THE FERTILIZING CAPACITY OF BOAR SEMEN STORED IN THE PRESENCE OF GLYCEROL AT 20, 5 AND -79°C

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Summary. Three experiments have been carried out to investigate the mechanism by which glycerol reduces the fertilizing capacity of boar spermatozoa during storage, and any relationship between this effect and the relatively poor conception rates obtained with deep-frozen boar semen.

The loss of fertilizing capacity in the presence of glycerol was shown to be temperature-dependent. Fertilizing capacity was significantly reduced after 6 hr of incubation at 20°C in the presence of 5 or 10% glycerol, although there was no detectable change in motility or morphology of the spermatozoa. By contrast, there was no effect on fertilizing ability after storage for 6 hr at 5°C or for 30 min at 20°C in the presence of these concentrations of glycerol. Removal of the seminal plasma and a large proportion of the free cytoplasmic droplets did not prevent the reduction in fertilizing capacity. When semen was frozen and thawed, attempts to minimize the exposure of spermatozoa to glycerol did not lead to fertilization when the thawed semen was inseminated through the cervix. It is suggested that at ambient temperature glycerol causes damage to boar spermatozoa which is exacerbated by the stress of freezing and thawing.

INTRODUCTION

The inclusion of glycerol in diluents used for the storage of boar semen causes a reduction in conception rates (Polge, 1956, 1959; Settergren, 1958; King & MacPherson, 1966; Graham, Rajammanan, Schmehl, Maki-Laurila & Bower, 1971a). In the present paper, experiments are described which sought to determine the mechanism of the harmful effect of glycerol, and any relationship between this effect and the relatively poor conception rates which have been obtained with deep-frozen boar semen (Polge, 1956; Settergren, 1958; Pursel, Johnson & Gerrits, 1969; Pursel & Johnson, 1971).

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MATERIALS AND METHODS

Semen was collected from six boars (one Landrace, two Large White, one Large Black and two Large White $\times$ Essex) of proven fertility. The diluent used contained 2 g glycine, 0.242 g tris (hydroxymethyl) aminomethane and 25 ml egg-yolk in 100 ml diluent. The pH was adjusted to 7.2 with HCl and streptomycin sulphate and benzyl penicillin, 750 i.u. per ml, were added. When glycerol was included, it replaced water in the diluents. Estimates of the percentage of motile spermatozoa were made on thin semen smears, using a phase-contrast microscope fitted with a warm stage. The stage and all slides, coverslips and pipettes were warmed to 37°C. Samples of the treated spermatozoa were fixed in buffered formol-saline (Hancock, 1957). Giemsa preparations were made by a modification of the method of Hancock (1952). If the semen had been diluted, it was necessary to remove egg-yolk and glycerol before making the smears. This step was achieved by fixing the sample in citrate buffer which contained 2% (v/v) formaldehyde and carefully centrifuging and washing the spermatozoa. The spermatozoa were smeared on a clean slide, which was then dried at 37°C and left overnight in the Giemsa stain described by Hancock (1952).

Ovulation time in the experimental gilts was controlled by use of methallibure (Aimax, I.C.I. Ltd) and gonadotrophins, as described previously (Polge, Day & Groves, 1968; Polge, Salamon & Wilmut, 1970). Autopsy was carried out 8 to 10 hr after ovulation and fertilization was assessed by examination of whole-mount preparations of the eggs. After fixation overnight in acetic alcohol (3:1, ethanol:acetic acid), the eggs were stained with 1% natural orcein in 45% aqueous acetic acid and examined with a phase-contrast microscope.

