

## Kinetics of changes in plasma membrane related to apoptosis and necrosis in bovine sperm cells at different incubation times

Weber Beringui FEITOSA<sup>1</sup>  
 André Monteiro da ROCHA<sup>1</sup>  
 Camilla Mota MENDES<sup>1</sup>  
 Marcella Pecora  
 MILAZZOTTO<sup>1</sup>  
 Marcelo Demarchi GOISSIS<sup>1</sup>  
 Cassia Cestari DELBONI<sup>1</sup>  
 José Antonio VISINTIN<sup>1</sup>  
 Mayra Elena Ortiz D'Ávila  
 ASSUMPTÃO<sup>1</sup>

1- Laboratory of *In Vitro* Fertilization, Cloning and Animal Transgenesis of  
 Department of Animal Reproduction College of Veterinary Medicine and Animal  
 Science, University of Sao Paulo, Sao Paulo, Brazil

### Abstract

Incubation time induce damages in sperm cells by necrosis and/or apoptosis. The aim of this study was the evaluation of changes in plasma membrane related to apoptosis and necrosis in bovine sperm cells through 2 hours of incubation. Sperm cells were incubated at 5% (v/v) CO<sub>2</sub> in air for 0, 30, 60, 90 and 120 minutes. After each period, sperm cells were incubated with fluorescent probes Yo-pro and propidium iodide (PI) to detect change in plasma membrane related to apoptosis and necrosis respectively. Using Yo-pro/PI assay, three different subpopulations of sperm cells were detected by flow cytometry: a) necrotic sperm cells (PI<sup>+</sup> and Yo-pro<sup>-/+</sup>); b) apoptotic sperm cells (Yo-pro<sup>+</sup> and PI) and c) living cells (Yo-pro<sup>-</sup> and PI). The percentage of live cells (plasma membrane integrity) significantly decreases over 2 hour of incubation, on the other hand, the percentage of necrotic and apoptotic cells increase during incubation. Changes in plasma membrane integrity were correlated to incubation time. While live cells were negatively correlated with the increase of incubation time, necrosis and apoptosis were positively correlated. It was also observed that necrosis was the main damage in sperm cells in all incubation times. In conclusion, incubation time induces changes in plasma membrane integrity related to necrosis and apoptosis, whether necrosis is present in higher quantity in all incubation times.

**Key words:**  
 Spermatozoa.  
 Cell death.  
 Flow cytometry.  
 Cell damage.  
 Bull.

### Correspondent address:

meoaa@usp.br; Tel: + 55 11 3091 7665;  
 Fax: + 55 11 3091 7412  
 University of Sao Paulo, College of Medicine  
 Veterinary and Animal Science, Department  
 of Animal Reproduction. Av. Prof. Orlando  
 Marques de Paiva, 87 - Cidade Universitária  
 São Paulo, SP - Brazil. CEP: 05508 270

Recebido para publicação: 15/04/2006  
 Aprovado para publicação: 29/05/2008

### Introduction

Use of frozen-thawed semen became routine in artificial insemination and in vitro fertilization. After thawing, a considerable number of spermatozoa do not survive and considerable numbers of surviving cells are affected structurally or functionally. This implies in reduction of fertilization rates, because success of fertilization depends on the amount of competent spermatozoa that survive until penetration in the oocyte.<sup>1</sup>

Several methods used to estimate fertility are based on in vitro evaluation of semen physical characteristics. However, it was demonstrated that these conventional techniques are not able to estimate accurately and repeatedly the fertility of a semen

sample.<sup>2,3</sup> Additionally, it was demonstrated that high fertility semen analysed by conventional test is not necessarily related with high reproductive rates. This indicates that these characteristics can not be truly associated with the reproductive capacity and fertility.<sup>4,5</sup> Thus, evaluation of semen based on these tests may allow the undesired use of semen from animals with low fertility.

Studies demonstrated that sperm cells exhibit characteristics of apoptotic cells. Apoptosis is a physiologically programmed cellular death characterized by shrinkage of the cell, reduction of cytoplasmic volume, condensation of chromatin, DNA cleavage and nuclear fragmentation. In contrast, necrosis is characterized as a result of injuries that cause increase of the cellular volume and

rupture of membrane.<sup>6</sup>

A significant negative correlation was reported between the proportion of apoptotic cells and sperm viability or motility in ejaculated semen.<sup>7,8,9,10</sup> Additionally, the presence of apoptosis in sperm cells has been correlated with infertility.<sup>11,12,13</sup>

Evaluation of apoptosis and necrosis in sperm cells would be a good indicative of the semen quality. Thus, the aim of this study was to investigate the kinetics of changes in post-thaw bovine sperm plasma membrane integrity, associated to apoptosis and necrosis through 2 hours of incubation.

