

Evaluation of glucose as a cryoprotectant for boar semen

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Fertility parameters of boar spermatozoa were evaluated in vitro, after freeze-thawing the semen in three different extenders containing permeable and non-permeable cryoprotectants: A (111.0mM Tris, 31.4mM citric acid, 185.0mM glucose, 20 per cent egg yolk, 3 per cent glycerol and 100 iu/ml penicillin G); B (200mM Tris, 70.8mM citric acid, 55.5mM glucose, 20 per cent egg yolk, three per cent glycerol and 100 iu/ml penicillin G); C (200mM Tris, 70.8mM citric acid, 55.5mM fructose, 20 per cent egg yolk, 3 per cent glycerol and 100 iu/ml penicillin G). The freeze-thawing techniques were the same for each extender. Eight ejaculates from four boars were obtained; the sperm-rich fraction of each ejaculate was extended in each of the three media at a final concentration of 400×10^6 sperm/ml, loaded into 0.5 ml straws and frozen at a rate of 30°C/minute to -196°C. The straws were thawed at 60°C for eight seconds. Sperm motility, acrosomal integrity and in vitro sperm penetration through the zona pellucida of gilt oocytes matured in vitro were evaluated. The motility of unfrozen spermatozoa was 93.1 per cent compared with 60.7 per cent, 48.2 per cent and 35 per cent for sperm frozen in extenders A, B and C respectively; these values were all significantly different ($P < 0.05$). There was no significant decline in sperm motility after incubation for 30 minutes in extender A, but there were significant decreases in sperm motility after 30 minutes of incubation in B and C. The percentage acrosomal integrities were 97.2 per cent for the control and 45.5 per cent, 30.3 per cent and 16.8 per cent for the frozen-thawed spermatozoa in extenders A, B and C respectively. The results of the in vitro penetration assay were 80.7 per cent when using control spermatozoa, and 42.2 per cent, 18.4 per cent and 3.3 per cent when using frozen-thawed spermatozoa in extenders A, B and C respectively

THE need to store semen from superior boars of certain bloodlines, and the need for the international exchange of frozen semen, has led to many attempts to develop methods for the commercial use of frozen boar semen (Pursel and Johnson 1975, Fiser and Fairfull 1990). Despite these efforts, conception rates with frozen semen are still below those obtained with fresh semen. At temperatures below 10°C, boar spermatozoa are highly sensitive to cold shock (Gilmore and others 1996), when the molecular organisation of the lipid matrix of the plasma membrane is disrupted irreversibly (Bamba and Cran 1992).

The survival of living cells during cryopreservation is dependent on the interaction between the cryoprotectant and the rates of cooling and thawing (Mazur 1985, Fiser and others 1993). Glycerol is the permeable cryoprotectant most commonly used for boar semen; its mechanism of action is not well understood, but it seems to regulate cellular dehydration by replacing intracellular water osmotically. This reduces the cellular volume and minimises the formation of intracellular ice crystals, although glycerol is a poor membrane stabiliser and, at high concentrations, causes membrane fusion (Anchordoguy and others 1987, Parks and Graham 1992). Low molecular weight non-permeable cryoprotectants, such as galactose, glucose, sucrose, trehalose or other sugars, have not been widely used in freezing boar spermatozoa, although they are considered to favour dehydration before cooling, and cause less intracellular ice crystal formation. The cryoprotective effects of sugars on sperm cells may differ according to the molecular weight of the sugars (Anchordoguy and others 1987, Molinia and others 1994); these compounds do not enter the cell and thus increase the effective osmolarity of the media and serve to protect the integrity of the acrosome (Gilmore and others 1996, Woelders and others 1997).

To evaluate cryopreservation techniques, various quality traits of the cryopreserved spermatozoa must be investigated. The aim of this work was to evaluate the freeze-thawing of boar spermatozoa, using a high concentration of glucose as a non-penetrating cryoprotectant, and glycerol as a penetrating cryoprotectant. The efficiency of the method was evaluated by studying the motility of the sperm, the integrity of the

acrosome, penetration in vitro by the sperm of pig oocytes matured in vitro.

MATERIALS AND METHODS

Semen collection

Semen was obtained from the ejaculates of four boars of proven fertility by the gloved hand method. The sperm-rich fraction was diluted 1:2 with Beltsville Thawing Solution extender (BTS) (Magapor) (Pursel and Johnson 1975). The samples were brought to the laboratory at 30°C and used the same day. Eight replicates of each experimental procedure were carried out, using one ejaculate per week.

