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Association of sperm DNA fragmentation index with demographics, semen parameters and outcomes of infertile male with repeated intrauterine insemination failure referred for intracytoplasmic sperm injection: a cohort study

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ABSTRACT

Objective. Integrity of sperm DNA was linked to successful intracytoplasmic sperm injection (ICSI) outcome. Sperm DNA fragmentation index (DFI) is a method to assess spermatozoa function and DNA damage. The current study examined DFI association with the demographic and seminal fluid parameters among males with mild oligo/asthenozoospermia, and we looked for clinical implications of DFI on the outcome of ICSI (in terms of fertilization rates, number of good quality embryos, and clinical pregnancy).

Patients and Methods. A prospective cohort study recruited 73 infertile couples with mild oligo athenospermia referred after repeated IUI failure for ICSI. Three sets of data were recorded: demographic criteria including male age, smoking status, and body mass index (BMI), Semen samples analysed for standard seminal fluid analysis, and DFI, which was categorized into 3 sub-groups: ICSI outcome was recorded

Results. DFI was positively correlated with age and BMI. Motile and morphologically normal sperms correlated moderately and negatively to DFI ($r = -0.45, -0.41$). Progressively motile and morphologically normal sperm showed a moderate negative relationship to DFI ($r = -0.45, -0.41$) $p < 0.0001$, 0.03, sluggish and immotile sperm showed a weak positive statistically meaningful link to DFI ($r = 0.3, 0.31$). Low DFI cases had the highest fertilization rates and numbers of good-quality embryos. Moderate and high DFI categories were highest in the negative pregnancy group; $p < 0.0001$.

Conclusions. Meaningful link of DFI to infertile male demographic and seminal fluid parameters allows reducing modifiable factors and selecting best sperm candidate to improve ICSI outcome. Further studies are needed to unveil diagnostic, therapeutic and prognostic implication of DFI.

INTRODUCTION

Infertility is a common problem that accounts for approximately 15% of the general population and means the failure to achieve a clinical pregnancy after 12 months of unprotected sexual intercourse [1]. It can be caused by male factors, female factors, or unexplained infertility. About one-third of infertility cases are attributed to sperm defects [2]. Fertilization involves integrating the DNA genetic

material within the sperm and egg cells. Chromatin integrity is a pivotal requirement in transferring genetic code safely to guarantee adequate and healthy embryonic development from implantation to full-grown foetus [3, 4].
Sperm chromatin fragmentation is associated with a reduction in the reproductive capacity of human spermatozoa [5]. It appears that higher sperm chromatin fragmentation is negatively linked to sperm parameters such as motility and morphology [6]. Earlier works discussed that DNA fragmentation rates of infertile patients were significantly higher than those of fertile couples, and it inversely impacts the clinical outcomes for Assisted Reproductive Technique (ARTs) [7, 8]. Similarly, a recent meta-analysis found that sperm DNA damage is associated with a lower pregnancy rate in IVF/ICSI [9].
Sperm DNA damage is multifactorial, including genetic, environmental, and extrinsic factors (such as recreational drugs, smoking, bad working environment, obesity, and the presence of testicular varicocele) all increase the risk of sperm DNA damage [10,11]. Identifying those risk factors is of utmost importance to predict the outcome of pregnancy [12]. Several methods can be utilized to determine DNA damage in spermatozoa; one is the sperm DNA fragmentation index (DFI) [13], which examines the functional assessment of male sperm fertility. Other methods include (chromatin structure assay (SCSA) testing, Terminal deoxynucleotidyl transferase dUTP, neck end labelling (TUNEL) assay, and Single-cell gel electrophoresis (COMET) assay) [12, 13].
The DFI identifies potential damage to the sperm based on its DNA integrity and damage; it is regarded as an important indicator of semen quality assessment quality [14].
The present study aimed to evaluate some important risk factors associated with increased DFI and the clinical implications of DFI on the outcome of ICSI.