## Materials and Methods

### *Chemicals and reagents*

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tissue culture media 199 (TCM 199 - HEPES and Bicarbonate) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, USA).

### *Semen incubation*

Straws samples from the same animal (Lagoa da Serra, Sertãozinho, Brazil) were thawed by water bath immersion at 37 °C for 30 seconds and centrifugated in discontinuous Percoll gradient (90% and 45% with SPERM-TALP medium [Parrish et al., 1988]) for 30 minutes at 600 x g. Pellets were resuspended in SPERM-TALP media and centrifugated for 5 minutes at 200 x g. After washing, motility was evaluated and concentration was calculated. Sperm cells were resuspended in IVF media (TALP-STOCK, 0.6% (w/v) BSA-V, 0.2 mM, sodium pyruvate, 50 µg/ml gentamicin) at a final concentration of 5 x 10<sup>6</sup> sperm/ml and incubated at 39 °C in a humidified atmosphere of 5% (v/v) CO<sub>2</sub> in air for 2 hours. Sub-samples were taken at 30 minutes intervals for flow cytometry measurements.

### *Apoptosis and necrosis assessment*

The fluorescent probe Yo-Pro (Molecular Probes) was used to detect changes in plasma membrane integrity

related to apoptosis. Fluorescent probe propidium iodide (PI) was used to detect changes in plasma membrane related to necrosis. Sperm cells at concentration of 1 x 10<sup>6</sup> were diluted in 1 ml of PBS added of 2 µl of Yo-Pro (100 µM) and then incubated for 20 minutes at room temperature. After this period, it was added 10 µl of PI (6 µM) and incubated for 10 minutes at room temperature. Flow cytometry analysis was performed immediately after incubation.

### *Flow cytometry*

A total of six replicates with different samples semen were analyzed using FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The green-fluorescent (Apoptosis – Yo-Pro) and red-fluorescent (Necrosis – IP) were detected using FL1 and FL3 detectors respectively. A total of 10 000 events were analyzed and fluorescent signals were recorded after logarithmic amplification. Cell doublets and debris were excluded using an FL3-A versus FL3-W gate and all data were evaluated using the WinMDI 2.8 software.

### *Statistic analysis*

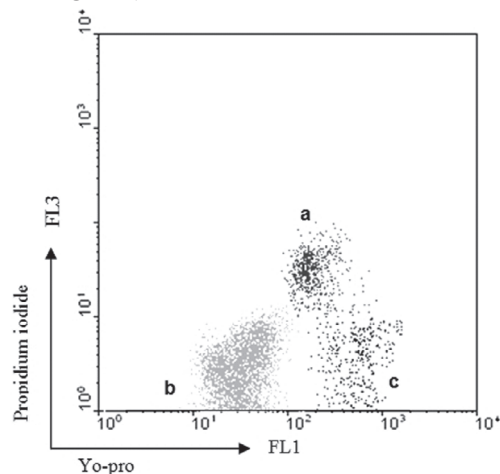
Statistical analyses were performed using PROC GLM and PROC REG of the *Statistical Analysis Systems* (SAS) package (SAS institute Inc., Cary, NC, USA). Dependent variables were plasma membrane integrity (live), necrosis and apoptosis rate. Independent variables were incubation times 0, 30, 60, 90 and 120 minutes. Differences were considered significant if  $P < 0.05$ .

## Results

Apoptosis is distinguished from necrosis by morphological and biochemical changes, including condensation and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm and loss of membrane asymmetry. The fluorescent probe Yo-pro has the ability to penetrate apoptotic cells because of permeability changes associated with the loss in asymmetry of plasma membrane. Thus, Yo-pro green

fluorochrome enters apoptotic cells and binds nucleic acids while propidium iodide (PI) is excluded from these cells.

