Effect of glycerol on the penetrating ability of fresh ram spermatozoa with zona-free hamster eggs

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Summary. The presence of 10% glycerol in diluted semen for 30–90 min before preincubation stimulated sperm penetration of zona-free eggs. Prolongation of semen storage in medium with glycerol did not further improve penetration ability. The penetrating activity of spermatozoa depended on the concentration of glycerol and incubation time. These results indicate that the presence of glycerol accelerates induction of the acrosome reaction which could be one of the causes of lower conception rate after insemination with deep-frozen ram semen.

Introduction

It is generally accepted that the lower conception rate achieved when using deep-frozen semen is caused by damage to the spermatozoa in the course of the freezing–thawing procedure, even if a cryoprotectant is used. Glycerol is widely used as a cryoprotective agent and its effect on human spermatozoa has been studied by Tulandi & McInnes (1984). These authors concluded that glycerol negatively affects sperm motility even before freezing at concentration of 2% and higher. Jeyendran *et al.* (1984) compared the cryoprotective effect of glycerol with that of a zwitterion buffer system, TESTCY (mixture of TRIS and TES). Human spermatozoa that were cryopreserved in the presence of glycerol showed greater motility immediately after thawing than did those preserved in the presence of TESTCY. However, after 2 h in culture, TESTCY-treated spermatozoa possessed higher motility than did glycerol-treated ones. Since the work of Hanada & Chang (1972) the ability of zona-free hamster eggs to interact with alien spermatozoa *in vitro* has been used as a test to evaluate sperm fertilizing ability. Jeyendran *et al.* (1984) assessed the fertilizing potential of frozen human spermatozoa by their ability to fuse with zona-free hamster eggs. After thawing, TESTCY-treated human spermatozoa exhibited better fusion than glycerol-treated spermatozoa. Jeyendran *et al.* (1985) reported a poor penetration rate of glycerol-treated cryopreserved human spermatozoa in comparison with untreated fresh control semen.

The aim of the present study was to analyse the effect of glycerol upon capacitation and the acrosome reaction in freshly ejaculated ram spermatozoa by assessment of their interaction with zona-free hamster eggs.

Materials and Methods

Ram spermatozoa and zona-free hamster eggs were allowed to interact as described by Pavlok & Fléchon (1985). The effect of concentration and of duration of exposure to glycerol, time of preincubation and kinetics of fusion were evaluated.

Mature hamster females were superovulated with injections of 100 i.u. PMSG (Bioveta Ivanovice, Czechoslovakia) per female and 100 i.u. hCG (Praedyn, Spofa Praha, Czechoslovakia) 72–78 h later. At 16–18 h after hCG injection the hamsters were killed and their oocytes isolated from the oviducts. Cumulus oophorus was removed with hyaluronidase (SEVAC Praha, Czechoslovakia) at a concentration of 190 TRU (turbidimetric units)/1 ml culture medium. The zona pellucida was dissolved by 0.02% α -chymotrypsin (Koch-Light Laboratories Ltd, Colnbrook,

U.K.). The details of the method were as described in detail by Pavlok (1981) and Majerčíak *et al.* (1982). The medium TCM 199 (ÚSOL Praha, Czechoslovakia) used for sperm capacitation, in-vitro fertilization, and dissolving of enzymes, was completed with 0.91 mM-sodium pyruvate (SERVA, Heidelberg, FRG), 3.25 mM-calcium lactate (B.D.H., Poole, U.K.), lyophilized bovine serum proteins, 20 mg/ml (SEVAC, Praha, Czechoslovakia), 25 i.u. penicillin (K-salt)/ml, and 25 i.u. streptomycin sulphate/ml. The pH after equilibration with 5% CO₂ and 95% air was 7.2–7.4.

Spermatozoa were collected with an artificial vagina from 2 rams. After collection each ejaculate was divided into two halves. The first ones were treated as follows.

