A simple zeta method for sperm selection based on membrane charge

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Objective: The objectives were: [1] to develop a simple zeta potential method for sperm isolation; and [2] to analyze the sperm maturity, morphology, kinematic, and DNA parameters.
Design: The phenomenon of sticky sperm adhering to slide surfaces was adapted for collecting charged sperm.
Setting: Clinical and academic research environment.
Patient(s): Discarded colloid-washed sperm from routine laboratory testing (n = 8).
Intervention(s): Sperm were centrifuged in serum-free medium and collected for analyses.
Main Outcome Measure(s): Kinematic parameters, DNA integrity, and maturity.
Result(s): The percentages of mature (73.0% ± 0.5% vs. control 63.5% ± 0.5% SEM) and DNA intact sperm (85.0% ± 0.3% vs. 69.5% ± 0.5%) increased in the male factor subgroup. Strict normal morphology (19.3% ± 0.1% vs. 10.0% ± 0.1%), hyperactivation (7.0% ± 0.1% vs. 3.6% ± 0.1%), and progressive motility (29.1% ± 0.1% vs. 19.9% ± 0.1%) increased by twofold.
Conclusion(s): The zeta method improved sperm parameters associated with increased fertilization and pregnancy after assisted reproduction procedures. Manipulation from the attaching–detaching process stimulated sperm metabolism without causing premature acrosome reactions. Total motility was unchanged suggesting a lack of association between total motility and zeta potential. (Fertil Steril® 2006;85:481–6. ©2006 by American Society for Reproductive Medicine.)

Key Words: Spermatozoa maturation, zeta potential, membrane charge, chromatin structure and DNA integrity, electrophoresis

The selection of mature sperm with intact DNA is an important step for assisted reproduction technology (ART) procedures. Immature sperm with protamine deficiency (1–3) injected into oocytes exhibited failed decondensation or abnormal unwinding of DNA (4). At present, various sperm separation or isolation methods exist to select sperm cells. These include swim-up methods (5), two-layer discontinuous gradient centrifugation (6, 7), pentoxifylline wash (8), test-yolk buffer (9), sedimentation methods (10), polyvinylpyrrolidone (PVP) droplet swim-out (11), electrophoresis (12, 13), and fluorescence cell sorting methods (14). However, a simple method to select for mature sperm is lacking. Mature sperm possess an electric charge of −16 to −20 mV (15), which decreases with capacitation (16, 17) or exposure to uterine neuraminidase and follicular fluid (FF) (18). This electric charge has been termed zeta potential (electrokinetic potential) and is defined as the electric potential in the slip plane between the sperm membrane and the surroundings (15).

Sticky sperm adhering to the glass slide surface in protein-free medium have always been considered a nuisance to personnel examining sperm motility. The idea was to use this electrostatic charge attraction property to immobilize highly charged sperm while washing away lesser charged sperm and debris. Hence, the objectives were to develop a simple method based on the zeta potential to isolate sperm and to analyze the sperm for differences in maturity, morphology, kinematic, and DNA integrity parameters. The results will help expand the number of isolation methods for the selection of quality sperm for ART procedures.

MATERIALS AND METHODS

Preparation of Sperm

Sperm of men (n = 8 cases) in an ART program were washed using the two-gradient colloid (Isolate; Irvine Scientific, Santa Ana, CA) procedure (6, 7). Testicular, epididymal sperm aspiration, and donor sperm cases were not included in the study. The procedures were approved by the

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Loma Linda University Institutional Review Board. The sperm washing procedure consisted of layering the semen on top of a discontinuous two-layer 90:45 colloid gradients in a 15-mL polystyrene centrifuge tube. The bottom layer was 1.5 mL of the 90% colloid solution and the upper layer was 1.5 mL of 45% colloid solution.

The tubes of gradients were centrifuged at 300 × *g* for 20 minutes and each resultant pellet was added with 1.0 mL of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered synthetic human tubal fluid (HTF) medium (Irvine Scientific) supplemented with 5% serum substitute supplement (Irvine Scientific). Each tube of diluted sperm was recentrifuged and the final pellet was resuspended in 0.4 mL of serum-supplemented HEPES–HTF medium.

