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Correlation between sperm motility and hypoosmotic swelling test on cryopreserved goat semen

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Abstract: The hypoosmotic swelling test (HOST) has been proved to be a good tool for evaluating the membrane integrity of spermatozoa of various domestic animals including goats, cattle, horses, and swine. The aim of this study was to evaluate the correlation between HOST and sperm motility on cryopreserved goat semen. Two adult Parda Alpina breed males were used, totalizing 8 semen collections for each one. Semen was diluted and packaged into 0.25 mL straws. Semen was cooled to 5 °C for 1 hour and pre-freezing was performed in liquid nitrogen vapor for 15 minutes. After this period, the straws were immersed in liquid nitrogen. Samples were thawed in water bath at 37 °C for 30 seconds; then, sperm motility assessment, hypoosmotic swelling test (HOST) and thermoresistance test were performed. Sperm motility and HOST showed high correlation (r = 0.62; P < 0.0001) fitted to a mathematical formula (Sperm Motility = 6.43995 + 1.11884 HOST) by a regression analysis. In conclusion, HOST proved to be a good complementary test to support the analysis of goat sperm motility; however, it should not be used as a unique test for sperm quality assessment.

Keywords: Caprine, Cryopreservation, Statistical assessment

Correlação entre motilidade espermática e teste hiposmótico no sêmen caprino criopreservado

Resumo: O teste hiposmótico (HOST) tem provado ser uma boa ferramenta para avaliar a integridade funcional da membrana plasmática dos espermatozoides de várias espécies domésticas, incluindo caprinos, bovinos, equinos e suínos. O objetivo deste estudo foi avaliar a correlação entre o HOST e a motilidade espermática no sêmen caprino criopreservado. Dois machos adultos da raça Parda Alpina foram utilizados, totalizando oito coletas de sêmen para cada um. O sêmen foi diluído e envasado em palhetas de 0,25 mL. O sêmen foi resfriado a 5 °C durante 1 hora e o congelamento foi realizado em vapor de nitrogênio líquido durante 15 minutos. Após esse período, as palhetas foram imersas em nitrogênio líquido. As amostras foram descongeladas em banho-maria a 37 °C por 30 segundos e, então, a avaliação de motilidade, teste hiposmótico (HOST) e teste de termorresistência foram realizados. Motilidade e HOST apresentaram alta correlação (r = 0,62, P < 0,0001), ajustada a uma fórmula matemática (motilidade espermática = 6,43995 + 1,11884 HOST) por análise de regressão. Conclui-se que o HOST provou ser um bom teste complementar para a análise da motilidade dos espermatozoides caprinos, contudo, não deve ser utilizado como único teste para avaliação da qualidade espermática.

Palavras chave: Caprinos, Criopreservação, Avaliação estatística

Introduction

Correlation analysis is an important tool for different areas of knowledge, not only as an end result, but as one of the steps for using other analysis techniques (Lira & Neto, 2006). The commonly known method to measure the correlation between two variables is the Pearson's Linear Correlation Coefficient (Lira, 2004).

Population correlation coefficient (parameter) ρ and its sample estimate r (Expression 1) are closely related to the bivariate normal distribution (Lira, 2004).

$$r_{x,y} = \frac{\sum_{i=1}^{n} x_i y_i}{\sqrt{\sum_{i=1}^{n} x_i^2 \sum_{i=1}^{n} y_i^2}} \quad (1)$$

Correlation coefficients indicate the existence of some type of relationship between variables. However, to investigate how this relationship happens, the most suitable method is the regression analysis, where the relationship can be expressed in mathematical form by an equation which links the variables. From all the equations that can be traced through the group of points on the scatter diagram, the function that best fits the model is the one with the lowest sum of squared distances (Least Squares Method).

In many occasions the computerized analysis of semen is not possible, thus complementary tests that can improve the accuracy of the analysis may be used. Commonly, the assessment of the sperm membrane is used as a tool for evaluating the semen, such as hypoosmotic swelling test (Fonseca et al., 2001), supravital test (Rodrigues et al., 2012) and others.

