VETERINÁRIA NOTÍCIAS

DOI: http://dx.doi.org/10.14393/VTV-v23n1-2017.2

A comparative study of three different dyes evaluating the physical integrity of the plasma membrane of cryopreserved bovine spermatozoa

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ABSTRACT

The morpho-functional nature of the plasma membrane assists to transport nutrients into the interior of spermatozoa, which requires the physical integrity of the membrane. This present study aims to compare three different dyes used to determine the integrity of the plasma membrane of bovine spermatozoa after freezing. The dyes tested were eosin 3% (p/v), trypan blue 0.4% (p/v), and propidium iodide (1.5 mM). Two sample groups were formed for the research. In the first group, one ejaculate was collected from each 11 animals, and in the second group, ten ejaculates were obtained from one animal. The fresh semen was evaluated using microscopy. The post-freezing samples were assessed using microscopy and flow cytometry. The results are presented in the form of average viability percentages ± standard deviations. The values membrane integrity, for the dyes eosin, trypan blue, and propidium iodide were 56.8±8.3%; 56.1±7.9%; 54.6±9.1%; respectively. There were no significant differences between the groups ($p \geq 0.001$). In comparative analysis of the techniques, flow cytometry enabled a greater number of cell evaluations, compared to phase contrast microscopy, with less variability and faster sample evaluation, hence adding value to the microscopy tests.

Keywords: eosin, trypan blue, propidium iodide.

Introduction

The spermatozoon plasma membrane involves the entire cell and defines the limit between the cytosol and the extracellular environment (ALBERTS et al., 2004). The semi-permeable nature of the membrane enables the maintenance of a chemical gradient of ions and other soluble components that are essential for the viability of the cell. The morpho-

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Artigo recebido em: 06/07/2016. Aceito para publicação em: 20/03/2017.

functional nature of the plasma membrane enables the transport of nutrients into the interior (FLESCH AND GADELLA, 2000), the control of internal osmolarity (LAGARES et al., 1998), and the biochemical functioning of acrosome reaction (GADELLA AND COLENBRANDER, 2003).

A variety of techniques to evaluate the integrity of the plasma membrane has been reported in the literature. Although the method described by Blom, (1950), employing vital staining with eosin dye (3%, w/v), is one of the most practical. Furthermore, the use of fluorescent dyes provides greater contrast and lower variability, compared to classical coloration procedures (NEILD et al., 2005), and the propidium iodide (PI) dye is highlighted for research use due to its ease preparation and the simplicity of the technique (GRAHAM et al., 1990). In addition, flow cytometry can be applied to quantify dead cells by means of brilliant red dye fluorescence (JONES AND SENFT, 1985; HARRISON AND VICKERS, 1990), with high accuracy and repeatability, and provides improved robustness of evaluations when performed together with phase contrast microscopy (EVENSON et al., 1993).

This present study aims to determine the integrity of cryopreserved bovine spermatozoa plasma membranes, using three different vital dyes. Motility and motion were evaluated in three different stages – fresh, post-dilution and post-freezing stages – to standardize the semen samples on procedures freezing effects. In this context, the phase contrast microscopy employed eosin 3% (p/v) and trypan blue 0.4% (p/v), while flow cytometry used propidium iodide (1.5mM).

Materials and methods

It has been obtained 21 ejaculates from bovines (aged 5-10 years). Prior to freezing, the ejaculates contained a minimum of 50% of

spermatozoa with progressive motility. All the samples were collected and frozen according to the protocol established by the Alta Genetics Reproduction Center (Alta Genetics Ltda., Uberaba, Brazil). The samples have been thawed as described in the Manual for Andrological Examination and Evaluation of Animal Semen (CBRA, 1998).

In order to evaluate the physical integrity of the plasma membrane, three different dyes were applied. Firstly, eosin (3% w/v, Sigma Chemical Co., St. Louis, MO, USA), following the procedure described by Blom (1950), using 10 µL of dye and 10 µL of semen, placed on a slide with a cover slip and analyzed by phase contrast microscopy at a magnification of x400, in which red-colored spermatozoa were considered to have plasma membrane lesions. Then, trypan blue (0.4% w/v, Sigma Chemical Co.), using 10 µL of dye and 10 µL of semen, placed on a slide with a cover slip and analyzed by phase contrast microscopy at a magnification of x400, in which blue-colored spermatozoa were considered to have plasma membrane lesions. Finally, the propidium iodide (1.5 mM, PI 4170, Sigma Chemical Co.) sperm membrane integrity was evaluated after diluting samples 1:20 in phosphated buffer solution (PBS)(250 µL semen in 5 mL PBS) at 98.6°F. A sample of 200µL was added to tubes containing 30 µL propidium iodide and 1 mL PBS, which were kept at room temperature in the dark for 10 min and then assessed with a flow cytometer (FACScan, Becton Dickinson®, San Jose, CA, USA). The red fluorescence (FL3) was collected through a 650-nm long-pass filter, after a 640-nm long-pass filter. Data cells were collected in list mode using BD CellQuest Pro version 4.0 software (Becton Dickinson ®, San Jose, CA, USA) conforming a protocol reported by Vasconcelos et al. (2010).

