Journal of Assisted Reproduction and Genetics
https://doi.org/10.1007/s10815-020-01910-7

REPRODUCTIVE PHYSIOLOGY AND DISEASE

Association between early embryo morphokinetics plus transcript levels of sperm apoptotic genes and clinical outcomes in IMSI and ICSI cycles of male factor patients

Esmat Mangoli[1] · Mohammad Ali Khalili[1] · Ali Reza Talebi[1] · Seyed Mehdi Kalantar[2] · Fatemeh Montazeri[2] · Azam Agharahimi[1] · Bryan J Woodward[3]

Received: 2 June 2020 / Accepted: 30 July 2020

Abstract

Purpose The aim was to assess the correlation of sperm apoptotic transcript levels with cleavage stage embryokinetic and pregnancy outcomes of intracytoplasmic morphologically selected sperm injection (IMSI) and ICSI methods in patients with male factor infertility.

Material and methods Eighty male factor cases were divided into ICSI and IMSI groups. ICSI was done routinely, and for IMSI, sperm was selected at high magnification and injected. On day 3, time-lapse parameters were evaluated, and the best embryos were transferred and followed to delivery. In addition, sperm DNA fragmentation and apoptotic transcript levels were quantified using reverse transcription Q-PCR between the groups.

Results IMSI selected spermatozoa had lower DNA fragmentation and apoptotic transcript levels compared with ICSI ($p < 0.0001$). Moreover, all cytokinetic variables and cleavage abnormalities were noticeably different between groups ($p < 0.0001$); the rates of clinical outcomes were higher in the IMSI group. The transcript levels of Caspase 3 showed a moderate negative correlation with s2 and s3 (rs = − 0.57, $P = 0.008$ and rs = − 0.51, $p = 0.021$, respectively) in the IMSI group. However, there was no relationship between sperm apoptotic transcript levels and clinical outcomes in two groups.

Conclusions Sperms selected at high magnification showed lower DNA fragmentation and apoptosis genes transcript. Also, better embryo kinetics and clinical outcomes were confirmed in IMSI than ICSI groups. Some time-lapse parameters may be associated with transcript levels of apoptosis genes. Therefore, these noninvasive techniques may be unique in assisting couples with male factor infertility.

Trial registration This trial retrospectively registered on 4 July 2020 (IRCT20180130038561N1).

Keywords ICSI · IMSI · MSOME · Time-lapse monitoring · Apoptosis · Transcript levels

Introduction

Intracytoplasmic sperm injection (ICSI) is commonly used for the treatment of male factor infertility or repeated fertilization failure after conventional IVF [1]. Optimally, sperm cells with high chance of fertilization and subsequent embryo growth would be used for assisted reproductive technology (ART). These spermatozoa would be viable, mature, with high DNA integrity, and structurally competent. In ICSI, routine sperm selection is based on motility and gross morphology at low magnification. Advanced sperm selection techniques might enables further selection of the appropriate sperm for use in ART [2]. Motile sperm organelle morphology examination (MSOME) is a noninvasive method for specific evaluation of motile spermatozoa. MSOME with the use of the Nomarski differential interference contrast (DIC) optics at > 6000× allows for, a better three-dimensional view of the sperm head and selection for intracytoplasmic morphologically selected sperm injection (IMSI) [3]. Selection criteria include the normalcy of the head, neck, and midpiece [3, 4].

✉ Mohammad Ali Khalili
khalili59@hotmail.com

[1] Department of Reproductive Biology, Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

[2] Abortion Research Center, Yazd Reproductive Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

[3] X&Y Fertility, Leicester, UK

Published online: 07 August 2020

Embryo selection is another challenge in the ART program. Despite different methods for embryo selection, such as metabolic profiling, O2 respiration, aneuploidy screening, and gene expression analysis, selection based on morphology is still an approved method [5, 6]. Assessment of the embryo quality at different stages may help to determine the embryos with the best potential for implantation. Embryo selection using time-lapse imaging (TLI) involves numerous different cellular events and dynamic parameters, the time of cell divisions and the time between cell divisions. Also, normal cleavage pattern and existence of cleavage abnormality of embryos are assessed in details [7]. Several recent studies have addressed the potential of TLI in clinical selection of competent embryos [8–10].

Evaluation of sperm DNA integrity, in addition to routine sperm parameters, could add further information on the quality of spermatozoa. Clinical evidences now point to sperm DNA damage as a detrimental factor to reproductive outcomes, with spermatozoa of infertile men having more DNA damage than fertile men [11–13]. Apoptosis is an essential process in both spermatogenesis and spermiogenesis and regulated with two families of genes including *bcl2* and *caspase*. The *Bcl2* family consists of antiapoptotic factors (*bcl2*, *bcl-xl*) and proapoptotic factors (*Bax*, *Bid*). The Caspase family is present in the cell as a group of inactive proenzymes that become active at the apoptosis process leading to destruction of proteins like poly (ADP-ribose) polymerase (PARP), lamins, and histone [14–16].

The ART procedures bypass the natural selection process, subsequently increasing the chance of spermatozoa with abnormal genomic material fertilizing a mature oocyte. To our knowledge, there has been no report on the apoptotic gene pattern of spermatozoa that are selected for ICSI and IMSI. In this study, for the first time, we aimed to compare the DNA fragmentation and apoptosis transcript levels of spermatozoa selected for ICSI and IMSI. In addition, the embryo kinetics with TLI and clinical outcomes were compared in couples with male factor infertility.

Materials and methods

Patient selection

This prospective randomized trial was performed between May 2016 to May 2018, at the Research and Clinical Center for Infertility in Yazd. This study was approved by the Ethics Committee of our institute (IR.SSU.MEDICINE.REC.1394.338) and retrospectively registered in the Iranian Registry of Clinical Trials (IRCT20180130038561N1). All patients provided informed consent. For this study, 80 couples with male factor infertility undergoing ICSI treatment with antagonist protocol for control ovarian hyperstimulation (COH) (female mean age 30.3 ± 3.5 and male mean age 35.7 ± 4.7) participated. Patients randomly were divided into two groups of IMSI and ICSI (40 cases in each group). Randomization was performed using computer-created random numbers. The patients, embryologists, and physicians were not blinded to the allocated treatment groups. The inclusion criteria were male factor infertility (asthenozoospermia, asthenoteratozoospermia, teratozoospermia), at least 3 years of primary infertility, healthy female without fertility problems, 38 years or younger with a basal FSH < 10 IU/ml, BMI between 25 and 30, and with the minimum of six mature oocytes. To achieve the maximum number of spermatozoa in the sperm selection process, patients with nonejaculated spermatozoa, oligoasthenoteratozoospermia (OAT), and ejaculates below 1×10^6 spermatozoa/ml were excluded. Also, women with > 2500 pg/mL estradiol levels at the time of triggering, the cases with no embryos for transfer, and total freeze cycles were excluded (Fig. 1).

Semen analysis and preparation

Semen was obtained by masturbation after 3–5 days of sexual abstinence. In liquefied semen, classical parameters of sperm concentration, motility, morphology, and vitality were assessed in 200 spermatozoa for each sample in accordance with the World Health Organization guideline [17]. Sperm count and motility were evaluated via the Makler chamber on light microscopy (Olympus Co. Tokyo, Japan). The viability and morphology were assessed by Eosin and Diff-Quick staining, respectively.

Sperm preparation was performed on two-layer concentration gradients [17]. Briefly, liquefied semen was gently layered on the top of the 80% and 40% PureSperm® (NidaCon Laboratories AB, Gothenburg, Sweden) layers with the help of a sterile glass Pasteur pipette and centrifuged at 300*g* for 10 min. The resulting sperm pellet was diluted with 1-ml Hams F10 supplemented with 5 mg/ml albumin, mixed well, and centrifuged at 300*g* for 5 min. Sperm pellet was once again resuspended with 1-ml medium and centrifuged again at 300*g* for 5 min. The final sperm preparation was suspended in 500 μl of the medium.