In tropical and subtropical areas, goats have proved to be a good option for production of milk, meat, and leather. As breeds expand, assisted reproductive technologies are necessary to assess and improve the efficiency of reproduction. In male goats, semen analyses are based upon methods developed in other domestic species. Thus, it becomes necessary to test and adapt techniques to the peculiarities of goat semen in the case of the hypoosmotic swelling test (HOST) that is used to verify sperm membrane functional integrity (Fonseca et al., 2001 & Fonseca et al., 2005).

When exposed to hypoosmotic solutions, biochemically-active spermatozoa increase their volume in order to establish equilibrium between the fluid compartment within the spermatozoa and the extracellular environment. Swelling provokes alterations in both cell size and shape that can be evaluated by using a phase contrast microscope (Cabrita et al., 1999). This swelling process promotes a spherical expansion of the sperm membrane covering the tail, thus forcing the flagellum to coil inside the membrane. Coiling of the tail begins at the distal end of the tail and proceeds toward the midpiece and head as the osmotic pressure of the suspending media is lowered (Jeyendran et al., 1984).

Therefore, the aim of this study was to evaluate the correlation between HOST and sperm motility on cryopreserved goat semen.

Material and methods

Two males Parda Alpina breed, with good body condition, approximately three years old were used. Collection period was from April to May of 2010, in the beginning of the breeding season of the caprine species. Semen, 1 ejaculate/animal every 2 days for three weeks, totaling 8 ejaculated/animal, was collected by artificial vagina, using for teaser an oestrous restrained doe.

After collection, the following physical analyses of semen were done: sperm motility (%), vigor (0-5) and mass movement (0-5) and, samples for evaluations of sperm morphology and sperm concentration were separated.

Mass movement was evaluated with a drop of semen of each ejaculate at 37 ^{o}C in light microscopy (100X). Sperm motility and vigor were evaluated in a light microscopy (100X) using a cover slip previously warm to 37 ^{o}C over a semen drop.

Samples of semen were conditioned in microcentrifuge tubes containing 1 mL of Hancock solution Brazilian College of Animal Reproduction [CBRA] (1998) for sperm morphological analysis through humid preparation with aid of phase contrast microscope (1000X). Two hundred cells were counted per ejaculate to measure the percentage of sperm defects, as major and minor defects according to CBRA (1998).

After physical examination, 10 µL of fresh semen was diluted in 1.99 mL of Hancock solution to determine the sperm concentration (haemocytometer method), to calculate the

number of doses and the final volume of diluent to be added.

The semen dilution was performed using a commercial extender (Table 1). The final dilution was done and the samples were packaged into 0.25 mL straws with 50 million sperms.

Table 1 - Chemical composition of the extender (BIOXCELL – IMV®)

Components	g/L	Components	g/L	Components	g/L
Tris	2.3	Glycine	0.2	Lincospectin 100	0.383
Sodium Citrate	6.2	Anhydrous Glucose	0.5	Glycerol	40.2
Potassium Chloride	0.8	Taurine	0.005	Hydrate of calcium lactate	0.7
Fructose	1.2	Gentamicin sulphate	0.24	Soy Lecithin	1.5
Monohydrate lactose		Tylosin tartarate	0.33	Monohydrate citric acid	2.5
Ultra-pure water q.s.p.	1000 mL		0.8		

Semen was packaged into 0.25 mL straws with 50 x 10^6 sperm. Straws were placed in a 20 mL test tube coated with refill (plastic bag) and placed into a 240 mL plastic container containing 125 mL of absolute ethyl alcohol. The container was placed horizontally inside a refrigerator, with internal temperature at 5 °C, with cooling rate of -0.38 °C.min^{-1} for 45 minutes and 15 minutes of equilibration time (Fürst et al., 2005).

Pre-freezing was performed for 15 minutes in liquid nitrogen vapor with pre-freezing rate of -10.7 °C.min^{-1}, reaching a temperature of -155 °C; for this, straws were placed 5 cm above liquid nitrogen (Chirineá et al., 2006). After this period, straws were submerged in liquid nitrogen for final freezing of semen, and stored in liquid nitrogen (-196 ºC) for 10 days.

