VIABILITY OF FRESH AND FROZEN BULL SPERM COMPARED BY TWO STAINING TECHNIQUES

[*Viabilidade de espermatozóides bovinos frescos e criopreservados comparados por duas técnicas de coloração*]

Felipe-Pérez YE[1], Juárez-Mosqueda ML[2], Hernández-González EO[3], and Valencia JJ[1,*]

[1]Departamento de Reproducción, [2]Departamento de Morfología, Facultad de MedicinaVeterinaria y Zootécnia-Universidad Nacional Autónoma de México y [3]Departamento de Biología Celular CINVESTAV- IPN, México DF, México 07360.

ABSTRACT - Semen cryopreservation results in sublethal damage to sperm due to membrane deterioration and an increased number of spermatozoa that undergo acrosome reaction, these damages lead to fertility reduction. The objective of this study was to compare two methods of staining in order to evaluate bull sperm viability after cryopreservation. A single batch of fresh and frozen semen from eight Holstein bulls was obtained for assessment of sperm viability and acrosomal status. Fresh and frozen-thawed semen samples were stained with eosin-nigrosin (EoNig) and the triple staining technique (TriSt) and evaluated under a light microscope. The results showed a significant decrease ($P < 0.05$) in the number of viable cells after cryopreservation with both techniques. A significant difference ($P < 0.05$) in viability on both fresh and frozen thawed sperm was observed when the two stains were compared. Differences in viability among bulls using both staining techniques were found on fresh samples, whereas in frozen-thawed sperm no differences were observed with the TriSt ($P < 0.05$). A marked decline (41.4 ± 11 S.D., $P < 0.001$) in the mean number of acrosome intact live sperm was observed after cryopreservation using TriSt. In contrast, the EoNig technique overestimated the mean number of living sperm that could maintain their fertilizing ability on the frozen-thawed semen samples. In frozen-thawed semen TriSt showed a mean number of 33.4 ± 6.2 S.D. Concluding that EoNig as well as the TriSt, are useful tools for evaluating fresh semen samples, but when evaluating cryopreserved bovine semen, the TriSt offers more reliable results.

Keywords: Cryopreservation, capacitation, mammalian spermatozoa, viability, acrosome reaction, staining techniques.

RESUMO – A criopreservação do sêmen resulta em danos não letais ao esperma devido a deterioração da membrana e aumento no número dos espermatozóides que apresentam reação acrossômica, e estes danos resultam em redução na fertilidade. O objetivo deste estudo foi comparar dois métodos de coloração para avaliação da viabilidade de espermatozóides de bovinos após criopreservação. Uma amostra de sêmen fresco e criopreservado de oito touros da raça Holandesa foi obtida para determinação da viabilidade espermática e estado do acrossomo. As amostras de sêmen fresco e criopreservado foram coradas com eosina-nigrosina (EoNig) e pela técnica de tripla coloração (TriSt) e avaliados por microscopia óptica. Os resultados revelaram uma redução significante ($P < 0,05$) no número de células viáveis após a criopreservação com ambas as técnicas. Uma diferença significante ($P < 0,05$) na viabilidade do sêmen fresco e criopresercado foi observada pela comparação das duas técnicas. Diferenças na viabilidade entre touros usando as duas técnicas de coloração foram encontradas nas amostras frescas, enquanto no sêmen criopreservado nenhuma diferença foi observada com TriSt ($P < 0,05$). Uma marcada redução (41, 4 ± 11 D.P., $P < 0.001$) no número médio de acrossomos intactos em espermatozóides vivos foi observada com TriSt. Por outro lado, a técnica EoNig superestimou o número médio de espermatozóides vivos que poderiam manter sua capacidade de fertilização nas amostras de sêmen criopreservado. No sêmen congelado, o TriSt apresentou média de 33,4 ± 6,2 D.P. Concluindo, a EoNig e a TriSt são ferramentas úteis para a avaliação de amostras de sêmen fresco, mas para a avaliação do sêmen bovino criopreservado, o TriSt fornece resultados mais confiáveis.

Palavras-Chave: Crio preservação, capacitação, espermatozóides de mamíferos, viabilidade, reação acrossômica, técnicas de coloração.

