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Assessment of the interaction between straw size and thawing rate and its impact on *in vitro* quality of post-thaw goat semen[1]

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ABSTRACT - The objective of this study was to analyze interactions between different straw sizes and thawing rates on the post-thaw goat semen parameters. Twenty-one ejaculates (seven per animal) were collected from three stud bucks by using an artificial vagina. After evaluation, the semen was extended in Tris-egg yolk-glycerol and packed in 0.25 and 0.50 mL straws, followed by storage in liquid nitrogen. Thawing was performed using two different rates: 37 ºC/1 min and 55 ºC/7 s. The interaction between the 0.5-mL straw and the thawing rate of 55 ºC/7 s promoted higher progressive motility. When the effect of straws alone was analyzed, it was verified that the use of the 0.50 mL straw promoted better conservation than the 0.25 mL one for progressive motility and acrosomal integrity, after the frozen-thawing procedures. Optimal results for progressive motility were achieved when goat semen was frozen in 0.5 mL straws and thawed in water at 55 ºC/7 s.

Key Words: *Capra hircus*, cryopreservation, semen processing

Introduction

Cryopreservation as a technique for goat semen storage supports a genome resource bank for an indeterminate period of time. Nevertheless, freezing and thawing induce detrimental effects on the ultra-structure, biochemistry, and functional integrity of the sperm (Watson, 2000), resulting in a reduction of motility, membrane integrity and fertilizing ability (Purdy, 2006). Many factors, such as freezing rate (Chemineau et al., 1991), extender (Hashemi et al., 2007), cryoprotectant (Lopes et al., 2009), dilution rate (Evans & Maxwell, 1987; Ritar et al., 1990a) and packaging method (Maxwell et al., 1995; Paulenz et al., 2004) affect the quality of frozen-thawed semen from different species. However, for goats, packaging in 0.25 and 0.5 mL French plastic straws does not seem to affect sperm motility (Ritar et al., 1990b).

Thawing rate is another important factor that affects semen quality parameters (Tuli et al., 1991). Traditionally, straws containing goat semen are thawed at 37 ºC in a water bath for 12-30 s (Watson, 2000; Cabrera et al., 2005), but another slow protocol (5 ºC/2 min in water bath) was demonstrated with lesser efficiency (Deka & Rao, 1987). Tuli et al. (1991) observed significantly higher progressive motility on goat semen thawed at 70 ºC/7 s, compared with the thawing rates of 37 ºC/2 min or 40 ºC/20 s.

Many studies have been conduced to assess the influence of different packaging methods on sperm survival, using different thawing procedures for boars (Eriksson & Rodriguez-Martinez, 2000), fish (Richardson et al., 2000), rams (Paulenz et al., 2004), and dogs (Nöthling & Shuttleworth, 2005). For goats, however, the majority of the studies report the effect of different thawing rates or packaging methods solely and there is a lack of information regarding a possible interaction between these factors. This interaction could increase the survival rate of sperm after thawing as well as the efficiency of goat semen cryopreservation. Therefore, the objective of this study is to investigate the interactions between straws of different sizes and different thawing rates and their impact on the post-thaw goat semen parameters.

Material and Methods

Experimental protocols and animal care were approved by the research committee of the Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, Brazil. Semen was collected from three bucks, one Savannah and two Boer. They were healthy and sexually mature, aging approximately two years. Goats were raised on a farm located in the rural area of Mossoró (5º11' S, 37ºW, and an altitude of 16 m), northeast Brazil, and maintained under

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extensive management with free access to the native vegetation, without restrictions of feed and water. However, two days before the semen collection, animals were housed in a common covered shelter, separated from females. The experiment was conducted from May to July 2009.