In Exp. 1, an examination was made of the effect of storage time, temperature and glycerol concentration on the fertilizing ability of the semen. Three concentrations of glycerol were used and the storage conditions were varied in three consecutive experimental groups. The concentrations of glycerol were 0, 5 and 10% in the diluted semen. That volume of whole ejaculate which contained 1.0×10^{10} spermatozoa was diluted to 50 ml with glycine-yolk-tris diluent at 30°C, before being allowed to cool to 20°C. In Group 1, the diluted semen was cooled to 5°C over a 3-hr period before further dilution (1:1) with diluent containing glycerol. The semen was stored in the presence of glycerol for 6 hr at 5°C before insemination. In Groups 2 and 3, the diluted semen was stored at 20°C for an equivalent 3-hr period before diluent which contained glycerol was added. In Group 2, the semen was stored in the presence of glycerol at 20°C for 6 hr before insemination whereas in Group 3, insemination was completed within 30 min of the addition of diluent which contained glycerol. Insemination was carried out 30 hr after the injection of HCG (Gestyl, Organon Ltd) and ovulation was expected to occur 40 to 42 hr after this injection (Dziuk & Baker, 1962). Each gilt was inseminated with 1.0×10^{10} spermatozoa in 100 ml. A total of fifty-four gilts was used, eighteen in each group and six for each glycerol concentration. One ejaculate was used to inseminate a gilt in each of the three glycerol concentrations within one storage condition.

There are potentially harmful enzymes in seminal plasma (Mann, 1964) and the cytoplasmic droplets contain many of the lysosomal enzymes (Dott & Dingle, 1968; Allison & Hartree, 1970). It is possible that glycerol renders the spermatozoa liable to attack by these enzymes.

A technique was employed in Exp. 2 by which the seminal plasma and a large proportion of the free cytoplasmic droplets were removed, and an estimate was made of the effect of glycerol on semen treated in this way. When the semen was collected for this experiment, the first sperm-rich fraction was collected into 800 ml of a diluent which rapidly inhibited motility (Salisbury & Graves, 1963). The collection was continued and the second ejaculatory wave was collected as whole semen. Centrifugation of the diluted semen at 600 *g* for 15 min induced good sedimentation of the spermatozoa while the droplets remained in suspension (Table 2). Control semen and the centrifuged sperm pellet were diluted with glycine-yolk-tris diluent to contain 1.0×10^{10} spermatozoa in 50 ml. The partly diluted semen was incubated at 20°C for 3 hr before dilution 1:1 with diluent containing 20% glycerol. Twelve gilts, six per treatment, were inseminated with control or centrifuged semen which had been stored for 6 hr at 20°C in the presence of glycerol.

In Exp. 3, groups of gilts were inseminated with semen that had been frozen and thawed. The factors which were varied were: the time of insemination relative to ovulation, the temperature at which the diluent containing glycerol was added and the temperature at which the semen was stored after it had been thawed until it could be used for insemination. In Groups 1 and 4, the sperm-rich fraction was used while in the other groups whole ejaculate was used in an attempt to increase the number and concentration of the spermatozoa in the samples to be inseminated. Whole ejaculates were centrifuged at 500 *g* for 25 min, the plasma was removed and the sperm pellet was resuspended to a volume of 40 ml with glycine-yolk-tris diluent. The sperm-rich fraction was diluted 1:1 with diluent which did not contain glycerol. Final dilution (1:1) with diluent containing 15% glycerol was carried out at 20°C (Groups 1 and 4) or 10°C (Groups 2, 3, 5 and 6). One hour after dilution with diluent which contained glycerol, the diluted semen was placed in 2-ml ampoules and cooled at 1°C per min to -10°C, 3°C per min from -10 to -35°C and then quickly to -79°C. The semen was thawed by shaking the ampoules in water at 37°C and the ampoules were stored at 30°C (Groups 1, 2, 4 and 5) or 10°C (Groups 3 and 6). When storage was to be at 10°C, care was taken to transfer the ampoules to water at 10°C as the last ice disappeared. The interval between thawing and insemination was about 30 min. Insemination was carried out either 16 hr before (Groups 1, 2 and 3) or at the time of ovulation (Groups 4, 5 and 6) and all gilts were slaughtered 8 hr after the expected time of ovulation.