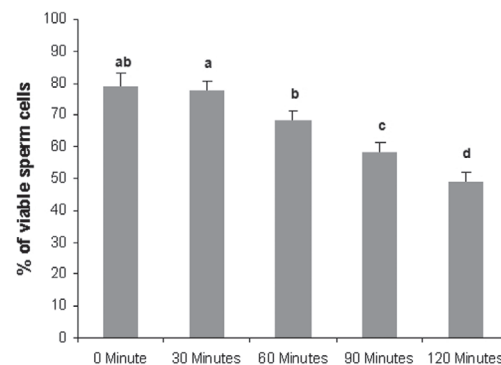
The combined use of Yo-pro and PI probes provides an efficient method for apoptosis detection<sup>14,15</sup>. Typical cytogram of bovine sperm cells labeled with Yo-pro/PI is shown in figure 1. Using Yo-pro/PI, three different subpopulations of sperm cells were detected: a) necrotic sperm cells (PI positive and Yo-pro negative or positive); b) apoptotic sperm cells (Yo-pro positive and PI negative) and c) living cells (Yo-pro and PI negative)



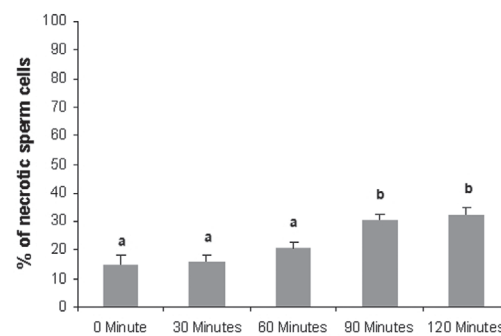
**Figure 1** - Flow cytometry cytogram of frozen/thawed sperm cells showing permeability to Yo-pro and to PI: a) necrotic cells are Yo-pro<sup>-and/or+</sup> and PI<sup>+</sup>; b) living cells are Yo-pro<sup>-</sup> and PI<sup>-</sup> and c) apoptotic cells are Yo-pro<sup>+</sup> and PI<sup>-</sup>

Over 2 hours incubation, a dramatic decrease of approximately 29% was observed in the live sperm cells subpopulation (Yo-pro and PI negative), ranging from an average of 78.8% at 0 hour to 49.2% at 2 hours of incubation ( $P < 0.0001$ ) as shown in figure 2. On the opposite, the percentage of necrotic sperm cells increased significantly from 14.8 % at 0 hour to 32.4 % at 2 hour of incubation ( $P < 0.0004$ ), as shown in figure 3.

In the same way, incubation induced a statistically significant increase in the proportion of apoptotic sperm ( $P < 0.0004$ ). Only 5% of sperm cells showed permeability to Yo-Pro-1



**Figure 2** - Percentage of live sperm cells (plasma membrane integrity) in different periods of incubation. Different letters indicate significant differences among the incubation periods ( $P < 0.001$ )

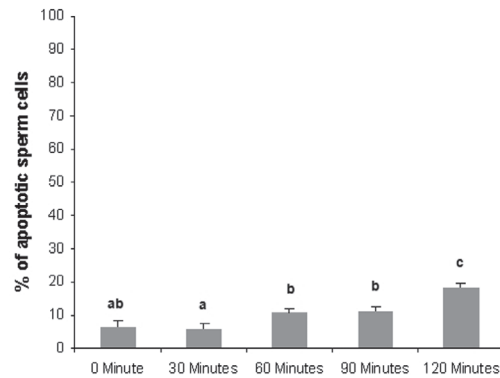


**Figure 3** - Percentage of necrotic sperm cells in different periods of incubation. Different letters indicate significant differences among the incubation periods ( $P < 0.001$ )

immediately after thawing (0 hour of incubation), whereas after 2 hours, this proportion reached 15% (Figure 4).

When necrosis and apoptosis were evaluated in each incubation time, it was observed that necrosis was present in higher quantity at 0 ( $P < 0.0019$ ), 30 ( $P < 0.0001$ ), 60 ( $P < 0.0001$ ), 90 ( $P < 0.0001$ ) and 120 ( $P < 0.0001$ ) minutes of incubation.

Significant effects were observed when data were submitted to linear regression analysis (Figure 5). For live sperm cells the equation obtained was:  $y = -0.2747x + 83.451$  ( $P < 0.0001$ ) and  $R^2$  0.7247. Necrotic sperm cells data produced the equation:  $y = 0.165x + 12.39$  ( $P < 0.0001$ ) and  $R^2$  0.6214. Apoptotic sperm cells were estimated by the equation:  $y = 0.1036x + 4.159$  ( $P < 0.0001$ ) and  $R^2$  0.632.



