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Objective assessment of the cryoprotective effects of dimethylformamide for freezing goat semen ☆

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ABSTRACT

The aim of this work was to assess the cryoprotective effects of dimethylformamide (DMF) for freezing goat semen, using an objective analysis by computer-assisted sperm analysis (CASA). Twenty-one ejaculates (seven per animal) were collected from three stud bucks with the aid of an artificial vagina and immediately evaluated for gross and microscopic characteristics. The semen was diluted in two steps with a Tris–egg yolk extender containing 6% glycerol or 6% DMF, frozen in 0.50-mL straws, and stored in liquid nitrogen. Samples were accessed for sperm morphology, sperm membrane structural and functional integrity, and by CASA, immediately after thawing. There were differences ($P < 0.05$) between glycerol and DMF with regard to subjective progressive motility (23.9 ± 2.2% vs. 16.6 ± 2.0%), objective progressive motility (3.5 ± 0.4% vs. 1.8 ± 0.3%), linearity (53.9 ± 1.6% vs. 48.1 ± 1.4%) and amplitude of lateral head (2.3 ± 0.1 vs. 2.9 ± 0.1 mm), which confirmed the efficiency of glycerol. In conclusion, dimethylformamide could be used as an alternative cryoprotectant for goat semen freezing. However it was showed that no benefits were derived by using dimethylformamide to replace glycerol at an equal 6% concentration.

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Introduction

Just over 60 years have passed since the first successful experiment on freezing goat sperm [34]. In spite of all this time, protocols for goat semen cryopreservation continue to be developed due to the wide range of results found for sperm motility, considered the parameter of choice to determine the degree of sperm damage inflicted by the cryopreservation procedure [3,9,18,19]. These results have varied from low values as 6% up to 62%, being the last reached with the use of antioxidants into the diluents [6,7,27].

The improvement of buck semen cryopreservation technologies requires in-depth knowledge of the properties of current extenders [29]. Thus, many membrane-permeable cryoprotectants (glycerol, dimethyl sulfoxide, ethylene glycol, and propylene glycol) and their combinations, have been tested for buck sperm [20,32], but the most frequently used penetrating cryoprotectant is yet glycerol. Nevertheless, glycerol is somewhat toxic to spermatozoa [17] and may induce osmotic damage [26]. The addition of glycerol by itself may cause certain structural damage and, hence, low motility of spermatozoa [28] that could result in a lower fertility rate when artificial insemination is used.

In stallions [2], rabbits [24], and boars [5], amides had been suggested as alternative cryoprotectants for semen freezing, primarily for individual males who were more sensitive to the toxic effects of glycerol [37]. Cryoprotective effects of amides are due to their lower molecular weights (73.09) and viscosities in comparison with glycerol (molecular weight 92.05), and for their higher membrane permeability, thereby reducing the possibility of cellular damage caused by osmotic stress [4,11]. Moreover, addition of the methyl (CH3) radical into the amide molecule increases its permeability through the sperm cell membrane and improves the efficiency of its cryoprotective action [5].

In goats, it was previously demonstrated that dimethylformamide (DMF) was not superior to glycerol as a cryoprotectant for goat semen, but in this study sperm characteristics were only subjectively analyzed [31]. It is important to emphasize that assessment of subjective motility has a limited fertility predictive value mainly because this subjective estimation can be affected by the observer's training and experimentation [36]. Nowadays, artificial insemination stations are adopting computer-assisted semen analysis (CASA) to increase objectivity in determination of sperm motility [18]. This tool could help to elucidate the patterns of motion of the goat sperm cryopreserved with DMF and detect

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subtle differences between this cryoprotectants and the glycerol. This information would be useful to prove if DMF could be used as an alternative cryoprotectant for goat semen freezing.

The aim of the current study was to compare the effects of glycerol and DMF in cryopreservation of goat semen based on post-thaw motility and velocity patterns evaluated objectively by CASA, sperm morphology and plasma membrane structural and functional integrity.

Materials and methods

Experimental protocols and animal care were approved by the research committee of the Universidade Federal Rural do Semi-Árido, Mossoró, Brazil.

Experimental animals

Semen was collected from three mature stud bucks (2 years of age) of good health and proved fertility capacity, being one Savannah and two Boer. They were raised on a farm located in the rural area of Mossoro (5°11′S, 37°W, and an altitude of 16 m), Brazilian Northeast. The goats were maintained at extensive management and fed with forage crop based on Caatinga forest, free water and supplemented with complete mineral mixture. Two days before the semen collection, they were housed in a common covered shelter separated from females. The experiment was conducted from May to September 2009.

