Protein quantification in frozen swine semen filtered with Percoll

Quantificação de proteínas em sêmen suíno congelado filtrado com Percoll

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Abstract

In this study, the protein concentration (PC) in fresh and frozen semen samples from three breeds was determined using discontinuous Percoll gradients. Ejaculates (n = 34) from Large White, German Pietrain, and Duroc-Jersey boars were cryopreserved. After thawing, Percoll gradients were used to process the samples. Protein extraction was performed through the Bradford method. The absorbance of the solution was measured using a microplate spectrophotometer at 562 nm and aliquots were analyzed after solvation. PC were evaluated using the absorbance values of the lowest dilution within the calibration range. PC was higher in fresh samples (0,169 mg/ml) compared to frozen samples (0,127 mg/ml). Among breeds, Duroc samples showed the highest PC (0,163 mg/mL), followed by Large White (0,146 mg/mL), and German Pietrain (0,129 mg/mL). Therefore, it was shown that cryopreservation caused a decrease in PC.

Keywords: Cryopreservation. Gradient. Protein. Semen. Swine.

Resumo

Neste estudo, a concentração de proteína em amostras de sêmen fresco e congelado de três raças foi determinada utilizando gradientes descontínuos de Percoll. Ejaculados (n = 34) de machos Large White, German Pietrain e Duroc-Jersey foram criopreservados. Após o descongelamento, gradientes Percoll foram utilizados para processar as amostras. A extração de proteínas foi realizada pelo método de Bradford. A absorbância da solução foi medida utilizando espectrofotômetro de microplacas a 562 nm e as alíquotas foram analisadas após solvatação. As concentrações de proteína foram avaliadas utilizando os valores de absorbância da diluição mais baixa dentro da faixa de calibração. A concentração de proteína foi maior nas amostras frescas (0,169 mg/ml) em comparação às amostras congeladas (0,127 mg/ml). Entre as raças, as amostras Duroc apresentaram maior concentração de proteína (0,163 mg/ml), seguida pela Large White (0,146 mg/mL) e German Pietrain (0,129 mg/ml). Demonstrou-se, portanto, que a criopreservação causou diminuição na concentração protéica.

Palavras-chave: *Criopreservação. Gradiente. Proteína. Sêmen. Suínos.*

Introduction

Artificial insemination (AI) using frozen-thawed semen is not widely adopted in commercial pig breeding due to its generally low fertility (Johnson et al., 2000). This reduced fertility is attributed to changes in sperm physiology and morphology during the cryopreservation (Yeste, 2016). However, despite the expected challenges in fertility and efficiency, germplasm banks and the acceleration of genetic adaptation at central and multiplier levels of the herd encourage the use of frozen semen (Waberski et al., 2019).

Numerous studies have explored the impact of laboratory procedures, such as semen cryopreservation. This technique has an impact on the biochemical, cellular and molecular processes carried out by the sperm (Upadhyay et al., 2021). It has been observed that protein concentration is dynamic and influenced by these processes (Bogle et al., 2017). Furthermore, investigations involving fresh and frozen-thawed pig semen have identified cryogenic damage as being multifactorial, with altered proteins associated with different stages of the cell development (Chen et al., 2014).

To understand the mechanisms of cryogenic damage, various methods have been employed to detect changes in esoteric cell proteins. Such studies have revealed differences in proteins in human (Zhang and Xiong, 2013), fish (Zilli et al., 2005), and sheep sperm (Li et al., 2011) after cryopreservation. Thus, a shared need for protein studies across different fields is a rapid and accurate method to estimate protein concentration. The Bradford Assay (Bradford, 1976) has become a preferred choice in many laboratories due to its simplicity, speed, and sensitivity compared to other methods. This test is also less prone to interference from common reagents and non-protein components of biological samples (Khramtsov et al., 2021). It is based on the direct binding of Coomassie Brilliant Blue G-250 (CBBG) dye to proteins at the arginine, tryptophan, tyrosine, histidine and phenylalanine residues. The assay responds mainly to arginine residues. The anionic CBBG binds to these residues, producing a maximum absorbance of 595 nm, while the free dye in solution has a maximum absorbance of 470 nm. The assay is monitored at 595 nm on a spectrophotometer and measures the CBBG complex bound to the protein, resulting in a shift in the absorption peak (Grintzalis et al., 2015).

