Zeta potential vs apoptotic marker: which is more suitable for ICSI sperm selection?

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Abstract

Purpose Selection of appropriate sperm is considered as a decision making point in the ICSI procedure. Canonically, sperm selection is based on morphology and motility. Recent advances in this field, have shown that, this procedure can be assisted by further selection based on membrane surface charge (Zeta potential) and surface apoptotic marker (phosphatidylserine externalization) using magnetic activated cell sorter (MACS). Based on the literature, both these procedures improve quality of selected sperm population. Therefore, this study aims to compare the efficiency of these two procedures.

Methods Semen samples were collected from 36 fertile and infertile (teratozoospermic and/or asthenozoospermic) individuals. Sperm DNA fragmentation, protamine deficiency and morphology were assessed by TUNEL, CMA3 and papanicolaou staining in unprocessed, MACS and Zeta processed samples.

Results Although both MACS and Zeta were able to separate a higher percentage of sperm with normal morphology, and lower DNA fragmentation and protamine deficiency compared to

unprocessed, MACS procedure could significantly isolate, a greater percentage of sperm with normal acrosome and protamine content compared to Zeta procedure.

Conclusion Both MACS and Zeta procedures improve the quality of the selected spermatozoa for ICSI. However, MACS procedure is more efficient in individuals with severe male factor infertility to select sperm with normal acrosome and protamine content but concern regarding transfer of MACS beads into the oocyte remains to be resolved.

Keywords Zeta · MACS · DNA fragmentation · Protamine deficiency · Morphology

Introduction

The continuously expanding applications of ICSI technique in the last decade and suboptimal success rate associated with this techniques demands future research, on different steps of this technology including sperm selection procedure [1, 2]. During in vivo conception, a spermatozoon destined to fertilize the oocyte has to perform various physiological functions and pass different barriers including the zona pellucida. However, during ICSI all or most of these steps are bypassed [3]. Therefore, researchers are working towards presenting an ideal sperm selection technique to improve the ICSI outcomes, as an important step on ICSI technique due to limited number of oocytes available for insemination. Commonly, semen is processed by swim up or density gradient centrifugation (DGC). Subsequently, a spermatozoon is selected based on motility and morphology and injected into an oocyte [4, 5]. Therefore, IMSI (Intra cytoplasmic morphologically-selected sperm injection) was proposed to select the most ‘normal’ looking sperm for insemination. Despite early positive results, recent studies have underestimated the value of this procedure and future meta-analysis, may shed more light, on this procedure [6].

Capsule In the two novel sperm selection procedures for ICSI, the efficiency of both DGC-zeta and MACS-DGC procedures to recovered sperm with intact DNA and normal morphology were similar. However, MACS-DGC was more efficient to recover sperm with normal protamine content and acrosome morphology.

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Furthermore, Avendona et al. [7] stated “in infertile men, sperm with apparently normal morphology may have DNA fragmentation and presence of an increased proportion of normal sperm with damaged DNA was negatively associated with embryo quality and pregnancy outcome after ICSI”. On the contrary, healthy babies have been born through ICSI technique using sperm with abnormal morphology such as round head sperm or sperm with abnormal axoneme [8]. Therefore, in addition to sperm motility and morphology, selection of sperm based on sperm surface characteristics or functional aspect of sperm physiology has been proposed.

In the past few years, our group with an interest in the novel sperm selection procedures has assessed several novel sperm selection procedures including hyaluronic acid binding method, Zeta, Magnetic-Activated Cell Sorting (MACS) and hypo-osmotic sperm selection procedures [9–13]. Therefore, we have proposed that one approach for selection of an ideal sperm selection procedure is comparison of these techniques *in vitro*, prior to comparison of these techniques at the clinical level. Therefore, the aim of this study was comparison of Zeta and MACS procedures for selection of normal sperm. It has been shown that a mature sperm, possess a static surface electrical charge around -16 to -20 mV, termed Zeta potential which decreases upon capacitation [14, 15]. Therefore, it has been proposed that sperm selection based on electrical charge may improve ICSI outcomes. We previously reported that combining density gradient centrifugation (DGC) with Zeta procedure can select sperm with intact chromatin and DNA, and can improve ICSI outcomes [11]. Other have also verified this conclusion, through the selection of sperm based on Zeta potential in a micro fluid/electrostatic filed [16].

In somatic cells, externalization of phosphatidylserine (EPS) from inner to outer layer of plasma membrane is considered as one of early signs of apoptosis [17]. However, this process is also considered as part of normal process during capacitation. Therefore, assessment of EPS before capacitation may be considered as a sign of early apoptosis while post capacitation may be considered as a sign of capacitation [18]. We previously showed that selection of sperm by MACS before DGC, improves the quality of selected sperm population [12]. In addition, it has been previously suggested that the selection of sperm by MACS can improved ICSI outcomes [19].

