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Conventional Magnification Suffices for High-quality Sperm Selection When Embryologist Experience Is Optimized: A Comparison with MSOME

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ABSTRACT

The ability to choose the appropriate sperm is essential to enhance the assisted reproductive technologies results. This study evaluated the performance of five embryologists in selecting sperm using conventional magnifications. In phase 1, sperm selection was done by an embryologist working on single sperm selection at ×400 magnification. Phase 2 involved 5 embryologists who selected sperm at ×200 and ×400 magnifications, twice for three semen samples, that their normal morphology confirmed at 2%, 4%, and 6%. Morphometric parameters with motile sperm organelle morphology examination (MSOME) and Sperm DNA fragmentation were assessed with sperm chromatin dispersion. The mean Cassuto score of selected sperm in phases 1 and 2 were 4.72± 0.94 and 4.02± 1.74, respectively. Data from phase 1 showed 95.3% and 1.7% placed in class I and no-class group (Sperm containing post acrosomal vacuoles). Morphometric evaluation of single sperm showed results of embryologist B was similar to phase 1 in terms of class I (100%) and no- class (0%) groups. Compared to raw semen, qualitatively single sperm selection by 5 embryologists significantly diminished sperm DNA fragmentation (16.73± 1.94 *vs* 22.26 ± 2.52, p< 0.00). It was comparable to previous phase (14.66± 3.21 *vs* 20 ± 3, p< 0.015). The sperm selection based at conventional magnification had lower DNA fragmentation than the raw semen samples (16.73± 1.94 *vs* 22.26± 2.52, p< 0.000). Standard magnification offers adequate resolution for embryologists to distinguish sperm with subtle morphological defects. In addition, ×400 magnification can lead to spermatozoa selection with high morphometric parameters and low DNA fragmentation. Furthermore, the findings highlight the critical role of embryologist training and experience as a practical approach to optimizing high-speed sperm selection with existing equipment.

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Introduction

Sperm morphology plays an important role in predicting pregnancy outcomes and live birth rate (Zhang *et al.*, 2021). Among the morphologic parameters of the sperm head, large nuclear vacuole (LNV), aberrant head ellipticity, angularity, and circularity have the strongest correlation with lower DNA integrity (Wang *et al.*, 2019; Villani *et al.*, 2022; Utsuno *et al.*, 2013). Therefore, efforts to improve morphology assessment can lead to sperm selection with lower DNA fragmentation. Intracytoplasmic sperm

injection (ICSI) is one of the assisted reproductive technologies (ART) that overcomes infertility by choosing spermatozoa at conventional magnification (×200 or ×400) using an inverted microscope. In this way, the priority is generally given to morphologically normal motile spermatozoa with vacuole-free heads or those with small vacuoles (WHO, 2021). Efforts to improve the morphological evaluation led to the development of the motile sperm organelle morphology examination (MSOME). MSOME with a resolution of 0.32 µm and ultra-high magnification provides a three-dimensional view to select better sperm cells with respect to morphological parameters (Lukaszuk *et al.*, 2022; Cassuto *et al.*, 2009). Huang *et al.* claimed that well-trained embryologists have the same ability in selecting spermatozoa with intact DNA in conventional sperm selection as natural sperm selection by hyaluronic acid binding method (Huang *et al.*, 2015). A trained embryologist should be able to select proper spermatozoa to improve clinical outcomes in ART (Shapiro *et al.*, 2023; Racowsky *et al.*, 2023). In order to maintain global ART success, there is a need to evaluate embryologists' performance in the clinical setting. In this study, we will evaluate the morphometric criteria of sperm selected by different embryologists at conventional magnifications of ×200 and ×400 and compare them with the criteria obtained with MSOME. The aim of this study was to evaluate the agreement between sperm selection performed by trained embryologists using conventional microscopy and the results obtained using the more advanced and higher-resolution MSOME technique.