Controlled ovarian hyper stimulation

The ovarian stimulation was similar to our previous study [18]. Stimulation protocol was started with recombinant human follicle-stimulating hormone (Gonal-F, Serono, Geneva, Switzerland 150–225 IU, subcutaneously) for 5 days. When the mature follicle (≥ 14 mm) was confirmed with transvaginal sonography, GnRH antagonist (Cetrotide) (Merck, Serono, Germany, 0.25 mg/daily, subcutaneously) was injected. Then, follicular growth was checked by transvaginal

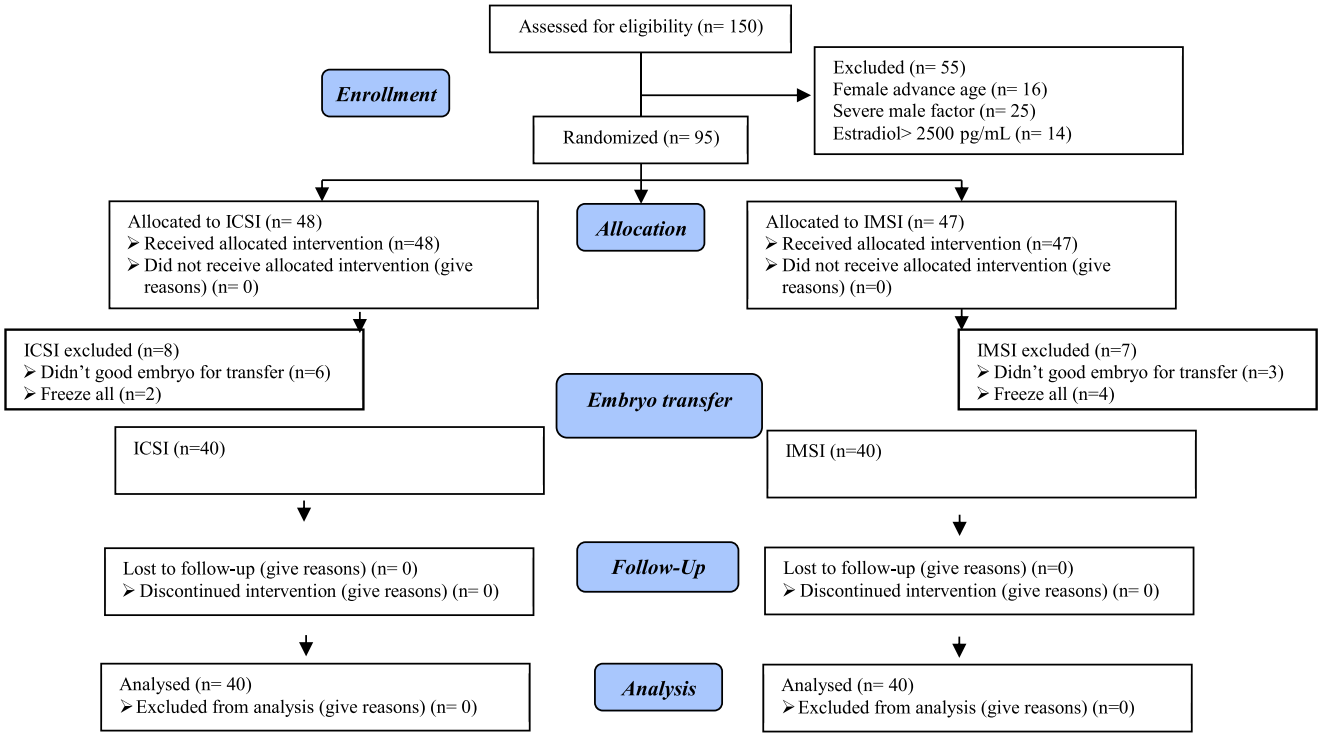

Fig. 1 Flowchart of participants' allocation, treatment, follow-up, and analysis

ultrasound and 10,000 IU of human chorionic gonadotrophin (HCG) (Pregnyl®; Organon, Oss, the Netherlands) was injected when at least three follicles reached a diameter of ≥ 17 mm. Ovum pickup was performed using ultrasound guidance 36 h after the trigger injection.

Laboratory procedure

After aspiration, cumulus-oocyte complexes (COCs) were incubated in culture medium (G-IVF; Vitrolife, Kungsbacka, Sweden) covered with oil (Ovoil; Vitrolife, Sweden) at 37 °C and 6% CO2 for 3 h. Following oocyte denudation, whether ICSI or IMSI was performed. ICSI was performed using prepared spermatozoa. The motile spermatozoa with normal morphology (normal head without cytoplasmic droplets or tail defects) were selected at × 400 magnification and then injected into mature oocytes [19, 20].

In the IMSI group, sperm selection was performed as described before [19, 21]. Briefly, spermatozoa were loaded in the polyvinyl pyrrolidone (PVP, Irvine Scientific) droplet in the glass-bottom culture dish (GWSt 1000; Will Co. Amsterdam, The Netherlands). The motile spermatozoa were carefully assessed under an inverted microscope equipped with a heated stage and differential interference contrast (DIC) optics and Nikon Digital Sight DS-Ri1 Camera at × 6000 magnifications. According to Cassuto and Barak method [4], established detailed classification scoring scale was set ranging between 6 and 0 points according to the normalcy of the head (2 points if normal), the symmetry of the base (1 point if normal), and the absence of vacuole (3 points if absent). Three grades of scorings were, therefore, established: class I: high-quality spermatozoa with calculated score of 4–6; class II: medium-quality spermatozoa with calculated score of 1–3; and class III: low-quality spermatozoa with calculated score of 0 (Fig. 2). Selected good quality spermatozoa (classes I and II), aspirated in the microinjection pipette, and then transferred into the collection drop (G-MOPS; Vitrolife, Kungsbacka, Sweden), were covered with paraffin oil in a plastic culture dish (Nunc 153006). Then, mature oocytes were loaded in the dish and were injected. For both groups, the injected oocytes were washed and cultured overnight in droplets of G1 (Vitrolife Co., Sweden) in a standard incubator.

Embryo culture

Fertilization was evaluated 16–18-h postinsemination. All normally fertilized zygotes were then transferred to a nine-well embryo culture dish (Primo Vision, Vitrolife, Sweden) containing 40 μl of G-1 medium and covered with mineral oil and equilibrated overnight in 37 °C and 6% CO2. Each culture dish was placed in TLI (Vitrolife) in a standard incubator. The monitoring system repeatedly took photographs of the embryos using an inverted microscope every 10 min, and the total scan of embryos was taken every 20 min, for 2 days.