For the first washing procedure, culture medium enriched with 10% glycerol (SERVA, Heidelberg, FRG; 5 and 10% respectively in Exp. 1) was used. After dilution to 1:8–1:10, semen samples were stored for about 10 min (30, 60, 90 or 120 min in Exp. 2) at room temperature and then the samples were centrifuged for 10 min at 600–800 g. The supernatant was discarded and the washing procedure was repeated once more using culture medium without glycerol. Spermatozoa were preincubated in small amounts of 40–50 µl in special microtubes at 37.5°C in an humidified atmosphere of 5% CO₂ and 95% air in Petri dishes. The sperm concentration at that time was 0.4–0.8 × 10⁹/ml. Preincubation lasted 4–6 h (0, 2, 4, 6, 8 and 10 h in Exp. 3). Co-incubation of spermatozoa and zona-free eggs was for 16 h (1.5, 3, 4.5 or 16 h in Exp. 4).

The second halves of the ejaculates were treated in the same manner but using culture medium without glycerol for both washings.

At the end of culture, the eggs were mounted under a coverslip supported by two strips of vaseline, fixed with 25% aceto-alcohol overnight, stained with 2% aceto-orcein and observed under a Zeiss phase-contrast microscope (Amplival). The criterion for penetration was the identification of swollen sperm heads or male pronuclei with sperm tails in the cytoplasm as described previously by Pavlok & McLaren (1972). The percentage of penetrated eggs in total number of eggs and the average sperm count in each penetrated egg (polyspermy) were evaluated. Each experiment was repeated at least 3 times.

Results

In all experiments it was found that glycerol stimulates penetration activity of fresh ram spermatozoa with zona-free hamster eggs.

Experiment 1: effect of glycerol concentration

The untreated spermatozoa showed lower penetration rates and polyspermy than did glycerol-treated spermatozoa. The penetration activity was greater when medium enriched with 10% glycerol was used than when the medium contained only 5% glycerol (Table 1).

Table 1. Effect of glycerol concentrations on penetration of zona-free hamster eggs by fresh ram spermatozoa preincubated for 5–6 h

Glycerol conc. (%)	Total no. of eggs (%) penetrated	Sperm no. per penetrated egg
0 (control)	116 (52.6)	1.57
5	119 (58.0)	1.62
10	123 (63.4)	2.02

Experiment 2: effect of duration of exposure of spermatozoa to glycerol

The penetration activity depended on the period of incubation. Glycerol-treated spermatozoa showed the highest penetration activity after incubation for 30 min. Longer periods of storage induced a gradual decrease of penetration activity and after incubation with glycerolated medium for 120 min the penetration activity was lower than in untreated (control) samples (Table 2).

Table 2. Effect of exposure time to glycerol on penetrating ability of zona-free hamster eggs with fresh ram spermatozoa preincubated for 4–5 h

Exposure time (min)	Total no. of eggs (%) penetrated	Sperm no. per penetrated egg
0 (control)	128 (28.9)	1.32
30	83 (79.5)	1.82
60	83 (74.7)	1.73
90	80 (53.6)	1.53
120	81 (22.2)	1.16

Table 3. Effect of glycerol on the penetrating ability of fresh ram spermatozoa preincubated for different times

Glycerol conc. (%)	Time of preincubation (h)	Total no. of eggs (%) penetrated	Sperm no. per penetrated egg
0 (control)	0	142 (2.1)	1.00
	2	138 (14.5)	1.30
	4	137 (36.5)	1.50
	6	134 (55.2)	1.77
	8	137 (37.2)	1.69
	10	131 (26.0)	1.59
10	0	145 (22.8)	1.15
	2	137 (48.9)	1.58
	4	135 (68.1)	2.22
	6	142 (45.1)	1.97
	8	133 (19.5)	1.50
	10	138 (5.8)	1.25

Table 4. Effect of glycerol on the kinetics of zona-free hamster egg penetration with fresh ram spermatozoa preincubated for 4–5 h

Glycerol conc. (%)	Sperm-egg incubation (h)	Total no. of eggs (%) penetrated	Sperm no. per penetrated egg
0 (control)	1.5	110 (26.4)	1.14
	3.0	110 (36.4)	1.18
	4.5	102 (44.1)	1.31
	16.0	107 (72.9)	1.85
10	1.5	108 (64.8)	1.46
	3.0	111 (82.9)	1.92
	4.5	110 (91.8)	2.31
	16.0	103 (94.2)	2.54

Experiment 3: effect of glycerol on the ability of ram spermatozoa to penetrate zona-free hamster eggs

Glycerol reduced the time necessary for preincubation and glycerol-treated spermatozoa gained the ability to penetrate zona-free eggs earlier. Spermatozoa that were untreated and not preincubated penetrated at a very low level. Maximal activity was achieved after 6 h preincubation and the penetrating ability was still present after preincubation for 10 h. When the spermatozoa were treated with glycerol, penetration occurred without preincubation, and maximal penetration was after 4 h preincubation. However, the decrease of penetrating ability occurred earlier and after preincubation for 10 h glycerol-treated spermatozoa showed very low penetration (Table 3).

