Flow Cytometric Sperm Chromatin Structure Assay as a tool to predict Intrauterine Insemination Outcome in Infertile Couples due to Mild Male Infertility

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Abstract: **Introduction:** Infertility affects approximately 15% of couples trying to conceive and a male cause is believed to be a sole or contributing factor in approximately half of these cases**.** Damage to the genetic component of spermatozoa seems to play the main role in a majority of cases where current approaches fail to reveal the specific cause of male infertility. As Sperm chromatin/DNA integrity is essential for the accurate transmission of paternal genetic information, a normal sperm chromatin structure is important for sperm fertilizing ability. Sperm chromatin structure assay (SCSA) is one of the most efficient and successful assay for providing both diagnostic and prognostic evaluations of man's potential for infertility. **The aim of this study** was to evaluate SCSA as prognostic indicator of the outcome of intrauterine insemination (IUI) in infertile couples due to male causes. **Methods:** Sixty couples with mild male factor infertility, semen samples on the day of IUI were collected. Semen analysis was performed using computer aided semen analysis (CASA) technique. The SCSA was performed using acridine orange (AO) followed by flow cytometric analysis. **Results:** The successful outcome was 23.3%, with no significant difference between successful cases (14 cases) and unsuccessful cases (46 cases) as regards CASA parameters, while for SCSA there was a significant difference between 2 groups. The successful cases for clinical pregnancy showed a DNA fragmentation index (DFI) 23.6±4.4%) while the group which failed to induce pregnancy was 30.4±4.9%. There was a highly significant negative correlation between the pregnancy rates and the DFI%, where no male couple with DFI ≥30% contributed to an ongoing pregnancy. In the other hand, except for total sperm count, all data from semen analysis were poorly associated with the outcome of IUI. **Conclusion**: The SCSA has proven its importance in demonstrating DNA damage as a cause of infertility in men with mild male factor and the subsequent events in the ongoing pregnancy.

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1. Introduction:

Male factor infertility is the sole cause of infertility in approximately 20% of infertile couples, with an additional 30% to 40% secondary to both male and female factors (Mosher and Pratt, 1991, Thonneau *et al.,* 1991). Current means of evaluation of male factor infertility remains routine semen analysis. However, approximately 15% of patients with male factor infertility have a normal semen analysis (Agarwal and Allamaneni, 2005).

Spermatogenesis is a complex process of male germ cell proliferation and maturation from immature diploid to mature haploid spermatozoa,(deKretser *et al.,*1998) where damage of sperm DNA or its chromatin structure can occur at any step (Erenpreiss *et al.,*2006). It has been proposed that sperm DNA integrity could be a possible fertility predictor used as a supplement to the traditional sperm parameters.

There are several different levels of sperm chromatin abnormalities that are important to consider: 1) damage to the actual DNA physical integrity in the form of single-stranded or double-stranded DNA strand breaks, 2) nuclear protein defects that may interfere with histone to protamine conversion and subsequent DNA compaction, and 3) chromatin structural abnormalities causing altered tertiary chromatin configuration (Evenson *et al.,* 2002). Approximately 85% of the DNA-incorporated histones are exchanged with transitional proteins, which are then exchanged with protamines during spermatogenesis. The result is a highly packaged chromatin structure, with apparently no DNA transcription or RNA translation taking place. The capacity of the maturing sperm cells for DNA repair is greatly reduced, and their ability to respond to damage by undergoing programmed cell death is progressively lost (Micinski *et al.,* 2009).

Reports attempting to relate sperm chromatin/DNA damage with conventional semen analysis parameters indicate that spermatozoa from patients with abnormal sperm count, motility and morphology have increased levels of DNA damage (Bungum *et al.,* 2011). Moreover, sperm DNA defects may have a possible negative impact on the outcome

of assisted reproductive techniques (ARTs) (Saleh *et al.,* 2003)

Currently, three major tests of sperm DNA fragmentation are most frequently used, including the Comet assay (single-cell gel electrophoresis), (Morris *et al.,* 2002, Lewis and Agbaje, 2008, Enciso *et al.,* 2009) the terminal deoxynucleotidyltransferase-mediated dUDP nick end-labeling (TUNEL) assay (Gorczyca *et al.,* 1993) and the sperm chromatin structure assay (SCSA) (Evenson *et al.,*1980, Evenson*et al.,*2002). Comet, TUNEL and SCSA all label single- or double-stranded DNA breaks; however, unfortunately, most of the available techniques for detection of sperm DNA damage provide limited information on the nature of the DNA lesions detected .

