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Original Article

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Comparison of the Efficiency of Magnetic-Activated Cell Sorting (MACS) and Physiological Intracytoplasmic Sperm Injection (PICSI) for Sperm Selection in Cases with Unexplained Infertility

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Abstract

Background: The cases with unexplained infertility may have an abnormality in their sperm chromatin structure. Sperm selection methods can be used to separate sperm with low DNA fragmentation. The purpose of this study was to compare the efficacy of physiological intracytoplasmic sperm injection (PICSI) with magnetic-activated cell sorting (MACS) in assisted reproductive techniques in cases with unexplained infertility.

Methods: The semen samples were collected from couples with unexplained infertility. After semen analysis and sperm DNA fragmentation (SDF) evaluations, samples were prepared with swim-up method. The rates of SDF in different fractions including raw semen (n=20), swim-up (n=20), only motile sperm after swim-up (swim-up selection) (n=20), MACS sperm selection (n=20), only motile sperm after MACS (MACS selection) (n=20), and PICSI sperm selection (n=16) were evaluated. Also, the main sperm characteristics and fine morphology of sperm suspension after MACS were assessed. Statistical analysis was performed using GraphPad Prism. The $p<0.05$ was considered statistically significant.

Results: DNA fragmentation index (DFI) values in PICSI and MACS groups were significantly reduced as compared to the swim-up group. The rate of this reduction was more pronounced in MACS (58.20±13.02) than PICSI (36.57±15.52) group. Also, our results showed that MACS resulted in decreased sperm motility, with no alteration in their fine morphology.

Conclusion: MACS was found to be more efficient in reduction of SDF rates than PICSI. However, none of the sperm selection techniques can not totally eliminated the spermatozoa with DNA fragmentation in the final sperm sample.

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Introduction

Initial diagnosis of male infertility includes the assessment of the semen quality performed according to the WHO guidelines. While impaired semen characteristics are indications of male infertility, normal spermogram results, on the other hand, do not necessarily guarantee an

acceptable fertilizing potential. It is estimated that roughly 15% of men with normal semen analysis profiles experience infertility (1). Sperm DNA assessment can provide information about the successful completion of spermatogenesis as well as the integrity of the genetic material that will be transmitted to the progeny. Both fertilization and embryo quality as well as embryo viability and progression of pregnancy would be affected by the sperm DNA integrity (2). It has been reported that DNA fragmentation index (DFI) is a useful biomarker for male infertility diagnosis. However, over 60% of men with unexplained infertility may have an abnormality in their chromatin structure. In unexplained infertility, sperm DNA testing can therefore be used as an additional marker of sperm quality to help during couples counseling (3).

Recently, advanced sperm selection techniques introduced the concept of enrichment of the spermatozoa with intact DNA before ICSI performance. These methods aim to overcome the limitations of classical sperm selection procedures (4). Such sorting is done according to sperm surface charge (selection of spermatozoa by electrophoresis and Z potential), apoptosis pathway (selection of spermatozoa by magnetic-activated cell sorting), sperm membrane maturity (hyaluronic acid binding), and ultra-high magnification (IMSI) (5). Duarte et al. showed that zeta potential analysis could improve progressive motility, morphology, DNA integrity, and maturity of sperm, thereby increasing the percentage of good-quality embryos and pregnancy rates (5). Magnetic-activated cell sorting (MACS) de-selects the apoptotic sperm based on the phosphatidylserine residues on their membranes. The surface of apoptotic cells can be targeted with labeled annexin V which specifically binds to this phospholipid (6). Reduction of apoptotic spermatozoa as well as DNA fragmentation within the ejaculate by means of the MACS system have recently been documented (7). Physiological intracytoplasmic sperm injection (PICSI) is also another method that selects spermatozoa based on the presence of hyaluronic acid (HA) receptors on sperm membrane. This glycocalyx is an integral component of the extracellular matrix of the cumulus oophorus and is composed of alternating repeats of D-glucuronic and N-acetyl D-glucosamine residues (8). In humans, oocytes are naturally surrounded by HA during the fertilization process, and it is the environment where natural sperm selection takes place. Furthermore, HA receptors are present in mature spermatozoa, and at least three hyaluronan binding proteins are involved in sperm maturation, acrosome reaction, motility, hyaluronidase activity, and sperm-zona binding (9). Jakab et al. reported that HA-bound spermatozoa complete the process of spermiogenesis with cytoplasmic extrusion and demonstrate enhanced levels of testis-expressed HSPA2 as a chaperone protein (10). Furthermore, HA-bound spermatozoa are also devoid of DNA fragmentation and the apoptotic marker, caspase 3. Most significantly, HA-bound spermatozoa display a reduced frequency of chromosomal aneuploidies in comparison to their nonbinding counterparts (11). Each of these biochemical and molecular parameters of developmental maturation play a critical role in the paternal contribution to successful pre-implantation embryo development.