The results of Exps 1 and 2 were treated by analysis of variance using weighted means. The percentages were converted to angles by the inverse sine transformation (Steel & Torrie, 1960) and the means presented are the retransformed values. Some tables of results contain a record of 'number of eggs examined', and the difference between this number and the number of corpora lutea includes eggs not recovered, those that it was not possible to classify and those which were considered to be 'immature'. Of the 1049 eggs examined

during these experiments, seventy were found to be immature (6.67%). The occurrence of such eggs after gonadotrophin treatment has been noted previously (e.g. Spalding, Berry & Moffit, 1955; Hunter, 1964, 1966).

RESULTS

Experiment 1

The effect of glycerol concentration on the fertilizing ability of semen stored at 20°C depended on the length of exposure of the spermatozoa to glycerol. The addition of 5 and 10% glycerol significantly reduced the proportion of eggs fertilized after a 6 hr exposure to glycerol at 20°C (Table 1, $P < 0.05$), whereas there was no effect of these concentrations in Group 3 when the spermatozoa were only exposed to glycerol for 30 min. There was a non-significant tendency

Table 1. The effect of storage condition and glycerol concentration on the fertilizing capacity of boar semen

	Group 1 (semen stored at 5°C for 6 hr)			Group 2 (semen stored at 20°C for 6 hr)			Group 3 (semen stored at 20°C for 30 min)		
Glycerol concentration (%)	0	5	10	0	5	10	0	5	10
No. of ovulations	76	83	88	119	68	71	84	73	87
No. of eggs examined	64	71	70	84	54	48	61	40	64
No. of eggs fertilized	26	20	45	77	19	11	51	23	48
Percentage fertilized	30.4	22.1	71.2	91.2	32.0	34.9	92.4	53.6	72.9

for 10% glycerol to increase the proportion of eggs fertilized after storage of the semen at 5°C. After storage for 6 hr at 20°C, the proportion of motile spermatozoa was 75% (range 60 to 80%) whereas after storage at 5°C, the average was 60% (range 50 to 80%). There was no effect of glycerol concentration on the percentage of motile spermatozoa. The only morphological change observed was swelling of a proportion of the acrosomes of spermatozoa which had been stored at 5°C. There was no evidence of change due to the presence of glycerol, even after 6 hr of storage at 20°C when the fertilizing ability of the spermatozoa was significantly reduced.

Experiment 2

The proportion of spermatozoa with droplets attached was rather high in both control and centrifuged samples, being 80% in one sample (Table 2). There was no apparent relationship between fertilizing ability and the proportion of spermatozoa with droplets attached. Whereas 63% of the free droplets could be identified in control samples, only 19% were present in centrifuged samples and it seems reasonable to assume that virtually all of the seminal plasma would be removed during the washing procedure.

There was no effect of partial removal of free droplets on the fertilizing capacity of spermatozoa stored at 20°C in the presence of 10% glycerol for 6 hr (Table 3). The proportion of eggs fertilized was, however, rather higher than after the same treatment in Exp. 1.

Table 2. The distribution of cytoplasmic droplets in control and centrifuged samples of boar semen

Boar	Control			Centrifuged		
	Spermatozoa with droplets attached (%)	Free droplets/100 spermatozoa	% free droplets present	Spermatozoa with droplets attached (%)	Free droplets/100 spermatozoa	% free droplets present
A	80	13	65	60	5	13
B	40	39	65	10	18	20
B	8	59	64	7	26	28
C	30	41	59	53	7	15
Mean	40	38	63	33	14	19

Experiment 3

It was estimated that 20% of the spermatozoa which had been frozen and thawed were motile at the time of insemination so that the number of motile spermatozoa inseminated ranged from 5.4×10^9 to 1.0×10^{10} . A spermatozoon had penetrated the ooplasm and stimulated the resumption of meiosis in one

Table 3. The fertilizing capacity of control and centrifuged samples of boar semen after storage at 20°C in the presence of 10% glycerol for 6 hr

	Control	Centrifuged
No. of corpora lutea	79	61
No. of eggs	56	44
No. fertilized	25	20
Percentage fertilized	40.7	44.2

egg recovered from a gilt in Group 1 (Table 4). The sperm head was swollen and the meiotic spindle was in the second anaphase. No other egg had been penetrated nor were there any spermatozoa attached to or embedded in a zona pellucida. The number of spermatozoa found in the flushings from the oviducts was negligible.

