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Testicular versus ejaculated spermatozoa in ICSI cycles of normozoospermic men with high sperm DNA fragmentation and previous ART failures

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Summary

As a part of male assessment, conventional sperm parameters including morphologic features have been dedicated as major factors influencing fertilisation and pregnancy rates in assisted reproductive technology (ART). Genomic integrity of spermatozoa has also been found to influence fertility prognosis, and hence, sperm DNA fragmentation index (DFI) has been adopted by many centres to document this entity. Despite several suggested approaches, there is lack of universal consensus on optimising fertility outcomes in males with high sperm DFI. In this context, the results from cycles using testicular spermatozoa (TESA) obtained by aspiration were compared with those of ejaculated spermatozoa (EJ) in normozoospermic subjects with high sperm DFI and previous ART failures. Clinical (41.9% versus 20%) and ongoing pregnancy rates (38.7% versus 15%) were significantly better and miscarriages were lower in TESA group when compared to EJ group. Sperm DFI should be a part of male partner's evaluation following unsuccessful ART attempts. When high DFI is detected (>30%), ICSI using testicular spermatozoa obtained by TESA seems an effective option particularly for those with repeated ART failures in terms of clinical, ongoing pregnancies and miscarriages even though conventional sperm parameters are within normal range.

Introduction

Infertility of unknown origin with normal semen is a common clinical problem affecting 6 to 27% of men attempting to conceive (Collins & Crosignani, 1992). In this population, several complementary tests are needed to find the cause, where sperm DNA integrity is an increasingly recognised factor for fertilisation capacity and embryo development (Sakkas & Alvarez, 2010). Despite normal semen parameters, 8% of infertile men have abnormal sperm genomic integrity (Aitken, 2006). Examination of sperm chromatin integrity reveals underlying causes of male fertility dysfunction including single-stranded and double-stranded breaks (Hamada *et al.*, 2012).

In the literature, accumulating evidence suggests examination of the sperm DNA integrity to obtain a healthy pregnancy through assisted reproductive techniques (ART). A recent opinion by the Practice Committee of the American Society for Reproductive Medicine (ASRM) acknowledged that determining the values of sperm DNA fragmentation might be clinically informative for *in vitro* fertilisation/intra-cytoplasmic sperm injection (IVF/ICSI) outcomes (Practice Committee of the American Society for Reproductive Medicine, 2015). The threshold of DNA fragmentation index (DFI) is quite important, as lower rates (<%30) have been strongly related to natural conception and success in intrauterine insemination (IUI) (Evenson & Wixon, 2008), whereas higher rates (>30%) have been linked with decreased pregnancy odds in IVF (Zhao *et al.*, 2014). On the other hand, the results obtained from ICSI cycles were more favourable and independent from DFI (Zhao *et al.*, 2014; Osman *et al.*, 2015). Even in cases with markedly high DFI (>50%), the pregnancy rate with ICSI was found to be similar to cases having lower DFI (15%) (Dar *et al.*, 2013). In ICSI cycles,

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selection of morphologically normal and motile spermatozoa was suggested as a process decreasing the likelihood of DNA-fragmented spermatozoa (Lewis *et al.*, 2013; Maettner *et al.*, 2014; Osman *et al.*, 2015). However, potential success rates offered by ICSI are also limited in terms of miscarriages with high DFI (Zhao *et al.*, 2014). It has been proposed that mature oocytes with functional DNA repair mechanisms have the ability to repair sperm DNA damage to some extent (Brandriff & Pedersen, 1981; Matsuda & Tobari, 1988). However, in the presence of high DFI, oocyte recovery mechanism may be unable to overcome paternal damage and thus embryo quality will be compromised (Sakkas & Alvarez, 2010).

Several approaches have been suggested to overcome high DFI-related drawbacks in ART. Usually, modifiable causes such as varicocele, infections, smoking and chemical intakes are treatable before beginning a new ART cycle. However, when no cause can be found, alternative methods are reasonable to bring DFI under its pathological threshold (ranging between 12% and 36.5% with the TUNEL technique) (Duran *et al.*, 2002; Henkel *et al.*, 2003, 2004). Oral intake of antioxidants (OAs) (Zini *et al.*, 2009; Abad *et al.*, 2013) or use of testicular spermatozoa obtained with either testicular sperm aspiration (TESA) or testicular sperm extraction (TESE) (Greco *et al.*, 2005; Esteves *et al.*, 2015), using recurrent ejaculations before fertilisation (Raziel *et al.*, 2001) or sperm selection techniques including intracytoplasmic morphologically selected sperm injection (IMSI) (Antinori *et al.*, 2008), PICSI (Parmegiani *et al.*, 2012) or magnetic cell sorting (Sharma *et al.*, 2015) has been attempted to enhance outcomes. Yet, none of these interventions, alone or combined, have been unequivocally proven to be of clinical value to overcome the potential detrimental effect of high DFI on ART outcomes (Practice Committee of the American Society for Reproductive Medicine, 2015). In this study, we aimed to compare the pregnancy outcomes of couples undergoing ICSI using either testicular or ejaculated spermatozoa in normozoospermic male subjects with high DFI and previous ART failures. The primary outcome was to compare clinical pregnancy rates (CPR), whereas the secondary outcome was ongoing pregnancy rates (OPR).