To obtain an optimal concentration of proteins, it is imperative to separate high-quality cells that are presumed to be structurally complete (Arias et al., 2017). For this, the Percoll technique can be used, which with centrifugation makes it possible to separate mobile sperm with membranes by density, intact plasma and acrosomal cells of immotile sperm and other cells (Noguchi et al., 2015). Building on these considerations, this study aims to investigate the filtration of frozen semen to determine its impact on protein concentration before and after this procedure in different breeds.

Material and methods

The boars chosen for this study were part of "Zentla Farm" located in Veracruz, Veracruz, Mexico. Boar selection was based on the following criteria: 2 to 4 years old, clinically healthy, without pathologies associated with the male reproductive tract, and with a proven history of fertility after conventional AI with fresh extended semen. In addition, boars followed a routine schedule of vaccination, deworming, and vitamins (A, D, and E) tailored to pigs. Boars were housed in individual pens and fed twice daily with 1.5 kg of concentrate containing 15% crude protein and 1.2 Mcal of metabolizable energy.

All procedures involving animals were performed according to the guidelines established by the Bioethics Committee of the School of Veterinary Medicine and Animal Sciences of Veracruz University (approval registration number COBIBA011/2021).

Sampling and evaluation of samples

Twelve ejaculates were collected by the gloved hand technique as described (Rillo et al., 1996) from three boars of the breeds (Large White, German Pietrain, and Duroc-Jersey) obtaining a total of 36 ejaculates. The samples obtenided were filtred through gauze to discard the mucus and any contaminants (Rillo et al., 1996). Furthermore, the ejaculates were transported in a cooler set at 4 °C to the Animal Reproductive Biology Laboratory of the Universidad Veracruzana. Were put refrigerated at

16 °C for 24 hours once. Gross motility was estimated based on the vigor of the spermatic waves, with values between 0 (very little or no swirl activity) and 5 (intense swirling, rapid dark and light waves), according to the classification of Salamon and Maxwell (1995). Ten samples with gross motility values below 3 were discarded. Individual progressive spermatozoa motility was evaluated as percentage (Maxwell and Evans, 1990). Sperm quantitation was performed by sperm cell counting in a Neubauer chamber, where a small sperm sample was placed until the counting area was completely covered. The samples were cryopreserved using the protocol proposed by Westendorf et al. (1975) and modified by Gutiérrez-Pérez et al. (2009). The straws were thawed in a laboratory bath at 37 °C for 30 seconds.

Percoll gradient filtration

Filtering through Percoll gradients was performed as follows. An 80% Percoll stock solution with a density of 1.123 g/ml was prepared by adding eight parts (v/v) of 100% Percoll (Sigma-Aldrich) with two parts (v/v) of Dulbecco's modified eagle's medium (DMEM; Sigma-Aldrich), 0.01 g/L gentamicin sulphate, and 6mM HEPES buffer (Sigma-Aldrich). The 80% stock solution of Percoll had a pH of 7.4 and an osmolarity of 280-320 mOsm/kg H_2O (Lima et al., 2015). The 40% Percoll was prepared by mixing the above prepared 80% stock solution with DMEM (1X). Semen was enriched by the discontinuous Percoll density gradient method. Briefly, 1 ml of each gradient was added to a 15 ml conical centrifugation tube. The 80 and 40% Percoll gradients were added as the lower and upper layers, respectively. Then, 1 ml semen was added to the top of the conical centrifugation tube and centrifuged for 20 minutes at 750 RCF at 24 °C (Rawat and Sharma, 2019).