**Figure 4** – Percentage of apoptotic sperm cells in different periods of incubation. Different letters indicate significant differences among the incubation periods ( $P < 0.001$ )

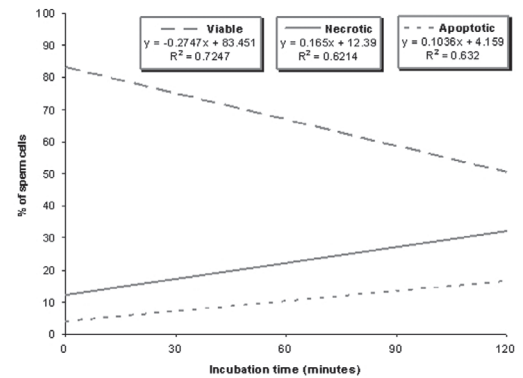
## Discussion

Functional evaluations of plasma membrane integrity potentially characterize the quality of spermatozoa. Several tests were reported for evaluating plasma membranes as supravital staining techniques<sup>16,17</sup> or hypo-osmotic swelling test<sup>18</sup>. Even though these methods can efficiently discriminate viable from dead or damaged cells, they fail in detect early phases of membrane dysfunction or initial phases of apoptosis.<sup>19,20</sup>

Apoptosis is a physiologic regulated process of cell death and is distinguished from necrosis by biochemical and morphological changes. While necrosis results from injury that causes swelling and membrane rupture, apoptosis causes compactation and fragmentation of nuclear chromatin, shrinkage of the cytoplasm and loss of membrane asymmetry.

Thus, Yo-pro has the advantage to detect early phases of membrane alterations because penetrates apoptotic cells with permeability changes, due to the loss in asymmetry of the plasma membrane. Additionally, Yo-pro is inexpensive, quick and easy to perform and provide interesting information on membrane modification related to the apoptotic process.<sup>15</sup>

Our results are in agreement with previous studies which demonstrate that a variable proportion of ejaculated live spermatozoa undergoes apoptosis under in



**Figure 5** – Linear regression analysis between incubation time and percentage of live, necrotic or apoptotic sperm cells

vitro incubation.<sup>21,22,23</sup> On the other hand, it differs from studies which incubation did not induce apoptosis in sperm cell.<sup>24,25</sup> This experiment clearly showed an increased permeability of plasma membranes to Yo-pro in living sperm cells during 2 hours of incubation. Moreover in the present study it was found highly significant association between the increase of incubation time and the percentage of apoptotic sperm cells.

The decrease of sperm quality during incubation may be due to reactive oxygen species (ROS). Despite the physiological effect of ROS in sperm cells, unbalanced ROS production by sperm causes damage.<sup>26</sup> High ROS concentrations causes lipid peroxidation of sperm plasma membranes and induce apoptosis, resulting in alteration of sperm function and fertilizing capacity<sup>23,27</sup> and DNA damage<sup>28</sup>. Additionally, a recent study has been suggested that ROS are responsible for damages to sperm cells during incubation.<sup>23</sup>

However, based on the fact that the percentage of necrotic sperm cells increase during incubation while percentage of apoptotic cells remains unchanged, apoptotic sperm cells has been considered a transitory step between cell viability and necrosis.<sup>29,30</sup>

The results of the present study are in opposition to results from Januskaukas, Johannisson and Rodrigues Martinez<sup>29</sup> and Muratori et al.<sup>30</sup> Despite the fact that the percentage of necrotic sperm cells was higher than percentage of apoptotic sperm cells in all incubation times, they behaved in similar way.

The percentage of necrotic and apoptotic sperm cells increased in same way during incubation, generating similar  $R^2$  after regression analyses ( $R^2 = 0.6214$  and  $R^2 = 0.632$ , respectively).

In conclusion, the present study showed that incubation time induces decrease on the percentage of live cells as consequence of necrosis and apoptosis. Further research is necessary to elucidate what is really responsible for the damage in sperm cells during

incubation and mainly, whether necrotic sperm cells results from transitory apoptotic sperm cells or if necrotic and apoptotic sperm cells are not related during incubation.

## Acknowledgements

This study was supported by The State of Sao Paulo Research Foundation (FAPESP), fellowship 03/10234-7 and grant 03/07456-8.