[*] Corresponding author. E-mail: jjvm@servidor.unam.mx, Facultad de Medicina Veterinaria y Zootécnia-Universidad Nacional Autónoma de México. México D. F. CP 04510

INTRODUCTION

Cryopreserved semen management for artificial insemination is a crucial step towards obtaining acceptable pregnancy rates. Thereby the main goal of semen evaluation is to predict its fertilizing ability (Britto et al., 2003) and several cell staining techniques have been developed to evaluate sperm damages caused by cryopreservation, such as the eosin-nigrosin staining (Rodríguez-Martínez, 2000). As a result, 50% of bull sperm viability has been established and accepted as the usual percentage of sperm damaged during cryopreservation (Watson, 1955 & 2000; Rasul et al., 2007). Nonetheless, the surviving sperm population might have morphological defects that reduce and impairs their fertilizing ability (Correa et al., 2007), and it is well known that cryopreserved semen presents sublethal damages and fertility reduction (Ricker et al., 2006; Correa et al., 2007).

Once sperm are ejaculated, they must undergo structural and metabolic modifications, known as capacitation, which are acquired through their passage inside the female reproductive tract (Pereira et al., 2000). Capacitation confers sperm the ability to gain hyperactive motility, interact with the oocyte zona pellucida, undergo acrosome reaction and finally initiate oocyte plasma membrane fusion (Rajesh & Preeti, 2004; Yanagimachi, 1994). Acrosome reaction allows sperm to penetrate the oocyte zona pellucida, and involves fusion of the outer acrosomal membrane with the plasma membrane, in order to ensure the release of acrosomic enzymes (Pereira et al., 2000). Therefore, sperm must retain an intact acrosome, so that acrosome reaction can occur at the proper time in order to perform fertilization, however, false acrosome reaction occurs during the freezing and thawing process (Bedford, 1990; Hou et al., 2002).

The main cause of cell damage during cryopreservation is attributed to intracellular ice formation (Mazur, 1984), producing sperm membrane deterioration and loss of acrosome integrity (Szasz, 2000) and although, glycerol is commonly used as an intracellular cryoprotectant, it exerts osmotic and toxic effects on the cryopreserved cells, to the point of been contraceptive for sperm of many species (Hammerstedt et al., 1990; Hammerstedt & Graham, 1992; Burh et al., 2001; Jeyendran et al., 1985; Wundrich et al., 2006).

Recently, the level of acrosome reacted sperm exposed to cryopreservation has been used to estimate sperm function as a response to cryopreservation and several assays have been developed to detect morphological and functional integrity of plasma membrane and sperm acrosomal membrane (Petrunkina et al., 2004; Troup et al., 1994; Peña et al., 2001, 2003; Januskauskas et al., 2000; Rathi et al., 2001). Integrity of acrosomal function is of crucial importance to normal fertilization, because acrosomeless, membrane damaged and cryocapacitated sperm cannot fertilize oocytes (Mazur, 1984, Hou et al., 2002; Januskauskas et al., 2000). Therefore, it is important to analyze frozen-thawed semen for sperm viability and simultaneously determine acrosome integrity (Thomas et al., 1997). The most common method to determine sperm viability is using an eosin-nigrosin (EoNig) sperm smear at the field level (Rodriguez-Martínez, 2000; Britto et al., 2003). However, other methods, like the triple staining (TriSt), could constitute a better option than EoNig, since TriSt is able to differentiate not only live from dead sperm, but also their acrosomal status at the same time (Talbot & Chacon, 1981; Garde et al., 1992).

Therefore, the aim of this study was to compare sperm viability of Holstein bull semen using two staining techniques, the usual EoNig viability stain and the TriSt in fresh and frozen-thawed samples.

MATERIALS AND METHODS

A single batch of fresh and frozen semen from eight Holstein bulls of proven fertility was obtained from a Semen Processing Center in México. The semen was collected with an artificial vagina. All fresh semen samples included in the study showed 70% motility, mass motility rates from 3 to 4, and less than 20% morphological abnormalities. One aliquot of 1 mL of fresh semen was separated and evaluated, while the rest of the ejaculate was frozen.