PATIENTS AND METHODS

Study design

This prospective cohort study was conducted at the Infertility Center in Kamal Al-Samarrai Hospital – Centre for Infertility Diagnosis and Assisted Reproductive Technologies in Baghdad, Iraq, among patients recruited via simple random sampling. The study was performed from April 2023 to April 2024. The Ethics Committee/College of Medicine; Mustansiriyah University (IRB No. 44 / January / 2023). All participants gave written consent.

Study population

Seventy-three infertile couples with male factor infertility, specifically the seminal fluid analysis mild oligo/asthenozoospermia in the whole sample, came to the outpatient clinic, and the decision for the ICSI program was made. Three sets of data were recorded: demographic criteria, including age, smoking status, and male body mass index (BMI). Semen samples were collected and analysed for standard seminal fluid analysis and DFI. Finally, the outcome of ICSI was recorded (fertilization rates, number of good quality embryos and clinical pregnancy rates).

Inclusion criteria

All female partners were nulliparous, non-smokers, with an age range from 20-40 years, and had a BMI between 18.5-30 kg/m^2. All were ovulating females with patent fallopian tubes, previous 3 intrauterine insemination failures (IUI) failure.

Exclusion criteria

1. Any cause of female factor infertility was excluded; those with previous uterine surgery or smokers were omitted.
2. Male partners who gave testicular sperm or who had diabetes or other systemic illnesses, varicocele, prostatitis, fever, medications, recent exposure to X-rays, drug abuse, and exposure to toxic chemicals. Full histories were recorded for both couples, specifically regarding the duration and causes of infertility. Drug histories were also evaluated. A pelvic examination was also performed. On day 2 or 3 of the cycle, hormonal investigations were done on the female partner.

Patient and public involvement

Iraqi female attending the Infertility centre in Kamal Al-Samarrai Hospital – Centre for Infertility Diagnosis and Assisted Reproductive Technologies in Baghdad, Iraq.

Semen examination and analysis

Semen samples were collected by masturbation after abstaining for three to five days. The sperm concentration and motility of all samples were assessed based on the World Health Organization (2010) guidelines [16]. Semen parameters including

(Semen volume (ml) after liquefied, concentration (10^6/ml), progressive motility (%), non-progressive motility (%), immotile sperms (%), morphologically normal sperms (%), and number of round cells (10^6/ml)

DNA fragmentation test (DFI)

Semen samples were analysed for fragmented DNA by Sperms DNA fragmentation kit (DFI) test based on the sperm chromatin dispersion (SCD) technique, provided by halo view DNA Fragmentation Kit; Shivani Scientific Industries (India). It involves a controlled DNA denaturation process to facilitate the subsequent removal of spermatozoon-containing proteins. In this way, the normal spermatozoa will create halos formed by loops of DNA at the head of the sperm, while sperms with damaged DNA will not form such halos. DFI was categorized in the following groups: low (15%), medium (15-30%), and high more than (30%).

Test summary

The semen sample is prepared as follows:

1. Prepare a working solution of Giemsa from the concentrated stock solution (Distilled water, Phosphate Buffered Saline, and Giemsa Stock Solution).
2. Sample Preparation: Immediately prepare a smear of the semen-agarose mixture on the agarose-coated slide and place the coverslip. Keep the slide at 4 °C to allow the smear to solidify.
3. Denaturation & Lysis: Overlay with Slide off the coverslip, then overlay with denaturation solution, then with distilled water, then buffer solution.
4. Fixing & Staining.
5. Observe the slide under a compound microscope at 40X Brightfield.

DNA Fragmentation Index (DFI %) = DNA Fragmented Spermatozoa / Total Sperm Counted (Minimum 200) Brightfield [17].

