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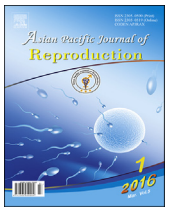

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Effect of cooling to different sub-zero temperatures on boar sperm cryosurvival

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ABSTRACT

Objective: To compare different cooling temperatures before ice formation on pig sperm quality, before and after cryopreservation.

Methods: Semen diluted in BF5 was cooled from 23 °C to 5 °C (1% glycerol, 200×10^6 cells/mL). Sperm were packaged in plastic straws, and maintained at +5 °C per 16 h. 1. Freezing point of diluted spermatozoa was determined by exposing straws to nitrogen vapors. 2. Straws (at +5 °C) were further cooled to −3 °C, −5 °C, and −7 °C, and rewarmed. 3. Straws (at +5 °C) were further cooled to −3 °C and −5 °C, then frozen and stored in liquid nitrogen, and one month later thawed. Progressive motility (PM), viability (Eosin/Nigrosine), plasma membrane functionality (HOST), and acrosome integrity (phase-contrast microscopy) were assessed.

Results: 1. Freezing point was −8.2 ± 0.3 (mean ± SEM); one of the ejaculates froze at different temperature from that of the others ($P < 0.05$). 2. PM (%) was 75%, 71%, 63%, and 40% ($P < 0.05$); viability (%) was 90%, 89%, 89%, and 81% ($P < 0.05$); HOST (%) was 49%, 43%, 40%, and 25% ($P < 0.05$); Acrosome integrity (%) was 90%, 89%, 83%, and 81% for +5, −3, −5, and −7 °C respectively. 3. PM (%) was 35%, 37%, and 39%; viability (%) was 57%, 60%, and 63%; HOST (%) was 22%, 22%, and 22%; acrosome integrity (%) was 86%, 85%, and 86% for +5, −3, and −5 °C respectively.

Conclusions: Cooling of pig sperm to −7 °C (no freezing) damaged sperm function and structure; in contrast, cooling to either −3 °C or −5 °C did not change pig sperm survival after freeze-thawing.

1. Introduction

Boar sperm cryopreservation, producing both good sperm cryosurvival and high on-farm fertility, is still a problem to solve. Two basic protocols for freeze-thawing, with some modifications, are still used [1,2]. During cryopreservation sperm plasma membrane suffers a series of changes in fluidity due to changes in temperature: when it decreases plasma membrane moves progressively from liquid-crystalline to gel phase, when temperature increases plasma membrane becomes hyper fluid adopting a hexagonal arrangement [3]. Most of the changes associated to cooling occur from 20 °C to 0 °C but additional phase transitions could take place at sub-zero temperatures [4,5].

For this, some have proposed to cool down the sperm beyond the traditional cooling temperature (4–5 °C) to allow sperm plasma membrane to accommodate those changes in fluidity without losing selective permeability. Regarding this approach, a number of attempts have been carried out with variable degree of success. Cooling to −2 °C or −5 °C before freezing has improved cryosurvival of buck, ram and boar spermatozoa [6–8]; in contrast, cooling to −3 °C produced no effect on equine sperm cryosurvival [9]. Variation in lipid composition of sperm plasma membrane between animal species [10] could explain those results.

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Some protocols for pig sperm cryopreservation, employing freezing machines, have incorporated cooling to −5 or −6 °C before ice formation [11,12]. However, the effect of cooling to other sub-zero temperatures, around the freezing point of common sperm diluents (about −5 °C), has not been tested.

The objective of this work was to compare the effect of cooling to different sub-zero temperatures, before freezing, on pig sperm cryosurvival.

2. Material and methods

2.1. Semen samples

Semen was collected by the gloved-hand method from 6 boars housed under the same feeding, sanitary and activity conditions; immediately after collection each ejaculate was diluted 1:1 (v/v) with a commercial diluent and transported at about 30 °C.

2.2. Semen processing

Semen arrived to the laboratory after 90 min approximately, it was left at room temperature to temperate, it was then centrifuged for 10 min at 500 ×*g* and supernatant was removed. Pellet was resuspended in BF5 freezing medium [1] without glycerol (400 × 10^6/mL). One mL of sperm in BF5 was taken and mixed (1:10, v/v) with BTS at 38 °C, then sperm assessment was carried out.