Materials and methods

Patient selection

Electronic database of an assisted reproduction centre between April 2014 and November 2015 was analysed to document eligible subjects. According to the IVF centre's policy, DFI was measured prior to a new ART cycle in all male subjects who have had at least 2 previous unsuccessful ART cycles despite normal sperm parameters. All male subjects with high DFI (>30%) were offered to undergo TESA procedure due to the IVF centre's policy to improve the ART outcomes (TESA group) in their subsequent cycle. Remaining subjects who refused to undergo TESA were included as controls (EJ group). The following inclusion criteria were established for the couple: (i) at least 2 unsuccessful ART attempts; (ii) women between 18 and 40 years of age (iii) sperm count with at least 15 million ml^{-1}; (iv) high DFI levels (>30%) in semen specimens at admission. An unsuccessful ART attempt was defined as failure to achieve pregnancy despite the transfer of at least one good-quality embryo in a fresh ART cycle.

Exclusion criteria were included: (i) oligozoospermia (<15 million ml^{-1}) and azoospermia; (ii) male subjects with obvious abnormalities noted in their medical history, physical examination, endocrine profile and with evidence of subclinical genital infections, leukocytospermia, cryptorchidism, cancer, chemotherapy or radiotherapy history or severe varicocele; (iii) women with a history of poor response to ovarian stimulation or fulfilling the Bologna criteria for expected poor responders (Ferraretti *et al.*, 2011); (iv) pre-implantation genetic screening (PGS), cryopreserved/thawed embryo transfer cycles; (v) apparent uterine or tubal pathology; (vi) subjects with genetic disorders; (vii) male subjects defined as heavy smokers (>20 cigarettes/day), (viii) couples with fertilisation failure history. There were no oocyte donation cycles included in the analysis. Cycle cancellations were performed due to lack of ovarian response, or no available embryos for transfer. Pregnancy rates per started cycle were recorded for all subjects.

Sperm collection and DNA fragmentation analysis

All parameters were evaluated during initial admission. Basic sperm parameters (concentration, motility and morphology) were evaluated in all samples according to the World Health Organization criteria (WHO, 2010). The materials were then processed for the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay, which was performed with tetramethylrhodamine-labelled dUTP and according to the manufacturer's instructions. Briefly, the procedure includes fixation, permeabilisation, labelling with TUNEL mixture and analysis steps. Each sample was washed three times in phosphate-buffered saline (PBS). Each suspension was transferred into a microwell plate, where the fixation solution composed of paraformaldehyde was added. After incubation period, permeabilisation solution containing Triton X was used during resuspension. Labelling was performed with manufacturer's specific 'TUNEL

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reaction mixture'. For internal control procedure, both negative and positive samples were prepared in each reaction set, using agent causing extreme DNA fragmentation. Analysing step involved fluorescence microscopic (Olympus BX61 with Bioview automated imaging analyser) approach to document the percentage of cells with positive apoptotic signal (Tesarik *et al.*, 2004). On each slide, approximately 300 to 500 cells were counted, and the percentage of spermatozoa with fragmented DNA (DFI) was calculated.

TESA procedure

Local anaesthetic (2% prilocaine, 5 ml) was injected into the area around the spermatic cord. Aspiration was then performed in the centre as well as in the upper and lower poles of each testicle using a 23-G needle. Constant negative pressure was applied to the syringe when the needle reached the centre of the testis and aspiration was performed at different angles in each puncture location. Testicular sperm aspiration was performed unilaterally in all patients. As there was no significant difference between two testes in physical examination, the right testis was chosen for TESA depending on the surgeon's routine preference. When adequate number of spermatozoa with normal morphology was obtained, it was accepted as a successful TESA procedure and terminated. The aspirated tissue from each location was placed on a separate slide, air-dried and stained with May-Grünwald Giemsa. Pathologic investigation was not routinely performed. The same senior urologist performed all TESA procedures.

