Embryonic development in rabbits after insemination with spermatozoa stored at 37, 5 or -196°C for various periods

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Summary. Rabbit spermatozoa stored at 37°C for 5 hr, 5°C for 5 hr or 5 days, and -196°C for 5 hr, 5 days, or 1 year were used to artificially inseminate does induced to ovulate normal or excess numbers of eggs. Fertility and subsequent embryonic development were examined. Frozen spermatozoa stored for 5 hr fertilized fewer oocytes than those stored for 5 days or longer. Fewer embryos were recovered on Day 6 than on Day 2. Blastocysts were smaller when spermatozoa were stored for 5 days, were frozen, or were placed in superovulating does, but no statistically significant differences in the % of fetal survival were found in the groups of normally ovulating does. Embryonic wastage associated with storage of spermatozoa resulted from a reduced fertilization rate and/or increased embryonic mortality before implantation.

Introduction

Sperm fertility in all species is difficult to assess because defective as well as physiologically fit spermatozoa can initiate embryonic development; however, only oocytes fertilized by nondefective spermatozoa generally develop to the fetal stage. Previous studies have shown that in some species, ageing of gametes causes mutations and aberrant chromatin behaviour which increases embryonic mortality (Salisbury, 1965, 1967, 1968; Salisbury & Hart, 1970, 1975). Furthermore, ageing of rabbit spermatozoa in the male tract for up to 49 days results in mortality of preimplantation and post-implantation embryos; after 49 days the spermatozoa are unable to initiate development (Tesh & Glover, 1968). Spermatozoa aged in the female tract can initiate embryonic development for only 31 hr, but even during this period postimplantation mortality increases (Tesh, 1969). In contrast, rabbit spermatozoa aged *in vitro* for 8 days at 5°C increase in fertility for the first 2 days; thereafter, fertility declines (Stranzinger & Paufler, 1971). Successful low temperature (-196°C) preservation of rabbit spermatozoa resulting in viable young has been documented (O'Shea & Wales, 1969; Stranzinger, Maurer & Paufler, 1971; Maurer & Stranzinger, 1971), but effects of long periods of storage at -196°C on fertilization and subsequent embryo development have not been examined. Furthermore, factors contributed by the female are not usually considered when spermatozoa are examined for fertility.

This investigation was undertaken to determine the effects of storing rabbit spermatozoa at 37, 5, or -196°C for various periods on the fertilizing ability of spermatozoa in does ovulating normally and those induced to superovulate, and on the development of the embryo.

Materials and Methods

Two ejaculates were collected once weekly within 20 min from each of 12 fertile White Vienna rabbits with an artificial vagina. Semen volume and motility per ejaculate were recorded, and samples

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having 50% or more progressive motility were pooled. The semen was extended with three parts of a tris-egg yolk-dimethyl sulphoxide extender (Stranzinger *et al.*, 1971) and divided into 6 aliquots. Aliquot 1 was stored for 5 hr at 37°C before insemination. Aliquots 2 and 3 were stored for 5 hr and 5 days, respectively, at 5°C. Glycerol (6% of the extender volume) was added to Aliquots 4, 5 and 6, and the extended semen was frozen in pellets (0.15 ml) on solid CO₂ (Stranzinger *et al.*, 1971) and stored in liquid nitrogen for 5 hr, 5 days, and 1 year, respectively. Semen collection and storage were replicated eight times from November to June. Progressive motility estimates and live/dead staining (Beatty, 1957) were performed for all semen samples before insemination, except that a live/dead stain was not used for spermatozoa stored for 1 year.

Two hundred and ten mature female rabbits of the White Vienna breed were randomly divided into 30 equal groups. The does in 12 of the groups were induced to superovulate as described by Maurer & Foote (1971). Does in the remaining 18 groups were induced to ovulate by an i.v. injection of 2.5 mg pituitary LH (Armour Baldwin). Five hours after receiving the LH injection, all does were inseminated intravaginally with a semen volume of 0.4–0.6 ml so that each doe received an average of 20×10^6 (range $11\text{--}36 \times 10^6$) motile spermatozoa. At 30–48 hr after the LH injection one female of each group (representing sperm storage time, storage temperature, and superovulation or nonsuperovulation) was killed and 4- to 16-cell embryos were recovered to determine the fertilization rate. Blastocysts were recovered from the same groups at 6 days to estimate preimplantation mortality. To determine total embryonic wastage resulting from storing spermatozoa at different temperatures and for various periods, the remaining nonsuperovulating does and those that were pseudopregnant were subjected to laparotomy on Days 32 or 33 to determine the number of corpora lutea or corpora albicantia.

