Retention of membrane charge attributes by cryopreserved-thawed sperm and zeta selection

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Received: 8 January 2007 / Accepted: 31 May 2007 / Published online: 26 July 2007
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Abstract

Purpose Mature sperm can be selected based on their negative zeta electrokinetic potential. The zeta selection of cryopreserved sperm is unknown. The objective was to study the effect of zeta processing on the morphology and kinematic parameters of cryopreserved-thawed sperm.

Methods Colloid-washed sperm ($N=9$ cases) were cryopreserved for 24 h, thawed and diluted in serum-free medium in positive-charged tubes. After centrifugation, the tubes were decanted, serum-supplemented medium was added and the resuspended sperm were analyzed. Untreated sperm and fresh sperm served as the controls.

Results There were improvements in strict normal morphology in fresh (11.8 ± 0.3 versus control 8.8 ± 0.3 %, mean $\pm$ SEM) and thawed (8.7 ± 0.2 versus control 5.4 ± 0.2 %) sperm after zeta processing. Percent sperm necrosis was

reduced after zeta processing (66.0 ± 0.6 versus unprocessed 74.6 ± 0.3 %). Progression decreased by 50% but not total motility after zeta processing of thawed sperm.

Conclusions The results suggested that the cryopreservation process did not impact the sperm membrane net zeta potential and higher percentages of sperm with normal strict morphology, acrosome integrity and reduced necrosis were recovered. The zeta method was simple and improved the selection of quality sperm after cryopreservation but more studies would be needed before routine clinical application.

Keywords Spermatozoa maturation · Zeta potential · Membrane charge · DNA integrity · Electrophoresis

Introduction

A recent report demonstrated improvements in washed sperm quality after selecting for negatively charged sperm using a zeta potential processing method [1]. Specifically, the percentages of sperm with strict normal morphology, hyperactive motility and forward progression almost doubled in the zeta processed sperm group. However, information is lacking for cryopreserved-thawed sperm processed with the zeta method. Such information is important and applicable in assisted reproduction technology (ART) cases involving frozen sperm derived from donor banks or urological procedures.

Selection of normal quality sperm is crucial for success in ART procedures. Currently, sperm is randomly selected for intracytoplasmic sperm injection (ICSI) based on a reasonable shape, motility without consideration for strict normal morphology [2]. Increasing the percentage of sperm with strict normal morphology is associated with an increase in sperm with normal DNA [3, 4] and enhances

Cryopreserved-thawed sperm processed using the zeta membrane-charge method showed improvements in strict normal morphology, acrosome and reduced necrosis.

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fertilization and pregnancy outcomes [5–8]. A simple method to select for cryopreserved-thawed sperm with normal strict morphology based on membrane charge is proposed here.

A mature sperm is defined as a haploid X- or Y-bearing unflagellated reproductive cell called a gamete where its condensed nucleus chromatin structure contains protamines and approximately 15% histones, and its cell membranes contain epididymal proteins that impart a net negative charge (−16 to −20 mV). This charge is termed zeta potential or electrokinetic potential which is defined as the electric potential in the slip plane between the sperm membrane and the surrounding medium [9]. The negative charge causes the sperm to adhere to surface material in protein-free medium. In this study, the goal was to use differences in membrane zeta potential of frozen-thawed sperm to select for normal quality sperm. The objective was to compare sperm morphology, kinematic, and apoptosis-necrosis parameters before and after zeta processing of cryopreserved-thawed sperm. It is hoped that the information obtained will assist in the development of new protocols for improving sperm selection for ART treatment.

Materials and methods

Preparation and analyses of sperm

Computer-aided sperm analyses (CASA) were carried out on nine ejaculated specimens derived from an assisted reproductive technology program. Untreated sperm concentration in all specimens was at least over 20 million/ml with over 50% total motility and over 4% strict normal morphology. Donor sperm, testicular and epididymal sperm were not included in this study. The procedures were approved by the Loma Linda University Institutional Review Board. Basically, an aliquot from each semen specimen was analyzed and sperm kinematic parameters measured using the Hamilton-Thorn HTM-C (Hamilton-Thorn, Danvers, MA) computer CASA system [10]. Sperm strict normal morphology and acrosome intactness were determined using the Tygerberg strict criteria method [5, 6] and the Spermac method [11] respectively and described below.

Each semen specimen was washed using the two-gradient colloid (isolate; Irvine Scientific, Santa Ana, CA) centrifugation procedure [12]. Basically, this wash procedure consisted of layering the semen on top of the discontinuous two-layer 90% bottom: 45% top colloid gradients in a 15 ml polystyrene centrifuge tube. Each tube was centrifuged at $300 \times g$ for 10 min and each resultant pellet was resuspended with 1.5 ml HEPES-buffered synthetic human tubal fluid medium (HTF, Irvine Scientific,

Santa Ana, CA) supplemented with 5% serum substitute supplement (Irvine Scientific, Santa Ana, CA). The resuspended sperm were centrifuged and each final pellet was resuspended in 0.5 ml serum-supplemented HEPES-HTF medium.