IVF outcome

Seventy-three female partners whose male partners had asthenospermia are (underwent an ICSI program by GnRH antagonist protocol and Fresh embryo transfer (ET). After ovum pickup and assessment of the oocyte and embryo. The number, quality, and maturity of the oocytes retrieved were assessed according to the ESHRE grading system [18]. Oocytes were inseminated using ICSI sperm injection (16-18 h). After oocyte injection by sperm, the fertilization rate in ICSI (formation of the pronuclei per 100 oocytes injected) was assessed [19]. After that, the embryo quality was assessed during development on day 3 before ET, according to Gardner and Schoolcraft in 1999 [20]. 1-3 embryos were transferred approximately 48 h (6-8 cell stages) after fertilization according to ASRUM embryo transfer guidelines according to the patient's age and embryo quality [21].

Follow-up of the study groups

Luteal phase support with 400 mg progesterone vaginal suppositories inserted twice per day for 2 weeks (Cyclogest pessaries, L.D. Collins & Co. Ltd. UK) on the oocyte retrieval day and continued for up to 12 weeks of pregnancy. Clinical pregnancy was defined as a positive pregnancy test with ultrasound visualization of cardiac activity of the intrauterine gestation sac [22].

Statistical analysis

The data normality was checked using the Shapiro-Wilks test. Student t-test and least significant difference-LSD were used to compare significant differences across different means. The chi-square test was used to compare significant differences between percentages. Pearson's correlation test examined the strength of association between different variables. All tests used the Statistical Analysis System- SAS (2018) program. A P-value of less than 0.05 was set as the level of significance for all tests.

RESULTS

In **Table 1**, the study demographic criteria with respect to DFI categories were described. Cases with low DFI were significantly higher among age groups > 40 years. Cases with moderate and high DFI were significantly higher among cases aged 30-39 years; $p < 0.0001$. Cases with normal BMI had the highest percentage of low DFI and high DFI, while overweight cases scored highest in moderate DFI. All the differences were highly significant, with $p < 0.0001$. Non-smokers had the highest percentage of low DFI, while smokers had the highest percentage in moderate and high DFI with $p < 0.0001$.

Table 2 was correlated with the age and BMI of the study participants, showing a significant positive relationship ($r = 0.46, 0.52$) $p < 0.05$, respectively.

Table 1. *Distribution of study participants by demographic characteristics.*

Parameters	Low DFI (< 15%)	Moderate DFI (15 – 30%)	High DFI (> 30%)	P- value
Age (Year)				
< 30	0 (0.00%)	10 (13.70%)	5 (6.84%)	
30- 39	2 (2.73%)	26 (35.62%)	12 (16.44%)	0.0001**
≥ 40	3 (4.11%)	8 (10.96%)	7 (9.59%)	
BMI Level (kg/m^2)				
Normal	3 (4.11%)	7 (5.59%)	10 (13.70%)	
Overweight	2 (2.74%)	25 (34.25%)	6 (8.22%)	0.0001**
Obese	0 (0.00%)	12 (16.441%)	8 (10.96%)	
Smoking status				
Current smoker	0 (0.00%)	31 (42.47%)	13 (17.81%)	0.0001**
Non-smoker	5 (6.84%)	13 (17.81%	11 (15.07%)	

**p ≤ 0.01.

Table 2. *Correlation of DFI versus demographic, seminal, and ICSI outcome.*

Variable	DFI (%)	
	r	P-value
Demographic criteria		
Age (Year)	0.46	0.001
BMI (kg/m^2)	0.52	0.001
Semen parameters	3 (4.11%)	8 (10.96%)
Semen volume (ml)	0.03	0.817
Concentration (106/ml)	-0.16	0.174
Progressive motility (%)	-0.45**	0.0001
Non-progressive motility (%)	0.30**	0.009
Immotile (%)	0.31**	0.008
Normal morphology (%)	-0.41	0.03
Round cell (106/ml)	0.17	0.143
ICSI Outcome		
Clinical pregnancy	-0.11 NS	0.317
Fertilization rate	0.002 NS	0.991
No. of Grade I embryo	0.08 NS	0.477

