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Factors affecting staining to discriminate between bull sperm with greater and lesser mitochondrial membrane potential

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ABSTRACT

The sperm mitochondrial membrane potential (MMP) is usually evaluated using the JC-1 dye. This study aimed to verify the effect of incubation temperature (25 °C or 38 °C), incubation time (10, 30, and 45 min), JC-1 stain concentration (0.2 μM, 2 μM, 8 μM, 12 μM), and the presence of glycerol (6.6% compared with 0%), on the capacity of the stain to discriminate between sperm with high mitochondrial membrane potential (hMMP) and low mitochondrial membrane potential (lMMP) in fresh and frozen bull sample by both flow cytometry and epifluorescence microscopy. The temperature (38 °C for 10 min) and the dye concentration (8 μM and 12 μM) resulted in a greater proportion of hMMP ($P < .05$). The incubation for 45 min at 38 °C resulted in a significant reduction of hMMP in samples stained with JC-1 dye at 8 μM and 12 μM ($P < .01$). A longer incubation time (45 min) and greater dye concentration (8 μM and 12 μM) resulted in an increased proportion of hMMP sperm in cryopreserved samples. Fresh sperm incubated with glycerol had a hMMP ($P < .05$). Data for the present study indicate that the optimal incubation temperature was 38 °C, with an incubation time differing between fresh (10–30 min) and cryopreserved sperm (at least 45 min). Furthermore, the JC-1 dye concentration used that could reliably detect the proportion of hMMP sperm was 2 μM in fresh samples, and at least 8 μM in cryopreserved sperm.

1. Introduction

Sperm mitochondria are considered to be the site where the adenosine-triphosphate (ATP) for sperm motility is produced (Silva and Gadella, 2006; Amaral et al., 2013). There is, however, increasing evidence of the important role of these intracytoplasmic organelles in calcium homeostasis (Nichols and Ferguson, 2002), such as in sperm maturation and capacitation (Cordoba et al., 2006; Aitken et al., 2004; Shivaji et al., 2009; Ramio-Lluch et al., 2011). Furthermore, mitochondrial membrane potential (MMP) appears to be useful in predicting sperm fertilization capacity in humans (Kasai et al., 2002).

Among the different dyes used to evaluate the activity of sperm mitochondria, the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) has become popular in recent years, due to its ability to differentiate sperm with greater and lesser mitochondrial inner transmembrane potential (Smiley et al., 1991; Cossarizza et al., 1993). In contrast to the Rhodamine 123 and Mito Tracker fluorochromes, which are transported and accumulated in active mitochondria emitting fluorescence, the JC-1 dye is taken up by all active mitochondria emitting a green fluorescence, but when metabolized in high

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concentrations there is a J-aggregate precipitation with an orange-red fluorescent emission (Smiley et al., 1991; Cossarizza et al., 1993; Gillan et al., 2005). This property allows for the differentiation, within an ejaculate, of sperm with greater (red-orange fluorescence) and lesser (green fluorescence) MMP. Use of the JC-1 dye is considered the more rigorous method for the functional evaluation of mitochondria (Garner et al., 1997).

The JC-1 dye is considered a metabolic stain, thus, the discrimination between sperm with lMMP and hMMP is largely affected by the condition in which sperm were stained and incubated (Garcıa-Macıas et al., 2005; Martinez-Pastor et al., 2010). The lack of guidelines for the use of the JC-1 dye for sperm MMP evaluation resulted in differences in the protocols for: 1) incubation temperature, between 25 °C (Gravance et al., 2000), 37 °C (Garner et al., 1997; Guthrie et al., 2008), and 38 °C (Pena et al., 2003; Guthrie and Welch, 2006; Bollwein et al., 2008; Ortega-Ferrusola et al., 2008; Goodla et al., 2014); 2) incubation time, from 10 min (Guthrie and Welch, 2006), to 10–15 min (Condorelli et al., 2012), 30 min (Garner et al., 1997; Bollwein et al., 2008), 40 min (Baumber et al., 2000; Gravance et al., 2000; Pena et al., 2003; Ortega-Ferrusola et al., 2008; Goodla et al., 2014), and 45 min (Garner and Thomas, 1999), and 3) dye concentrations, from 0.153 µM (Bollwein et al., 2008), to 0.3 µM (Guthrie and Welch, 2006), 1.5 µM (Ortega-Ferrusola et al., 2008, 2009), 2 µM (Garner and Thomas, 1999; Baumber et al., 2000; Gravance et al., 2000), 3 µM (Pena et al., 2003), 7.65 µM (Garner et al., 1997; Guthrie et al., 2008), and 12 µM (Goodla et al., 2014).

