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Research Article

Assessment of Individual Sperm Proteins as a Reliable Method for Determination of the Cryopreservation Technique of Choice

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Abstract

Spermatozoa cryopreservation is an integral part of assisted reproductive technology, which is used for the management of infertility and some other medical conditions. Various cryopreservation procedures have been introduced. The most frequently applied techniques for comparing these procedures are subjective and objective cellular biology techniques. This manuscript represents the author's viewpoint regarding the importance of applying the molecular biology assessment of proteins as markers of physiological parameters and fertilizing ability of post-thawing spermatozoa.

Keywords: Human spermatozoa; Cryopreservation; Vitrification; Sperm Proteins

Introduction

Sperm cryopreservation is the main strategy to reserve male fertility after cytotoxic chemotherapy. It is also important for establishment of donor banks and in the practice of assisted reproductive technology when the woman wants the sperm in a different time or place after the donation, or to avoid repeated surgical extraction in cases of obstructive azoospermia [1].

The conventionally applied cryopreservation technique is based on the use of permeating cryoprotectants and slow freezing rate. However, many studies have documented potential toxic effects of this method, including a negative impact on the sperm genetic material. Thus, the vitrification technique, which relies on the avoidance of permeating cryoprotectants and ultra-rapid cooling has been developed, with many studies showing its results comparable to those of conventional slow cryopreservation [1-3].

However, numerous spermatozoa proteins have recently been identified, and comparison between sperm proteins before and after various treatments has come into focus of research, because various sperm proteins are involved in the regulation of sperm physiological parameters and the fertilizing ability before and after cryopreservation [4,5].

Until now, there is little evidence about the changes of sperm proteins that may result from the cryopreservation procedure. The research in this area is advancing rapidly, helping researchers to resolve the molecular bases of cryopreservation- induced defective sperm functions [5].

The results obtained before by Zilli research group, who used two dimensional polyacrylamide gel electrophoresis (2-DE) and matrix associated laser desorption / ionization time-of-flight (MALDI-TOF) mass spectrometry to verify whether the protein expression of sea bass sperm was affected by cryopreservation, stated that the protein profiles

differed between fresh and frozen / thawed spermatozoa, as revealed by visual inspection and by image analysis software. They identified 163 spots in fresh sperm; among them, 13 were significantly decreased and 8 were absent in cryopreserved spermatozoa [6].

In addition, the generation of reactive oxygen species (ROS) associated with cryopreservation could be responsible for mammalian sperm damage and the limited value of stored semen in artificial insemination [7]. Increased ROS generation by itself was found to affect human spermatozoa proteins in terms of expression and degradation [8]. A recent study revealed twenty seven proteins which differed significantly between control and post- thawing human spermatozoa. These proteins are thought to be involved in various sperm physiological processes, hence, spermatozoa dysfunction after cryopreservation was suggested to be due to protein degradation and or modification [9].

Furthermore, it was reported before that the actin band in western blotting differs between fresh and post-thawing sperm [10], which might reflect the affection of individual proteins by the process of freezing and thawing. Accordingly, the functions of such proteins would be affected post-thawing, which would ultimately affect the functionality and fertilizing ability of the sperm.

Based on the above mentioned experiences with human and non human sperm, the affection of sperm proteins would be an important target to compare different cryopreservation techniques. Investigating the important key proteins that are involved in the control of various sperm physiological parameters, and the degree of their affection by various cryopreservation techniques, would provide an evidence based data regarding the functional ability of the sperm post-thawing as well as which technique of cryopreservation would be associated with less harm. Examples of such key proteins would be focal adhesion kinase and prohibitin.

Prohibitin (PHB) is a 30-kDa protein, consists of two highly homologous subunits, PHB1 and PHB2, which assemble into a ring like structure in the mitochondrial inner membrane. Absence of PHB in somatic cells was found to be associated with mitochondrial membrane depolarization and increased generation of ROS. Significant positive correlations were found among PHB expression, mitochondrial membrane potential, and sperm motility in normozoospermia, asthenozoospermia, and oligoasthenozoospermia samples [11]. Together, these observations suggest that PHB expression is an indicator of sperm quality, and that PHB is important for sperm motility, and sperm mitochondrial function.