The recovered ova were counted and examined microscopically to determine whether cleavage had occurred. The Day-6 blastocysts were counted, classified as typical (spherical) or degenerate (partly or completely collapsed), and perpendicular diameters on the spherical blastocysts were measured using an ocular micrometer. The numbers of ovulation points or corpora lutea were recorded. The fertilization rate was calculated by dividing the number of embryos that had cleaved by the total number of ova recovered. The percentage of blastocysts and fetuses was calculated by dividing the number of each by the total number of corpora lutea. Blastocyst volume was calculated by $V = 4.19r^3$, in which the value of r was one-fourth of the sum of the two diameters.

Statistical evaluation. The doe was considered to be the experimental unit. The effects of sperm motility, % of spermatozoa unstained, and total number of spermatozoa were assessed by analysis of variance. The data, expressed as percentages and considered for comparable groups, were transformed using the arc-sine transformation and assessed by analysis of variance.

Results

Sperm motility and viability

The details of the spermatozoa used for the inseminations are presented in Table 1. Storage time affected motility and viability only at 5°C as spermatozoa stored for 5 days had lower motility ($F_{1,14} = 7.1$; $P < 0.05$) and viability ($F_{1,14} = 8.0$; $P < 0.05$) than those stored for 5 hr. Spermatozoa stored at 37°C for 5 hr had a significantly lower motility ($F_{1,14} = 6.1$; $P < 0.05$) and viability ($F_{1,14} = 9.0$; $P < 0.05$) than those stored at 5°C for 5 hr; similarly, spermatozoa stored at -196°C had significantly lower motility ($F_{2,45} = 7.0$; $P < 0.01$) and viability ($F_{2,37} = 20.5$; $P < 0.001$) than those stored at 37 or 5°C.

Effects on fertilization

Spermatozoa frozen for 5 hr were significantly less fertile ($F_{2,36} = 3.7$; $P < 0.05$) than those stored at 37 and 5°C (Table 2). A significant ($F_{1,48} = 5.4$; $P < 0.05$) temperature $\times$ time interaction between spermatozoa stored at -196 and at 5°C was found between 5 hr and 5 days of storage: the fertilization rate of frozen spermatozoa increased whereas that for spermatozoa stored at 5°C decreased with time. No other treatment effects were found.

Table 1. Mean $\pm$ S.E.M. motility, percentage unstained (alive), and total number of rabbit spermatozoa used to inseminate does after storage at different temperatures and for various intervals

Sperm storage		Vol. inseminated (ml)	% sperm. motile	% sperm. unstained (alive)	Total no. of motile sperm. inseminated ($\times 10^6$)
Temperature	Time				
20°C (unextended)	0 hr	—	64 ± 2	78 ± 2	—
20°C (extended)	0 hr	—	64 ± 1	82 ± 1	—
37°C	5 hr	0.4	57 ± 3	67 ± 4	21.0 ± 1.0
5°C	5 hr	0.4	64 ± 1	81 ± 2	24.0 ± 1.1
	5 day	0.6	37 ± 3	72 ± 3	20.4 ± 0.9
	1 year	0.6	43 ± 2	—	24.3 ± 0.9
-196°C	5 hr	0.6	44 ± 2	57 ± 2	23.7 ± 1.1
	5 day	0.6	45 ± 1	57 ± 3	25.3 ± 1.3
	1 year	0.6	43 ± 2	—	24.3 ± 0.9

Table 2. Fertilization rates (mean $\pm$ S.E.M.) for rabbit spermatozoa stored at several temperatures and times and inseminated into does (7/group) ovulating (a) normal or (b) excess numbers of eggs