Intracytoplasmic morphologically selected sperm injection (IMSI) is another sperm selection technique which allows non-invasive selection of the spermatozoa with ×6000 magnification. At this magnification, spermatozoa with the most normal-like morphology without vacuoles can be selected (12). Recent studies reported that IMSI could improve the outcome of selection of sperm with low level DNA fragmentation and pregnancy (13). Also, men with the etiology of teratozoospermia seem to get the best outcome from this technique (14).

In previous studies, the efficacy of PICSI and MACS methods for sperm selection was evaluated and it was indicated that these techniques can be suitable for the selection of spermatozoa with low level of DNA damage in patients undergoing ICSI (15). The purpose of this study was to compare the efficacy of PICSI versus MACS in sperm selection for cases with unexplained infertility. The subjects had normal semen, and chromatin integrity and fine anatomical features of their spermatozoa were assessed.

Methods

Study design and population: A total of 20 semen samples specimens were collected from Iranian men referred to Andrology Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran from October 2019 to February 2020. Ethical approval was obtained from the ethics review board for clinical research (IR.TUMS.MEDICINE.REC. 1397.475). Informed consents were obtained from

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males who were admitted to the andrology laboratory for routine sperm analysis. The couples with female infertility were excluded, and men with unexplained infertility and below 35 years of age were enrolled in the study. Assessment of semen analysis was based on WHO criteria (16). The abstinence period was 2-7 days. The mean of sperm concentra-tion, progressive motility, and normal morphology was 45.3±7.8, 50.25±9.30, and 4.75±0.63, respectively. The design of study is illustrated in figure 1. Briefly, following liquefaction for 30 *min* at 37℃, one small aliquot of semen was served for evaluation of DFI. Then, sperm preparation was done using swim-up protocol according to WHO guidelines. The harvested suspension was aliquoted into several groups including swim-up, PICSI, and MACS. In the PICSI group, motile sperm bound to HA spot was only selected, but in swim- up and MACS groups, the motility was not 100%. In these groups, the suspension of sperm after swim-up and MACS was added into culture media in ICSI dishes. Then, motile sperm was only aspirated with an injection needle (about 100 spermatozoa) which resulted in the formation of two other groups, namely swim-up selection and MACS selection. Therefore, six groups for DNA status were evaluated including "semen", "swim-up" "MACS", "PICSI", "swim-up selection", and "MACS selection" to create a better match for comparison with PICSI group.

HA-mediated ICSI sperm selection using PICSI dishes: The methods of PICSI-mediated sperm selection are as follows; A PICSI dish (Origio, Denmark) with orienting lines, each ending with round HA-coated spot, was selected in order to facilitate sperm selection. Next, 5-10 *µl* of washing media (Ham's F10 with 10% human serum albumin) was added to each ring at 37℃ for 5-15 *min*, under sterile conditions and covered up with 3-4 *ml* of mineral oil (Irvine Scientific, USA). Thereafter, 1-5 *µl* of sperm suspension was prepared in sperm washing medium (Irvine Scientific, USA) and placed into the media drop around the HA-coated spot. Finally, the motile sperm cells, bound to HA spot, were aspirated using microinjection needle and discharged to agars for evaluation of DNA fragmentation by SCD staining (15).

Magnetic–activated cell sorting (MACS): MACS Separator Kit (Miltenyi Biotec, Germany) was used for elimination of apoptotic sperm First, sperm samples, previously prepared by swim-up,

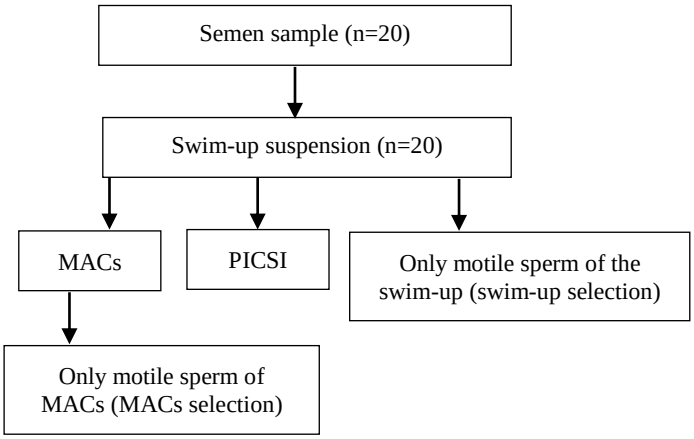

Figure 1. Flowchart of the design of study. MACS: Magnetic-activated cell sorting, PISCI: Physiological intracytoplasmic sperm injection

