

SPERM DNA INTEGRITY TEST AND ASSISTED REPRODUCTIVE TECHNOLOGY (ART) OUTCOME

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Summary

The objective of the study was to investigate the influence of sperm DNA fragmentation index (DFI) by DNA integrity test on pregnancy outcome and pregnancy loss after assisted reproductive technology (ART) procedure: autologous intracytoplasmic sperm injection (ICSI), donation eggs ICSI, and intrauterine insemination (IUI). We investigated men from 531 couples undergoing autologous ICSI procedure (n=416), from couples undergoing donation eggs procedure (n=39) and IUI (n=76). We performed the following interventions: semen analysis, DNA integrity test, embryo scoring by Gardner and Schoolcraft grading system (1999). The study showed no statistically significant differences between the group regarding pregnancy rate ($\chi^2=0.55$; $p>0.05$; OR=1.25, 95% CI 1.23-1.46; $p>0.05$). However, with increased levels of DFI, the number of pregnancy losses became higher (including biochemical pregnancies and spontaneous abortions) at OR=5.65 (95% CI 4.32-7.11; $p=0.05$). We examined the percentage of grade I blastocysts (by Gardner and Schoolcraft, 1999) before donation eggs embryo transfer and found a statistically significant correlation with both the DFI ($\chi^2=7.80$; $p<0.05$) and sperm morphology ($\chi^2=6.14$; $p<0.05$). Analysis of the relationship between DFI and IUI output (clinical pregnancy, miscarriage) revealed significant correlations in both directions: between DFI and pregnancy rate after IUI ($\chi^2=6.29$; $p<0.05$) and between the DFI and pregnancy development after IUI ($\chi^2=6.87$; $p<0.05$). The three group categories (autologous, heterologous ICSI procedures and IUI) studied showed that sperm samples with DFI>27% were associated with increased risk of early pregnancy loss. Men with infertility should undergo DNA fragmentation assay in addition to the standard semen analysis. When DFI exceeds 27%, ICSI should be a method of choice, even in cases where the conventional parameters of semen analysis tests are normal.

Key words: sperm DNA fragmentation, male infertility, ICSI, pregnancy rate, pregnancy loss

Introduction

The male factor is one of the most common causes of infertility, assuming that defects in sperm account for 30% to 50% of clinical cases of infertility [1-3]. Sperm abnormalities, including decreased sperm concentration and motility, and abnormal sperm

morphology forms, lead to reduced likelihood to achieve natural pregnancy. Before 1992, severe abnormalities of sperm parameters were not fully addressed by current procedures for treatment of infertility as intrauterine insemination (IUI) and conventional in vitro fertilization (IVF) procedure. The establishment of intracytoplasmic sperm injection (ICSI) in 1992 revolutionized the treatment of male infertility, allowing males with a single living sperm, regardless of other parameters, to become candidates for treatment of infertility [4]. However, in many clinics, using ICSI, the outcome of the procedures is below expectations. The first reports appeared on higher success rate of surgical procedures with extracted sperm [5, 6], and the lack of correlation between conventional sperm parameters. Unsuccessful ICSI cycles became the focus of debate for reproductive specialists.

In clinical practice, the etiology of unsuccessful ICSI cycles is most often associated with the female partner's age [7], regardless of sperm parameters. Therefore, many couples, failing to complete the assisted reproductive technology (ART) procedure are offered egg donation. The development of abnormal embryos [6] and blastocysts is associated with abnormal sperm parameters [8-11]. Results from assessment of sperm DNA fragmentation (DFI) by analyzing sperm chromatin structure (DNA Integrity test), have impact on both natural pregnancy [10, 11] and that achieved through ART. Pilot studies have shown that high levels of DNA fragmentation (DFI>27%) decrease fertility potential in patients undergoing ART procedure [12], even in men with completely normal standard sperm parameters [13]. Therefore, the lack of correlation between conventional parameters of sperm and DNA fragmentation [5, 9, 14, 15] determine DNA fragmentation as a potential source of male infertility in normozoospermic men. That is why further evaluation of sperm DNA fragmentation is required in the study of male infertility [16].