The resuspended sperm were divided for processing using the zeta method or left untreated (colloid-washed control). Sperm kinematic parameters were measured using the Hamilton-Thorn HTM-C (Hamilton-Thorn, Danvers, MA) computer-aided sperm analysis (CASA) system (7). The normal reference values for total motility, forward progression, and hyperactivation were greater than 50%, 25%, and 3%, respectively. The sperm normal morphology was analyzed using the Tygerberg strict criteria method developed by Kruger and colleagues (19, 20) as described. Sperm maturity and DNA integrity were determined using the aniline blue assay (21–26) and the acridine orange test (27–31), respectively, and details presented in the following sections.

Zeta Potential Sperm Processing Method

The zeta method should be carried out immediately as sperm cells become less negatively charged with the onset of capacitation (16, 17). It was essential to use a new centrifuge tube for this method as the electrostatic charge was maximal and if needed, could be verified using electrostatic voltmeters (AlphaLab Inc., Salt Lake City, UT; All-Spec Industries, Wilmington, NC). The washed sperm (0.1 mL) was pipetted into the tube and diluted with 5 mL of serum-free HEPES–HTF medium. To ensure that the tube was positively charged (+2 up to +4 kV at 1 inch), the tube was placed inside a latex glove up to the cap and grasping the cap, the tube was rotated two or three turns and rapidly pulled out.

Each tube was kept at room temperature (22°C) for 1 minute to allow adherence of the charged sperm to the wall of the centrifuge tube. Hold each tube by the cap and avoid grounding the tube. It was noteworthy that glass centrifuge tubes tended to permit more sperm adherence when compared with polystyrene tubes. However, glass tubes should be rinsed and soaked before use to reduce contaminants.

After 1 minute, the tubes were centrifuged at 300 × *g* for 5 minutes and each tube was simply inverted to drain out nonadhering sperm and other cell types. Excess liquid was blotted off at the mouth of each tube. Note that centrifugation would not alter the net charge on each tube. Serum-supplemented HEPES–HTF medium (0.2 mL) was pipetted into each tube allowing the medium to trickle down the side of the tube to neutralize the charge on the wall of the tube and detach the adhering sperm. The collected medium at the bottom of each tube was repipetted and used to rinse the wall of the same tube several times to increase the number of recovered sperm. The use of culture medium with a higher percentage of serum or discharging the tube might improve recovery of detached sperm in low sperm concentration situations. Aliquots of the detached sperm were analyzed and the results compared with the control sperm data.

Spermac Stain for Sperm Morphology and Acrosome Intactness

The Spermac stain was used to stain the sperm for the morphology as well as the acrosome intactness analyses, as previous reported (32, 33). Briefly, for each tube of processed sperm, a sperm smear was made on a glass slide and air dried. The dried smear was fixed in formalin (fixative I) provided in the Spermac kit (Stain Enterprises, Onderstepoort, S. Africa, distributed by Sepal Reproductive Devices, Sudbury, MA) for 5 minutes at room temperature (22°C). Each slide with fixed sperm was rinsed and stained in solution A for 2 minutes before being rinsed with water. Each slide was stained in solution B for 1 minute, rinsed and stained in solution C for 1 minute, followed by more rinsing. The stained slides were air dried for 10 minutes before being analyzed in oil immersion (×1,000) and bright field light microscopy.

The assessment of normal morphology was carried out using the Tygerberg strict criteria method (19, 20) on at least 100 sperm. A sperm was classified as strict normal when the head was oval with the acrosome occupying 40%–70% of the head, absence of midpiece and tail defects, and absent or negligible cytoplasmic droplets with the appropriate head dimensions. The normal reference value for this parameter was 14% or more of analyzed sperm meeting the strict normal morphology criteria (19).

The percentages of sperm with intact acrosome were determined from the same set of Spermac-stained morphology slides (33). Sperm with an intact acrosome was stained green at the anterior acrosomal region with a continuous membrane. 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. The normal reference value for this parameter was 45% or more sperm with intact acrosome (33).

Acridine Orange Test for Sperm With Unfragmented or Intact DNA

The acridine orange test was used to determine the percentage of sperm with fragmented or damaged DNA (27–31). A normal specimen typically has 70% or more sperm with intact DNA. The acridine orange test was carried out

in a darkened room to reduce photo-bleaching of the stained sperm by light. An aliquot (0.1 mL) of either zeta processed or colloid-washed (control) sperm was carefully pipetted into a tube containing 0.2 mL of HCl-acidified Triton X-100 (31) and held for 30 seconds followed by the addition of 0.3 mL of a 1% acridine orange (United States Biochemical Corp., Cleveland, OH) in saline solution. After 1–5 minutes at room temperature (22°C), a drop of the stained sperm was placed on a glass slide, covered with a cover slip, and examined in an ultraviolet (UV) fluorescent microscope at ×500 magnification. A total of 100 cells was analyzed for each slide. Special care was taken by narrowing down the UV light diaphragm or initially adding a drop of slowfade buffer (Molecular Probes Inc., Eugene, OR).