Freezing and Thawing

The freezing protocol was performed as mentioned elsewhere (Martínez et al., 2006), briefly: The first semen dilution was made on 50% skimmed milk and antibiotics; 75 mg of Lincomycin and 125 mg of Gentamicin per Lt of milk, then the sample was maintained at 4°C. The second dilution was made after four hours with skimed milk, antibiotics and 8% of glycerol, so the extender was added every 20 minutes until reaching the desired volume. The diluted semen was automatically packaged into 0.5ml straws, containing $45x10^6$ sperm. Finally, straws were placed inside an automatic vapor

nitrogen freezer, where temperature was lowered from 4 to -196°C, and frozen samples were plunged into liquid nitrogen for storage.

Thawing process was carried out after a week of cryopreservation, so then three straws of semen per bull were placed into a water bath at 35°C for 20 sec.

Eosin-nigrosin staining

This method has been used as a routine staining in order to evaluate sperm viability (World Health Organization, 1992, Rodríguez-Martínez , 2000), briefly, after washing sperm samples in saline solution (154mM NaCl) at 37°C, one drop of the suspension containing $35x10^6$ sperm/mL was placed on a tempered glass slide, which was mixed with one drop of EoNig solution (0.2g of eosin and 2g of nigrosin were dissolved in a buffered saline solution [153mM NaCl and 9.65mM NaH_2PO_4, pH 7.4], mixed for 2 hrs at room temperature and filtered to obtain the staining media). The mixture was smeared on the glass slide and let air dried. The samples were observed under a light microscope. Eosin penetrates in non viable cells which appear red. Nigrosine offers a dark background facilitating the detection of viable, non stained cells. Four smears were performed from each ejaculate, in fresh and also in frozen-thawed samples.

Triple staining

This method was performed by Talbot & Chacon (1981) for human sperm and modified for application on different domestic animal sperm. The staining protocol for bovine sperm was done according to Martínez et al., (2006), briefly: samples of fresh and frozen-thawed bovine sperm suspensions were washed by centrifugation (500 x g) and resuspension with PBS (137mM NaCl, 2.7mM KCl, 9.6mM NaH_2PO_4, 1.4mM KH_2PO_4, pH 7.4). Sperm were concentrated at $35x10^6$ cells/mL, an aliquot of 0.5mL was added to an equal volume of Trypan blue at 2% in PBS and incubated at 35°C in a water bath for 15 min. Thereafter, sperm samples were washed 3 times in PBS or until the supernatant appeared clear. Live and dead sperm can be differentiated by the vital stain Trypan blue, which penetrates into the post acrosomal region of dead cells. Sperm pellets were then fixed in 3% glutaraldehyde in caccodilic buffer (0.1M, pH 7.7) for 30 min at 4°C, next, samples were washed twice with distilled water and placed on a glass slide, making a smear of each sample which were air dried. Smears were incubated in Bismark brown (8% in aqueous solution, pH 1.8) at 37°C for 5 min. Then, smears were washed with distilled water and incubated for one minute on Rose Bengal (8% in 0.1M Tris, pH 5.3) at 37°C. Finally, smears were washed in distilled water and let air dried.

Classification criteria for patterns obtained with TriSt for human sperm are: pink acrosome and blue post acrosomal region indicate acrosome intact dead sperm; pink acrosome and brown post acrosomal region indicate acrosome intact live sperm (AILS); white acrosome and blue post acrosomal region indicate acrosome reacted dead sperm and white acrosome with brown post acrosomal region indicate acrosome reacted live sperm (ARLS) (Talbot & Chacon, 1981).

Slides were observed under a bright field microscope at 100X and assessed for the number of sperm that were alive at the time of fixation and at the same time the number of sperm that had undergone acrosome reaction. A total of 100 cells were counted from each smear (Salisbury & Mercier, 1945), having 4 smears analyzed from each bull, either from fresh or frozen-thawed semen samples.

Statistical analysis

Data were analyzed using Sigma Stat 3.1 (SISTAT software, Inc., CA, USA). The Analysis of Variance (ANOVA) test was used to analyse individual differences among sperm viability parameters. The Holm-Sidak method was used for multiple comparison procedure, however when samples failed the equal variance test a Krushkal-Wallis test was used. AILS and live sperm means were compared performing the Paired-T test. Unless otherwise indicated, data are presented as means ± S.D. Differences were considered to be significant if the calculated probability of their occurrences by chance was less than 5% ($P<0.05$).