Materials and Methods

Subject selection

The study was based on a cohort of consecutive infertile couples undergoing ART at the centre for ART procedure (Medical Center Clinical Institute for Reproductive Medicine – Pleven, Bulgaria), during the period January 2010 –

December 2014. A total number of 531 ART procedures: 416 autologous ICSI cycles, 39 donation egg procedures, and 76 IUI procedures (28 donor sperm procedures and 48 autologous IUI procedures) were included. The inclusion criteria for the female partners were baseline follicle-stimulating hormone (b-FSH) <12.5 IU/l for both autologous procedures.

Semen collection and preparation

Semen sample were collected by masturbation after 3 to 7 days of sexual abstinence. After complete liquefaction at 36.6 C° for 30 minutes, 5 µL of each sample was loaded in an improved Neubauer counting chamber (Marienfeld Superior, Germany) where conventional sperm analysis of concentration was carried out. Morphology was assessed according to strict Kruger criteria, and motility was measured. All results were classified according to the World Health Organization (WHO) 2010 criteria [17].

DNA integrity test

Sperm DNA damage was evaluated by flow cytometry after staining with acridine orange. On the day of analysis, the samples were defrosted and immediately tested. All buffers and samples were kept at 4 to 6 C° and the test was performed in a box with ice. After washing with TNE (0.15M NaCl, 0.01M TrisHCL, 1mM EDTA, pH 7.4) buffer, the sperm concentration was adjusted to approximately 1-2x10⁶ cells/ml and 200 µL of the sample was placed in 12x75 flow cytometry test tubes. After that, 400 µl of low-pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15M NaCl and 0.08M HCl was added for 30 sec., immediately followed by 1.2 ml staining buffer (phosphate-citrate buffer, pH 6.0) with 6 mg/l acridine orange (AO, chromatographically purified; Sigma-Aldrich, USA). The cells acquired were analysed using a FACScan flow cytometer (Becton Dickinson). A total of 5000 events were accumulated for each measurement at a low flow rate. A 488 nm light source was used. The study of the sperm chromatin was based on the properties of metachromatic AO staining. Acridine orange emitted green light upon binding to double-stranded DNA (native) and red light when binding to a single-stranded DNA. Data was analyzed by FlowJo software (TreeStar, Inc, Ashland, OR). Computer gates were used to determine the proportion of spermatozoa with increased levels of red and green fluorescence. The results were presented as DNA

fragmentation index (DFI), high DNA stainability (HDS).

ART procedures

ICSI

Oocytes for recombinant FSH (rec-FSH - Gonal F, Merck Serono; Puregon, Organon) were retrieved from women after ovarian stimulation for 7-11 days. When at least one follicle reached a diameter >18 mm, 10 000 IU of human chorionic gonadotropin (hCG - Pregnyl, Organon) was administered to induce follicle maturation. Extraction of eggs was performed 36 hours later under vaginal ultrasound control by follicle aspiration.

IUI

Fallopian tubes patency was proved by either hysterosalpingography (HSG) or laparoscopy. Female patients were stimulated by clomiphene citrate (Clostilbegyt) for 5 days and human menopausal gonadotropin (hMG, Merional) 75 IU of day 8±3 for 4 days.

When two or more follicles measured more than 18 mm, 10 000 IU hCG (Choriomon, Pregnyl) was applied to stimulate follicular maturation. After 36 hours, a raw semen sample treated by the double-density gradient method was prepared. The procedure for washing and dilution was performed under Supra Sperm media (Origio) and 1 ml aliquot of this sperm sample was used for insemination.

Pregnancy outcomes

A biochemical pregnancy (BP) was defined as a plasma β -hCG concentration of >5 IU/l, 14 days after embryo transfer (ET). A clinical pregnancy (CP) was defined as the presence of gestational sac detected by ultrasound and with a heartbeat 3 weeks after a positive result from a β -hCG test. Pregnancy loss was defined as miscarriage before gestation week 12.

Embryos scoring

The Gardner and Schoolcraft (1999) grading system was used to evaluate blastocysts expansion. A grade 1 blastocyst was assumed to have a blastocoel cavity of less than half of the volume of the embryo.

