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Evaluation of Sperm DNA Fragmentation Index (DFI) for the Outcome of Assisted Reproductive Technique in Infertile Male

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ABSTRACT

Background: *The objective of this study was to determine whether there is any possible relationship between DFI with Assisted Reproductive Technique outcome and also with age and semen parameters.*
Methods: *This was a case study of limited number of patients .15 male infertile patients undergoing ICSI were included in the present study. SCSA and ART were performed on semen aliquots taken from the same ejaculate. Semen parameters (count & morphology), Fertilization rate, Embryo quality and Biochemical pregnancy rates were also correlated to SCSA (DFI) parameter. Statistical analysis was also performed using SPSS version.*
Results: *Significant difference was observed between the DFI of the subject and normality at a significant level of 1% (p= 0.000).The results showed no correlation between DFI and fertilization rates, biochemical pregnancy. But a weak correlation was observed between DFI and embryo quality and embryo quality with fertilization rate, biochemical pregnancy. For IUI, the DFI above 25% resulted in negative pregnancy. One ongoing pregnancy was achieved for ICSI with DFI levels 34.33%.*
Conclusion: *DFI can be used as an independent predictor of pregnancy in case of IUI. SCSA test can be considered as accessory to the standard semen analysis in infertile men. In ART, pregnancy loss and time spending can be avoided based on SCSA (DFI) and move on to ICSI for better result.*
Keywords: *Sperm Chromatin Structure Assay, DNA Fragmentation Index, Intra cytoplasmic Sperm Injection, Intra Uterine Insemination.*

INTRODUCTION

A large number of couples, all over the world are unable to produce children in spite of unprotected sexual co-habitation. According to World Health Organization 2000, about 20% anomalies are responsible for male factor and 27% anomalies are

related to both partners. The genetic integrity of the spermatozoan is essential for normal embryo development. Now there are infertility clinics to help in diagnosis and corrective treatment of some of these disorders and enable these couple to have children. However, if the corrections are not possible the couples should be assisted to have children through assisted reproductive techniques (ART).

Congenital, genetic or immunological factors, infections of the genital tract, varicoceles, acquired urogenital abnormalities and endocrine disturbances are all the causes of male infertility. Causes of male infertility are grouped under three categories in that Pre-testicular alter the normal hormonal milieu for sperm development and sensitivity to free radical damage. Testicular and Post testicular induce significant damage, increase reactive oxygen species and also effect sperm transit time. Male infertility can also be caused by low semen quality and number of different morphological characteristics.

The mammalian sperm chromatin structure is highly organized, condensed and compacted. This type of highly organized nature of the sperm chromatin protects the paternal genome during the transport through the reproductive track [1, 2].The sperm cell DNA is more exposed than the DNA of the other cells in the body. The sperm head contain minimal amount essential bio molecules that required to sustain its journey to the egg. Female egg contains many DNA repair mechanism that can mask the effects of damaged sperm DNA during the early stages of embryonic development.

The sperm DNA damage may arise from combination of chromatin remodeling [3] oxidative stress [4] and abortive apoptosis [5]. Sperm DNA fragmentation refers to the level of damaged DNA that present inside the sperm head. High sperm DNA fragmentation doesn't appear to affect the fertilization but it affect the embryo cleavage and subsequent blastocyst development. DNA fragmentation levels are closely correlated with IUI, IVF and ICSI miscarriage and pregnancy rates. High sperm DNA fragmentation is found in men with normal semen parameters and also with poor semen parameters. Increased sperm DNA fragmentation is associated with oxidative stress, defects in topoisomerase activity, infection, leukocytospermia, drug use, cigarette smoking, and exposure to environmental and occupational pollutants, advanced age, varicocele, and elevated testicular temperature.

The SCSA is one of the diagnostic and prognostic evaluations of the male's potential for subfertility or infertility. It detects the abnormal chromatin structure consisting of sperm DNA fragmentation index (%DFI) and abnormal nuclear proteins (%HDS). It was invented 30 years ago by Evenson and measures the Susceptibility of sperm chromatin to DNA denaturation in situ induced by low pH treatment. The assay is based on the metachromic properties of a DNA binding fluorescent dye, acridine orange (AO). AO intercalates with DNA and emits green fluorescence when bound to intact, double stranded DNA and red fluorescence when bound to single strand, fragmented DNA. The SCSA however quantifies the fluorescent signals

by flowcytometry. Upon excitation by laser light, emitted red and green fluorescent signals from individual cells are detected by photo multiplier tubes. Routinely, 5000 individual sperm cells are analyzed in a few minutes.