Analyses of post-washed fresh sperm before zeta processing

An aliquot from each tube of washed fresh sperm was analyzed using the computer CASA system and the kinematic parameters recorded [10]. Sperm smears were prepared and strict normal morphology with acrosome status determined [11]. Analyses of sperm necrosis and apoptosis were carried out using the Hoechst 33348: propidium iodide dual-stain fluorescent method briefly outlined below [13, 14].

Spermac stain for strict normal morphology and acrosome integrity

Strict normal morphology was assessed using the spermac stain method (Stain Enterprises, Onderstepoort, S. Africa, distributed by Sepal Reproductive Devices, Sudbury, MA). A sperm smear was spread on a glass slide, air-dried and fixed in formalin (Fixative I) for 5 min at room temperature (22°C). The slide with fixed sperm was rinsed and stained in Rose Bengal-based solution A for 2 min. The slides were rinsed and stained in Orange G-based solution B for 1 min. This was followed by rinsing, staining in Janus Green-based solution C for 1 min and a final rinse. Each slide was air-dried for 10 min and analyzed in oil immersion (1,000 $\times$) bright field light microscopy.

The Tygerberg strict criteria method [5, 6] was used to analyze the morphology of at least 100 sperm. A sperm was classified as normal when the head was oval with the acrosome occupying 40–70% of the head, absence of midpiece and tail defects and absent or small cytoplasmic droplets with the appropriate head dimensions.

The percentages of sperm with intact acrosome were determined from the same set of Spermac-stained morphology slides. Regardless of the shape of the sperm head, sperm were considered as having an intact acrosome when: (1) the anterior acrosomal region stained green and (2) the dark green thickened “rubber band” border forming a semicircle line at the tip of the head remained unbroken or continuous [11]. The posterior postacrosomal region of each sperm head was red-pink in coloration. Sperm lacking the red-pink counter-stain in the posterior head region were indicative of inadequate staining and were not counted. Sperm with non-intact acrosome (reacted or defective) showed peeled acrosomal membranes, spotting, irregular

thickness in the green band or partial green coloration. Another type of sperm with non-intact acrosome (missing acrosomal enzymes) was stained either white or red at the acrosomal region without any green color at the head. For each sperm smear, at least 100 sperm cells were analyzed and the percentage of sperm with intact acrosome was calculated by dividing the number of sperm with intact green acrosome over the total number of sperm analyzed and multiplied by 100.

Dual-stain fluorescence assay

The percentages of apoptotic and necrotic sperm cells were analyzed by means of a previously published dual fluorescence assay procedure [13, 14]. The procedure involved mixing a drop of washed sperm with a drop of Hoechst 33342 stain (10 μ M HO342 or bisbenzimidazole, Sigma Chemical Co., St. Louis, MO dissolved in saline) placed on a glass slide. This was followed by mixing the sperm with a drop of propidium iodide stain (32 μ M PI, Sigma Chemical Co., St. Louis, MO dissolved in saline) and a cover slip placed over the mixture. The stained sperm were analyzed after a minute using an ultra-violet (UV) epifluorescent microscope. Each analysis was completed within 1–2 min for accuracy due to increased fluorescence of the Hoechst 33342 stained sperm with time. Three types of sperm were distinguishable: live sperm (clear or slightly blue-stained at the postacrosomal region), apoptotic sperm (completely blue head) and necrotic sperm (red-pink head) [14]. Sperm with faintly blue acrosomal tips were considered apoptotic [15] while sperm that were half blue and half red were considered necrotic.

Zeta potential sperm selection process

The zeta selection process [1] is defined as an electrostatic procedure that selects for sperm with a net negative zeta potential charge due to acquired epididymal proteins localized on the membrane surface. The initial step in the experiment design consisted of equally dividing each washed sperm specimen into two portions. One portion was cryopreserved as outlined below while the remaining portion was subjected to zeta processing. Zeta processing [1] was carried out immediately to avoid confounding factors due to capacitation-related changes in the sperm membrane electropotential [16, 17]. The washed sperm from each specimen was pipetted into a new centrifuge tube and diluted with 5 ml of serum-free HEPES-HTF medium. To ensure that the tube was positively charged, the tube was wrapped inside a polypropylene plastic sheet, rotated two or three turns and rapidly pulled out. The positive electrokinetic potential charge was verified using a surface DC

electrostatic voltmeter (AlphaLab Inc., Salt Lake City, UT). Typically, a tube of washed fresh sperm was -3.9 KV at 1 in. which became $+1.3$ KV at 1 in. after electron-stripping with the plastic sheet.