Ovarian stimulation protocol

All couples were subjected to ICSI, and all sperm injections were performed with fresh specimens. One ART cycle of each patient was included in the study. Ovarian stimulation was carried out using recombinant FSH (Gonal-F, Merck Serono, Istanbul) with or without human menopausal gonadotropin adjunction (hMG) (Menogon, Ferring, Istanbul) beginning from the second day of the menstrual cycle with a starting dose ranging between 150 and 300 IU day^{-1} according to the patient's ovarian reserve. GnRH antagonist (Cetrotide, Merck Serono, Istanbul) was introduced (0.25 mg day^{-1}) on the sixth day (fixed antagonist protocol) and continued throughout ovarian stimulation. When at least three follicles were ≥ 18 mm, recombinant human chorionic gonadotropin (rhCG) (250 μg; Ovitrelle, Merck Serono, Istanbul) was used for final oocyte maturation. Transvaginal ultrasound-guided oocyte retrieval and embryo transfer procedure were performed as described elsewhere (Pabuccu *et al.*, 2015). Clinical pregnancy was defined as the presence of a gestational sac with an embryonic pole and a positive heart beat at 7 weeks of gestation, and an ongoing pregnancy was defined as the presence of an intrauterine sac with an embryonic pole demonstrating cardiac activity at 10 weeks of gestation.

Statistical analysis

Data analysis was performed using SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, United States). Continuous data were described as mean ± SD (standard deviation) for normal distributions and median (minimum and maximum value) for skewed distributions. Categorical data were described as the number of cases (%). Mean differences between groups were compared by Student's t–test, whereas Mann–Whitney U-test was applied for the comparison of median values. Nominal data were analysed by Pearson's chi-square or Fisher's exact test, where applicable. Correlation analysis was conducted using a Spearman's rank test. A p value less than 0.05 was considered statistically significant.

Results

A total of 92 couples who had undergone at least two unsuccessful ART cycles and whose male partner had high sperm DFI (>30%) despite normal semen count were selected out of 1675 total admissions during the study period. Unexplained infertility was the only indication for all couples. A total of 71 subjects fulfilled the inclusion criteria (71/92), and testicular spermatozoon was used in 31 of them during ICSI. The remaining 40 couples refused to undergo TESA, and ICSI was carried out with ejaculated spermatozoa (EJ group).

Basal demographic characteristics including age, BMI, infertility duration, AFC and serum AMH levels were similar as shown in Table 1. The total progressive motile sperm count (TPMSC), number of subjects with sperm morphology >4% and mean DFI (%) of groups are also comparable (Table 1). Cycle characteristics and cancellations were similar between the groups (Table 2). Despite similar fertilisation and implantation rates, pregnancy outcomes in terms of CPR and OPRs were significantly higher in TESA group when compared to controls (Table 3). According to Spearman's correlation analysis, in the overall group, a significant *negative correlation* between sperm DFI% and spermatozoa with normal morphology% ($r = -0.625$, $P < 0.001$) was found as shown in the scatterplot graph (Figure 1).

Discussion

The goal of sperm DFI measurement is to add more prognostic information to the counselling and planning

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Table 1 Demographic characteristics of groups

	TESA (*N* = 31)	EJ (*N* = 40)	*P* Value
Age (years) Mean±SD	33.0 ± 3.9	33.9 ± 3.7	0.348
BMI of women (kg m^{-2}) Mean±SD	24.3 ± 2.9	25.1 ± 2.2	0.227
No of previous attempts, n Mean±SD	3.2 ± 1.6	4.0 ± 1.2	0.018*
Duration of infertility (years) Mean±SD	8.2 ± 2.8	8.8 ± 3.4	0.453
AFC (in both ovaries), n Mean±SD	8.3 ± 2.2	7.8 ± 2.6	0.286
AMH (ng ml^{-1}) Mean±SD	2.2 ± 0.8	2.0 ± 1.0	0.338
TPMSC ($\times 10^6$) Median (min–max)	54.7 (5–200)	45 (11–87)	0.424
No of subjects with sperm morphology >4%[a] n (%)	12 (38.7)	17 (42.5)	0.513
DFI (%) Mean±SD Median (min–max)	44.8 ± 10.8 41 (32–71)	41.7 ± 8.2 41.5 (30.7–65.6)	0.177

TESA: testicular sperm aspiration, EJ: ejaculated spermatozoa, BMI: Body mass index, AFC: Antral follicle count, AMH: Anti-Mullerian Hormone, TPMSC: total progressive motile sperm count, DFI: DNA fragmentation index. Results are given in terms of mean (±SD) or median (min–max) (independent sample t-test, Mann–Whitney U-test). [a]According to Kruger's criteria. **P* < .05.