Table 4. The fertilizing capacity of boar semen which had been frozen and thawed

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
No. of gilts	6	4	4	5	5	6
Av. no. of sperm. inseminated $\times 10^{10}$	2.7	5.0	3.2	2.8	3.9	3.1
Av. volume inseminated (ml)	115	80	80	120	80	80
No. of ovulations	75	49	44	137	67	101
No. of eggs examined	63	38	28	78	45	71
No. of eggs fertilized	1	0	0	0	0	0

DISCUSSION

In Exp. 1, the effect of glycerol on the fertilizing ability of boar spermatozoa varied with the length of exposure to glycerol and with temperature (Table 1). A similar temperature-dependent effect of glycerol on boar spermatozoa was noted during a study of motility, when it was found after storage for 4 days that glycerol improved motility at 5°C, but clearly reduced the percentage of motile spermatozoa after storage at 15°C (Foote, Young, Turkheimer & Bratton, 1959). There have been considerable variations in the results of experiments carried out to define the effect of glycerol on the fertilizing ability of boar spermatozoa. Successful results following long distance transport of boar semen have been achieved by incubation of the semen at 5°C in the presence of 5% glycerol for 45 to 76 hr. Twenty-one gilts were inseminated and fifteen of these farrowed (Niwa, Mizuho, Soejima & Geing, 1964). By contrast, a study of the fertilizing ability of boar semen stored for up to 10 hr at 7°C in the presence of 0, 2, 5 or 7% glycerol revealed a lower conception ratio after storage of semen in the presence of 5 or 7% glycerol (Graham *et al.*, 1971a). The variation in the results reflects differences in the number and concentration of spermatozoa which were inseminated. When large numbers were inseminated, there was no reduction in fertilizing capacity after storage at 5°C (Niwa *et al.*, 1964; see also Table 1 in this paper), whereas when smaller numbers were inseminated a reduction in fertilizing capacity was apparent (Graham *et al.*, 1971a).

The effect of glycerol on boar spermatozoa is clearly temperature-dependent. At 37°C, boar spermatozoa are rapidly immobilized by glycerol whereas at 20°C, motility is not affected as quickly but fertilizing capacity is lost within a few hours. At 5°C, the loss of fertilizing capacity occurs over a longer period. By contrast, when bull semen is stored at 5°C, incorporation of glycerol in the diluent increases the conception ratio (see Melrose, 1962).

The mechanism by which glycerol reduces the fertilizing capacity of boar spermatozoa is not known. As exposure to glycerol for 6 hr at 5°C or for 30 min at 20°C did not cause a reduction in fertilization (Table 1), the reduction caused by exposure for 6 hr at 20°C must involve action on the semen rather than on the female reproductive tract. It is probable that glycerol acts directly on the spermatozoa as removal of seminal plasma and a large proportion of the free cytoplasmic droplets did not prevent a reduction in fertilizing capacity (Table 3). No change, however, was detected in the motility or morphology of spermatozoa incubated for 6 hr at 20°C in the presence of 5 or 10% glycerol, despite the fact that the fertilizing capacity of semen stored under these conditions was significantly reduced. Semen from the same boars was subjected to the treatments applied in Exp. 1 and spermatozoa were examined by electron microscopy (Jones, 1973). Changes were observed in the acrosomes of spermatozoa stored in the presence of 5 or 10% glycerol at 5°C, although the fertilizing capacity of semen stored under these conditions was not affected significantly. By contrast, no morphological change was detected which could be associated with the considerable loss of fertilizing ability of spermatozoa stored at 20°C in the presence of these concentrations of glycerol.