Sperm storage		Mean $\pm$ S.E.M. no. of CL	Embryos recovered (%)	Cleaved embryos (%)	1-cell and/or degenerating embryos†
Temperature	Time				
<i>(a) Nonsuperovulating does</i>					
37°C	5 hr	8.6 $\pm$ 0.8	99 $\pm$ 3	81 $\pm$ 14	19
5°C	5 hr	11.1 $\pm$ 0.9	87 $\pm$ 10	87 $\pm$ 13	13
	5 day	8.5 $\pm$ 1.2	83 $\pm$ 7	44 $\pm$ 17	56
-196°C	5 hr	8.9 $\pm$ 1.2	96 $\pm$ 4	45 $\pm$ 12	55
	5 day	10.3 $\pm$ 0.8	92 $\pm$ 7	80 $\pm$ 14	20
	1 year	8.9 $\pm$ 1.5	94 $\pm$ 3	69 $\pm$ 16	31
<i>(b) Superovulating does</i>					
37°C	5 hr	34.4 $\pm$ 5.2	97 $\pm$ 4	70 $\pm$ 9	30
5°C	5 hr	41.9 $\pm$ 12.7	88 $\pm$ 9	61 $\pm$ 13	39
	5 day	27.9 $\pm$ 6.0	68 $\pm$ 10	43 $\pm$ 20	57
-196°C	5 hr	44.4 $\pm$ 6.6	72 $\pm$ 10	42 $\pm$ 14	58
	5 day	25.6 $\pm$ 5.3	80 $\pm$ 14	55 $\pm$ 20	45
	1 year	34.3 $\pm$ 4.3	94 $\pm$ 10	68 $\pm$ 13	32
<i>Summary of main effects*</i>					
<i>(a)</i>		9.4 $\pm$ 1.1	92 $\pm$ 6	68 $\pm$ 14	32
<i>(b)</i>		34.8 $\pm$ 6.7	83 $\pm$ 9	56 $\pm$ 15	44
37°C		21.5 $\pm$ 3.0	98 $\pm$ 3	75 $\pm$ 12	25
5°C		22.4 $\pm$ 5.2	82 $\pm$ 9	59 $\pm$ 16	41
-196°C		22.1 $\pm$ 3.3	88 $\pm$ 8	60 $\pm$ 15	40
5 hr		24.9 $\pm$ 4.6	90 $\pm$ 7	64 $\pm$ 13	36
5 day		18.1 $\pm$ 3.3	81 $\pm$ 9	55 $\pm$ 12	45
1 year		21.6 $\pm$ 2.9	94 $\pm$ 6	68 $\pm$ 14	32

* Includes temperature $\times$ time interaction of 5 and -196°C and 5 hr and 5 days.

† Standard error is the same as for cleaved embryos.

Effects on blastocyst development

Significant ($F_{1,72} = 35$; $P < 0.001$) embryo loss occurred between Day 2 (cleavage stage; Table 2) and Day 6 (blastocyst stage; Table 3). Sperm storage time, storage temperature, and type of female inseminated did not significantly ($P > 0.10$) affect the percentage of blastocysts recovered or their morphological appearance.

Blastocyst volumes (Table 3) were significantly affected by superovulation ($F_{1,415} = 23.7$; $P < 0.001$), storage time ($F_{2,295} = 11.4$; $P < 0.001$), and storage temperature ($F_{2,415} = 6.1$; $P < 0.05$). Blastocysts from nonsuperovulating does were larger than those from superovulating females except for the spermatozoa stored for 5 days at 5 or -196°C . Smaller blastocysts resulted when the spermatozoa were stored for 5 days at 5 or -196°C regardless of uterine environment. Blastocysts which resulted from spermatozoa stored at -196°C for 1 year were equal in volume to those from spermatozoa stored for 5 hr. Blastocysts which developed from spermatozoa stored at 37 or -196°C for 5 hr were smaller than those resulting from spermatozoa stored at 5°C for 5 hr.