Statistical analysis

Statistical analysis was performed using Statgraphics plus 2.1 software for Windows. The data are presented as mean \pm standard deviation

(mean \pm SD). Statistical significance was set at $p \leq 0.05$.

Results

ICSI procedure

We investigated data of 416 couples undergoing autologous ICSI procedure and 39 couples undergoing donation eggs procedure. In the first group, the males' mean age range was 35.16 \pm 5.7 years, and that of the female partners was 34.42 \pm 4.24 years. We registered 189 positive pregnancy tests and 227 negative tests on day 14 after ET. The pregnancy rate outcome was 45.4% (Table 1).

The female patients were grouped according to age and the pregnancy rate was registered for each group. Results were as follows: group 1 - 61 patients, age <29 years, pregnancy rate 52.46%; group 2 - 179 patients, age range 30-35 years, pregnancy rate 48%; group 3 - 122 patients, age range 35-40, pregnancy rate 46.7%; and group 4 - 53 patients, age 40-43 with a pregnancy rate 13%.

Regarding the pregnancy loss after a positive test on day 14 after ET, we registered 122 clinical pregnancies with delivery (67.4%). Pregnancy loss was registered in 59 women - 24 BP (3.2%) and 35 missed abortions (19.4%).

Regarding DFI, the patients were divided into two groups depending on the cut-off value of DFI (27%). No statistically significant difference was seen between the DFI value and the pregnancy rate ($\chi^2=0.55$; $p>0.05$; OR=1.25; 95% CI 1.23-1.46).

Increased values of DFI were associated with higher rates of pregnancy loss (OR=5.65; 95% CI 4.32-7.11; $p<0.05$), including biochemical pregnancies and spontaneous abortions (SPA). In the group of DFI<27%, the pregnancy loss rate was 27% (14% BP; 13% SPA), while in the group of DFI >27%, the pregnancy loss rate was twice as high (63%).

To evaluate the embryo quality, we used the scoring system of Gardner and Schoolcraft. According to that system, the cut-off value of grade I blastocyst is 40%. Two groups were formed according to this value, above and below a value of 40%, respectively, and the DFI values for paternal spermatozoa in the two groups were compared. From sperm with normal DNA fragmentation, 87.23% of embryos were obtained, and of these, 64.6% were classified as grade I quality, and 35.4% were of low quality.

Table 1. Comparison of couple characteristics (data from 531 ART procedures), sperm parameters, DNA damage, embryo quality and pregnancy outcome between pregnant and non-pregnant couples by cycle type (IUI, ICSI and donation egg ICSI)

	IUI		ICSI		Donation egg ICSI	
	Pregnant	Not Pregnant	Pregnant	Not Pregnant	Pregnant	Not Pregnant
Cycles included (n)	14	62	189	227	15	24
Male age (years)	31.05 ±7.3	44±5.5	35.3±5.7	36.2±5.8	—	—
Sperm concentration (million/ml)	68.85 ±24.4	27.17±1.59	50.79±3.4	48.25±4.48	59.4±5.6	51±5.6
Progressive motility (a+b, %)	55.79 ±18.7	52.33±20.4	39.9±9.6	41.6±11.6	45.6±5.6	43.5±5.6
Sperm morphology (normal forms, %)	—	—	3.7±2.03	3.4±2.4	3.6±5.6	3.2±5.6
DFI (%)	8.86±7.14	15.09±9.4	14.18±8.9	15.85±10.12	16.69±5.6	15.34±5.6
HDS (%)	5.85±3.01	9.65±2.99	10.7±6.62	11.4±5.08	7.35±5.6	7.72±5.6
Female age (years)	32.7±7.3	36.5±7.2	33.7±3.8	35.3±4.48	41±5.6	45.2±5.22
Oocytes retrieve (n)	—	—	15.94	12.5	21±5.6	18±5.6
Blastocysts grade I (%)	—	—	68±24.8	66.7±27	65±5.6	54±5.6
Pregnancy rate (%)	18.8	—	45.4	—	55.2	—

The distribution in the embryos group, obtained from sperm cells with DFI>27% was similar: 54.7% of blastocysts were classified as good quality embryos. No statistically significant difference was found for the DFI of the paternal spermatozoa in the two groups (p>0.05).

