of the fourteen embryos. The karyotypes of eleven female embryos had a normal 44,XX complement of chromosomes. There was only one 44,XY male embryo. A 45,XX cell line with an extra acrocentric chromosome was found in one 12-day embryo from an aged doe. One 24-day female embryo, from a young doe, had a modal number of forty-two chromosomes in 35% of the metaphases. The numbers of metaphases examined containing <40, 40, 41, 42, 43, 44, 45 and >45 chromosomes were 11, 13, 13, 24, 39, 153, 26 and 8, respectively. The sex ratio (7% male : 93% female) deviated significantly ($P<0.005$) from the expected ratio.

DISCUSSION

The level of progesterone used in the present studies was reported to maintain pregnancy in ovariectomized does (Wu & Allen, 1959). The doses of oestrogen chosen were found to maintain CL in X-ray irradiated rabbit ovaries (Keyes & Nalbandov, 1967). The amount of progesterone needed to maintain pregnancy in ovariectomized does can be reduced if a small amount of oestrogen is given (Asdell, 1964). Zarrow (1961) suggested that an optimal effect could be obtained by using a progesterone–oestrogen combination in a ratio of 750:1. However, supplemental progesterone and/or oestrogen failed to improve reproduction in aged rabbits. This failure to improve reproductive rates suggests that a deficiency in circulating progesterone was not the limiting factor causing embryonic death in aged rabbits. The initial decline in reproductive performance in mice has been attributed to failure of luteal support of the uterus (Harmon & Talbert, 1970). The CL in the aged does in Exp. 3 were smaller than those in the young does at 24 days *post coitum* but this was thought to be the result of the increased embryonic death and not the cause. The fact that subsequent studies indicated peripheral plasma progestin levels to be similar in young and old rabbits (Spilman, Larson, Concannon & Foote, 1972) supports this concept.

Pregnancy was blocked in all does given 4 μg oestradiol starting on Day 3 in Exp. 1. It is likely that ovum transport was affected (Greenwald, 1967). Rabbit ova normally enter the uterus 48 to 56 hr after ovulation and 90% are in the uterus by 64 hr (Harper, 1964). Pregnancy blockage was avoided in the second experiment by starting the treatment 2 days later on Day 5 and by using a lower dose.

The lower superovulatory response in the aged does was expected (Beatty, 1958; Maurer, Hunt & Foote, 1968). The ability of ova from young and aged does to develop equally *in vitro* agrees with previous studies and with the results of reciprocal transfers of ova from young and aged does to young and aged recipients (Maurer & Foote, 1971). However, increased arrested cleavage has been reported *in vivo* at 60 hr in old does (Adams, 1970).

Our findings that the decline in reproductive capacity was not primarily due to a reduction in ovulation rate agrees with reports in the hamster (Thorneycroft & Soderwall, 1969) and in some rodents (Talbert, 1968). Aged does at 12 and 24 days *post coitum* had 71·3% and 76·7% as many CL, 48·4% and 45·6% as many implantation sites and only 14·0% and 20·0% as many viable embryos, respectively, as did the young does (Table 2). The number of CL represented as implantation sites, 76·5% and 91·5%, in the young does at 12 and 24 days, respectively, was significantly ($P<0·025$) greater than the 48·8% and 59·2% observed in the aged does. At 12 and 24 days *post coitum*, 68·7% and 81·2%, respectively, of the CL were represented as viable embryos in young does compared to 12·6% and 19·5% in aged does ($P<0·005$).

A reduction in the number of total follicles and an increase in atretic follicles with increasing age has been noted in several species (Talbert, 1968). The larger number of follicles in young rabbits, particularly towards the end of the gestation period, has previously been reported (Adams, 1968) and may be

important in maintaining CL function through oestrogen production (Keyes & Nalbandov, 1967).

The total numbers of embryos and viable embryos were approximately the same at both 12 and 24 days *post coitum*, indicating that most of the embryonic deaths had occurred by 12 days *post coitum*. Since dead embryos that were not completely resorbed were found at 24 days *post coitum*, however, the process of embryo wastage was not complete by Day 12. Adams (1970) reported that prenatal loss in rabbits mainly occurred after Day 10. In aged mice, most of the embryonic deaths occurred in the early postimplantation period (Talbert, 1971).

Chromosomal analysis was only possible on tissue from viable embryos and no embryos were examined before 12 days *post coitum*. Most of the embryonic mortality occurred before 12 days *post coitum* in the present study and therefore any chromosomal anomalies associated with embryonic mortality would have gone undetected. Cytological analyses at earlier stages are indicated.

The poor reproductive efficiency in aged rabbits noted in this study could not be attributed entirely to ovulation rate, fertilization rate, ovum potential, CL function, circulating levels of progesterone and oestrogen, or to chromosomal anomalies of the fetuses. Some other defect in the uterine environment is presumably implicated.

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