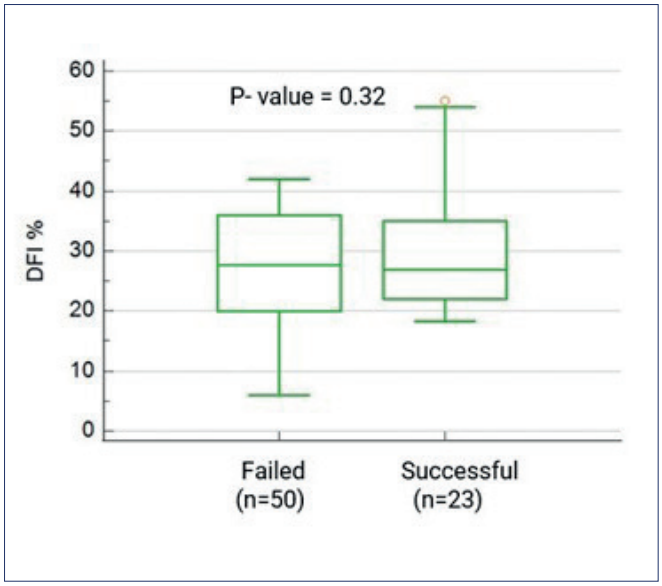

Figure 1. *No statistically significant values were found in DFI levels between cases with negative and positive pregnancy at ICSI cycles.*

The correlation of DFI *versus* seminal fluid parameters showed an insignificant positive link to seminal volume and round cells (r = 0.03, 0.17) p > 0.05. Sperm concentration showed an insignificant inverse link to DFI (r = -0.16) p > 0.05. The percentage of progressively motile sperm and sperm morphology showed a moderate negative relationship to DFI (r = -0.45, -0.41) p = 0.0001, p = 0.03, while the percent of non-progressive and immotile sperm showed a weak positive statistically meaningful link to DFI (r = 0.3,0.31), p = 0.009, 0.008, respectively.

Age and BMI had no impact on ICSI outcomes as p > 0.05 while smoking status significantly impacted the number of grade 1 embryos. Likewise, the seminal fluid concentration, volume, and the number of progressive motile sperms showed an insignificant effect on ICSI outcome; p > 0.05. The percentage of morphologically normal sperms significantly improved fertilization rates and the number of good-quality embryos with p < 0.049 and 0.024, respectively. Finally, cases with low DFI had the highest fertilization rates and numbers of good-quality embryos with p < 0.042 and 0.0006, respectively (**Table 3**).

The effect of the DFI category on clinical pregnancy was interesting, as shown in **Table 4**. The highest percent (72.91) of cases who achieved pregnancy were those with moderate DFI with a mean (of 18.44 ± 1.08), followed by those with high DFI (17.39%) with a mean (of 34.75 ± 2.05).

The highest percentage of couples who failed to achieve pregnancy were those who had moderate

Table 3. *The distribution of social characteristics and seminal fluid analysis according to fertilization and number of good quality embryos.*

Variable		Fertilization rate Mean ± SD	P-value	No. of Grad 1 embryo Mean ± SD	P-value
Age (years)					
< 30		68.74 ± 2.80	0.865	5.40 ± 1.10	0.217
30-39		65.93 ± 3.02		3.67 ± 0.46	
≥ 40		67.19 ± 4.09		3.72 ± 0.44	
BMI Level (Kg/m^2)					
Normal		66.11 ± 2.79	0.587	4.45 ± 0.93	0.078
Overweight		68.22 ± 3.91		3.18 ± 0.31	
Obese		65.21 ± 2.20		5.05 ± 0.74	
Smoking status					
Current smoker		65.99 ± 2.87	0.521	3.50 ± 0.31	0.0358 *
Non smoker		68.07 ± 2.58		4.86 ± 0.76	
Seminal fluid parameters					
Concentration (106/ml)	> 15	61.62 ± 2.73	0.09	4.69 ± 0.31	0.06
	< 15	67.94 ± 2.35		3.90 ± 0.43	
Volume	> 1.5 cc	-	--	-	---
	< 1.5 cc	66.81 ± 2.01		4.04 ± 0.36	
Progressive motility (%)	> 32	63.39 ± 3.70	0.25	3.17 ± 0.28	0.08
	< 32	68.39 ± 2.37		4.44 ± 0.51	
Normal morphology (%)	> 4	75.56 ± 7.83	0.0498*	2.33 ± 0.37	0.024*
	< 4	65.58 ± 1.98		4.28 ± 0.40	
DFI category					
Low (< 15%)		77.60 ± 9.17		4.58 ± 0.65	
Moderate (15-30%)		64.76 ± 2.63	0.0415 *	4.09 ± 0.46	0.0006 **
High (> 30%)		68.34 ± 3.14		1.00 ± 0.00	

