# Sperm DNA Fragmentation Index and Intracytoplasmic Sperm Injection Outcome in Egyptian Couples

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**Sperm of infertile men mostly contain more DNA damage than their fertile counterparts. Sperm DNA damage might have a negative effect on the fertility potential of such patients. This study measures the percentage of spermatozoa with nuclear DNA fragmentation in semen samples used for intacytoplasmic sperm injection (ICSI), before and after sperm processing and investigates the association between sperm DNA fragmentation index (DFI) and ICSI outcome (embryo grade and clinical pregnancy). Sperm DFI was measured, by TUNEL, in fresh semen samples obtained from thirty male partners of infertile couples, seeking ICSI treatment. The mean sperm DFI before and after processing was 4.5% and 8.83% respectively. Sperm DFI increased significantly after processing (*P*< 0.001) regardless the type of processing used (*P*= 0.877). The correlation between sperm DFI, before and after semen processing, and clinical pregnancy was statistically insignificant (*P*=0.689 and *P*=0.631 respectively). DFI of sperm after semen processing showed a significant negative correlation with embryo grading at day 3 (r = - 0.232, *P* = 0.002). In conclusion, sperm DFI increases significantly after processing and correlates negatively with embryo grade.**

The World Health Organization (WHO) defined clinical infertility as a disease of the reproductive system defined by failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild *et al.,* 2009).

The term "male infertility" does not constitute a defined clinical syndrome, but rather, a collection of different conditions exhibiting a variety of etiologies and diagnoses. In men, oligozoospermia, asthenozoospermia, teratozoospermia and azoospermia are the main causes of infertility, and these account for 20% –25% of cases (Poongothai *et al.,* 2009).

DNA integrity is defined as the absence of single stranded or double stranded breaks and absence of nucleotide modifications in the DNA. The loss of integrity in sperm DNA may occur at any level from the transformation of the spermatogonial germ cells to the ejaculated sperm, thereby the DNA damage may be present in the testicular sperm, epididymal sperm or the ejaculated sperm. DNA damage occurring during sperm transit and storage in the epididymis or post ejaculation cannot be repaired by sperm, because post spermiogenesis there is negligible transcription and translation (Shamsi *et al.,* 2009).

Several mechanisms generating DNA fragmentation have been postulated: (1) In the testis as part of the apoptotic process, also known as the abortive apoptosis theory. (2) During chromatin compaction, especially during replacement of histones by protamines, the defective maturation theory. (3) Following release from the testis, oxidative stress is thought to be the main mechanism responsible for the occurrence of DNA fragmentation and DNA base oxidation (Steger *et al.,* 2011)

These mechanisms of DNA damage are *in vivo* but the clinically induced DNA damage in assisted reproduction procedures constitutes an additional concern for in vitro conceptions. These techniques require sperm preparation and processing in which the seminal plasma is removed by centrifugation and washing which makes sperm more

vulnerable to oxidative damage (Venkatesh *et al.,* 2011).

Several methods are currently available to evaluate SDF (Steger *et al.,* 2011) including: sperm chromatin structure assay (SCSA), acridine orange test, sperm chromatin dispersion test (also known as Halosperm), in situ nick translation assay, single-cell gel electrophoresis assay (also known as COMET) and terminal deoxynucleotidyltransferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL)

The TUNEL technique is a direct quantification of sperm DNA breaks, by incorporating- flourescin labeled- dUTP at single-stranded and double stranded DNA breaks in a reaction catalyzed by the enzyme TdT. The DNA breaks, based on the incorporated dUTP, can then be measured using bright field or fluorescent microscopy as well as flowcytometry. Sperm are then classified as TUNEL positive (flourescin labeled) or negative and expressed as a percentage of the total sperm (Ribas-Maynou *et al.,* 2013)

In this study, we evaluated the percentage of spermatozoa with nuclear DNA fragmentation, before and after semen processing for ICSI and investigated its association with ICSI outcome as regards embryo grade and clinical pregnancy.

## Materials and Methods

### Study Population

This study was performed on 30 semen samples, before and after processing from 30 male partners of infertile couples undergoing ICSI trials at El-Shatby hospital, Alexandria University, between February and September 2013. Couples, with middle aged (30 -50 years, mean ± SD: 37.47 ± 5.06 years) non-azoospermic men, whose female partners were younger than 35 years old (mean ± SD: 31.5 ± 4.39 years) were included. All of them were suffering from primary infertility for at least 3 years (mean ±SD: 7.03 ± 3.71 years) and were consulted on whether to perform IVF or ICSI, after describing the details of both procedures to them, and all chose ICSI. Subjects with autoimmune diseases (e.g. type I diabetes mellitus), history of chemotherapy and or radiotherapy were excluded (to avoid known causes of DNA damage).

