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Relationship of sperm DNA fragmentation, apoptosis and dysfunction of mitochondrial membrane potential with semen parameters and ART outcome after intracytoplasmic sperm injection

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Abstract
Purpose The objective of this study was to assess the relationship of DNA damage, apoptosis and dysfunction of mitochondrial membrane potential (MMP) in ejaculated spermatozoa with semen parameters (sperm concentration, motility and normal morphology) and to evaluate their effects on assisted reproductive technology (ART) outcomes after intracytoplasmic sperm injection (ICSI).
Methods Semen parameters in 120 infertile couples who underwent ICSI treatment were routinely analyzed and examined for the incidence of sperm DNA fragmentation (DF) by the sperm chromatin dispersion test (SCD). Whereas the incidences of sperm apoptosis and dysfunction of MMP were assessed by flow cytometry. The correlation among different sperm factors and ART outcomes was evaluated statistically.
Results Sperm parameters were negatively related to DF (motility and normal morphology, $p < 0.01$), apoptosis (concentration, motility and normal morphology, $p < 0.01$, $p < 0.05$ and $p < 0.05$, $p < 0.01$ respectively), and dysfunction of sperm MMP (concentration, motility and normal morphology, $p < 0.01$). DF also showed a positive correlation with apoptosis and dysfunction of sperm MMP ($p < 0.05$, and $p < 0.01$ respectively). However, there was no significant correlation among DF, apoptosis and dysfunction of sperm MMP with ART outcomes, except early apoptosis which showed significant ($p < 0.05$) negative correlation with pregnancy rate.
Conclusion In the present study; DF, apoptosis and dysfunction of sperm MMP indicated negative relationship with sperm parameters. Although there was a negative correlation between early apoptosis and pregnancy rate, no significant correlation was observed between these parameters and ICSI outcomes.

Keywords Apoptosis · Mitochondrial membrane potential · DNA damage · ICSI

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Introduction

Occasionally semen analysis is unable to predict the fertilizing capacity of spermatozoa, because some factors such as sperm nuclear DNA integrity, the presence of apoptotic spermatozoa, mitochondrial dysfunction [1] and increased ROS production [2] are not assessed in conventional semen analysis. This might be partially responsible for the low

fertilization rates in assisted reproductive technology (ART) [3–6].

Routine semen parameters cannot show the defects of sperm DNA. It has been observed in men with normal spermiogram who are still infertile that the cause could be linked to abnormal sperm DNA [7]. Moreover in the ICSI technique, there is always the risk of using spermatozoa with damaged DNA [8]. A negative correlation between DNA fragmentation (DF), fertilization rate ($r = -0.70$; $p = 0.03$) and embryo quality ($r = -0.70$; $p = 0.03$) after IVF and ICSI has been reported [9]. The percentage of DF was lower in infertile men who initiated a clinical pregnancy after assisted conception [10–12] than in those who did not [13–15].

Moreover, apoptosis is a mode of cellular death that induces a series of cellular morphological and biochemical alterations leading to cell suicide [16, 17]. Recent studies have demonstrated apoptosis-like conditions (or abortive apoptosis), including single-stranded DNA damage, the presence of activated caspases, and externalization of phosphatidyl serine on the sperm plasma membrane in ejaculated human spermatozoa [18, 19]. Chen et al. have assessed the relationship between apoptosis and male infertility, and showed a significant correlation between them. This relation was also observed in a mouse model [20, 21].

In addition, motile sperm are essential for the fertilizing capacity of spermatozoa and depend directly on the activity of the mitochondria [22]. A recent study has shown that the loss of mitochondrial membrane potential (MMP) and decrease in energy may lead to decreased sperm motility [23]. Therefore, dysfunction of MMP can be a symptom of male infertility [24].

Finally, as most available research evaluates the relation of only one intracellular sperm parameter with classical sperm parameters and clinical or laboratory outcomes, this study was setup to assess first; the relationship among different sperm intracellular parameters such as DF, apoptosis, low MMP and routine sperm parameters (motility, normal morphology and sperm concentration) and secondly, the effect of these factors on ICSI outcomes such as; fertilization rate, embryo quality score, embryo cleavage score, and pregnancy rate.