The straws were thawed in water bath at 37 °C for 30 seconds, placed in microcentrifuge tubes and homogenized for immediate analysis of sperm motility, by phase contrast microscopy at 100X magnification.

In the thermoresistance test the thawed semen was incubated at 37 °C for three hours, and the sperm motility were assessed at 0, 60, 120 and 180 minutes by the same observer, by phase contrast microscope (100X).

The functional integrity of the sperm membrane was evaluated by the hypoosmotic swelling test (HOST), using a hypoosmotic solution of 100 mosmol.kg^{-1}. To prepare the hypoosmotic solution, 9 g fructose and 4.9 g trisodium citrate were dissolved in 1000 mL of deionized water (Revell, Mrode, 1994 & Fonseca et al., 2001).

The withdraw of semen aliquots for HOST and thermoresistance test were performed at the same time, every 60 minutes an aliquot of 20 µL of semen was added to 1 mL of hypoosmotic solution and incubated for 30 minutes in water bath at 37 °C. Later, 0.5 mL Hancock solution was added to the samples to fix them.

Each sample was mounted between glass slide and cover slip and examined in phase contrast microscope (1000X) analyzing 100 sperm per sample. The spermatozoa were classified by the presence or absence of coiled tail. The result was determined as a percentage (Expression 2).

$$HOST\ \% = (\%\ \text{change in tail after HOST}) - (\%\ \text{change in tail before HOST}) \quad (2)$$

From each semen collection (n=16), five semen samples were analyzed, totalizing 80 observations. For statistical analysis, data were submitted to arcsine transformation (Expression 3) and submitted to correlation analyses, Pearson's correlation coefficient was calculated (Prob > |*r*| under H0: *Rho*=0), the significance of correlation was checked by applying "*T* test" using

PROC CORR of Statistical Analyses System (SAS, 2002). For a best understanding of relationship between variables a regression analyses was performed using PROC REG (SAS, 2002). Significance level adopted was $\alpha = 0.05$.

$$y' = arc\ sin\sqrt{y} \quad (3)$$

Results and discussion

Interpretation of the *r* value depends on the objectives of its use and the reasons why it is calculated. According to Callegari-Jacques (2003), the correlation coefficient can be assessed qualitatively as follows: if $0.00 < |r| < 0.30$, low linear correlation exists; if $0.30 \leq |r| < 0.60$, linear correlation is moderate; if $0.60 \leq |r| < 0.90$, there is a high linear correlation; if $0.90 \leq |r| < 1.00$, there is very high linear correlation.

Based on this, sperm motility and HOST showed low to moderate correlation in each time of incubation. Nevertheless, considering all data, of 0 to 180 minutes, a high correlation could be noted (Table 2).

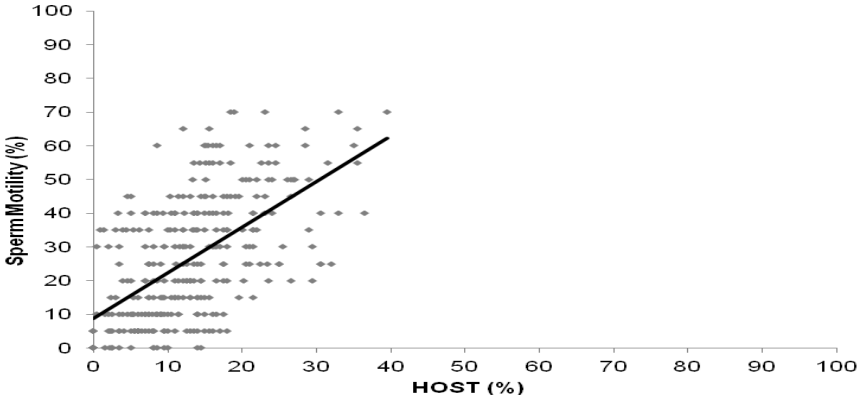
Figure 1 shows the distribution of sperm motility and HOST values. The regression analysis that best fitted the correlation data was a linear function (Expression 4). The R^2 value of 0.3875 indicates that proposed model can explain 38.75% of total variability.