SCSA is a flow cytometric test where sperm DNA breaks can be evaluated indirectly through the DNA denaturability. The assay measures the susceptibility of sperm DNA to acid-induced DNA denaturation in situ, followed by staining with the fluorescent dye acridine orange. Through specific SCSA software, a scatter plot is created, showing the ratio of green and red sperm. The percentage of red sperm is called DNA fragmentation index (DFI) (Evenson *et al.,* 2002). It is also shown that 25–40% of infertile men may have normal standard sperm characteristics according to WHO criteria, but a DFI% >20–30%. (Bungum *et al.,* 2007,Giwercman *et al.,* 2010).

Additionally, infertile couples using IUI were 7.3 times more likely to achieve pregnancy delivery if the DFI was <30% in a metanalysis (Evenson and Wixon, 2006). Given these findings, measures of sperm DNA integrity appear to have a high predictive value for IUI outcomes.

The aim of this study was to evaluate SCSA as prognostic indicator of the outcome of intrauterine insemination (IUI) in infertile couples due to male causes.

2. Subjects and Methods:

Study design and setting:

This study was conducted from May 2012 to May 2013, at Clinical Pathology and Obstetrics and Gynecology Departments, Zagazig University Hospitals, Egypt. This study comprised 60 couples with mild male factor infertility excluding female causes of infertility.

Ethical issues:

The study was approved by the institution Ethics Review Board, and written informed consent was obtained from all participants.

Male partners with mild male factor infertility, only one of the following parameters below the following normal lower reference limit according to WHO criteria 2010, (i)Sperm concentration ≥ $15x10^6$ (ii) Motility (grade I+ grade II) ≥ 32% (iii)Normal forms≥ 4%

Female partner inclusion criteria was: (i) Age ≤ 37 years; (ii) Basal FSH < 9.5IU/l; (iii)BMI (18 - 24) kg/m2; (iv)Regular cycle (24 - 35) days; (v)Both ovaries are present.

All female members of this study were subjected to the following for controlled ovarian stimulation: On cycle days 3 to 7, women were given Femara 2.5 mg/day (letrozole-Novartis) with 1–2 ampoules (75–150 IU) of HMG (menogon, ferring) day 5. Ovarian and endometrial responses monitored by vaginal ultrasonography on cycle days 9 to 13 and 5000–10000 IU of HCG (Choriomon; IBSA) administered when at least one follicle is (17-18mm) in mean diameter. Standard IUI performed 36 hours after administration of HCG.

On the day of IUI semen samples were collected by masturbation after 2-5 days of abstinence, at the day of oocyte retrieval or insemination. Routine semen analysis was performed using CASA technique according to the WHO recommendations (2010).After semen analysis, within 1 h from the time of ejaculation, 100 µlof the raw semen sample was frozen at - 80°C for later SCSA analysis.

Semen samples were processed by swim up technique (Ricci *et al.,* 2009), A 1 ml aliquot of the prepared semen sample was used for insemination with a Cashmed catheter (catalogue no. IUI 18; Cashmed, Surrey, UK).

Female partners were examined for positive pregnancy test by quantitative B.HCG level in serum on day 14 and positive cases were followed clinically to 12 weeks of pregnancy. Pregnant cases were followed for 12 weeks to confirm ongoing pregnancy.

Specific laboratory tests:

On the day of analysis, the samples were quickly thawed and analyzed immediately. Sperm DNA damage was measured by SCSA using acridine orange according to (Evenson and Wixon, 2006) followed by FCM analysis using FAC scan flow cytometry (BD Immunocytometry Systems, San Jose, CA, USA). An aliquot of the semen sample (15-65 µl) was diluted to a concentration of 2×10^6 sperm/ml in TNE buffer to a total of 200 µl in a Falcon tube. Immediately, 400 µl of acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% v/v Triton X-100, pH 1.2) was added to the Falcon tube. After exactly 30 seconds, 1.20 ml of Acridine Orange (AO)-staining solution was added, containing 6 µg AO (chromatographically purified; Polysciences, Warrington, PA, USA) per ml buffer (0.037 M citric acid, 0.126 M Na2HPO4, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0). The samples were analyzed using a FACScan flow cytometer.

Data acquisition of 5000 events was initiated exactly 3 min after the addition of acid detergent

solution using the BD CellQuest Pro version 4.0 software (BD Biosciences). Data were transferred to Microsoft excel software, Computer gates are used to determine the proportion of spermatozoa with increased levels of red fluorescence (denatured single-stranded DNA) and green fluorescence (native double-stranded DNA) for calculation of X mean (red fluorescence) and Y mean (green fluorescence) and calculation of the percentage of sperm with an abnormally high DNA stainability(HDS) which represents another distinct population in semen that characterizes immature spermatozoa with incomplete chromatin condensation. The extent of DNA denaturation was expressed in terms of the DFI%, which is the ratio of red to total (red plus green) fluorescence intensity, i.e. the level of denatured DNA over the total DNA (Evenson *et al.,*2002).