Semen collection and initial evaluation

One month before starting the experiments, all bucks were trained for semen collection with the aid of an artificial vagina (43 °C) connected to a glass graduated tube using an induced-estrus doe as teaser. Each buck was collected twice a week. Immediately after collection, the ejaculates were maintained immersed in a warm water bath at 37 °C. Semen assessment was performed within approximately 15 min, and only those semen samples with at least 80% sperm progressive motility were selected for freezing. A total of 21 ejaculates (seven per animal) were used in this experiment.

Color, aspect and volume were evaluated in fresh semen. Microscopic criteria such as sperm progressive motility (%) and mass activity (0–5 scale) were performed subjectively by light microscopy (Nikon, Eclipse E200, Tokyo, Japan) under 100× magnification. Structural integrity of plasma membrane was established by analyzing a slide stained with Bromo-phenol Blue under light microscopy (400×), counting 200 cells per slide. Following initial assessment, a 10 μL semen aliquot was diluted in 2 mL of buffered formalin (10%) and sperm concentration (sperm $\times 10^6$ mL^{-1}) was determined using a Neubauer counting chamber. For sperm morphology evaluation, 200 sperm cells from random fields in Bengal Rose smears were analyzed by light microscopy, under 1000× magnification. Total sperm defects were counted in 200 cells, following classification as primary or secondary [23]. For the evaluation of sperm membrane integrity, a hypo-osmotic swelling test (HOST) was performed immediately after the semen collection, using a citric acid and fructose hypo-osmotic solution (100 mOsm/L). A total of 200 spermatozoa were counted using a phase-contrast microscope at 400× magnification, and spermatozoa presenting swollen coiled tails were considered as presenting a functional sperm membrane [13].

Semen processing

An extender consisting of 3.028 g Tris–hydroxymethyl-amino-methane, 1.78 g monohydrated citric acid and 1.25 g D-fructose, dissolved in 100 mL of distilled water, was used [33]. The osmosis of this solution was 295 mOsm/L and the pH 6.6. Two and a half percent of this solution was subsequently replaced by egg-yolk.

Semen was initially divided in two aliquots and extended in Tris–egg yolk at room temperature (32 °C). Samples were kept in an isothermal box and transported to the laboratory. After 40 min, temperature into the isothermal box reached 15 °C (−0.30 °C/min), and the samples were transferred to a refrigerator for a further 30 min, where it reached 4 °C at −0.37 °C/min. Progressive motility was evaluated yet at 4 °C. After cooling, one semen aliquot was added to Tris–egg yolk plus glycerol in a final concentration of 6%, and the other was added to Tris–egg yolk plus DMF in a final concentration of 6%. Final dilution resulted in a sperm concentration of 150×10^6 sperm/mL. Each sample was packed into previously marked 0.5-mL ($n = 4$) plastic straws, which were placed horizontally in a thermal box for 5 min, 5 cm above the liquid nitrogen (N_2) level, reaching a temperature closer to −70 °C in the vapor. The straws were plunged into liquid N_2 for storage.

After 1 month, samples were transported to the Integrated Center for Biotechnology (NIB/UECE – Fortaleza, CE, Brazil) for thawing and further analysis. The straws were removed from the liquid nitrogen and randomly thawed on a water bath at 37 °C/1 min 7 days after freezing. Finally, straws were removed, dried, the plug cut off and the contents pushed out into a glass vial that stood in a water bath at 37 °C. Semen samples (two straws per treatment) were immediately evaluated for sperm progressive motility, morphology and membrane integrity.

Computer-assisted semen analysis

Thawed semen was also evaluated by CASA in accordance with previous recommendations. Briefly, a 10 μL aliquot of semen sample was placed on a pre-warmed Makler counting chamber (Sefi Medical Instruments Ltd., Haifa, Israel), allowed to settle for 1 min, maintained at 37 °C and examined in a phase-contrast microcopy system (Olympus BH-2, Tokyo, Japan), with stroboscopic illumination coupled to a video camera adapted to the Sperm Class Analyzer (SCA version 3.2.0; Microptic S.L., Barcelona, Spain). The settings of the instrument were temperature, 37 °C; frame rate, 25 frames/s; minimum contrast, 75; straightness threshold, 80%; low velocity average pathway (VAP) cutoff, 10; and medium VAP cutoff, 45. Three nonconsecutive randomly selected microscopic fields were scanned. The parameters analyzed were number of counted cells, total motility (%), progressive motility (%), velocity average pathway (VAP; μm/s), velocity straight line (VSL; μm/s), curvilinear velocity (VCL; μm/s), amplitude of lateral head (ALH; μm), beat cross frequency (BCF; Hz), straightness (STR; %), and linearity (LIN; %) [12].

