

Effects of Sperm DNA Fragmentation on Semen Parameters and ICSI Outcome Determined by an Improved SCD Test, Halosperm

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Abstract

Background: Sperm DNA fragmentation is known as an important cause of male infertility. The influence of sperm DNA damage on reproductive potential has been subject of many studies indicating various results and remaining the subject controversial. In this study, we investigated differences of the semen parameters and intracytoplasmic sperm injection (ICSI) outcome according to sperm DNA fragmentation levels (DFLs) of patients.

Materials and Methods: The DFLs were determined by Halosperm, a new improved sperm chromatin dispersion (SCD) test. Patients were grouped as low DNA fragmentation group (LFG $\leq 30\%$) and high fragmentation group (HFG $>30\%$).

Results: Our analysis showed that semen parameters including concentration of untreated sperm and motility of prepared semen were low in HGF, whereas other parameters were not different. Sperm DNA fragmentation levels decreased in both groups after semen preparation by density gradient technique.

Conclusion: No difference was detected on ICSI outcomes (fertilization, embryo development, embryo cleavage, embryo quality and pregnancy rates) between two group.

Keywords: Assisted Reproductive Technique, DNA Fragmentation, ICSI, Sperm

Introduction

The effect of sperm DNA fragmentation on infertility has been subject of several studies. Previously, 10-20% DNA fragmentation was reported in ejaculated spermatozoa (1). The infertile men with poor sperm motility and morphology have suggested to possess increased DNA fragmentation levels when compared to men with normal semen parameters (2, 3). Interestingly, men with normal semen parameters has also suggested to have high DNA fragmentation levels (DFL), which can be one of the reasons of unexplained infertility (4).

Aberrant chromatin packaging during spermatogenesis, defective apoptosis before ejaculation, or excessive production of reactive oxygen species (ROS) cause DNA fragmentation in sperm cells, however, the mechanisms underlying the situation has not been clarified yet (5-7).

Controversies still present on the effects of sperm DNA damage on reproductive outcome. Some investigators indicated that clinical pregnancy was affected adversely by sperm DNA damage

in the cases of intracytoplasmic sperm injection (ICSI) (8, 9). Moreover, fertilization achieved by a sperm having fragmented DNA may cause poor embryonic development, decreased implantation and pregnancy rates, and recurrent pregnancy losses (8-12). On the contrary, some others suggested that sperm DNA damage was ineffective on fertilization, embryo quality, and pregnancy rates in cases of *in vitro* fertilization (IVF) and ICSI (13, 14).

Several tests are available to measure sperm DNA fragmentation levels including TUNEL, comet assay, DNA Breakage Detection-Fluorescence In Situ Hybridization (DBD-FISH) test, the chromomycin A3 test, SCSA test and Sperm Chromatin Dispersion (SCD) test (15-20). The halosperm is a new improved SCD test based on the principle that sperm with fragmented DNA does not produce halo of dispersed DNA loops which is characteristic of sperm with nonfragmented DNA.

In this study, our aim is to evaluate the differences

Received: 8 Mar 2010, Accepted: 16 Jun 2010

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Royan Institute
International Journal of Fertility and Sterility
Vol 4, No 2, Jul-Sep 2010, Pages: 73-78

of semen parameters and ICSI outcome between the patients having low and high sperm DFLs assessed by new Halosperm test.

Materials and Methods

Study design

Patients undergoing their first ICSI attempt because of male factor infertility, < 39 years of age and having > 2 metaphase II oocytes were included in our prospective study. A total of 60 patient's sperm samples were analyzed in where 10 of the samples were oligozoospermic (<20 mil/ml sperm concentration), 8 of those were asthenozoospermic (<50% total motility), 17 of samples were teratozoospermic (<4% morphology) and 25 of those showed more than one anomaly. Fragmentation levels above 30% and below 30% considered as high fragmentation group (HFG; n=24) and low fragmentation group (LFG; n=36), respectively (21). We have taken an agreement form from the patients.

Semen analysis

Both the semen analysis (WHO, 1999) and DNA fragmentation assesment were performed on the day of oocyte pick-up. Specimens were collected by masturbation after 3-5 days of sexual abstinence and analysis were performed after liquefaction with a phase contrast microscope (Nikon E 400, Japan). Sperm concentrations, morphology, motility rates and acrosomal status were assessed before and after semen preparation. Sperm motilities were classified as grade A, B, C, D and at least 100 spermatozoa were scored with $\times 40$ objective. Total motility rates were calculated as the total of A, B and C motility rates. Sperm morphology and acrosomal index were assessed according to Kruger's strict criteria after staining with Diff-3 stain (GCC Diagnostics, UK). Acrosomes, comprising 40-70% of the sperm head, having no vacuole with a smooth surface were classified as a normal acrosome and acrosomal index were defined as the percentage of sperm cells having normal acrosome.