The highest percentage of high-quality embryos (72.13%) were observed in the female age group below 29 years. This percentage was 65.4% in the group with age range 30-35 years, and in the 36-40 and over 40 age groups it was 57.02% and 63.3%, respectively. In this distribution, we found a weak correlation between the age of female patients and embryo quality ($\chi^2=4.64$; $r=0.10$; $p<0.05$). This suggests that it was not the age of females that determined the embryo quality but rather the cause of infertility and the stimulation procedure protocol. After using long-down stimulation protocol, the mean number of the eggs retrieved was 13.5 eggs per patient (SD±9.8). The age distribution of females is shown on Table 2.

Table 2. Distribution between female age and number of retrieved eggs by ICSI procedure

Eggs (n)	<10	10-15	>15	Total
Female age				
<29 years	6.28%	1.21%	7.25%	14.73%
30-35 years	18.85	10.63%	13,74%	43.24%
36-40 years	14.2%	5.50%	9.42%	29.23%
>40 years	8.94%	1.45%	2.42%	12.80%
Total	48.31%	18.84%	32.82%	100%

We found a statistically significant relationship between the number of oocytes, obtained after stimulation and the age of the patient ($\chi^2=22.8$; $p<0.001$), as well as between the number of oocytes per female and pregnancy outcome ($\chi^2=10.62$; $p<0.05$; Table 3).

A statistically significant correlation between sperm morphology and pregnancy outcome after ICSI procedure was not found ($\chi^2=0.46$; $p>0.05$), nor was a correlation found with the occurrence of spontaneous abortions after ART ($\chi^2=2.47$; $p>0.05$).

This study found statistically significant correlation ($\chi^2=12.19$; $p<0.05$) between sperm morphology and DFI. The distribution between DFI and sperm morphology is shown on Table 4.

Regarding sperm motility, a weak statistical dependence on DFI was found ($\chi^2=2.47$; $p<0.05$). The distribution between DFI and sperm progressive motility is shown on Table 5.

Table 3. Distribution between the number of eggs per female and pregnancy outcome after ICSI

Eggs/female	Pregnancy outcome	Pregnant	Not pregnant	Total
<10		18.07%	30.12%	48.19%
10-15		9.4%	9.4%	18.8%
>15		18.07%	14.94%	33.01%
Total		45.54%	54.46%	100%

Table 4. Distribution between DFI and sperm morphology (ICSI procedure)

DFI (%)	Morphology (normal forms, %)	>4%	0-3%	Total
<26.9%		41.35%	45.67%	87.02%
>27%		2.88%	10.1%	12.98%
Total		45.23%	54.77%	100%

Table 5. Distribution between DFI and sperm progressive motility (ICSI procedure)

DFI (%)	Motility (a+b, %)	>32%	<32%	Total
<26.9%		72.36%	14.66%	87.02%
>27%		9.38%	3.61%	12.98%
Total		81.73%	18.27%	100%

Donation eggs ICSI procedure

The data were collected from 39 heterologous ICSI donation egg procedures. The mean age of the recipients was 41±5.22 years. The pregnancy rate was 55.2%, and 66.7% clinical pregnancies were achieved. In 33.3% of the cases, reproductive failures were registered (miscarriages or biochemical pregnancies).

We found no significant difference between DFI and pregnancy rate of heterologous ICSI donation egg procedures ($\chi^2=1.41$; $p>0.05$) and there was a weak statistical correlation between DFI and pregnancy loss ($\chi^2=3.49$; $p<0.05$; $r=0.25$; $p=0.03$; Table 6).

When examining the impact of sperm parameters on the outcome of ICSI procedures with donated eggs, a statistically significant correlation between sperm morphology and outcome of heterologous ICSI procedures ($\chi^2=0.50$; $p>0.05$) was not found (Table 7).

Evaluation of percentage of grade I blastocysts (by Gardner and Schoolcraft, 1999) before ET revealed a statistically significant correlation with both DFI ($\chi^2=7.80$; $p<0.05$) and sperm morphology ($\chi^2=6.14$; $p<0.05$).