Therefore, considering that both Zeta and MACS procedures, can improve quality of sperm selection population, the aim of this study is to compare the efficiency of these two procedures for future sperm selection procedure.

Materials and methods

This study was approved by the Institutional Review Board, and informed consents were signed by all the individuals who

provided semen samples. Semen samples were collected from 26 individuals with male factor infertility with teratozoospermia and/or asthenozoospermia and 10 fertile individuals who participated in the embryo donation program at Isfahan Fertility and Infertility Center, as the control group. Oligozoospermia individuals were not included in this study, since sufficient numbers of sperm were required to complete the required analysis. Semen samples were collected through the process of masturbation in sterile containers after 3–4 days of sexual abstinence. Sperm concentration, motility, morphology and volume of each ejaculate were analyzed according to World Health Organization guidelines-2010 [20]. All the materials used in this study were purchased from Merck (Germany), unless otherwise stated in the text.

Experimental design

Initially, each sample was divided into three portions. One portion was washed with Ham's F10+10 % albumin and labeled as the “unprocessed group” as control group. The second portion was subjected to the “DGC- Zeta procedure” and the third portion was used for “MACS-DGC procedure”. Therefore, following MACS-DGC and DGC- Zeta procedures, percentage of sperm with abnormal morphology, protamine deficiency and DNA fragmentation were defined using papanicolaou staining, Chromomycin A3 staining (CMA3) and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), (for detail see Kheirollahi-Kouhestani et al.) [11], respectively. These tests were also carried out on unprocessed samples (Fig. 1).

DGC-Zeta procedure

Following density gradient centrifugation (DGC) in absence of serum, Zeta procedure was carried out according to Chan et al.

Fig. 1 Flow diagram of overall experiment design. *CMA3* Chromomycin A3; *TUNEL* TdT mediated dUTP Nick-End Labeling; *DGC* Density Gradient Centrifugation; *MACS* Magnetic-Activated Cell Sorting

(2006) and Kheirollahi-Kouhestani et al. (2009) [11, 14]. Briefly, sperm concentration was set at five million per ml in each tube. Positive charge was induced by putting the tube inside latex glove up to the cap. Then, the tube was grasped by the cap and it was rotated two or three turns. The tube was rapidly removed from the glove. Each tube was kept at room temperature for 1 min to allow adherence of the charged sperm to the surface of the tube. The medium within the tube was then removed and discarded to eliminate non-adhering sperm. Then, the surface of the tube was rapidly washed by 1 ml of Ham's F10+albumin 10 % to neutralize the charge and wash down the adhered sperm to the bottom of each tube. Then, papanicolaou staining, CMA3 and TUNEL assay were carried out on this fraction.

MACS-DGC procedure

Magnetic-Activated Cell Sorting (MACS) is a suitable procedure used for selecting the desired cells based on membrane surface markers. Normally, phosphatidylserine (PS) present in internal layer of plasma membrane. Externalization of phosphatidylserine (EPS) to the outer membrane of sperm can be assessed by Annexin V, a protein that binds specifically to EPS and enables the identification of EPS positive cells. Briefly, semen samples were washed with Ham's F10+ 10 % Albumin and then samples were diluted to ten million per ml. Then, 100 μ l of each semen sample was mixed with one hundred microliter of micro beads solution (Milteny Biotec; India) and then, this mixture was incubated for 15 min at room before being loaded to MACS column (Milteny Biotec; India). Annexin positive sperm was retained within the column while annexin negative sperm were allowed to pass the column. Then, DGC procedure was carried out on Annexin negative samples before papanicolaou staining, CMA3 and TUNEL assay.

TUNEL and CMA3 staining

Briefly, after fixation of each sample with 4 % methanol-free formaldehyde, a commercial detection kit (Apoptosis Detection System Fluorescein, Promega, Mannheim, Germany) was used for the detection of DNA fragmentation according to Kheirollahi-Kouhestani et al. [11]. In addition, each sample were fixed in Carnoy's solution and stained for 20 min with 100 μ l of 0.25 mg/ml CMA3 (Sigma Co., St. Louis, MO, USA) solution [12]. In each group around 500 sperm were counted.

Statistical analysis

Results are expressed as means $\pm$ SEM. Levene's test for equality of variances and Shapiro-Wilk were carried out to assess normal distribution. For statistical analysis, the repeated measures ANOVA were used. P-values of less than 0.05 were considered significant.

Results

Table 1 shows the descriptive analysis of semen parameters in three groups; total population, fertile, and infertile groups. The mean ages of male in this study were 35.00 ± 1.00 . Figure 2 indicates comparison of mean percentages of DNA fragmentation (TUNEL assay), protamine deficiency (CMA3staining), abnormal morphology, and normal acrosome in unprocessed, MACS and Zeta procedures in total population, fertile, and infertile groups.