Semen samples were prepared by two-layer (90% and 45%) discontinuous PureSperm (Nidacon, Sweden) gradient technique. 1 mL of semen samples were added on top of two layers and centrifuged at 400 g for 20 minutes. Thereafter, 400 μ L of the pellet containing the selected spermatozoa were collected from the bottom of the conical-based tubes (Falcon 2095, Becton Dickinson, USA). Pellets of the same patient were collected and suspended in 5 mL of G-IVF medium (Vitrolife, Kungsbacka, Sweden). The suspensions were centrifuged at 500 g for 6 min. Supernatants were discarded and cen-

trifugation was repeated after suspension with G-IVF medium. Finally supernatants were discarded and pellets were resuspended in 0.5 mL medium for further examination.

Sperm DNA fragmentation assessment

Sperm DNA fragmentation were assessed before and after semen preparation with Halosperm kit (INDAS laboratories, Spain). An aliquot of semen sample was diluted to 10^6 /mL in phosphate-buffered saline (PBS). Agarose in eppendorf tubes provided in the kit were placed in a water bath at 90°-100°C for 5 minutes to fuse the agarose, and then in a water bath at 37°C. After 5 minutes of incubation at 37°C, 60 μ L of the diluted semen sample were added to the eppendorf tube and mixed with the fused agarose. 20 μ L of semen-agarose mixture were pipetted onto slides precoated with agarose provided in the kit and covered with a 24 \times 24 mm coverslip. The slides were placed on a cold plate in the refrigerator (4°C) for 5 minutes to let the agarose to produce a microgel with the sperm cells embedded in. The coverslips were removed and the slides were immersed horizontally in an acid solution prepared by mixing 80 μ L of HCl provided in the kit with 10 mL of distilled water and incubated for 7 minutes. Slides were horizontally immersed in 10 mL of the lysing solution for 25 minutes. After washing 5 minutes with distilled water, the slides were dehydrated in increasing concentrations of ethanol (70%, 90%, 100%) for 2 minutes and then left for drying. Slides were covered with a mix of Wright's staining solution (Merck, Germany) and PBS (1:1) (Merck, Germany) for 5-10 minutes. Slides were washed in tap water and allowed to dry. A minimum of 100 spermatozoa per sample were scored under the $\times 100$ objective in brightfield microscopy. Sperm with large halo (thickness is similar or larger than the length of the smallest diameter of the core) and sperm with medium-sized halo (thickness between: greater than 1/3 of the smallest diameter of the core and less than the smallest diameter of the core) were classified as 'spermatozoa having no fragmentation'. Spermatozoa with a small halo (thickness were similar or smaller than 1/3 of the smallest diameter of the core) and without halo were classified as 'spermatozoa having DNA fragmentation'.

Ovarian stimulation and ICSI procedure

Ovulation induction of female partners were short or long gonadotrophin-releasing hormone (GnRH) analogue suppression protocol or a GnRH antagonist protocol using human menopausal gonadotrophins or recombinant follicle

stimulating hormone. When the dominant follicle reached 17 mm size, 5000 or 10000 IU of human chorionic gonadotropin (hCG) injection were administered. Cumulus-oocyte complexes were retrieved 36 hours after the administration of hCG by the guidance of transvaginal ultrasound. Microinjection procedure was performed as described previously by Van Steirteghem et al. 1993 (22). Sequential media of Sage is used for the culture of oocytes and embryos. Presence of two pronuclei (2PN) and two polar bodies in the oocyte cytoplasm 16-18 hours after ICSI were defined as fertilization. Fertilized oocytes were checked 48 and 72 hours after ICSI for embryonic development. The cleavage status and the quality were assessed with an inverted microscope (Olympus IX71, Japan) according to the scoring criteria of Ziebe et al., 1997 (23). Embryos having fragmentation levels of <10%, 10-20% with uneven blastomeres, 20-50% and >50% were classified as grade A, B, C and D respectively. Grade A and B were classified as good-quality embryos, grade C and D were classified as poor-quality embryos. Embryos having 2-6 cells on day 2 were defined as normally cleaved embryos, otherwise defined as abnormally cleaved. Embryo development rates were defined as the percentage of cleaved embryos fertilized normally.

