GENETICS AND REPRODUCTION

Colloidal Centrifugation for Dog Sperm Selection Prior Cryopreservation with Glycerol.

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ABSTRACT

Background: Dog ejaculates are produced in three fractions: the first and third fractions contain prostatic fluid, which is harmful for cryopreservation. The second fraction is spermatozoa-rich. Collecting the second fraction of the ejaculate is complex; hence, colloidal centrifugation plus the addition of glycerol at three different concentrations (4, 6, and 8%) as cryoprotectant was evaluated as a method for sperm selection.

Methods: In this study, 72 seminal samples from 6 crossbred dogs were evaluated at the University of Cuenca-Ecuador. Two aliquots were made from each sample, one for conventional centrifugation (Group 1), the other for colloidal centrifugation (Group 2). After centrifugation, each aliquot was subdivided into three parts, and 4, 6, and 8% glycerol were added, respectively. Three sperm evaluations were made (initial, pre-thawing, and post-thawing).

Results: Sperm concentration was similar between the initial sample (IS) and Group 1 ($P>0.05$) after centrifugation, whereas fewer spermatozoa where observed in Group 2 ($P<0.05$). Evaluation of pre-thawing individual progressive motility (IPM) revealed that the spermatozoa in Group 1 underwent a 30% motility reduction in comparison to the IS and Group 2. Post-thawing IPM within the groups with different concentrations of glycerol (4, 6, and 8%) which underwent previous centrifugation with Percoll (Group 2), showed statistical differences ($P<0.05$). However, the HOS test produced similar percentages.

Conclusions: Colloidal centrifugation prior freezing ensures better sperm selection and purification.

Key words: *colloidal centrifugation; semen; dogs; glycerol*

INTRODUCTION

The interest in canine reproduction has remarkably increased worldwide in recent years. Accordingly, artificial insemination with fresh semen has become common practice. However, the outcome of dog (*Canis lupus familiaris*) semen cryopreservation does not meet the expectations due to external factors, like processing, dilution, and freezing of samples (Sánchez *et al.*, 2017), as well as common factors to the species; amount, fraction, and purity of the ejaculates (Strzezek and Fraser, 2009), which affect post-thawing seminal quality.

The quality of collected samples is essential for cryopreservation in terms of purity, which is complicated due to the three fractions of dog ejaculation. The first and third fractions are originated in the prostatic gland and are void of spermatozoa. Additionally, they have components that interfere with freezing. The second fraction, which is spermatozoa-rich, is the one that should be collected (Goericke *et al.*, 2011).

Separating these noxious components of the ejaculate (first and third fractions) from the spermatozoa-rich portion (second fraction), has encouraged researchers to address this issue through mechanical centrifugation mainly. However, these studies produced more, but low-quality spermatozoa (Chatdarong *et al.*, 2010). In order to improve sperm quality, Morrell and Rodriguez (2016) used colloidal centrifugation to separate live spermatozoa from other spermatozoa with different maturity stages or dead, as well as non-sperm cells. They concluded that this method would allow them to produce post colloidal centrifugation seminal samples with higher numbers of viable and pure spermatozoa, thus improving the statistics of cryopreservation.

Another factor that can affect the quality of post-thawing semen is cryoprotectants. Glycerol is the most commonly used cryoprotectant in species of zootechnical interest. It can be added either in a single step followed by cooling at 4 °C, for 1-2 h (Rota *et al.*, 1999) or two-step procedure, for 1.5-2 h total (Peña and

Linde, 2000). However, high molecular weight glycerol is toxic in high concentrations, and though it has been studied in other species, the data in relation to dogs are contradictory (Belala *et al.*, 2016). Therefore, it is important to determine the proper glycerol dose that should be used for cryopreservation of dog semen.

The goal of this paper was to assess colloidal centrifugation before cryopreservation as a way to select and purify spermatozoa, using three different concentrations of glycerol (4, 6, and 8%) in dog semen.