Materials and Methods

Study design

This study was conducted from September to December 2023 in two phases. It was approved by the Ethics Committee of Yazd University of Medical Sciences (IR.SSU.MEDICINE.REC.1400.308). Written consent was obtained from infertile couples referred to Yazd Infertility Center, and the surplus semen specimens were used. 1,380 sperm cells from 9 patients were evaluated based on the WHO qualitative criteria (WHO, 2021), by an embryologist trained for the MSOME technique. The required sample size was estimated as 461 sperms considering a test power of 90%, confidence interval of 95%, and type- 1 error of 5%. The sample size was calculated considering previous study (Zhang *et al.*, 2021), and 100- 150 sperm cells were evaluated from each prepared sample at ×400 magnification. Next, the morphometric parameters of selected spermatozoa and the presence and location of vacuoles were assessed by MSOME. Phase 2 was done to assess the performance of 5 embryologists (codes A-E) in sperm selection. A sample from each of 6 patients and 40 spermatozoa per patient was selected for ICSI (Fig. 1).

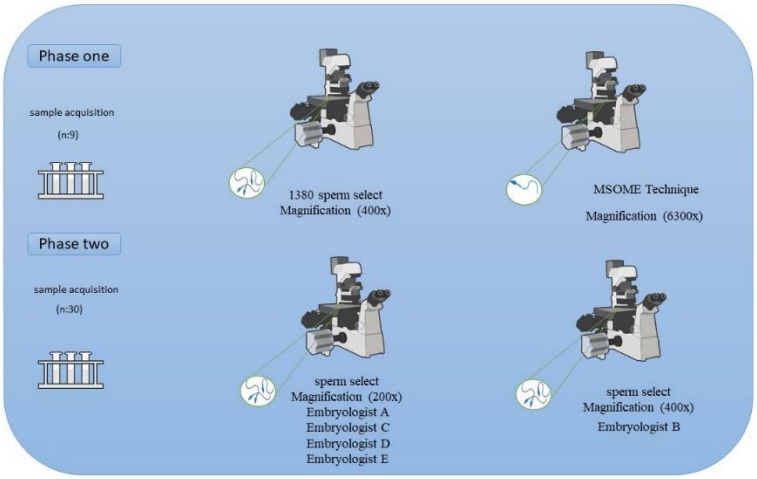

Fig. 1. Overview of the study phases. Briefly, in Phase 1, sperm selection was performed by a single embryologist who carried out single-sperm selection under magnifications of ×400 and ×6300 (MSOME). In Phase 2, five embryologists participated in the study and selected spermatozoa under conventional magnifications of ×200 and ×400.

Preparation of semen sample

Semen samples from patients selected for the ICSI protocol were obtained by masturbation after 2 to 7 days of sexual abstinence. After liquefaction, semen analysis was performed according to the WHO guidelines 2021(WHO, 2021). The motile sperm fraction was extracted by density gradient centrifugation using a 1 mL semen sample overlaid on top of 2ml bilayer solution of pure sperm 40% and 80%, centrifuged at 300 g for 15 min. The pellet was washed with 1ml human tubal fluid (HTF) medium (Irvine Scientific) to collect pure spermatozoa. The final pellet was resuspended with 500µl medium.

Sperm parameters

Phase 1 was conducted on the morphologically normal sperm selected from samples with morphologies confirmed at 2%, 4% and 6%. This test was performed by the same embryologist who evaluated sperm parameters in phases 1 and 2. Morphologically normal spermatozoa were selected under routine ICSI conditions at ×400 magnification to be injected into oocytes (WHO, 2021). Intact spermatozoa with a regularly contoured oval head containing 40% - 70% of the acrosomal region, a regularly slender midpiece followed by a thin tail, were considered normal (WHO, 2021). Two oval-shaped droplets containing polyvinylpyrrolidone (PVP) 7% (Irvine Scientific, USA, 90121) were placed in an ICSI dish, covered with 1.5 ml mineral oil to prevent evaporation. Then, 1µl of the prepared sample was loaded in 5µl droplet of PVP. The dish was placed on the heating plate (37˚C) of an inverted microscope. The selected sperm were transferred to 1µl droplet of SynVitro Flush placed in a glass bottom dish (GWSt 1000; Will Co.) designed to evaluate morphometric parameters by MSOME. The selected spermatozoa were transferred to the PVP one by one, and an image of each sperm was captured. Then, sperm cells were scored by Cassuto's scoring system (Cassuto *et al.,* 2009). In this system, several major and minor morphological characteristics of spermatozoa are assessed. Briefly, the major criteria include sperm head morphology (shape and size), the presence and size of vacuoles, and the morphology of the sperm head base. The minor criteria include evaluation of the acrosome, tail insertion, and the presence of cytoplasmic droplets. Based on this scoring system, spermatozoa are classified into three categories. Class I includes spermatozoa with a normal head and a maximum of two additional abnormalities that are not located in the head region. Class II includes spermatozoa with a normal head but presenting more than two abnormalities. Class III includes spermatozoa that exhibit abnormalities in the sperm head, regardless of the presence of other defects (Cassuto *et al.,* 2009). They were also evaluated in terms of morphometric parameters, including length and width, number of vacuoles and largest diameter of vacuoles, angularity and circularity of the head using MSOME. Furthermore, the sperm DNA integrity was evaluated by sperm chromatin dispersion (SCD) assay.