The DNA fragmentation index (DFI) is the proportion of sperm containing fragmented DNA and is calculated from a histogram obtained from the ratio between red and red+green fluorescence. The SCSA is not a replacement for the semen analysis. The patient can feel confident that he has all the information available from the results of both the tests about his chances of initiating a pregnancy through ART.

MATERIALS AND METHODS

Subjects

The case study was based on 15 infertile couples undergoing ICSI at infertility clinic during the period from July 2013 to March 2014. Maximal care was exerted to include patients with normal female partners. All subjects were asked to provide semen samples after 3 to 5 days of ejaculatory abstinence. Semen samples were produced by masturbation directly in to a sterile plastic container.

Semen Preparation

Routine andrological examination of the semen (volume, count, motility, morphology) has been performed according to WHO criteria (WHO, 2010). After semen analysis, within one hour from the time of ejaculation, 250\500 µl of fresh raw semen samples was aliquoted equally in to 2ml labelled cryotubes with the patient name. Then the tubes were put in to a metal cane and in to the liquid Nitrogen tank and transferred to andrology centre for SCSA analysis.

Sperm Chromatin Structure Assay (SCSA)

SCSA was performed according to the procedure described by Evenson and coworkers in 2000&2002. The samples were individually removed and thawed at 37ºC for 30 seconds. An aliquot was transferred to TNE buffer to a final concentration 1-2 X 10^6 / ML**.** 200µL of this sperm suspension is mixed with 400µL solution of 0.1% Triton X-100 at pH 1.2. After 30 seconds 1.20 ml of AO staining solution is added. Stain cells were analyzed by fluorescence activated cell sorter scan flow cytometer.5000 sperm are analyzed at an event rate of 100-200 cells / sec. If the event rate is above 250, a new sample must be prepared to ensure the precise equilibrium between the AO dye and the sperm. Due to the low concentration of one of the sample, the report is based on the analysis of 3000 cells. When excited with a 488 nm light source, acridine orange bound to double stranded DNA emits green fluorescence and acridine orange bound to single stranded DNA emits red fluorescents. The extent of DNA denaturation in terms of the DFI, which is the ratio of red to total (red+green) fluorescence intensity, that is, the level of denatured DNA over the total DNA. The DFI value (%DFI) was calculated for each sperm cell in a sample and the resulting DFI frequency profile was obtained.

Statistical Analysis

Statistical analysis was performed by using SPSS 11.5. Statistically significant term was used to denote a two sided test with a *p* value of <0.05.

The significant variation from the standard protocol for semen parameters such as age, count and morphology were test verified by using one sample case t-test. The 15 patient semen samples were subjected to DFI analysis to verify whether the DFI% of the individual significantly deviated from the standard protocol of 6.5% DFI and data was analyzed using one sample case t-test. The Pearson correlation was also calculated between DFI with semen parameters (age, count and morphology) and reproductive outcome (pregnancy rate, fertilization rate, embryo quality).

RESULTS

In the present case study, assessment of DNA damage by SCSA was carried out in a total of 15 couples undergoing infertility treatments, followed by an attempt to understand it's influence on reproductive outcome. Out of 15 one underwent IUI and 14 ICSI.

Semen Analysis

All collected semen samples were liquefied within 30 minutes and their macroscopic examination revealed that they were in accordance with the standard described by WHO, 2010. Two out of fifteen were severe oligoesthenoteratospermia (SOAT) and their morphology was less than 4%. The statistical analysis one sample t-test from table (1&2) and figure(1&2) reports revealed that there is no significant difference between the observed values of count and morphology in relation to standard values at a significant level of 0.05. The patients cannot be considered as fertile based only upon the normal semen analysis. Aliquots from 15 ejaculates assessed using the SCSA and their individual reports are included in this study. In our study, only DNA fragmentation Index (DFI) was taken into consideration.