MATERIALS AND METHODS

This research was done at the biotechnology laboratory of the University of Cuenca-Ecuador, located on coordinates 3° 4´53.93´´ south latitude, and 79° 4´ 39.59´´, west longitude. Altitude: 2 671 m above sea level; precipitation: 800-2 000 mm; relative humidity: 80%; and temperatures: 7-12 °C.

A number of 12 seminal samples were collected from 6 medium-sized clinically healthy 3-5 year-old crossbred dogs, with a body condition (BC) of 5 (1-9 scale) (Baldwin *et al.*, 2010), whose ejaculates met the minimal standard parameters for cryopreservation of the samples (Restrepo *et al.*, 2009). Semen collection was made by mechanical compression (digital manipulation). The first two fractions of the ejaculate were collected. These 12 seminal samples were divided into two aliquots, one for each experimental group (Group 1 and Group 2).

Two experiments were made. The first included two types of centrifugation before freezing the seminal sample collected (Group 1, Group 2):

- Group 1: The seminal sample was centrifuged using the conventional method, at 700 g/10 min.
- Group 2: The aliquot of the seminal sample was centrifuged in a Percoll column (60%), at 700 g/10 min, at first. Then the pellet was suspended in fertilization medium *in vitro*, and centrifuged at 350 g/10 min.

In the second experiment, three concentrations of glycerol (4, 6, and 8%) used a cryoprotectants of seminal samples in Group 1 (conventional centrifugation), and Group 2 (colloidal centrifugation), in experiment 1. Twelve 0.25 cm^3 straws were packed per glycerol group (G1a, G1b, G1c, G2a, G2b, G2c) (Fig. 1), which allowed post-freezing evaluation of the 72 samples.

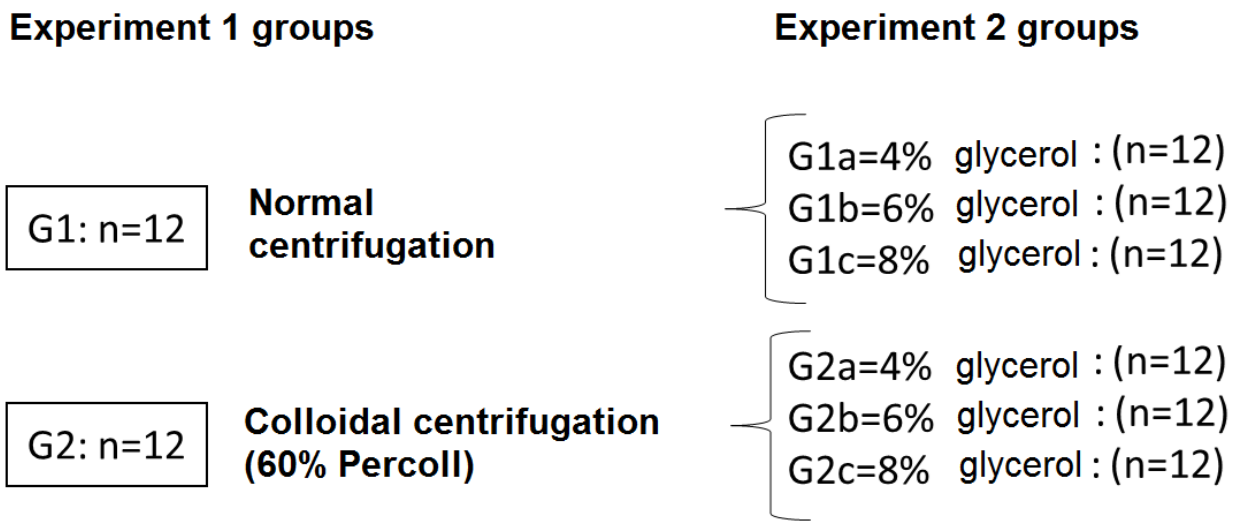

Fig. 1. Protocol of experiments 1 and 2 in the study

Dilutants

Dilutant A was made containing 0.13 g of citric acid; 0.24 g of Tris; 0.1 g of fructose; 2 ml of egg yolk; and 8 ml of ultra-pure water. Dilutant B was made by adding dilutant A glycerol to 4, 6, and 8% concentrations.