Meanwhile, focal adhesion kinase protein family (FAK) appears to have a direct role in protein tyrosine phosphorylation of spermatozoa, which may occur via two pathways, the canonical protein-kinase A pathway and a calcium-stimulated pathway. This protein tyrosine phosphorylation activity is a very important step in sperm capacitation process, which is required to render the sperm competent to fertilize an oocyte [12].

In a practical experience of the author, cryopreservation of swim-up- prepared human spermatozoa with conventional slow freezing and permeating- cryoprotectants- free vitrification showed different degrees of sperm proteins affection (Figure 1).

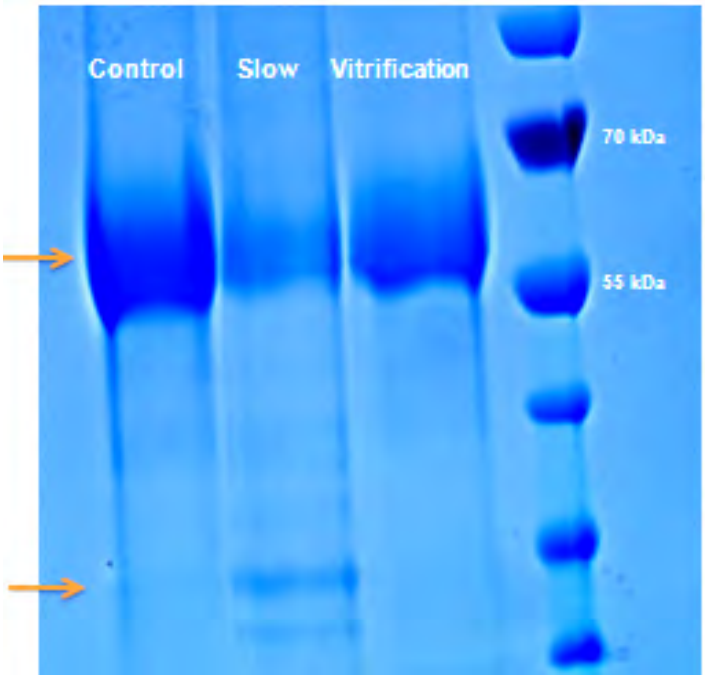

Figure 1. Protein extraction and separation in SDSPage showed a different bands' pattern among control non- frozen, slow and vitrification post- thawing spermatozoa. The most obvious band in all specimens was a protein band between 55- 70 kDa. This band is denser in control than in vitrification, and denser in vitrification than in slow post- thawing spermatozoa protein extracts. The differences were visible by inspection as well as statistically significant after analysis with image-lab analyzer software ($P < 0.05$).

Although no individual proteins were assessed, the application of such a simple molecular biology technique, SDSPage, was able to show significant differences between fresh and post-thawing spermatozoa, as well as between both cryopreservation techniques, regarding the isolated sperm proteins (Figures 1 and 2). Of course further application of mass spectrometry and proteomics analysis and or western blotting would provide more precise data about the individual affected proteins and the roles they play to control the physiological parameters and fertilizing ability of the sperm. However, such a level of basic and simple investigation still was able to provide a reliable evi-

dence that vitrification is superior over conventional slow cryopreservation regarding the degree of affection of sperm proteins, where conventional slow freezing was associated with more significant sperm protein degradation (Figures 1 and 2).

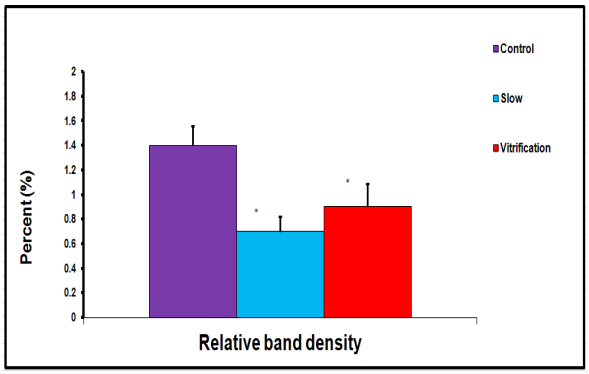

Figure 2. Relative band densities before and after cryopreservation. For this protein band, the relative density was decreased from ± 1.4 in control to ± 0.7 in slow freezing (50% reduction, with significant difference P = 0.003), and to ± 0.9 in vitrification (36% reduction, with significant difference P = 0.009). A significant difference was also found between both cryopreservation techniques (P = 0.042). Asterisks indicate significant differences between marked columns with each other as well as with the control.