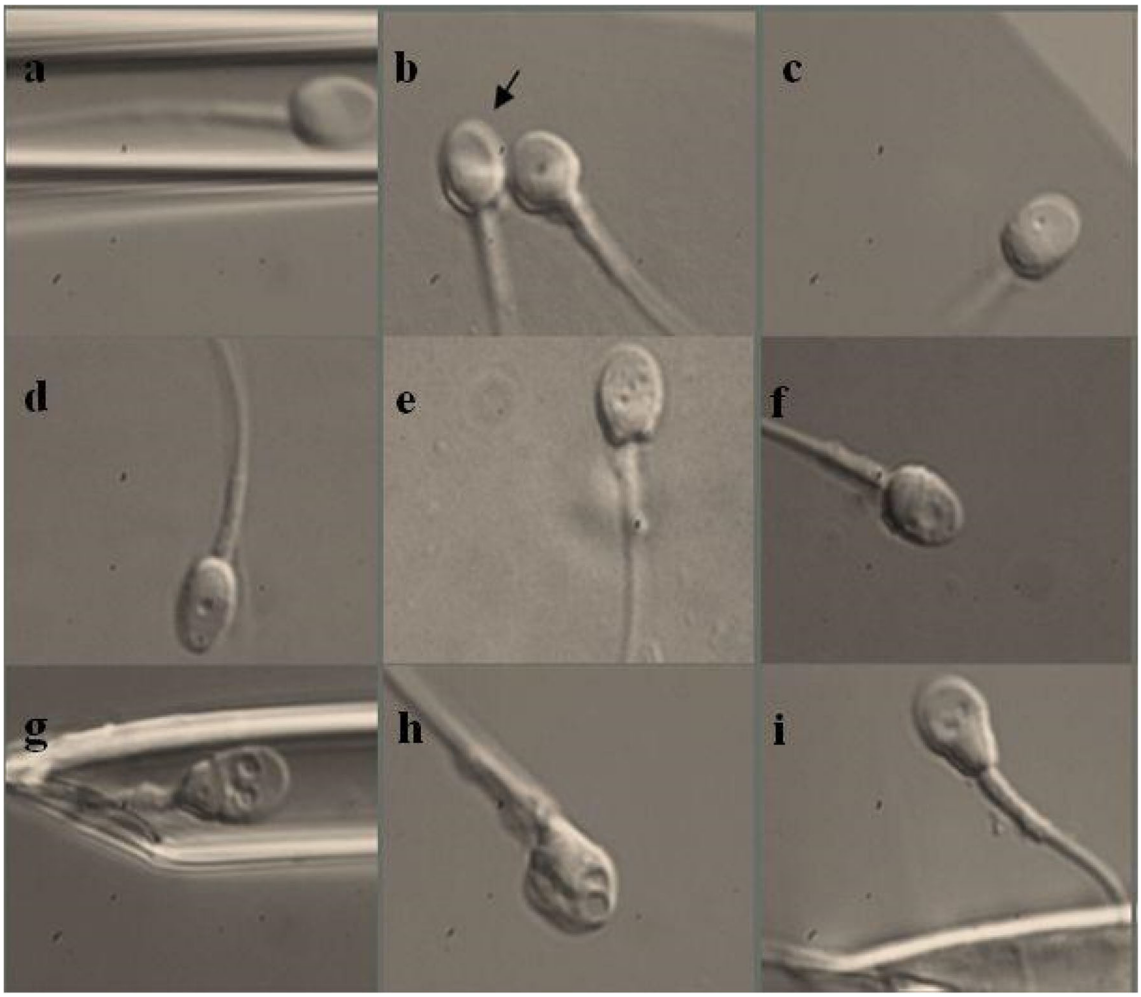

Fig. 2 Classification of spermatozoa selected at × 6000 magnification into 3 different categories. Class I—good quality spermatozoa, class II—worse quality spermatozoa, and class III—poor quality spermatozoa. **a**, **b**, **c**—spermatozoa of class I; **d**, **e**, **f**—spermatozoa of class II; **g**, **h**, **i**—spermatozoa of class III [4]

Embryo selection with TLI

Three days after culture, the embryos were evaluated by looking at the images using the Embryo viewer (Vitrolife) software. The following early kinetic markers were evaluated: PN fading (pnf), time to 2 cells (c) (t2), 3c (t3), 4c (t4), 5c (t5), 6c (t6), 7c (t7), and 8c (t8) and relative timing parameters s1, cc2, s2, and s3. Duration of the second cycle (cc2; t3–t2) and the final kinetic parameters looked where the time to complete first synchronous division s1 (t2–tPNf), the time to complete second synchronous divisions (s2:t4–t3) and the time to complete third synchronous divisions (s3:t8-t5). In addition, some cleavage anomalies specifically monitored were (A) uneven blastomere size at the two-cell stage [22], (B) reverse cleavage (RC), where a blastomere was reabsorbed after cleavage [23]. (C) Direct cleavage (DC) is where a single blastomere divided directly from 1 to 3 cells in less than 5 h [24]. Also, the rate of embryos arrest was assessed. The best embryos were selected according to an algorithm, as defined recently [19, 21, 25]. Briefly, after deselecting the embryos with abnormal morphology and cleavage abnormality, according to the timing variables t5 (48.5–56.6 h), s2 (t4–t3 < 0.76 h) and cc2 (t3–t2 < 11.9 h) [25], two embryos with ideal morphokinetic parameters were selected and transferred for each patient. Surplus good embryos were cryopreserved at the same day.

Clinical outcomes

Patients were followed up for biochemical pregnancy (positive b-hCG, 14 days after embryo transfer in the serum), clinical pregnancy (detection of one or more gestational sac with a fetal heartbeat in ultrasound after 6 weeks), and the live birth rate (LBR) (the percentage among the numeral of live births and the total of embryos transferred). Also, the implantation rate (IR) (the fraction among the numbers of gestational sacs and the sum of transferred embryos) was assessed [26].

Evaluation of sperm DNA fragmentation

The terminal deoxynucleotidyl transferase–mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL) was used to evaluate sperm DNA fragmentation (SDF). In both groups, immediately after sperm selection for injection, 100 spermatozoa were selected (for ICSI group at × 400, for IMSI at × 6000) and loaded in the drop of Hams F10 on the slide, air-dried, and fixed in methanol (RT, 30 min) and left in the freezer until staining. The slides were washed in phosphate-buffered saline (PBS, pH 7.4). 0.1% Triton X-100 (Merck, Germany) and 0.1% sodium citrate (on ice, for 2 min) were used for permeability. The samples were washed with PBS; then, 30 ml of TUNEL mixture (Roche, USA) was added to all samples and incubated for 60 min at 37 °C in a humid chamber in dark. After that, the slides were washed three times with PBS and finally assessed with fluorescence microscope under × 100 eyepiece objective [27, 28]. The sperm cells with fragmented DNA (TUNEL+) showing bright green color; while, the nuclei of the normal cells (TUNEL−) were pale green. A total of 100 spermatozoa were counted, and the rates of TUNEL+ spermatozoa were reported as the percentage.

Evaluation of sperm apoptosis transcript levels

For the ICSI group (control), 100 spermatozoa from prepared sample were selected at × 400. For the IMSI group (sample), 100 spermatozoa from prepared sample were selected with MSOME criteria at × 6000, both samples were then washed with PBS and collected in sterile microtubes and stored at – 80 °C for further analysis.

Extraction of total RNA and first strand cDNA synthesis

QuantiTect®, RNeasy Micro kits (Qiagen, Europe) was used for purification of total RNA. The RNA concentration was determined by Nano Drop spectrophotometer and adjusted to a concentration of 1000 ng/μL. cDNA was then synthesized using RevertAid First Strand cDNA synthesis kit (Thermo Fisher scientific Inc.) in the same day according to the manufacturer's guide. The reverse transcription was performed in 20 μL reactions for 60 min at 42 °C, followed by 70 °C for 5 min to inactivate the reverse transcriptase. The reverse transcription reaction product was directly used in quantitative PCR (qPCR) in a separate step to amplify the targets.

Real time polymerase chain reaction

Using specified primers (Table 1), the relative transcript levels of some apoptotic genes (Bcl2, Bax, Caspase 3) were evaluated by quantitative real time PCR (qRT-PCR). GAPDH gene was used as internal control. The PCR run was carried out according to QuantiTect SYBR Green RT-PCR kit (Applied Biosystems, UK, Lot no:1201416) on an ABI 7500 RT-PCR system (Applied Biosystems) using the following program: stage 1: 95 °C for 5 min and stage 2: 95 °C for 20s, 58 °C for 30 s, 72 °C for 20 s for a total of 40 cycles. It was continued by a melt curve step at 95 °C for 15 s, 58 °C for 1 min, and 95 °C for 15 s. All samples were run in duplicate to reduce the sampling error, and the mean value of each duplicate was used for all further calculations. Reverse transcriptase minus samples and no template controls were run together with the main samples. Verification of Amplicon specificity and size were performed by 2% agarose gel and product length as well as by a melting curve analysis. The relative transcript levels were then calculated by a mathematical model, which included an efficiency correction for real time PCR efficiency of the individual transcripts [14, 29] as follows: ratio = (E target) ΔCt target (control-sample)/(E ref) ΔCt ref (control-sample). The relative transcript levels of a target gene were determined from the real time PCR efficiency (E) and the threshold cycle difference for an unknown sample versus a control (Ct control-sample). For each gene, cDNA dilution curves were generated and used to calculate the individual real time PCR efficiencies [$E = 10(-1/\text{slope})$].

Statistical Analysis

The sample size was considered by comparison between two proportions. Considering the significance of 5% and the test power of 80% and, according to the maximum amount of standard deviation of transcript levels of gene 2.4 and to achieve a significant difference of at least 1.5 units, 41 cases were placed in each group [30]. Data normalization was performed with Kolmogorov–Smirnov test; Results were expressed as mean ± standard deviation (SD) for normal numeric variables, median ± interquartile range for nonparametric variables and percentage for categorical variables. The means of samples with normal distribution and sufficient size were compared by independent samples test parametric test (independent samples *t* test). In case of nonnormal distribution, the medians were compared by Mann–Whitney test. Categorical variables were compared using chi-square. A Spearman's rank correlation was used to analyses relationships between apoptotic transcript levels and early cleavage timing in the developing embryos. Statistical analysis was performed using the Statistical Package for the Social Sciences 20 (SPSS Inc., Chicago, IL, USA) with significance set at $P < 0.05$.