Materials and methods

Sample collection

This study was approved by the local Ethics Committee at Royan Institute (Tehran, Iran) and was conducted from 2010 until 2011 at the Institute's Assisted Conception Unit. During this time, a total of 120 patients signed an approved informed consent and were enrolled in our study. On the day of follicular puncture, semen samples were collected by masturbation into sterile plastic containers after 3–4 days of sexual abstinence. Semen samples were first liquefied at room temperature for 30 min, and then analyzed for sperm concentration and motility by light microscopy. The percentage of morphologically abnormal spermatozoa was evaluated by routine Papanicolaou staining. Samples with volume 1 ml, total motility 2 % and concentration 2×10^6/ml were also excluded from the study.

Semen preparation

In this study, 2 ml of collected samples were divided into two parts. One part was diluted with Hams F10 (Sigma-Aldrich, USA) supplemented with 5 % human serum albumin (HSA, Vitrolife, Sweden). The diluted semen was washed twice by centrifugation for 5 min at 220 g. The supernatant was removed and 1 ml of fresh medium supplemented with 5 % HSA was gently added to the final pellet not disturbing the pellet. The motile spermatozoa were allowed to swim up for 1 h into the overlaying medium at 37° C in a 5 % CO_2 incubator. The motile sperm were used for the ICSI technique. The second part of the sample was centrifuged at $200 \times g$ for 5 min. Then, 10×10^6 sperm of the pellet were resuspended in 0.5 ml of Hams F10 and used for DNA damage, MMP and apoptosis evaluation.

Determination of DNA fragmentation

To assess sperm DNA fragmentation, sperm chromatin dispersion test (SCD) was used according to the previous studies by Fernandez et al. [25, 26]. In brief, an aliquot of a semen sample was diluted in phosphate-buffered saline (PBS) to 10 million/ml. 30 µl of this suspension was mixed with 70 µl of 1 % low-melting point aqueous agarose (Fermentas, Life Sciences, Canada). Then total mixture was put on a glass slide which was coated with 0.65 % standard agarose (Cinagen, M7730, Iran), covered with the coverslip (24–60 mm), and placed at 4 °C for 4 min. After that, in order to make a single-strand DNA motif, coverslips were removed gently and slides were immediately immersed horizontally in a tray with freshly prepared acid denaturation solution (0.08 N HCl) for 7 min at 22 °C in the dark. These slides were respectively transferred to a tray with neutralizing and lysing solution 1 (0.4 M Tris, 0.8 M DTT, 1 % SDS, and 50 mM EDTA, pH 7.5) and neutralizing and lysing solution 2 (0.4 M Tris, 2 M NaCl, and 1 % SDS, pH 7.5) for 10 and 5 min at room temperature. Then slides were washed in tris–borate–EDTA buffer (0.09 M tris–borate and 0.002 M EDTA, pH 7.5) for

2 min, slides dehydrated in ethanol (70, 90 and 100 %) for 2 min each and air dried. Finally slides were stained with Wright solution (Merck, Darmstadt, Germany) and PBS (Merck) at a 1:1 ratio for 5–10 min and washed in tap water then allowed to dry and observed by light microscopy [25].

For this study, a minimum of 200 spermatozoa per sample were scored under the light microscope (100× objectives). According to previous research [26], five SCD patterns were existed: (1) sperm cells with large halos, (2) sperm cells with medium-sized halos, (3) sperm cells with very small-sized halo, (4) sperm cells without a halo, and (5) sperm cells without a halo and degraded. The sperm with large-to-medium size halos were considered as healthy sperm with no fragmented DNA, whereas sperm with small size halos or without a halo and degraded were considered as damaged sperm with fragmented DNA (Fig. 1).