One month before starting the experiments, all the males were trained for semen collection in an artificial vagina (43 °C) connected to a glass graduated tube using an induced-estrus doe as a teaser (Silvestre et al., 2004). Semen was collected from each buck twice a week. Immediately after collection, the ejaculates were maintained immersed in a warm water bath at 37 °C until their assessment. Semen assessment was performed within approximately 15 min, and only those semen samples presenting at least 80% spermatozoa showing individual 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 the fresh semen. Microscopic criteria such as sperm progressive motility (%) and mass activity (0–5 scale) were subjectively analyzed by light microscopy (Nikon, Eclipse E200, Tokyo, Japan) under 100x magnification. All the samples were evaluated by the same operator. Percentage of live spermatozoa was established by analyzing a slide stained with Brome-phenol Blue under light microscopy (400×), counting 200 cells per slide (Derivaux et al., 1980). Following the initial assessment, a 10-μL semen aliquot was diluted in 2 mL of buffered formalin (10%), and the 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 were analyzed in Bengal Rose smears by light microscopy, under 1000x magnification. The total sperm defects were counted in 200 cells, in which acrosomal integrity was also evaluated (Njenga et al., 1999). For the evaluation of sperm membrane integrity, a hypo-osmotic swelling test (HOST) was performed immediately after the collection of semen, using a sodium citrate and fructose hypo-osmotic solution (100 mOsm/L). A total of 200 spermatozoa were counted using a phase-contrast microscope at 400x magnification, and spermatozoa with swollen coiled tails were considered as presenting a functional sperm membrane (Fonseca et al., 2005).

An extender consisting of 3.028 g Tris-hydroxymethyl-aminomethane, 1.78 g monohydrated citric acid and 1.25 g D-fructose dissolved in 100 mL of distilled water was used (Siqueira et al., 2009). The osmotic pressure 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 extended in Tris-egg yolk at room temperature (32 ºC). Samples were kept in an isothermal box and transported to the laboratory. After 40 min, the temperature in the isothermal box reached 15 ºC (–0.30 ºC/min), and the samples were transferred to a refrigerator for 30 more minutes, where they reached 4 ºC at 0.37 °C/min. After cooling, the semen was added to Tris-egg yolk plus glycerol 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 (n = 4) and 0.25 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 close to -70 ºC in the vapor. Finally, the straws were plunged into liquid nitrogen for storage up to the thawing moment (Sundararaman & Edwin, 2008).

The straws were removed from the liquid nitrogen and randomly thawed seven days after freezing. A couple of straws (one of 0.5 and another of 0.25 mL) of each sample were thawed on a water bath at 37 ºC/1 min, and other pair from the same sample on a water bath at 55 ºC/7 s, following another 30 s at 37 ºC. Finally, straws were removed and dried, the plug was cut off, and the contents pushed out into a glass vial that was in a water bath at 37 ºC. The semen samples were immediately evaluated for sperm progressive motility, live sperms, morphology, and acrosomal and membrane integrity as previously described.

For statistical analysis, 21 replicates were performed for each treatment. The results were expressed as mean ± SEM (standard error of the mean) and evaluated through the SAS (Statistical Analysis System, version 6.10), Percentage data were ArcSin transformed. To evaluate the effect of different factors and their interactions on the variables considered in this study, the data were subjected to the analysis of variance using the General Linear Model procedure (GLM). The Friedman test was applied to evaluate differences between different treatments (straw size × thawing temperature) with regard to sperm characteristics, except for sperm motility, which was evaluated by Kruskal-Wallis test. For all statistical analysis, significance was established at $P<0.05$.

Results and Discussion

Fresh goat semen was yellowish in color and milky in aspect. The total volume of ejaculates was 1.1±0.4 mL, with a sperm concentration of $2.4\pm1.0\times10^9$ spermatozoa/mL. Sperm progressive motility of fresh semen was 94.9±1.7%, and mass activity was 3.9±0.1. Percentage of live sperm was 92.7±2.0% and sperm with normal morphology was 76.2±1.8%, in which 99.8±0.1% spermatozoa presented intact acrosome. These findings are considered within the normal range for goat species, according to Salviano & Souza (2008).

A total of 30.8±2.0% sperms with functional membrane integrity were found in the HOST for fresh semen, which demonstrates that membrane damage still exists in the beginning of the experiment. It is important to emphasize that the animals were raised under extensive management in a tropical area, and hence, under thermal stress. The high temperatures found in the low latitudes affect the sperm quality and production in ruminants (Entwistle, 1992). Thermal stress is associated with oxidative stress and the generation of reactive oxygen species (ROS), responsible for the loss of membrane integrity, impaired cell function and decreased motility of the sperm. For goats, the ratio of unsaturated to saturated fatty acids in the sperm membranes is higher than in other species, making the membranes more susceptible to peroxidative damage in the presence of ROS (Bucak et al., 2009). These facts were probably associated to the low values found for HOST in fresh semen.