Table 1 The genes (*Bax*, *Bcl2*, *Caspase3* (apoptotic genes) and *GAPDH* (housekeeping gene)) and their product length

Accession numbers	Gene	Primer sequence	PCR product size (bp)	qPCR efficiencies (%)
NM_ 001291428	*Bax*	Forward:5′CGGAACTGATCAGA ATC-3′	124	94
		Reverse:5′GACATCAGTCGCTT CAGTG-3′		
NM_000633	*Bcl-2*	Forward:5AGAAAGCAGGAAAC CTGTGG-3′	126	96
		Reverse: 5′-GATAGCAGCACAGG ATTGGA-3′		
NM_032991	*Caspase 3*	Forward: 5′-CAGCCCATTTCTCC ATACG-3′	128	94
		Reverse: 5′-GCCTCACCACCTTT AGAAC-3′		
NM_ 001289746	*GAPDH*	Forward: 5′-TGGTATCGTGGAAG GACTCA-3′	132	97
		Reverse: 5′-CCAGTAGAGGCAGG GATGAT-3′		

Results

Initially, 150 male infertile cases enrolled in the study. Of those, 95 patients had the inclusion criteria on the day of oocyte retrieval and were randomized into two groups. Forty-seven cases in the IMSI group and forty-eight cases in the ICSI group. A total of 55 cases were excluded initially were advance maternal age (n = 16), severe male factor (n = 25), and estradiol levels > 2500 pg/mL at the time of triggering (n = 14). Also, 7 patients from the IMSI group and 8 cases from the ICSI group were excluded, because they had no suitable embryos for transfer. All other participants were available and followed to terms (Fig 1). The couples were referred to the ICSI program with male factor etiologies. Sixty-two (77.5%) patients were undertaken for the first time, whereas 18(22.5%) cases were undergone ART treatment for two or more cycles. No differences were detected between male and female ages, sperm parameters, male factor subgroups, serum AMH, estradiol, and the number of oocytes between the two groups (Table 2). In ICSI group, a total of 400 COCs were collected, of which 356 denuded oocytes were MII and microinjected. A total of 265 (74.43%) oocytes fertilized normally, and 220 (61.79%) embryos were formed. In IMSI group, a total of 350 COCs were collected, and 330 MII oocytes were microinjected. A total of 260 (78.78%) fertilized normally, and 245 (74.24%) embryos developed. For each patient, two of the best embryos according to the morphokinetic parameters were selected and transferred, whilst supernumerary good quality embryos were vitrified.

According to Table 3, TLI analysis showed that all of the early developmental events were significantly earlier in ICSI than IMSI ($p < 0.0001$) (Table 3). For evaluation of cleavage abnormality, significant relationships were recorded between groups in the percent of fragmentation, multinucleation, uneven blastomers, and reverse cleavage abnormalities ($p < 0.05$) (Table 4). IMSI had lower percentage of abnormalities when compared with ICSI, but there were minor differences for direct cleavage abnormality; the presence of vacuoles in the embryos and embryo arrests between the groups ($p > 0.05$) (Table 4).

According to the data of transferred embryos, almost all of the early developmental events were significantly different between the groups ($p < 0.0001$), except for t5, cc2, and s2 with no differences ($p = 0.06$, $p = 0.30$, $p = 0.86$, respectively) which match up with the algorithm. The trend of chemical pregnancy, implantation, clinical pregnancy, and live birth were higher in the IMSI group. But, only the implantation rate was significantly higher in sample group ($p = 0.01$; Fig. 3).

According to the TUNEL assay, there was a significant difference in the percent of spermatozoa with DNA fragmentation between the groups ($p < 0.0001$). The percentage of spermatozoa with DNA fragmentation was lower in IMSI than in ICSI patients (Fig. 4). Other part of this study evaluated the mRNA levels of two proapoptotic-associated genes (Bax and Caspase 3) and antiapoptotic-associated gene (Bcl2) by comparative real-time RT–PCR. The findings showed there was a significant increase in the transcript levels of BAX, Caspase 3, and the proportion of BAX/Bcl2 in ICSI group compared with IMSI. Also, the transcript levels of Bcl2 gene were lower in the ICSI group (Fig. 5).

Correlation between transcript levels of sperm apoptotic genes and embryo kinetic parameters were measured to consider the relationship between these two variables in the embryo development. The data showed a moderate negative

Table 2 Laboratory characteristic of patients between ICSI and IMSI groups

Lab characteristics	ICSI group (n = 40)	IMSI group (n = 40)	P value
Male age (year)*	34.7 ± 4.6	35.5 ± 5.3	0.34
Sperm count (× 10^6) #	30.0 ± 41.0	40.0 ± 60.0	0.44
Progressive motility (%) #	20.0 ± 20.0	20.0 ± 25.0	0.12
Nonprogressive motility (%) #	10.0 ± 0.0	10.0 ± 0.0	0.21
Immotile (%) #	70.0 ± 20.0	60.0 ± 30.0	0.12
Normal morphology (%) #	1.0 ± 1.0	1.0 ± 1.0	0.10
Round cell (× 10^6) #	7.0 ± 9.0	7.0 ± 8.0	0.50
Female age (year)*	30.3 ± 4.0	30.4 ± 3.7	0.76
Estradiol (pg/ml)*	1232.0 ± 349.5	1258.6 ± 394.9	0.50
AMH (ng/ml)*	1.9 ± 0.4	1.9 ± 0.6	0.52
Number of COC#	8.0 ± 4.0	7.0 ± 3.0	0.10
Number of MII oocytes #	7.5 ± 3.2	7.0 ± 2.0	0.08
Number of 2PN #	6.0 ± 3.0	6.0 ± 2.0	0.09
Fertilization rate*	74.4 ± 14.6	76.4 ± 16.1	0.08
Male factor subgroups:*			
Asthenozoospermia	10 (25)	12 (30)	0.83
Asthenoteratozoospermia	20 (50)	20 (50)	
teratozoospermia	10 (25)	8 (20)	

Data were presented as *mean ± SD and number (%); Data were presented as # median ± I.Q

COC cumulus oocyte complex, *MII* metaphase II, *2PN* two pronuclear

correlation between transcript levels of *Caspase3* with the s2 and s3 (correlation coefficient [rs] = − 0.57, P = 0.008, and rs = − 0.51, p = 0.021, respectively) among IMSI group. Transcript levels of sperm apoptotic genes had no correlation with embryo kinetic timing in ICSI group (Table 5). Also, the findings indicated that transcript levels of sperm apoptotic genes were not correlated with embryo cleavage abnormalities, like reverse cleavage, direct cleavage, and multinucleation in both groups (p > 0.05). In addition, no association was observed between transcript levels of sperm apoptotic genes with clinical outcomes in the groups (p > 0.05). There was also no correlation between TUNEL assay

Table 3 Kinetic data of all embryos in two study groups

kinetic markers (hours postICSI)	ICSI embryos (n = 220)	IMSI embryos (n = 245)	P value
tPNf	19.2 ± 1.6	24.1 ± 3.0	< 0.0001*
t2	20.7 ± 1.9	26.1 ± 3.4	< 0.0001*
s1	1.5 ± 0.7	1.9 ± 0.7	< 0.0001*
t3	28.4 ± 5.0	34.7 ± 4.9	< 0.0001*
cc2	7.0 ± 4.4	9.3 ± 2.9	< 0.0001*
t4	31.8 ± 4.9	37.2 ± 5.0	< 0.0001*
s2	3.6 ± 4.5	1.5 ± 0.7	< 0.0001*
t5	40.4 ± 7.0	48.7 ± 5.1	< 0.0001*
t6	42.0 ± 6.5	50.0 ± 4.3	< 0.0001*
t7	49.9 ± 5.2	54.0 ± 4.2	< 0.0001*
t8	52.9 ± 5.0	58.8 ± 3.6	< 0.0001*
s3	15.8 ± 7.7	10.8 ± 4.7	< 0.0001*