Assessment of mitochondrial membrane potential

JC-1 staining was used to determine MMP by flow cytometry. A stock solution (1 mg/ml) of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylben-zimidazolyl-carbocyanine iodide (JC-1, T3168, Molecular Probes, Eugene, USA) in dimethylsulfoxide (DMSO, D8779, Sigma, USA) was prepared and stored at −20 °C. At the time of testing, one milliliter of the sperm suspension was mixed with 2.0 ml of warmed JC-1 stock solution and incubated for 30 min at 37 °C in the dark. Finally to assess the functionality of MMP the samples were washed in PBS and analyzed by flow cytometry (BD FACS Calibur, Becton Dickinson, San Jose, CA, USA) [27]. In healthy cells with high mitochondrial, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. Whereas in unhealthy cells with low MMP, JC-1 remains in the monomeric form, which shows only green fluorescence. Both JC-1 aggregates and monomers exhibit green fluorescence (peak emission at 527 nm) which is measured in the FL1 channel (530 nm). JC-1 aggregates show a red spectral shift (peak emission at 590 nm), and are measured in the FL2 channel (585 nm) and cells with altered mitochondrial function will remain bright in the FL1 channel, but will have reduced FL2 160 intensity (Fig. 2).

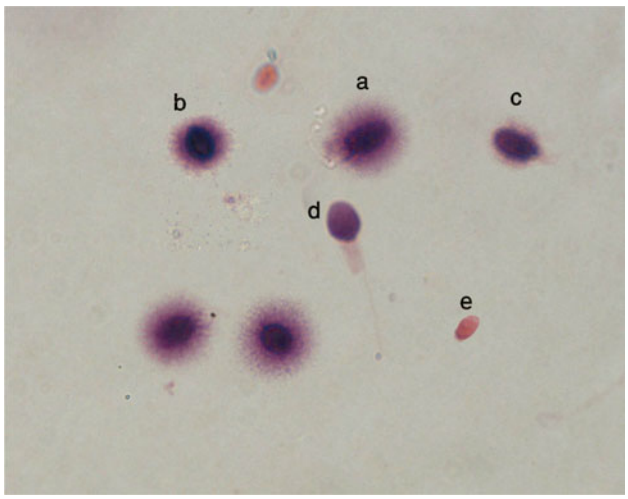

Fig. 1 The sperm chromatin dispersion test sperm with different size halos. Sperm show **a** the nuclei with large size halo, **b** the nuclei with medium size halo that were considered with nonfragmented DNA, whereas **c** the nuclei with small size halo, **d** without halo, **e** without a halo and degraded which were considered sperm with fragmented DNA

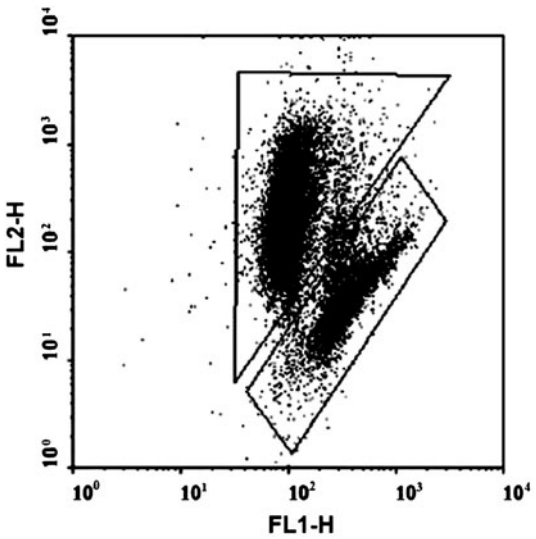

Fig. 2 Scatter gate and sperm cells stained by JC-1. JC-1 aggregates are measured in FL2 channel and sperm with low mitochondrial membrane potential are measured in FL1 channel

Assessment of apoptosis

In this study, apoptosis was assessed using the Annexin V-FITC apoptosis detection kit (APOAF-50TST, Sigma, St. Louis, MO, USA). An aliquot of semen specimen that contained 1×10^6 spermatozoa was washed twice ($300 \times g$, 10 min, 4° C) in PBS. To each cell suspension, 5 µl of annexin V-FITC conjugate and 10 µl of propidium iodide solution was added. After 15 min cells were analyzed by flow cytometry (BD FACS Calibur, Becton Dickinson, San Jose, CA, USA) [28] (Fig. 3).