Extended semen presented 93.8±1.7% of progressive motile sperm, while after the cooling process and addition of glycerol, semen showed a significant reduction of this parameter to 70.5±1.7% (P<0.05). It is clear that chilling reduces the motility of the sperm, whereas glycerol induces osmotic and structural damages in the sperm, which results in reduction of goat semen kinematic parameters (Sundararaman & Edwin, 2008).

The cryopreservative process greatly affected (P<0.05) sperm progressive motility and the percentage of live spermatozoa (Table 1). As related before, both thermal stress and the cryopreservation process cause changes in the membrane integrity, and the Brome-phenol Blue staining technique is based on the permeability of the membrane. Cryopreservation still causes phase transitions of the lipids in the membrane of spermatozoa (Woelders, 1997). This vulnerability is due to lipids and proteins in the membrane that are not covalently linked and can move easily (Parks & Graham, 1992).

Although a significant decrease (P<0.05) was found for membrane integrity after the cryopreservation process, it was clearly demonstrated that these values after thawing became closer than those found in fresh semen. It must be emphasized that the semen had already been damaged before the cryopreservation process, as demonstrated by the low membrane integrity percentages in fresh semen. It is known that frozen-thawed sperm were mobile but damaged because the plasma membrane is more sensitive than the nucleus and locomotor (mid-piece) parts of the cell, a finding in agreement with previous studies (Salamon & Maxwell, 1995; Celeghini et al., 2008). Therefore, after thawing, with the addition of cryoinjuries, a great number of spermatozoa died (Salamon & Maxwell, 1995). These data

Table 1 - Mean values (± SEM) for characteristics of frozen goat semen packed into 0.25 or 0.50 mL straws and thawed at 37 ºC/1 min or 55 ºC/7 s (n = 21)

Sperm parameter	Fresh semen	0.25 mL		0.50 mL	
		37 ºC/1 min	55 ºC/7 s	37 ºC/1 min	55 ºC/7 s
Progressive motility (%)	94.9±1.7a	32.2±2.1bc	30.4±2.2c	36.3±2.1bc	37.2±2.2b
Live sperm (%)	92.7±2.0a	16.2±1.9b	17.2±2.0b	15.2±1.9b	15.6±2.0b
Membrane integrity (%)	30.8±2.0a	22.8±1.9b	21.6±2.0b	23.4±1.9b	24.1±2.0b
Normal sperm (%)	76.2±1.8a	62.4±1.4b	65.4±1.4b	60.9±1.4b	63.4±1.4b
Total defects (%)	23.9±1.7a	37.6±1.4b	34.6±1.4b	39.1±1.4b	36.6±1.4b
Acrosomal integrity (%)	99.8±0.1a	99.4±0.1a	99.4±0.1a	99.7±0.1a	99.5±0.1a

a,b Within a row, values with different superscripts differ (P<0.05).
SEM - standard error of the mean.

Table 2 - Mean values (± SEM) for frozen-thawed goat sperm characteristics comparing the effect of thawing rate and straw size separately (n = 21)

Seminal parameters	Thawing rate*		Straw size	
	37 ºC/1 min	55 ºC/7 s	0.,25 mL	0.50 mL
Progressive motility (%)	34.3±1.4	34.1±1.5	31.4±1.5b	36.9±1.4a
Live sperm (%)	16.0±1.4	17.0±1.5	17.1±1.5a	15.9±1.4a
Membrane integrity (%)	23.2±1.3	22.7±1.3	22.4±1.3a	23.5±1.3a
Normal sperm (%)	61.6±0.1	64.2±1.0	63.7±1.0a	62.1±1.0a
Total defect (%)	38.4±0.1	35.8±1.0	36.3±1.0a	37.9±1.0a
Acrosomal integrity (%)	99.6±0.1	99.4±0.1	99.4±0.1b	99.6±0.1a

* No differences (P>0.05) were observed between thawing rates.
a,b Within a row, values with different superscripts differ (P<0.05).
SEM - standard error of the mean.

suggest that membrane integrity can be as important as motility to predict the fertility rates.

The packaging of goat semen made in a 0.5 mL straw in association with the thawing rate performed at 55 ºC/7 s promoted higher progressive motility (P>0.05). These results agree with those found for dogs, in which an interaction between 0.5 mL straw with a thawing rate performed with high temperature (70 ºC/5 s) promoted the best post-thaw semen quality (Nöthling & Shuttleworth, 2005).

