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Evaluation of two different boar semen freezing protocols and their effects on semen quality after thawing

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Abstract

The aim of this study was to evaluate the quality of boar sperm using two different freezing techniques. The protocols were proposed by Westendorf and Paquignon. In both methods the seminal plasma is removed. In the protocol Paquignon the seminal plasma is removed at environmental temperature and the Westendorf protocol suggests that the plasma withdrawal should be made at 15ºC. The sperm suspension was performed using a cooling extender comprised of egg yolk and sugar (glucose - Paquignon and lactose - Westendorf). The freezing extender was added to the presence of glycerol for both methods and Orvus ES Paste on the methodology of Westendorf. The freezing curve comprised cooling from 5 to -5ºC at 3ºC per min and then from -5 to -140ºC at 40ºC per min. The sperm motility and vitality, viability, osmotic resistance, total morphological abnormalities, abnormal tail, head and acrosome, the presence of proximal cytoplasmic droplet and motility degradation rate were evaluated. The method proposed by Westendorf showed greater sperm viability ($P < 0.05$) and fewer changes in midpiece. There was no difference ($P > 0.05$) between the techniques for the other parameters. It was concluded that the method proposed by Westendorf could be indicated in future research for frozen boar semen.

Keywords: biotechnology, boar semen, centrifugation, freezing, reproduction.

Introduction

The technique of semen cryopreservation is an important strategy for the preservation and better utilization of genetic material of great value. In addition, it facilitates the exchange of genetic material between countries and also the preservation of many endangered species (Johnson *et al*., 2000). In swine, the use of frozen semen has not been applied to the production system due to its poor quality when thawed and the lower pregnancy rate and litter size compared to fresh semen. This fact can be explained mostly because the sperm membrane of swine is different from other species due to its lower amount of cholesterol (Paulenz *et al*., 1999). These latter two events are responsible for low sperm viability after thawing.

Whereas cooled semen between 15 and 18ºC is preserved up to three to five days without losses in the sperm quality, with an annual farrowing average rate above 88% and over 10 total born piglets; with frozen semen the birth rates do not reach these values (Romero *et al*., 2004). Thus, the use of boar frozen semen represents only 1% worldwide, and even though it has been available since 1975, a major breakthrough in commercial application has not yet occurred (Saravia *et al*., 2005; Großfeld *et al*., 2008).

Different methodologies can be used for freezing boar semen, most of them are adaptations of the methods proposed by Paquignon *et al*. (1974) and Westendorf *et al*. (1975). Thus, the need to study these methodologies containing such adjustments aiming to improve the quality of thawed boar semen as well as its future applicability in large scale in the production system of pigs is advisable.

The main difference between the two protocols is the moment when the seminal plasma is removed. Regarding the Paquignon methodology the seminal plasma is withdrawn immediately after the sperm collection, while in the Westendorf methodology, the seminal plasma is removed only when the cooling curve achieves 15ºC. Some authors have reported that the previous contact with the seminal plasma is beneficial to the cryotolerance of the semen (Johnson *et al*., 2000; Muiño-Blanco *et al*., 2008). On the other hand, some studies have shown that the removal of seminal plasma immediately after collection of semen is an available technique to improve the quality of frozen-thawed semen (Eriksson *et al*., 2001; Kawano *et al*., 2004; Okazaki *et al*., 2009).

The aim of this study was to evaluate sperm quality parameters after thawing of the boar semen frozen using two modified techniques proposed by Paquignon *et al*. (1974) and Westendorf *et al*. (1975).

Material and Methods

Semen samples were collected in July of 2010 from four boars of proven fertility belonging to São Paulo Farm, located in the city of Oliveira - MG, Brazil and three from the Porcine Experimental Center at the Department of Animal Science, Federal University of

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Lavras (UFLA), Lavras, MG, Brazil totalizing seven animals and seven ejaculates (one ejaculate from each boar). The ejaculates were collected by the gloved hand with the aid of a dummy collection method in a graduate bottle/flask (ml) with 500 ml capacity, preheated at 37°C and protected by an isothermal container (thermal cup collection; Hancock and Howell, 1959).

