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Cryopreservation of boar sperm comparing different cryoprotectants associated in media based on powdered coconut water, lactose and trehalose ☆

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ABSTRACT

In swine spermatozoa, the damage caused by cryopreservation is more severe than other species, provoking reduced potential for fertilization. Adjustments in the freezing extender composition may be an important alternative to increase its efficiency. The objective of this study was to test the efficiency of different cryoprotectant solutions during cryopreservation of swine semen with a controlled cooling curve. Three cryoprotectant solutions (5% dimethylformamide, 3% glycerol and the combination of these two cryoprotectants) were used in association with three base media (powdered coconut water, lactose and trehalose), constituting nine different treatments. The semen was frozen using a controlled-rate freezer (TK-3000). After thawing, semen was evaluated for total sperm motility, vigor, morphology, plasma membrane integrity and acrosome integrity. Cryopreservation with the controlled curve using an automated system showed satisfactory results, guaranteeing practicality and repeatability for the process of freezing swine sperm. With this curve, the solutions of lactose, trehalose and powdered coconut water associated with glycerol, as well as the solution of coconut water containing dimethylformamide, presented higher quality of sperm compared to the other solutions. Powdered coconut water associated with dimethylformamide appears as a new solution for swine sperm cryopreservation. The freezing controlled curve used in this study allowed standardization of the cryopreservation technique.

Introduction

Semen cryopreservation is successful in many mammalian species, but is still inefficient in swine. High individual variation of the ejaculate among boars [18] and among sperm subpopulations [1,10,20], as well as the composition of the spermatozoa and even the season [2], prevent a predictive analysis of resistance to freezing for a sample of swine semen. These variations make it difficult to establish a standardized freezing protocol.

Cryopreservation of semen can cause biochemical and functional damage to the spermatozoa, leading to a reduction in motility, viability and transport in the female genital tract [15]. However, in swine spermatozoa, this damage is more serious, provoking reduced potential for fertilization.

Some reports have shown that the cryo damage induced by freezing and thawing can be minimized by adding the lipoproteins, or optimizing cooling rate and cryoprotectant [23]. According to Gómez-Fernández et al. [11], adjustments in the freezing extender composition could be an important alternative to increase the efficiency of semen cryopreservation in this species. Corcuera et al. [8] highlight the importance of non-permeable and permeable cryoprotectants, a combination that is commonly used. According to the authors, non-permeable cryoprotectants, such as sugars, protect the cell membrane from volume changes upon freezing and thawing, whereas permeable cryoprotectants, such as glycerol, are very effective in lowering the intracellular water freezing point and reduce intracellular ice crystal formation.

In the attempt to minimize and control damaging effects on swine sperm during the freezing process, the objective of this work was to evaluate the cryopreservation of boar semen in nine cryoprotectant solutions formed by the addition of dimethylformamide (DMF), glycerol (GLY) and their association, in media based

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on powdered coconut water, lactose and trehalose, using an automated cooling curve.

Materials and methods

Ethics in experimentation

The study was performed according with EU Directive 2010/63/EU for animal experiments, as solicited by the Cryobiology Journal.

Reagents and media

All components of the media were acquired from Sigma–Aldrich (St. Louis, MO, USA), and the media were prepared under sterile conditions with milli-Q water. The Beltsville Thawing Solution-BTS [19] with penicillin and streptomycin added was used as an extender to transport fresh semen. The cooling extender was composed of 80% lactose (11%) and 20% fresh egg yolk. The freezing extenders were constituted from 5% DMF, 3% GLY and the combination of these two cryoprotectants, associated with three base media: (a) 67.4 g ml^{-1}powdered coconut water (ACP-103®, pH 7.00, 300 mOsm/kg; the coconut water was extracted from the fruit in sterile form and lyophilized, without further manipulations; produced at the University of Ceará State – UECE, Brazil); (b) 250 mM trehalose; (c) 11% lactose. The freezing solutions had 20% of egg yolk and 1.5% of Equex STM (Nova Chemical Sales Inc., Scituate, MA, USA) added, forming nine treatments.