The statistical analysis t-test reports revealed that there was a significant difference between the DFI of the subjects and the normality at a significant level of 1% (p=.000) (Table 3 & figure 3). From the figure, it speaks out the fact that DNA damage in infertile male is higher than the fertile one. It was further evident that a majority of the subjects had DNA damage below a threshold of 27% by SCSA. Only four subjects had DNA damage above 27% by SCSA. Out of 4, only one resulted in ongoing pregnancy after ICSI had the DFI value 34.33%. The present study demonstrates that even men with high levels of DNA damage can become biologically fathers, when DFI exceeded 27. One couple treated with IUI, no pregnancy was seen when the DFI exceeded 27% (DFI= 27.08%).

The Pearson correlation was calculated between DFI with age and semen parameters (count and morphology). From the table (4) and graph (figure 4, 5&6), it represents that there is no significant difference was observed between DFI with age and semen parameters. From table 5, the correlation was calculated between DFI and reproductive outcome. We couldn't find no relationship between the DFI and fertilization rates, biochemical pregnancy (Figure7&8). But a weak correlation was observed between DFI and Embryo quality ($r_=$ -.238). Similarly a weak correlation was observed between Embryo quality with fertilization rate and biochemical

pregnancy($r_=$ -.287; $r_=$ -.297). This show that if the embryo quality poor, there is a perfectly predictable decrease on fertilization rate and biochemical pregnancy. In the present study the grade 1 quality embryo has a better chance of getting pregnancy than the other grades (Figure8). While exploring the relationship between sperm DNA damage and reproductive outcome in the context of ART, it is imperative to understand the extent of DNA damage in individual spermatozoa and how different sperm processing techniques can remove DNA damaged sperm.

When %DFI above 25% go on to IVF, or even better, ICSI treatment. DFI can be used as an independent predictor of pregnancy in couples undergoing IUI. when the %DFI is >25% the SCSA suggest to do changes in lifestyle and/or medical intervention to reduce this value and include SCSA test as a accessory to the standard semen analysis in infertile men.

Table 1: One- Sample t- Test for count

	N	Mean	Std. Deviation	Std Error Mean
Count	15	59.4000	63.79633	16.47214

One- Sample Test

	Test value = 39		
	T	Sig. (2-tailed)	Mean Difference
Count	1.238	0.236	20.40000

Table 2: One sample t- test for morphology

	N	Mean	Std. Deviation	Std Error Mean
Morphology	15	6.1333	4.53347	1.17054

One- Sample Test

	Test value = 4		
	T	Sig. (2-tailed)	Mean Difference
Morphology	1.823	0.090	2.13333

Table 3: One sample t- test for DFI

	N	Mean	Std.Deviation	Std Error Mean
DFI	15	22.5487	10.49644	2.71017

One- Sample Test

	Test value = 6.5		
	t	Sig. (2-tailed)	Mean Difference
DFI	5.922	0.000	16.04867

Table 4: Correlation of DFI with semen parameters and age

		DFI	Age	Count	Morphology
DFI	Pearson-correlation	1	0.157	0.365	-0.099
	Sig.(2-tailed)		0.577	0.181	0.725
	N	15	15	15	15
Age	Pearson-correlation	0.157	1	0.687	0.609
	Sig.(2-tailed)	0.577		0.005	0.016
	N	15	15	15	15
Count	Pearson-correlation	0.365	0.687	1	0.806
	Sig.(2-tailed)	0.181	0.005		0.00
	N	15	15	15	15
Morphology	Pearson-correlation	-0.099	0.609	0.806	1
	Sig.(2-tailed)	0.725	0.016	0.00	
	N	15	15	15	15

Table 5: Correlation of DFI with reproductive outcome

		DFI	Fertilization Rate	Embryo Quality	Biochemical Pregnancy Rate
DFI	Pearson-correlation	1	0.361	-0.238	0 .094
	Sig.(2-tailed)		0.205	0.412	0.748
	N	14	14	14	14
Fertilization Rate	Pearson-correlation	0.361	1	-0.287	0.450
	Sig.(2-tailed)	0.205		0.320	0.107
	N	14	14	14	14
Embryo Quality	Pearson-correlation	-0.238	-.287	1	-0.297
	Sig.(2-tailed)	0.412	0.320		0.302
	N	14	14	14	14
Biochemical Pregnancy Rate	Pearson-correlation	0.094	0.450	-0.297	1
	Sig.(2-tailed)	0.748	0.107	0.302	
	N	14	14	14	14