were added with 20 *µl* of annexin V microbeads and 1X binding buffer, mixed gently and incubated for 15 *min* at 24℃. Then, the samples were centrifuged at 300 *g* for 10 *min*, the supernatant was removed completely, and sperm pellet was resuspended in 500 *µl* of 1 X binding buffer. For MACS, a MiniMACS column (Miltenyi Biotec, Germany) was placed in the MACS separator. The column was prepared by washing with 500 *µl* of 1X binding buffer. The sperm suspension was added in 500 *µl* 1X binding buffer on the column. Then, the cell suspension was passed through the column drop by drop (15). The harvested suspension was evaluated for motility, DNA fragmentation, and motile sperm organelle morphology examination (MSOME).

Motile sperm organelle morphology examination (MSOME): For evaluation of fine morphology of spermatozoa, extracted from MACS, the motile spermatozoa were examined under digital inverted microscope using Nomarski optical system that increases magnification by X6600. In brief, 1 *µl* of sperm suspension was mixed with 5 *µl* of 8% PVP (Vitrolife, Sweden) which was placed on a sterile glass bottom dish (Willco wells BV, Netherlands). Images of the spermatozoa were captured with differential interference contrast (DIC) microscopy and Nikon Digital Sight DS-Ri1 Camera. The morphological evaluation was performed according to Cassuto–Barak classification. Spermatozoa were classified into three groups of high (Class I), medium (Class II), and low-quality (Class III) based on the head shape, presence of vacuoles, and the shape of the head base (17).

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Sperm DNA fragmentation: The samples were slowly mixed with pre-warmed agarose gel and 20 *µl* of this solution was placed on the precoated glass slide in swim-up and MACS groups. In other groups, 10 *µl* of heated low melt agarose was loaded on percolated glass slide immediately after sperm aspiration with injection needle; then, the selected spermatozoa were discharged. The glass slide was covered with coverslip and it was removed after 5 *min*. Next, the slides were incubated in a DNA unwinding solution as a denaturing solution. The acid solution is a soft DNA denaturant, which melts the DNA double helix when it contains massive DNA breakage. These DNA breaks behave as starting points of denaturation which subsequently move along the DNA helix.

If the sperm DNA molecule is massively broken, most of the genome will be denatured, whereas non-fragmented DNA will remain intact. Accordingly, chromatin nucleoids of sperm without fragmented DNA showed large halos of dispersed DNA. Conversely, when lysis was performed on sperm chromatin with massively broken DNA, which are susceptible to denaturation, dispersal of DNA in microgel was not observed or occurred only to a limited extent. Finally, after a brief wash and dehydration in increasing ethanol baths, the sperm chromatin was stained for visualization under bright-field microscopy. The spermatozoa were scored under the light microscope (×1000) and big or medium halos were considered as spermatozoa without DNA fragmentation while fragmented spermatozoa showed small or no halos (18).

Statistical analysis: Statistical analysis was performed using GraphPad Prism. The data were checked for normality of distribution by applying the D'Agostino-Pearson normality test. Statistical significance was assessed using one-way ANOVA (Tukey's post hoc), paired t-test or Wilcoxon test and $p<0.05$ was considered statistically significant. Data were expressed as mean ± standard deviation.

Results

DNA fragmentation index: Figure 2 demonstrates DFI values of each group measured after initial swim-up. As it is seen in the figure, DFI values in swim-up selection, PICSI, and MACS groups were found to be significantly reduced as compared with the initial swim-up group. On the other hand, the DFI values were similar between and among groups in which motile spermatozoa were processed and compared (Figure 1). In additional analyses, DFI reduction rate (the second DFI/the first DFI×100) between semen and swim-up preparations was calculated. Reduction rates of swim-up, swim-up selection, PICSI, and MACS groups were 28.9±14.93, 36.66±12.50, 58.04±9.76, and 70.70±10.39, respectively. Also, after swim-up, PICSI, and MACS were found to provide an additional DFI reduction of 36.57±15.52 and 58.20±13.02, respectively and these values were found to be significant ($p<0.0001$, one-way analysis of variance, Tukey's post hoc).

