EFFECT OF STORAGE OF RABBIT SPERMATOZOA AT −79°C ON THEIR SUBSEQUENT TRANSPORT AND FERTILITY IN THE RABBIT DOE

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Abstract

Twenty-four does were inseminated with either 3×10^6 or 30×10^6 motile spermatozoa, either recently collected or after storage at −79°C. The females were killed 18 hr after insemination, and ova and spermatozoa recovered from their reproductive tracts. Fewer spermatozoa were recovered from does inseminated with 3×10^6 than with 30×10^6 motile sperm. There was no significant difference between fresh and stored semen in numbers of spermatozoa recovered.

The number of sperm in the oviducts was small; mean numbers were 650 and 166 per oviduct from inseminates of 30×10^6 and 3×10^6 cells respectively. Despite this, 85% of the ova cleaved on culture for 23 hr at 37°C, with again no difference between fresh and stored semen.

Measurement of the number of supplementary spermatozoa showed that fewer sperm were associated with ova recovered from does inseminated with stored cells than with those from does inseminated with fresh semen.

In the rabbit, freezing and storage of spermatozoa had no effect on their subsequent transport in the female genital tract.

I. INTRODUCTION

Reconstituted skim milk powder has been used as the basis of a diluent for frozen storage of rabbit spermatozoa (O'Shea and Wales 1969). With this technique the percentage of spermatozoa that were motile was lower than that in recently collected semen, and, although reasonable fertility was obtained, litter size was smaller following insemination with stored cells.

In the rabbit, insemination with comparatively few spermatozoa results in high fertility (Wales *et al.* 1965; Wales and O'Shea 1968). It was postulated that any defects in fertilization due to frozen storage of the spermatozoa might be more readily seen when inseminating with small numbers of cells.

The present investigation was a study of the effect of freezing rabbit spermatozoa on their subsequent transport through the female tract, and on their fertility, including a comparison of the effects of inseminating with 3×10^6 or 30×10^6 motile spermatozoa.

One problem in contrasting fresh and frozen spermatozoa is the higher percentage of dead cells in the stored semen. When equal numbers of motile spermatozoa are inseminated the total numbers will be greater for the stored sample. However, in the rabbit the cervix acts as a barrier ensuring that few dead cells penetrate to the

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uterus (Noyes *et al.* 1958; Bedford 1971). For this reason it was felt that the vaginal spermatozoa may form a different population and they were not considered when comparing fresh and frozen semen.

II. Materials and Methods

Semen was collected with an artificial vagina, and only those ejaculates with good initial motility and density were used. For insemination with fresh spermatozoa an aliquot of semen was diluted with a washing diluent consisting of 20 mM sodium phosphate buffer (pH 7), 5 mM potassium chloride, 2 mM magnesium chloride, 10 mM fructose, 114 mM sodium chloride, and penicillin (30 mg/100 ml). The remainder of the spermatozoa were diluted, frozen using the final cooling and freezing solutions described by O'Shea and Wales (1969), and stored overnight in solid carbon dioxide. Six-month-old white virgin does, which had been caged individually for at least 4 weeks, were injected intravenously with human chorionic gonadotrophin (50 i.u.) at the time of insemination. One vial of the frozen semen was thawed, centrifuged at 1200 *g* for 10 min, and the cells washed with the diluent described above. The washed cells were suspended in diluent and incubated at 37°C for 10 min before an estimate of the percentage of motile spermatozoa was made. The rest of the stored semen was then thawed, diluted or concentrated as required, and the spermatozoa deposited in the vagina with a plastic insemination pipette.

Following insemination the does were caged separately and killed by intravenous injection of sodium pentobarbitone at the times given for each experiment. Ligatures were applied to the tract *in situ* to divide it into fallopian tubes, uteri, cervices, and vagina. Particular care was taken to leave the external os of the cervix with the vagina.

To recover both spermatozoa and ova the oviducts were flushed from the uterine end into embryological watch glasses, previously coated with silicone to reduce sperm losses (Braden 1953), with a phosphate-buffered medium (pH 7·35). This consisted of Brinster's BMOC-2 medium (Brinster 1969) with the potassium dihydrogen orthophosphate and sodium bicarbonate replaced with 20 mM sodium phosphate buffer. The solution was forced through each oviduct as two 0·5-ml flushings, with 1 ml of air being injected through the tube between the two fluid washings (Mattner and Braden 1963). Ova were recovered under a binocular microscope.

