J. Reprod. Fert. (1968) **15**, 87–92

EMBRYONIC SURVIVAL SUBSEQUENT TO CULTURE OF RABBIT SPERMATOZOA AT 38° AND 40° C

P. J. BURFENING AND L. C. ULBERG

Department of Animal Science, North Carolina State University, Raleigh, North Carolina, U.S.A.

(*Received* 18*th November* 1966, *revised* 20*th January* 1967)

Summary. Following a preliminary experiment, split ejaculates of rabbit semen, incubated for 3 hr at 38° or 40° C, were examined and inseminated separately into the uterine horns of rabbits mated 4 hr previously to vasectomized males. Eggs were recovered and examined for evidence of fertilization 30 hr *post coitum*. Following return of the eggs to the oviduct their survival was estimated by counting the implantation sites at 9 days *post coitum*. There was no evidence of any effect of treatment temperature on fertilizing capacity of semen, but embryonic survival rate was higher (75%) in horns inseminated with semen incubated at 38° C than in those inseminated with semen incubated at 40° C (53%). Neither fertilization rate nor embryonic survival rate was significantly correlated with semen quality.

INTRODUCTION

Recently, Bishop (1964) pointed out that most investigators consider the female responsible for embryonic death. There are, however, reports which indicate that spermatozoa can be influenced so that the fertilization rate is not affected but the rate of embryonic death is increased. Examples of such treatments are X-rays (Amoroso & Parkes, 1947; Dott, Ishida & Chang, 1966; Edwards, 1957; Rowson & Dott, 1964), various alkylating agents (Jackson, Fox & Craig, 1961), ageing of spermatozoa at 4° C, *in vitro* (Salisbury, 1965) and elevated temperature (Howarth, Alliston & Ulberg, 1965). Young (1929) subjected guinea-pig spermatozoa, retained in the epididymis, to elevated temperatures and found an increase in both the number of stillbirths and abortions. Walton (1930), studying the effects of temperature and length of storage period on rabbit spermatozoa, observed a decrease in litter size in females inseminated with spermatozoa stored at 40° C compared with females inseminated with spermatozoa stored at 37° C. He attributed these differences to increased fertilization failure, but had no measure of fertilization or rate of embryonic survival. Howarth *et al.* (1965) found a significant decrease in the rate of embryonic survival of eggs fertilized with spermatozoa capacitated in does maintained at an elevated ambient temperature. However, the direct effect of temperature on the spermatozoa could not be separated from a possible maternal effect. This separation is achieved in the present study.

MATERIALS AND METHODS

A breeding colony of New Zealand White rabbits was maintained at about 21° C, 65% relative humidity and 12 hr of daily illumination. The study was divided into two experiments.

In the first experiment a series of ejaculates was collected from a male. Each ejaculate was diluted to 2 ml with modified Krebs–Ringer phosphate solution (Lardy & Phillips, 1943). The diluted semen was then evaluated by a haemocytometer for concentration and scored for percentage motility (0 to 100), progressive motility (1 to 5) and percentage of live spermatozoa by the eosin–nigrosin technique (Beatty, 1958). The diluted ejaculate was then split; each half was placed in a 15×45 mm glass vial and sealed with a rubber stopper. One vial of semen was incubated at 38° C and the other at 40° C for 3 hr to form two treatment groups. These incubation temperatures are comparable to rectal temperatures of does maintained at normal (21° C) and elevated (32° C) air temperatures (Alliston, Howarth & Ulberg, 1965). At the end of the incubation period the spermatozoa were re-evaluated for percentage motility, progressive motility and percentage live. Before insemination a further dilution to approximately $1{\cdot}0 \times 10^7$ cells/ml was made.

Females, mated 4 hr previously to a vasectomized male, were subjected to mid-ventral laparotomy and inseminated with 0·1 ml of each treated sample, using one treatment to each uterine horn. At 30 hr *post coitum* (p.c.) another mid-ventral laparotomy was performed, the reproductive tract exposed and the number of ovulation sites on each ovary was determined. The infundibular end of the oviduct was intubated with a polyethylene tube and the eggs were flushed into a watch glass with modified Krebs–Ringer phosphate solution. The eggs were examined through a stereomicroscope at $10 \times$ magnification for evidence of cleavage; all cleaved eggs were considered fertilized and re-deposited into their original oviduct. At 12 days p.c. the female was autopsied and the number of implantation sites was determined, by visual observation, as a swelling of the uterine horn. Each implantation site was dissected and all embryos with a visible heart beat were considered to be normal. A third group, run concurrently as controls, was inseminated with diluted fresh semen.