Embryo transfers were performed by the guidance of abdominal ultrasound on day 2 or 3. Serum β -hCG levels were measured 12 days after transfer. A ≥ 15 mIU/ml β -hCG value on the 12 nd, and a

doubled β -hCG value on the 14 th day indicated a positive pregnancy.

Statistical analysis

SPSS for Windows 10.0 software package was utilised for the statistical analysis. Chi-square test and Mann Whitney U test was used to evaluate the rates and proportions and differences between the groups respectively. The results were evaluated in 95% confidence interval and the statistical significance was defined as, $p < 0.05$. The values of parameters are mean \pm standard deviation.

Results

Patient characteristics, including the female age (30.1 ± 4.4 and 31.8 ± 4.5), the male age (33.1 ± 4.8 and 34.4 ± 4.1), the number of oocytes retrieved (13 ± 6.8 and 12.2 ± 6.1) and the embryos developed (8.6 ± 3.9 and 7.7 ± 4.4) were similar in LFG and HFG respectively. All the patients included were having oligo (10) / astheno (8) / teratozoospermia (17), where 25 of the samples showed more than one anomaly.

Firstly, the semen parameters (morphology, motility and concentration) were compared between HFG and LFG in untreated and prepared semen samples. In untreated semen samples, lower sperm concentrations were observed in the HFG than LFG ($p < 0.05$). However, parameters including morphology, acrosomal index, total motility and progressive motility rates were found similar between the groups (Table 1).

Table 1: Mean values \pm SD of semen parameters in LFG and HFG groups

	LFG (fr $\leq 30\% \pm$ SD)	HFG (fr $> 30\% \pm$ SD)
Untreated semen parameters (%)		
DNA fragmentation rate	26.2 \pm 6	34 \pm 10.2
Total motility rate	68.6 \pm 13.8	59.3 \pm 20.8
Progressive motility rate	18.6 \pm 10.6	11.6 \pm 10.2
Normal morphology rate	1.4 \pm 1.3	0.8 \pm 1.4
Acrosomal index	80 \pm 12.6	69.2 \pm 19.6
Sperm concentration (mil/ml)	70.7 \pm 54.4	28.7 \pm 36.4 *
Semen parameters after preparation (%)		
DNA fragmentation rate	16.3 \pm 4.5	15 \pm 7.4
Total motility rate	91.8 \pm 4.6	75.6 \pm 18.4 *
Progressive motility rate	51.6 \pm 16	29 \pm 21.1 *
Normal morphology rate	2.1 \pm 1.9	1.2 \pm 1.1
Acrosomal index	86 \pm 8.8	74 \pm 12.1
Sperm concentration (mil/ml)	45 \pm 40	22 \pm 29.1
Difference in DNA fragmentation	9.9	19

* Statistically significant at $p < 0.05$

Table 2: Mean values ± SD of ICSI outcome in LFG and HFG groups

ICSI outcomes	LFG (fr ≤ 30% ± SD)	HFG (fr > 30% ± SD)
Number of oocytes collected	13 ± 6.8	12.2 ± 6.1
Number of oocytes injected	10.6 ± 5	9.4 ± 4.5
Mean number of oocytes fertilized	8.6 ± 4.3	7.9 ± 4.3
Fertilization rate (%)	84 ± 0.18	81 ± 0.14
Mean number of embryos developed	8.6 ± 3.9	7.7 ± 4.4
Embryo development rate (%)	100 ± 0.0	95 ± 0.13
Normally cleaved embryo rate (%)	94 ± 0.08	88 ± 0.22
Good quality embryo rate(%)	83 ± 0.27	70 ± 0.24
Number of embryos transferred	2.4 ± 0.68	2.5 ± 0.85
Pregnancy rate (%)	68	71

No statistically significant difference was obtained

After sperm preparation, motility rates (total and progressive motility) declined in the HFG ($p < 0.05$) as shown in Table 1. Additionally, the fragmentation levels significantly reduced in both groups after semen preparation (9.9 % in the LFG and 19% in the HFG).

Secondly, ICSI outcome were compared between LFG and HFG groups. No significant differences were observed in parameters including fertilization (84% and 81%), embryo development (100% and 95%), normally cleaved (94% and 88%) and good quality embryo rates (83% and 70%) between LFG and HFG respectively ($p > 0.05$) (Table 2). In addition, pregnancy rates were not different between the groups (68% and 71% respectively) ($p > 0.05$) (Table 2).