The uteri were flushed with physiological saline into plastic tubes in the same manner as the oviducts. Each cervix was flushed from the cranial end with 1 ml of physiological saline and the upper vagina was washed with 3 ml of saline. Washings containing the spermatozoa were stored at −15°C for subsequent direct counting of cells (Lightfoot and Salamon 1970) by means of Fuchs–Rosenthal haemocytometers of depth 0·2 mm. Centrifuging of dilute suspensions of spermatozoa in silicone-coated, but necessarily narrow tubes, in an attempt at concentrating the suspensions (Braden 1953) resulted in high and variable losses of spermatozoa, and was not used.

Following recovery from the oviduct, ova were taken up in a small drop of fluid, placed on a cavity slide, and examined for supplementary spermatozoa. A count was made of any spermatozoa that could be seen with gentle rolling of the ova. Where the zona pellucida and perivitelline space could not be seen clearly, the ova were excluded from the comparison of effects of treatments on the number of supplementary spermatozoa. The number of spermatozoa seen was called an index, as it was not an attempt to measure the total number associated with an ovum. Ova were then transferred to a small drop of BMOC-2 culture medium under light paraffin and incubated for 23 hr at 37°C (Brinster 1969). Following culture the ova were examined for cleavage.

The statistical significance of the effects of the treatments on the numbers of spermatozoa recovered were determined by analysis of variance, after transformation of the data according to the formula $\log_{10}$ (count +2). The data for the index of supplementary spermatozoa were analysed using the method of unweighted means for disproportionate subclass numbers (Snedecor 1956).

III. Results

(*a*) *Spermatozoa in Tract at 6, 12, and 24 hr after Insemination*

Because there were no reports in the literature of the numbers of spermatozoa recovered from the doe after insemination with frozen semen, a preliminary experiment

was carried out. Does were inseminated with either 1×10^6 or 10×10^6 motile thawed spermatozoa and killed at 6, 12, or 24 hr after insemination. There were no significant treatment effects although trends towards higher recovery of cells at 12 hr and following insemination with 10×10^6 spermatozoa were present.

This result meant that spermatozoa could be recovered from the female tract when as few as 1×10^6 motile stored cells had been inseminated.

TABLE 1

EFFECT OF DILUENT AND CONCENTRATION OF SPERMATOZOA ON THE TRANSPORT OF 10×10^6 MOTILE THAWED SPERMATOZOA IN THE RABBIT DOE

Values are means of duplicates of three ejaculates

Treatment	Inseminate concentration (spermatozoa per ml)	Location	Spermatozoa recovered	
			Arithmetic count †	Log (count +2)‡
Concentrated and resuspended in phosphate–saline	5×10^6	Oviducts	44 (31–62)	1·65
		Uteri	453 (94–1120)	2·51
		Cervices	385 (0–810)	2·12
		Vagina	437 (0–1500)	2·00
Concentrated	50×10^6	Oviducts	91 (0–156)	1·74
		Uteri	1841(156–8520)	2·83
		Cervices	281(125–500)	2·39
		Vagina	1357 (94–3560)	2·75
Concentrated and resuspended in milk diluent	5×10^6	Oviducts	26 (0–94)	0·83
		Uteri	187 (0–655)	1·56
		Cervices	250 (0–690)	1·73
		Vagina	452 (0–1590)	1·83

Summary of the analysis of variance of the data after transformation to $\log_{10}$ (count +2)

Source of variation	D.F.	Variance ratio
A. Treatment		
Phosphate saline *v.* milk	1	7·84**
Sperm concentration	1	13·07***
B. Location in genital tract		
Linear	1	7·99**
Quadratic	1	5·24*
Cubic	1	3·61
C. Differences between ejaculates	2	6·87**
Interactions:		
A×C	4	4·72**
Others	24	0·32
Between-does within-treatment error variance	36	*0·5184*

*$P<0·05$. **$P<0·01$. ***$P<0·001$.

†Mean, with range in parentheses. ‡Mean.

(*b*) *Effect of Diluent on Sperm Transport*

A possible confounding factor in comparing recently collected semen and frozen spermatozoa was any effect of the different diluents in which the cells were

suspended. Therefore, the effect on sperm transport of the presence at insemination of the milk diluents used in freezing rabbit semen was investigated by inseminating does with frozen semen which had undergone one of three treatments. The thawed semen was centrifuged at 1200 *g* for 10 min. and the supernatant removed. One portion of the residue was diluted to 5×10^6 motile spermatozoa per millilitre with milk diluent (one part washing diluent, four parts cooling diluent, and five parts freezing diluent), another portion with washing diluent, and a third was diluted to 50×10^6 motile spermatozoa per millilitre with washing diluent.

For each of three ejaculates, two does were inseminated with 10×10^6 motile spermatozoa from one of the above three treatments and killed 18 hr after insemination. Spermatozoa and ova were recovered from each of the 18 does used.