In spite of the better interaction between the 0.5 mL straw and the thawing at 55 ºC/7 s, when data were grouped to assess the isolated effect of thawing rates or straws, no differences were verified between thawing performed at 37 ºC/1 min and at 55 ºC/7 s for all assessed parameters (Table 2). Despite these results, Lahnsteiner (2000) related thawing rate as the most sensitive parameter during semen cryopreservation. This author recommended performing the thawing at high temperatures in order to avoid recrystallization, because the warming damage occurs when the spermatozoa pass through the critical zone of -50 through -15 ºC or -5 ºC. Similarly, spermatozoa suffer osmotic stress, when the duration of thawing is insufficient for the outflow of excess cryoprotectants from the cell and the spermatozoa swells and lyses as the medium dilutes abruptly on account of melting of the extracellular ice (Andrabi, 2007).

Thawing rates used in the present study do not seem to be different in promoting spermatozoa damages, probably because 37 ºC/1 min and 55 ºC/7 s for goat semen do not produce sufficient differences in the amount of large ice crystals formed during recrystallization, which are very deleterious for spermatic cells. In fact, for goat semen, different temperatures have been used in protocols of thawing by different authors (Tuli et al., 1991; Deka & Rao, 1987; Khalifa & El-Saidy, 2008; Sundararaman & Edwin, 2008). On the other hand, studies have demonstrated that a fast thawing rate results in better post-thaw quality when compared with slower thawing for stallions, rams (Watson, 1990), bulls (Pace et al., 1981), boars (Eriksson & Rodriguez-Martinez, 2000) and dogs (Nöthling & Shuttleworth, 2005). However, there is a plateau in the relation between thawing rate and sperm survival; nevertheless, by increasing the temperature from 50 to 70 ºC, sperm motility could not be further improved (Woelders & Malva, 1998). The authors of the present study hypothesize that a plateau from 37 ºC to 55 ºC would not affect the semen quality of the goats either. However, attention to temperature and timing becomes much more critical at temperatures greater than 37 °C, as these high temperatures can result in sperm mortality if performed improperly. Thus, thawing semen at 37 °C is more suitable under practical conditions of artificial insemination and the risk of overheating the thawed semen is also lower.

Otherwise, when the effect of straw size was compared alone, sperm progressive motility and acrosomal integrity was significantly better preserved with the use of 0.5 mL straws (Table 2). The surface-to-volume ratio of the package has important implications for the cooling, freezing, and thawing rates of semen. Semen packaging is also important for practical reasons, as it determines both the means of identification of each dose of semen and how it may be arranged for storage in the liquid nitrogen container (Maxwell et al., 1995).

The pattern of cooling and initiation of ice crystallization depends, among other factors, on the straw size. The 0.25 mL straw has a higher surface-to-volume ratio than the 0.50 mL one, which increases the opportunity for post-thaw temperature changes of the semen within the straw. Such changes can compromise the post-thaw recovery of live spermatozoa and thus decrease fertility (Johnson et al, 1995).

Different lipids have different phase transition temperatures and some types of lipid aggregates in domains of gel-like (frozen) lipids, thus excluding other lipid types that remain in the liquid-crystalline (melted) state (Woelders, 1997; Medeiros et al, 2002). In the cryopreservation process, membrane proteins are also excluded from these gel domains and consequently find themselves in a non-physiological lipid environment (Medeiros et al, 2002). This is believed to impair the function of membrane proteins that are necessary for structural integrity or ion metabolism (Watson, 2000), which was also probably associated to the lower preservation of the progressive motility and acrosomal integrity with the 0.25 mL straw use as observed in the present study.

The results in this study disagree with a previous study (Ritar et al., 1990b), which concluded that there is no difference between goat sperm frozen in 0.25 (33% post-thaw motility) and 0.50 mL straws (34% post-thaw motility). In addition, bull semen packaged in 0.25 and 0.50 mL straws containing 10×10^6 total spermatozoa/straw and thawed at 37 ºC/30 s in both cases, provided similar conception rates (Johnson et al., 1995).

Numerical differences found for progressive motility after thawing between 0.25 and 0.5-mL straws in the present research were really low, and probably, there are few biological implications on total fertility. Furthermore, the dilution rate was exactly the same for both groups (150×10^6 sperm/mL), which determined an equal proportion of extender and sperms for all the treatments.

Conclusions

It is recommended that goat semen be frozen in 0.5 mL straws and thawed in water at 55 ºC/7 s.

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