RESULTS

Two sperm patterns were observed when the EoNig stain was used, dead and live sperm (Figure 1). TriSt showed four patterns on bull sperm samples, which slightly differed from those referred to human sperm (Talbot & Chacon, 1981). Color patterns observed on the acrosome reacted sperm were: light blue acrosome and blue post acrosomal region in acrosome reacted dead sperm (ARDS), and light brown acrosome and pink-brownish post acrosomal region in acrosome reacted live sperm (ARLS). Color patterns observed in the acrosome intact sperm were as follows: pink acrosome and pink-brownish post acrosomal region in acrosome intact live sperm (AILS), and pink acrosome and blue post acrosomal region in acrosome intact dead sperm (Figure 2).

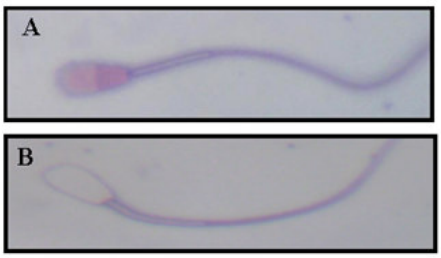

Figure 1. Patterns of bull sperm stained with the eosine-nigrosin technique. A) Represents the dead sperm and B) the live sperm.

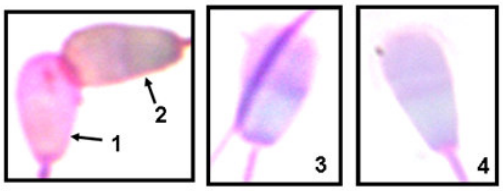

Figure 2. Patterns of bull sperm stained with the triple staining technique. 1) Acrosome intact live sperm, 2) acrosome reacted live sperm, 3) acrosome intact dead sperm and 4) acrosome reacted dead sperm.

A significant decrease in the number of viable sperm after cryopreservation was observed with both techniques (P< 0.05). For the TriSt method, the total number of live sperm was determined by adding AILS to ARLS (Table 1).

Differences on sperm viability of fresh samples among bulls (P<0.05) were detected using both staining techniques, except when the frozen-thawed samples were evaluated by TriSt. EoNig staining revealed that in fresh samples, only one bull scored below the mean number of sperm viability (bull 1), six were close to the mean (bulls 2-6) and two were above (bulls 7 and 8); in contrast when TriSt was performed, the bull that was below the mean obtained a better score. Furthermore, when the frozen-thawed samples were evaluated by EoNig, one bull remained below the mean number of living sperm (bull 1), four were close to the mean (bulls 3-6) and three were above (bulls 2, 7, and 8). Nonetheless, when TriSt was performed on the frozen-thawed samples no statistical differences were observed between bulls (Table 2).

According to the TriSt results, a dramatic decline (41.4 ± 11, P<0.001) in the mean number of AILS after cryopreservation was observed, compared with the mean number of AILS of fresh samples (74.8 ± 10), less than half of the population remained AILS after thawing (33.4 ± 6.2). However, the mean number of living sperm (EoNig) and AILS (TriSt) in fresh samples did not differ (Figure 3).

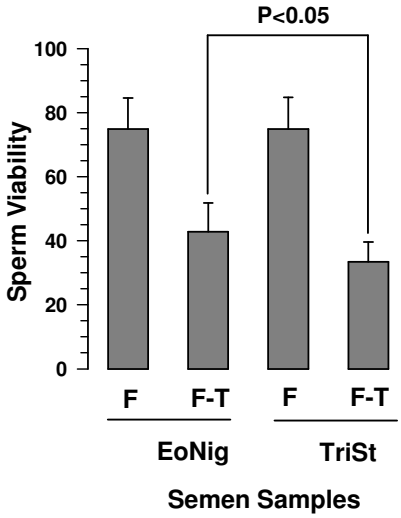

Figure 3. Comparison of bull sperm viability of fresh and frozen-thawed samples obtained with EoNig and TriSt. Line connecting bars indicates statistical differences between means (Paired-T test). EoNig shows data of live sperm, TriSt shows data of acrosome intact live sperm. F = Fresh, F-T = Frozen-Thawed, EoNig = Eosin-Nigrosin, TriSt = Triple Staining.

