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Sperm chromatin structure assay in prediction of in vitro fertilization outcome

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SUMMARY

Sperm DNA fragmentation index (DFI) assessed by sperm chromatin structure assay is a valuable tool for prediction of fertility in vivo. Previous studies on DFI as predictor of in vitro fertilization (IVF) outcome, based on relatively small materials, gave contradictory results. The present study examines, in a large cohort, the association between sperm DFI and the outcome of IVF/ICSI procedure. The study is based on 1633 IVF or ICSI cycles performed at the Reproductive Medicine Centre, Skåne University Hospital, Malmö, Sweden, between May 2007 and March 2013. DFI values were categorized into four intervals: DFI ≤ 10% (reference group), 10% < DFI ≤ 20%, 20% < DFI ≤ 30%, DFI > 30%. For the three latter intervals, the following outcomes of IVF/ICSI procedures were analyzed in relation to the reference group: fertilization, good quality embryo, pregnancy, miscarriage, and live births. In the standard IVF group, a significant negative association between DFI and fertilization rate was found. When calculated per ovum pick-up (OPU) Odds Ratios (ORs) for at least one good quality embryo (GQE) were significantly lower in the standard IVF group if DFI > 20%. OR for live birth calculated per OPU was significantly lower in standard IVF group if DFI > 20% (OR 0.61; 95% CI: 0.38–0.97; $p = 0.04$). No such associations were seen in the ICSI group. OR for live birth by ICSI compared to IVF were statistically significantly higher for DFI > 20% (OR 1.7; 95% CI: 1.0–2.9; $p = 0.05$). OR for miscarriage was significantly increased for DFI > 40% (OR 3.8; 95% CI: 1.2–12; $p = 0.02$). The results suggest that ICSI might be a preferred method of in vitro treatment in cases with high DFI. Efforts should be made to find options for pharmacologically induced reduction of DFI. The study was based on retrospectively collected data and prospective studies confirming the superiority of ICSI in cases with high DFI are warranted.

INTRODUCTION

Infertility is a profound medical and social problem affecting one in six couples trying to become pregnant (Templeton *et al.*, 1990). Up to 50% of the infertility problems are described to be related to a male factor (Comhaire, 1987). Investigation of the male partner in the infertile couple is traditionally based on the conventional semen analysis, which includes assessment of sperm concentration, motility, and morphology. The analysis has, however, a limited value both as a diagnostic tool and as a guide to selection of the therapeutic procedure (Bonde *et al.*, 1998; Jequier, 2004). To overcome these limitations, a number of new sperm tests have been developed, perhaps most promising being those assessing sperm DNA integrity (reviewed in (Erenpreiss *et al.*, 2006)). Among them, sperm chromatin structure assay (SCSA), introduced by Evenson (Evenson *et al.*, 1980), is based on a rather standardized methodology and has been shown to be of clinical value (Spano *et al.*, 2000; Bungum *et al.*, 2007). It has been shown that DNA fragmentation index (DFI) as measured by SCSA is a relatively independent predictor of male sub-fertility in vivo (Giwercman *et al.*, 2010). The chance of conception achieved by intercourse or by intra-uterine insemination decreases already at DFI levels above 20% and approaches zero when DFI exceeds the level of 30% (Spano *et al.*, 2000; Bungum *et al.*, 2007). These findings indicate that DFI is a clinically useful marker of male infertility. A further question is to what degree sperm chromatin integrity affects the outcome of in vitro-assisted reproductive technology (ART) (Evenson *et al.*, 1999; Larson *et al.*, 2000; Larson-Cook *et al.*, 2003). It is agreed that even spermatozoa with high DFI can be used to achieve pregnancy with help of in vitro techniques i.e., in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Gandini *et al.*, 2004; Bungum *et al.*, 2007), but it is still unclear whether the chance of pregnancy is related to the level of DFI.