Table 1 shows the mean numbers of spermatozoa recovered and a summary of the analysis of variance. There was a significant interaction between the diluent and ejaculate effects. When the mean square of this interaction was used as error the effects of diluent and of sperm concentration were not significant. There were fewer cells present in the oviducts than in the other parts of the tract.

As only small numbers of supplementary spermatozoa were noticed in the ova, a more thorough examination of their numbers was included in the next experiment.

(*c*) *Effects of Freezing on Sperm Transport*

To compare recently ejaculated and frozen semen, two does per group were inseminated with either 3×10^6 or 30×10^6 motile fresh or thawed spermatozoa and killed 18 hr later. Three different collections of semen were used to give a total of 24 does inseminated. Mean numbers of spermatozoa recovered from each part of the female tract, together with a summary of the analysis of variance are given in Table 2.

Significantly fewer spermatozoa were recovered when does were inseminated with 3×10^6 than with 30×10^6 motile spermatozoa, although the difference varied in extent between ejaculates. There appeared to be a decreased number recovered with frozen spermatozoa but this was not significant when the mean square of the ejaculate interaction was used as error in the analysis. With one ejaculate frozen semen resulted in a higher number of spermatozoa recovered whereas the other two ejaculates gave the opposite result. There were more spermatozoa in the uterus than in the other two sites.

Ova recovered from does inseminated with fresh semen had a significantly higher index of supplementary spermatozoa than those from does inseminated with frozen cells ($P < 0{\cdot}01$). When 30×10^6 motile spermatozoa were inseminated the mean numbers of spermatozoa seen per ovum were 2·0 and 0·69 for ova from does inseminated with fresh and frozen semen respectively. After insemination with 3×10^6 spermatozoa the corresponding values were 1·85 and 0·25. There was no effect of number of spermatozoa inseminated.

Despite the low numbers of sperm found in the oviducts high cleavage rates were obtained. Of the 81 ova collected from does inseminated with fresh spermatozoa and cultured, 71 cleaved, while with frozen semen 56 of 68 ova cleaved during culture. There were no effects on fertility of number of sperm inseminated or from frozen storage of semen.

IV. Discussion

Although the motility of individual spermatozoa was decreased by storage, this had no effect on their distribution through the female genital tract. In the ewe, Lightfoot and Salamon (1970) concluded that the low fertilization rate following

Table 2

RECOVERY OF SPERMATOZOA FROM THE GENITAL TRACT OF THE RABBIT DOE 18 HR AFTER INSEMINATION WITH FRESH OR FROZEN SEMEN

Values are means of duplicates of three ejaculates

Semen	Motile spermatozoa in inseminate	Location	Spermatozoa recovered: Arithmetic count †	Spermatozoa recovered: Log_{10} (count +2)‡
Fresh	30×10^6	Oviducts	1224 (530–2500)	3·02
		Uteri	13538(1120–27170)	3·95
		Cervices	4061 (620–11390)	3·46
	3×10^6	Oviducts	543 (0–1190)	2·26
		Uteri	8447 (0–34940)	2·90
		Cervices	2741 (0–11390)	2·64
Frozen	30×10^6	Oviducts	1374 (0–6290)	2·33
		Uteri	5034 (60–14660)	3·16
		Cervices	1113 (0–2750)	2·55
	3×10^6	Oviducts	120 (0–500)	1·48
		Uteri	1040 (0–2280)	2·39
		Cervices	593 (0–1500)	2·00

Summary of the analysis of variance of the data after transformation to log_{10} (count +2)

Source of variation	D.F.	Variance ratio
A. Effect of freezing	1	12·17**
B. Number of spermatozoa inseminated	1	15·14***
C. Location in genital tract		
Linear	1	2·41
Quadratic	1	8·45**
D. Differences between ejaculates	2	14·40***
Interactions:		
A×D	2	7·66**
Others	27	0·48
Between-does within-treatment error variance	36	*0·7581*

*$P<0·05$. **$P<0·01$. ***$P<0·001$.

† Mean, with range in parentheses. ‡Mean.

insemination with pellet frozen ram semen was due to the failure of the normal pattern of transport leading to low numbers of motile spermatozoa in the genital tract. Another difference between these species is the absence of effect of sperm concentration on fertility in the rabbit (Wales *et al.* 1965; Wales and O'Shea 1968),

compared with the depressing effect of sperm dilution on fertility in the ewe (Lightfoot and Salamon 1970; Salamon and Lightfoot 1970). This is paralleled by the smaller cervical population seen in ewes inseminated with more dilute sperm suspensions (Lightfoot and Salamon 1970), as contrasted to the doe, where sperm transport is not altered by varying sperm concentration in the inseminate.