Each positive-charged tube was kept at room temperature (23°C) for 1 min to allow adherence of the charged sperm to the wall of the centrifuge tube. Each tube was held by the cap and care taken to avoid grounding the tube. After 1 min, each tube was centrifuged at $300\times g$ for 2 min and inverted to drain out non-adhering sperm and other cell types. Excess liquid was blotted off at the mouth of each tube. Serum-supplemented HEPES-HTF medium (0.2 ml) was pipetted into each tube to neutralize the charge on the wall of the tube and detach the adhering sperm. The collected sperm suspension at the bottom of each tube was analyzed for CASA, morphology, necrosis parameters and the results recorded as zeta-processed post-washed fresh sperm.

Cryopreservation and thawing of post-washed fresh sperm

The remaining portion of each washed fresh sperm specimen not processed by the zeta method was cryopreserved using a standard sperm freezing protocol provided by the freezing medium manufacturer (Catalog No. 90128, Irvine Scientific, Irvine, CA). The freezing medium contained test-yolk buffer, glycerol and gentamycin. Briefly, an equal volume of washed sperm was mixed with an equal volume of freezing medium in a freezing vial and refrigerated for 1 to 1.5 h. The vial of cooled sperm was stored submerged in liquid nitrogen for 24 h.

The frozen sperm specimens were rapidly thawed in a 37°C water bath for 10 min. After 10 min, a drop of thawed sperm was removed for CASA, morphology and DNA parameters analyses and the results recorded in the post-washed thawed sperm before zeta process category. To remove the cryoprotectant, the content of each vial was pipetted into a 15 ml centrifuge tube, centrifuged at $300\times g$ for 10 min, and the pellet resuspended in 0.3 ml serum-supplemented HEPES-HTF medium. The electrokinetic potential charge on the centrifuge tube was assessed. Typically the charge was about -2.0 kV at 1 in. The zeta process of electron stripping was carried out as described above and the resultant charge would be about $+1.3$ kV at 1 in. The tube of sperm was kept from grounded contacts for 1 min and centrifuged at $300\times g$ for 2 min. Each tube was inverted to drain out non-adhering sperm. Excess liquid was blotted off at the mouth of each tube and serum-supplemented HEPES-HTF medium (0.2 ml) was pipetted into each tube. The sperm suspension was analyzed for CASA, morphology and DNA parameters, and the results recorded as post-washed after zeta-processed thawed sperm.

Note that the present study focused on applying the zeta process selection on thawed sperm and did not examine the effects resulting from first zeta processing the sperm and then followed by cryopreservation. The reason was mainly due to the low sperm concentration after each zeta selection process which meant inadequate numbers for a cryopreservation study.

Statistical analysis

The results of the sperm parameter measurements were expressed as mean $\pm$ SEM (standard error of the mean). The significance of the means was tested using Students' *t* test statistic. A value of $P<0.05$ was considered significant.

Results

The percentages of sperm with strict normal morphology increased ($P<0.05$) after the zeta method processing of both the fresh (34% improvement) and cryopreserved-thawed (62% improvement) washed sperm specimens (Table 1). The percentage of sperm with an intact acrosome also increased (40% enhancement) after zeta processing of thawed sperm. There were decreases in sperm necrosis (–12%) and apoptosis (–64%) after zeta processing of the thawed sperm.

In terms of motility, progression was decreased (50%) but not total motility after zeta processing of thawed sperm. In contrast, there was increased progression (40%) observed in zeta processed fresh sperm. Hyperactive movement decreased (–47%) while linearity increased (21%) in fresh sperm after zeta processing. There were no other

differences observed in the remaining parameters. As expected, the cryopreservation process decreased (–57%) the total motility from 69.8 ± 0.6 to $41.0\pm0.4\%$.

The recovery rate of cryopreserved-thawed sperm after zeta processing was 8 to 10% of the original concentration.

Discussion

In the area of clinical ART, the main concern arising from the use of cryopreserved sperm is the reported freeze-related decrease in strict normal morphology and sperm necrosis leading to lower fertilization and IVF/ICSI pregnancy outcome [18]. The results of the present study demonstrated that improvements in strict normal morphology and reduced sperm necrosis could be achieved after zeta potential processing of the cryopreserved-thawed sperm. A direct association exists between these parameters and treatment outcome [3–8]. Furthermore, the percentage of sperm with an intact acrosome also increased suggesting that the zeta processing would not trigger premature acrosome reactions. The data suggested that the cryopreservation process, which involved low temperatures and exposure to glycerol and egg yolk protein, did not impact the sperm membrane net zeta potential. This was important as the crux of the zeta potential method was the negative electrokinetic charge required for selection [1, 9].

