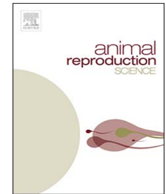
ARTICLE IN PRESS

Animal Reproduction Science xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci



# A simple flow cytometry protocol to determine simultaneously live, dead and apoptotic stallion spermatozoa in fresh and frozen thawed samples

M.C. Gil[a], C. Ortega Ferrusola[b], L. Anel-López[b], J.M. Ortiz-Rodriguez[a], M. Alvarez[b], P. de Paz[c], L. Anel[b], F.J. Peña[a,*]

[a] *Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain*
[b] *Department of Animal Medicine and Surgery, University of León, Spain*
[c] *Department of Molecular Biology, University of León, Spain*

## ARTICLE INFO

*Keywords:*
Stallion
Spermatozoa
Caspase 3
Ethidium homodimer
H33342

## ABSTRACT

Spermatozoa undergo apoptotic changes during the cryopreservation process. These changes, recently termed spermptosis, resemble the cryopreservation induced delayed onset of cell death observed after thawing of somatic cells. Due to its importance in cryobiology, methods to easily identify spermptotic cells are warranted. In this study, a well-validated method for identification of spermatozoa with caspase 3 activity was compared with use of the combination of Hoechst 33342 (H-42) and ethidium homodimer (Eth-1). Live, dead and apoptotic spermatozoa assessed with each method were compared using descriptive statistics and method agreement analysis. No differences were observed in the percentages of spermatozoa in each of the categories investigated with each method. Moreover the method agreement analysis indicated there were consistent findings using both methods The combination H-42/Eth-1 can be successfully used to determine apoptosis in addition to dead and live spermatozoa. Moreover the intensity of H-42 fluorescence (bright and dim populations) allows for distinguishing of live and dead sperm cells.

## 1. Introduction

Flow cytometry is increasingly used in clinical andrology in stallions (Love, 2016; Munoz et al., 2016). Numerous protocols have been developed in the last decade allowing the simultaneous assessment of multiple parameters in the spermatozoa. Developments in flow cytometry have allowed for an enhanced understanding of the biology of spermatozoa, particularly in the fields of clinical spermatology and sperm biotechnology. One of the aspects recently discovered with the aid of flow cytometry, relate to the dependence of the stallion spermatozoa on oxidative phosphorylation to obtain energy for motility and sperm house-keeping functions (Gibb et al., 2014; Pena et al., 2015; Plaza Davila et al., 2015; Davila et al., 2016). This dependence also results in a greater production of the superoxide anion ($O_2^{-\bullet}$), that if deregulation of redox homeostasis occurs, leads to oxidative stress, accelerated senescence of the spermatozoa and ultimately death of the cell (Aitken et al., 2012b; Morillo Rodriguez et al., 2012; Gallardo Bolanos et al., 2014; Martin Munoz et al., 2015; Munoz et al., 2016; Ortega-Ferrusola et al., 2017). This accelerated sperm senescence has been attributed to changes resembling apoptosis and the term spermptosis was proposed to refer to this biological phenomenon (Gallardo Bolanos et al., 2014; Ortega-Ferrusola et al., 2017). A similar form of cell death after cryopreservation has also been

* Corresponding author at: Veterinary Teaching Hospital, Laboratory of Equine Spermatology and Reproduction, Faculty of Veterinary Medicine, University of Extremadura, Avd de la Universidad, s/n 10003, Cáceres, Spain.
*E-mail address:* fjuanpvega@unex.es (F.J. Peña).

https://doi.org/10.1016/j.anireprosci.2017.12.009
Received 16 October 2017; Received in revised form 8 December 2017; Accepted 14 December 2017
0378-4320/ © 2017 Elsevier B.V. All rights reserved.

described in somatic cells and is termed cryopreservation induced delayed onset cell death (Baust et al., 2001; Baust et al., 2016). These changes have been described in relation to cryopreservation but also may be attributable to stallion subfertility. Due to the importance of these changes, numerous assays have been developed for the identification of cell states using flow cytometry (Martinez-Pastor et al., 2010; Pena et al., 2016b; Ortega-Ferrusola et al., 2017). Many of these assays require the use of three different probes, usually the H-42 probe, to identify spermatozoa and eliminate non-sperm debris from the analysis. If simpler protocols, with minimal spectral overlap, can be developed the assessment of apoptotic changes could be facilitated.