Semen processing

At the laboratory, sperm motility was estimated subjectively under a phase-contrast microscope and sperm concentration was evaluated by a haemocytometer. Each ejaculate was split into four aliquots and then centrifuged for 15 minutes at 700 g; the supernatant was discarded and the pellet of each fraction was diluted. One fraction was diluted in BTS to produce a final sperm concentration of 4×10^8 sperm/ml and then held at a temperature of 18°C as a control sample; the other three fractions were resuspended, each in a different extender (A, B or C), to produce a final sperm concentration of 8×10^8 sperm/ml and were then frozen as described below. Extender A contained 111.0mM Tris, 31.4mM citric acid, 185.0mM glucose, 20 per cent egg yolk, 100 iu/ml penicillin G (Harayama and others 1992); extender B contained 200mM Tris, 70.8mM citric acid, 55.5mM glucose, 20 per cent egg yolk, 100 iu/ml penicillin G (Cordova and others 1997); extender C contained 200mM Tris, 70.8mM citric acid, 55.5mM fructose, 20 per cent egg yolk, 100 iu/ml penicillin G (Woelders and others 1997). The sperm suspensions were placed in a container filled with water at room temperature (20°C), which was in turn placed in a cool room maintained at 4°C so that the suspensions cooled slowly, reaching 4°C after two hours. The samples were then mixed with an equal volume (also at 4°C) of each of the three extenders, supplemented with 6 per cent glycerol (v/v), to

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produce final concentrations of 3 per cent glycerol and 4×10^8 sperm/ml.

Freezing and thawing

Each semen sample was loaded into 0.5 ml straws (L'Aigle) and sealed with polyvinyl alcohol (PVA) powder. The straws were then placed horizontally on a stand, 3 cm above the surface of liquid nitrogen, where they cooled at a rate of 30°C per minute (Fiser and Fairfull 1990); when they reached -120°C , as measured with a thermocouple (Speedomax; Leeds and Northrup), the straws were plunged into liquid nitrogen (-196°C) in a liquid nitrogen storage tank and left there for seven days. They were thawed at a rate of 1200°C per minute by immersing each straw in a water bath at 60°C for eight seconds (Fiser and others 1986, Fiser and Fairfull 1990). After thawing, the samples were diluted 1:3 with BTS at 30°C .

Evaluation of the frozen-thawed sperm

Sperm motility was assessed subjectively, immediately after thawing, in a phase contrast microscope at 400 X, and after the sperm had been incubated for 30 minutes at 37°C in an atmosphere of 5 per cent carbon dioxide in air (thermoreistance test). Acrosomal integrity was evaluated by incubating 1 ml of the sperm for 10 minutes with $2 \mu\text{l}$ of 300nM Liso Tracker (Molecular Probes), an organelle-specific fluorescent staining technique. More than 200 spermatozoa in each replicate of each cryopreservative combination were observed in an epifluorescence microscope. This technique is based on the fact that the probe accumulates in cellular compartments with a low internal pH and can be used to investigate Golgi-derived organelles (Haugland 1996, Thomas and others 1997).

The sperm's fertilising capacity was assessed by in vitro fertilisation (IVF) assays with gilt oocytes matured in vitro (Crosby and Barros 1999, De los Reyes and others 2001). Frozen-thawed and unfrozen control semen were selected by using a 30 to 60 Percoll (P-4937; Sigma) gradient and centrifuged at 700 g for six minutes. The sperm concentration was determined with a haemocytometer, and the spermatozoa were diluted in fertilisation medium (De los Reyes and other 2001) supplemented with 2mM caffeine and 4.5mM calcium chloride to induce sperm capacitation. Spermatozoa were added to 50 μl drops containing matured oocytes, at a final sperm concentration of 2×10^6 spermatozoa/ml and coincubated for 20 hours at 38°C in an atmosphere of 5 per cent carbon dioxide in air. At the end of the incubation period the oocytes were fixed with 5 per cent neutral formalin, stained with 200 $\mu\text{g}/\text{ml}$ of propidium iodide and examined with a confocal microscope (LSM-410 Axiovert; Zeiss). Penetrated eggs were defined as eggs having sperm heads in the perivitelline space or in the oocyte cytoplasm, or containing a sperm pronucleus (Crosby and Barros 1999).