Inclusion of 5 to 10% glycerol in the medium causes a reduction in the

motility and oxygen uptake of boar spermatozoa incubated at 37°C (Iida, 1964; Sanford, King & MacPherson, 1972). Incubation of bull semen under these conditions caused a similar but smaller reduction in activity (Sanford *et al.*, 1972). Addition of diluent containing glycerol to bull and boar semen disrupts the normal distribution of cations (Yassen & Foote, 1967; Hood, Foley & Martin, 1970) and causes loss of glutaminoxaloacetic transaminase (GOT) into the extracellular fluid (Crabo, Bower, Brown, Graham & Pace, 1971). Loss of GOT from boar spermatozoa was more rapid when diluent which contained glycerol was added at 37°C than when it was added at 5°C. The authors assumed that loss of GOT from the spermatozoa indicated the occurrence of damage to the cytoplasmic membrane.

Fowl spermatozoa in diluents which contain 5%, or more, of glycerol are completely infertile when the semen is deposited in the vagina (Polge, 1951), although the fertilizing capacity is partly restored if the glycerol is removed by dialysis (Polge, 1951) or if the semen is deposited in the uterus (Allen & Bobr, 1955). When the glycerol concentration is reduced rapidly *in vitro*, most fowl spermatozoa are killed and it has been suggested that similar damage might occur when the semen is deposited in the female reproductive tract (Polge, 1951). As the uterovaginal junction constitutes a barrier to the passage of spermatozoa in the chicken (Allen & Bobr, 1955), it seems probable that the spermatozoa damaged by the rapid removal of glycerol in the vagina cannot pass into the uterus, whereas some of those damaged in the uterus can survive and achieve fertilization. It seems unlikely that penetration of glycerol into boar spermatozoa would be incomplete after 6 hr at 5°C or 30 min at 20°C, so the reduction in fertilizing ability after 6 hr in the presence of 5 or 10% glycerol at 20°C could not be due entirely to damage caused by rapid removal of glycerol after insemination.

There was a marked contrast between the revival of about 20% of the frozen and thawed spermatozoa and the almost complete absence of fertilization in Exp. 3. Although autopsy was performed only 8 hr after insemination, very few thawed spermatozoa were found in flushings from the region of the oviduct or the uterotubal junction; after the insemination of freshly collected semen considerable numbers of spermatozoa persist at the uterotubal junction for at least 24 hr (Rigby, 1966).

The attempts in Exp. 3 to reduce the exposure of spermatozoa to glycerol at the higher temperatures which had been shown to be particularly damaging did not lead to fertilization. By contrast, a large proportion of the eggs were fertilized if the frozen and thawed semen was deposited in the oviduct (Polge *et al.*, 1970) or if the final concentration of glycerol was reduced and the semen was deposited in the uterus (Wilmot & Polge, 1971). Pregnancies have been obtained after insemination through the cervix of boar semen frozen and thawed by various methods (Crabo & Einarsson, 1971; Graham *et al.*, 1971a, b; Pursel & Johnson, 1971.) There are considerable differences in the techniques employed, but all use relatively low concentrations of glycerol (0 to 3%). Inclusion of higher concentrations of glycerol caused progressively greater damage to the acrosomal region of the spermatozoa (Wilmot & Polge, 1971, 1972). It is possible that, as suggested by measurement of GOT release (Crabo

et al., 1971), addition of diluent which contains glycerol causes slight damage to the cytoplasmic membranes before freezing and that this damage is exacerbated by the stress of freezing and thawing. Although spermatozoa stressed in this manner can survive in the oviduct and achieve fertilization, they are not able to survive in the uterus or to pass from the uterus into the oviducts.

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