The aim of this study was to develop a simple protocol to assess apoptotic changes, using a combination of two probes with minimal spectral overlap (H-42 and Eth-1). The use of this combination of probes was validated by comparing with use of a classical, and well proven assay for caspase 3 activation (Moran et al., 2008; Ortega-Ferrusola et al., 2008; Ortega Ferrusola et al., 2009; Gallardo Bolanos et al., 2014).

## 2. Material and methods

### 2.1. Reagents and media

The H-42 (Excitation: 350 nm, Emission: 461 nm) probe (Ref: H3570); Cell Event Caspase-3/7 Green (Excitation, 502 nm; Emission: 530 nm) (Ref: C10423) and Eth-1 (Excitation, 528 nm; Emission, 617 nm) (Ref E1169) were purchased from Fisher Scientific Spain (Madrid, Spain). All other chemicals were purchased from SIGMA (Madrid, Spain).

### 2.2. Semen collection and processing

Three ejaculates per horse were collected from seven fertile stallions. The samples were obtained on a routine basis (three collections/week) during the 2016 breeding season. Stallions were maintained according to institutional and European regulations (Law 6/2913 June 11th and European Directive 2010/63/EU). Ejaculates were obtained and processed (fresh and frozen/thawed) using protocols that have been previously published in detail (Rodriguez et al., 2011; Morillo Rodriguez et al., 2012; Martin Munoz et al., 2015; Ortega-Ferrusola et al., 2017).

### 2.3. Staining for determination of live and dead cells and caspase 3 and 7 activity

This protocol has been developed in the laboratory where the present research was conducted and has been extensively described in previous publications (Gallardo Bolanos et al., 2014; Martin Munoz et al., 2015; Munoz et al., 2016; Ortega-Ferrusola et al., 2017). In brief, stock solutions of CellEvent (2 mM in DMSO), Eth-1 (1.167 mM in DMSO), and Hoechst 33342 (1.62 mM in water) were prepared. Spermatozoa ($5 \times 10^6$/mL) in 1 mL of PBS were stained with 1 μL of CellEvent, and 0.3 μL of Hoechst 33342 and incubated 25′ in the dark at r.t. Then 0.3 μL of Eth-1 was added to each sample and after incubation for 5 additional minutes the samples were immediately evaluated using the flow cytometer. Representative cytograms of the assay and gating strategy are shown in Fig. 1.

### 2.4. Flow cytometry

Flow cytometry analyses were conducted in a MACSQuant Analyzer 10 (Miltenyi Biotech), equipped with three lasers emitting at 405, 488, and 635 nm and 10 photomultiplier tubes (PMTs): V1 (excitation 405 nm, emission 450/50 nm), V2 (excitation 405 nm, emission 525/50 nm), B1 (excitation 488 nm, emission 525/50 nm), B2 (excitation 488 nm, emission 585/40 nm), B3 (excitation 488 nm, emission 655–730 nm (655LP + split 730)), B4 (excitation 499 nm, emission 750 LP), R1 (excitation 635 nm, emission 655–730 nm (655LP + split 730)), and R2 (excitation 635 nm, emission filter 750 LP). The system was controlled using MACS Quantify software. The quadrants or regions used to quantify the frequency of each sperm subpopulation depended on the particular assay. Forward and sideways light scatter was recorded for a total of 50,000 events per sample. Gating the sperm population after Hoechst 33342 staining eliminated non-sperm events.

The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. The data were analyzed using Flowjo V 10.2 Software (Ashland, OR, USA). Unstained and single-stained controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications (Pena et al., 2003; Gallardo Bolanos et al., 2014; Martin Munoz et al., 2015).

### 2.5. Statistical analyses

The normality of the data was assessed using the Kolmogorov-Smirnoff test. Because the data were normally distributed, the results were analyzed using an ANOVA followed by use of a Tukey *post-hoc* test for pair-wise comparisons (using SPSS 19.0 software for Mac). Differences were considered significant when $P < 0.05$. The results are displayed as the mean ± SEM. Bland and Altman Plots, a method agreement analysis technique, of the two staining protocols were also constructed. The differences between paired measurements of the same samples were calculated, and the mean of the differences (d) were used to estimate the average bias of one method relative to the other (Nagy et al., 2003; Pena et al., 2005). The 95% limit of agreement was calculated as $d \pm 2$ SD, where SD is the standard deviation of the differences between paired measurements. These calculations were performed using Microsoft Excel for Mac OS X and SPSS for Mac.

Download English Version:

https://daneshyari.com/en/article/8404034

Download Persian Version:

https://daneshyari.com/article/8404034

Daneshyari.com