Preparation of the two aliquots

Diluent A was added to the total volume of the ejaculate in a 1:1 proportion. A volume of 100 µl was added for microscopic analysis. The remaining volume was divided into two similar aliquots, one for conventional centrifugation (Group 1), and the other for centrifugation with Percoll at 60% (Group 2).

Percoll column at 60%

A volume of 900 µl pure Percoll (37 °C) was poured into an Eppendorf tube, and 100 µl of TALF 10 x medium were added. Then 335 µl of the medium were transferred into an Eppendorf tube, and 165 µl of FIV-SOF medium were added to make 60% Percoll.

Centrifugation

The aliquot in Group 1 (seminal sample) was centrifuged at 700 g/10 min, then the pellet (250 µl) was collected, and dilutant A was added in a 1:1 proportion.

The aliquot in Group 2 underwent automatic sperm selection by centrifugation in a previously prepared Percoll column (60%), at 700 g/10 min. The pellet (250 µl) was placed in 500 µl of fertilization medium (IVF), and it was centrifuged at 350 g/10 min, in order to remove the residues of Percoll. Finally, diluent A was added to 250 µl of pellets, at a 1:1 concentration.

Evaluation of seminal quality

After a 1:1 dilution of the initial sample, a volume of 100 µl was analyzed for pre-freezing sperm quality, according to Restrepo *et al.*, (2009), which included

- Individual progressive motility (IPM): A volume of 10 µl of the seminal sample diluted 1:1 was observed through a 40x microscope. The assessment values were expressed in percents.
- Vitality: Assessment was made by means of eosin-nigrosin staining. A mix was made of 5 µl of the seminal sample and 5 µl of eosin-nigrosin, then it was observed through a 40x microscope.
- Morphology: Evaluation was made to 100 spermatozoa, which were classified into normal and abnormal. The specimens with alterations in the head, mid part, and tail, were considered abnormal.
- Concentration: A volume of 95 µl of water was added to 5 µl of the seminal sample and mixed in an Eppendorf tube. Then, 10 µl of the mix were placed in Neubauer chamber, and were allowed to rest for 1 min. Then, five quadrants were chosen.
- Membrane integrity and functionality (HOS): The HOS test was performed to evaluate this parameter. Volumes of 20 µl of the fluid semen and 100 µl of HOS solution were mixed in Eppendorf tubes for homogenization. Then, the mix was kept in lukewarm water bath for 30 min. Finally, a drop of the preparation was placed on the slide and the contents were observed through the microscope to check the number of spermatozoa with folded and coiled tails.

Balance phase

The three aliquots (G1a, G1b, and G1c) from Group 1 (conventional centrifugation), and the three (G2a, G2b, and G2c) from Group 2 (colloidal centrifugation), were poured into a receptacle containing water at 15 °C/30 min, and then placed in the refrigerator, at 5 °C/1 h. That procedure helped lower the temperature to 5 °C, keeping the seminal samples with dilutant A solely.

Addition of the cryoprotectant (dilutant B)

The aliquots from dilutant B containing 4, 6, and 8% glycerol were stored in the refrigerator for 1 h to lower their temperatures to 5 °C.

Following the balance time of the aliquots in Groups 1 and 2 with the seminal samples and dilutant A, the total corresponding diluent B was added in four parts every 20 min.

Packing

Straws (0.25 cm^3) had been stored in the refrigerator 30 min before to reach 5 °C prior packing. The straws were manually loaded into the refrigerator to maintain the freezing chain. Sealing was done with metal bearings.