Table 1. Bull sperm viability compared by two staining techniques. Different letters within rows and columns indicate significant differences between means of semen samples and staining techniques; a and b P= 7.2 E-07, b and c P= 5.7 E-09, c and d P= 3.45 E-011. TriSt data shows the mean number of acrosome intact live sperm added to acrosome reacted live sperm.

Staining Technique	Semen samples	
	Fresh	Frozen-Thawed
Eosin-Nigrosin	74.84 ± 9.72 [a]	42.84 ± 8.92 [b]
Triple Staining	83.62 ± 8.45 [c]	37.25 ± 8.25 [d]

Table 2. Individual bull sperm viability compared by EoNig and TriSt techniques in fresh and frozen-thawed samples. Different letters in rows within a column indicate significant differences between means; P<0.05. TriSt data shows the mean number of acrosome intact live sperm added to acrosome reacted live sperm.

Bulls	Eosin-Nigrosin		Triple Staining	
	Semen samples		Semen samples	
	Fresh	Frozen-Thawed	Fresh	Frozen-Thawed
1	52.0 [a]	26.5 [a]	89.8 [e]	31.5 [a]
2	73.8 [b]	51.5 [b]	84.8 [d]	49.5 [a]
3	75.5 [b]	38.0 [c]	78.3 [b]	30.8 [a]
4	75.8 [b]	40.8 [c]	73.8 [a c]	40.3 [a]
5	79.0 [b]	41.3 [c]	75.5 [a b]	30.8 [a]
6	79.3 [b]	40.3 [c]	78.0 [b]	48.8 [a]
7	80.0 [b c]	53.0 [b]	93.8 [f]	29.0 [a]
8	83.5 [c]	51.5 [b]	92.3 [f]	37.5 [a]

DISCUSSION

TriSt differentiates sperm viability and simultaneously acrosomal status, indicating the number of sperm morphologically able to perform fertilization (AILS), while EoNig considers that the whole number of living sperm could be able to fertilize. When EoNig staining smears are evaluated, non stained sperm cells were consider alive, whereas, partially and totally stained cells were considered dead, since their plasma membrane was altered and allowed stain penetration.

TriSt implies the use of the following dyes: Trypan blue, which is a diazo dye derived from toluidine, a negatively charged vital stain and has a great binding affinity for various membranes and tissues, but it is used to selectively color dead cells, yet it remains to be determined under what circumstances the dye may color membranes. Rose Bengal is an aromatic anionic dye that binds to proteins, and has more affinity to less hydrophobic domains. Bismark brown is a soluble organic diazo die that belongs to the group of heteropolycyclic dyes, and it stains acid mucins.

The results indicate that the human sperm patterns obtained by TriST were different form the bull sperm, Talbot & Chacon (1981) reported white or non stained acrosomal region for the acrosome reacted sperm, in contrast acrosome reacted bull sperm acquired a light color from the Trypan blue (dead sperm) or Bismark brown (live sperm).

One possible explanation to the differences observed on the TriSt patterns between human and bull sperm is that after the acrosome reaction event, the inner acrosomal membrane and its associated components are exposed, therefore the staining dyes might have different affinity due to the reorganization of glicoproteins on the inner acrosomal membrane which differs within species. Furthermore, the different glycoproteins expressed on the inner acrosomal membrane suffer various modifications on their sugar content, which many authors have suggested is a critical event for facilitating sperm-egg interaction (Holt, 1995; Riley, et al., 2002; Shetty, et al., 2003; Cummersom, et al., 2005).

Recent reports indicate that the sperm membrane integrity is influenced by centrifugation (Weiss, et al., 2004), therefore another possible explanation for the different staining patterns obtained on bull sperm is that the juxtaposition of the plasma membrane with the nuclear membrane over the equatorial region was disrupted by the centrifugation process and allowed the diffusion of the dyes in that portion of the head (Maxwell & Johnson, 1997).

It is important to have real images of the different patterns obtained with TriSt, in order to have a reference for future experiments. Even though, there are many reports on the use of the TriSt technique, to the best of our knowledge, this is the first time that TriSt patterns for bull sperm are shown.