Semen collection

Semen from three adult commercial hybrid boars with known fertility and proven artificial insemination success were used. Three ejaculates from each animal presenting more than 80% total motility, 70% spermatozoa with intact plasma membrane and less than 35% pathologic spermatozoa were used in the experiment.

Freezing protocol

The gel-free semen fraction was diluted 1:1 (v/v) in BTS, remaining for 1 h at 24 °C in a hermetic box (Minitüb® DTC-17, Germany). In the laboratory, an aliquot of 100 μL of diluted semen was separated for evaluations of total sperm motility, vigor and integrity of the plasma membrane and acrosome. After 1 h, the semen was centrifuged at 800*g* for 10 min, and the supernatant was poured off. A sufficient quantity of the cooling extender was added to the pellet to reach the concentration of 450×10^6 spermatozoa/mL. The nine freezing solutions were added separately, so that the concentration reached 300×10^6 spermatozoa/mL. The samples were placed in cryoprotectant solutions at 24 °C and kept at the same temperature until all treatments were packaged in 0.25 mL French straws (Minitüb, Tiefenbach, Germany). All treatments were loaded in 20 min, at most. Then, the straws were immediately placed in the freezing machine (TK-3000 Modular SE, Brazil) and started the cooling curve. The temperature was reduced by 0.25 °C min^{-1} until it reached 5 °C, remaining at this temperature for 2 h before starting the negative curve, with a reduction of 20 °C min^{-1} until −120 °C. Finally, the straws were plunged into liquid nitrogen for storage at −196 °C. The samples were stored in liquid nitrogen for at least one week before evaluations. Thawing was performed in a water bath at 36 °C for 30 s. The thawed semen samples were re-suspended with 0.9% saline solution at the same temperature in the proportion of 1:4 (v/v) and immediately evaluated for motility, vigor and membrane integrity. After 5 min acrosome integrity was evaluated. A simplified schematic representation of the freezing process and analysis of semen is shown in Fig. 1.

Evaluations of frozen-thawed sperm

Each ejaculate was evaluated in triplicate (27 straws were evaluated for each treatment), and the following evaluations were carried out: total sperm motility, vigor and sperm morphology in a phase contrast microscope (Nikon Eclipse E200); and integrity of the plasma membrane and of the acrosome by fluorescent probes in an *epi*-fluorescent microscope (Nikon Eclipse E200/C-SHG1). The evaluations were performed by a technician qualified in the laboratory routine.

Evaluation of sperm motility and vigor

Sperm motility (0–100%) and vigor (0–5) were evaluated subjectively with a sample of 7 μL of thawed semen on a heated slide under cover slip. The slides and cover slips were heated on a digital table and digital thermal platinum (Neovet, Uberaba, Brazil) adapted to the microscope.

Evaluation of sperm morphology

An aliquot of 200 μL of thawed diluted semen was placed in a 1.5 mL tube with 500 μL of 4% formalin solution for later evaluation. An aliquot of 7 μL was placed on a slide with a cover slip and 200 spermatozoa were analyzed. Sperm pathologies were divided into head, midpiece and tail region defects.

Evaluations of the integrity of plasma membrane and acrosome

Sperm membrane integrity was assessed using 6-carboxy-fluorescein diacetate (C-FDA) and propidium iodide (PI) (Invitrogen Molecular Probe®, Eugene, OR, USA), as described by Carvalho et al. [7]. Two hundred spermatozoa were analyzed under an *epi*-fluorescent microscope and classified into intact (head stained with green fluorescence) and damaged (head stained with red fluorescence) plasma membrane. Acrosome status was examined using fluorescent probe isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and PI, as described by Carvalho et al. [7]. Sperm labeled in red with PI were considered alive. Living cells were classified as acrosome reacted, if the acrosome had uniform FITC-PNA green fluorescence, or as acrosome intact, if no fluorescence was visible.