Freezing

The straws were placed on racks, 5 cm above liquid nitrogen, for 10 min. Then they were introduced in the liquid nitrogen, at -196 °C.

Statistical analysis

A randomized block design (RBD) was used in a 2x3 bi-factorial experiment. The blocks were formed by the six dogs each, from which two seminal samples were collected. SPSS for Windows, version 25® was used for statistical analysis. The data were analyzed by analysis of variance and the Tukey's range test, 5% significance. At first, the three instances of seminal assessment (initial, pre-freeze, and post-freeze) were considered. Then, the three concentrations of glycerol were assessed (4, 6, and 8%) as cryo-protectants of the seminal samples previously centrifuged by two different methods: conventional (G1), and Percoll column (G2).

RESULTS AND DISCUSSION

The application of conventional centrifugation (Group 1) revealed no statistically differences between the sperm concentration achieved and the initial seminal sample (P>0.05). However, Group 2 (Percoll centrifugation at 60%) showed fewer spermatozoa after the process, compared to the initial sample and Group 1 (P <0.05) (Fig. 2A). The reduction observed in the number of spermatozoa after the colloidal centrifugation was described by Gharajelar *et al.* (2016), who affirmed that the sperm selection pressure is greater with fewer, but high-quality spermatozoa recovery. Similar results were achieved by Macías *et al.* (2009), when explaining colloidal centrifugation. They observed that the initial concentration of 100x106/ml dropped to 20x106 spermatozoa/ml.

Today, conventional centrifugation is used to eliminate the prostatic fraction of the ejaculate. However, it might lead to structural damages at the acrosome and the plasmatic membrane of spermatozoa, considerably reducing sperm motility (Rijsselaere *et al.*, 2002). This concept might explain why individual progressive motility (IPM) of spermatozoa in Group 1 (conventional) assessed after centrifugation, had an approximately 30% decrease compared to the initial seminal sample and the sample from Group 2 (60% Percoll) (Fig. 2B). These results match the reports made by Dorado *et al.* (2013), who said that colloidal centrifugation of canine semen improved IPM significantly (P<0.001). The rise in mortality observed in G2 might be attributed to the colloid type used for centrifugation, which, according to Morrell and Rodriguez (2016), permits researchers to select a subpopulation of motile spermatozoa with normal morphologies and intact membranes. However, other authors, like Varela *et al.* (2015) say that centrifugation *per se* generates a reduction in sperm motility, which may have affected the spermatozoa in Group 1 of the experiment (Fig. 2B).