Statistical analysis

The data were tested for normality and homogeneity, followed by application of one-way ANOVA with Tukey's post-test for multiple

comparisons, by GraphPad Prism 4 software. The differences were considered to be statistically significant when p≤0.001.

Results and discussion

All the ejaculates were evaluated before and after freezing, considering the following parameters: total motility, progressive motility, and vigor. The mean values obtained are provided in Table 1. In the groups of semen tested at the three different stages (fresh, post-dilution, and post-freezing), in terms of the percentages of spermatozoa showing total and progressive motility, were observed significant differences between the treatments (p≤0.001).

Table 1. Values of the evaluation parameters for semen obtained from different steps of the freezing-thawing process (21 ejaculates, n=21).

Ejaculate	Motility(%)		Vigor
	Total	Progressive	
Fresh	70.4±4.8[a]	66.1±3.9[a]	4.1±2.4[a]
Post-dilution	69.6±5.2[a]	64.9±4.6[a]	3.8±1.8[a]
Post-freezing	41.3±7.4[b]	34.3±6.1[b]	3.1±1.3[b]

The data are given as means ± standard deviations. Different superscript letters, in the same column, indicate significant differences between the treatments (p≤0.001).

Regarding the evaluation of vigor, the values obtained for the semen from the twenty one ejaculates were 4.1, 3.8, and 3.1 for the fresh, post-dilution, and post-freezing semen, respectively. In this context, statistically significant differences (p≤0.001) were only found between the post-freezing group and the other groups.

These results, as described on table 1, confirm the experimental design as stated above in material and methods that consist in motility samples bigger or equal to 50%.

The challenge faced by the sperm cell during the freezing process is not its ability to withstand a storage temperature of 320.8°F, but rather its

capacity to tolerate the changes that occur during the intermediate temperature range (5 to -76°F), through which it must pass twice (during freezing and thawing). This is due to the formation of intracellular ice (MAZUR, 1984). The extent of the damage depends on the degree of ice formation and the size of the crystals. In this context, large crystals can result in mechanical damage to the cells, directly influencing the motility of the sperm, and are one of the main causes of cell death during freezing. On the other hand, small crystals might not be prejudicial to the cell. However, during the thawing process, the growth of these small crystals due to recrystallization can cause severe damage (WATSON, 1995).

The results of the study demonstrate that there was a reduction in the percentage of motile cells after freezing/thawing, which could have been a consequence of the mechanical damage caused by large crystals. Nevertheless, the values obtained for the different parameters were in accordance with the guidelines for post-freezing bovine semen, whereby progressive motility and vigor should be ≥30% and ≥3, respectively (CBRA, 1998).

Adverse changes in the structure and integrity of membranes have been associated with reduced fertility of semen that has been frozen and subsequently thawed. The composition of phospholipids is asymmetrical, with phosphatidylcholine and sphingomyelin concentrated on the external side of the lipid bilayer, while phosphatidylserine and phosphatidylethanolamine are concentrated inside. During cryopreservation, there is translocation of the phospholipid structure, with externalization of phosphatidylserine in a process known as lipid scrambling. Moreover, the mechanisms involved in the scrambling, phosphorylation of tyrosine, and efflux of cholesterol observed for cryopreserved spermatozoa differ from capacitation. However, as in capacitation, cryopreservation results in greater membrane fluidity, with exposure of binding sites to external molecules, in order that the cell can capacitate easier and/or faster (GADELLA AND COLENBRANDER, 2003). In addition, it can also lead to

lesions in the membrane structure, which explains the differences between the values obtained from fresh and post-freezing semen.

Using the eosin dye to evaluate twenty one ejaculates, there was a significant difference ($p \leq 0.001$) between the fresh and post-freezing semen in terms of the percentages of cells with intact membranes (82.7±7.4 and 56.8±8.3, respectively).