Statistical analysis

Analysis of data was performed using Sigma Stat for Windows version 3.11 (Systat software, Inc, USA). One-way repeated measures ANOVA was used to compare the effects of different treatments. The data are given as mean values ± standard deviation. When ANOVA revealed a significant effect, the treatments were compared by Tukey test. A difference of $P < 0.05$ was considered significant.

Results

Evaluations of sperm motility and vigor

The percentages of motile sperm (Table 1) were superior when coconut water was used with DMF and glycerol, and when glycerol was the cryoprotectant with trehalose or lactose. The percentages of motile sperm were superior when glycerol was the cryoprotectant with coconut water (38.88 ± 10.39), lactose (37.22 ± 7.85) or trehalose (36.11 ± 11.67) and when coconut water was used with DMF (30.27 ± 7.55). Sperm vigor presented the highest values in the treatments that used lactose (2.66 ± 0.43), coconut water (2.56 ± 0.44) or trehalose (2.44 ± 0.56) associated with glycerol.

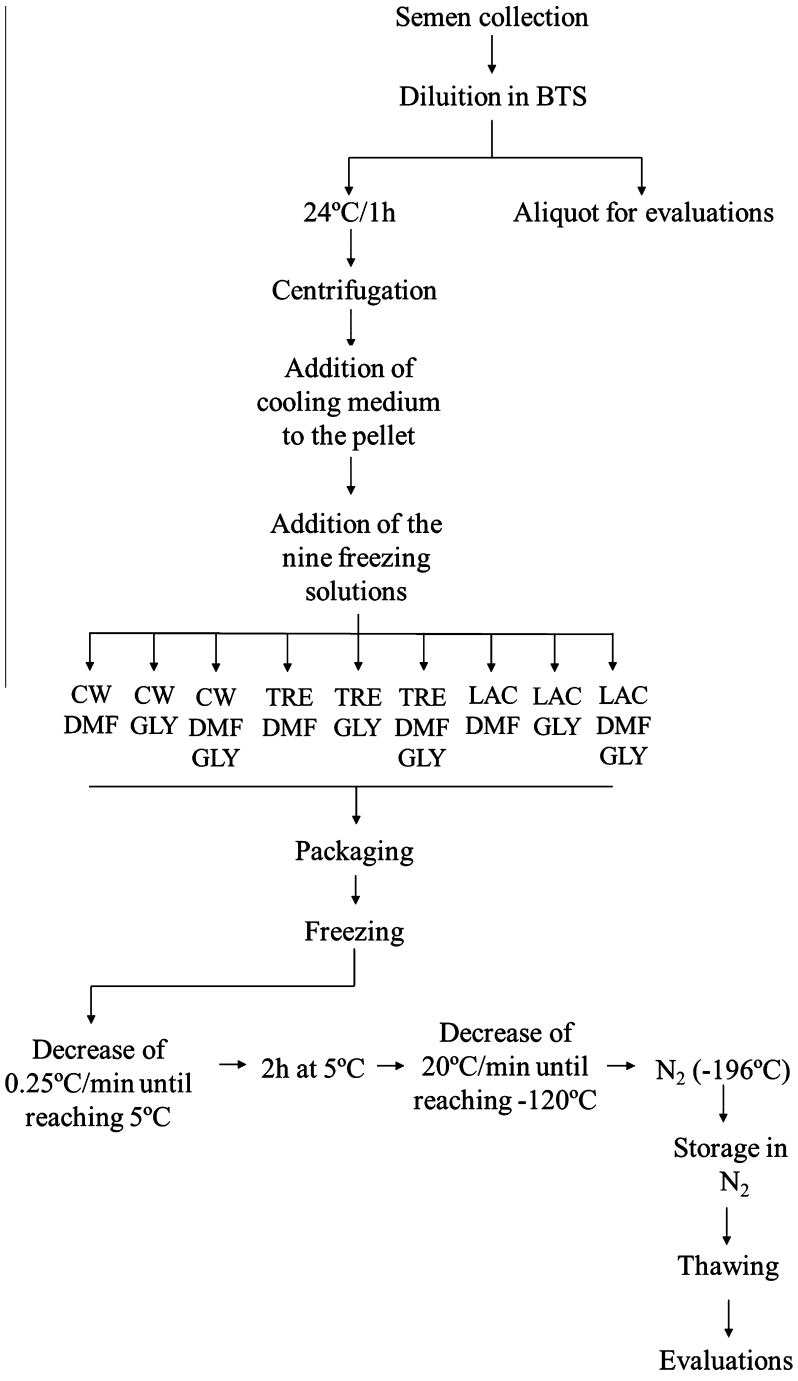

Fig. 1. Scheme for cryopreservation of swine sperm with different cryoprotective solutions in automated system (TK-3000 SE Modulator). BTS: Beltsville Thawing Solution; CW: coconut water; DMF: dimethylformamide; GLY: glycerol; TRE: trehalose; LAC: lactose; N_2: liquid nitrogen.

When the percentage of total pathological spermatozoa was evaluated, the values remained significantly similar, as well as when the different categories of defects were evaluated separately.

Evaluation of plasma membrane and acrosome integrity

The treatments that maintained major number of both intact membranes were (Table 2) those that had glycerol as the only cryoprotectant, independent of the base extender, along with the treatment that associated coconut water with DMF.