Our data assessed by EoNig revealed that after thawing less than 50% of sperm population is able to survive. Nevertheless, the most important aspect of this study reveals that the mean number of cryopreserved sperm that could actually perform fertilization, the acrosome intact living sperm, is about one third of the population. Altogether our data indicates that the EoNig technique is a useful

tool for evaluating sperm viability of fresh semen, however it does not provide enough information about the sperm acrosomal status, therefore it overestimates the proportion of cryopreserved cells that is able to fertilize. In that regard the TriSt technique is a method that significantly contributes to conventional sperm assessment tests in predicting viability and simultaneously acrosome status of cryopreserved bull semen. Therefore TriSt should be considered as a powerful tool to be incorporated as a routine test for cryopreserved semen analysis.

Anytime semen from different males is obtained for experimental purposes, it is commonly usual to find differences between individuals, even within the same breeds. Our results show that when EoNig was performed to evaluate sperm viability, one of the bulls scored below the mean, despite of having gone through the selection process, where this sample scored at least 70% of motility and 3-4 of mass motility. If only the EoNig assay had been considered, this sample should have been withdrawn, not only from the experiment, but also from the semen processing center. However, when the same sample of this bull was analyzed by TriSt, the mean number of viable sperm from the fresh sample scored within the acceptable parameters of inclusion and did not show statistical differences with the rest of the bulls in the frozen-thawed samples. The latest could be a consequence of the TriSt process which allows a better differentiation of the sperm patterns, since it provides a clear background on the smear.

There are various staining protocols developed to assess the quality of sperm used in assisted reproductive techniques, although most of them require of highly sophisticated equipment. Different fluorescent probes are tested for sperm, which require a fluorescence microscope in order to expose treated cells to distinct excitation and emission properties, or computer assisted programs such as CASA, which are not easily available. Hoechst 33258, Yo-Pro-1, PI, SYBR-14, CTC are among the most commonly used to detect sperm damages after thawing (Silva & Gadella, 2006). Furthermore, the use of fluorescent stains requires special handling care, storage and a proper disposable container.

Previous work done by Garner et al. (1994) to assess bovine sperm viability using SYBR-14 and PI, obtained an average media of 50.3% of living sperm in frozen semen, even after they removed damaged sperm by a filtration method. In contrast, by using the EoNig staining, a 42.84 ± 8.92 SD percentage of viable cells on frozen-thawed bovine semen could be determined in the present work. Since, results on viability of bovine semen using either staining method do not show statistical differences, we do not find any advantage on the use of fluorescent reagents over the EoNig staining.

On the other hand, our results are not in agreement with those reported by the use of fluorescent probes to determine the percentage of viable acrosome-intact cryopreserved bovine sperm. Research done by Nagy et al. (2003), using SYBR-4 combined with PE-PNA and PI aid by the flow citometer, showed an increased proportion of acrosome intact cells following freeze-thaw, detecting a mean value of 59.36±12.38 SD. This result differs from the one obtained in this work by the TriSt, which determined a 33.4±6.2 SD of AILS. These differences could be explained by the unequal conditions on the entire methodology, by instance the extenders used on the previous experiments are different, plus the storage time of cryopreserved semen was not specified and it is well known that the flow citometer is able to distinguish only those cells that can pass by the column one at the time, and discharges those that go through the column overlapping or in a different angle, therefore this estimations are not one hundred percent accurate.

The later indicates that the TriSt technique could be easily adopted by a small laboratory since it does not require neither of highly sophisticated equipment nor expensive material.

Garde et al. (1992) made adaptations to the TriSt technique to evaluate ovine semen and compared their results with the application of different evaluation techniques, and concluded that TriSt offers advantages over the rest of the staining techniques used for acrosomal status of cryopreserved samples. The present results are in accordance to those reported for Garde et al. (1992), since the advantages of the triple staining technique on evaluating the number of sperm cells that have undergone acrosomal reaction has been taken as a useful parameter to evaluate spermatic function in response to the cryopreservation process for sperm of different species, among those are human, canine, equine and bovine sperm (Peña, 2001; 2003; Rathi, 2001; Martínez et al, 2006)

CONCLUSION

In conclusion, our study demonstrated that both staining techniques, the EoNig as well as the TriSt, can be consider as useful tools for evaluating fresh bovine semen. However, when frozen thawed bovine semen is evaluated, EoNig over estimates the proportion of living sperm. Furthermore, the use of

the TriSt offers more reliable results, since this technique allows to accurately determine the proportion of AILS, which is an important parameter to be consider as a crucial point in the evaluation of a freezing-thawing procedure on predicting the fertilizing ability of bovine semen prior to their use in AI.