Full history was taken from all participants; including smoking habits, drug and medical history. All subjects enrolled in this study signed a written informed consent before participation. Details that might disclose the identity of the subjects under study were omitted. The study received approval of the Medical Ethics Committee of the Faculty of Medicine, Alexandria University and the practical work has been carried out in accordance with the code of Ethics of the World Medical Association (1964 Declaration of Helsinki and its later amendments).

### Semen Collection and Analysis

Routine semen analysis was performed for all male partners participating in the present study. Semen specimens were obtained by masturbation after 3-5 days abstinence. After liquefaction, manual semen analysis was performed according to World Health Organization (WHO) 2010 guidelines (WHO, 2010). Seminal volume was determined using a graduated tube. Sperm concentration was assessed using Improved Neubauer counting chamber and expressed in millions/ml. Sperm motility was assessed in at least 200 spermatozoa within 1 hour from liquefaction and expressed as percent of total motile sperm. Sperm viability was assessed by Eosin stain, particularly in samples with poor motility. Approximately 200 spermatozoa per slide were assessed for the percentage of normal and abnormal morphology.

### Sperm Processing

Semen preparation was done by either the swim up technique or density gradient centrifugation, depending on the initial semen quality. Normal semen samples were subjected to the swim up technique, while poor quality samples were subjected to density gradient centrifugation.

In the swim up technique, 1 ml of semen was placed in a sterile 15-ml conical centrifuge tube, and 1.2 ml of supplemented medium was gently layered over it. The tube was inclined at an angle of about 45°, to increase the surface area of the semen–culture medium interface, and incubated for 1 hour at 37 °C. Then, the uppermost 1 ml of medium, which contained highly motile sperm cells, was removed. This was diluted with 1.5–2 ml of supplemented medium. The sample was centrifuged at 300*g* for 5 minutes and the supernatant was discarded. The sperm pellet was re-suspended in

0.5 ml of supplemented medium for assessment of sperm concentration, progressive and total motilities.

Regarding density gradient centrifugation, the density-gradient medium was prepared in a test-tube by layering 1 ml of 40% (v/v) density-gradient medium over 1 ml of 80% (v/v) density-gradient medium (*SpermGrad™ Vitrolife, Sweden, ref 10102*). SpermGrad™ is bicarbonate and HEPES buffered medium containing silane-coated, colloid silica particles. The semen sample was mixed well. One ml of semen was placed above the density-gradient media and centrifuged at 300*g* for 20 minutes. The supernatant was removed and the sperm pellet was re-suspended in 5 ml of supplemented medium to aid removal of contaminating density-gradient medium, and then finally centrifuged at 300*g* for 5 minutes. The washing procedure was repeated. The final pellet was re-suspended in supplemented medium (*Global Total™ LifeGlobal, Canada, product number H5GT-030*) by gentle pipetting (López *et al.,* 2013).

Sperm concentration, progressive and total motilities were assessed post processing. For counting spermatozoa, improved Neubauer haemocytometer, used with special thick cover- slips, was loaded with the processed sample and assessed within 10 minutes to avoid evaporation. The concentration of spermatozoa was calculated per ml. Sperm counts were assessed in both chambers of the haemocytometer at × 400 magnification. Moreover, slides were examined to assess the motility of all spermatozoa within a defined area of the field at × 400 magnification. Two hundred spermatozoa were being scored in two stages; progressive (PR) first, followed by non-progressive (NP) and immotile (IM) from the same area. Progressively motile spermatozoa are those moving actively, either linearly or in a large circle, regardless of speed. Non-progressive motility includes all other patterns of sperm motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed. Immobility means no sperm movement at all. Total motility includes both progressive and non progressive non-progressive motilities. The average percentage of each motility grade was calculated and approximated to the nearest whole number, i.e. PR, NP and IM. Only intact spermatozoa, defined as having a head and a tail, were assessed, i.e., motile pinheads were not counted (WHO, 2010). Then the specimen was used directly for ICSI (López *et al.,* 2013).