Experiment 4: effect of glycerol on kinetics of penetration

Untreated spermatozoa penetrated the zona-free eggs gradually during the culture (16 h), while glycerol-treated spermatozoa penetrated mostly during the first 4.5 h of culture. This was deduced from differences between values after 4.5 h and 16 h. For untreated spermatozoa the difference was 28.8% (72.9–44.1%) penetrated eggs and 0.54 (1.85–1.31) spermatozoa per egg were calculated. For glycerol-treated spermatozoa the equivalent differences were 2.4% (94.2–91.8%) penetrated eggs and 0.23 (2.54–2.31) spermatozoa per egg (Table 4).

Discussion

After glycerol treatment the absolute number of ram spermatozoa that were able to penetrate zona-free hamster eggs increased in a very short interval. It can be therefore supposed that glycerol accelerates and synchronizes the induction of the acrosome reaction.

One of the factors which could affect the course of capacitation and the acrosome reaction is the binding of fatty acids to glycerol, although there is no direct evidence for this possibility. Free fatty acids function as a decapacitator and after their removal (e.g. by binding to albumin) the sperm membranes are destabilized and the acrosome reaction induced (Lui & Meizel, 1977; Aonuma *et al.* 1982; Juetten & Bavister, 1983). For free fatty acids binding to glycerol the equilibrium of reaction 'membrane phospholipids → lysophospholipids + free fatty acids' is destroyed and lysophospholipids, several of which function as guinea-pig acrosome reaction-stimulating factors (Fleming & Yanagimachi, 1981), could better be applied.

In view of the widespread use of glycerol in diluents for deep freezing of semen, it is of interest that ram spermatozoa can utilize it under aerobic conditions. According to Hutton *et al.* (1980) and Jones & Stevenson (1983), glycerol could be linked to glycolytic pathways at the triose phosphate stage. Triose phosphates are converted into lactic acid which can be further oxidized. These steps require oxygen and therefore glycerol stimulates respiration in semen of rams (Mann & White, 1957) and bulls (Halangk *et al.*, 1985). Regarding the induction of the acrosome reaction, the metabolic pathway is less certain, because an increase of penetration ability is apparent even after a short incubation.

The acrosome reaction could also be stimulated by osmotic pressure changes of medium, because the osmotic pressure of the culture medium increased several times after glycerol addition. A stimulatory effect of hyperosmolarity on the acrosome reaction of human spermatozoa has been shown by Aitken *et al.* (1983) and Oliphant & Brackett (1973) concluded that glycerol affects negatively sperm motility and fertilization of intact mouse ova.

In the light of the present results it seems that a lower conception rate after using frozen-thawed semen could be caused by acceleration of the capacitation events. Crozet & Dumont (1984) suggest that the acrosome reaction occurs at the surface of the zona pellucida in ewes. Hunter *et al.* (1980) detected spermatozoa in sheep oviducts 6–8 h after insemination. Hunter & Nichol (1983) found fertilized eggs 22–24 h after mating at the onset of oestrus. At least for some of the cryopreserved

spermatozoa therefore the acrosome reaction could be induced earlier, perhaps in a reservoir of the female genital tract even before ovulation and so spermatozoa could be released as fully acrosome-reacted or the acrosome reaction is achieved during the passage through the female genital tract. It may be for this reason that Salamon & Lightfoot (1967), using frozen-thawed ram semen, obtained a higher percentage of fertilized eggs after intrauterine insemination than after cervical insemination. Similarly, Armstrong & Evans (1984) achieved higher conception rates after intrauterine insemination with deep-frozen ram semen than after cervical insemination.

Further experiments concerning assessment of the fertilizing ability of glycerol-treated ram spermatozoa *in vivo* in relation to the time of ovulation are in progress.

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