Statistical analysis

Twenty-one replicates were performed for each treatment. The results were expressed as mean ± SEM. Data were checked for normality by Shapiro–Wilk test, and for homoscedasticity by Levene's test using the univariate procedure of the Statistical Analysis System (SAS 6.10, SAS Institute Inc., Cary, NC, USA). Data were analyzed by General Liner Model (GLM). Comparisons among different cryoprotectants on seminal parameters were analyzed by Tukey test. To evaluate the individual effect of the animals and its interactions with cryoprotectants effect on studied variables, data were evaluated by Fisher's PSLD test. For all statistical analysis, a significant difference of 5% was considered.

Results

Fresh and extended semen

Fresh goat semen was yellowish in color and milky in aspect. Total volume of ejaculates was 1.1 ± 0.1 mL, with a sperm concentration of $2.4 \pm 0.2 \times 10^9$ spermatozoa/mL. Sperm progressive motility of fresh semen was 95.0 ± 2.0%, and mass activity was 3.9 ± 0.2. Percentage of sperm presenting intact membrane was 90.7 ± 3.5% and sperm with normal morphology was 76.1 ± 1.7%, being 33.0 ± 1.8% with functional membrane integrity. Total morphological defects were found in 23.9 ± 1.7%, being 0.6 ± 0.2% classified as primary and 23.2 ± 1.6% as secondary. Extended semen presented 93.8 ± 2.0% progressive motile sperms. Immediately after addition of the extender plus cryoprotectant at 4 °C, a decrease to 70.5 ± 2.0% and 77.9 ± 2.0%, respectively, in progressive motile sperm for glycerol and DMF was detected ($P < 0.05$).

Post-thaw sperm characteristics

After thawing procedure, there were no significant differences ($P > 0.05$) between cryoprotectants for live sperms, morphology and membrane integrity (Table 1). Sperm motility patterns (CASA data) of the frozen-thawed semen are shown in Table 2. A significant difference ($P < 0.05$) between two cryoprotectants evaluated was found for end points assessed by CASA, including progressive motility, LIN and ALH. The proportion of sperm in the four populations was established on Table 3. In general, better glycerol preservation was observed in the kinematic characteristics when compared to dimethylformamide ($P < 0.05$). There were no significant interactions between individual goats and cryoprotectants.

Discussion

After the addition of cryoprotectants in goat semen at 4 °C, subjective motility was better preserved with the use of DMF. These results are similar to that found in canine semen [21]. It is known that the addition of a cryoprotectant to a suspension could affect its hydraulic conductivity and interfere with the osmotic stress to which cells are exposed during cooling and freezing cycles [14]. Because at that temperature, osmotic pressure assisted by DMF addition is less deleterious to sperm than that caused by glycerol [21].

However, post-thaw results demonstrated that sperm velocity patterns, as evaluated by CASA (progressive motility, LIN, ALH), were better preserved in the use of glycerol than DMF. These results were contrary to those previously reported for stallions [2], rabbits [24] and boars [5] but were similar to that reported for bull [15] and dog sperm [21]. In the latter species, it was hypothesized that differences in sperm susceptibility to the cryoprotectants can affect the adaptation of substances for various species, perhaps due to unknown toxic conditions. It was also suggested that differences among species in the quantity and type of phospholipids could interfere with stability of the sperm membrane during cryopreservation [16].

Table 1
Mean values (±SEM) for frozen-thawed goat sperm characteristics cryopreserved with glycerol or dimethylformamide (DMF) as cryoprotectants.

Sperm parameter	Glycerol	DMF
Structural membrane integrity (%)	13.1 ± 6.3	19.4 ± 5.8
Functional membrane integrity (%)	11.8 ± 3.2	11.1 ± 2.9
Normal sperm (%)	64.3 ± 3.1	67.9 ± 2.8
Abnormalities (%)	35.7 ± 3.1	32.1 ± 2.8
Primary defects (%)	0.8 ± 0.4	0.6 ± 0.4
Secondary defects (%)	34.9 ± 2.9	31.5 ± 2.7

No significant differences were identified between treatments ($P > 0.05$).

Table 2
Mean (±SEM) motility end points, measured by CASA, of frozen/thawed goat semen ($n = 21$) cryopreserved with glycerol or dimethylformamide (DMF) as cryoprotectants.