Data were calculated using independent t test and presented mean ± standard deviation (SD)

Pnf PN fading, *t2* = first cleavage (2-cell stage), *t3* second cleavage (3-cell stage), *t4* 4-cell stage, *t5* 5-cell stage, *t6* 6-cell stage, *t7* 7-cell stage, *t8* 8-cell stage. The time to complete first synchronous divisions s1 (t2–tPNf), durations of the second cycle (cc2; t3–t2), the time to complete second synchronous divisions (s2:t4–t3), and the time to complete third synchronous divisions (s3:t8–t5). *p value < 0.05 was significant

Table 4 Frequency and percentage of cleavage abnormalities between ICSI and IMSI groups

Cleavage abnormalities	ICSI group (n = 220)	IMSI group (n = 245)	OR (95% CI)	P value
Fragmentation	157 (71.3)	151 (61.6)	0.656 (0.473–0.911)	0.01*
Multinucleation	66 (30.0)	23 (9.3)	0.233 (0.149–0.364)	< 0.0001*
Uneven blastomers	82 (37.2)	23 (9.3)	0.174 (0.112–0.270)	< 0.0001*
Reverse cleavage	82 (37.2)	49 (20.0)	0.421 (0.296–0.599)	< 0.0001*
Direct cleavage	13 (5.9)	16 (6.5)	1.033 (0.553–1.932)	1.00
Vacuoles	13 (5.9)	12 (4.8)	0.762 (0.383–1.517)	0.48
Arrest	22 (10.0)	16 (6.5)	0.632 (0.357–1.118)	0.12

OR, odds ratio; *CI*, confidence interval, *p value < 0.05 was significant. The values within parenthesis represent %

with embryokinetics parameters and clinical outcomes in both groups of IMSI and ICSI ($p > 0.05$).

Discussion

Comparison of embryokinetic between IMSI and ICSI

This study was set up to assess if there were any benefits of the IMSI procedure compared with conventional ICSI in patients with male infertility. To the best of our knowledge, only one study has evaluated the developmental dynamics of IMSI-derived embryos [31]. Also, there are few studies comparing the embryo kinetics between IMSI and ICSI cycles. The results showed that all of the early developmental events were significantly beneficial in IMSI group, when compared to ICSI. According to the TLI parameters, the timings of divisions for embryos derived from IMSI were similar to the Herrero's study [32] that declared the average timing of embryo division. But, the timing of division in the ICSI group was different and occurred earlier. Also, the rates of cleavage abnormality (fragmentation, multinucleation, uneven blastomere and reverse cleavage) were significantly lower in the embryos derived from IMSI procedure. Confirming our findings, Knez et al. [31], for the first time, evaluated the influence of vacuoles on the morphology and dynamics of early developmental events in preimplantation embryos. They concluded that the size and number of vacuoles had an influence on developmental dynamics of IMSI-derived embryos and reaching to blastocyst stage [31].

It has been reported that too slow or too fast embryo development may have metabolic and/or chromosomal defects [33–35]. However, it also depends on the circumstance of each laboratory, like culture media and temperature. When ICSI was done with abnormal sperm, especially the presence of vacuoles, it was related to late embryo development. Slow embryonic development with poor progression to cleavage is generally considered to be due to sperm DNA damage or aneuploidy of the embryo [35, 36]. Studies showed that spermatozoa with large vacuoles are associated with high level of sperm chromatin dispersion (SCD), chromatin condensation defect, and packaging abnormality. They suggested the use of high magnification sperm selection prior to microinjection to deselect score-0 spermatozoa, while improving the ART outcomes [37–39]. Embryos that are formed from cases with severe male infertility and testicular spermatozoa are usually accompanied by delayed embryo kinetic parameters and more prone to aneuploidy [36, 40]. Sperm with abnormal chromatin may negatively influence not only the fertilization and early cleavage, but also embryonic capability by interfering with embryonic genome activation. Sperm centrosome plays the

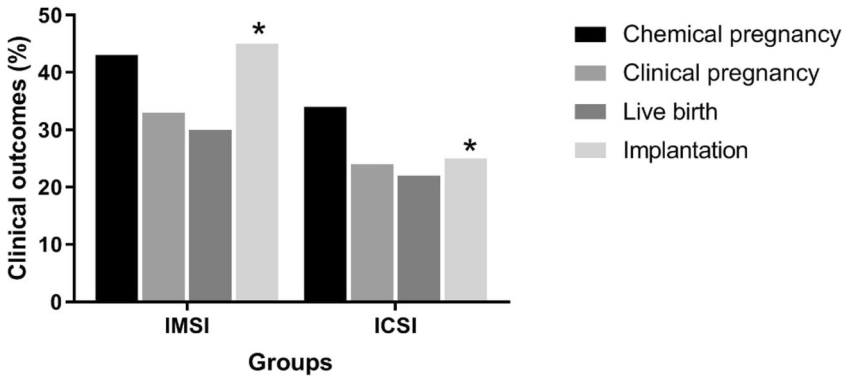

Fig. 3 Comparison of clinical outcomes between the groups. Chi-square test, *p < 0.05 was significant

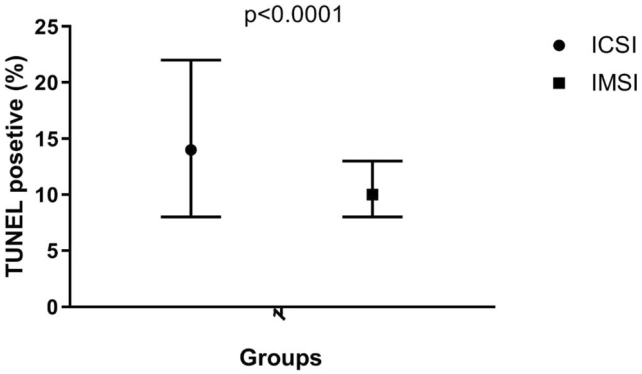

Fig. 4 Comparison of sperm apoptosis range between the groups. Mann–Whitney test, median ± I.Q, p value < 0.0001 was significant

main role in the embryo division. Defects in the centrosome of abnormal spermatozoa or in the testicular spermatozoa were associated with delayed first cleavage, slow embryo development, and reduced morulae and blastocyst formation [40, 41]. However, embryonic genome activation does not occur until after the 8-cell stage. So, the real value of a sperm selection method would be the effect not only on early cleavage events, but also on compaction.

Our results showed embryos that were derived from ICSI had rapid cleavage with higher cleavage abnormalities and poor clinical outcomes when compared with IMSI. According to the results, it might be considered that ICSI embryos had more chromosomal errors and genetic defects than IMSI. Studies declared that IMSI effects occur only as a positive later paternal effect. The late paternal effect is characterized by poor embryo development to blastocyst stage, implantation failure, and pregnancy loss, associated with sperm abnormalities at the level of DNA and chromatin. Our previous study showed that the abnormal sperm parameters and chromatin alteration affect the normal embryo kinetics in ICSI program [18]. Although, DNA damage cannot be recognized with selection of spermatozoa at high magnification,

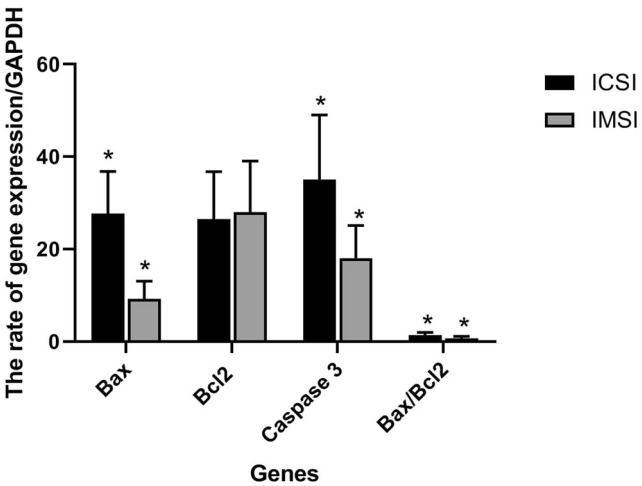

Fig. 5 Comparison of apoptotic transcript levels (mean ± SD) between two groups. Independent Samples t test, *p value < 0.05 was significant