Statistical analysis

The results obtained with the different cryopreservation media were analysed by analysis of variance, the ejaculates being considered the replication factor. The percentages were transformed by the Bliss arcsine formula (Sokal and Rohlf 1981). The differences were evaluated with a Tukey test, and statistical significance was assumed at $P < 0.05$.

RESULTS

Table 1 summarises the mean values of the motility, acrosome integrity and in vitro sperm penetration through the zona pellucida of unfrozen control, and frozen-thawed spermatozoa.

Sperm motility

Frozen-thawed spermatozoa showed a significant ($P < 0.05$) decrease in the percentage of sperm with progressive motil-

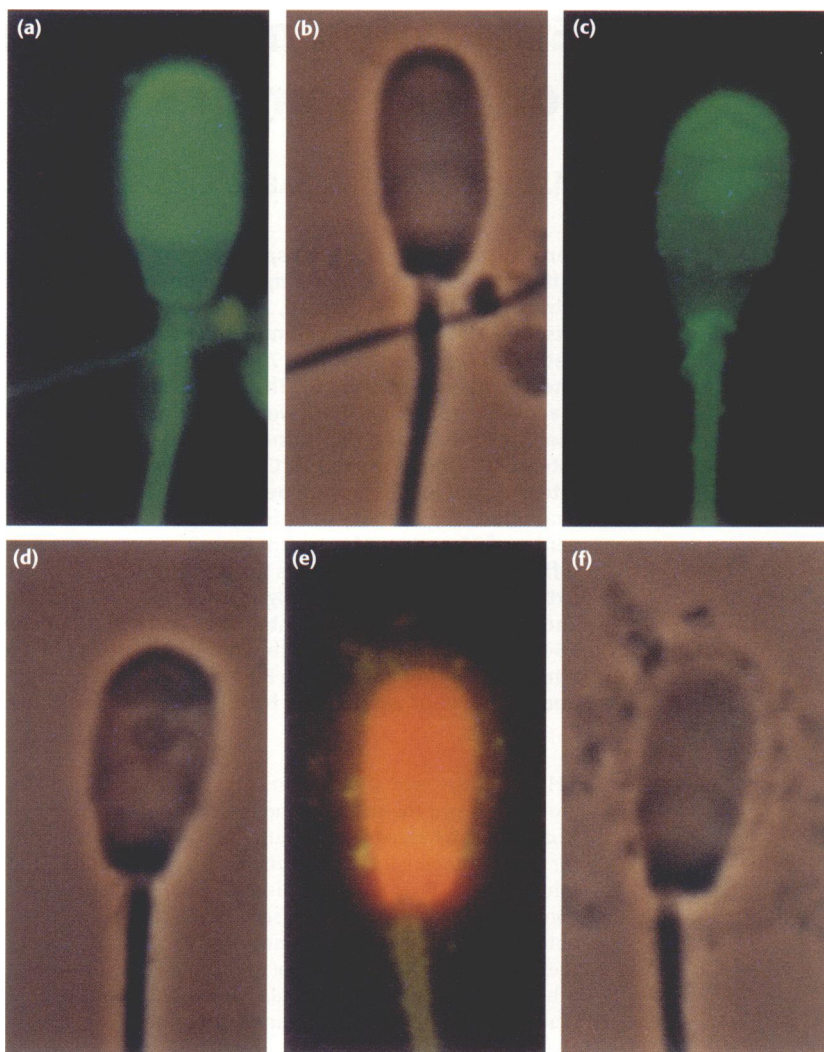


FIG 1: Frozen-thawed boar sperm incubated for 10 minutes with an organelle-specific fluorescent stain and propidium iodide, and studied by epifluorescence and phase-contrast microscopy. The acrosome appears intact (a, b), slightly modified (c, d) and completely modified in a dead (red) sperm (e, f)

ity compared with the control spermatozoa. After 30 minutes of incubation, the motility of the sperm showed no significant decline when medium A was used as the cryopreservative, but with media B and C there was a significant decrease ($P < 0.05$) in sperm motility after 30 minutes of incubation.