Ovarian stimulation and conventional ICSI procedure

Ovarian stimulation was carried out using down regulation protocol as previously described [29, 30], and oocyte retrieval was performed by ultrasound-guided follicle aspiration, 36–38 h after hCG administration. The oocytes

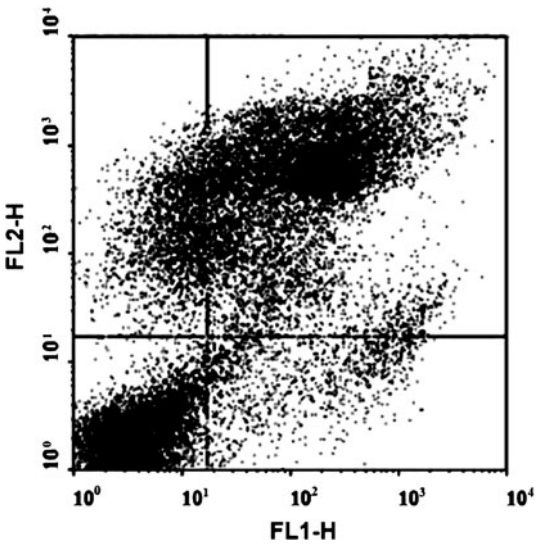

Fig. 3 Annexin V-FITC/PI staining and flow cytometric analysis of amounts of apoptosis in sperm cells. The *x* axis (FL1) reflects annexin V–FITC fluorescence; the *y* axis (FL2) reflects PI fluorescence. In each panel, *lower left quadrant* shows cells which are negative for both annexin V-FITC and PI, *lower right* shows annexin V positive cells which are in the early stage of apoptosis, *upper left* shows only PI positive cells which are dead, and *upper right* shows both annexin V and PI positive, which are in the stage of late apoptosis or necrosis

underwent intracytoplasmic sperm injection (ICSI) and were cultured in G-1 (version 3; Vitrolife, Kungsback, Sweden) supplemented with 10 % recombinant human serum albumin (rHSA; Vitrolife) for 2–3 days. On the day 2 or 3, the growth and quality of each embryo were scored. Finally, dependent upon the patients' embryos; a maximum of two or three embryos with the best morphology and growth rate were selected and cultured for 20 min up to a maximum of 2 h in Embryo-Glue (Vitrolife) prior to embryo transfer. The embryo transfer was then performed with a Labotect catheter (Labotect, Straberg, Germany).

Evaluation of fertilization rate in ICSI patients

In each patient, fertilization rate was defined as the number of normal fertilized oocytes that contained two pronuclei (2PN) divided by the total number of mature oocytes (MII) $\times$ 100.

Evaluation of embryo quality and embryo cleavage score in ICSI patients

In this study, embryos were divided as grade A–D according to certain morphological criteria. Grade A was defined as 7–8 cells with no fragmentation and equal size blastomeres; grade B was defined as 5–6 cells, 10 % fragmentation and equal size blastomeres; grade C was defined as 3–4 cells, 20–50 % fragmentation and non-uniform size blastomeres; and grade D was considered to be 2 cells, 50 % fragmentation with non-uniform size blastomeres. Finally, scores were assigned as follows: 4 (grade A), 3 (grade B), 2 (grade C), and 1 (grade D), and embryo quality score in each patient was determined by dividing the total score of embryos to the total number of embryos.

To score embryo cleavage, the numbers of blastomeres in each embryo were counted and scored on day 2 as follows: grade 4 was an embryo with 5–6 cells; grade 3 was an embryo with 4 cells; grade 2 was an embryo with 2–3 cells; and grade 1 was an embryo with 1–2 cells. Whereas on day 3, embryo growth was scored as follows: grade 4 was an embryo with 8–10 cells; grade 3 was an embryo with 6–7 cells; grade 2 was an embryo with 4–5 cells; and grade 1 was an embryo with 2–3 cells. Finally, embryo cleavage score in each patient was determined by dividing the total score of embryos to the total number of embryos.

Evaluation of pregnancy rate

Patients were tested for serum β-hCG assay 15 days after embryo transfer. If the pregnancy test was positive, the luteal support was continued until 12 weeks of gestation. Clinical pregnancy rate was then defined as the number of pregnant patients to the total number of treated patients.