There was no statistically significant difference in the percentage of grade I quality blastocysts between autologous and heterologous

procedures ($p>0.05$).

IUI

Data about 48 couples undergoing autologous procedure of IUI, and 28 women who underwent insemination with donor sperm (76 treatments altogether) were analyzed.

The average age of the men involved in IUI was 31.08±7.1 years, and that of the women was 32.6±5.5 years. The oldest female patient was 43 years old.

The pregnancy rate in autologous procedures was 18.18%, and for the IUI donor sperm procedures it was 17.8%. The distribution between DFI and IUI outcome is shown on Table 8.

The highest pregnancy rate was registered in the group aged 36-40 years - 24.1%. We found no statistically significant relationship between women's age and the IUI success rate ($\chi^2=2.72$; $p>0.05$).

Regarding the distribution of pregnancy loss rate after IUI procedure no correlation was established with the age of the female patients ($\chi^2=1.31$; $p>0.05$). In the IUI group, 14 positive tests recorded, of which nine clinical pregnancies ended with delivery (64.29%), and miscarriage was registered in five women (35.71%).

When examining the relationship between DFI and IUI outcome (clinical pregnancy, miscarriage) significant correlations were found in both directions: between DFI and pregnancy rate after IUI ($\chi^2=6.29$; $p<0.05$) on the one hand, and between the DFI and pregnancy development after IUI, on the other hand ($\chi^2=6.87$; $p<0.05$).

Depending on the outcome, the male patients were divided into two groups: fertile and infertile. A statistically significant difference was found between the two groups for sperm

concentration/ml ($p<0.01$; Student's t-test=2.07). In the fertile group, the DFI was significantly lower than that in the infertile group ($p<0.05$). As to the fertile group, the HDS was also significantly lower than that in the infertile patient group ($p<0.05$; $r=0.5$; t-test=1.51).

Comparison between DFI in the pairs with clinical pregnancy and those with spontaneous abortion revealed a statistically significant difference ($p<0.05$; t-test=1.58).

Table 6. Distribution between DFI and pregnancy rate (donation eggs ICSI procedure)

ICSI outcome \ DFI%	Pregnant	Not pregnant	Total
<26.9%	47.3%	42.11%	89.47%
>27%	7.89%	2.33%	10.53%
Total	55.26%	44.74%	100%

Table 7. Distribution between sperm morphology and pregnancy rate (donation eggs ICSI procedure)

ICSI outcome \ Morphology (normal forms, %)	Pregnant	Not pregnant	Total
<4%	36.84%	26.32%	63.16%
>4%	18.42%	18.42%	36.84%
Total	55.26%	44.74%	100%

Table 8. Distribution between DFI and IUI outcome

IUI outcome \ DFI	Clinical pregnancy	SPA	Total
<26.9%	64.29%	14.29%	78.57%
>27%	0.0%	24.43%	21.43%
Total	64.29%	35.71%	100%

Discussion

ICSI

The mean age of our male and female patients undergoing fertility treatment was higher than 30. Most of the female patients were 30-35 years old, with a relatively good pregnancy rate. Regarding the number of oocytes retrieved after a long down stimulation protocol, data showed that most of the females fell in the group in which up to 10 eggs per female were retrieved. There was also a negative correlation between the number of oocytes and the age of the patient, as could be expected. The results showed that the highest chance of achieving pregnancy was registered in the group of women from whom 10 to 15 were retrieved. The number of oocytes determined the pregnancy rate. However, no correlation was

found between the oocyte number and pregnancy loss rate.

These data suggest a successful model of stimulation to retrieve an optimal number of 10 eggs in order to achieve a high pregnancy rate with ICSI procedure.

The study found no correlation between DFI and registration of pregnancy on day 14 after ET.