### Assessment of Sperm DNA Fragmentation by TUNEL (Caglar *et al*, 2007)

Sperm DNA fragmentation was detected in 30 semen samples before and after processing (i.e. 60 samples) by the In Situ Cell Death Detection Kit, with Fluorescein label, (Roche Diagnostics, version 16.0, Cat. No. 11684795910, GmbH, Mannheim, Germany), following the manufacturer's recommendations. This technique is based on the labeling of DNA strand breaks. Briefly, after smearing spermatozoa on slides, at a concentration that avoids their overlapping, they were air- dried and fixed with a freshly prepared fixation solution (4% paraformaldehyde in PBS, pH 7.4) for 1 h at 15 - 25°C. The slides were subsequently washed in PBS (phosphate buffer saline) and incubated in freshly prepared permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice (2-8°C). Slides were, then, rinsed twice with PBS before being incubated with FITC- labeled terminal deoxyribonucleotidyl transferase (TdT). The slides were, finally, covered and incubated in a humidified atmosphere for 60 min at 37°C in the dark. After labelling, slides were rinsed twice in PBS.

For standardization of the assay, controls were included in every experiment; for the negative control, the TdT was omitted from the reaction mix (i.e. 50 µl of label solution only were added). The positive controls were prepared by incubating the sperm cells for 20 minutes at room temperature with 50 units/ml DNAse I (Boehringer Mannheim, Mannheim, Germany) prior to incubation with the TUNEL reagents.

In each slide, the total number of spermatozoa and the percentage of cells with fragmented DNA were determined by analysing each microscope field using both light and fluorescence microscopy. Stained cells were quantified on Olympus BX51TRF fluorescence microscope, with an excitation wavelength in the range of 450 – 500 nm and detection in the range of 515 – 565 nm (green fluorescence). A minimum of 300 spermatozoa per slide were assessed.

The percentage of spermatozoa with fragmented DNA was calculated as the number of TUNEL positive green fluorescing nuclei (FITC-labeled) in relation to the total number of sperm nuclei counted and the result was referred to as DNA fragmentation index or DFI-TUNEL (%).

### Intracytoplasmic sperm injection, Embryo Grading (Stylianou *et al.,* 2012) and Transfer

After sperm injection into metaphase II oocytes, embryos were graded into 3 categories according to a) the number of blastomeres, b) evenly sized blastomeres, and c) the percentage of fragmentation into A (good quality embryos), B (moderate quality) and C (poor quality). Embryos were, finally, transferred on day 3.

### Clinical Pregnancy Detection

Beta hCG testing was done to all female partners 2 weeks after embryo transfer (ET) and ultrasound detection of fetal sac(s) and pulsations was performed 28 days after ET.

### Statistical Analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Kruskal Wallis test was used to study the relationship between % sperm DFI in processed semen and embryo quality on day 3, while Mann Whitney test was used to compare between any two independent variables. Wilcoxon signed ranks test was used to compare between semen parameters pre and post-processing. Spearman coefficient was used to get the correlation between day 3 embryo quality and the percentage of sperm DFI in processed semen. Significance of the obtained results was judged at the 5% level (Binu *et al.,* 2014).

## Results

This study determined the percentage of spermatozoa with fragmented DNA (DNA fragmentation index, or DFI) in semen samples used for ICSI, before and after sperm processing. After obtaining the semen samples, they were processed by either the swim-up technique (n=14) or by density gradient (n= 16), depending on the quality of the semen.

The mean sperm DFI before and after processing was 4.5% and 8.83% respectively (Table 1). Comparing semen parameters before and after processing, it was found that sperm count, motility and morphology improved after processing (count Z= 4.794 $P<0.001$; motility Z=3.228, $P<0.001$; morphology Z=4.58, $P<0.001$) as shown in table 2, however, sperm DFI increased significantly after processing ($P<0.001$) regardless the type of processing used (Z= 0.155, $P= 0.877$) as shown in table 1 & Fig 1.

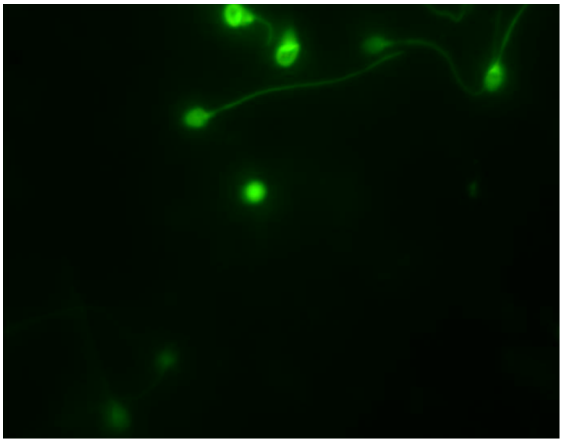

**Figure 1.** Sperm cells examined for DNA damage by TUNEL assay. The upper sperm cells are TUNEL positive (green fluorescence) while the lower 2 sperm cells are TUNEL negative under fluorescent microscope.