Statistical analysis

In our study, we determined sample size based on primary information of the correlation among the study variables. A medium to small effect size ($r = 0.25$) was computed based on a pilot study. By considering the confidence level equal to 0.95, a power equal to 0.80, and utilizing G-Power 3.1.2 software (Franz Faul, Universita, Kiel, Germany), the sample size computed at least 95 cases. In consideration of the possibility of some inadequate responses, we increased the sample size to 120 cases. Data analysis was performed using SPSS version 15.0 software. Pearson correlation test was used to assess correlation of DF, apoptosis and MMP with rates of lab and clinical outcomes, and routine sperm parameters. $p < 0.05$ was considered as a significant level.

Result

The minimum, maximum and mean $\pm$ SD of routine semen analysis (concentration, motility, normal morphology), sperm intracellular parameters (early and late apoptosis, MMP, DF), fertilization rate, embryo quality, and cleavage score of 120 patients who were candidate for ICSI treatment are summarized in Table 1. The correlation

Table 1 Mean of sperm intracellular factors, routine classical sperm parameters, fertilization rate, embryo quality, and cleavage score in infertile patients who were candidates for ICSI treatment

Sperm parameters	Minimum	Maximum	Mean ± SD
Sperm concentration ($\times 10^6$/ml)	2	150	46.33 ± 28.88
Sperm motility (%)	2	50	28.88 ± 11.15
Sperm normal morphology (%)	0	20	10.41 ± 5.75
Early apoptosis (%)	0.35	24.02	5.50 ± 4.84
Late apoptosis (%)	2.80	91.93	23.85 ± 15.39
Low MMP (%)	12.74	95.01	48.09 ± 21.15
DF (%)	2	99	27.78 ± 24.63
Fertilization rate (%)	0	100	69.07 ± 28.79
Embryo quality score	1.33	4	2.69 ± 0.39
Embryo cleavage score	1	4	2.05 ± 0.65

Note: Data are shown as mean ± SD

among different sperm factors such as apoptosis, low MMP, DF and classical sperm parameters is also summarized in Table 2. As a result, a significant ($r = 0.475$; $p < 0.001$) correlation was observed between early and late apoptosis. Late apoptosis was positively correlated with low MMP ($r = 0.458$; $p < 0.001$). DF showed a significant direct correlation with late apoptosis ($r = 0.233$; $p = 0.010$) and with low MMP ($r = 0.428$; $p < 0.001$).

Sperm concentration was also negatively correlated with early apoptosis ($r = -0.260$; $p = 0.004$), late apoptosis ($r = -0.210$; $p = 0.021$) and MMP ($r = -0.286$; $p = 0.002$). Moreover, sperm motility showed a negative correlation with early apoptosis ($r = -0.193$; $p = 0.035$), late apoptosis ($r = -0.427$; $p < 0.001$), MMP ($r = -0.479$; $p < 0.001$) and DF ($r = -0.249$; $p = 0.006$). Normal sperm morphology only showed negative correlation with low MMP ($r = -0.498$; $p < 0.001$), DF ($r = -0.261$; $p = 0.004$), early apoptosis ($r = -0.212$; $p = 0.020$) and late apoptosis ($r = -0.262$; $p = 0.004$). Follow-up of patients showed a total number of 27 out of 120 patients who were pregnant with gestational sacs (22.5 %).

As seen in Table 3, no significant correlation was observed between semen parameters (sperm concentration, motility and normal morphology), DF, apoptosis and low MMP of sperm with laboratory ICSI outcome (fertilization rate, embryo quality score, embryo cleavage score) and pregnancy rate, except early apoptosis which showed significant ($r = -0.180$; $p = 0.049$) negative correlation with pregnancy rate.

Discussion

Semen analysis is one of the most popular tests for the evaluation of classical sperm parameters. These tests do not provide any information to specialists about intracellular factors such as DF, MMP reduction, and apoptosis. Knowing this information in association with classical sperm parameters can be useful in predicting treatment results.