Our results confirmed data reported by other researchers [18-20]. The presence of sperm with damaged DNA has a negative impact only on pre- and post-implantation embryo development [21, 22]. This phenomenon, known as ART bypass, occurs during ICSI procedures with spermatozoa with damaged chromatin. High DFI does not predict successful treatment by ICSI but we found a statistically significant relationship between DFI>27% and pregnancy loss. When

comparing the DFI in fertile and infertile patients in relation to the percentage of grade I blastocysts obtained, no statistically significant correlation was found. This reduction of the pregnancy loss rate after ICSI could be attributed to previous treatment with antioxidants (Lycopene, Astaxanthin)[23].

According to literature data, HDS has no predictive value for success with ICSI [24], which agrees with the data we obtained.

Sperm parameters (concentration, motility, morphology) have no predictive value on ICSI procedure outcome, either. A statistically significant correlation was found with respect to motility and morphology, as compared to the registered pregnancy rate after the ET.

The comparison between fertile and infertile men involved in ICSI procedures showed no significant difference between sperm parameters between the two groups. Probably, conventional sperm parameters reflect functional sperm ability.

Hence, the main conclusion is that choosing the best quality sperm cells is crucial for the success of ICSI procedure. However, this has no influence on events after achieving pregnancy.

No correlation was found between pregnancy rate outcome and sperm parameters and DFI in the group undergoing donation egg procedure. Our results showed an important correlation between DFI and the quality of blastocysts.

The correlation between DFI and occurrence of SPA after heterologous ICSI procedure was weak. These data confirmed the assumption of the leading role of functional properties of the egg in terms of pregnancy outcome in heterologous procedures.

IUI

Sperm DNA fragmentation has been shown to be an independent predictor of success in couples undergoing intrauterine insemination [25]. According to the Georgetown fertility study [25], the chances of occurrence of pregnancy are greatly reduced in DFI>30% (OR=8.0). In this study, DFI>30% was shown to be associated with the highest rate of spontaneous abortions. The period of pregnancy was shorter in DFI<15%. A value of DFI of 20-25% seems to be a clinically significant threshold for natural conception. Bungum M. et al. [26] reported that the IUI success declined in cases of DFI>20% and approached zero when DFI was 30%. Giwercman [27] believes that for men with poor sperm morphology, the DFI threshold for reduced

fertility was 20%.

Our study found a relatively lower success rate of IUI procedures in older females. This also explains the lower success rate in the group with heterologous (donor) procedure. With IUI, a significant correlation was found between the DFI and the procedure outcome as regards registered pregnancies. In relation to miscarriages, HDS was significantly lower in the group of men from couples with registered pregnancy. When comparing DFI between men from couples with clinical pregnancy and those with miscarriages, a significant difference was established.

Of the sperm parameters, only the concentration of sperm/ml proved to be associated with pregnancy rate after IUI.

Conclusions

It could be concluded that DFI is a predictive factor for ART outcome. DFI can be used as an independent predictor of pregnancy and childbirth in couples undergoing IUI. We can argue that the ICSI procedure allows avoiding failure in case of damaged semen chromatin. High DFI does not exclude successful ICSI, but BP and miscarriages could be of two-fold higher incidence in ICSI procedures when the DFI exceeds 27%.

The three groups of patients undergoing autologous and heterologous ICSI procedures and IUI in our study showed that DFI>27% was associated with increased risk of early pregnancy loss.

Infertile males should undergo DNA fragmentation assay in addition to standard semen analysis. When DFI exceeds 27%, ICSI should be a method of choice, even in cases where the conventional parameters of the semen analysis are normal. This study showed that DFI was higher than 27% in 13% of the patients.

Concerning HDS, it seems that this parameter had a predictive value only on the IUI outcome.

The biological explanation of the good results after ICSI in cases of high DNA fragmentation needs to be further clarified. In this regard, it could be assumed that donor eggs possess a good capacity for repair in case of sperm DNA fragmentation. Our study showed an increased risk of early pregnancy loss in cases with high levels of DNA fragmentation in ART (ICSI, IUI). High levels of DNA fragmentation are associated with a smaller percentage of blastocysts grade I

after ICSI. It seems that the choice of morphologically normal and functional quality sperm increases the chances for successful pregnancy. The choice of a low sperm DNA fragmentation provides a high percentage of good quality blastocysts and a successful course of pregnancy.

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