BMI: body mass index; DFI: DNA Fragmentation Index.

Table 4. *Association between DFI fragmentation category and clinical pregnancy.*

DFI Category DFI Category	Positive clinical pregnancy (n = 23)		Negative clinical pregnancy (n= 50)		P-value
	No. (%)	Mean ± SD	No. (%)	Mean ± SD	
Low (< 15%)	2 (4.70)	9.53 ±0.61	3 (6.00%)	11.84 ±0.73	
Moderate (15 – 30%)	16 (72.91%)	18.44 ±1.08	27 (54.00%)	23.08 ±1.69	0.0001**
High (> 30%)	4 (17.39%)	34.75 ±2.05	20 (40.00%)	37.22 ±2.63	

DFI (54%) with a mean (23.08 ± 1.69), followed by high DFI cases (40%) with a mean (37.22 ± 2.63). Although the DFI category affected ICSI outcome yet the mean DFI % across non-pregnant and pregnant did not. In **Figure 1** the mean DFI% level was statistically insignificant between the two, with a p = 0.32.

DISCUSSION

Most of the infertile men in the current study had moderate DFI (60.3%), followed by high DFI (32.9%) finally, and 6.8% of them had low DFI. In correlation analysis, DFI showed significant positive correlations with advancing age and BMI, which aligned with earlier works [23, 24].

The relation with age can be attributed to various oxidative stress exposures, environmental pollution, infection, and smoking, which inversely affect sperm integrity [25-27].

BMI's relationship to increasing DFI was controversial among scholars; a recent metanalytic study declared that the evidence behind that link is insufficient and requires further work [28].

Study strengths

The present study highlighted DFI's vital role in male fertility among Iraqi males with strict inclu-

sion and close follow-up. Moreover, it has included many confounders that inversely affect fertility, including age, BMI, and smoking.
Furthermore, the study comprehensively discussed the advantages of DFI in conjunction with SFA to shed more light on its roles. DFI can help select the best sperms and embryos that have the lowest DFI, which improves success odds for the ART technique. It can spot couples that are at risk of fertilization failure, allowing an explanation or insight for those with repeated pregnancy losses or failed implantation. Moreover, it allows therapeutic interventions; many aggress that DFI can be reduced with antioxidants, changing lifestyles, and quitting smoking, allowing a tailored ART strategy. The reduction of OS in couples with high DFI upscales their chances of conception both naturally or by assisted reproduction technique. Thus, DFI has a valuable diagnostic, therapeutic, and prognostic role in male fertility.

Study limitations
Since the inclusion was so tight, the sampling size was relatively small. A multicentric study would bring better insight. Finally, the study type is another limitation; we recommend a case-control to verify DFI impact in normospermia males.

