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

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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_2 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^+ and Yo-pro$^{-/+}$); b) apoptotic sperm cells (Yo-pro$^+$ and PI^-) and c) living cells (Yo-pro$^-$ 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.

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.[1]

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.[2,3] 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.[4,5] 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.[6]

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

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 (FSC) were obtained from Gibco (Grand Island, NY, USA).

Semen incubation

Straws samples from the same animal (Lagoa da Serra, Sertaozinho, 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 ìg/ml gentamicin) at a final concentration of 5×10^6 sperm/ml and incubated at 39°C in a humidified atmosphere of 5% (v/v) CO_2 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 1×10^6 were diluted in 1 ml of PBS added of 2?l of Yo-Pro (100?M) and then incubated for 20 minutes at room temperature. After this period, it was added 10?l of PI (6?M) 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[14,15]. 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)

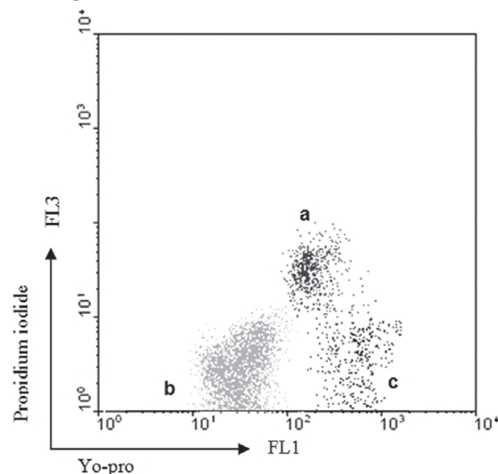

Figure 1 - Flow cytometry cytogram of frozen/thawed sperm cells showing permeability to Yo-pro and to PI: a) necrotic cells are Yo-pro$^{-and/or+}$ and PI^{+}; b) living cells are Yo-pro^{-} and PI^{-} and c) apoptotic cells are Yo-pro^{+} and PI^{-}

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

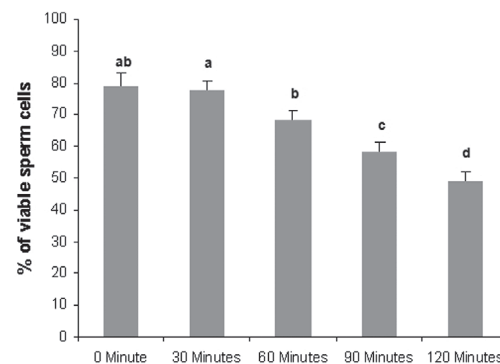

Figure 2 – Percentage of live sperm cells (plasma membrane integrity) in different periods of incubation. Different letters indicate significant differences among the incubation periods (P < 0.001)

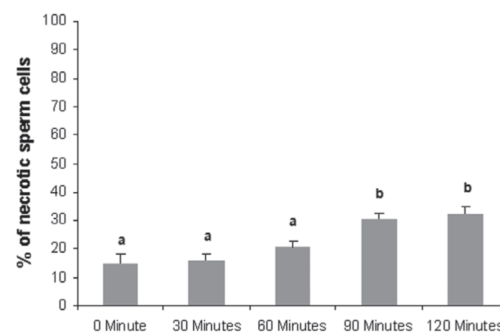

Figure 3 – Percentage of necrotic sperm cells in different periods of incubation. Different letters indicate significant differences among the incubation periods (P < 0.001)

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.

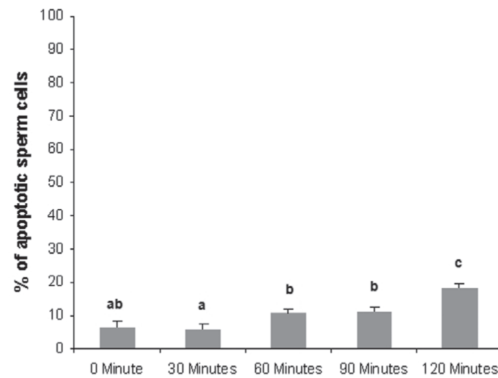

Figure 4 – Percentage of apoptotic sperm cells in different periods of incubation. Different letters indicate significant differences among the incubation periods ($P < 0.001$)

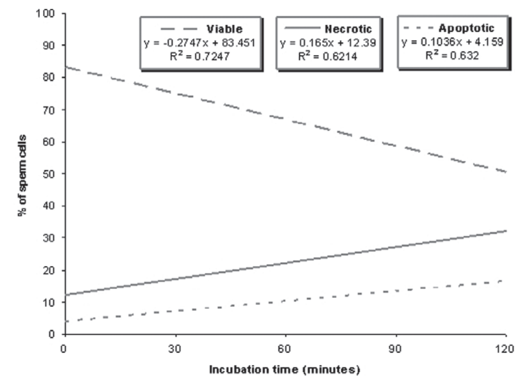

Figure 5 – Linear regression analysis between incubation time and percentage of live, necrotic or apoptotic sperm cells

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[16,17] or hypo-osmotic swelling test[18]. 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.[19,20]

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.[15]

Our results are in agreement with previous studies which demonstrate that a variable proportion of ejaculated live spermatozoa undergoes apoptosis under in vitro incubation.[21,22,23] On the other hand, it differs from studies which incubation did not induce apoptosis in sperm cell.[24,25] 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.[26] High ROS concentrations causes lipid peroxidation of sperm plasma membranes and induce apoptosis, resulting in alteration of sperm function and fertilizing capacity[23,27] and DNA damage[28]. Additionally, a recent study has been suggested that ROS are responsible for damages to sperm cells during incubation.[23]

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.[29,30]

The results of the present study are in opposition to results from Januskaukas, Johannisson and Rodrigues Martinez[29] and Muratori et al.[30] 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

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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 espermatozóides bovinos durante 2 horas de incubação. Os espermatozóides foram incubados a 5% (v/v) CO_2 em ar por 0, 30, 60, 90 e 120 minutes. Depois de cada periodo, 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-$pro^{-/+}$); b) células espermáticas em apoptose (Yo-pro^+ and PI^-) e c) células espermáticas vivas (Yo-pro^- and PI^-). A porcentagem de células vivas (membrana plasmática integra) significativamente diminui durante 2 horas de incubação, por outro lado, a porcentagem de espermatozóides 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.

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