2.3. Semen assessment

Progressive motility was assessed subjectively under light microscopy; a smear stained by Eosin/Nigrosine (EN) was employed to assess viability and normal/abnormal spermatozoa [13] under light microscopy using the 10× and 20× objectives, 200 cells were counted for each determination.

Sperm plasma membrane functionality was assessed by the hypo-osmotic swelling test (HOST) as follows: 100 µL of diluted semen were mixed (1:1 v/v) with 100 µL of a hypo-osmotic solution (60 mOsm/kg), this mix was kept in a water bath at 38 °C for 30 min, and then 30 µL of glutaraldehyde (0.4%) were added to immobilize the spermatozoa; 200 cells were counted under phase-contrast microscopy using the 100× objective.

To assess acrosome integrity, 100 µL of sperm in BTS were taken and mixed (1:1 v/v) with 100 µL of glutaraldehyde (0.4%); percentage of cells showing a smooth and well-defined apical ridge was calculated after counting 200 spermatozoa in phase-contrast microscopy using the 100× objective.

Sperm concentration was estimated by counting spermatozoa in the Neubauer chamber employing a dilution 1:200 (sperm: formaldehyde saline solution).

Progressive motility, viability, plasma membrane functionality, and acrosome integrity were assessed before and after (1) cooling–rewarming, and (2) freeze-thawing.

2.4. Cooling of spermatozoa

Diluted spermatozoa were slowly cooled from 23 °C to 5 °C at a rate of 0.04 °C/min; when diluted sperm reached 7 °C, BF5 with glycerol was added in three fractions to obtain a final concentration of 200 × 10^6 sperm/mL and 1% glycerol; diluted spermatozoa were packaged in 0.5 mL plastic straws that were sealed with PVA. Straws were put inside glass tubes that were positioned into a special recipient filled with saline water (10% w/v; 500 mL approx.); in this way, straws were kept in vertical position and dry, thus avoiding the stressful step of drying the straws before freezing. That recipient was introduced in a commercial refrigerator. Temperature inside the straws was monitored by means of a digital thermometer (Traceable VWR, Texas USA). Straws were kept at +5 °C overnight.

2.5. Experimental design

In the first stage, freezing point of diluted spermatozoa was determined by exposing the straws (n = 50, 10 per each ejaculate from 5 boars) to nitrogen vapors, 4 cm above the level of liquid nitrogen. Temperature was monitored by means of a thermocouple positioned inside each straw; readouts were saved in a computer. For each frozen straw, the release and the plateau of latent heat of fusion were registered.

In the second stage, straws (at +5 °C) were further cooled to (1) −3 (0.19 °C/min), (2) −5 (0.15 °C/min), and (3) −7 °C (0.12 °C/min), and rewarmed immediately to 38 °C; straws at +5 °C served as control. To cool the straws to sub-zero temperatures, the special recipient employed to carry the straws during cooling from 23 to 5 °C into the refrigerator, was introduced into an insulated box filled with crushed saline ice (10% w/v) at −12 °C; this method has been previously validated in our laboratory. Twelve ejaculates from 6 boars, 3 straws per treatment plus one straw as monitor, were used in this stage.

In the third stage, straws (at +5 °C) were further cooled to (1) −3 and (2) −5 °C, frozen in nitrogen vapor 4 cm over liquid nitrogen level for 15 min, and stored in liquid nitrogen for at least one month; straws at +5 °C served as control. Fourteen ejaculates from 6 boars, 3 straws per treatment plus one straw as monitor, were used in this stage. Straws were thawed by immersion in water at 38 °C for 30 s; the content of each straw was poured in dry tubes into the water bath.

2.6. Statistical analysis

Data of freezing point was analyzed by the Kruskal–Wallis test to look for possible differences between ejaculates. Data of (1) cooling–rewarming and (2) freeze-thawing were analyzed by ANOVA to look for possible differences between cooling treatments. Data expressed as percentages were arcsine transformed to normalize them before analysis. The general linear model procedure from the Statistica for Windows 5.5 software (StatSoft Inc., Tulsa OK, USA, 2000) was used.

3. Results

3.1. First stage – freezing point

Freezing point was −8.2 ± 0.3 (mean ± SEM); however, one of the ejaculates froze at different temperature from that of others. The freezing range between straws was −5.1 °C to −11.2 °C (Table 1).