Table 1. Effect of semen processing on sperm DNA fragmentation index

| | Before Processing | After Processing | *P value |
|---|---|---|---|
| DFI (%) | 3.0 (0.0 – 30.0) | 6.0 (1.0 – 33.0) | <0.001 |

Abnormally quantitative data expressed as median (min. – max.) and was compared using Wilcoxon signed ranks test.

*: Statistically significant at $P \leq 0.05$

Table 2. Effect of semen processing on sperm count, motility and morphology

| | Raw semen | Processed semen | *P value |
|---|---|---|---|
| Count (million/ml) | 39.0 (7.0 – 126.0) | 41.0 (10.0 – 150.0) | <0.001* |
| Total motility (%) | 70.0 (26.0 – 95.0) | 72.50 (25.0 – 100.0) | 0.001* |
| Morphology (normal, %) | 7.0 (2.0 – 8.5) | 8.0 (3.5 – 9.5) | <0.001* |

Data expressed as median (min. – max.) and was compared using Wilcoxon signed ranks test

*: Statistically significant at $P \leq 0.05$

There was no statistically significant correlation between sperm count (r=- 0.126, *P*= 0.506) or morphology (r=- 0.035, *P*= 0.853) and sperm DFI before semen processing. However, a statistically significant moderate negative correlation was found between sperm motility and DFI of sperm before processing (r=-0.516, *P*= 0.003). Moreover, there was no statistically significant correlation between % DFI of sperm before processing and male age (r=-0.119, *P*=0.53) or period of infertility (r = -0.113, *P*= 0.552).

To investigate the possible association between sperm DFI and ICSI outcome, embryo quality and clinical pregnancy rates were assessed. As regards the relationship between embryo grade and % DFI of sperm in processed semen, the DFI ranged between 1-25% in class A (good quality embryos) with a mean ± SD of 5.69 ± 5.03, while in classes B (moderate quality embryos) and C (poor quality embryos) it ranged between 1-33% (mean ± SD: 9.78±9.19) and 3-20% (mean ±SD= 7.89 ± 5.73) respectively, with a statistically significant difference between class A and the other 2 classes. Moreover, there was a significant negative correlation between percent DFI of sperm in processed semen and embryo grade at day 3, (r = -0.232, *P*=0.002), (Table 3).

Table 3. Relationship between sperm DNA fragmentation index and embryo quality 3 days post- processing.

| | Embryo Quality on Day 3 | | | *P* value |
|---|---|---|---|---|
| | Good (A) (n = 133) | Moderate (B) (n = 36) | Poor (C) (n = 9) | |
| % Sperm DFI in Processed Semen | 3.0 (1.0 – 25.0) | 6.0 (1.0 – 33.0) | 6.0 (3.0 – 20.0) | <0.001* |
| Sig.bet. Grps | | A-B* | | |
| $r_s$ (*P*) | | -0.232* (0.002*) | | |

Data expressed as median (min. – max.) and was compared using Kruskal Wallis test.

Sig bet. groups was done using Mann Whitney test. $r_s$: Spearman coefficient. *: Statistically significant at $P \leq 0.05$

Grade (A) embryos: good quality embryos

Grade (B) embryos: moderate quality embryos

Grade (C) embryos: poor quality embryos

Clinical pregnancy occurred in 50% of cases (n=15). The correlation between sperm DFI, before or after semen processing, and clinical pregnancy was statistically insignificant (Z = 0.4, *P*=0.689 and Z= 0.48, *P*=0.631 respectively) (Table 4).

Table 4. Correlation between sperm DNA fragmentation index and clinical pregnancy after intracytoplasmic sperm injection from processed semen.

| | Clinical Pregnancy | | |
|---|---|---|---|
| | Negative (n=15) | Positive (n=15) | *P value |
| Sperm DFI Pre-processing (%) | 3.0 (0.0 – 30.0) | 3.0 (1.0 – 8.0) | NS |
| Sperm DFI Post-processing (%) | 6.0 (1.0 – 33.0) | 6.0 (1.0 – 20.0) | NS |

Data expressed as median (min. – max.) and was compared using Mann Whitney test

*: Statistically significant at *P* ≥ 0.05 is not significant (NS).