Interpretation and comparison with other literature
Both paternal age and BMI failed to have a meaningful relationship to fertilization rates and the number of good quality embryos. As for smoking, it did not affect fertilization rates; however, it reduced the number of good-quality embryos. Taniguchi *et al*. discussed that paternal smoking will not affect fertilization and the number of good-quality embryos; still, it can affect the timing of the early stages of embryonic life [29].
The current data showed a negative association of DFI with sperm progressive motility and morphology, which was in line with Lourenço metanalysis, which declared that higher oxidative stress was related to higher DFI. A dysfunctional mitochondrial function and higher rates of apoptosis among sperms with higher DFI were suggested underlying causes [30].
Ferrigno *et al*.'s [31] results confirmed that abnormal sperm morphology was the best predictor for higher DFI concentration. Their study has further discussed an interesting concept: not all morphological normal sperms have normal DFI; sperms with high DFI were also reported in that category. Analysis showed that cases with low DFI had the highest fertilization rates and the number of good-quality embryos. The adverse effect of fragmented DFI on sperm was evidenced by reduced fertilization rate; those cases with higher DFI have less sperm capable of fertilization [32, 33].
Furthermore, the number of blastocytes produced was also reduced. Oleszczuk *et al*. discussed that 20% DFI increases the odds of having low-quality embryos [34], which is attributed to dysfunctional sperm kinetics that led to poor-quality embryos [35].
Some studies set a DFI value of <15% for better fertilization and pregnancy rates. Others declared that DFI > 30 was linked to lower rates of implantation and pregnancy progression [36, 37].
The current study showed that the highest percent of cases who achieved pregnancy were those with moderate DFI, followed by those with low DFI; conversely, the highest percentage of those who failed to get pregnant had moderate and high DFI. The study of Zhang *et al*. [38] confirmed the lack of a meaningful link between DFI *vs* cleavage, fertilizations, implantations, and clinical pregnancy rates in ICSI cycles. Moreover, no link was found between miscarriage and life birth rates [38].
Zhu *et al*. looked at the impact of high DFI sperms on the outcome of fresh and frozen embryo transfer cycles in the ICSI cycle. They confirmed no significant difference between biochemical and clinical pregnancy rates as well as abortion rates in the studied groups. However, the delivery rate was higher in normal FDI cases among fresh embryo transfer [39].
Repalle *et al*. study examined the DFI effect on life birth rates among cases with unexplained infertility undergoing ICSI. Based on implantation and clinical pregnancy rates, they showed no difference in high and low DFI groups. Interestingly, in the adjusted regression model, DFI was a predictor of low cumulative life birth rates and higher abortion rates [40].
Braga *et al*. examined the impact of sperm DFI and oocyte quality, which is an important confounder of ICSI outcomes. Presumably, oocytes are responsible for repairing sperm DNA during the transition of the oocyte embryo. Their results showed that cases with low DFI (less than 30%) yielded higher good-quality embryos, higher fertilization, and pregnancy rates irrespective of ova quality. On the other hand, cases with high DFI positively correlate with abortion rates [41].
Although an insignificant relation was found between DFI and ICSI outcomes still, the outcome

with respect to abortion and life delivery rates was reduced among high DFI cases [41, 42].
DNA fragmentation plays an important role in male infertility; it should be suspected whenever the IVF outcome is not good with a normal semen analysis [43-45].

CONCLUSIONS

The present study demonstrated a correlation between DFI (DNA fragmentation index) and demographic characteristics of infertile males and seminal fluid parameters. This correlation provides an opportunity for therapeutic intervention by targeting modifiable variables that contribute to increased sperm DFI. In addition, the DFI category allows the simultaneous selection of the most suitable sperm candidate to enhance the outcome of ICSI. Additional research is required to reveal DFI's diagnostic, therapeutic, and prognostic significance.

COMPLIANCE WITH ETHICAL STANDARDS

Authors' contribution
A.F., W.N.: Validation, visualization, statistical analysis. Z.A.J.: Conceptualization, methodology, project administration, software, supervision, validation, visualization. All authors: Data curation, investigations, writing – original draft, writing – review & editing.

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Study registration
N/A.

Disclosure of interests
The authors declare that they have no conflict of interests.

Ethical approval
The protocol was approved by the Mustansiriyah ethics committee of Obstetrics and Gynecology/ Iraq (NO: 44 in January 2023).

Informed consent
Written informed consent was provided by all couples enrolled following the Declaration of Helsinki and local guidelines.

Data sharing
Data are available under reasonable request to the corresponding author.

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