ACKNOWLEDGEMENTS

This work was supported by a grant from UNAM (PAPIIT IN-206506, MLJM) and CONACYT (79921, EOHG). We thank Dr. W. Remberg and Dr. E. Camors (Semen Processing Center RECA, Querétaro, México) for providing the bull semen and Dra. Ma. Elena Reséndiz for technical assistance.

REFERENCES

Bedford J.M. 1990. Germ cells and fertilization. Reproduction in mammals. Austin and Short Ed. Cambridge University Press. 1: p128-163.

Brito F.C., Barth A.D., Bilodeau-Goeseels S., Panich P.L. & Kastelic P. 2003. Comparison of methods to evaluate the plamalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology 60: 1539-1551.

Buhr M.M., Fiser P., Bailey J.L. & Curtis E.F. 2001. Cryopreservation in different concentrations of glycerol alters boar sperm and their membranes. J Androl. 22: 961-969.

Correa L.M., Thomas A. & Meyers S.A. 2007. The macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to motility and morphology defects. Biol Reprod. Article in press.

Cummerson J.A., Flanagan B.F., Spiller D.G. & Johnson P.M. 2006. The complement regulatory proteins CD55 (Decay accelerating factor) and CD59 are expressed on the inner acrosomal membrane of human spermatozoa as well as CD46 (membrane factor protein). Immunology 118: 333-342.

Garde J., García A.C., Gutiérrez A. & Vázquez I. 1992. Triple tinción para valorar acrosomas normales y viabilidad espermática en semen ovino. Med. Vet. 9: 1-6.

Garner D.L., Johnson L.A., Yue S.T., Roth B.L. & Hugland R.P. 1994. dual DNA staining assessment of bovine sperm viability using SYBR-14 AND Propidium Iodide. J. Androl. 15:620-629.

Hammerstedt R.H., Graham J.K. & Nolan J.P. 1990. Cryopreservation of mammalian sperm what we ask them to survive. J Androl. 11: 73-88.

Hammerstedt R.H. & Graham J.K. 1992. Cryopreservation of poultry sperm: the enigma of glycerol. Cryobiology 29:26-38.

Holt W.V. 1995. The sperm plasma membrane, p.1-16. In: Fenichel P. & Parinaud J. Human Sperm Acrosome Reaction. Montrouge, John Libby Eurotext.

Huo L.J., Ma X.H. & Yang Z.M. 2002. Assesment of sperm viability, mitochondrial activity, capacitation and acrosome intactness in extended boar semen during long term storage. Theriogenology 58:1349-1360.

Januskauskas A., Jahannisson A., Soderquist L.K. & Rodríguez-Martínez H. 2000. Assesment of sperm characteristics post-thaw and response to calcium ionophore in relatioin to fertility in swedish dairy AI bulls. Theriogenology 53: 859-875.

Jeyendran R.S., Van-Der-Ven H.H., Pérez-Pelaez M. & Zaneveld L.J. 1985. Effect of glycerol and cryopreservation on oocyte penetratioin by human spermatozoa. Andrología 17:241-248.

Martínez C.O., Juárez-Mosqueda M.L., Hernández J. & Valencia J. 2006. Cryopreservation of bull spermatozoa alters the perinuclear theca. Theriogenology 66:1969-1975.

Maxwell W.M. & Johnson L.A.1997. Membrane status of boar spermatozoa after cooling or cryopreservation. Theriogenology 48:209-219.

Mazur P. 1984. Freezing of lliving cells: mechanisms and implications. Am. J. Physiol. 247:125-142.

Nagy S., Jansen J., Topper E.K. & Gadella B.M. 2003. A triple stain flow cytometric method to assess plasma and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol. Reprod. 68:1828-1835.

Peña A.I., Johannisson A. & Linde-Forsberg C. 2001. Validation of flow citometry for assessment of viability and acrosomal integrity of dog spermatozoa and evaluation of different methods of cryopreservation. J. Reprod. Fertil. 57:371-376.

Peña A.I., Luguilde L.L., Barrio M., Herradon P.G. & Quintela L.A. 2003. Effects of Equex from different sources on post-thaw survival, longevity and intracellular Ca concentration in dog spermatozoa. Therioigenology 59:1725-1739.