Table 5 Spearman's rank correlations analyzing the relationship between transcript levels of sperm apoptotic genes and embryo kinetics of individual embryos in the two groups

Kinetic parameters	ICSI (n = 220)			IMSI (n = 245)		
	BAX	*BCL2*	*CASP3*	*BAX*	*BCL2*	*CASP3*
tPNf	0.28	0.40	0.08	0.15	0.05	− 0.01
t2	− 0.20	− 0.10	− 0.17	0.21	0.07	0.05
s1	− 0.36	− 0.29	− 0.20	0.14	0.32	0.21
t3	0.21	0.32	− 0.03	0.22	0.11	0.01
cc2	0.39	0.37	0.04	− 0.17	− 0.04	− 0.18
t4	0.10	0.25	− 0.04	0.11	0.10	− 0.13
s2	− 0.31	− 0.18	− 0.07	− 0.07	0.29	− 0.57*
t5	0.29	0.28	0.22	− 0.06	0.06	0.30
t6	− 0.09	− 0.10	0.16	− 0.09	− 0.00	0.28
t7	− 0.13	− 0.22	0.12	− 0.05	0.31	0.16
t8	− 0.07	− 0.14	− 0.17	0.06	0.21	0.02
s3	0.10	− 0.20	− 0.23	0.07	0.18	− 0.51*

*s2 and s3 timing were a moderate negative correlation with transcript levels of *CASP3* in the IMSI group (P = 0.008 and P = 0.021, respectively). *p value < 0.05 was significant

Pnf PN fading, *t2* first cleavage (2-cell stage), *t3* second cleavage (3-cell stage), *t4* 4-cell stage, *t5* 5-cell stage, *t6* 6-cell stage, *t7* 7-cell stage, *t8* 8-cell stage. The time to complete first synchronous divisions s1 (t2–tPNf), durations of the second cycle (cc2; t3–t2), the time to complete second synchronous divisions (s2:t4–t3), and the time to complete third synchronous divisions (s3:t8–t5)

but, it is possible to identify sperm with intranuclear vacuoles. These structures may reflect better probability of molecular defects responsible for DNA damage.

Clinical outcomes

In this study, there were higher rates for normal fertilization and embryo formation in the IMSI group compared with the ICSI. Also, the rate of biochemical and clinical pregnancies, live birth, and implantation were higher in the IMSI compared with the ICSI group, but, the differences were insignificant, except for implantation. According to the results of a metaanalysis, IMSI not only significantly improved the percentage of top-quality embryos, implantation, and pregnancy rates, but also significantly reduced the miscarriage rates as compared with ICSI [42]. The data in this regard are still contradictory; according to a recent Cochrane study (2020), there is a weak evidence to support or reject the IMSI procedure, and mandate more high-quality clinical trial studies [43]. Knez and colleagues [31] confirmed significantly higher implantation and clinical pregnancy rates with IMSI in the severe teratozoospermia patients. In contrast, some studies reported no differences between ICSI and IMSI with regards to fertilization, implantation, and pregnancy rates as well as

miscarriage rates in unselected infertile patients and two previous ICSI failures [44–47]. These findings can be explained by the fact that during ICSI, morphological assessment of the sperm nucleus takes place at × 400. These conflicting results might have occurred due to differences in inclusion criteria, stimulation protocols, seminal and oocyte qualities, along with many other confounding variables within the IVF cycles. On the other hand, this technique is not recommended for all ART patients. Recent studies reported that IMSI is preferential for both severe male factor patients and repeated ICSI failures [19, 21, 48]. It is noteworthy that more prospective randomized trials are required to confirm the superiority of IMSI over conventional ICSI in certain infertile populations.

Sperm DNA fragmentation

The capacity of human spermatozoa to fertilize and produce a good-quality embryo with a high implantation and developmental potential depends on its DNA integrity. During spermatogenesis, harmful effects could occur that affect the chromatin condensation and DNA integrity. Avendano and coworkers [49] demonstrated that DNA fragmentation in morphologically normal spermatozoa had a significant negative effect on embryo quality and pregnancy outcome in ICSI patients. Interestingly, our TUNEL results showed that spermatozoa that were selected with MSOME presented lower percentage of DNA fragmentation, and the generated embryos were of better quality than ICSI embryos. However, there was no correlation between sperm DNA fragmentation with embryo kinetics and clinical outcomes. This difference may be a result of the mixed origin of infertility. Moreover, it is difficult to isolate the real influence of SDF in clinical settings because, while sperm DNA integrity can be evaluated, the oocyte repair capacity remains a problem.

It is well established that high-quality gametes are required to produce top-quality embryos, and that both the sperm and oocyte genomes contribute to the embryonic genome [50]. A number of studies have shown that abnormalities in the paternal DNA and chromatin adversely impact on embryo quality [11, 51–53]. In line with our study, Kohn et al. [54] showed morphologically abnormal spermatozoa is likely to be related to reduce sperm DNA integrity.

The literature in this regard is conflicting, some authors recommended that measurement of sperm DNA fragmentation and chromatin integrity is an important prognostic tool for various natural conception and ART practice [11, 55]. Also, other studies showed that some of the sperm parameters and high SDF levels had influential roles on embryo kinetics in patients with male factor infertility. They suggested application of advance methods for selecting spermatozoa with intact chromatin [12, 18].

Apoptotic genes transcript levels profile

In the present study, for the first time, the transcript levels of apoptotic genes in IMSI and ICSI spermatozoa were evaluated for patients with male infertility. The results showed the transcript levels of proapoptotic genes were significantly lower in the IMSI group compared with ICSI. On the other hand, the transcript levels of the antiapoptotic gene was higher in the IMSI group than ICSI. In other words, sperm selection at high magnification may cause selected spermatozoa with healthy DNA and reduced transcript levels of apoptotic genes. Also, this result confirmed our findings in terms of TUNEL staining. We do not have information on how genes are expressed and proteins formed in the spermatozoa following this level of transcription of apoptotic genes. As already mentioned, sperm DNA damage may be repaired by the oocyte genome. Current data may not be useful and practical, but this is the first step in examining the expression of sperm apoptosis genes and their effects on embryo quality and clinical outcomes in IMSI and ICSI cycles. This was the first study that evaluated sperm apoptotic genes transcript levels in IMSI and ICSI groups and their correlations with embryo kinetics and clinical outcomes. The findings showed a moderate negative correlation between transcript levels of *Caspase 3* with s2 and s3 in the IMSI group. We did not notice this relationship in the ICSI group, which could be related to the speed of divisions in this group that was faster than normal. Also, the transcript levels of proapoptotic genes were significantly higher in this group. But, in the IMSI group, the speed of division was within normal range according to Herrero et al. [32], and the transcript levels of proapoptotic genes were lower. It was observed that with increasing transcript levels of Caspase 3, the time of second and third synchronization were decreased.

There was no relationship between sperm apoptotic transcript levels and clinical outcomes in two groups. It was better to culture embryos in TLI to blastocyst stage and evaluate them. This was one of the limitations of our study. In addition, the low sample size and limitation of time and budget could affect the results. Therefore, a large study in this field is needed.

Conclusion

Sperm selection with MSOME parameters and IMSI can improve the embryo quality and clinical outcomes. Based on timing parameters and cleavage patterns made possible by TLI, present data agree that the IMSI procedure has better embryo kinetics and clinical outcomes than ICSI. In addition, there was lower percentage of DNA fragmentation and transcript levels of apoptotic genes in MSOME selected spermatozoa. There was a moderate negative correlation in transcript levels of *Caspase 3* with s2 and s3 timings in IMSI group.

Moreover, there was no relationship between apoptosis gene transcript levels and clinical outcomes in two groups. So, these noninvasive techniques can be applied for advancing the success rates in assisted male reproduction.

Acknowledgments This study was extracted from the PhD thesis of Esmat Mangoli. The authors thank Dr Hossein Falahzadeh, Professor of Biostatistics, for statistical assistance. We acknowledge the staff of the ART and Molecular Genetics laboratories in Yazd Reproductive Sciences Institute for their great contribution to all the laboratory work and data gathering.