Table 2 Ovarian stimulation outcomes of groups

Mean±SD	TESA (*N* = 31)	EJ (*N* = 40)	*P* Value
Duration of ovarian stimulation (days)	10.9 ± 1.5	11.6 ± 1.5	0.084
Peak serum estradiol (pg ml^{-1})	2423 ± 840	2388 ± 1076	0.888
Serum progesterone on the day of hCG (ng ml^{-1})	0.79 ± 0.29	0.78 ± 0.67	0.885
Peak endometrial thickness (mm)	10.3 ± 1.8	10.10 ± 1.44	0.541
No of retrieved oocytes, n	8 ± 3.2	7.8 ± 2.3	0.821
Mature oocytes (MII), n	6.4 ± 3.1	5.5 ± 2.6	0.206

HCG: human chorionic gonadotropin. Results are given in terms of mean (±SD).

Table 3 Reproductive outcomes of groups

	TESA (*N* = 31)	EJ (*N* = 40)	*P* Value
Fertilisation (%) Mean±SD	74.1 ± 20.7	71.1 ± 26.9	0.619
Implantation (%) Mean±SD	24.7 ± 33.2	15.00 ± 30.3	0.205
Clinical pregnancy rate per started cycle, n (%)	13 (41.9)	8 (20)	0.045*
Ongoing pregnancy rate per started cycle, n (%)	12 (38.7)	6 (15)	0.023*
Miscarriages, n (%)	1 (3.2)	7 (17.5)	–
Cycle cancelations, n (%)[a]	1 (3.2)	5 (12.5)	–

ET: Embryo transfer. **P* < 0.05. [a]all cycle cancellations were after oocyte retrieval due to poor embryo quality.

process, so as to guide couples particularly those with repeated unsuccessful ART attempts. Even though a threshold value is yet to be elucidated, depending on the unfavourable reproductive outcomes of men with high DFI, different approaches have been applied up to date. In this study, we documented significantly favourable reproductive outcomes when testicular spermatozoon was used during ICSI than those of ejaculated spermatozoa in normozoospermic men with high DFI.

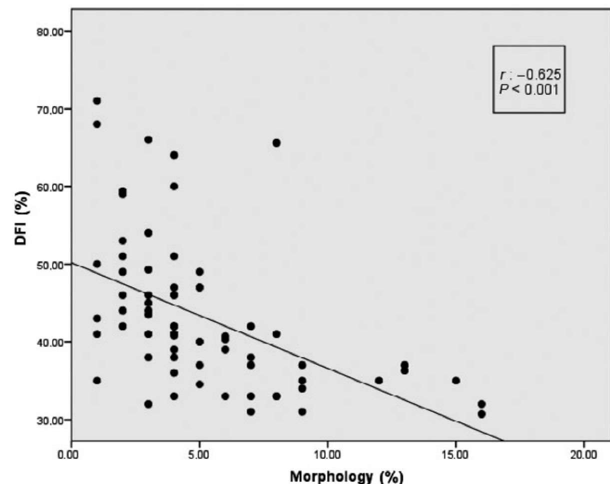

Fig. 1 Scatterplot Showing Correlation Between DFI (%) and sperm morphology (%).

To overcome high DFI-related drawbacks, ICSI with ejaculated spermatozoa has been used. This technique solely revealed 47.9% clinical (Bungum *et al.*, 2007) and

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20.6% ongoing (Speyer *et al.*, 2010) pregnancies in cases with high DFI (>30%) utilising sperm chromatin structure assay (SCSA). In idiopathic infertility with high sperm DFI (25–50%), 38.2% of clinical pregnancies was achieved using single cell gel electrophoresis (COMET) assay (Simon *et al.*, 2013). In our study, when compared to the literature, slightly lower implantation (15%) and ongoing pregnancies (20%) were detected with TUNEL assay in EJ group. These results could be attributed to our study population as all subjects had at least two unsuccessful ART attempts despite ICSI with ejaculated spermatozoa and transfer of good-quality and quantity embryos in the presence of a young female partner. Based on these characteristics, reproductive outcomes with ejaculated spermatozoa are not satisfactory in men with high DFI particularly in those with repeated ART failures.