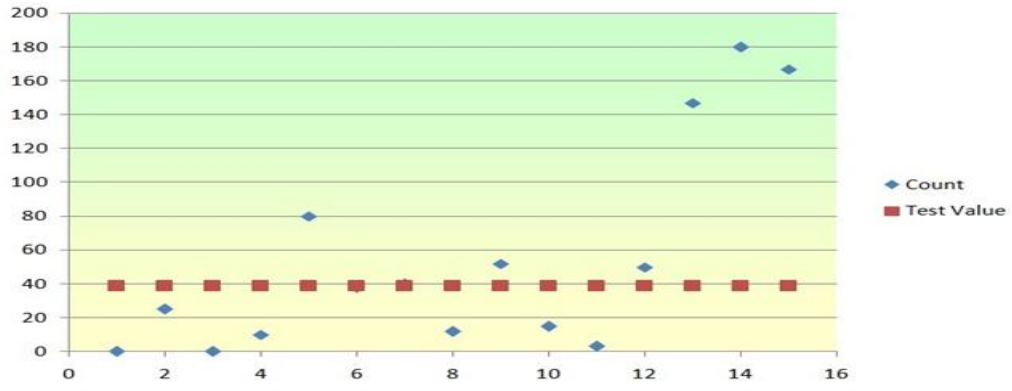

Figure 1: Standard graph of t- test for count

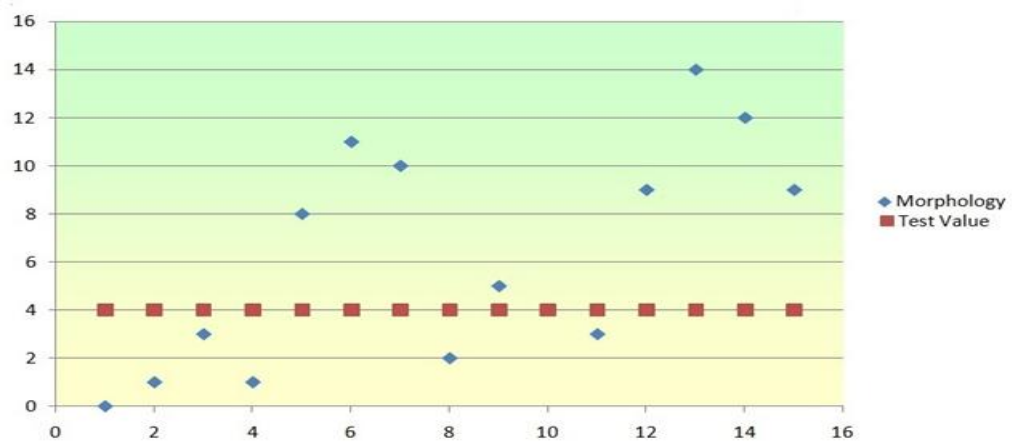

Figure 2: Standard graph of t-test for morphology

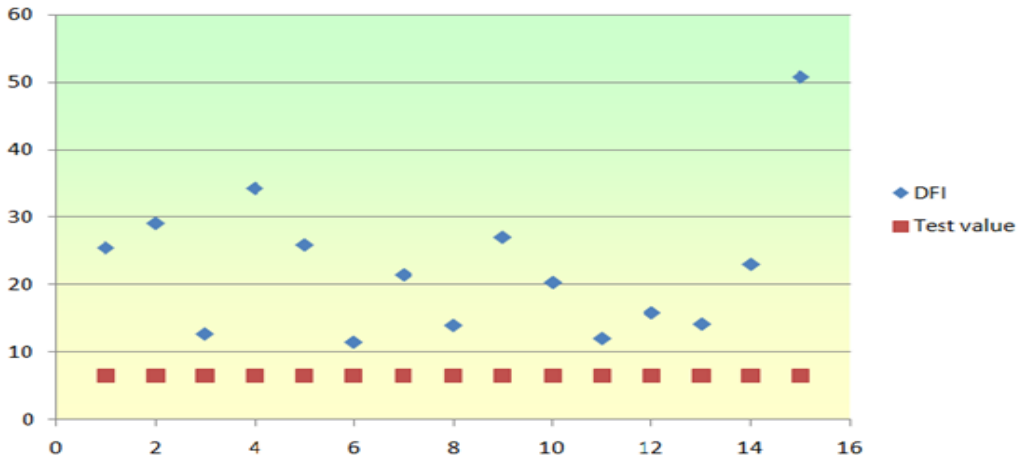

Figure 3: Standard graph of t- test for DFI

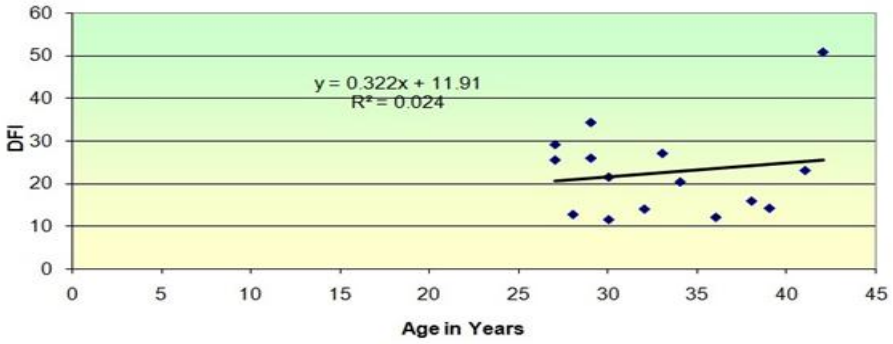

Figure 4: Standard graph of DFI v/s age

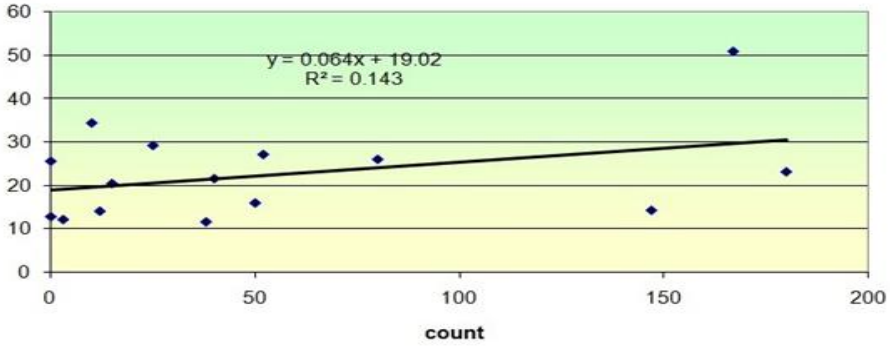

Figure 5: Standard graph of DFI v/s count

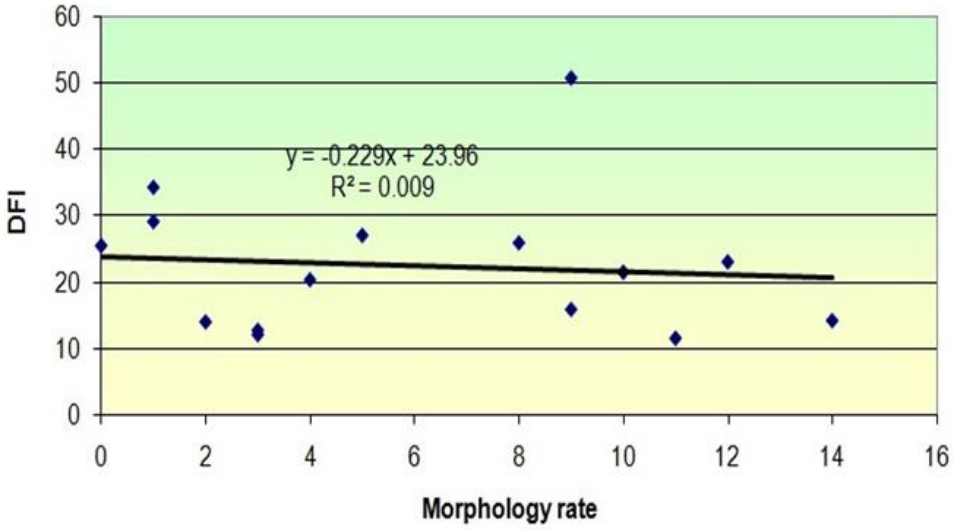

Figure 6: Standard graph of DFI v/s morphology

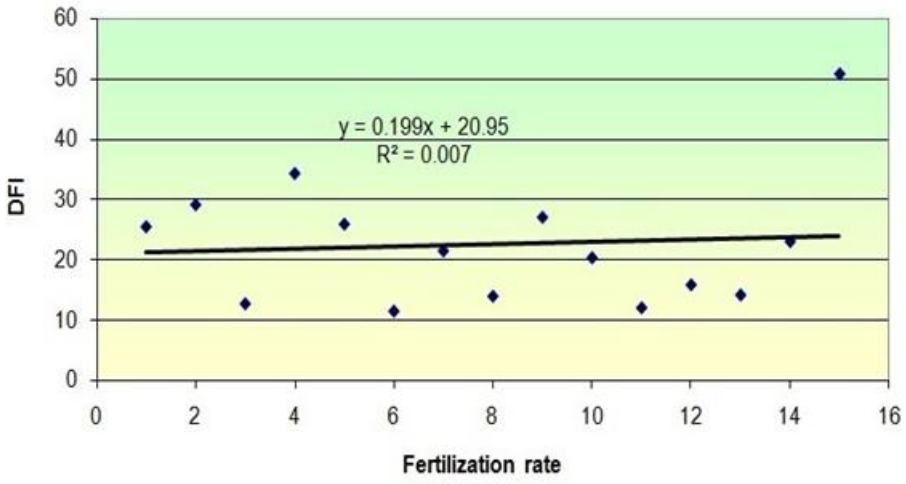