Protein extraction

Protein extraction was performed as previously described, following the Bradford methodology (Bradford, 1976). Briefly, 5 µL of the semen sample and 250 µL of the dye solution were added to the wells. The plate was incubated at 37 °C for 5 minutes and the absorbance of the solution was measured using a microplate spectrophotometer at 595 nm. When performing the BA, 200 µL of a dye solution was added to 25 µL of the sample. The mixture was incubated at 37 °C for 30 minutes in a thermal shaker. In this case, the absorbance of the solutions was evaluated at 562 nm and aliquots were analyzed immediately after solvation. All samples were diluted 1:1, 1:3, 1:9, and 1:27 in water. Samples of concentrated nanoparticles (batches NP9 and NP10) were diluted 1:5, 1:15, 1:45, and 1:135. Protein concentrations were calculated using the absorbance values of the lowest dilution within the calibration range. Since the bovine serum albumin concentrations obtained by UV spectrometry and gravimetric analysis were different, both were used to measure nanoparticle concentration. To construct the calibration curve for gelatin, the concentration was measured by gravimetric analysis. The concentration of nanoparticles was calculated by direct (concentration of purified nanoparticles) and indirect methods (Figure 1).

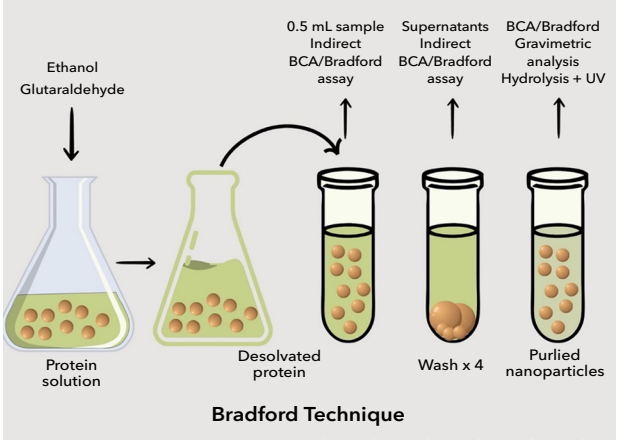

Figure 1 - Schematization of the Bradford method.

Statistical analyses

Statistical analyses were performed with the software SAS (SAS Institute. Cary, North Carolina, US) following the GLIMMIX procedure. The statistical model included the fixed effects of the type of semen (frozen and fresh), breed (Large White, Pietrain, and Duroc), and their interactions. Residual analysis was performed to test homogeneity and normality using the UNIVARIATE procedure. Post-hoc comparisons were performed using Tukey's HSD method. Statistical differences were established when $p < 0.05$.

Results

The protein difference between cryopreserved and fresh semen samples was 0.046 mg/mL, with fresh semen having the highest ($p < 0.001$) protein concentration (Table 1). Additionally, results in Table 2 show that breeds influence protein concentration between fresh and frozen semen. Fresh semen from Duroc and Large White breeds averaged (0.184 mg/dL) the highest protein concentration. Frozen samples from the three breeds averaged (0.123 mg/dL) the lowest concentration among the samples. Interestingly, no differences were detected among fresh samples of the Pietrain breed with all the frozen samples. Differences ($p = 0.002$) among the three breeds regardless of the type of semen were detected. It was found that the Duroc and Large White breeds had the highest protein concentrations (0.163 and 0.146 mg/mL, respectively) compared to the Pietrain breed (0.130 mg/mL) (Figure 2).

Table 1 - Protein concentration (mg/mL) of frozen and fresh swine semen

Protein concentration	Type of semen		p-value
	Fresh	Frozen	
Mean[1]	0.169[a]	0.123[b]	< 0.001
Standard error of the mean	0.006	0.004	
Maximum	0.224	0.149	
Minimum	0.121	0.089	

Note: [1]Means within a row with different superscripts ([a, b]) differ by $p < 0.001$.

Table 2 - Protein concentration (mg/mL) of fresh and frozen swine semen from three breeds

Protein concentration	Large White		Duroc		Pietrain		p-value
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	
Mean[1]	0.174[ab]	0.119[c]	0.194[a]	0.132[c]	0.141[bc]	0.118[c]	0.037
SEM	0.007	0.005	0.012	0.008	0.009	0.005	
Maximum	0.224	0.149	0.198	0.149	0.195	0.168	
Minimum	0.121	0.089	0.184	0.119	0.083	0.110	

Note: [1]Means within a row with different superscripts ([a, b, c]) differ by $p < 0.05$. SEM = standard error of the mean.