Acrosomal integrity

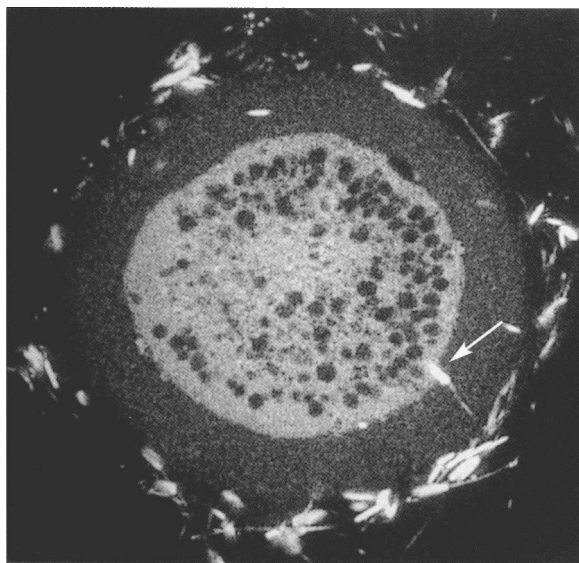
Structurally intact and altered acrosomes, as judged by the fluorescence exhibited after treatment with 300nM Liso

TABLE 1: Mean (sd) percentages of sperm mobility in frozen-thawed boar semen immediately after thawing and after incubation at 37°C for 30 minutes, and mean (sd) percentages of spermatozoa with an intact acrosome and mean (sd) percentages of oocyte penetration in vitro, in semen frozen in three different extenders, compared with control unfrozen semen

Extender	Initial motility	Motility after 30 minutes	Acrosomal integrity	Penetration
Control	93.1 (1.9) ^a	92.2 (1.9) ^a	97.2 (1.9) ^a	80.8 (10.4) ^a (59/73)
A	60.7 (6.1) ^b	51.8 (3.4) ^b	45.5 (5.1) ^b	42.2 (21.3) ^b (46/109)
B	48.2 (6.6) ^b	39.8 (3.5) ^c	30.3 (8.0) ^c	18.3 (13.5) ^c (20/109)
C	35 (10.1) ^c	25.7 (7.7) ^d	16.8 (8.6) ^d	3.3 (8.9) ^d (3/90)

Different superscripts in the same column indicate significant differences ($P < 0.05$)

FIG 2: In vitro matured and in vitro fertilised pig oocyte. Confocal photomicrograph of the oocyte stained with 200 µg/ml propidium iodide. There is a sperm in the perivitelline space (arrow) and many other spermatozoa are bound to the outer surface of the zona pellucida



Tracker, are shown in Fig 1. The principal changes in the frozen-thawed sperm were the degradation, swelling and loss of acrosomes. In all the media the freeze-thawing procedure had a damaging effect on the acrosome ($P < 0.05$) compared with unfrozen spermatozoa (Table 1), but the proportions of spermatozoa with acrosomal abnormalities were different in the different cryopreservation media ($P < 0.05$); there were fewest changes with extender A, and the greatest amount of abnormalities were observed using extender C.

Sperm penetration through the zona pellucida

A total of 381 inseminated eggs were studied with the confocal microscope. The sperm penetration is shown in Fig 2. The best penetration rate was obtained with unfrozen spermatozoa, followed by those frozen-thawed in medium A, and lower rates were obtained when using media B and C. The differences were all significant ($P < 0.05$).

DISCUSSION

Glycerol has been widely used as a low molecular weight permeable cryoprotectant (Polge and others 1949, Crabo and Einarsson 1971, Pursel and others 1973, Almlid and Johnson 1988, Fiser and others 1993), but there have been few investigations of the use of sugars at high concentrations for cryopreserving boar spermatozoa. In this study, glucose was used as a low molecular weight non-penetrating cryoprotectant, which in theory should generate fewer problems with osmolarity (Palasz and Mapletoft 1996). To freeze the boar spermatozoa, two different glucose concentrations were used in extenders A and B (185.0mM and 55.5mM respectively) and 55.5mM of fructose instead of the glucose in extender C, all with 3 per cent glycerol. The results for sperm motility showed that glucose could have a positive cryopreservative effect for boar spermatozoa, possibly by stabilising the sperm plasma membrane and facilitating good dehydration, thus avoiding intracellular ice crystal formation. The motility values observed with medium A were higher than those described by Fiser and Fairfull (1990) and Fiser and others (1993), who used the same freezing and cooling rates and 3 per cent glycerol, but with Beltsville F5 freezing medium (Pursel and Johnson 1975), with a lower glucose concentration than medium A. Cordova and others (1997) observed higher sperm motility percentages than in this study, using a medium like medium B and analysing motility after selecting sperm with a higher proportion of motile and morphologi-

cally normal spermatozoa. Woelders and others (1997) showed that sugars help to protect sperm against the damage induced by rapid cooling rates, and it has been found that monosaccharides are more suitable than disaccharides for preserving the motility of ram spermatozoa (Molinia and others 1994).