In phase 2, after dish preparation, 40 morphologically normal sperm cells were selected from two droplets at conventional magnification, and 20 sperms were transferred into a glass-bottom dish to evaluate morphometric parameters at 63,000× magnification. Also, 20 spermatozoa were collected for assessment of DNA fragmentation. Sperm selection was performed twice for three semen samples, with their normal morphologies confirmed at 2%, 4%, and 6%. In this way, each embryologist performed sperm selection six times.

For sperm morphology parameters, sperms selected at ×200 or ×400 magnifications. Fresh motile spermatozoa were used to evaluate morphometric parameters by MSOME (Cassuto *et al.,* 2009). Each spermatozoon was scored between 0-6 according to: (2× Head) + (3× Vacuole) + (1× Base). Each spermatozoon with 4-6 score, 1-2, and 0 were categorized as Class I, II, and III, respectively. Therefore, top-quality spermatozoa with normal base, absence of acrosomal vacuole, and regularly contoured head with no extrusion or invagination of nuclear membrane received a score of 6, and the poorest-quality spermatozoa scored 0 (Cassuto *et al.*, 2009). The selected sperm were transferred to 5 µl droplets of PVP one by one. For sperm head evaluation, digital images of each sperm were captured at ×6300 magnification using an inverted microscope equipped with high-power Nomarski differential interference contrast (DIC)

optics (Nikon ECLIPSE TE300S) (Fig. 2).

In addition to the sperm length and width, the longest diameter of the vacuole, lateral symmetry, and circularity of the sperm head, ellipticity (length/ width ratio), and angularity of each spermatozoon were evaluated. Sperm length was defined as the distance between the junction of the midpiece and base and the tip of the sperm head. Sperm width indicated the longest distance between two lateral parts of the sperm head. This distance corresponds to the small diameter of an oval sperm head (Fig. 2)