Pereira R.J.T.A., Tuli R.K., Wallenhorst S. & Holtz W. 2000. The effect of Heparin, caffeine and calcium ionofore A2387 on in vitro induction of the acrosome reaction in frozen-thawed bovine and caprine spermatozoa. Theriogenology 54:185-192.

Petrunkina A.M., Gröpper B., Töpfer-Petersen E. & Günzel-Apel A.R. 2004. Volume regulatory function and sperm membrane dynamics as parameters for evaluating cryoprotective efficiency of a freezing extender. Theriogenology 63:1390-1406.

Rajesh K.N. & Preeti B.R. 2004. Role of tyrosine phosphorilation in sperm capacitation/acrosome reaction. Reprod. Biol. Endocr. 2:75.

Rasul Z., Ahmed N. & Anzar M. 2007. Antagonist effect of DMSO on the cryoprotection ability of glycerol during cryopreservation of buffalo sperm. Theriogenology 68:813-819.

Rathi R., Colenbrander B., Bevers M.M. & Gadela B.M. 2001. Evaluation of in vitro capacitation of stallion spermatozoa. Biol. Reprod. 65:462-470.

Ricker J.V., Linfor J.J., Delfino W.J., Kysar P., Scholtz E.L., Tablin F., Crowe J.H. & Ball B.A. 2006. Equine sperm membrane phase behavior: the effects of lipid-based cryoprotectants. Biol. Reprod. 74:359-365.

Riley R.C., Kemper C., Leung M. & Atkinson J.P. 2002. Characterization of Human membrane cofactor protein (MCP, Cd46) on spermatozoa. Mol. Reprod. Dev. 62:534-546.

Rodríguez-Martínez H. 2000. Evaluación de semen congelado. Métodos tradicionales y de actualidad. IVIS. P.1-5.

Salisbury G.W. & Mercier E. 1945. The Reliability of Estimates of the Proportion of Morphologically Abnormal Spermatozoa in Bull Semen. J. Anim. Sci. 4:174-178.

Shetty J., Wolkowicz M.J., Digilio L.C., Klotz K.L., Jayes F.L., Diekman A.B., Westbrook V.A., Farris E.M., Hao Z., Coonrod S.A., Flickinger J.C. & Herr J.C. 2003. SAMP14, A novel acrosomal membrane associated, glycophosphatiddylinositol-anchored member of the Ly-6/Urokinase- type plasminogen activator receptor superfamily with a role in sperm-egg interaction. J. Biol. Chem. 278:30506-30515.

Silva P.F.N. & Gadella B.M. 2006. Detection of damage in mammalian sperm cells. Theriogenology 65:958-978.

Szasz F., Sirivaidyapong S., Chen F.P., Voorhout W.F., Marks A., Colenbrander B., Solti L. & Gadella B.M. 2000. Detection of calcium ionophore induced membrane changes in dog sperm as a simple method to predict the cryopreservability of dog semen. Mol. Reprod. Dev. 55:289-298.

Talbot P. & Chacón R. 1981. A triple staining thechnique for evaluating normal acrosome reaction of human sperm. J. Exp. Zool. 215:201-208.

Thomas C.A., Garner D.L., Dejarnette J.M. & Marshall C.E. 1997. Fluorometric assessment of acrosomal integrity and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 56:991-998.

Troup S.A., Lieberman B.A. & Matson P.L. 1994. The acrosome reaction to ionophore challenge test: assay reproductibility, effect of sexual abstinence and results for fertile men. Hum. Reprod. 9:2079-2073.

Watson P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. 7:787-891.

Watson P.F. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60/61:481-492.

Weiss S, Janett F, Burger D, Hässig M, Thun R. & Schweiz A.T. 2004. The influence of centrifugation on quality and freezability of stallion semen. Theriogenology 146:285-293.

World Health Organization. 1992. WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. 3rd ed, Cambridge, The Press Syndicate of the University of Cambridge.

Wundrich K., Paasch V., Leicht M. & Glander H.J. 2006. Activaton of caspaces in human spermatozoa during cryopreservation and immunoblot study. Cell Tissue Bank 7:81-90.

Yanagimachi R. 1994. Mammallian fertilization. In: Physiology of reproduction. Edited by Knobil ENJ. New York , Raven Press. p.189-317.