Because of the results from the first experiment in which only one male was used, it was decided to replicate the experiment in such a way that effects of different males, ejaculates and females could be determined. The same three treatment groups (control, 38° C and 40° C) were used but with four ejaculates from each of four males. Each ejaculate was used to inseminate two females. Tyrode's solution (Cameron, 1950), containing 1 mg bovine plasma albumen-fraction V, 50 units of penicillin and 50 μg streptomycin sulphate/ml, was used to dilute the semen. Otherwise, insemination procedures in the two experiments were the same. Number and size of implantation sites were determined at 9 days p.c. by means of a mid-ventral laparotomy, instead of autopsy at 12 days, as in the first experiment. Counts of normal foetuses were made at autopsy 25 days p.c.

Embryonic survival was calculated from the proportion of fertilized eggs which formed implantation sites at 9 days following return to the oviduct. For

the statistical analysis, each surviving egg was accorded a value of 1, while each of the missing eggs was assigned a value of 0. The procedure for unequal subclass numbers (Harvey, 1960) was followed.

RESULTS

Data from the first experiment (Table 1) show that there was no difference in the fertilization rates when the spermatozoa were incubated at 38° C or 40° C. However, significantly ($P<0{\cdot}05$) more (22·1%) of the eggs fertilized with spermatozoa incubated at 38° C formed implantation sites at 12 days p.c. than did the eggs fertilized with spermatozoa incubated at 40° C. Also, fewer of the implantation sites on the side inseminated with spermatozoa incubated at 40° C

TABLE 1

RATE OF EMBRYONIC SURVIVAL (%) FOR VARIOUS STAGES OF DEVELOPMENT SUBSEQUENT TO FERTILIZATION WITH SPERMATOZOA INCUBATED FOR 3 HR AT TWO DIFFERENT TEMPERATURES

Stage of development	*Incubation temperatures of spermatozoa*					
	Control		38° *C*		40° *C*	
Recovery rate $\frac{\text{Total ova recovered}}{\text{Total corpora lutea}}$	57/57	(100)	57/61	(93·4)	74/75	(98·7)
Fertilization rate $\frac{\text{Cleaved ova}}{\text{Total ova}}$	56/57	(98·2)	56/57	(98·2)	71/74	(95·9)
Pre-implantation survival $\frac{\text{Implant sites}}{\text{Cleaved ova}}$	35/56	(62·5)	36/56	(64·3)	30/71	(42·2)*
Post-implantation survival $\frac{\text{Normal embryos}}{\text{Implant sites}}$	33/35	(94·3)	33/36	(91·7)	24/30	(80·0)
Total survival $\frac{\text{Normal embryos}}{\text{Cleaved ova}}$	33/56	(58·9)	33/56	(58·9)	24/71	(33·8)**

*$P<0{\cdot}05$, **$P<0{\cdot}01$ by Chi-square.

(80·0%) contained embryos with a heart beat than did the side inseminated with spermatozoa incubated at 38° C (91·7%). This difference was not significant. Nevertheless, it resulted in a significant ($P<0{\cdot}01$) difference of 25·1% in the total survival. These data, although from only one male, suggested that spermatozoa could be influenced before fertilization by a 2° C increase in temperature which, while having no effect on fertilization rate, decreased pre-implantation embryonic survival.

Data from the second experiment again indicated that an increase of 2° C during incubation had no effect on the fertilization rates (Table 2). Eggs fertilized with spermatozoa incubated at 38° C had significantly ($P<0{\cdot}01$) more implantation sites at 9 days than those fertilized with the 40° C spermatozoa. The difference of 21·6% between treatment groups was almost the same as that observed in the first experiment (22·1%).

Also there was a significant ($P<0{\cdot}01$) difference between males in the number of embryos that formed implantation sites. The embryonic survival rate for spermatozoa (combined 38° C and 40° C) from the four individual males was 86·7%, 62·5%, 44·8% and 51·6%. There was no significant difference between

TABLE 2

RATE OF EMBRYONIC SURVIVAL (%) AT VARIOUS STAGES OF DEVELOPMENT AND ANALYSIS OF VARIANCE OF 9-DAY SURVIVAL WITH SPERMATOZOA INCUBATED AT TWO DIFFERENT TEMPERATURES

Stage of development	*Incubation temperatures of spermatozoa*					
	Control		38° *C*		40° *C*	
Recovery rate $\frac{\text{Total ova recovered}}{\text{Total corpora lutea}}$	123/130	(94·6)	55/56	(98·2)	58/64	(90·0)
Fertilization rate $\frac{\text{Cleaved ova}}{\text{Total ova}}$	116/123	(94·3)	52/55	(94·5)	58/58	(100)
Pre-implantation survival $\frac{\text{Implant sites}}{\text{Cleaved ova}}$	66/116	(56·9)	39/52	(75·0)	31/58	(53·4)
Post-implantation survival $\frac{\text{Normal foetuses}}{\text{Implant sites}}$	50/59	(84·7)	15/32	(46·9)	24/31	(77·4)
Total survival $\frac{\text{Normal foetuses}}{\text{Cleaved ova}}$	50/106	(47·2)	15/46	(32·6)	24/54	(44·4)