Table 2 Correlation among different sperm factors such as; apoptosis, low mitochondrial membrane potential (MMP), DNA fragmentation (DF) and classical sperm parameters in infertile patients who were candidates for ICSI treatment

Sperm parameters	Early apoptosis	Late apoptosis	Low MMP	DF	Sperm concentration	Sperm motility	Sperm normal morphology
Early apoptosis	–	$r = 0.475$	$r = 0.174$	$r = 0.121$	$r = -0.260$	$r = -0.193$	$r = -0.212$
		$p < 0.001$	$p = 0.057$	$p = 0.188$	$p = 0.004$	$p = 0.035$	$p = 0.020$
Late apoptosis	$r = 0.475$	–	$r = 0.458$	$r = 0.233$	$r = -0.210$	$r = -0.427$	$r = -0.262$
	$p < 0.001$		$p < 0.001$	$p = 0.010$	$p = 0.021$	$p < 0.001$	$p = 0.004$
Low MMP	$r = 0.174$	$r = 0.458$	–	$r = 0.428$	$r = -0.286$	$r = -0.479$	$r = -0.498$
	$p = 0.057$	$p < 0.001$		$p < 0.001$	$p = 0.002$	$p < 0.001$	$p < 0.001$
DF	$r = 0.121$	$r = 0.233$	$r = 0.428$	–	$r = -0.100$	$r = -0.249$	$r = -0.261$
	$p = 0.188$	$p = 0.010$	$p < 0.001$		$p = 0.280$	$p = 0.006$	$p = 0.004$
Sperm concentration	$r = -0.260$	$r = -0.210$	$r = -0.286$	$r = -0.100$	–	$r = 0.370$	$r = 0.463$
	$p = 0.004$	$p = 0.021$	$p = 0.002$	$p = 0.280$		$p < 0.001$	$p < 0.001$
Sperm motility	$r = -0.193$	$r = -0.427$	$r = -0.479$	$r = -0.249$	$r = 0.370$	–	$r = 0.617$
	$p = 0.035$	$p < 0.001$	$p < 0.001$	$p = 0.006$	$p < 0.001$		$p < 0.001$
Sperm normal morphology	$r = -0.212$	$r = -0.262$	$r = -0.498$	$r = -0.261$	$r = 0.463$	$r = 0.617$	–
	$p = 0.020$	$p = 0.004$	$p < 0.001$	$p = 0.004$	$p < 0.001$	$p < 0.001$	

Table 3 Correlation between apoptosis, mitochondrial membrane potential (MMP) and DNA fragmentation (DF) with laboratory and clinical ICSI outcome in infertile patients

	Early apoptosis	Late apoptosis	Low MMP	DF	Concentration	Motility	Normal morphology
Fertilization rate	$r = -0.082$	$r = -0.072$	$r = -0.083$	$r = -0.175$	$r = -0.024$	$r = 0.091$	$r = -0.176$
	$p = 0.371$	$p = 0.436$	$p = 0.365$	$p = 0.056$	$p = 0.794$	$p = 0.321$	$p = 0.054$
Embryo quality score	$r = -0.026$	$r = -0.094$	$r = -0.019$	$r = 0.002$	$r = -0.059$	$r = -0.117$	$r = -0.095$
	$p = 0.777$	$p = 0.305$	$p = 0.840$	$p = 0.985$	$p = 0.526$	$p = 0.202$	$p = 0.300$
Embryo cleavage score	$r = -0.098$	$r = -0.091$	$r = 0.011$	$r = 0.055$	$r = -0.052$	$r = -0.091$	$r = -0.113$
	$p = 0.289$	$p = 0.325$	$p = 0.902$	$p = 0.548$	$p = 0.573$	$p = 0.321$	$p = 0.221$
Pregnancy rate	$r = -0.180$	$r = -0.143$	$r = 0.087$	$r = -0.010$	$r = 0.042$	$r = 0.122$	$r = 0.0001$
	$p = 0.049$	$p = 0.119$	$p = 0.344$	$p = 0.916$	$p = 0.650$	$p = 0.183$	$p = 0.992$

In this study, the relationship of DF, mitochondrial defects and apoptosis with classical sperm parameters, fertilization rate, embryo quality score and pregnancy rate have been investigated. Studies regarding sperm parameters and their correlation with evaluated factors have clearly shown that the damage to MMP and increase in membrane defects have important associations with sperm motility and leads to the reduction of motility.

This issue confirms that the mitochondria and electron chain located in its membrane act as energy and ATP source for the sperm [31] and any problems in performances would significantly affect sperm motility. This is also true for sperm morphology; and sperm with worse appearances have shown lower membrane potential than those with good appearances [32].