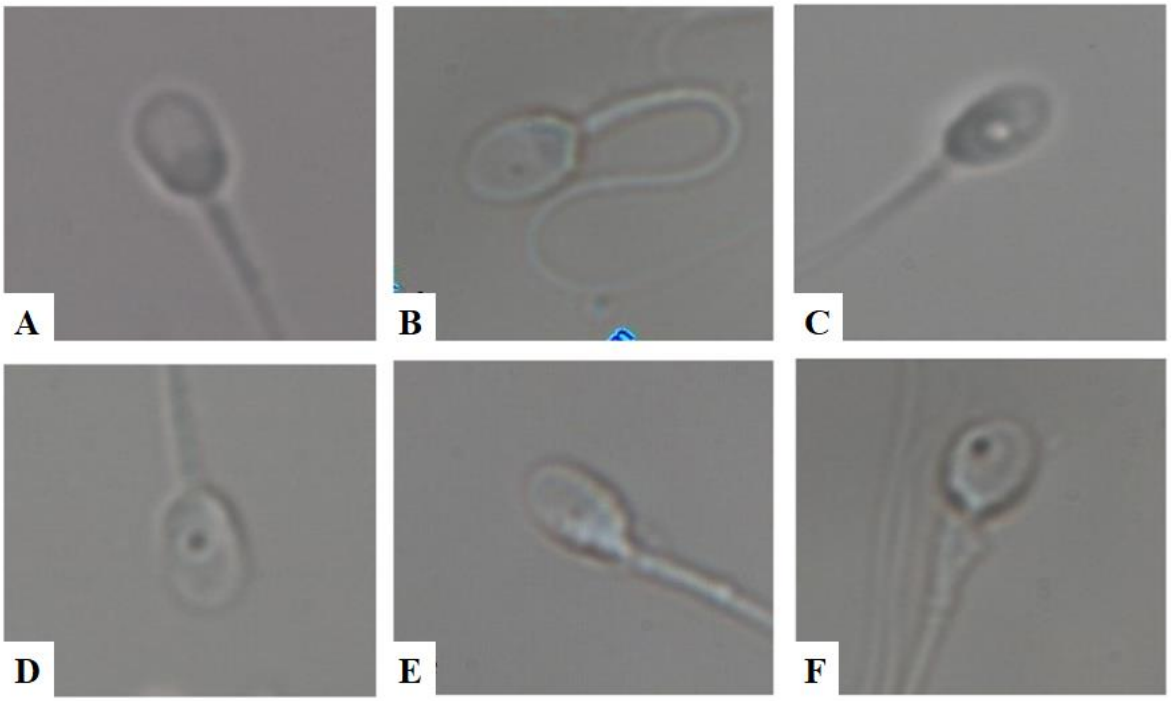

Fig. 2. Assessment of sperm fine morphology using MSOME technology: A) Normal spermatozoa; B) Angularity in base; C) Post acrosomal vacuole; D) Vacuole in equatorial segment; E) Midpiece is not aligned with major axis of sperm head; F) Tapering midpiece.

Sperm DNA fragmentation

Sperm DNA fragmentation was assessed by SCD test using Halo sperm or SDFA kit (IVFCO, Iran, 82130). 50 µl of sample was mixed with pre-warmed agarose gel. Then, 20 µl of suspension was placed on a pre-coated glass slide, covered by a 22 × 22 coverslip, and kept at 4˚c for 5 min. After that, the coverslip was removed, and the glass slide was submerged in solutions A and B for 7 and 15 min, respectively. After washing the glass slide with distilled water for 2 min, the slide was dehydrated by increasing the concentration of Ethanol and airdried. Next, the slide was stained with solutions C, D, and E for 4, 3, and 2 min, respectively. Finally, each spermatozoon was scored by light microscopy (×1000). For the prepared sample, a total of 200 sperm were scored. Spermatozoa with large- or medium-halo were considered without DNA fragmentation. The presence of no halo was scored as fragmented DNA sperm. DNA fragmentation Index (DFI) was presented as the percentage of sperms with DNA fragmentation (Tandara *et al.*, 2014).

Statistical analysis

The results are presented as numbers and percentages. The statistical analysis of the data was performed in SPSS software version 20. Chi-square and Exact test were utilized for the comparative evaluation of the morphometric parameters of selected sperms. The embryologists were compared with each other using the one-way analysis of variance (ANOVA). Tukey post-test was used to resolve the differences. $p< 0.05$ denoted statistically significant. Figure 3 was illustrated using GraphPad Prism 10.1.2.

Results

Sperm parameters evaluation

A total of 1.380 sperms from nine prepared samples at ×400 magnification (three prepared samples for each morphology Index) were evaluated according to the WHO guidelines (WHO, 2021). Morphological characterization of each isolated sperm was evaluated according to the scoring scale proposed by Cassuto (Table 1). In summary, Table 1 relates to phase one of the study. As mentioned, in this phase, a trained embryologist selected sperms at 400x magnification, and then the selected sperms were evaluated at 6000x magnification. The results showed that in samples with grade 2 morphology, 88.2% of the selected sperms were class 1, in samples with grade 4 morphology, 95.4% of the selected sperms were class 4, and in samples with grade 6 morphology, 99.6% of the selected sperms were Class I (Cassuto *et al.*, 2009). The ellipticity of the selected spermatozoa was also evaluated at 400x magnification and at 6000x magnification according to the Kasuto classification. The results showed that most of the selected spermatozoa in all morphological grades (2%, 4% and 6%) had ellipsoids in the normal range of 1.3 to 1.8 (78.2%, 82.8% and 83.2%, respectively), and there was no significant distribution difference between the three morphological groups (Table 2).

Table 1. The classes of selected sperms between different morphologies according to Cassuto scoring system.

Normal morphology	Class I	Class II	No class	P- value
2%	280 (88.6)	23 (7.3)	13 (4.1)	0.000 *
4%	551 (95.8)	17 (3)	7 (1.2)	
6%	484 (99.0)	1 (0.2)	4 (0.8)	
Total	1315 (95.3)	41 (3)	24 (1.7)	

*Based on Chi-square test; p< 0.05; Value inside parenthesis is (%).