Table 2 – Sperm motility (%), hypoosmotic swelling test (HOST; %) and Pearson's correlation coefficient between both variables of thawed goat semen:

	Thawed semen				
	0 min.	60 min.	120 min.	180 min.	General[d]
Motility (Mean ± SEM)	45.2 ± 1.6	31.4 ± 1.6	19.8 ± 1.5	9.1 ± 0.9	
HOST (Mean ± SEM)	19.7 ± 0.9	13.3 ± 0.7	10.6 ± 0.7	8.4 ± 0.7	
Correlation pairs[a] (*n*)	80	80	80	80	320
Correlation coefficient (*r*)	0.24[c]	0.49[b]	0.47[b]	0.56[b]	0.62[b]

SEM = Standard error of mean; [a] Pairs of variables used in the Pearson's correlation analyses; [b] $P < 0.0001$ (*T*-test); [c] $P < 0.05$ (*T*-test); [d] Correlation coefficient calculated by all data of 0 to 180 minutes.

Figure 1 – Dispersion graphic of sperm motility and HOST of cryopreserved goat semen; $R^2 = 0.3875$ ($P < 0.0001$).



$$Sperm\ Motility = 6.43995 + 1.11884\ HOST \quad (4)$$

The basic assumption for using the Pearson's correlation coefficient is the linearity of relationship between the two variables. A second assumption hypothesizes that the variables involved are random and are measured at least on an interval scale. A third assumption is that the two variables have a combined bivariate normal distribution, which is equivalent to say for each X data, the variable Y is normally distributed. This assumption is required to make statistical inferences (hypothesis test and confidence interval), and it is essential for small samples (Bunchaft & Kellner, 1999), but decreases in importance as the sample size increases, which is justified by the Central Limit Theorem for multivariate distributions (Johnson & Wichern, 1988).

Nevertheless, in most situations, the bivariate distributions of interest do not have a normal distribution (Snedecor & Cochran, 1980). Thus, it is possible to make a transformation of data so that approximates the combined bivariate normal distribution. Thereby, it becomes possible to estimate ρ on the new scale. One of the goals of the data transformation is the correction of non-normality and also variance homogeneity of the variables involved in the analysis (Siqueira, 1983).

One of the important properties of the membrane, including sperm membrane, is the selective transport of molecules that can be observed when a cell is exposed to hypoosmotic conditions, allowing the entry of water into the intracellular environment until the osmotic balance. This process occurs only in viable sperm plasma membrane. Due to the influx of water occurs cellular edema, viewed by winding of the tail, location with the greatest susceptibility to cellular edema (Jeyendran et al., 1984).

Hypoosmotic swelling test is based on the observation that a sperm with an intact cell membrane, when placed in hypoosmotic solution allows water to pass through the cell membrane to restore the osmotic balance between the intracellular and extracellular fluids. With the influx of water into the cell, there is an increase of cell volume (edema), with subsequent folding of the tail (Jeyendran et al., 1984).

Correlation between sperm motility and HOST already has been reported in both fresh and cryopreserved semen of domestic animals; commonly, it varies from moderate to high correlation. In fresh semen, high correlation coefficients varying from r = 0.75 in equine (Neild et al. 1999) to r = 0.94 (Kumi-Diaka 1993) in canine have been reported. In cryopreserved semen moderate correlation (r = 0.57) was found in equine (Neild et al. 1999), in goats, moderate and high correlations (r = 0.53 and r = 0.78) were found by Santos et al. (2006) and Castilho et al. (2009), respectively.

Hypoosmotic swelling test should not be used as single parameter for goat sperm quality assessment (Bispo, 2009). In this study, Pearson's correlation coefficients found varied from low to high correlations, besides regression analysis showed 38.75% of adjustment for the model, which enhances this hypothesis.

Nevertheless, as a supplemental test HOST showed to be a good parameter of sperm quality, which is supported by others authors. Since the sperm motility analysis, singly, does not seem to be able to predict goat semen fertility (Castilho et al. 2009), additional tests such HOST become important to assist goat sperm evaluation for artificial insemination programs.

In conclusion, HOST should not be used as a unique test for sperm quality assessment. However, it proved to be a good complementary test to support the analysis of sperm motility of goat semen, particularly in cases in which the analyses with fluorescent dyes could not be performed.

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