In conclusion, together with the cellular biology techniques used for the assessment of post- thawing spermatozoa, the application of various molecular biology techniques would provide more information that can be reflected on the clinical practice in the field of sperm cryopreservation and assisted reproductive technology.

Materials and Methods of author's experimental data

After ethical approval and consents were taken, three semen samples were collected according to WHO criteria from male subjects between 25 and 40 years of age. The samples were collected by masturbation after at least 48 hours of sexual abstinence. Semen analysis was performed according to the published guidelines of the World Health Organization [1]. Samples were classified according to the following lower reference limits: 15 million spermatozoa / ml, 32 % progressive motility and a minimum of 4 % morphologically normal spermatozoa.

Each semen sample was diluted 1:2 with pre-warmed (37 Cº) Quinn's sperm wash medium (Sage Media, Trumbull, CT, USA) and transferred into a conical centrifuge tube (Becton Dickinson, NJ, USA) and centrifuged at 300 g for 10 minutes. The supernatant was carefully removed and discarded. The sperm pellet was resuspended in 1 ml of the same medium by gentle pipetting, followed by centrifugation again for 10 minutes at 300 g. After removing and discarding the supernatant, 1 ml of pre-warmed (37 Cº) HTM +1% SSS was gently placed over the pellet, without disturbing it, followed by incubation for 60 minutes, at 37 Cº and 6% CO2 atmosphere, in oblique position (45º). After incubation, the tube was handled gently and returned to up-right position and the uppermost 500 µl medium was removed into a sterile Eppendorf tube where the highly motile sperms are present [1].

Each swim-up preparation was afterwards divided into three equal parts; one is fresh control, one for subsequent conventional slow freezing and one for subsequent vitrification. Slow freezing and vitrificatin were performed according to the guidelines [1].

Protein extraction and SDS polyacrylamide gel electrophoresis

Fresh, slow cryopreserved and vitrified spermatozoa of the same sample and concentration were centrifuged at 300 g for 10 minutes. The supernatant parts were removed and discarded, while the cellular pellets were resuspended in 100 µl RIPA lysis buffer supplemented with 10% animal component- free protease inhibitor cocktail (Sigma, Munich, Germany), with vigorous shaking, vortex and sonication, when needed, to disrupt the pellet.

Protein concentration and protein amount in each sample were determined by the Bradford method. The standard curve was obtained using blank water and serial protein concentrations of 50- 1600 µg / ml of bovine serum albumin (BSA). After duplicates of serial dilutions of each sample (6 µl) were equilibrated with colour reagent (100 µl) (Bio-Rad laboratories GmbH, München, Germany) for 10 minutes at room temperature, the absorbance measurements were done using a double- beam UV- visible spectrophotometer. The standard curve was plotted and the protein concentration in each sample was determined relative to the standard curve.

Equal amounts of protein were subjected to SDS- polyacrylamide gel electrophoresis together with 10 µl of pre- stained page ruler protein marker (10 - 170 kDa) (Thermo Fisher Scientific, Bonn, Germany). The separated protein bands were stained in the gel using Coomassie blue (Thermo Fisher Scientific, Bonn, Germany). The stained gels were then scanned and densities of the bands were determined by Image- Lab analyzer software (Life Science Research, BioRad, München, Germany). The relative band densities were calculated by dividing the actual band density (obtained by the image - lab analyzer) by the average of the three bands of each sample (control and post- thawing bands). The results were visibly detected as well as by the statistical calculations. For statistical analysis, Excel data sheet (Microsoft office 2007) for calculation of mean and standard deviation (SD) was used. Comparison between the three treatments was done using a Prism6Demo program

for determination of significant differences, using non-paired T-test, where P values less than 0.05 were considered significant.

Conflict of Interests

No special funding was provided for the development of this work.

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