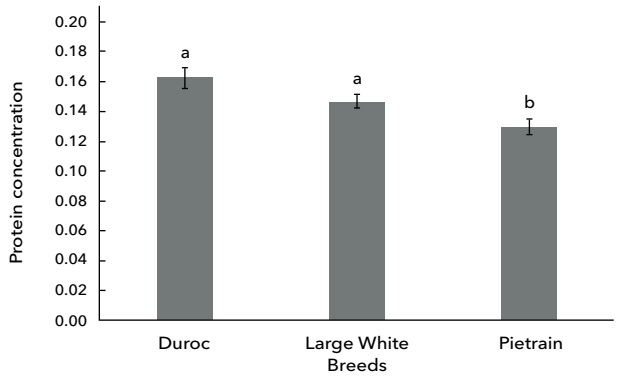

Figure 2 - Protein concentration (mg/dL) of swine semen from different breeds

Note: Columns with different superscripts ([a, b]) differ by $p < 0.05$.

Discussion

Despite the technological advances, cryopreservation reduces survival, acrosome integrity, motility and fertility of frozen-thawed sperms (Johnson et al., 2000). In this study, cryopreservation negatively affected protein concentration, frozen samples had lower protein concentration compared to fresh semen. Different studies have focused on the alteration in protein quantification following cryopreservation in diverse species. In bovines, has been shown that cell membrane proteins are lost through the freeing-thawing process (Ollero et al., 1998), including a decrease in the amount of heat shock protein 90 in bulls (Zhang and Xiong, 2013). A study in boars

found that up to 41 proteins, involved in multiple proces-ses such as sperm premature capacitation, adhesions, energy supply, and fertilization (e.g. A-kinase anchor protein 3, superoxide dismutase 1, and outer dense fibre 2) decreased in frozen-thawed sperm (Chen et al., 2014). Although our study did not analyze specific proteins, the low protein concentration found after cryopreservation may be an indicator of low integrity and fertility potential from the sperm cells.

The Percoll density gradient method is based on sperm cell density, regarding the maturation stage and integrity of sperm cells as male gametes with a normal nucleus have greater density (Lee et al., 2009). In our study, semen analyzed with 80% Percoll had greater protein concentration than samples at 40%. A study performed by Patriani et al. (2019) showed that the highest sperm motility as well as the lowest abnormalities and mortality were found in those samples processed with the densest Percoll gradient. According to a recent study on boars, those semen samples with high motility and optimal morphology had a higher concentration of the major seminal plasma PSP-I and cathepsin B (Lazari et al., 2019), two proteins associated with correct sperm functionality. These findings explain that spermatozoa processed in Percoll 40% might have lower sperm traits due to lower protein concentration compared to sperms processed with Percoll 80% which recovered optimal quality spermatozoa.

Duroc and Pietrain boars produced semen with the highest and lowest protein concentrations in fresh and frozen-thawed samples, respectively. Our results were similar to a study aimed to determine the influence of the breed of boars on protein concentrations (and other metabolites) in seminal plasma (Žaja et al., 2016). In that study, the Pietrain breed produced samples with one of the lowest protein concentrations compared to Swedish Landrance, German Landrance and a hybrid breed. It's noteworthy that the Duroc breed was not evaluated. Considering these findings, it could be logical to think that the Pietrain breed has a suboptimal cryotolerance compared to the other breeds. This hypothesis gains strength when considering the study by Žaja et al. (2016) in which semen from Pietrain boars had the lowest total antioxidative status among the analyzed breeds. Antioxidant factors are essential to reduce the detrimental effects of cryopreservation on sperm quality and improve post-thaw fertility (Riesco et al., 2021).

More research is needed to enhance the cryopreservation of boar sperm as AI is one of the most used reproductive biotechnologies in the swine industry. By improving the use of AI, it is possible to improve the genetic gain among swine farms.

Conclusion

In this study, cryopreservation influences the protein concentration in porcine ejaculates, since there is a decrease in cryopreserved samples compared to fresh diluted samples. Additionally, the Duroc and Large White boars produced semen with the highest protein concentration among the studied breeds.

Acknowledgments

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