Figure 7: Standard graph of DFI v/s Fertilization rate

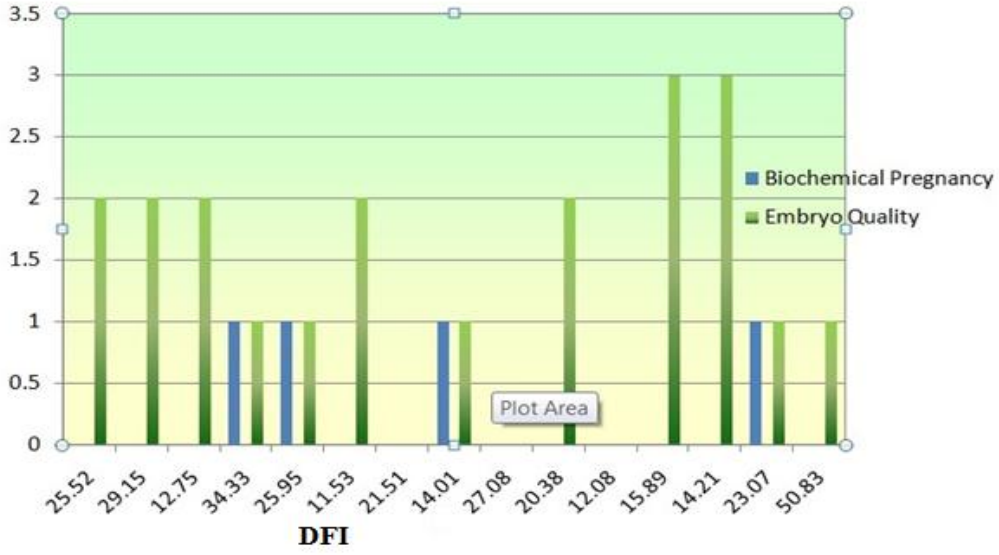

Figure 8: Standard graph of DFI v/s Embryo quality and Biochemical pregnancy

DISCUSSION

In the present study, SCSA analysis was performed on semen aliquot taken from the ejaculate used for ART. Three of the 15 couples receiving ICSI had DFI levels above 27% and only one resulted in ongoing pregnancy had DFI 34.33%. One of the 15 underwent IUI had DFI level 27.08% and resulted in negative pregnancy. Duran *et al*., (2002) and Sahel *et al*.,(2003) have reported in their two recently published studies that pregnancy loss after IUI when DFI level high. In a previous study by Bungum *et al*., (2004) also showed successful fertilization and ongoing full term pregnancies despite high levels (>27%) of DFI in the particular sample used for ICSI when compared to the traditional IVF method .The result from our study was contrary to the three SCSA papers published to date [6,7,8] no clinical pregnancies were achieved using IVF or ICSI, when the DFI exceeded 27% or 28% and an