The values of parameters indicates mean ± standard deviation of data.

Discussion

Conflicting results are present on the effects of sperm DNA fragmentation on semen parameters and the requirement for sperm chromatin assays in routine laboratory examinations. Some studies reported no correlation between sperm DNA fragmentation and semen parameters (24, 25) whereas others observed a negative correlation in some and/or all parameters (26, 27). Recently, Cohen-Bacrie et al., 2009 reported a negative correlation between rapid progression rate and DNA fragmentation determined by TUNEL, but no correlation between sperm DNA fragmentation, sperm morphology (head, acrosome and intermediate piece) and concentration (28). The chromatin condensation has been suggested a valuable parameter to assess male fertility and used in routine laboratory investigations of semen in the cases of assisted reproduction (29).

Several methods are available to detect sperm DNA fragmentation including Halosperm, an improved SCD test developed by Fernández (20). There is limited data in the literature comparing the differences in semen parameters of patients having different DNA fragmentation levels determined by Halosperm. By using this technique, a negative correlation was found between all semen parameters (motility, morphology, concentration) and DNA fragmentation levels determined by Halosperm (30). In our study, we compared the differences between the LFG and HFG in untreated and prepared semen. The sperm concentrations in the untreated semen samples and the motility rates (total and progressive motility) reduced after semen preparation in the HFG however other parameters did not change. Thus, reduction of sperm concentration may indicate an apoptotic process present in those semen samples. In addition we found both total and progressive motility increased more in LFG than HFG after semen preparation suggesting that semen samples having lower DNA fragmentation levels benefits more from semen preparation by means of sperm motility.

Sperm DNA fragmentation levels reduced significantly in both groups after preparation proposing that semen preparation by density gradient technique helps to eliminate the sperm cells having fragmented DNA therefore decrease the fragmentation levels in semen.

In the second part of the study, we compared ICSI outcome of the groups including fertilization, embryo development, embryo quality, embryo cleavage and pregnancy rates. No correlation were reported between DNA fragmentation and fertilization rate using SCSA or comet assay (4, 8, 31), on the contrary, an inverse relationship were found in the studies utilizing Halosperm and SCD test

(30, 32). In our results we found no differences in fertilization rates, embryo development, embryo quality (good and bad quality), and embryo cleavage (normally and abnormally cleaved) rates between the groups. According to these results it may be suggested that embryo's developmental potential is not affected by DNA fragmentation level of the semen until day 3 of development as the embryonic genome is activated after day 3 (33), that oocytes and embryos can repair sperm DNA damage (34) and because of the patient population selected. It may also be considered that, the sperm having fragmented DNA were eliminated and the best sperm was chosen with higher probability to have an intact DNA for the ICSI procedure. Our results confirm the results of other studies where different methods used for DNA fragmentation assessment. These investigators concluded that fertilization, embryo development and subsequent pregnancy were not affected by DNA fragmentation of the sperm population (35, 36).

Additionally, we examined the relationship between DNA fragmentation and pregnancy outcome. A recent meta-analysis and several other studies including studies using Halosperm and SCD test did not observe a relationship between sperm DNA damage and pregnancy rates (30, 32, 35, 37). On the contrary, in other studies a negative correlation between sperm DNA fragmentation and pregnancy rates were reported (9, 38). We observed no differences in pregnancy rates between the groups after ICSI. The lack of association between sperm DNA damage and pregnancy rates may be the result of selecting best sperms for ICSI and choosing the best scoring embryos for transfer. This may reduce the risk to use a sperm having fragmented DNA and therefore potential adverse effects of sperm DNA damage on pregnancy rates may not be observed.

Conclusion

Our results show that, semen parameters including initial sperm concentration and motility after preparation were decreased in patients having high DNA fragmentation whereas no differences were observed in other parameters. Semen preparation by density gradient technique decreased the sperm percentage of fragmented DNA, causing a higher probability to choose a sperm with intact DNA. None of the ICSI outcome parameters including fertilization, embryo development, quality and cleavage and pregnancy rates were affected by sperm DNA fragmentation levels. More detailed studies are required to observe the effects of sperm DNA fragmentation on semen param-

eters and ICSI outcome.

Acknowledgements

Study's financial support is done by Ferticenter IVF Unit. There is no conflict of interest in this article.

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