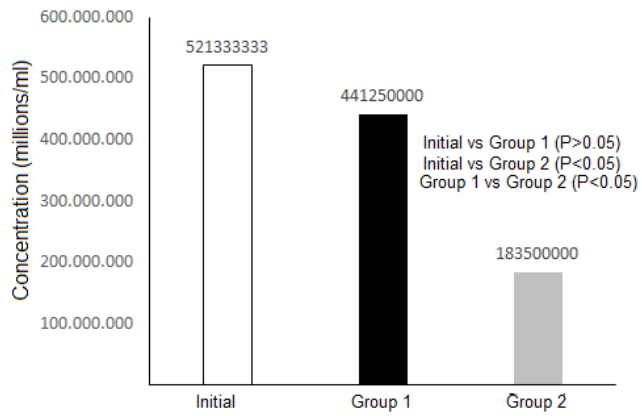

Figure 2A

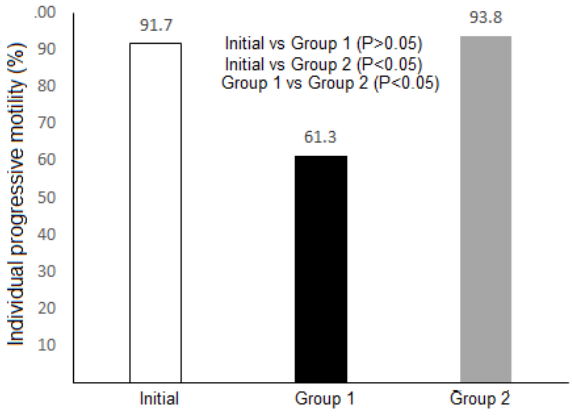

Figure 2B

Fig. 2A. Averages of initial concentration vs. Group 1 (conventional) and Group 2 (60% Percoll), after centrifugation. Fig. 2B. Average of initial individual progressive motility (IPM) vs. the IPM from Groups 1 and 2 after centrifugation. Tukey's test (5%).

The kind of centrifugation used in the experiment (G1) (conventional and G2 (60% Percoll) had no incidence in the number of live spermatozoa achieved (P>0.05) (Fig. 3A). A similar sperm vitality outcome was reported by Restrepo *et al.* (2011), when assessing different colloid concentrations used during centrifugation. In contrast, other papers describe lower values of sperm vitality for Percoll centrifugation (Crespo *et al.*, 2015; Dorado *et al.*, 2013; and Gálvez *et al.*, 2015).

A similar behavior was observed in terms of malformation analysis (abnormalities) in the initial samples and the ones observed in G1 (conventional) and G2 (60% Percoll) (Fig. 3B). These data support the reports made by Gálvez (2015), but differ from other reports made by Dorado *et al.* (2013) who determined that the conventional centrifugation method increases the number of sperm abnormalities when compared to colloidal centrifugation (P <0.05). Although in this paper no statistical differences are seen between Groups 1 and 2, Group 2, which showed a minor percentage of malformations.

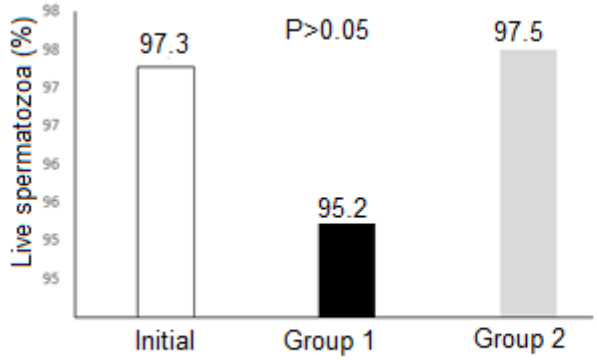

Figure 3A

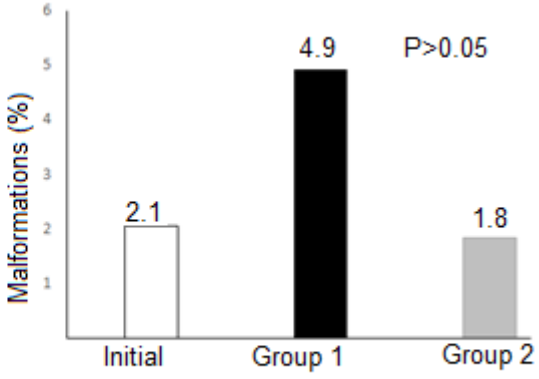

Figure 3B

Fig. 3A. Compared averages of live spermatozoa in the samples: initial, Group 1 (conventional) and Group 2 (60% Percoll), after centrifugation. Fig. 3B. Percentage of malformations observed after centrifugation in the initial sample, Group 1, and Group 2. Tukey's test (5%).

Post-thaw individual progressive motility (PIM) in Group 1 (conventional centrifugation) did not show any statistical differences (P>0.05) between the groups, at different concentrations of glycerol used as cryoprotectants (G1a=4 %; G1b=6 %; G1c=8 %) (Fig. 4ª), similar to Silva *et al.* (2002); Herrera *et al.* (2012); Carlotto *et al.* (2011) and Uribe *et al.* (2011), who achieved no significant results (P>0.05), regarding the percentage of post-thawing individual progressive motility upon evaluation of different glycerol concentrations.