Sperm with DNA that became fragmented or denatured to single strands stained orange-red at the head. Healthy sperm with double-stranded DNA appeared green (27–31). The percentage of sperm with intact double-stranded DNA (normal integrity) was calculated by dividing the number of green-stained sperm over the total number of sperm and multiplying by 100.

Acidic Aniline Blue Assay for Sperm Nuclear Maturity

The aniline blue assay (21–26) was used to identify immature sperm with persistent histones and defects in histone-protamine replacement that would affect orderly sperm DNA uncoiling in the ooplasm (1, 21). Aliquots of zeta-processed sperm and control sperm were separately smeared on glass slides and air dried. The sperm smears were fixed in formalin solution (fixative I solution, Spermac stain, Sepal Reproductive Devices) for 5 minutes, rinsed in water, and stained in 5% aniline blue in 4% acetic acid (pH 3.5) solution (21) for 5 minutes. After staining, the slides were rinsed in water and air dried. The slides were examined using oil immersion at ×1,000 magnification in bright field illumination. Immature sperm characterized by nuclear histone proteins (lysine-rich) stained dark blue, whereas mature sperm with protamines (cysteine and arginine-rich) remain clear and unstained (21–26). The percent sperm maturity was calculated from the ratio of the number of unstained sperm to the total number of sperm analyzed and multiplied by 100. A percent maturation of more than 75% was regarded as normal (21–26).

Statistical Analysis

The results of the sperm parameter measurements were expressed as mean ± SEM (standard error of the mean). The significance of the means was tested using Student's *t* test statistic. A value of $P<.05$ was considered significant.

RESULTS

The percentage of sperm with strict normal morphology was almost doubled (19.3% ± 0.1% vs. 10.0% ± 0.1% control) after processing using the zeta method when compared with the control (Table 1). Similarly, sperm hyperactive motility and forward progression were increased almost twofold in the zeta processed sperm group recategorizing the sperm group from below to above normal reference values. The

TABLE 1

Sperm parameters after processing using either the zeta potential method or untreated control.

Parameter	N	Control	Zeta processed
All inclusive group			
Total motility (%)	800	74.8 ± 0.1	78.3 ± 0.2
Forward progression (%)	800	19.9 ± 0.1	29.1 ± 0.1[a]
Hyperactivation (%)	800	3.6 ± 0.1	7.0 ± 0.1[a]
DNA normal integrity (%)	800	81.0 ± 0.1	89.0 ± 0.1[a]
Strict normal morphology (%)	800	10.0 ± 0.1	19.3 ± 0.1[a]
Acrosome intactness (%)	800	45.3 ± 0.2	51.9 ± 0.2
Aniline blue maturity (%)	800	73.3 ± 0.2	79.6 ± 0.2[a]
Male factor groups			
Total motility (%)	400	68.8 ± 0.2	69.8 ± 0.4
Forward progression (%)	500	13.0 ± 0.1	26.6 ± 0.3[a]
Hyperactivation (%)	500	0.6 ± 0.1	4.6 ± 0.1[a]
DNA normal integrity (%)	200	69.5 ± 0.5	85.0 ± 0.3[a]
Strict normal morphology (%)	400	5.4 ± 0.1	17.4 ± 0.1[a]
Acrosome intactness (%)	500	37.6 ± 0.3	49.8 ± 0.3[a]
Aniline blue maturity (%)	400	63.5 ± 0.5	73.0 ± 0.5[a]

[a] Different from control, $P<.05$.

Chan. Zeta method for sperm selection. Fertil Steril 2006.

improvements in these three parameters were more pronounced when a subset of the data representing male factor specimens were analyzed.

Smaller but significant improvements were observed for the DNA normal integrity and the aniline blue sperm maturity parameters after processing the sperm using the zeta method. As before, the improvements were more prominent for the group of male factor infertility specimens. Interestingly, there was no improvement in percentage of total motility, but there was only a modest increase in the percentage of acrosome intact sperm after zeta processing.