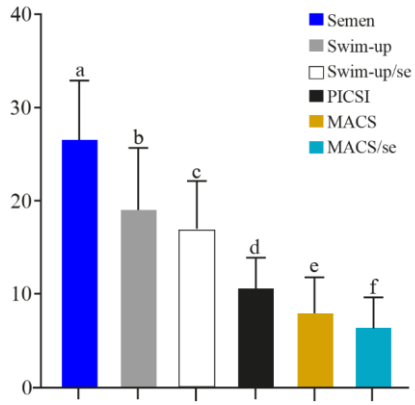

Figure 2. The DNA fragmentation index in different groups. a *vs.* b, c, d, e, f <0.0001, b *vs.* c=0.7, and e *vs.* f=0.9, b *vs.* d <0.0001, c *vs.* d=0.008, b and c *vs.* e <0.0001, d *vs.* e=0.5, d *vs.* f=0.1 (one-way analysis of variance, Tukey's post hoc)

The percentage of motile spermatozoa, bound to HA spot, was 22.00±22.38. In four samples, no spermatozoa were attached to hyaluronic acid. Therefore, 16 samples were included in PICSI group. There was no correlation between HBA and DFI in semen (p=0.8, r=-0.04), swim-up (p= 0.19, r=-0.30), and PICSI (p=0.4, r=-0.20) groups.

The motility rate of prepared sperm cells decreased after MACS selection (84.75±8.92 to 69.75±17.08, p=0.001). However, the percentage of normal morphology and fine normal morphology was similar. These data indicated that MACS method can improve DFI without improving the sperm parameters and quality (Table 1).

Correlation of DFI with other parameters: In this study, no correlation was found between DFI and motility and morphology in normal semen. The correlation between DFI of semen and swim-up suspension was significant ($p<0.001$ and r=0.73, CI: 0.43-0.88). In addition, there was a significant

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Table 1. Comparison of sperm quality before and after MACS

Sperm parameters	Swim-up	MACS	p-value
DFI [a]	19.00±6.77	8.00±3.92	p=0.0001
Progressive motility [b]	84.75±8.92	69.75±17.08	p=0.002
Normal morphology [a]	5.65±0.87	5.65±1.13	p=0.99
Sperm fine morphology (Class I) [a]	34.00±16.54	32.00±8.03	p=0.73

a: Paired t-test, b: Wilcoxon test, DFI: DNA fragmentation index, MACS: Magnetic–activated cell sorting

correlation between DFI of PICSI group with the DFI of semen (p=0.02 and r=0.57, CI:0.10-0.83) and swim-up suspension (p=0.01 and r=0.59, CI: 0.13-0.84). Furthermore, the correlation between the DFI of MACS group with the DFI of semen (p<0.001 and r=0.73, CI: 0.42-0.88) and swim-up suspension (p<0.001 and r=0.74, CI: 0.44-0.89) was significant.

Discussion

Intracytoplasmic sperm injection (ICSI) has been used to overcome sperm disorders in assisted reproductive technology (ART) programs, but overall live birth rate has not exceeded 30% (19). In these situations, the presence of intracellular and nuclear disorders, which cannot be estimated by semen analysis, may be one of the causes of male infertility (20). Detailed evaluation of embryo development can be one of the appropriate strategies to determine whether sperm DNA fragmentation can be considered as one of the biomarkers of sperm quality (21). Although studies indicated that there can be possible negative impact of sperm DNA fragmentation on embryological parameters and implantation potential (22, 23), others did not report such an impact of sperm DNA damage on early embryonic development and clinical pregnancy rate (24, 25). Recently, Ribas-Maynou et al. showed a negative but insignificant trend of implantation and live birth rates in samples with high DFI (26).

Both terminal uridine nick-end labeling (TUNEL) and sperm chromatin structure assay (SCSA) are nowadays considered to be the gold standards for identification of spermatozoa with DNA damage. However, being complex in nature, time consuming and expensive, these techniques cannot be routinely performed in all andrology laboratories worldwide. Sperm chromatin dispersion (SCD) test was another assay with similar sensitivity to allow widespread assessment of DNA damage using available equipment, such as bright-field microscopy (27).

In addition, the efficiency of sperm selection techniques is still debatable. In general, sperm selection in ICSI procedure is performed by assessing its morphology and viability under the microscope by an embryologist worldwide. So far, several techniques for better sperm selection have been introduced including IMSI, MACS, PICSI, and Zeta potential. Sperm selection with lower DFI is crucial in improving the outcome of ART and sperm selection by MACS and PICSI provides the possibility to choose the sperm with normal DNA status for ICSI program (28). So far, studies have compared each of these sperm selection techniques with raw semen sample; therefore, effectiveness of existing techniques in reducing the rate of DFI is still less discussed. The purpose of this study was evaluating the efficacy of MACS and PICSI techniques simultaneously on selection of spermatozoa with the lowest rate of DFI.