Availability of data and material The data is available in SPSS format.

Authors' contributions Conception and design of study: Esmat Mangoli, Mohammad Ali Khalili, Ali Reza Talebi, Seyed Mehdi Kalantar. Acquisition of data: Esmat Mangoli, Fatemeh Montazeri, Azam Agharahimi. Analysis and interpretation of data: Esmat Mangoli, Mohammad Ali Khalili, Seyed Mehdi Kalantar. Drafting the manuscript: Esmat Mangoli. Revising the manuscript: Mohammad Ali Khalili, Bryan J Woodward. Approval of the version of the manuscript to be published: Esmat Mangoli, Mohammad Ali Khalili, Ali Reza Talebi, Seyed Mehdi Kalantar, Fatemeh Montazeri, Azam Agharahimi, Bryan J Woodward.

Funding information This study was supported by funding from Shahid Sadoughi University of Medical Sciences.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The study was approved by the Ethics Committee of our institute (IR. SSU.MEDICINE.REC.1394.338).

Consent to participate All patients provide informed consent to participate in study.

Consent for publication All patients signed informed consent to publish data.

References

1. Teixeira DM, Barbosa MA, Ferriani RA, Navarro PA, Raine-Fenning N, Nastri CO, et al. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. Cochrane Database Syst Rev. 2013;7:CD010167. https://doi.org/10.1002/14651858.CD010167.pub2.
2. Ghazali S, Talebi AR, Khalili MA, Aflatoonian A, Esfandiari N. Large nuclear vacuoles in spermatozoa negatively affect pregnancy rate in IVF cycles. Iran J Reprod Med. 2015;13(7):425–32.
3. Bartoov B, Berkovitz A, Eltes F, Kogosowski A, Menezo Y, Barak Y. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. J Androl. 2002;23(1):1–8. https://doi.org/10.1002/j.1939-4640.2002.tb02595.x.
4. Cassuto NG, Bouret D, Plouchart JM, Jellad S, Vanderzwalmen P, Balet R, et al. A new real-time morphology classification for human spermatozoa: a link for fertilization and improved embryo quality. Fertil Steril. 2009;92(5):1616–25. https://doi.org/10.1016/j.fertnstert.2008.08.088.
5. Armstrong S, Arroll N, Cree LM, Jordan V, Farquhar C. Time-lapse systems for embryo incubation and assessment in assisted reproduction. Cochrane Database Syst Rev. 2015;2:CD011320. https://doi.org/10.1002/14651858.CD011320.pub2.
6. Alhelou Y, Mat Adenan NA, Ali J. Embryo culture conditions are significantly improved during uninterrupted incubation: a randomized controlled trial. Reprod Biol. 2017;18:40–5. https://doi.org/10.1016/j.repbio.2017.12.003.
7. Chamayou S, Patrizio P, Storaci G, Tomaselli V, Alecci C, Ragolia C, et al. The use of morphokinetic parameters to select all embryos with full capacity to implant. J Assist Reprod Genet. 2013;30(5):703–10. https://doi.org/10.1007/s10815-013-9992-2.
8. Tejera A, Aparicio-Ruiz B, Meseguer M. The use of morphokinetic as a predictor of implantation. Minerva Ginecol. 2017. https://doi.org/10.23736/S0026-4784.17.04098-9.
9. Dominguez F, Meseguer M, Aparicio-Ruiz B, Piqueras P, Quinonero A, Simon C. New strategy for diagnosing embryo implantation potential by combining proteomics and time-lapse technologies. Fertil Steril. 2015;104:908–14. https://doi.org/10.1016/j.fertnstert.2015.06.032.
10. Siristatidis C, Komitopoulou MA, Makris A, Sialakouma A, Botzaki M, Mastorakos G, et al. Morphokinetic parameters of early embryo development via time lapse monitoring and their effect on embryo selection and ICSI outcomes: a prospective cohort study. J Assist Reprod Genet. 2015;32(4):563–70. https://doi.org/10.1007/s10815-015-0436-z.
11. Zheng WW, Song G, Wang QL, Liu SW, Zhu XL, Deng SM, et al. Sperm DNA damage has a negative effect on early embryonic development following in vitro fertilization. Asian J Androl. 2017;20:75–9. https://doi.org/10.4103/aja.aja_19_17.
12. Wdowiak A, Bakalczuk S, Bakalczuk G. The effect of sperm DNA fragmentation on the dynamics of the embryonic development in intracytoplasmatic sperm injection. Reprod Biol. 2015;15(2):94–100. https://doi.org/10.1016/j.repbio.2015.03.003.
13. Cho CL, Agarwal A. Role of sperm DNA fragmentation in male factor infertility: a systematic review. Arab J Urol. 2018;16(1):21–34. https://doi.org/10.1016/j.aju.2017.11.002.
14. Fesahat F, Sheikhha MH, Kalantar SM, Tabibnejad N, Firouzabadi RD, Saeedi H, et al. Developmental competence and apoptotic gene expression patterns of mature and immature human oocytes retrieved from controlled ovarian stimulation cycles. Reprod Biol. 2018;18(1):27–32. https://doi.org/10.1016/j.repbio.2017.11.002.
15. Grunewald S, Paasch U, Wuendrich K, Glander HJ. Sperm caspases become more activated in infertility patients than in healthy donors during cryopreservation. Arch Androl. 2005;51(6):449–60. https://doi.org/10.1080/014850190947813.
16. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science. 1998;281(5381):1312–6.
17. Organization WH. WHO laboratory manual for the examination and processing of human semen. 2010.
18. Mangoli E, Khalili MA, Talebi AR, Ghasemi-Esmailabad S, Hosseini A. Is there any correlation between sperm parameters and chromatin quality with embryo morphokinetics in patients with male infertility? Andrologia. 2018;50(5):e12997. https://doi.org/10.1111/and.12997.
19. Mangoli E, Khalili MA, Talebi AR, Agha-Rahimi A, Soleimani M, Faramarzi A, et al. IMSI procedure improves clinical outcomes and embryo morphokinetics in patients with different aetiologies of male infertility. Andrologia. 2019;51(8):e13340. https://doi.org/10.1111/and.13340.
20. Faramarzi A, Khalili MA, Soleimani M. First successful pregnancies following embryo selection using time-lapse technology in Iran: case report. Iran J Reprod Med. 2015;13(4):237–42.
21. Mangoli E, Khalili MA, Eftekhar M, Macchiarelli G, Palmerini MG. First successful live birth following the use of MSOME and time lapse for sperm and embryo selections in a patient with severe

male factor infertility: A case report. J Gynecol Obstet Hum Reprod. 2019;48(10):883–5. https://doi.org/10.1016/j.jogoh.2019.03.004.