increased miscarriage rate has been associated with high levels of DFI [9,10]. Similarly, Boe-Hansen *et al*., (2006) reported ongoing pregnancy had DFI value 47.9% supports our findings. One of the recent studies by Bungum and coworkers (2004) have shown successful fertilization and ongoing full- term pregnancies despite high levels (>27%) of DFI in the particular sample used for ICSI. Gandini *et al.,* (2004) hypothesized that the sperm DNA integrity becomes particularly relevant when fertilization occurs under more natural circumstance or in conventional IVF. This was also reported by Bungum and coworkers, who showed that semen samples with DFI above 27% were more likely to result in biochemical pregnancy using ICSI, compared to the traditional IVF method.

In our study, the statistical analysis t-test reports showed a significant difference between DFI of the subject and the normality at 1% level. It has been shown that the fraction of abnormal sperm detected by SCSA is higher in men with infertility problems [11, 12, 13].

No Pearson correlation was observed between DFI with semen parameters, age and biochemical pregnancy. But a weak correlation was observed between DFI and embryo quality(r = -.238) and embryo quality with fertilization rate and biochemical pregnancy(r = -.287; r = -.297). This showing that if the embryo quality poor, there is a perfectly predictable decrease on fertilization rate and biochemical pregnancy. In the present study the grade 1 A quality embryo has a better chance of getting pregnancy than the other grades. Sun *et al.*, (1997) and Lopus *et al.*, (1998) both reported a negative association between strand breaks in sperm DNA and fertilization rate. Host *et al*., (2000) suggested that in case of ICSI the technicians select only spermatozoa with normal morphology and thereby reduce the risk of introducing spermatozoa with strands breaks. But this was however be questioned, since traditional sperm parameters such as sperm count and morphology have proven to be poorly correlated to the DFI [14, 15, 16].

In our opinion, it is not surprising that the invasiveness of the ICSI procedure allows even low quality sperm the chance to initiate a successful pregnancy. In case of ICSI, Successful pregnancies reported even using testicular spermatids [17] is quite different from that acquired by sperm after spermatogenesis and Epididymal maturation. The sperm is focused into the oocyte and the possibility of selecting an unwanted, genetically defective sperm is much higher than the normal way or in conventional IVF. ICSI may be bypassing all natural hurdles for sperm selection. We cannot assess the repair capability of the oocyte and do not know if there are oocytes capable of repairing even heavily DNA damaged sperm. We are aware that our case study is limited as the total number of patients who underwent ART was too small. Additional large-scale trials are needed to confirm the results of the present study. DFI can be used as an independent predictor of pregnancy in couples undergoing IUI. When DFI exceeds 27%, ICSI should be the preferred method.

CONCLUSION

Sperm Chromatin is a very complex structure, and its capability to decondense is one of the essential criteria for considering a spermatozoon to be fertile. DNA integrity in sperm is essential for the accurate transmission of genetic information. Several methods are currently used to assess DNA damage. However, except for SCSA no other assays have cut-off point between normal levels in the average fertile population and the minimal levels of sperm DNA integrity required to achieve pregnancy. The statistical analysis reports revealed that there is no significant difference between semen parameter and the standard values. But in case of DFI a significant difference (p=.000) was found that showed the DNA damage in infertile male is higher than the fertile male. No correlation was observed between DFI and embryo quality and between embryo quality with fertilization rate and biochemical pregnancy. From our findings we conclude that the grade 1 quality embryo has better chance of getting pregnancy than the other. In case of ART, based on SCSA test results pregnancy loss can be decreased and avoid spending time in unsuccessful IUI treatment and move on to ICSI for better success

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