ANALYSIS OF VARIANCE

38° C and 40° C treatments			*Control, 38° C and 40° C treatments*		
Source of variation	*df*	*MS*	*Source of variation*	*df*	*MS*
Total (corrected)	109		Total (corrected)	225	
Treatments	1	2·2437**	Treatments	2	1·517384
Males	3	0·9005**	Males	3	0·00000407
Ejaculates in males	4	0·0675	Females in males	28	0·46900**
Females in ejac. males	8	0·2695	Treatment × males	6	0·70541**
Error	93	0·1926	Error	186	0·147702

** $P < 0·01$.

TABLE 3

EFFECT OF INCUBATION ON SEMEN QUALITY

Source	*Percentage live*		*Percentage motile*		*Progressive motility*	
	df	*MS*	*df*	*MS*	*df*	*MS*
Treatments	2	550·50	2	5243·5**	2	11·0455**
Before incub. vs. After incub.	1	1097·40**	1	10459·2**	1	22·0910**
After incub. 38° C vs. After incub. 40° C	1	3·60	1	27·8	1	0·00
Males	5	1258·21**	5	162·7	5	0·5032
Treatment × males	10	217·42	10	299·4**	10	1·0915*
Error	39	106·09	48	138·8	48	0·4531
Means ± S.E.						
Before incubation	72·90 ± 2·48		65·45 ± 2·05		3·68 ± 0·461	
After incubation 38° C	63·90 ± 4·67		37·95 ± 2·75		2·45 ± 0·453	
After incubation 40° C	62·75 ± 4·44		39·54 ± 3·36		2·45 ± 0·599	

** $P < 0·01$.

ejaculates from the same male or between females inseminated with semen from the same ejaculate.

For an unexplained reason, possibly because of unparalleled differences among control females, fewer eggs (56·9%) were implanted than for the 38° C experimental group (75·0%). Consequently, the importance of the experimental treatments is obscured by the addition of the control rabbits to the data.

Observations made at 25 days p.c. may lack biological significance due to manipulations at 9 days p.c. However, the number of implantation sites resulting in normal foetuses at 25 days p.c. was significantly ($P<0·05$) lower in the 38° C group than in the control or 40° C groups.

Table 3 indicates a highly significant decrease in all three measurements of semen quality during the 3-hr incubation period, but no difference could be detected in semen quality at the end of the period due to incubation temperature. There were no significant correlations between any measure of semen quality and fertilization or embryonic survival rates. All correlations were between −0·2188 and 0·0897. This means that observations of semen characteristics, as measured in this study, can not be used to predict with any degree of accuracy the subsequent fertilization or embryonic survival rates.

DISCUSSION

The results of this study are in agreement with those of Howarth *et al.* (1965) who found a significant decrease in the pre-implantation embryonic survival of ova fertilized by spermatozoa subjected to an elevated temperature *in utero*. So it appears that spermatozoa can be directly affected by elevated temperature to cause a decrease in subsequent embryonic survival rate without affecting the fertilization rate.

These observations are also in accord with reports of other forms of stress on spermatozoa. For example, when rabbit spermatozoa were irradiated *in vitro* with a dose between 400 and 800 r of either X-ray or cobalt 60 (Amoroso & Parkes, 1947; Chang, Hunt & Romanoff, 1957) the embryos fertilized with the irradiated spermatozoa had an increased embryonic death rate. The majority of the embryos fertilized with irradiated spermatozoa failed to develop beyond the blastocyst stage (Dott *et al.*, 1966). Similar results have also been observed in mice (Edwards, 1957) and cattle (Rowson & Dott, 1964). Triethylene melamine (TEM) in rats and mice did not interfere with motility or fertilizing capacity of spermatozoa, but no litters were produced (Bateman, 1960). All these agents presumably act on the deoxyribonucleic acid (DNA) of the spermatozoa. When bull spermatozoa were aged *in vitro* at 4° C there was an increase in the embryonic death rate associated with increased storage time (Salisbury & Flerchinger, 1961). These spermatozoa had a decreased DNA content (Salisbury, Birge, De La Torre & Lodge, 1961). It is generally assumed that the chromatin of the mature spermatozoa is inert, that is to say, that it is not actively taking part in cellular metabolism; however, Graves & Salisbury (1963, 1966) have shown that labelled glycine, thymidine, glucose and fructose could be incorporated into the DNA of the spermatozoa *in vitro*.

An increase of 2° C may alter the metabolism of the chromatin in such a way as to cause a change in the DNA content similar to that observed in spermatozoa

under other forms of stress. As proposed by Howarth *et al.* (1965), this change in the DNA could be expressed as a dominant lethal mutation resulting in early death of the embryo. If this is, in fact, what happens it would account for the lack of correlation between the measures of semen quality (live–dead ratio and motility) and embryonic survival.

ACKNOWLEDGMENTS

Contribution from the Animal Science Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper No. 2287 of the Journal Series. The technical assistance of Mrs M. B. Walton is acknowledged.

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