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Effectiveness of human spermatozoa biomarkers as indicators of structural damage during cryopreservation

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ABSTRACT

Human spermatozoa cryopreservation techniques are used to maintain and protect male fertility in cases such as infertility and malignancy treatments. However, during cryopreservation, the spermatozoa's metabolic rate is reduced and they undergo dramatic functional and structural changes owing to exposure to cryoprotectants and freezing-thawing procedures. While the effects of cryopreservation on cells are documented, to date the induced cryodamage on structural and/or functional sperm biomarkers is not well established at multivariate scale. To address this question, we performed basic sperm analysis, sperm DNA fragmentation assessment, spontaneous acrosome reaction measurement, and cytoskeleton evaluation after thawing samples from subjects with normal and low-quality semen. A cryodamage rate was used to determine the effects of the freeze-thaw process on spermatozoa. In addition, a Principal Component Analysis (PCA) was used for data reduction and to evaluate sperm-specific patterns during the cryopreservation process. We found that the vitality, progressive motility and sperm count from low-quality samples after cryopreservation show higher damage rates (≥40%) than in normal sperm samples. However, cytoskeleton, DNA, tail and mid-piece and acrosome display the highest cryodamage rates (~50–99%) and are equally susceptible to cryopreservation-induced damage in both low- and normal-quality semen samples. Overall, the evaluation of these parameters provides meaningful information about different aspects of sperm functionality after cryopreservation.

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1. Introduction

Cryopreservation of human spermatozoa is a widely used method in current assisted reproduction techniques (ARTs) [41]. Advances in cryopreservation techniques applied to human gametes preserve individual fertility, avoiding pathological effects after radio- or chemotherapy treatments such as testicular failure, ejaculatory dysfunction, subfertility and sterility due to gonad removal or permanent damage to germ cells [34]. Likewise, sperm structure and integrity are necessary for fertilising an oocyte, and changes in functional morphology must be recorded after freeze-thawing processes.

During cryopreservation, sperm undergo dramatic changes in their intra- and extracellular environment associated with two main factors: on the one hand, the addition and removal of cryoprotective agents, and on the other hand, the formation of ice-crystals [31,34]. The most commonly reported cryoinjuries on human spermatozoa involve a marked reduction in viability [16,32] and motility [4,14]. Further freezing-thawing results in damage to plasma membrane fluidity and integrity [3,17,29], oxidative stress leading to lipid peroxidation [19,36], variations in acrosomal state and content [2,14,33], or changes in mitochondrial functionality [2,18], chromatin decondensation [2] and DNA fragmentation [2,5].

Moreover, it has been reported that the severity of damage during the freeze-thaw cycle varies considerably between different individuals and different samples from the same individual [13,20,26]. Previous reports showed higher DNA damage/chromatin modification in morphologically abnormal spermatozoa than in those with a normal morphology during freeze-thaw processes

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[25]. Other findings have indicated that freeze-thawing causes significant alterations in sperm chromatin, morphology and membrane integrity in both fertile and infertile men [15]. Nonetheless, DNA in spermatozoa from fertile samples has been shown to be unaffected by cryopreservation using cryoprotectants [35]. By contrast, significant sperm damage was recorded in infertile samples induced by freezing-thawing procedures [8]. Therefore, determining whether the effects of cryopreservation depend on the quality of semen samples is an issue that remains to be resolved. In this context, little is known to date about the simultaneous assessment of DNA integrity, acrosome status, motility, viability and morphology, because in these reports the cryodamage effects have mainly been evaluated separately [7,21,27,32]. The purpose of this study was to identify a subset of the most useful biomarkers for assessing post-thaw sperm quality from subjects with normal and low-quality semen samples.

2. Material and methods

2.1. Semen collection and analysis

The participants in our study were 33 men (30–36 age range) undergoing fertility treatment at the Hospital la Fe Andrology Unit (Valencia, Spain). Approval for the present study was obtained from the Hospital la Fe Research Ethics Committees in accordance with the principles of the Declaration of Helsinki.

Semen samples were collected by masturbation in a clean universal container after a sexual abstinence period of between 3 and 7 days. The samples were liquefied at 37 °C during 20–30 min. Basic sperm parameters (motility, morphology, concentration and vitality) were assessed in accordance with World Health Organization (WHO) guidelines (2010) [42].

Two groups were defined, according to the results obtained in the basic sperm analysis, to test for cryopreservation damage: (i) a normal semen group with 16 samples from normozoospermic subjects and, (ii) a low-quality group with 17 samples.

