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P1

Multicolor protocol for evaluation of apoptosis or damage to the plasma membrane of the spermatozoa

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Apoptosis is a type of programmed cell death characterized by a series of morphological changes, the participation of caspases, release of cytochrome C mitochondrial and nuclear fragmentation. In the cryopreservation process stress occurs, which can lead to aggregation and procaspases activation inside the cell leading to apoptosis. The externalization of phosphatidylserine is used by many andrologists as a marker of apoptosis, although it can also occur without necessarily being an apoptotic event, as in sperm capacitation or because of the cryopreservation process. Thus, the aim of this study was to develop a semen evaluation methodology combining phosphatidylserine translocation and identification of activated caspase. We used the probes Hoechst 33342 (H342, Sigma), propidium iodide (IP, Sigma), and fluorochromes: FITC associated with caspase 3/7 (molecular probes) and APC associated with annexin V (BD Bioscience). Twelve stallions were used; semen was collected by artificial vagina model Botucatu

(Botupharma), and immediately after collection semen was filtered to remove the gel and diluted with a milk diluent (Botu-Semen, Botupharma) to a concentration of $50 \times 10^6/\text{mL}$. The sample was then divided into two equal portions, one for positive control and other negative control. Positive control was subjected to $300 \mu\text{M}$ hydrogen peroxide for induction of lesions for 5 min; then 2 washes were performed at $300 \times g$ for 5 min. Finally both positive and negative controls were used for the production of the intermediate. For each group $200 \mu\text{L}$ dilution of semen ($5 \times 10^6/\text{mL}$) was added $5 \mu\text{L}$ of Hoechst 3342 ($100 \mu\text{g}/\text{mL}$) $5 \mu\text{L}$ propidium iodide ($50 \mu\text{g}/\text{mL}$) $5 \mu\text{L}$ of Annexin V-APC (according to manufacturer's protocol, BD bioscience) and $1 \mu\text{L}$ FITC-caspase3/7 (according to manufacturer's protocol, Molecular Probes), the samples were incubated for 30 min at 35°C in the dark. A total of 10000 gated-events were analyzed per sample by flow cytometry, BD LSR Fortessa™ (Becton Dickinson, Mountain View, CA, USA). Statistical analysis was performed using GraphPad Prism 5 (2007); linear regression was carried out to determine the ratio between treatments. For membrane integrity, the estimated coefficient of determination $R^2 = 0.9331$; for caspase activated $R^2 = 0.9914$, while for positive cells annexin, $R^2 = 0.9992$. The conclusion is that it is possible to use this multicolored combination for this evaluation in which apoptosis is differentiated from sperm membrane damage.

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