Statistical analysis:

Statistical analysis was performed using the SPSS 14.0 software (SPSS Inc., Chicago, IL). mean, standard deviation, median and range were used for descriptive statistics, as appropriate. To test inter-group differences in non parametric results, the Mann Whitney test was applied. Continuous parametric variables were tested with Student t-test. Correlation between parameters is expressed by(r) .A *P* value ≤0.05 was considered as statistically significant.

3. Results:

The results of IUI were 46 (76.7%) cases failed to achieve pregnancy and 14 cases (23.3%) with clinical pregnancy, 2 of them failed to achieve ongoing pregnancy (abortion).

As regard the correlation between CASA and SCSA parameters before processing there was only a significant correlation between the absolute count of semen and HDS (r 0.6) (Table 1).

When we compared CASA parameters between the positive andnegative groups for clinical pregnancy we found no significant difference between 2 groups in all parameters except for count of sperms/ml there wassignificant relationship, *P* value 0.03 (Table 2). The same results were obtained when we compared group of positive ongoing pregnancy (n=12) and those with negative outcome (n=46), only sperm count /ml showed significant difference, the *p* value 0.02.

Table (3) demonstrates the comparison between SCSA parameters in cases according to presence of clinical pregnancy, there was a highly significant deference between both groups as regard all parameters except for mean X. Table (4) shows the results of SCSA in cases of ongoing pregnancy, there was a highly significant difference between 2 groups as regard mean Y (*P*< 0.05), DFI and HDS (*P*<0.01).

As regards to two cases of abortion, they were an older age (38y and 40y). CASA parameters were lower semen volume, absolute count and SDI. The results of SCSA of 2 cases showed higher DFI% (24, 26%) and HDS% (4.9, 5) than other cases.

Table (1): Correlation between CASA and SCSA parameters in thestudied cases

Parameter	Count/ml	Absolute Count.	Motility%	Progressive Motility%	Normal forms%	TZI	SDI
	r	r	r	r	r	r	r
X	0.26	0.09	0.25	0.23	0.05	0.21	0.17
Y	0.29	-0.02	-0.24	-0.27	-0.08	0.12	-0.16
DFI	-0.01	0.18	0.37	0.25	-0.04	0.01	0.05
HDS	0.29	***0.60****	-0.02	-0.15	0.02	-0.21	0.04

X: red florescence **Y**: green florescence .**DFI**%: DNA fragmentation index. **HDS**%: high DNA stainability. **TZI:**teratoZoospermic index. **SDI**: sperm deformity index**.** *Significant correlation.

Table (2): Comparison of CASA parameters in +ve and –ve clinical pregnancy cases

Parameter	**Mean of +ve cases for clinical pregnancy N =(14)**	**Mean of -ve cases for clinical pregnancy N = (46)**	***P* value**
Age (y)	32.9±4.8	32.1±4.5	0.69
Volume (ml)	2.64±1.46	2.8±1.6	0.81
Count (mil/ml)	22.6±1.46	43.6±23.2	***0.03****
Absolute count (mil)	48.5±23.7	125.5±114.5	0.09
Motility %	35.7±19.1	50.0±15.7	0.05
TZI	1.37±0.18	1.34±0.21	0.76
SDI	0.91±0.18	0.95±0.15	0.93
	Median (Range)		
Progressive motility%	28(16-70)	24(16-85)	0.99
Normal forms%	10(3-32)	10(2-30)	0.68

TZI: Terato Zoospermic Index. **SDI**: Sperm Deformity Index.

**P* value < 0.05 significant difference.

Table (3): Comparison of SCSA parameters in +ve and –ve clinical pregnancy cases

Parameter	+ve cases for clinical pregnancy N = (14)	-ve cases for clinical pregnancy N = (46)	*P* value
X	102.1±1.41	104.2±0.92	0.72
Y	364.6±19.4	354.8±5.9	***0.02****
DFI%	23.6±4.4	30.4±4.7	***<0.001***
HDS %	4.86±0.70	5.6±0.6	***<0.001***

X: red florescence. **Y**: green florescence. **DFI**: DNA fragmentation index. **HDS**: high DNA stainability.
**P* value < 0.05 significant difference. ** *P* value< 0.01 highly significant difference.