After basic sperm analysis, each sample was divided into two aliquots for pre-freeze and post-thaw examination. Comprehensive sperm structure and functional evaluation was performed in both groups (normal and low-quality semen samples), including basic semen concentration, motility and morphology analysis, sperm vitality examination, sperm DNA fragmentation assessment, cytoskeleton evaluation, and spontaneous acrosome reaction measurement.

2.2. Freezing and thawing procedure

The second aliquot was prepared for cryopreservation following the manufacturers' instructions. Freezing Medium Test Yolk Buffer with glycerol and gentamicin (Irvine Scientific©, Santa Ana, CA, USA) was added slowly to semen samples to reach a 1:1 dilution. The mix of semen sample with cryoprotector was packaged into 1.8 ml cryotubes (Nunc, Brand Products, Roskilde, Denmark) and kept at room temperature for 10 min. The cryotubes were then kept at 4 °C for 30 min, and subsequently in liquid nitrogen vapour for 30 min. After that, the samples were plunged into liquid nitrogen (−196 °C) and stored during at least 10 days in liquid nitrogen.

Thawing was performed at room temperature. Once thawing was complete, the Freezing Medium (Irvine Scientific©, Santa Ana, CA, USA) was removed by adding a sperm wash medium (PureSperm® wash, Nidacon, Mölndal, Sweden) and centrifuging at 300×g for 10 min.

2.3. Sperm DNA fragmentation assessment

Assessment of sperm DNA fragmentation was performed using the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridinetriphosphate (dUTP) nick-end labelling (TUNEL) method (Roche, Mannheim, Germany) according to the manufacturer's instructions.

A drop of sample fixed in 1% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany) was smeared on a slide and air dried. The slides were then washed 3 times in PBS 1X and incubated in a permeabilisation solution (0.1% Triton X-100 with 0.1% sodium citrate in water) for 2 min at −20 °C. The slides were washed again in PBS 1X and incubated at 37 °C for 1 h in the dark with TdT enzyme and fluorescein isothiocyanate (FITC)-labelled dUTP provided in the kit. Finally, the slides were washed and mounted with 100 nM DAPI (4′,6-diamino-2-phenyilindole; Sigma-Aldrich Steinheim, Germany) and Vectashield mounting medium (Vector Labs, Burlingame, CA., Steinheim, Germany). TUNEL (Roche, Mannheim, Germany). They were then analysed using a Leica DMRB (Leica Microsystems, Germany) fluorescence microscope at 100×magnification. A minimum of 200 spermatozoa per sample were evaluated.

2.4. Immunocytochemistry of α-tubulin

The study of the tail cytoskeleton was performed by fluorescence immunocytochemistry on fresh and thawed fixed sperm (1% paraformaldehyde) (Sigma-Aldrich, Steinheim, Germany). After permeabilisation with 1% Triton X-100 in PBS 1X for 10 min, nonspecific bindings were blocked with 10% BSA for 1 h at room temperature. The samples were then washed in PBS 1X and incubated for 1 h at room temperature with mouse monoclonal anti α-tubulin (1:600 dilution, Sigma-Aldrich; Steinheim, Germany). The slides were rinsed in PBS 1X for 15 min and incubated with DyLight 488 donkey anti-mouse IgG (1:300 dilution. Jackson Inmuno Laboratories, West Grove, USA) for 1 h. The samples were then washed in PBS 1X and mounted with Vectashield mounting medium (Vector Labs, Burlingame, CA) with 100 nM 4′, 6-diamino-2-phenylindole (DAPI; Sigma-Aldrich, Steinheim, Germany).

The slides were examined using a Leica DMRB (Leica Microsystems, Germany) fluorescence microscope at 100× magnification. A minimum of 200 spermatozoa per sample were assessed. Three different tubulin labelling patterns were observed: total labelling (TubT), discontinuous labelling (TubD) and final labelling (TubF).

2.5. Spontaneous acrosome reaction measurement

Spontaneous acrosome reaction assessment was performed using Fluorescein Isothiocyanate-labelled *Pisum sativum agglutinin* (FITC-PSA) (Sigma-Aldrich, 203 Steinheim, Germany) following the protocol described elsewhere [25]. Samples fixed on a glass slide with absolute methanol were washed with double-distilled water, dried at room temperature and incubated during 30 min with 50 μl of FITC-PSA (50 μg/ml) at room temperature in the dark. The slides were then washed and mounted with Vectashield mounting medium (Vector Labs, Burlingame, CA) with 100 nM 4′, 6-diamino-2-phenylindole (DAPI; Sigma-Aldrich, Steinheim, Germany). A minimum of 200 spermatozoa per sample were evaluated using a Leica DMRB (Leica Microsystems, Germany) fluorescence microscope at 100× magnification.

2.6. Statistical analysis

The Kolmogorov-Smirnov goodness-of-fit tests were firstly

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