## Kinetics of changes in plasma membrane related to apoptosis and necrosis in bovine sperm cells at different incubation times

### Resumo

O tempo de incubação causa danos nas células espermáticas relacionados a necrose e/ou apoptose. O objetivo deste estudo foi avaliar as mudanças na membrana plasmática relacionadas a apoptose e necrose em espermatozoides bovinos durante 2 horas de incubação. Os espermatozoides foram incubados a 5% (v/v)  $CO_2$  em ar por 0, 30, 60, 90 e 120 minutos. Depois de cada período, as células espermáticas foram incubadas com as sondas fluorescentes Yo-pro e iodito de propideo (PI) para detectar mudanças na membrana plasmática relacionadas a apoptose e a necrose, respectivamente. Usando Yo-pro/PI assay, três subpopulações diferentes de células espermáticas são detectadas pelo citômetro de fluxo: a) células espermáticas em necrose ( $PI^+$  and Yo-pro<sup>-/+</sup>); b) células espermáticas em apoptose (Yo-pro<sup>+</sup> and PI<sup>-</sup>) e c) células espermáticas vivas (Yo-pro<sup>-</sup> and PI<sup>-</sup>). A porcentagem de células vivas (membrana plasmática íntegra) significativamente diminuiu durante 2 horas de incubação, por outro lado, a porcentagem de espermatozoides em necrose e apoptose aumentaram durante a incubação. As mudanças na integridade da membrana plasmática foram correlacionadas com o tempo de incubação. Enquanto as células vivas foram correlacionadas negativamente com o aumento do tempo de incubação, necrose e apoptose foram correlacionadas positivamente. Também foi observado que necrose foi o principal dano causado pelo tempo de incubação nas células espermáticas. Conclui-se que o tempo de incubação causa alteração na integridade da membrana plasmática relacionadas a necrose e apoptose nas células espermáticas, sendo que necrose foi observada em maior quantidade em todos os tempos de incubação.

### Palavras-chave:

Espermatozóide.  
Morte celular.  
Citômetro de fluxo.  
Dano celular.  
Touro.

### References

- 1 WATSON, P. F. The causes of reduced fertility with cryopreserved semen. **Animal Reproduction Science**, v. 60-61, p. 481-492, 2000.
- 2 CORREA, J. R.; PACE, M. M.; ZAVOS, P. M. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program **Theriogenology**, v. 48, p. 721-731, 1997.
- 3 RODRIGUEZ-MARTINEZ, H. Laboratory semen assessment and predication of fertility: still utopia? **Reproduction in Domestic Animals**, v. 38, p. 312-318, 2003.