22. Basile N, Vime P, Florensa M, Aparicio Ruiz B, Garcia Velasco JA, Remohi J, et al. The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. Hum Reprod. 2015;30(2):276–83. https://doi.org/10.1093/humrep/deu331.
23. Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Clinical significance of intercellular contact at the four-cell stage of human embryos, and the use of abnormal cleavage patterns to identify embryos with low implantation potential: a time-lapse study. Fertil Steril. 2015;103(6):1485–91 e1. https://doi.org/10.1016/j.fertnstert.2015.03.017.
24. Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, et al. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. Fertil Steril. 2014;102(5):1287–94 e5. https://doi.org/10.1016/j.fertnstert.2014.07.738.
25. Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. The use of morphokinetics as a predictor of embryo implantation. Hum Reprod. 2011;26(10):2658–71. https://doi.org/10.1093/humrep/der256.
26. Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, et al. The international glossary on infertility and fertility care, 2017. Hum Reprod. 2017;32(9):1786–801. https://doi.org/10.1093/humrep/dex234.
27. Mangoli E, Talebi AR, Anvari M, Taheri F, Vatanparast M, Rahiminia T, et al. Vitamin C attenuates negative effects of vitrification on sperm parameters, chromatin quality, apoptosis and acrosome reaction in neat and prepared normozoospermic samples. Taiwan J Obstet Gynecol. 2018;57(2):200–4. https://doi.org/10.1016/j.tjog.2018.02.006.
28. Talebi AR, Fesahat F, Mangoli E, Ghasemzadeh J, Nayeri M, Sadeghian-Nodoshan F. Relationship between sperm protamine deficiency and apoptosis in couples with unexplained repeated spontaneous abortions. Int J Reprod Biomed (Yazd). 2016;14(3):199–204.
29. Tabibnejad N, Sheikhha MH, Ghasemi N, Fesahat F, Soleimani M, Aflatoonian A. Association between early embryo morphokinetics plus cumulus cell gene expression and assisted reproduction outcomes in polycystic ovary syndrome women. Reprod BioMed Online. 2019;38(2):139–51. https://doi.org/10.1016/j.rbmo.2018.10.010.
30. Cavalcanti MC, Steilmann C, Failing K, Bergmann M, Kliesch S, Weidner W, et al. Apoptotic gene expression in potentially fertile and subfertile men. Mol Hum Reprod. 2011;17(7):415–20. https://doi.org/10.1093/molehr/gar011.
31. Knez K, Tomazevic T, Vrtacnik-Bokal E, Virant-Klun I. Developmental dynamics of IMSI-derived embryos: a time-lapse prospective study. Reprod BioMed Online. 2013;27(2):161–71. https://doi.org/10.1016/j.rbmo.2013.04.002.
32. Herrero J, Tejera A, Albert C, Vidal C, de los Santos MJ, Meseguer M. A time to look back: analysis of morphokinetic characteristics of human embryo development. Fertil Steril. 2013;100(6):1602–9.e1-4. https://doi.org/10.1016/j.fertnstert.2013.08.033.
33. Brezinova J, Svobodova M, Oborna I, Fingerova H, Dostal J, Krskova M. Embryo quality evaluation according to the speed of the first cleavage after IntraCytoplasmic Sperm Injection (ICSI). Ceska Gynekol. 2006;71(3):204–8.
34. Prados FJ, Debrock S, Lemmen JG, Agerholm AI. The cleavage stage embryo. Hum Reprod. 2012;27(S1):i50–71 27(s1):50-71.
35. Tesarik J. Paternal effects on cell division in the human preimplantation embryo. Reprod BioMed Online. 2005;10(3):370–5. https://doi.org/10.1016/s1472-6483(10)61798-1.
36. Desai N, Gill P, Tadros NN, Goldberg JM, Sabanegh E, Falcone T. Azoospermia and embryo morphokinetics: testicular sperm-derived embryos exhibit delays in early cell cycle events and increased arrest prior to compaction. J Assist Reprod Genet. 2018;35(7):1339–48. https://doi.org/10.1007/s10815-018-1183-8.
37. Cassuto NG, Hazout A, Hammoud I, Balet R, Bouret D, Barak Y, et al. Correlation between DNA defect and sperm-head morphology. Reprod BioMed Online. 2012;24(2):211–8. https://doi.org/10.1016/j.rbmo.2011.10.006.
38. Boitrelle F, Ferfouri F, Petit JM, Segretain D, Tourain C, Bergere M, et al. Large human sperm vacuoles observed in motile spermatozoa under high magnification: nuclear thumbprints linked to failure of chromatin condensation. Hum Reprod. 2011;26(7):1650–8. https://doi.org/10.1093/humrep/der129.
39. Boitrelle F, Guthauser B, Alter L, Bailly M, Wainer R, Vialard F, et al. The nature of human sperm head vacuoles: a systematic literature review. Basic Clin Androl. 2013;23:3. https://doi.org/10.1186/2051-4190-23-3.
40. Kahraman S, Sahin Y, Yelke H, Kumtepe Y, Tufekci MA, Yapan CC, et al. High rates of aneuploidy, mosaicism and abnormal morphokinetic development in cases with low sperm concentration. J Assist Reprod Genet. 2020;37(3):629–40. https://doi.org/10.1007/s10815-019-01673-w.
41. Niederberger C. Re: The Impact of Male Factor Infertility on Early and Late Morphokinetic Parameters: A Retrospective Analysis of 4126 Time-Lapse Monitored Embryos. J Urol. 2020;101097JU0000000000000110704. https://doi.org/10.1097/JU.0000000000001107.04.
42. Souza Setti A, Ferreira RC, Paes de Almeida Ferreira Braga D, de Cassia Savio Figueira R, Iaconelli A Jr, Borges E Jr. Intracytoplasmic sperm injection outcome versus intracytoplasmic morphologically selected sperm injection outcome: a meta-analysis. Reprod BioMed Online. 2010;21(4):450–5. https://doi.org/10.1016/j.rbmo.2010.05.017.
43. Teixeira DM, Hadyme Miyague A, Barbosa MA, Navarro PA, Raine-Fenning N, Nastri CO, et al. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. Cochrane Database Syst Rev. 2020;2:CD010167. https://doi.org/10.1002/14651858.CD010167.pub3.
44. Oliveira JB, Cavagna M, Petersen CG, Mauri AL, Massaro FC, Silva LF, et al. Pregnancy outcomes in women with repeated implantation failures after intracytoplasmic morphologically selected sperm injection (IMSI). Reprod Biol Endocrinol. 2011;9:99. https://doi.org/10.1186/1477-7827-9-99.
45. Balaban B, Yakin K, Alatas C, Oktem O, Isiklar A, Urman B. Clinical outcome of intracytoplasmic injection of spermatozoa morphologically selected under high magnification: a prospective randomized study. Reprod BioMed Online. 2011;22(5):472–6. https://doi.org/10.1016/j.rbmo.2010.11.003.
46. Gatimel N, Parinaud J, Leandri RD. Intracytoplasmic morphologically selected sperm injection (IMSI) does not improve outcome in patients with two successive IVF-ICSI failures. J Assist Reprod Genet. 2016;33(3):349–55. https://doi.org/10.1007/s10815-015-0645-5.
47. La Sala GB, Nicoli A, Fornaciari E, Falbo A, Rondini I, Morini D, et al. Intracytoplasmic morphologically selected sperm injection versus conventional intracytoplasmic sperm injection: a randomized controlled trial. Reprod Biol Endocrinol. 2015;13:97. https://doi.org/10.1186/s12958-015-0096-y.
48. Goswami G, Sharma M, Jugga D, Gouri DM. Can intracytoplasmic morphologically selected spermatozoa injection be used as first choice of treatment for severe male factor infertility patients? J Hum Reprod Sci. 2018;11(1):40–4. https://doi.org/10.4103/jhrs.JHRS_74_17.
49. Avendano C, Franchi A, Duran H, Oehninger S. DNA fragmentation of normal spermatozoa negatively impacts embryo quality and

intracytoplasmic sperm injection outcome. Fertil Steril. 2010;94(2):549–57. https://doi.org/10.1016/j.fertnstert.2009.02.050.

50. McDowell S, Kroon B, Ford E, Hook Y, Glujovsky D, Yazdani A. Advanced sperm selection techniques for assisted reproduction. Cochrane Database Syst Rev. 2014;10:CD010461. https://doi.org/10.1002/14651858.CD010461.pub2.
51. Acharyya S, Kanjilal S, Bhattacharyya AK. Does human sperm nuclear DNA integrity affect embryo quality? Indian J Exp Biol. 2005;43(11):1016–22.
52. Carrasco B, Arroyo G, Gil Y, Gomez MJ, Rodriguez I, Barri PN, et al. Selecting embryos with the highest implantation potential using data mining and decision tree based on classical embryo morphology and morphokinetics. J Assist Reprod Genet. 2017;34(8):983–90. https://doi.org/10.1007/s10815-017-0955-x.
53. Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod. 2006;21(11):2876–81. https://doi.org/10.1093/humrep/del251.
54. Kohn TP, Kohn JR, Lamb DJ. Role of Sperm Morphology in Deciding Between Various Assisted Reproduction Technologies. Eur Urol Focus. 2018;4(3):311–3. https://doi.org/10.1016/j.euf.2018.07.029.
55. Sadeghi MR, Lakpour N, Heidari-Vala H, Hodjat M, Amirjannati N, Hossaini Jadda H, et al. Relationship between sperm chromatin status and ICSI outcome in men with obstructive azoospermia and unexplained infertile normozoospermia. Romanian J Morphol Embryol. 2011;52(2):645–51.