The distribution of spermatozoa along the tract differs from that previously reported (El-Banna and Hafez 1970). The greater number in the uterus than in the cervix may be due to the technique used for sperm recovery or to the fact that the does were inseminated with small numbers of spermatozoa.

As shown in the present report and by Chang (1951), fertility can still be high in the rabbit with very few spermatozoa in the fallopian tube. The number of spermatozoa recovered from an oviduct does not necessarily reflect the total number transported to that site as they pass on into the peritoneal cavity (Mattner and Braden 1963; Thibault 1972).

A greater "flow rate" of fresh as compared to stored spermatozoa may be a cause of the different numbers of supplementary spermatozoa. Motile sperm were observed in the vicinity of ova from does inseminated with fresh semen but not when frozen semen was used. This greater motility may also mean a greater chance of sperm–egg collision. An alternative explanation is the greater degree of motility exhibited by individual fresh spermatozoa compared to motile thawed cells, as its own motility propels the spermatozoon through the cumulus oophorus and zona pellucida (Austin 1969). Reduced longevity of frozen spermatozoa, as suggested by Mattner *et al.* (1969) for sheep, was not seen in the present experiments. However, it is possible that the frozen spermatozoa can remain intact long enough to fertilize eggs but then rapidly deteriorate so that contact of further sperm with the fertilized ova is reduced.

The numbers of supplementary sperm seen in the present experiment, even with fresh sperm, are much lower than normal (Braden *et al.* 1954; Bedford 1966; Overstreet 1970). This is caused firstly by the method of counting the spermatozoa, so they correspond more nearly to the perivitelline count of Bedford (1971). Secondly the small number of accessory sperm must also be due to the low number of spermatozoa in the oviduct. That even some supplementary spermatozoa are present illuminates two points. When large numbers of supplementary sperm are present, excess numbers have reached the fallopian tube. Conversely their occurrence, however few, following insemination with 3×10^6 motile thawed spermatozoa indicates that the goal of looking at insemination with minimal numbers of thawed spermatozoa was not reached. Unfortunately if smaller numbers were inseminated, counting of spermatozoa in the oviduct would be very difficult.

From the results it is suggested that while the degree of motility of spermatozoa is important in penetration of the ovum, it is of little moment in sperm transport through most of the female tract. The frozen storage of rabbit semen has no effect on sperm transport in the female reproductive system.

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VI. REFERENCES

AUSTIN, C. R. (1969).—In "Reproduction in Domestic Animals". (Eds. H. H. Cole and P. T. Cupps.) 2nd Ed. pp. 364–5. (Academic Press, Inc.: New York.)
BEDFORD, J. M. (1966).—*J. exp. Zool.* **163,** 319.
BEDFORD, J. M. (1971).—*J. Reprod. Fert.* **25,** 211.
BRADEN, A. W. H. (1953).—*Aust. J. biol. Sci.* **6,** 693.
BRADEN, A. W. H., AUSTIN, C. R., and DAVID, H. A. (1954).—*Aust. J. biol. Sci.* **7,** 391.
BRINSTER, R. L. (1969).—In "The Mammalian Oviduct". (Eds. E. S. E. Hafez and J.R. Blandau.) pp. 419–44. (University of Chicago Press : Chicago.)
CHANG, M. C. (1951).—*Annali Ostet. Ginec.* **73,** 918.
EL-BANNA, A. A., and HAFEZ, E. S. E. (1970).—*Fert. Steril.* **21,** 534.
LIGHTFOOT, R. J., and SALAMON, S. (1970).—*J. Reprod. Fert.* **22,** 385.
MATTNER, P. E., and BRADEN, A. W. H. (1963).—*Aust. J. biol. Sci.* **16,** 473.
MATTNER, P. E., ENTWISTLE, K. W., and MARTIN, I. C. A. (1969).—*Aust. J. biol. Sci.* **22,** 181.
NOYES, R. W., ADAMS, C. E., and WALTON, A. (1958).—*Fert. Steril.* **9,** 288.
O'SHEA, T., and WALES, R. G. (1969).—*Aust. J. biol. Sci.* **22,** 709.
OVERSTREET, J. W. (1970).—*J. Reprod. Fert.* **21,** 279.
SALAMON, S., and LIGHTFOOT, R. J. (1970).—*J. Reprod. Fert.* **22,** 409.
SNEDECOR, G. W. (1956).—"Statistical Methods." 5th Ed. (Iowa State College Press: Ames.)
THIBAULT, C. (1972).—*Int. J. Fert.* **17,** 1.
WALES, R. G., MARTIN, L., and O'SHEA, T. (1965).—*J. Reprod. Fert.* **10,** 69.
WALES, R. G. and O'SHEA, T. (1968).—*Aust. J. biol. Sci.* **21,** 181.