All three dyes were only used in the case of the post-freezing semen. The values for membrane integrity were calculated from the average of 21 samples for each dye. There were no significant differences between these values (Table 2).

Table 2. Values of the evaluation of membrane integrity obtained from post thawing semen, using three different dyes. (n=21)

Ejaculate	Evaluation		
	Eosin	Trypan Blue	Propidium Iodide
Post-freezing	56.8±8.3[a]	56.1±7.9[a]	54.6±9.1[a]

The data are given as means ± standard deviations. Different superscript letters, in the same column, indicate significant differences between the treatments ($p \leq 0.001$).

The physical integrity of the plasma membrane is normally evaluated after staining the cells with suitable dyes. When the cells succeed in preventing the entry of dye, the membrane is considered intact. Thus, most of the dyes used have a high affinity for DNA; however, they only attach to it if there is a lesion in the membrane. In this context, if the plasma membrane of the spermatozoon is not functionally intact, the cell is not considered viable. Blom (1950) noticed that eosin dye could be used to identify the preservation or lesion of the membrane, with the cell acquiring a pink color in the case of lesion. Furthermore, other dyes such as YO-PRO-1 (which has excitation/emission wavelengths of 488/515 nm) (HARRISON AND VICKERS, 1990) and propidium iodide (PINTADO et al., 2000) can also be used to determine membrane integrity by means of flow cytometry,

which offers high precision and accuracy, likewise and straightforward evaluation of semen samples (EVENSON et al., 1993).

It is suitable to consider that these technologies require costly equipments, such as a flow cytometer and a phase contrast microscope, as well as the availability of fluorescent probes. In addition, these probes are not manufacture locally in Brazil ; thus, it must be imported, entailing lengthy bureaucratic processes (ARRUDA et al., 2011). Therefore, the expensive technologies become an obstacle to overcome and to use these probes as a routine in Brazil.

Even though all the dyes employed in this research (eosin, tryptan blue, and propidium iodide) can be used to investigate the integrity of the spermatozoon plasma membrane, two different techniques were adopted. For the first two dyes, phase contrast microscopy was employed, which enabled the analysis of around 200 spermatozoa per slide. In the case of propidium iodide, flow cytometry was used, enabling assessment of around 50,000 spermatozoa in each test, hence improving analytical robustness and providing greater accuracy and precision compared to the classical coloration methods employing phase contrast microscopy (NEILD et al., 2005). As a result, these described procedures allow introducing value to traditional microscopy tests.

Conclusions

The different dyes could be used in the routine evaluation of the plasma membrane integrity of bovine spermatozoon, without the loss of repeatability allowing greater accuracy of the results.

Acknowledgments

The authors are grateful for the support provided by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, Project no. APQ-01256-09) and Programa de Mestrado em Sanidade e Produção Animal nos Trópicos (PAPE, Project no. 2008/006).

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Estudo comparativo de três diferentes corantes para avaliação da integridade física da membrana plasmática de espermatozoides bovinos criopreservado

Resumo

O aspecto morfo-funcional da membrana plasmática facilita o transporte de nutrientes para o interior do espermatozoide, o que está ligado à integridade física da membrana plasmática. O presente trabalho objetivou comparar três diferentes corantes na avaliação da integridade física da membrana plasmática do espermatozoide bovino, pós-congelamento. Utilizando os corantes eosina 3% (p/v), azul de tripan 0,4% (p/v) e iodeto de propídio (1,5mM). Foram formados dois grupos para pesquisa. O primeiro, um ejaculado de cada 11 animais e um segundo grupo de dez ejaculados de um único animal. Foi realizada avaliação microscópica do sêmen fresco (motilidade total, motilidade progressiva, vigor e integridade da membrana plasmática). Das amostras congeladas foram avaliados todos os parâmetros descritos anteriormente, além da técnica de eosina, azul de tripan, e iodeto de propídio. Os resultados estão apresentados como média de porcentagem ± desvio padrão. Os valores para a integridade de membrana, utilizando os corantes eosina, azul de tripan, e iodeto de propídio, foram 56.8±8.3%; 56.1±7.9%; 54.6±9.1%, respectivamente. Não há diferença significativa entre os grupos ($p \geq 0.001$). Na análise comparativa das técnicas podemos observar que a citometria de fluxo, possibilita um número maior de avaliação celular, quando comparado à técnica de microscopia de campo claro, possibilitando uma menor variabilidade e maior rapidez na avaliação da amostra.

Palavras-chave: eosina, azul de tripan, iodeto de propídio.

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