In general, higher total sperm motility, vigor, and cells with intact membranes and without reacted acrosomes, were found in treatments that used glycerol as the cryoprotectant, independent of the combined base medium. However, when DMF was used together with coconut water as a base medium, the results were similar to those obtained with glycerol, showing no significant difference among these treatments, except for vigor.

Discussion

Solutions of lactose, trehalose and powdered coconut water associated with glycerol, as well as the solution of coconut water containing DMF, produced higher quality of sperm than the other solutions after swine semen cryopreservation using a controlled cooling curve. The use of an automated cooling curve provided satisfactory results and made the whole procedure highly practical.

Table 1
Results of evaluations of total sperm motility, vigor and morphology after thawing the samples of swine semen, testing nine different treatments frozen in an automated system.

Treatments	Sperm motility (%)	Vigor (0–5)	Abnormal cells (%)	Head defects (%)	Midpiece defects (%)	Tail defects (%)
Treatments	Sperm motility (%)	Vigor (0–5)	Abnormal cells (%)	Head defects (%)	Midpiece defects (%)	Tail defects (%)
Fresh semen	85 ± 3.53	3.61 ± 0.41	18.44 ± 9.67	1.27 ± 0.87	11.27 ± 6.71	5.88 ± 3.87
CW + DMF	30.27 ± 7.55^{ab}	2.00 ± 0.61^{b}	20.27 ± 8.83	3.35 ± 2.05	10.00 ± 6.93	6.92 ± 3.77
CW + GLY	38.88 ± 10.39^{a}	2.56 ± 0.44^{ab}	21.85 ± 10.39	3.22 ± 1.933	11.72 ± 7.23	6.87 ± 4.93
CW + DMF + GLY	21.11 ± 9.04^{b}	2.06 ± 0.54^{b}	20.57 ± 9.55	3.03 ± 2.57	9.20 ± 6.16	8.33 ± 5.57
TRE + DMF	19.44 ± 9.66^{b}	1.69 ± 0.58^{b}	19.83 ± 9.14	2.59 ± 2.19	9.38 ± 6.22	8.13 ± 5.96
TRE + GLY	36.11 ± 11.67^{ab}	2.44 ± 0.56^{ab}	20.18 ± 11.33	2.09 ± 2.26	10.48 ± 7.01	7.61 ± 5.25
TRE + DMF + GLY	19.44 ± 7.98^{b}	1.94 ± 0.54^{b}	20.88 ± 10.45	3.00 ± 3.33	9.70 ± 6.50	8.18 ± 5.71
LAC + DMF	24.05 ± 13.08^{b}	2.08 ± 0.59^{b}	21.50 ± 10.42	3.38 ± 3.58	10.00 ± 7.00	8.11 ± 4.95
LAC + GLY	37.22 ± 7.85^{a}	2.66 ± 0.43^{a}	21.35 ± 9.81	2.75 ± 2.24	11.64 ± 7.98	6.94 ± 4.64
LAC + DMF + GLY	18.77 ± 10.30^{b}	1.91 ± 0.71^{b}	22.00 ± 10.33	2.63 ± 2.63	10.03 ± 7.55	9.33 ± 4.84