Spermatozoa retrieved from the testis tends to have better DNA quality compared with ejaculated spermatozoa (Greco *et al.*, 2005; Moskovtsev *et al.*, 2010). As previously reported, post-testicular spermatozoa has higher DFI induced by oxygen radicals during passage of the spermatozoa through the seminiferous tubules and the epididymis (Sakkas & Alvarez, 2010). Supporting these data, ICSI with testicular spermatozoa yielded better implantation and pregnancy rates compared to ejaculated spermatozoa where both testicular and ejaculated spermatozoa revealed good-quality embryos (Greco *et al.*, 2005). Relatively higher clinical pregnancies (40%) were reported in patients with high DFI (Sakkas & Alvarez, 2010). Two very recent reports demonstrated that testicular spermatozoon was associated with higher CPRs and live birth rates compared to ejaculated spermatozoa, revealing at least 50% CPR in both studies (Esteves *et al.*, 2015; Mehta *et al.*, 2015). In our study, 41.9% CPR was detected in TESA group that is comparable but quite lower than those in the literature. This discrepancy is dedicated to our study population as all subjects had previous unsuccessful ART attempts. Moreover, higher number of retrieved oocytes (>10) in the above-mentioned studies contributes to more favourable results when compared to ours (<10 oocytes in all groups).

Increased sperm DNA damage has been associated with a 'late paternal effect' during the activation of male gene expression contributing to increased odds of miscarriage (Tesarik *et al.*, 2004). Successful fertilisation is more likely with ICSI rather than IVF, but miscarriage risk is still evident. In a recent meta-analysis, significantly increased miscarriage rate was reported even after ICSI in the presence of high sperm DNA damage (OR 2.68; 95% CI: 1.40–5.14; $P = 0.003$) (Zhao *et al.*, 2014). In our study, higher miscarriages were detected in EJ group when compared to TESA results that are quite comparable with those of Esteves *et al.* (2015). Similarly, Mehta *et al.* reported no single abortion in their cohort using testicular spermatozoa undergoing ART (2015). Based on these data, ICSI with testicular spermatozoa rather than ejaculate is associated with decreased odds of miscarriages in those with high DFI.

DNA repair ability of the oocyte is another important aspect in the presence of high sperm DFI. This ability likely depends on the quality of oocytes during ART (Meseguer *et al.*, 2011), and younger women may take the advantage of providing good-quality oocytes. In our study, relatively younger age of the female partner may be the possible explanation for 29.6% clinical pregnancies despite previous failed ART attempts. However, the exact capability of oocytes in the repair process or to what extent are still obscure points.

The ICSI procedure requires special attention to select optimal spermatozoa to be microinjected with better chances of embryological and clinical outcomes. Among the sperm processing techniques, O'Connell *et al.* (2003) determined that discontinuous gradient centrifugation (DGC) could select spermatozoa with lower nuclear damage. In addition, sperm DNA fragmentation significantly decreases ($P < 0.0001$) throughout the steps of semen preparation (fresh, after gradient centrifugation, washing and swim-up) in ICSI cycles (Sá *et al.*, 2015). In our clinic, DGC followed by swim-up is sequentially used for sperm processing for ICSI, which is previously documented as an effective method for decreasing sperm DNA fragmentation (Sá *et al.*, 2015).

Normal morphology of the spermatozoa is regarded as a surrogate marker for the quality of spermatogenesis. Poor morphology of spermatozoa is highly associated with increased DNA fragmentation (Moskovtsev *et al.*, 2009), supported by the results obtained from our study. Besides this clear converse correlation, particularly in those with infertility history, altered genomic integrity could also be the fact of apparently normal spermatozoa (Avendaño *et al.*, 2009). Moreover, in infertile population with normal sperm morphology, a highly significant negative correlation was also found between the percentage of spermatozoa with fragmented DNA and embryo quality (Avendaño *et al.*, 2010). As a result, novel techniques that allow accurate separation of viable and morphologically normal spermatozoa with intact DNA should be sought for optimising ICSI outcomes.

Another important point is the safety issues of the TESA procedure. The retrieval of testicular spermatozoa mandates a surgical intervention and provides potential risks such as bleeding, infection and impaired testicular function. In the current study, no single complication was noted within TESA procedure underlining the importance of adequate clinical experience to perform the procedure.

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Even though the relationship between DFI and male infertility is well documented, it seems that there is still a subject of controversy in daily practice. Even if the major limitation of the current study is lack of randomisation, which limits the scope of the conclusions, we infer that sperm DFI should be a part of male partner's evaluation following failed ART cycles despite normal sperm parameters unless previously documented. When high DFI is detected, testicular spermatozoa should be considered for ICSI rather than ejaculated spermatozoa in normozoospermic men with previous failed ART attempts.

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