As the membrane of the sperm contains abundant unsaturated fatty acid (PFU) [33] and also as mitochondrial activity leads to the release of free radicals such as H_2O_2 and O_2, defects in mitochondrial activity and excessive release of ROS could be causes of sperm membrane and morphology destruction [34].

These results are in the line with Espinoza et al. [32] who have shown that the evaluation of the mitochondrial membrane and its potential can be considered a test for semen quality and classical parameters. Barroso et al. [35] have also shown that MMP measurement can be a good test for the quality of human sperm.

In addition, the significant and inverse relationship of DF with motility and morphology was another notable issue in this study. It appeared that samples with fewer cells and lower motility indicate more DNA damage. Giwercman et al. [36] have shown that samples of low-quality semen highly expressed Fas, p53 and had DNA damage. On the other hand, men with normal semen parameters displayed high levels of DF, which could be a reason for unexplained infertility [37]. In contrast to these studies, Sakkas et al. [19] who used the TUNEL assay were not able to find any correlation between sperm DNA damage and motility [32]. Moreover, we should mentioned that for the assessment of sperm DF, both TUNEL and SCD tests are sensitive methods; and it seems that the SCD test gives a similar predictive value for sperm DF when compared with the TUNEL and sperm chromatin structure assays (SCSA) [37].

In our study, a direct correlation between DF and apoptosis, and an inverse correlation between classical parameters and apoptosis confirmed the results of Chen et al. [20] who reported a negative relationship between both motility and apoptosis, and also between morphology and apoptosis, with no relationship between sperm count and apoptosis [20]. However, these authors did not find any relationship between sperm count and apoptosis. Whereas, Oosterhuis et al. [38] observed a significant negative relationship between TUNEL positive cells and sperm concentration and linked low sperm count to high levels of apoptosis [38]. In our study, it has been clearly shown that apoptosis was considered a factor in DF induction and its increase had an obvious impact on the reduction of sperm quality.

Comparison of other intracellular parameters also indicated that sperm with high DF levels had a direct relationship with MMP reduction and late apoptosis. It seems that DNA damage and caspase activity during apoptosis could cause damage to the mitochondrial membrane and reduce its potential.

Finally, the relationship between fertilization rate, embryo quality, and pregnancy rate has been simultaneously studied with DF, MMP, early and late apoptosis and classical parameters. The results with an exception of early apoptosis showed no significant relationship with ICSI outcomes. The latter had no noticeable effects on lab parameters such as fertilization, embryo quality and cleavage rate, but reduced significantly pregnancy rate. The reason for this surprising result could be related to injection of sperm in an early apoptotic phase which is hard to differentiate from normal and even poor quality sperm. In general, as embryologist during ICSI procedure try to select good quality sperm even in poor semen samples, it

may be impossible to find exact correlation between sperm parameters and clinical outcomes in all ART clinics [39, 40]. In addition the capability of oocyte cytoplasm for repairing of sperm DNA damaged, may interfere in our finding and repaired even those injected spermatozoa with intracellular damage such as DF, apoptosis and dysfunction of MMP. This phenomena has also been reported by others [40]. No relationship between sperm parameters and ICSI outcomes has also been reported by other investigators such as Abu-Hassan et al. [41]. The latter investigators observed no relationship between DF and fertilization rates in patients who underwent ICSI [41].

In contrast, Freeman et al. [42] have shown that low-quality sperm can lead to decreased fertility in patients who undergo IVF and ICSI. Zini et al. [43] with a follow-up of ICSI patients have shown that sperm DNA damage is only associated with significantly increased risk of pregnancy loss after IVF and ICSI. The latter investigators in their study in 2005 have also shown that a high percentage of sperm with DNA damage (>30 %) is associated with an increased prevalence of multinucleated blastomeres after ICSI procedure, and they suggested that an increased sperm DNA damage may have an adverse effect on embryo quality [44].

In conclusion, our data indicated that in spite of a negative correlation between DF, apoptosis and MMP with semen parameters and also a negative significant correlation between early apoptosis and pregnancy rate, there was no correlation between these sperm intracellular parameters and ICSI outcomes.

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Conflict of interest None.

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