CW: coconut water; DMF: dimethylformamide; GLY: glycerol;
TRE: trehalose; LAC: lactose. In the columns, different letters represent a significant difference ($p < 0.05$) between treatments.

Table 2
Evaluations of the integrity of the plasma membrane and the acrosome carried out by staining swine semen samples with fluorescent probes, testing nine different treatments frozen in an automated system.

Treatments	Intact membrane (%)	Intact acrosome and viable sperm (%)
Fresh semen	81.05 ± 5.07	78.16 ± 5.20
CW + DMF	31.05 ± 12.75^{ab}	28.24 ± 14.00^{ab}
CW + GLY	37.02 ± 9.51^{a}	36.53 ± 11.03^{ab}
CW + DMF + GLY	16.41 ± 7.60^{b}	16.77 ± 10.35^{b}
TRE + DMF	20.75 ± 9.88^{b}	20.42 ± 10.04^{b}
TRE + GLY	40.58 ± 9.05^{a}	37.27 ± 10.49^{a}
TRE + DMF + GLY	18.5 ± 8.50^{b}	14.40 ± 8.25^{b}
LAC + DMF	22.83 ± 10.18^{b}	21.37 ± 9.19^{b}
LAC + GLY	41.75 ± 7.42^{a}	40.03 ± 7.62^{a}
LAC + DMF + GLY	19.30 ± 9.46^{b}	16.85 ± 9.83^{b}

CW: coconut water; DMF: dimethylformamide; GLY: glycerol; TRE: trehalose; LAC: lactose. In the columns, different letters represent a significant difference ($p < 0.05$) between treatments.

The addition of sugars to the freezing extender has beneficial effects on sperm membrane integrity after thawing, since these molecules provide energy for the sperm during incubation, and before freezing, because they maintain the osmotic pressure of the diluent, contribute to cellular dehydration and act as cryoprotectants [16]. In this experiment we did not observe any difference in the quality parameters of the cryopreserved swine sperm in response to the type of sugar added, because all the molecules associated with glycerol maintained the same standard of motility and integrity of plasma membrane and acrosome.

However, effects of the internal cryoprotectant on the sperm parameters were observed, with the best results obtained with the presence of glycerol. These results were similar to those observed by Bianchi et al. [4], using lactose and glycerol, maintaining 45% of spermatozoa with intact plasma membrane, although not with an automated curve. Better results were obtained by Malo et al. [16], comparing the trehalose, glucose and lactose sugars, combined with 3% glycerol, which provided total sperm motility of 59.07 ± 6.79% and 55.80 ± 3.71%, respectively, for lactose and trehalose. These results were higher than those found in this study for this parameter in the same sugar molecules (37.22 ± 7.85% and 36.11 ± 11.67%, respectively for lactose and trehalose). These findings confirmed that glycerol is the cryoprotectant of choice for freezing semen in most mammal species. In mouse sperm, Storey et al. [21] verified that omission of glycerol resulted in complete loss of membrane integrity in cryopreservation protocols.