The mean percentage of DNA fragmentation in unprocessed, MACS and Zeta procedures in total population were 17.7 ± 1.6 , 12.1 ± 1.7 and 9.8 ± 1.2 , respectively. This parameter showed a significant reduction following the MACS and Zeta procedures compared with the unprocessed sample ($p < 0.05$). In fertile group the mean percentage of DNA fragmentation in unprocessed, MACS and Zeta procedures were 15.8 ± 2.5 , 10.3 ± 2.5 and 10.6 ± 1.9 and in the infertile group were 18.5 ± 2.2 , 13.00 ± 2.3 and 9.5 ± 1.6 , respectively. In both groups a significant reduction in the mean percentage of DNA fragmentation sperm was observed in MACS and Zeta procedures compared to unprocessed or control group.

The mean percentages of CMA3-positive sperm in unprocessed, MACS and Zeta procedures in total population were 54.8 ± 2.1 , 42.8 ± 2.00 and 50.6 ± 1.8 respectively. This parameter showed a significant reduction following the MACS procedure when compared to the unprocessed and Zeta procedure ($p < 0.05$). In the fertile group, the mean percentage of CMA3-positive sperm in unprocessed, MACS and Zeta procedures were 46.7 ± 4.1 , 38.00 ± 2.8 and 48.9 ± 2.3 and in the infertile group, were 58.00 ± 2.3 , 44.4 ± 2.5 and 51.3 ± 2.4 , respectively. In both groups significant reduction was observed following the MACS-DGC procedure compared with the unprocessed and Zeta group ($p < 0.05$). In addition, we observed a significant reduction in the percentage of CMA3-positive sperm in Zeta compared with the unprocessed or control group in the infertile group.

The mean percentage of abnormal sperm morphology in the unprocessed, MACS and Zeta procedures in total population was 96.4 ± 0.6 , 94.00 ± 0.7 and 93.6 ± 0.9 respectively. This parameter in unprocessed, MACS and Zeta procedures in the fertile group were 91.1 ± 1.1 , 89.00 ± 1.3 and 86.7 ± 1.6 and, in the infertile group were 98.6 ± 0.2 , 96.00 ± 0.4 and 96.4 ± 0.4 , respectively. Comparison of the mean percentage of abnormal morphology showed a significant reduction in the percentage of abnormal sperm morphology following the MACS and Zeta procedures when compared with the unprocessed group ($p < 0.05$) in total population and infertile group.

The mean percentages of normal acrosome in the unprocessed, MACS and Zeta procedures in total population were 64.00 ± 3.6 , 72.5 ± 3.1 and 60.9 ± 3.3 , respectively. This parameter in unprocessed, MACS and Zeta procedures in fertile group were 81.9 ± 2.5 , 80.00 ± 3.5 and 67.5 ± 5.1 , and

Table 1 Descriptive information of semen parameters in each group

Groups	Parameter	Mean $\pm$ SE	Minimum	Maximum
Total population ($n=36$)	Concentration $\times 10^6$	73.6 $\pm$ 8.1	20	250
	Volume (ml)	3.6 $\pm$ 0.31	1	8.8
	Motility (%)	52.7 $\pm$ 2.4	8	70
	Abnormal morphology (%)	96.5 $\pm$ 0.6	84	100
Fertile ($n=10$)	Concentration $\times 10^6$	94.7 $\pm$ 20.7	30	250
	Volume (ml)	3.5 $\pm$ 0.7	1	7.9
	Motility (%)	54 $\pm$ 2.9	40	65
	Abnormal morphology (%)	91.1 $\pm$ 1.1	84	95
Infertile ($n=26$)	Concentration $\times 10^6$	65.5 $\pm$ 7.7	20	150
	Volume (ml)	3.6 $\pm$ 0.3	1.2	8.8
	Motility (%)	52.2 $\pm$ 3.2	8	70
	Abnormal morphology (%)	98.6 $\pm$ 0.2	96	100

in infertile group were 56.9 ± 4.3 , 69.4 ± 4.1 and 58.3 ± 4.1 , respectively.

Comparison of the mean percentage of normal acrosome showed a significant increase following the MACS when compared to the unprocessed sample and Zeta procedure ($p < 0.05$) in total population and infertile groups. However, the percentage of normal acrosome was significantly decreased following Zeta procedure compared to unprocessed sample in fertile group.