The comparative evaluation of vacuole features between different morphologies showed that as the morphology index increased, the rate of spermatozoa without vacuoles also increased; 39.2%, 69.4% and 73.4% in morphology 2%, 4% and 6%, respectively. The sperms with post-acrosomal vacuoles were categorized as a no-class group. 1.74% (n=4) of the selected sperms had deep post-acrosomal vacuoles. The mean score of selected spermatozoa in three morphologies, 2%, 4% and 6%, were 4.35±1.18, 4.75± 0.85 and 4.94± 0.79, respectively.

Table 2. Comparison of the ellipticity (ratio of sperm head length to width) of selected sperms between different morphologies according to WHO (2021).

Morphology	1.3 >	1.3-1.8	1.8 <	P-value
2%	34 (10.8)	247 (78.2)	35 (11.1)	0.042*
4%	64 (11.1)	476 (82.8)	35 (6.1)	
6%	54 (11)	407 (83.2)	28 (5.7)	
Total	152 (11)	1130 (81.9)	98 (7.1)	

*Based on Chi-square test; P-value < 0.05; Value inside parenthesis is (%).

In the second phase of this study, several embryologists (A-E) were asked to independently select sperms with morphology grades of 2%, 4%, and 6% under conventional magnification. Embryologist B achieved 100% class I sperm in all morphology grades (magnification 400 and work experience 12-20 years). In contrast, embryologist D selected only 47.2% class I sperm, and 25% of the selected sperms were in the unclassified category. Also, the sperms selected by embryologists C (55.8% class I) and E (64.3% class I) were significantly lower than those of embryologists A (83.8% class I) and B (Table 3). However, overall, the average number of sperm selected by different embryologists at conventional magnification was approximately 70%, and the average number of total sperm in the non-class group was 10.2% (Table 3). Statistical analysis confirmed highly significant differences among embryologists across all morphology grades (p= 0.000 for 2% and 4%, p= 0.001 for 6%, and p= 0.000 for total). Results showed that embryologists with more experience (12 to 20 years) had significantly higher mean Cassuto scores across all morphology grades (2%, 4%, and 6%) compared with less experienced embryologists (4 to 5 years). The most pronounced differences were observed in the 4% and 6% morphology groups, where experienced embryologists scored approximately 5.2- 5.3 versus 3.2- 3.7 for less experienced operators (p= 0.000 for all comparisons) (Tables 4-5).

Table 3. Comparison of the class of selected spermatozoa between different embryologists according to Cassuto scoring system.

Embryologist	Morph 2%			Morph 4%			Morph 6%			Total		
	Class I	Class II	No class	Class I	Class II	No class	Class I	Class II	No class	Class I	Class II	No class
A	18	4	4	20	0	2	24	0	2	62 (83.8)	4 (5.4)	8 (10.8)
B	30	0	0	22	0	0	24	0	0	76 (100)	0 0	0 0
C	16	6	4	8	16	4	24	6	2	48 (55.8)	28 (32.6)	10 (11.6)
D	10	2	8	10	10	6	14	8	4	34 (47.2)	20 (27.8)	18 (25)
E	18	12	4	20	6	0	16	8	0	54 (64.3)	26 (31)	4 (4.8)
P-value		0.000 *			0.000 *			0.001 *			0.000 *	

*Exact test; P-value < 0.05; non-class: sperm with post-acrosomal vacuoles; Value inside parenthesis is (%).

Table 4. Comparative evaluation of the selected sperm scores in different morphologies between the embryologists.

Embryologists	Score		
	2% Mean ± SD	4% Mean ± SD	6% Mean ± SD
A	3.92 ± 1.58	4.81 ± 1.73	4.92 ± 1.52
B	5.00 ± 0.98	5.72 ± 0.47	5.75 ± 0.44
C	3.38 ± 1.62	2.64 ± 1.47	3.87 ± 1.38
D	2.70 ± 2.38	2.92 ± 1.89	3.15 ± 1.68
E	3.58 ± 1.70	4.15 ± 0.88	4.16 ± 0.91
P-value	0.000 *	0.000 *	0.000 *

* Statistical analysis was performed based on one-way ANOVA; p< 0.05

Table 5. Comparative evaluation of the score of selected spermatozoa according to embryologists work experiences in different morphologies.