- 4 LINFORD, E.; GLOVER, F. E.; BISHOP, C.; STEWARD, D. L. The relationship between semen evaluation methods and fertility in the bull. **Journal of Reproduction and Fertility**, v. 147, p. 283–291, 1976.
- 5 XU, X.; POMMIER, S.; ARBOV, T.; HUTCHINGS, B.; SOTTO, W.; FOXCROFT, G. R. In vitro maturation and fertilization techniques for assessment of semen quality and boar fertility. **Journal of Animal Science**, v. 76, p. 3079–3089, 1998.
- 6 STRASSER, A.; O'CONNOR, L.; DIXIT, V. M. Apoptosis signaling. **Annual Review of Biochemistry**, v. 69, p. 217–245, 2000.
- 7 MARCHETTI, C.; OBERT, G.; DEFFOZEZ, A.; FORMSTECHE, P.; MARCHETTI, P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation, and cell viability by flow cytometry in human sperm. **Human Reproduction**, v. 17, p. 1257–1265, 2002.
- 8 PENA, F. J.; JOHANNISSON, A.; WALLGREN, M.; RODRÍGUEZ-MARTÍNEZ, H. Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. **Theriogenology**, v. 60, p. 677–689, 2003.
- 9 TAYLOR, S. L.; WENG, S. L.; FOX, P.; DURAN, E. H.; MORSHEDI, M. S.; OEHNINGER, S.; BEEBE, S. J. Somatic cell apoptosis markers and pathways in human ejaculated sperm: potential utility as indicators of sperm quality. **Molecular Human Reproduction**, v. 10, p. 825–834, 2004.
- 10 SAID, T. M.; PAASCH, U.; GLANDER, H. J.; AGARWAL, A. Role of caspases in male infertility. **Human Reproduction Update**, v. 10, p. 39–51, 2004.
- 11 ANZAR, M.; HE, L.; BUHR, M. M.; KROETSCH, T. G.; PAULS, K. P. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. **Biology of Reproduction**, v. 66, p. 354–360, 2002.
- 12 LEWIS, S. E.; AITKEN, R. J. DNA damage to spermatozoa has impacts on fertilization and pregnancy. **Cell and Tissue Research**, v. 322, p. 33–41, 2005.
- 13 HUANG, C. C.; LIN, D.; TSAO, H.; CHENG, T.; LIU, C.; LEE, M. Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. **Fertility and Sterility**, v. 84, p. 130–140, 2005.
- 14 IDZIOREK, T.; ESTAQUIER, J.; BELS, F.; AMEISEN, J. C. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. **Journal of Immunological Methods**, v. 185, p. 249–258, 1995.
- 15 MARTIN, G.; SABIDO, O.; DURAND, P.; LEVY, R. Cryopreservation induces apoptosis-like mechanism in bull sperm. **Biology of Reproduction**, v. 71, p. 28–37, 2004.
- 16 EVENSON, D. P.; DARZYNKIEWICZ, Z.; MELAMED, M. R. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. **The Journal of Histochemistry and Cytochemistry**, v. 30, p. 279–280, 1982.
- 17 GARNER, D. L.; JOHNSON, L. A. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. **Biol. Reprod.**, v. 53, p. 276–284, 1995.
- 18 JEYENDRAN, R. S.; VAN DER VEN, H. H.; PEREZ-PELAEZ, M.; CRABO, B. G.; ZANEVELD, L. J. D. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. **Journal of Reproduction and Fertility**, v. 70, p. 219–228, 1984.
- 19 JANUSKAUSKAS, A.; JOHANMSSON, A.; RODRIGUEZ-MARTINEZ, H. Assessment of sperm quality through fluorometry and sperm Chromatin structure assay in relation to field fertility of frozenthawed Semen from Swedish AI bulls. **Theriogenology**, v. 55, p. 947–961, 2001.
- 20 NAGY, S.; HALLAP, T.; JOHANNISSON, A.; RODRIGUEZ-MARTINEZ, H. Changes in plasma membrane and acrosome integrity of frozen-thawed bovine spermatozoa during a 4 h incubation as measured by multicolor flow cytometry. **Animal Reproduction Science**, v. 80, p. 225–235, 2004.
- 21 PAASCH, U.; GRUNEWALD, S.; FITZL, G.; GLANDER, H. J. Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. **Journal of Andrology**, v. 24, p. 246–252, 2003.
- 22 BARROSO, G.; TAYLOR, S.; MORSHEDI, M.; MANZUR, F.; GAVIÑO, F.; OEHNINGER, S. Mitochondrial membrane potential integrity and plasma membrane translocation of phosphatidylserine as early apoptotic markers: a comparison of two different sperm subpopulations. **Fertility and Sterility**, v. 85, p. 149–154, 2006.
- 23 CHAVEIRO, A.; SANTOS, P.; DA SILVA, F. M. Assessment of sperm apoptosis in cryopreserved bull semen after swim-up treatment: a flow cytometric study. **Reproduction in Domestic Animals**, v. 42, p. 17–21, 2007.
- 24 LACHAUD, C.; TESARIK, J.; CAÑADAS, M. L.; MENDOZA, C. Apoptosis and necrosis in human ejaculated spermatozoa. **Human Reproduction**, v. 19, p. 607–610, 2004.
- 25 MARTIN, G.; SABIDO, O.; DURAND, P.; LEVY, R. Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. **Human Reproduction**, v. 20, p. 3459–3468, 2005.
- 26 DE LAMIRANDE, E.; GAGNON, C. Impact of reactive oxygen species on spermatozoa: a balancing

act between beneficial and detrimental effects. **Human Reproduction**, v. 10, p. 15-21, 1995.

27 DURU, N. K.; MORSHEDI, M. S.; OEHNINGER, S. C. Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. **Fertility and Sterility**, v. 74, p. 1200-1207, 2000.

28 AITKTEN, R. J.; KRAUSZ, C. Oxidative stress, DNA damage and the Y chromosome. **Reproduction**, v. 122, p. 497-506, 2001.

29 JANUSKAUSKAS, A.; JOHANNISSON, A.; RODRIGUEZ-MARTINEZ, H. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. **Theriogenology**, v. 60, p. 743-758, 2003.

30 MURATORI, M.; MAGGI, M.; SPINELLI, S.; FILIMBERTI, E.; FORTI, G.; BALDI, E. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. **Journal of Andrology**, v. 24, p. 253-262, 2003.