CASA end points	Glycerol	DMF
Number of counted sperm ($\times 10^3$)	1.3 ± 0.1^a	1.2 ± 0.1^a
Total motility (%)	18.1 ± 1.9^a	13.4 ± 1.6^a
Progressive motility (%)	3.5 ± 0.4^a	1.8 ± 0.3^b
Curvilinear velocity (μm/s)	36.6 ± 1.6^a	36.4 ± 1.3^a
Velocity straight line (μm/s)	20.0 ± 1.1^a	17.6 ± 1.0^a
Velocity average pathway (μm/s)	25.5 ± 1.3^a	23.6 ± 1.1^a
Linearity (%)	53.8 ± 1.6^a	48.1 ± 1.4^b
Straightness (%)	77.5 ± 1.0^a	75.2 ± 0.9^a
Amplitude of lateral head (μm)	2.3 ± 0.1^a	2.9 ± 0.1^a
Beat cross frequency (Hz)	8.8 ± 0.5^a	9.9 ± 0.4^a

[a,b] Within a row, means without a common superscript differed ($P < 0.05$).

Table 3
Proportion of sperm in the four populations defined by CASA of goat semen cryopreserved using glycerol or dimethylformamide (DMF).

Sperm population	Glycerol	DMF
Rapid (%)	2.3 ± 0.4^a	1.6 ± 0.3^a
Medium (%)	3.0 ± 0.3^a	1.8 ± 0.3^b
Slow (%)	12.7 ± 1.5^a	10.0 ± 1.3^a
Static (%)	82.0 ± 1.9^a	86.6 ± 1.6^a

[a,b] Within a row, means without a common superscript differ ($P < 0.05$).

In goats, it was previously demonstrated that the addition of 7% glycerol or 5% DMF to a skim milk-based extender promoted numerically higher results for post-thawing subjective motility and vigor with the use of glycerol in spite of the absence of significant difference [31]. Nevertheless in the present study the evaluation of motion parameters in CASA system was performed, which is considered more precise than subjective estimation. Currently, quantitative data by CASA has allowed for detection of subtle changes in sperm motion, velocity and morphology, improving accuracy and efficiency on discrimination between treatments in laboratory studies of new extenders, cryoprotectants and others processes [1]. CASA eliminates errors and this is the reason why CASA instruments report lower values for percent of mass and progressive motility than manual estimates [8]. Otherwise, it is necessary to emphasize that CASA does not substitute subjective analyses, but only complements it. The majority of the studies that we have during the last 70 years are based on the subjective evaluation of sperm. Inevitably, the previous findings in the literature are the corner stone of our current knowledge and industry.

In the current research, sperm progressive motility was more affected with the DMF than the glycerol use. It is known that sperm motility is a central component of male fertility because of its importance on migration in the genital tract and gamete interaction for fertilization [35]. In humans the DMF exposition causes toxicant effects on sperm function and motility perturbation. DMF or *N*-methylformamide, a biotransformation product of DMF, is associated with adverse effects on sperm mitochondria [8], but how it happens it is not clear. It is known that mitochondrial function is one of the etiologic factors that are recognized for sperm motility reduction. The propulsive efficacy of sperm is primarily dependent on mitochondrial function; sperm mitochondria located in the sperm mid piece deliver the required energy for the generation and propagation of the flagellar wave. Male infertility can result from a significant decrease in the number of motile forms or from movement quality disorder [22].

The present study demonstrated that sperm linearity was better preserved in the use of glycerol, while DMF promoted better results for amplitude of lateral head. Linearity measures the departure of the cell track from a straight line; it is the ratio of VSL/VCL.

The ALH corresponds to the mean width of the head oscillation as the sperm swims. Both linearity and ALH seem to be indicators of sperm hyperactivation [25]. A study to determine the correlation between CASA parameters for goat semen and sperm migration in cervical homologous mucus demonstrated that linearity is correlated to sperm in vitro migration efficiency, where spermatozoa presenting values of LIN > 50% showed better migration [10]. Further investigations to define which parameter is most important for fertility in caprine species remains to be conducted.

Even if some sperm characteristics were better preserved in the use of glycerol, the present is a basic study that demonstrates the possibility of using DMF as a cryoprotectant for goat semen freezing. It is known that not only the nature but also the concentration of cryoprotectants could interfere in post-thawing results [17]. In addition, our team had recently demonstrated that goat semen cryopreserved in the use of 6% DMF provide a 27.3% pregnancy rate [30]. These results indicate that DMF has a potential as a cryoprotectant for goat semen, but other concentrations of this substance, and also other freezing protocols should be tested. Also, DMF in varying concentrations could be tested as an alternative cryoprotectant for the semen of bucks whose semen freezes poorly when glycerol is used, since that this positive effect was previously proved for stallion [2].

In conclusion, results demonstrated the possibility of using DMF as an alternative cryoprotectant for goat semen freezing. However it was showed that no benefits were derived by using dimethylformamide to replace glycerol at an equal 6% concentration, and other concentrations of this cryoprotectant should be tested.

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