## Discussion

There have been concerns about potential consequences of the use of DNA damaged sperm in ICSI, as this technique overrides the process of natural selection. Although there has been a significant amount of research on human sperm DNA integrity over the last decade, our understanding of the mechanisms of sperm DNA damage and their effects on reproductive outcomes is deficient (Schulte *et al.,* 2010)

The aim of this work was to evaluate the percentage of spermatozoa with nuclear DNA fragmentation, measured by TUNEL, in semen samples used for ICSI, before and after sperm processing. We also aimed at investigating the possible association between sperm DNA fragmentation index (DFI) and ICSI outcome (embryo grade and clinical pregnancy). Moreover, we examined the relationship between the percentage of sperm DFI and sperm characteristics. In addition, the effect of semen preparation on the integrity of sperm DNA and parameters of semen analysis was evaluated.

Sperm processing procedures could trigger increased production of reactive oxygen species (ROS) which begin to accumulate in the samples, and so, prolonged exposure of spermatozoa to ROS during processing, is mostly the cause of increased DFI post-processing.

There are several other studies which examined the relationship between DFI and semen parameters, fertilization rate, class of embryo cleavage and pregnancy outcome. In a cohort study by Lopez *et al.* (López *et al.,* 2013), only the first attempts at ICSI procedures with ejaculated sperm were included. They recruited 152 couples from February 2009 to January 2011. The average time seeking for pregnancy prior to coming at their clinic in this group of patients was 19.36±12.41 months (mean±SD). Only women with FSH levels lower than 10 IU /L and sperm samples with a concentration of 5 million sperm/ml or more were included. To avoid potential sources of bias, they excluded women over 42 years old and treatment cycles that resulted in a poor ovarian response (<3 mature oocytes collected) or those involving epididymal, testicular and cryopreserved

sperm samples. Male patients having varicocele, leukocytospermia or under pharmacological treatment were also excluded from the study. A non-significant correlation was found between sperm DNA fragmentation and fertilization and embryo rate (r = 0.070; *P*= 0.388; r= 0.083; *P*=0.304, respectively).

The prospective study of Benchaib et al. (Benchaib *et al.,* 2003) concerned 54 cycles of ICSI performed during a given period of 4 months. ART procedures involving cryopreserved sperm samples or testicular extracted sperm were excluded from this study. Only ICSI procedures with ejaculated sperm were included. Sperm DNA fragmentation was measured by TUNEL assay and correlated with semen and ART outcomes. A significant negative correlation was observed between DNA fragmentation and sperm concentration (r = -0.44, *P*<0.01); between fragmentation and sperm motility (r = -0.28, *P*<0.05) and between fragmentation and the percentage of atypical forms (r = -0.36, *P*<0.01). However the pregnancy rate was not influenced by sperm characteristics. No relationship between fragmentation and embryo quality was found .The fertilization rate did not seem to be influenced by sperm fragmentation (r = - 0.14, not significant for ICSI. However, when DNA fragmentation was divided into two categories according to a threshold value of 10%, the fertilization rate was significantly higher for DNA fragmentation below 10% (84.1 versus 70.7%, *P*<0.05).

On the other hand, the first goal of a comparison study, by Marchesi *et al.* (Marchesi *et al.,* 2010) was to determine the effect that semen processing has on sperm DNA integrity. The second goal was to assess which processing technique (modified swim-up versus density gradient centrifugation) results in a superior sample. DNA integrity was measured using a novel Toluidine Blue assay. Raw semen samples were collected from thirty-two male individuals and scored for routine semen analysis. Prior to discarding the specimens, identical aliquots were divided and processed by density gradient centrifugation and a modified swim-up technique. The Toluidine Blue Assay was used to analyze raw and processed samples. Both density gradient centrifugation and the modified swim-up techniques improved DNA quality compared to the unprocessed sample. However, the modified swim-up technique proved superior (i.e. the swim-up technique generated a sperm sample with better DNA integrity).

Among the main limitations of our study are the relatively small sample size, and the inability to follow up our female patients throughout their pregnancy to determine the miscarriage rate or live births rate in order to correlate it with sperm DFI.

From the findings of our study, we recommend re-evaluation of the current sperm processing techniques and addition of antioxidants to media used in processing to minimize sperm DNA damage and the potential transmission of genetic mutations in assisted reproductive cycles. Moreover, developing a simplified novel DNA integrity test for the sperm to be inseminated is crucial.

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