After collection the semen was taken to the Physiology and Pharmacology Laboratory at the Veterinary Medicine Department (DMV/UFLA) where the sperm-rich fraction was frozen according to the modified methodologies proposed by Paquignon *et al*. (1974) and Westendorf *et al*. (1975). Before freezing, the fresh semen quality of the ejaculates was evaluated by macroscopic analyzes of volume, smell and seminal aspect (milk or watery) and microscopic analysis of motility, vitality and sperm morphology. Only those ejaculates presenting more than 70% motility, vitality of at least three and morphological defects under 10% were used for the freezing process. Regarding the modified freezing methodology proposed by Westendorf *et al*. (1975), initially 25 ml of fresh semen were diluted in BTS® extender (Beltsville Thawing Solution - MINITUB of Brazil Ltda®) in the proportion of 1:1 (semen/extender). An adjustment to room temperature over a period of 90 min in the dark was done. Afterwards, the semen was cooled to 15ºC over 180 min and the seminal plasma was removed by centrifugation (800 g for 10 min). The sperm pellet obtained from this last step was then resuspended in extender cooling, comprising 80% of lactose solution at 11 and 20% of egg yolk solution in order to obtain a sperm concentration of 1.5 x 10^9 sperm/ml. The determination of sperm concentration was achieved by diluting the samples 1:100 (vol/vol) in formaldehyde-citrate buffered solution and the concentration was determined using a Neubauer cell counting chamber.

The semen was then stored at 5°C for 90 min. After this period, 5.0 ml of freezing extender, comprising 89.5% of cooling extender previously used, 1.5% of Orvus ES Paste (Equex-Paste, Ref.13560/0030, Minitüb Afüll-und Labortechnik GmbH & Co.KG) and 9% of glycerol were added in order to achieve a final concentration of 1 x 10^9 sperm/ml and a glycerol final concentration of 3%. The solution was homogenized and semen samples were placed in 0.5 ml straws containing 500 million sperm each. The straws were then introduced into nitrogen vapor (Taylor-Wharton, CP 300,'dry shipper'), following the freezing curve proposed by Murgas *et al*. (2001), which was 5 to -5ºC at a rate of 3ºC per minute, and from -5 to -140ºC at a rate of 40ºC per min. Subsequently, the straws were placed in liquid nitrogen (Cryometal, DS-18) at -196°C temperature until thawing.

Regarding the modified methodology proposed by Paquignon *et al*. (1974), immediately after sperm collection, semen samples was diluted in BTS® extender to obtain a final concentration of 0,6 x 10^9 spermatozoas/ml and centrifuged (800 g for 10 min) in aliquots of 10 ml for seminal plasma removal. Subsequently, the sperm pellet were resuspended in 4 ml of the cooling extender (5.67% of glucose and 22.5% of egg yolk), reaching a concentration of 1.5 x 10^9 sperm/ml. The aliquots of semen were then kept at room temperature (23ºC) for 120 min and then kept at 15°C for 180 min. After this time, each aliquot was added to 2.0 ml of freezing extender (91% of cooling extender and 9% of glycerol) and they were kept in the refrigerator at 5°C for 60 min. The solution was homogenized and semen samples were placed in 0.5 ml straws containing 500 million sperm each.

The freezing slope followed the same technique described by Murgas *et al*. (2001), previously reported for the Westendorf *et al*. (1975) methodology.

Thawing and sperm sample evaluation the thawed semen straw was performed by immersion in a water bath at 50°C for eight seconds. After thawing, the semen samples were resuspended in 1.0 ml of BTS® extender and incubated again at 37°C in a water bath, when motility and vitality were analyzed at 0, 30, 60, 90 and 120 min. At 0 and 120 min sperm viability, osmotic resistance test (ORT), sperm morphological changes and motility degradation rate (MDR) were also evaluated.

The sperm motility and vitality were measured using the average of two evaluators, who observed a drop of semen on a pre-heated slide at 37°C using an optical microscope (400X magnification). The motility value was given in percentage of motile cells and the vitality analysis took into account the intensity of movements, using a zero (low intensity) to five (high intensity) scale. Sperm viability was evaluated by mixing one drop of eosin-nigrosin solution with one drop of thawed semen on a slide on a warmplate at 37°C. The samples were examined under optical microscope (400X magnification). In a total of 200 cells, the percentages of live sperm (appearing colorless) and dead (absorbing dye and become rosy) were counted as reported by Mies Filho (1982).