The aim of the present study was to verify the effect of the temperature, incubation time, and stain concentration on the proportion of sperm with hMMP or lMMP evaluated using the JC-1 dye with fresh and cryopreserved bull semen using both flow cytometry and epifluorescence. The values recorded for MMP were also correlated with the sperm kinetic parameter and sperm membrane integrity, to detect possible relationships between these parameters.

2. Materials and methods

2.1. Animals and seminal screening

The study involved 16 Swiss Brown bulls (2–6 years old) owned by the Superbrown Consortium (Trento, Italy) used in regular artificial insemination (AI) services. The bulls were housed in individual box stalls at the Alpenseme AI Centre of the Provincial Breeders Federation of Trento (Ton, Trento, Italy). Semen was routinely collected twice a week using a pre-warmed vagina. After collection, the volume of the semen sample was measured by the weight. The concentration was determined using an Accucell photometer (IMV Technologies, L'Aigle, France) after dilution 1:100 with saline solution, as previously described (Gloria et al., 2014). The total sperm per ejaculate (TSE − as concentration x volume) was also calculated (Gloria et al., 2016).

Mass motility was estimated subjectively (magnification: X 400) at 37 °C using phase contrast microscopy after dilution with pre-warmed Bioxcell (IMV Technologies). Normal sperm morphology was evaluated after dilution at 100×10^6 sperm/mL with 0.9% NaCl solution and 3% glutharaldehyde using phase contrast microscopy (magnification: X 1000), as previously reported (Hancock, 1957; Contri et al., 2013).

Only ejaculates with a total number of sperm exceeding 3×10^9 sperm, with subjective motility ≥ 80% (Contri et al., 2013) and normal morphology > 80% (Gloria et al., 2014) were included in the study. For the trials, aliquots of raw semen were removed from ejaculates routinely collected for the artificial insemination services.

2.2. Semen cryopreservation

Raw semen was diluted with Bioxcell (IMV Technologies) at 80×10^6 sperm/mL, equilibrated for 3 h at 5 °C using a passive refrigerator, packaged in 0.25-mL straws, and frozen with a programmable nitrogen freezer (Digicool 5300, IMV Technologies) as previously reported (Muino et al., 2008). The straws were stored in liquid nitrogen until evaluation. Frozen-thawed samples were obtained by immersion of 10 straws/ejaculate for 5 min in a water bath at 38 °C.

2.3. Semen evaluation

2.3.1. Objective sperm motility

Motility and kinetic variables were objectively evaluated using a computer-assisted sperm analyzer (IVOS 12.3–Hamilton-Thorne Bioscience, Beverly, MA, USA). Before the analysis, the sample was diluted with a Hepes buffer solution (HBS – NaCl 130 mM, KCl 4 mM, fructose 14 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, Hepes 10 mM, bovine serum albumin 0.1%, and penicillin/streptomycin 10 µg/ml; pH 7.4, 310 mOsm – Thomas et al., 1998) at 30×10^6 sperm/mL and warmed at 38 °C for 5 min, then a 6-µl drop was loaded into a Makler chamber (Sefi Medical Instruments, Haifa, Israel), and 12 non-consecutive fields were analyzed. The CASA setting used was previously reported (Contri et al., 2010). After each assessment, the playback option was used for quality control for the correct acquirement of the field.

The following variables were considered: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, as VSL/VAP, %), and linearity (LIN, as VSL/VCL, %). Sperm with a VAP ≥ 80 µm/s and STR ≥ 75% were considered progressive.

2.3.2. Sperm membrane integrity

The membrane integrity was measured on the fresh and cryopreserved samples using propidium iodide (PI)/SYBR-14 dual stain