Interestingly, the results were consistent with a previous report by Glander and colleagues [19] which indicated that the electrophoretic motility (EPM) of negative-charged human sperm subjected to microelectrophoresis did not change after freezing in egg yolk based cryoprotectant. However, this did not preclude dynamic exchanges in

Table 1 Changes in cryopreserved-thawed sperm parameters ($N=9$ specimens) after processing with the zeta membrane charge method

Parameter	Semen	Post-wash			
		Fresh (Before zeta)	Fresh (After zeta)	Thawed (Before zeta)	Thawed (After zeta)
Total motility (%)	64.4 ± 0.3	69.8 ± 0.6	67.0 ± 0.6	41.0 ± 0.4	37.9 ± 0.6
Progression (%)	22.9 ± 0.3	20.9 ± 0.2^a	29.2 ± 0.5^a	27.6 ± 1.5^b	13.9 ± 0.3^b
Average path velocity (μ/s)	37.1 ± 0.2	45.1 ± 0.2	41.6 ± 0.2	30.3 ± 0.2	30.3 ± 0.1
Curvilinear velocity (μ/s)	45.0 ± 0.2	62.3 ± 0.3	55.0 ± 0.3	41.1 ± 0.3	41.1 ± 0.2
Hyperactivation (%)	0.2 ± 0	3.2 ± 0.1^a	1.7 ± 0.1^a	0.2 ± 0	1.1 ± 0.1
Linearity (%)	62.3 ± 0.1	49.0 ± 0.2^a	59.3 ± 0.2^a	61.3 ± 0.2	59.1 ± 0.4
Strict normal morphology	6.2 ± 0.2	8.8 ± 0.3^a	11.8 ± 0.3^a	5.4 ± 0.2^b	8.7 ± 0.2^b
Acrosome intact (%)	39.9 ± 0.7	53.2 ± 0.6	59.3 ± 0.7	35.6 ± 0.9^b	49.8 ± 0.9^b
Necrosis (%)	NA	40.3 ± 0.5	43.1 ± 0.4	74.6 ± 0.3^b	66.0 ± 0.6^b
Apoptosis (%)	NA	3.5 ± 0.1	2.5 ± 0.1	3.6 ± 0.1^b	1.3 ± 0.1^b

Values are presented as mean $\pm$ SEM.

^{a,b} For each row, values with similar superscripts are different. $P<0.05$

membrane protein components. Changes in protein composition in the membrane have been documented, for instance, a decrease in membrane P34H and an increase in alpha-tubulin after cryopreservation [20].

The mechanism involved in the zeta selection of sperm with strict normal morphology was based on the greater net negative charge of mature sperm due to membrane sialoglycoproteins [21, 22], specifically, gp20-CD52 glycopolypeptides, acquired during transit through the epididymis [23]. Researchers have correlated the maturity of sperm membranes with strict normal morphology, an parameter indicative of successful fertilization [7, 24, 25]. Disruptions in the sperm maturation process, for example, frequent ejaculations, would result in a less negative-charged sperm due to reduced membrane proteins. In contrast, sperm cells given time to replace somatic histones with protamines for chromatin condensation, a critical step in maturation, would additionally acquire epididymal proteins to yield a net negative zeta potential. The negative zeta potential property was recently applied by researchers using electrophoresis to select sperm for an in vitro fertilization procedure resulting in the birth of an infant [26].

In terms of improvements in the remaining parameters related to movement such as total motility, progression and velocities, the results were mixed. Total sperm motility and velocities were unaffected by the brief exposure to the serum-free condition or the manipulation from the attaching-detaching process suggesting a lack of association between these parameters and zeta potential [1]. Note that the sperm fraction selected by the zeta process consisted of both motile sperm and non-motile sperm with net negative membrane charge. In contrast to fresh sperm, the progressive motility of thawed sperm declined after zeta processing. This was likely due to increased sensitivity of thawed sperm to zeta processing resulting from a loss of plasma membrane asymmetry [27] and a conversion to less progressive non-linear hyperactivation movement [28]. In this study, the percentage of hyperactivation was numerically higher after zeta processing of thawed sperm but this was not statistically significant.

Currently, sperm is randomly selected for ICSI based on a reasonable shape and motility [2]. Moreover, sperm selection by high-magnification motile sperm organelle morphology examination (MSOME) [29], electrophoresis [30] and hyaluronic acid hydrogel have recently been proposed [31]. While the zeta method has shortcomings such as low sperm recovery, a requirement of uncapacitated sperm [16, 17] and the possible bias for X-bearing sperm [9, 21, 32, 33], nevertheless, the zeta method appears promising for selecting thawed sperm for ICSI. However, more studies are needed before its application in routine ART treatment.

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