On the contrary, the seminal samples centrifuged with 60% Percoll (Group 2), and frozen at different concentrations of glycerol (G2a=4 %; G2b=6 %; G2c=8 %) did show statistical differences (P <0.05), which coincided with the description made by Restrepo *et al.* (2011), who achieved statistical differences when applying two different concentrations of glycerol. Besides, it matched the reports of Mota *et al.* (2011) and Gharajelar *et al.* (2016), who claimed that the addition of glycerol to the cryopreservation process significantly increased the post-thawing motility percentage.

Assessment of the live post-thawing spermatozoa percentage revealed that there was a statistically significant difference (P <0.05) between the groups at different glycerol concentrations of seminal samples, which had been previously centrifuged in a conventional manner (G1) and 60% Percoll (G2) (Fig. 4B). These data matched the ones achieved by Uribe *et al.* (2011) and Cheuquemán *et al.* (2018), who obtained statistically significant results with the application of different glycerol concentrations, thus determining

that cryopreservation reduces post-thawing sperm vitality. However, this differs from the description made by Cardoso *et* (2003), who applied three concentrations (4, 6, and 8%) of glycerol, and achieved similar vitality percentages.

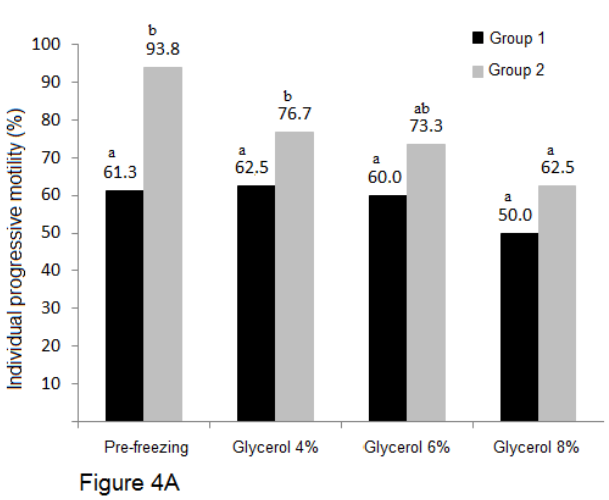

Figure 4A

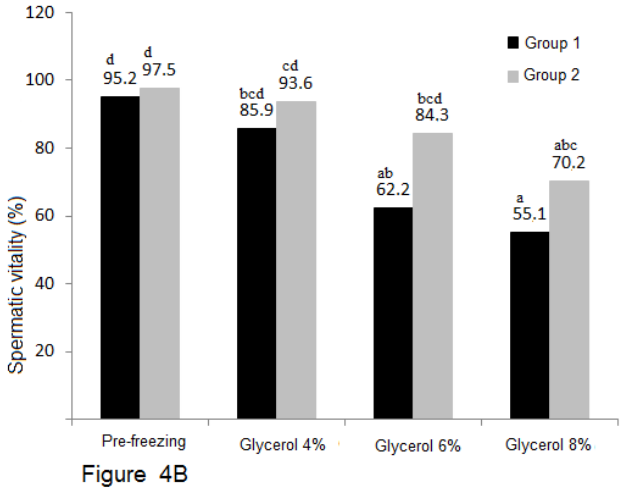

Figure 4B

Fig. 4A. Average of post-thawing individual progressive motility (IPM), using three different glycerol concentrations (G1a=4 %; G1b=6 %; G1c=8 %) in the two seminal samples previously centrifuged in different manners: Group 1 (conventional) and Group 2 (60% Percoll) Fig. 4B. Average of post-thawing live spermatozoa, after using three different glycerol concentrations (G1a=4 %; G1b=6 %; G1c=8 %) in the two seminal samples previously centrifuged in different manners: Group 1 (conventional) and Group 2 (60% Percoll) Tukey's test (5%). ab= difference between the groups

Statistical differences (P <0.05) were observed between Groups 1 and 2 corresponding to the abnormality percentages achieved in either group (Fig. 5A), which were similar to Silva *et al.* (2002) and Cardoso *et al.* (2003) in a comparison of glycerol concentrations at 4, 6, and 8%, with statistical differences (P <0.05), as in the study made by Restrepo *et al.* (2011), contrary to Uribe *et al.* (2011), who obtained (P>0.05) using different concentrations of glycerol.