The determination of motility degradation rate (MDR) was performed incubating 2.0 ml of thawed semen at 37°C in a water bath for a period of 2 h. The following formula for determining the motility degradation rate was applied: MDR (%) = (vitality in 10 min of incubation - vitality in 2 h of incubation)/(vitality in 10 min of incubation) x 100 (Salgueiro *et al*., 2003).

Osmotic resistance test (ORT) was conducted adding 100 µl of thawed semen in 1.0 ml of BTS® hyposmotic solution (100 mOs/l - two parts of distilled water and one of the extender). The mixture was incubated for 40 min at 37°C. After this time a differential count of 100 cells was conducted using phase contract microscopy (Nikon, Optiphot-2) (1000X magnification). The calculation of ORT (%) followed the formula proposed by Melo and Henry (1999): ORT = % of tail abnormalities after the osmotic resistance test - % of tail abnormalities before the osmotic resistance test.

The morphological sperm alterations were analyzed from the differential counting of 100

spermatozoa using phase contract microscopy (Nikon, Optiphot-2; 1000X magnification). The presence of proximal cytoplasmic droplets, tail alterations, head, acrosome, intermediate part and total abnormal spermatozoa were evaluated.

Statistical analysis

In the statistical analyses a randomized blocks design was used (experimental animals) with a split plot divided by the time of evaluation (evaluation time) under two different treatments (protocol used for the freezing procedure). The plot was represented by four straws.

The sperm viability, osmotic resistance and total morphological abnormalities data were subjected to variance analysis after square root transformation in order to achieve data normality (Shapiro Wilk). The averages were compared by the F test. Motility, vitality and specific morphological changes were evaluated by the nonparametric test of Kruskal-Wallis. All statistical procedures were performed using the SAS statistical software, SAS Institute, 1996.

Results

Comparing the modified methodologies proposed by Paquignon *et al*. (1974) and Westendorf *et al*. (1975), there was no difference (P > 0.05) in motility and vitality evaluations (Table 1).

Table 1. Motility (%) and vitality of thawed boar semen using different freezing methodologies (n = 7 animals).

Methodology	Time (minutes)					Average
	0	30	60	90	120	
- Sperm motility (%) -						
Westendorf	34.4	21.3	10.0	2.5	0.6	13.8
Paquignon	35.0	20.7	10.7	5.7	0.7	14.6
Average	34.7[a]	21.0[b]	10.4[c]	4.1[c]	0.7[c]	
P =	0.0001					
- Sperm vitality -						
Westendorf	3.00[a]	2.63[a]	1.50[b]	0.38[c]	0.13[d]	1.53
Paquignon	3.00[a]	2.57[b]	1.86[c]	0.71[d]	0.14[e]	1.66
Average	3.00	2.60	1.68	0.54	0.13	
P =	0.0001					

[a,b]Means followed by different letters in the line differ by the Kruskal-Wallis test (P < 0.05).

There were also no differences (P > 0.05) in the motility degradation test and both techniques showed high values (Westendorf = 96, 9% and Paquignon = 95, 2%). The viability test showed significant difference (P < 0.05) between the two methodologies (Table 2), being higher for the Westendorf protocol. No significant differences (P > 0.05) were found for osmotic resistance and total morphological changes. Regarding specific morphological changes, there were differences (P < 0.05) for midpiece alterations. The Westendorf methodology samples presented fewer abnormalities (0, 44) in compared to the Paquignon protocol (1, 21).

Table 2. Sperm viability (%), osmotic resistance (%) and total morphological abnormalities (%) of thawed boar semen using different freezing methodologies (n = 7 animals).