Basic semen analyses before sperm processing indicated a mean semen volume of 2.7 ± 0.1 mL and a mean sperm concentration of 47.7 ± 0.3 million/mL. The recovery rate of sperm cells after the zeta method was 8.8% of the original colloid-washed sperm population.

DISCUSSION

The zeta method of sperm processing was simple to perform, inexpensive, and permitted rapid recovery of sperm with improved sperm parameters, particularly strict normal morphology, DNA normal integrity, and aniline blue maturity. These parameters are associated with improved fertilization and pregnancy after intracytoplasmic sperm injection (ICSI) (21–31). Moreover, sperm progressive motility and hyperactivation were enhanced, suggesting that the brief exposure to the serum-free condition or the manipulation from the attaching–detaching process acted as a trigger to stimulate sperm metabolic activity without causing premature acrosome reactions. Greater progressive motility and hyperactivation are predictive of successful pregnancies after ART procedures (34–36). In contrast to the aforementioned parameters, total sperm motility was unchanged suggesting a lack of association between total motility and zeta potential. In this regard, preprocessing sperm using the two-gradient colloid wash was an important step to maximize the isolation of motile sperm.

The main advantage of the zeta method was that sperm cells were not exposed to high voltage electricity (13, 37, 38). In addition, the zeta method did not require the use of expensive electrophoresis equipment, Tris buffers, extreme pH environments, and UV irradiation. An interesting observation was that the twitching sperm immobilized to the inside surface of the tube could be seen using an inverted microscope with the tube held at the stage. Separate zones of attachment on the sperm surface (acrosome tip, postacrosomal region, midpiece, tail tip, or entire surface) could be ascertained by placing a drop of the sperm suspended in serum-free medium onto a glass slide and using phase contrast microscopy to immediately examine the sperm. Capacitated motile sperm from fertile patients showed more movement while partially adhered on the glass slide surface in contrast to uncapacitated sperm, which are completely immobilized with occasional twitching.

The design of the zeta method came from observing sperm adhering to the surface of glass slides when the culture medium was not supplemented with serum or albumin protein. When serum or albumin was added back to the medium, the sperm once again exhibited progressive movement suggesting that the added protein neutralized the charged glass surface and perhaps the net negative zeta potential charge on the sperm (12, 15, 38). The net negative electrical charge of the sperm surface has been documented for other species such as bovine (39–42), porcine (43), and chimpanzee (44). However, the occurrence of net positive charge sperm and the agglutination of positive and negative sperm remain unknown. The occasional sperm cell that adhere to the glass slide surface despite the presence of protein supplementation suggests a very high zeta potential on the sperm that perhaps is indicative of a state of supermaturation deserving of further exploration.

The sperm adhesion force involved Van der Waals, electrostatic, hydration repulsion, or specific charge–charge interactions at the slide surface. In this study, the sperm zeta potential or stickiness property was used to hold the sperm to the wall of the centrifuge tube. The sperm zeta potential was reported to be due to charged sialoglycoproteins (12, 17) in the sperm membrane. In the epididymis, prostasomes (45–47) link the three forms of negatively charged gp20-CD52 glycopolypeptides to the sperm plasma membrane by glycosylphosphatidylinositol (GPI) anchors (17, 45, 48, 49).

Interestingly, the sperm zeta potential charge becomes less negative after capacitation (16, 41). This is due to the release of the gp20-CD52 low charge form (17) with passage of time suggesting the need to carry out the zeta processing method as soon as possible. Other studies have indicated that an X-bearing sperm has a greater net negative charge than a Y-bearing sperm (15, 40, 42). Whether or not the zeta method results in recovery of more X-bearing sperm remains to be determined in future studies.

A drawback of the zeta method is the low recovery of processed sperm, hence, limiting its usefulness in oligozoospermic cases. Furthermore, the zeta method may not be useful for testicular or caput epididymal sperm that lack sufficient net electrical charge on the sperm membrane surface (43). In addition, this method, which depends on the electrical charge of the centrifuge wall, has not been tested in a very humid environment, which has been known to neutralize the electrical surface charge by the presence of fine water droplets.

Nevertheless, the zeta method appears promising and adds to the list of sperm isolation methods available in the clinical laboratory. An isolation process for quality sperm is important, particularly for the ICSI procedure, which presently

relies on sperm motility as the only criterion for the selection of sperm for oocyte injection. More studies are needed such as sequential zeta processing and type of centrifuge tube surface material for sperm adherence before its application in ART.

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