When DMF was used associated with lactose and trehalose or together with glycerol in all freezing solutions in this study, it became evident that sperm total motility, plasma and acrosomal membrane integrities were compromised. Only when DMF was associated with powdered coconut water was it possible to observe a beneficial effect, similar to the other cryopreservation solutions containing only glycerol. The protective action of this amide can be attributed to its molecular structure and to its ability to penetrate the cellular membrane, because its functional groups bond to the hydrogen of the water molecules and the highly hydrophilic nature of the amide molecule allows strong interaction with water, which reduces the intracellular formation of ice crystals [3]. The beneficial effect of coconut water on the spermatozoa is likely due to a neutral fraction that contains various anions and cations, free sugar, sorbitol and inositol, verifying that it does not contain any unknown substance with special properties [12]. Coconut water has been studied for some time as a diluent of goat semen and is often used in herds in the northeastern region of Brazil, with positive results [13].

Bottini-Luzardo et al. [5] demonstrated that use of fresh filtered coconut water did not show improvement in sperm motility, mitochondrial activity, plasma and acrosome integrity, compared to the use of deionized coconut water in swine cryopreservation. This occurs because the high concentration of Ca^{2+} present in fresh coconut water blocks the use of sugars also present during ATP production by mitochondria of spermatozoa [9]. Moreover, the excessive amount of Ca^{2+} may cause premature sperm capacitation and provoke sperm damage during freezing [5]. In this work, we demonstrated that use of powdered coconut water did not show deleterious effects on the sperm when the extender with coconut water also contained glycerol or DMF separately, and none of the parameters analyzed were lower than those observed in other base media. Other advantage is that the powder can be easily stored and readily sent to regions where fresh coconuts are not available. Moreover it is a standardized alternative of easy preparation and low cost, typically originating from tropical fruit [6].

Sperm morphology was not altered in any of the treatments, indicating that they were efficient in guaranteeing general stability of the spermatozoa. Similarly Wu et al. [23] by testing 14 different treatments, found no significant difference in sperm morphology.

A substantial proportion of alterations associated with the cryopreservation of swine semen can be attributed to the cooling of the sperm, more than the freezing–thawing processes [17]. Heat shock is prevented in many freezing protocols by slow and gradual cooling of sperm (about 1 °C min^{-1}) from 15 to 5 °C [22]. In the current work, the decline in temperature of 0.25 °C min^{-1} from 24 to 5 °C may have been one of the factors that contributed to the preservation of motility over 38.88 ± 10.39% and membrane integrity of 37.02 ± 9.51% in the treatments that used coconut water associated with glycerol.

When Kumar et al. [14] used the cooling curve with moderate decreases in temperature (−30 °C min^{-1} from −5 to −50 °C), the rates of sperm viability and acrosome integrity reached 48.2 ± 1.62 and 34.5 ± 1.76, respectively, while in this study these rates reached 41.75 ± 7.42 and 40.03 ± 7.62, in treatments with lactose and glycerol. These authors claim that this cooling rate is generally recommended as a successful process for sperm cryopreservation in boars, rams and bulls, using a minimal concentration of glycerol, compatible with preservation of fertilizing capacity. This negative curve was similar to that used in the current study, which decreased 20 °C min^{-1} from 5 to −120 °C, providing greater preservation of sperm motility and acrosome integrity when compared to the work of Kumar et al. [14]. Thus, we showed that the curve used in this work can also be recommended for cryopreservation of swine sperm.

In conclusion, we can infer that cryopreservation with a controlled curve using an automated system demonstrated satisfactory results, guaranteeing practicality and repeatability for the process of freezing swine sperm, consequently making it possible to have genetic material more widely available on a commercial scale. With this curve, the solutions of lactose, trehalose and powdered coconut water associated with glycerol, as well as the solution of coconut water containing DMF, presented a higher quality of sperm than the other solutions. The powdered coconut water associated with glycerol or DMF appears as a new option for swine sperm cryopreservation. Despite the encouraging results found in this study, experiments using more sensitive techniques to access the fertilization potential in semen samples, be they *in vivo*, such as artificial insemination, or *in vitro*, as with *in vitro* fertilization, are still necessary to prove the efficacy of the cryoprotectant solutions and adapted freezing technique. These will allow simultaneous evaluation of the most important requirements in the sperm cell for the fertilization process.

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