Methodology	Time (minutes)		Average
	0	120	
- Sperm viability (%) -			
Westendorf	69.3	47.9	58.6[A]
Paquignon	60.4	32.6	46.5[B]
Average	64.8[a]	40.2[b]	
CV[*] (%)	14.64		
- Osmotic resistance (%) -			
Westendorf	17.8	12.0	14.9
Paquignon	20.7	13.3	17.0
Average	19.2[a]	12.6[b]	
CV (%)	16.14		
- Morphological alterations (%) -			
Westendorf	16.3	20.4	18.3
Paquignon	15.7	22.3	19.0
Average	16.0[a]	21.3[b]	
CV (%)	11.36		

[a,b]Means followed by different lowercase letters in the row and capitals in the column differ by the F test (P < 0.05).
[*]Coefficient of variation.

Discussion

The results of motility and vitality obtained from thawed boar semen were different from those found by Bianchi *et al*. (2011) who obtained higher motility values in the frozen semen according to the Westendorf methodology. The authors explained this fact as being due to the plasma remaining with the semen during the cooling period.

The higher viability of sperm frozen by the Westendorf modified methodology is probably due to the largest exposure of spermatozoa to seminal plasma during the pre-freezing, resulting in a more natural cryoprotectant effect (Martins *et al*., 2005). Still, in the methodology proposed by Westendorf, the glycerol was added after the cooling curve reached 5°C, whereas in the Paquignom methodology this was added after the curve reached 15ºC. According to Almlid and Johnson (1988), the glycerol may have a cytotoxic effect when used in inappropriate conditions. Some studies have showed that glycerol concentrations between 2 and 4% are optimal for sperm viability when added at 5ºC (Almlid and Johnson, 1988; Fiser and Fairfull, 1990). Thus, the effect of most prolonged exposure of the semen with the glycerol in the Paquignon protocol could provide lower viability than the observed in the Westendorf protocol.

Both protocols include egg yolk and glycerol in the freezing extender composition. The glycerol minimizes the solution effect, increases the viscosity of the intra-and extracellular solutions so that the water leaves the cells more slowly despite the pressure from the adjacent ice and probably, it inserts between the sperm cells membrane phospholipids (Hammerstedt and Graham, 1992). According to Watson (1995) the presence of cryoprotectant substances of the egg yolk contained in the cooling extenders and the presence of glycerol in the freezing temperature, both at the same concentration, may have protected the sperm membrane both externally and internally.

The difference found in the results of the two study periods in the osmotic resistance test can be explained by the freezing process that may have caused damage to sperm cell membrane, making it more sensitive, especially after 120 min of thawing. The percentage of cells that are reactive to the test in assessment time 0 was lower than the percentage of motile sperm. Alvarenga (2009) working with cooled swine semen also found inferior levels of functional osmotic resistance than the levels of sperm motility. According to this author, there may be motile cells in semen, but those are less resistant to osmotic stress. Regarding the study of morphological specific alterations, the fewer midpiece abnormalities in the Westendorf methodology can be explained by the presence of Orvus ES Paste. According to Graham *et al*. (1971), this substance acts as an antioxidant and its addition decreases the damage to sperm caused by freezing and thawing processes in concentrations ranging from 0.5% to 2.0% (Westendorf *et al*., 1975; Pursel *et al*., 1978). As the plasma membrane, the middle piece is one of the most sensitive portions of sperm attacked by free radicals due to the presence of large numbers of mitochondria (Saleh and Agarwal, 2002). Thus, the Orvus ES Paste can more effectively maintain the midpiece physics cryoprotection, reflecting in a lower amount of changes. On other hand, according to Watson (1975) and Pursel *et al*. (1978) the function of the Orvus ES Paste is attached to the yolk egg, since this is a detergent that causes the emulsion and the dispersion of yolk lipids facilitating their interaction with the sperm membrane surface, which seems to be crucial for the egg yolk expected protective effect.

The results obtained in this study reinforce the importance of further research with frozen boar semen. To date, no technique has proved enough effectiveness in order to replace the use of cooled semen with frozen semen. In the future, research relating to different cryoprotectants or activating substances using boar semen after thawing will be useful for marketing of frozen boar semen.

Based on the results of this study, the modified freezing methodology proposed by Westendorf provided better sperm quality after thawing and it can be used as a basis for future studies with frozen boar semen.

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