Table 3. Developmental rate and volume (mean $\pm$ S.E.M.) of Day-6 blastocysts which resulted from the insemination of superovulating and nonsuperovulating does (7/group) with semen stored at several temperatures for various times

Sperm storage		Mean $\pm$ S.E.M. no. of CL	Blastocysts recovered (%)	Typical blastocysts (%)	Blastocyst volume (μ l)
Temperature	Time				
(a) <i>Nonsuperovulating does</i>					
37°C	5 hr	11.0 $\pm$ 1.1	68 $\pm$ 15	81 $\pm$ 10	23.5 $\pm$ 2.6
5°C	5 hr	10.6 $\pm$ 0.9	62 $\pm$ 16	59 $\pm$ 19	26.2 $\pm$ 3.0
	5 day	9.1 $\pm$ 0.6	44 $\pm$ 15	69 $\pm$ 19	9.3 $\pm$ 1.4
-196°C	5 hr	10.6 $\pm$ 1.0	60 $\pm$ 15	76 $\pm$ 17	24.5 $\pm$ 3.2
	5 day	9.3 $\pm$ 0.4	46 $\pm$ 16	69 $\pm$ 18	9.9 $\pm$ 1.6
	1 year	9.3 $\pm$ 0.8	32 $\pm$ 13	67 $\pm$ 21	29.2 $\pm$ 3.4
(b) <i>Superovulating does</i>					
37°C	5 hr	55.4 $\pm$ 9.5	57 $\pm$ 11	60 $\pm$ 14	12.7 $\pm$ 1.3
5°C	5 hr	44.1 $\pm$ 4.7	58 $\pm$ 13	60 $\pm$ 16	20.4 $\pm$ 1.2
	5 day	41.6 $\pm$ 5.3	51 $\pm$ 11	36 $\pm$ 14	8.1 $\pm$ 0.5
-196°C	5 hr	39.4 $\pm$ 7.4	44 $\pm$ 12	41 $\pm$ 16	13.9 $\pm$ 2.0
	5 day	38.6 $\pm$ 6.1	36 $\pm$ 12	54 $\pm$ 18	9.4 $\pm$ 1.2
	1 year	40.7 $\pm$ 5.9	59 $\pm$ 14	56 $\pm$ 14	13.1 $\pm$ 0.8
<i>Summary of main effects</i>					
(a)		6.0 $\pm$ 0.8	52 $\pm$ 15	69 $\pm$ 17	20.9 $\pm$ 1.3
(b)		43.3 $\pm$ 6.5	51 $\pm$ 12	51 $\pm$ 15	13.8 $\pm$ 0.5
37°C		33.2 $\pm$ 5.3	62 $\pm$ 13	70 $\pm$ 12	15.8 $\pm$ 1.2
5°C		26.4 $\pm$ 2.9	54 $\pm$ 14	54 $\pm$ 11	16.5 $\pm$ 1.1
196°C		24.6 $\pm$ 3.6	46 $\pm$ 13	60 $\pm$ 17	14.5 $\pm$ 0.8
	5 hr	28.5 $\pm$ 4.1	58 $\pm$ 14	63 $\pm$ 15	18.5 $\pm$ 0.7
	5 day	24.6 $\pm$ 3.1	44 $\pm$ 13	55 $\pm$ 17	8.9 $\pm$ 0.5
	1 year	25.0 $\pm$ 3.4	45 $\pm$ 13	61 $\pm$ 17	15.7 $\pm$ 1.0

Effects on fetal survival

There were no significant differences ($P > 0.10$) among groups regarding survival of conceptuses to term (Table 4), although does which were inseminated with frozen spermatozoa or spermatozoa stored for ≥ 5 days tended to have fewer young. The average litter size for does inseminated with spermatozoa stored at 37, 5, and -196°C was 6.0, 6.2, and 3.4, respectively; while, spermatozoa stored for 5 hr, 5 days or 1 year produced litter sizes of 5.6, 4.9, and 1.9, respectively.

Table 4. Embryonic survival to term in nonsuperovulating does (7/group) inseminated with spermatozoa stored at several temperatures for various times

Sperm storage		Mean $\pm$ S.E.M. no. of CL	Mean $\pm$ S.E.M. % of fetuses
Temperature	Time		
37°C	5 hr	9.4 $\pm$ 1.0	64 $\pm$ 17
5°C	5 hr	8.4 $\pm$ 1.0	68 $\pm$ 13
	5 day	10.7 $\pm$ 1.0	61 $\pm$ 12
-196°C	5 hr	9.9 $\pm$ 0.8	52 $\pm$ 17
	5 day	9.0 $\pm$ 0.6	39 $\pm$ 17
	1 year	7.6 $\pm$ 0.8	24 $\pm$ 16
<i>Summary of main effects</i>			
37°C		9.4 $\pm$ 1.0	64 $\pm$ 17
5°C		9.6 $\pm$ 1.0	64 $\pm$ 12
-196°C		8.8 $\pm$ 0.7	39 $\pm$ 17
	5 hr	9.2 $\pm$ 0.9	61 $\pm$ 16
	5 day	9.8 $\pm$ 0.8	50 $\pm$ 15
	1 year	7.6 $\pm$ 0.8	24 $\pm$ 16