Work Experience (Year)	Score		
	2% Mean ± SD	4% Mean ± SD	6% Mean ± SD
4-5 (C, D and E)	3.30 ± 1.88	3.22 ± 1.59	3.73 ± 1.42
12-20 (A and B)	4.50 ±1.53	5.27 ± 1.33	5.32 ± 1.20
P-value	0.000 *	0.000 *	0.000 *

*Statistical analysis based on one-way ANOVA; p< 0.05

Sperm DNA fragmentation

The results of the first phase demonstrated that compared to raw semen, qualitatively single sperm selection had significantly lower DNA fragmentation (20 ±3 *vs* 14.66± 3.21, p< 0.01). In phase 2, DFI was recorded between selected sperms and the sample population of raw semen (Fig.3). The results showed that sperm selection based on conventional magnification had lower DNA fragmentation than the raw semen samples (16.73± 1.94 *vs* 22.26 ±2.52, p< 0.000). The findings also showed that the reduction rate of DNA fragmentation of the selected sperm was 24.85%, similar to data obtained in the previous part of the experiment (26.7%). Insignificant differences were noticed between the DFI of single sperm selection carried out by the 5 embryologists (p= 0.09).

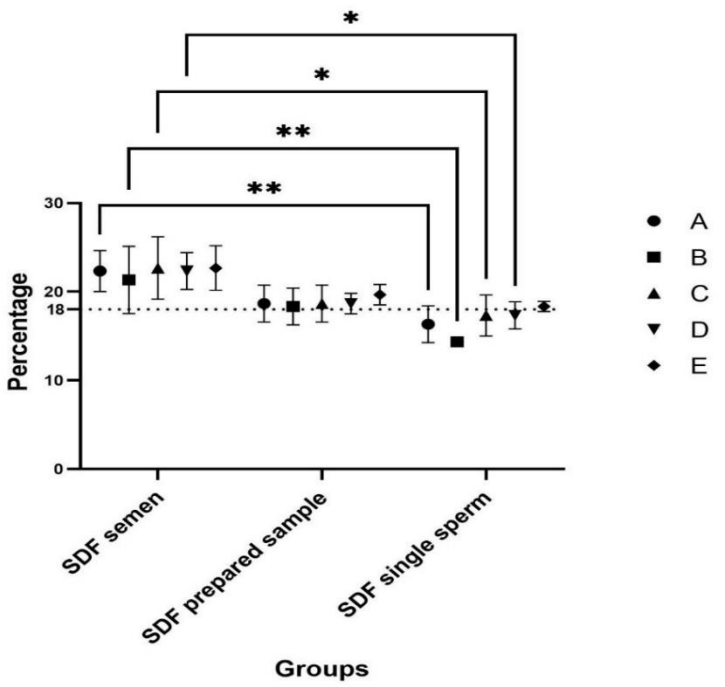

Fig. 3. Comparative evaluation of SDF between selected sperm and raw semen. *p< 0.05, **p< 0.01.

Discussion

Sperm selection with good morphology and low DNA fragmentation is an important challenge for embryologists. Therefore, considering the embryologist's performance in sperm selection can lead to identification of their capability in ART. Providing some approaches to address their general skill in sperm selection/ injection can improve the results of ART outcomes. The data in phase 1 showed that ×400 magnification offers enough resolution to deselect spermatozoa with medium to large size vacuoles. Also, the ICSI man can omit spermatozoa in class III at ×400 magnification during sperm selection.

Our findings showed that WHO criteria sperm selection can assist embryologists in selecting good-quality spermatozoa. Two embryologists (A and B) selected sperm with high scores in the morphometric parameters with low DNA fragmentation within an acceptable time frame. 10% of all selected sperms that was chosen by embryologist A contained a nuclear vacuole. In addition to the embryologist's skill in sperm selection, a recent study showed that optical resolution of 0.5 µm allows embryologists to get a satisfactory visualization of spermatozoa. Therefore, compared to an optical resolution of 0.64 µm for ×20 magnification, optical resolution of 0.41µm for ×40 objectives offers adequate resolution to distinguish subtle defects in sperm head as well as DNA integrity (Lukaszuk *et al.*, 2022).

In semen analysis, the morphology index is reported for raw semen, rather than for the prepared samples transferred to the PVP medium (WHO, 2021). Both the present study and Zhang's (2021) study showed that the optical resolution of the standard ICSI system enables well-trained embryologists to detect detailed information about the selected spermatozoa (Zhang *et al.*,2021). Thus, spermatozoa with large acrosomal and post-acrosomal vacuoles can be ignored in ICSI setting (Zhang *et al.*, 2021; Lukaszuk *et al.*, 2022). Compared to the semen samples, DFI was clearly diminished in the selected spermatozoa in both studies (Zhang *et al.*, 2021). Therefore, taking enough time to choose high-quality spermatozoa with suitable morphology can lead to selection of spermatozoa with low DNA fragmentation (Zhang *et al.*, 2021; Utsuno *et al*., 2013; Jakubik-Uljasz *et al.*, 2020).