Discussion

Recently, numerous procedures of sperm selection and preparation for ICSI have been proposed and some have been implicated in the ART centers [1, 2]. However, the efficiency of these techniques is still debatable and questionable. Despite implementation of these procedure and improved assisted reproduction technique (ART) outcomes, the success rates of these procedures are still limited. However, a

Fig. 2 Comparison of the mean percentages of protamine deficiency, DNA damage, normal acrosome and abnormal morphology, in the unprocessed, MACS-DGC and DGC-Zeta samples in total population, fertile and infertile group. Common letters show significant difference at $p < 0.05$

percentage of couples may face with failed fertilization and/or retarded embryos develop [21]. Therefore, selection of functional, viable sperm with intact DNA remains in the frontline of research in andrology or ART. Previous study has shown that DGC, routinely use for sperm preparation, isolates spermatozoa with higher mitochondria membrane potential and intact DNA, but its efficiency to remove apoptotic spermatozoa remains limited [22]. Furthermore, considering the fact that sperm which has entered process of apoptosis, is finally destined to DNA fragmentation, therefore, removal of apoptotic sperm by sperm selection techniques is of vital importance in assisted reproductive techniques, especially in the treatment of severe male infertility [22].

Two roles have envisaged for PS externalization in human spermatozoa. One as a sign of early apoptosis, as in somatic cell and the second as physiological process during acquisition of capacitation [23, 24]. The bi-functional roles PS in human spermatozoa makes interpretation and design of experiment very confusing [18, 23]. The use of magnetic cell sorting using annexin V-conjugated micro beads, at molecular level can mark and isolate sperm with translocation of membranes of PS [25]. On the other hand, functional and viable sperm with intact DNA can be selected based on the electrical charge of membrane, termed Zeta potential [11]. Previously, we independently evaluated the quality of sperm selected by Zeta and MACS procedures, and showed that both procedures can select viable sperm with higher quality compared to routine sperm processing or the DGC [11, 12]. Therefore, the aim of this study was to compare quality of sperm selected by Zeta and MACS procedures, in hope of showing which procedure is more efficient.

Considering that during ICSI technique, sperm is selected based on the morphology and vitality, therefore, we also assessed sperm acrosome status, as this structure is associated with ability of sperm to undergo acrosome reaction [26]. Indeed, absence of acrosome is associated with inability of sperm to induce oocyte activation [27]. Furthermore, pervious study show a weak correlation between acrosome activity and ability to induce oocyte activation. The results of this study showed that although both procedures were able to separate higher percentage of sperm with normal morphology compared to unprocessed samples, but MACS procedure could isolated significantly a greater percentage of sperm with normal acrosome compare to Zeta in total and infertile population.

Moreover, sperm DNA integrity has a fundamental role in reproductive process and ICSI outcome [28]. Two recent studies assessing DNA integrity with TUNEL assay have shown absence of pregnancy in individuals showing DNA fragmentation of greater than 20 % [29], and absence of birth in subpopulation with DNA fragmentation of greater than 15 % [30]. Similar conclusion was achieved by other researchers when using different procedure for assessing DNA

integrity [31, 32]. Sperm DNA fragmentation has been mainly attribute to abnormality in differentiating germ cells, commonly termed “abortive apoptosis” [33] and abnormality in replacement of histones by protamine during spermiogenesis commonly referred as “sperm immaturity” [34]. Therefore, in this study we assessed DNA fragmentation using TUNEL assay as an indicator of aborted apoptosis and protamine deficiency by CMA3 staining as a marker of sperm maturation in both Zeta and MACS sperm selection procedures. Both procedures selected sperm population with lower DNA fragmentation and protamine deficiency compared with unprocessed samples. It is interesting to see that percentage of sperm with abnormal protamine contents were reduced significantly in MACS compared to Zeta procedure, while percentage of DNA fragmentation were insignificantly lower Zeta compared to MACS procedure in infertile individuals.

An overall look at the results, it can be concluded that both of sperm selection procedures can select sperm with normal morphology, normal acrosome, normal protamine content and intact DNA. Although, some of these improvement are small and significant but considering the cut of values of 4 % for normal morphology [20], 40 % for protamine deficiency [35] and 15 % for DNA fragmentation [30], the observed improvement might result in substantial difference in clinical outcomes as has been previously reported. Therefore, the observed difference between the two procedures in term of each parameter may have significant effect on clinical outcomes. Despite the significant improvements observed between the two procedures within fertile and infertile group, percentage of improvement observed between fertile and infertile individuals was not significant (data not shown). This could be attributed to the fact that in this study, we did not assess individuals with severe male infertility since substantial number of sperm were required for different assessments post processing. On the other hand, this observation may be attributed to the general improvement occurring during the processing and further modifications of innovation are required to improve the quality of processed sample so that percentage of improvements are substantial higher in infertile individual. In this study, MACS procedure was more efficient in isolation of sperm with normal acrosome and protamine content and might have higher priority for sperm selection in severe teratozoospermic patients. However, in MACS procedure, despite being previously used for human ICSI, concern regarding transfer of MACS bead into the oocyte remains to be resolved.

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