Discussion

Fertilization failure and embryonic mortality before implantation accounted for most of the embryonic wastage associated with freezing and/or storing spermatozoa. Frozen spermatozoa stored for 5 hr fertilized proportionally fewer oocytes than did nonfrozen spermatozoa; however, storage of spermatozoa for 5 days or longer resulted in improved fertilizing ability. Although no reduction in sperm motility was found, fewer spermatozoa were seen in the mucin coat and zona pellucida of embryos recovered from does inseminated with spermatozoa frozen for 5 hr. Murdoch & O'Shea (1973) previously reported that fewer supplementary spermatozoa were associated with ova recovered from does inseminated with frozen spermatozoa than those from does inseminated with fresh semen. Freezing and thawing of mammalian cells have been reported to damage mitochondria, ribosomes, lysosomes, and the endoplasmic reticulum (Tappel, 1966; Quinn & White, 1966; Quinn, White & Cleland, 1969). The loss and return of the fertilizing ability of frozen spermatozoa might have been due to a time-related repair of damage caused by the freezing process and/or ice crystal formation changes with time causing less cellular damage during thawing (Rapatz & Luyet, 1961).

Storage of spermatozoa at 5°C for 5 days resulted in a reduction in fertility. This reduced fertility may have resulted from loss of enzymes, since protein loss and lysosomal release were reported for cooled spermatozoa and other cells (Tappel, 1966; Quinn *et al.*, 1969). Reduced fertility of frozen spermatozoa has also been reported following loss of glutamic oxaloacetic transaminase, which was used to monitor cellular integrity (Pace & Graham, 1971). In direct comparison, aged rabbit spermatozoa fertilized fewer oocytes than freshly ejaculated spermatozoa (Roche, Dziuk & Lodge, 1968). Freshly ejaculated spermatozoa may have a higher proteolytic enzyme activity which permits faster penetration of the zona pellucida. Further investigation is needed to determine cause and effect relationships.

Some (37%) of the embryonic loss occurring before the blastocyst stage resulted from the reduced fertilization rate, but additional embryonic mortality (12%) was probably related to defective spermatozoa. Comparison of the percentage of blastocysts classified as 'typical' with fetal development indicates that many of the blastocysts classified as being abnormal developed into fetuses and little postimplantation embryonic mortality can have occurred.

The significant decrease in blastocyst volume resulting from insemination of spermatozoa stored for 5 days at 5 or -196°C suggests possible damage to the genetic information system. However, no significant decrease in fetal survival occurred, and the decrease in volume may have been only a transient physical change or a temporary delay in development. A stoppage in growth of frog gastrulae is known to result from fertilization by aged spermatozoa (Hart & Salisbury, 1967) and can be corrected with RNA extract from gastrulae developed from oocytes fertilized by fresh spermatozoa. Furthermore, DNA loss was reported in cooled and frozen ram spermatozoa after a 6-hr incubation at 37°C , indicating cellular damage and lysosomal activation (Quinn *et al.*, 1969). The significant decrease in volume of blastocysts recovered from the superovulating does could be due to a nutritional deficiency. Uterine proteins have been reported to increase blastocyst volume (El-Banna & Daniel, 1972; Maurer & Beier, 1976), and an altered pattern of protein secretion in uteri from superovulating rabbits found (Kirchner, 1969). This altered pattern is consistent with the delayed secretion pattern found in oestradiol-treated rabbits (Beier, Kühnel & Petry, 1971).

Progressively fewer fetuses resulted from frozen spermatozoa stored for periods up to 1 year, but the differences were not statistically significant. The trend of increased embryonic wastage with storage time and temperature found in this study was similar to that previously reported (Stranzinger & Paufler, 1971; O'Shea & Wales, 1969; First, Stratman & Casida, 1963; Salisbury & Flerchinger, 1961). It is clear that some unphysiologically fit spermatozoa can at least initiate conception by fertilizing oocytes.

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