However, the HOS test produced similar percentages (Fig. 5B) of spermatozoa that reacted positively in the different groups (P>0.05), which are similar to the one determined by Carlotto *et al.* (2011), who observed no statistical differences using different concentrations of glycerol at different freezing times. However, these values are different from the reports made by Restrepo *et al.* (2011), who found significant differences upon applying various concentrations of glycerol, like Sánches *et al.* (2002) using glycerol as cryoprotectant.

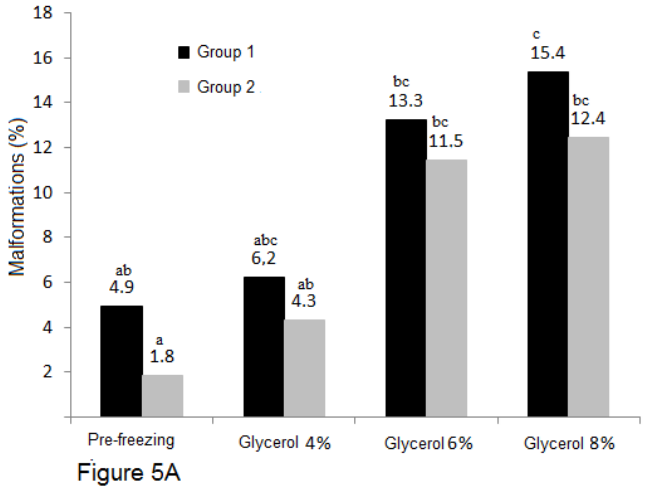

Figure 5A

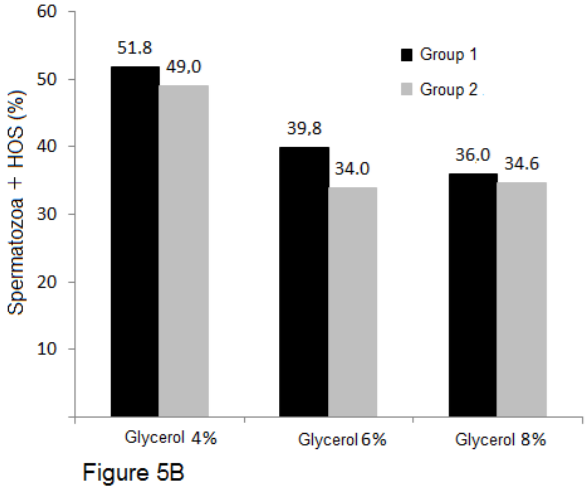

Figure 5B

Fig. 5A. Average of pre-freeze and post-thawing spermatozoa malformations using three different glycerol concentrations (G1a=4 %; G1b=6 %; G1c=8 %) in the two seminal samples previously centrifuged in different manners: Group 1 (conventional) and Group 2 (60% Percoll) Fig. 5B. Percentage of spermatozoa that reacted positively to post-thawing HOS test using three different glycerol concentrations (G1a=4 %; G1b=6 %; G1c=8 %) in the two seminal samples previously centrifuged in different manners: Group 1 (conventional) and Group 2 (60% Percoll) Tukey's test (5%). ab= difference between the groups

CONCLUSIONS

Centrifugation of the seminal sample collected with 60% Percoll before freezing is a technique that allows greater selection and sperm purification. Besides, the addition of 4% glycerol as cryoprotectant provides higher post-thawing cell protection percentages.

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