Although our results were in accordance with Zhang's (Zhang *et al.*, 2021), the rate of reduction in DFI was 25% in ours, compared to 46.1% in their study. The comparative assessment of DNA

fragmentation in raw semen between the two studies demonstrated that mean DFI was nearly three times higher in ours than in Zhang's study. Also, Avendano *et al,* showed that morphologically normal sperms in the motile sperm fraction of infertile men had high DNA fragmentation (Avendano *et al.,* 2009). The angularity of the sperm head is considered an abnormality that strongly correlates with lower DNA integrity (Utsuno *et al.,* 2013). In the best-case scenario, 50% of all sperms chosen by embryologists A and B were without angularity in the base and had regular oval heads. Therefore, detailed attention to the sperm base could lead to improvements in the nuclear quality of the selected sperm, especially for embryologist B (Fig. 1).

Due to the presence of a centriole in the midpiece, it is important to pay attention to the structure of the midpiece during sperm selection. Proper centrosomal function of the sperm is needed for aster and pronucleus formation (Ugajin *et al.,* 2010). Two abnormalities of the midpiece were observed among the sperm selected by embryologists C, D, and E. They noticed sperm cells with a tapering midpiece and with midpieces not aligned with the major axis of the sperm head (Fig.2). Considering that there were no sperm with these two abnormalities among the sperms selected by embryologists A and B, this precision indicates the higher skill and experience of them. This precision was observed in the performance of the embryologist who selected the sperms in phase 1. Another structural defect is the presence of vacuoles characterized as nuclear concavities of the sperm head covered by acrosomal and plasma membranes (Boitrelle *et al*., 2011). This structure is linked to defective chromatin condensation and double-strand DNA breaks (Boitrelle *et al*., 2011; Pastuszek *et al*., 2017). Especially, deep vacuoles and the ones located in the nucleus compartment or equatorial segment are characterized by higher variation in genomic copy number than non-vacuolated spermatozoa (Berkovitz *et al*.,2018). Therefore, omitting spermatozoa with post- acrosomal vacuoles is a major challenge for achieving higher fertilization and pregnancy outcomes.

In phase 2, without considering acrosomal vacuoles, 10% of the total selected sperms contained post- acrosomal vacuoles, while only 1.74% of selected spermatozoa showed nuclear vacuoles in phase 1. In the case of embryologist D, this figure reached close to 45% of the selected sperms in morphology 2%, and 25% of the all-selected sperms in three morphologies. Compared to embryologist B who chose the sperms at ×400 magnification, sperm selection was performed at ×200 magnification by embryologist D.

Therefore, the findings showed the embryologist B selected spermatozoa with low DFI by deselection of spermatozoa with large vacuoles, nuclear vacuole, and abnormal features of midpiece. Compared to the other embryologists, the higher quality of the selected spermatozoa by embryologist B can be attributed to sperm selection at ×400 magnification that provides adequate resolution. The majority of embryologists selected spermatozoa at ×200 magnification, especially in the group with morphology 2%. Although several studies suggested that the non-invasive MSOME technique can play a unique role for male factor cases (Mangoli *et al.,* 2020; Goswami *et al.,* 2018), proper utilization of this high technology can assist us in morphometrical data. Considering the relatively limited scale of our study, these findings should be interpreted with caution, and further research with larger sample sizes is warranted to confirm and expand upon the present results.

Conclusion

Sperm selection at ×400 magnification can offer adequate resolution for embryologists to distinguish spermatozoa with proper morphology and DNA integrity. Embryologists can benefit from ×400 magnification by deselecting spermatozoa with subtle defects, especially large vacuoles and abnormal base and midpiece. Periodic assessment of embryologist performance and adequate knowledge of equipment capabilities are vital to increase success in ART.

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Ethical approval

This study was approved by the Ethics Committee of Yazd University of Medical Sciences (IR.SSU.MEDICINE.REC.1400.308).

Data availability

The data are available on request.

Conflict of interests

The authors declare no competing interests.

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