Frozen-thawed spermatozoa survive for only a short time in the reproductive tract (Pursel and other 1978). The incubation in vitro of frozen-thawed spermatozoa at body temperature partially mimics the situation in vivo, and for that reason the thermoresistance test gives a better indication of the fertility of boar sperm than its immediate post-thaw motility (Larsson and Einarsson 1976). In this study, the mobility of the sperm decreased significantly in media B and C when the spermatozoa were incubated at 38°C for 30 minutes. Clarke and Johnson (1987) also reported a reduction in the motility of frozen ejaculated spermatozoa after they had been incubated. The beneficial effects of sugar supplementation on the post-thaw viability of spermatozoa have been reported by Aslam and others (1992). With medium A there was no significant decrease in progressive motility; the higher concentration of glucose in this medium may have been responsible for these results.

Acrosomal integrity is another indicator used to evaluate the success of freeze-thawing methods, because it is of paramount importance for the fertilising ability of spermatozoa. The acrosomal changes observed after cryopreservation contribute to the generally lower fertility rates obtained after artificial insemination with frozen semen than with fresh semen (Ström-Holst and others 1997, Holt 2000). The high percentage of acrosomal integrity in the control group (97.2 per cent) suggests that the buffer solution used (BTS) did not damage the spermatozoa. These results agree with those obtained with dog semen by Ström-Holst and others (1998), where the major damage to the structure of the acrosome occurred during freezing and thawing rather than during the initial dilution and cooling. The changes in acrosomal integrity were similar to the changes in motility. The proportion of spermatozoa with an intact acrosome was significantly higher in the semen cryopreserved with medium A, with media B and C giving poorer protection to the spermatozoa. The effect of sugars on acrosomal structure was investigated by Yildiz and others (2000), who reported that monosaccharides added to an extender, improved the viability and increased the percentage of intact acrosomes in dog sperm. In control unfrozen sperm the percentage of sperm with intact acrosomes was higher than the sperm's percentage motility, suggesting that even under physiological conditions, motility may not be an entirely reliable parameter of sperm integrity; poor motility does not always indicate cellular damage.

Even though some frozen-thawed spermatozoa are motile and have undamaged acrosomal membranes, their normal fertilising ability may be affected by freezing and thawing. The interaction between sperm and egg is a complex process that requires several sperm functions: initial recognition, attachment followed by binding, acrosome reaction and penetration of the zona matrix (Barros and others 1996, Crosby and Barros 1999, De los Reyes and Barros 2000). To measure the fertilising ability of the sperm, gilt oocytes matured in vitro were used, and the ability of the frozen-thawed sperm to cross the homologous zona pellucida was studied in vitro. Although Nagai and others (1988) reported that frozen spermatozoa cannot penetrate pig oocytes matured in vitro, the present results agree with those of Wang and others (1991), who observed that ejaculated semen frozen in Beltsville F5 extender could penetrate pig oocytes (Pursel and Johnson 1975). They used a higher sperm concentration (25 to 50 × 10⁶ spermatozoa/ml) than that used in this work (2 × 10⁶ spermatozoa/ml), which was chosen after preliminary experiments showed that penetration was higher with more than 1 × 10⁶

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sperm/ml; other researchers have used lower concentrations of sperm in fertilisation drops to avoid a high rate of polysperm, but it is possible that with frozen-thawed semen the concentration needs to be higher than with fresh semen.

There was a significant decrease in the sperm penetration rate after freezing and thawing, but when the IVF assay was performed there was a significant percentage of penetrated oocytes. Better rates of in vitro penetration were achieved when the spermatozoa were cryopreserved in medium A than when they were frozen in media B and C.

These results suggest that glucose, used as a low molecular weight non-penetrating cryoprotectant, could be an important adjuvant for the freezing of boar spermatozoa. The use of glucose or fructose alone at low concentrations, and especially fructose, did not provide a protective effect. The use of glucose in the extender in conjunction with glycerol could improve boar sperm survival and maintain its fertilising ability, in terms of normal motility, acrosomal integrity and penetration capacity in vitro.

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