Table (4): Comparison of SCSA parameters in +ve and –ve ongoing pregnancy cases

Parameter	Means of +ve cases for ongoing pregnancy N =(12)	Means of -ve cases for ongoing pregnancy N = (46)	*P* value
X	101.7±1.79	104.2±0.92	0.70
Y	358.1±9.5	354.8±5.9	***0.03****
DFI%	23.5±4.8	30.4±4.7	***<0.001***
HDS%	4.4±0.77	5.6±0.6	***<0.001***

X: red florescence. **Y**: green florescence. **DFI**: DNA fragmentation index. **HDS**: high DNA stain ability.
*P value < 0.05 significant difference. ** *P* value < 0.01 highly significant difference

4.Discussion

In our study 60 semen samples from males with mild male factor infertility were subjected to examination by CASA before processing semen sample prior to insemination by IUI. Aliquots of raw unprocessed semen samples were tested by SCSA flowcytometric analysis for DNA damage.

When we tried to correlate the parameters of CASA and SCSA study, we found only a positive correlation between HDS and absolute count of sperms, while no correlation could be found between all other parameters, our results was in contrast to Sills et al., 2004 who found a significant inverse correlations between high sperm concentration and DFI% and HDS%, also they found that both progressive motility and normal morphology were also strongly inversely correlated with DFI and HDS, this difference in results may be due to the difference in patients number between 2 studies. In another study by Larson *et al.,* 2003, they found no significant correlation between SCSA parameters and CASA parameters. The results were in agreement with Giwercman *et al.,*2003 who found that the association between sperm DNA damage and the traditional semen parameters is shown to be only weak to moderate, another study showed that 25–40% of infertile men may have normal standard sperm characteristics according to WHO criteria, but a DFI above 20–30% (Giwercman *et al.,*2010).

Among our cases there were two cases failed to obtain ongoing pregnancy (abortion), we noticed a lower semen volume and absolute count and elevated DFI% and HDS%, one study stated that men of couples with repeated spontaneous abortions have significantly higher DFI values (Carrell *et al.,*2003).

There was a highly significant negative correlation between the pregnancy rates and the DFI%, where no male couple with DFI ≥30% contributed to an ongoing pregnancy. This was the cut off set by different studies as (Evenson *et al.,*1999, Spano *et al.,*2000) where a larger sample was illustrated. In the study byBungum *et al.*(2007) the test group which contributed to pregnancy had a DFI ≤30 where the negative group for pregnancy had a DFI ≥30. But no cut off for HDS was set for the same groups and it didn't seem to be of predictive value. Another published study by Saleh *et al.*(2003) reached a similar conclusion. They found significantly higher DFI% levels in the couples who failed to obtain a pregnancy after IUI. This study was, however, based on 11 IUI couples only.

In a study by Boe-Hansen *et al.* (2006), it was found that ongoing pregnancy was achieved for only one sample with a DFI >27% and undergoing IVF treatment. The sample was only marginally above the 27% threshold level. Also in that study, the association of a low percentage of immature cells (HDS <10%) with a higher pregnancy rate was not observed.

In contrast to these previous reports of no reported pregnancy following assisted reproduction above a DFI of 28%, Bungum *et al.* (2004) reported an IUI pregnancy in a man with a DFI of 34%, and 13

pregnancies following IVF or ICSI with a DFI ≥27%, suggesting that these techniques can, in fact, overcome such a high level of abnormality. The authors, however, did not, specify the DFI of these individual cases.

In disagreement with this study, other studies found no or weak correlations between pregnancy rates and DFI% like (Tomlinson *et al.,*2001, Morris *et al.,*2002).

In all these reports the parameter of choice - regarding the correlation between DNA alteration and pregnancy rates- was in fact the DFI% and that was logical since it is the most reported parameter of SCSA analysis in all published studies, hence it can be compared to clarify its significance. Also, DFI represents a more complex indicator for the relationship between mean X (red florescence for denatured DNA) and the Y mean (green florescence for native DNA).

Among the data obtained throughout this study, means of the following parameters Y, DFI%, and HDS% showed significant relation to positive clinical pregnancy. While DFI% and HDS% were significantly correlated to proved ongoing pregnancy. In the other hand the ordinary data from semen analysis were poorly correlated to the outcome. This may implicate the need of proper techniques to assess DNA of sperms in the process of ART.

Recently, Evenson, 2013, stated that the SCSA is considered to be the most time- and cost-efficient, precise and repeatable DNA fragmentation assay. SCSA data are more predictive of male factor infertility than classical semen analyses.

Sperm DNA breaks are mainly thought to be a result of oxidative stress. Some reports demonstrating a positive effect of antioxidant therapy in men with a high DFI have been published; however, the study populations have been small and data conflicting (Bungum, 2012) In the future SCSA may also have the potential to give indications for a causal treatment of disturbances of male fertility.

Finally we can summarize our results into two main points. First, the SCSA has proven its importance in demonstrating DNA damage as a cause of infertility in men with mild male factor and the subsequent events in the ongoing pregnancy. The second result, was the relationship between the DFI% and the stability of pregnancy, one of great importance but certainly further separate studies are needed with larger sample and stabilization of the other factors that may interfere.

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