Our study first showed that the rate of DFI was comparatively decreased after swim-up. Swim-up alone could result in approximately 30% reduction in DFI as compared to raw semen sample. A similar study to ours also concluded that swim-up improved the rate of sperm DFI (29). Using swim-up, processed spermatozoa in PICSI and MACS group also indicated further significant improvements in the DFI reduction rate. Among the groups, our results showed the DFI reduction rate in PICSI group was about 60% and this reduction was found to be significantly different from swim-up alone and swim-up selection groups. Similar to our result, Parmegiani et al. reported the reduction rate of 68% after selection with PICSI. Although they reported better sperm morphology in PICSI selected group, there was not a significant im-

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provement in pregnancy and implantation rates (30). Miller et al. in a large cohort study concluded that PICSI did not improve the pregnancy and live birth and its wider utilization was not recommended (31).

Compared to swim-up group, DFI in the MACS group was found to be significantly decreased. The DFI of sperm after MACS sperm selection and PICSI sperm selection was found to be similar, but the reduction rate in MACS was found to be higher than PICSI. Lee et al. reported that MACS reduced DFI from 13.5 to about 10% (about 74%) in couples with unexplained infertility (32). Also, Bucar et al. reported 73.5% improvement in DFI than raw semen after MACS and swim-up. It is important to note that the same groups demonstrated a similar improvement by density gradient centrifugation (DGc) and swim-up (33). Their result was similar to our study.

During evaluation of sperm parameters after MACS sperm selection, it was found that motility was significantly decreased after using the technique. This may indicate a possible deleterious effect of MACS sperm selection on spermatozoa. On the other hand, the general morphology as well as fine morphology of spermatozoa were not different before and after MACS selection. In the case of PICSI, nearly all of the spermatozoa that were bound to HA had good morphology. Our data showed that MACS sperm selection can improve the DFI without improvement in sperm parameters. Lee et al. reported that MACS did not change the motility (32). However, similar to our study, Cakar et al. concluded that MACS significantly reduced both concentration and motility, ergo not suitable and practical for low sperm concentration. They also reported that although MACS improved the DFI, this reduction was not significantly different in comparison to swim-up and density gradient centrifugation (DGc) (34).

Horta et al. studied the effect of MACS in patients with normozoospermia and male factor infertility. There was not any significant improvement in terms of pregnancy, implantation, and miscarriage rates in their study. However, the major disadvantage of this study was that the SDF was not assessed in their research (35). Hasanen et al. in 2020 also compared the outcome of PICSI versus MACS for abnormal DFI in ICSI cases. They reported that laboratory as well as clinical data were similar, although they did not evaluate the DFI outcome after sperm processing in their study (15).

Dirican et al. reported that MACS method increased the embryo development and pregnancy rates in cases with oligoasthenoteratozoospermia. These cases had higher proportion of spermatozoa containing DNA breaks in which MACS could have eliminated the negative effects of abnormal SDF (36). Later, Delbes et al. used assays including hyaluronic acid binding assay, sperm chromatin structure assay (SCSA), chromomycine A3 staining, TUNEL, and comet assays to evaluate the sperm cells separated by MACS technique in all categories of semen samples. The data showed that MACS selected spermatozoa had good chromatin quality as measured by the TUNEL and SCSA assays (37). The reduction rate of DFI was high in MACS group when compared with PICSI group albeit without a significant difference in DFI between PICSI and MACS groups. In addition, the rates of reduction in both techniques seem to be dependent on DFI of semen. Therefore, patients with extremely high DFI may benefit less from these techniques. Similar to our conclusion, Esteves et al. after reviewing the literature concluded that none of the sperm selection techniques could completely eliminate the DNA fragmented spermatozoa (38). Lepine et al., in their meta-analysis, reported that it is still not clear whether advanced sperm selection strategies will likely improve the live birth rate. They recommended that more high quality studies are needed to come to final conclusion (39). The limitation of this study was the lack of clinical data from infertile men.

Conclusion

According to our results, the MACS technique is found to be more efficient than PICSI for sperm selection in ICSI setting. Although the data showed that both PICSI and MACS could not completely eliminate the sperm with fragmented DNA, MACS had more potential for DFI reduction rate. There are still debates on efficacy of these techniques in assisted reproduction programs.

Conflict of Interest

All authors have no conflicts of interest to declare.

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