In addition to pregnancy and the implantation rate, the outcome of IVF and ICSI can be assessed by fertilization rate and embryo quality. Whilst the impact of sperm DNA integrity on embryo development and implantation was confirmed by animal studies (Ahmadi & Ng, 1999; Penfold *et al.*, 2003), the findings in human studies are conflicting. While some of studies do not reveal any value of assessment of sperm chromatin damage in prediction of fertilization failure or pregnancy rate (Niu *et al.*, 2011), this association is clearly seen by others (Saleh *et al.*, 2003; Bungum *et al.*, 2007). A recent meta-analysis indicated an impact of high DFI on the pregnancy outcome after IVF or ICSI but no statistical significance was seen when SCSA specifically was evaluated as a method of assessment of DFI. Thus, the predictive value of SCSA was not confirmed for IVF or ICSI (Zhang *et al.*, 2015). These ambiguous results can, at least partly, be the effect of the small study sizes, lack of distinction between various types of ART and the use of different techniques for assessment of DFI. The present study evaluates in a larger sample the predictive value of DFI assessed by SCSA in relation to fertilization rate, embryo quality, pregnancy rate, the risk of miscarriage as well as probability of live birth following IVF and ICSI.

MATERIALS AND METHODS

Study design and patient population

This is a cohort study where data were analyzed retrospectively. The outcome of IVF treatments in regard to fertilization, embryo quality, pregnancy, miscarriage, and live birth were analyzed in relation to the level of DFI. The study is based on a database of 6660 consecutive IVF procedures performed at the Reproductive Medicine Centre (RMC), Skåne University Hospital, Malmö, Sweden, between May 2007 and March 2013. Generally, the criteria for being treated at this public university clinic included female age below 39 years at start of the treatment, female BMI preferably below 30 kg/m^2 as well as both partners being non-smokers. Four hundred and seventy-one cycles were excluded because donated and/or cryopreserved gametes were used or cryopreservation of all oocytes was performed. Furthermore, one hundred and twenty-nine cycles were excluded because non-ejaculated spermatozoa were used for fertilization. Among residual 6060 cycles, only the 2956 first attempts were included in order to avoid multiple involvement of the same couple. Finally 1829 of them (62%) had SCSA analysis performed. In 1820 of female partners at least one oocyte was aspirated. One hundred and sixty-six cycles where both standard IVF and ICSI were applied in the same cycle were also excluded. Additional 21 cycles were excluded because of miscellaneous data corruption. Finally, data on 1633 cycles were included in the statistical analysis.

During the sample collection period, saving aliquots of ejaculate used for IVF/ICSI for subsequent SCSA analysis was, gradually, introduced as clinical routine, which is the reason for 38% of treatments with no SCSA data. However, the selection of samples for SCSA was random, apart from those ICSI treatments where sperm concentration was below 1×10^6/mL or which no SCSA was done. This is, probably, the explanation as to why the participants and those excluded because of lack of SCSA data did not differ as considers age and sperm motility whereas sperm concentration is slightly higher in the former group. The data are presented in Table 1. Background characteristics for participants considering male and female age, female BMI, type of stimulation, follicle-stimulating hormone (FSH) total dose, and number of aspirated oocytes are given in Table 2. Among the 1107 embryo transfers, the 22 were performed as double embryo transfers (DET) and in the remaining 1085 cases, a single embryo was transferred (SET). Mean DFI value was 15.7% in the SET group and 15.4% in the DET group.

Table 1 The characteristics of participants and those excluded because of lack of sperm chromatin structure assay (SCSA) data

	Participants $n = 1633$	Non-participants because of lack of SCSA data $n = 1127$
Age (years), median/range	35/21-55	34/21-55
Sperm concentration ($\times 10^6$/mL), median/range	45/0.1-480	35/0.1-290
Sperm motility (%), median/range	67/0-100	70/0-100

Table 2 Background characteristics for participants

	DFI ≤ 10%	10% <DFI ≤ 20%	20% < DFI ≤ 30%	DFI > 30%
Male age (years), mean/SD	33.5/5.3	33.9/5.3	34.7/5.9	35.9/6.3
Female age (years), mean/SD	32.4/4.1	32.4/4.1	32.7/4.1	32.4/4.4
Female BMI (kg/m^2), mean/SD	23.5/3.1	23.5/3.3	23.3/3.2	23.6/3.6
Agonist/Antagonist/ Other (%)	69/31/0.3	66/34/-	64/36/-	72.4/27.6/-
FSH total dose (IU), mean/SD	1896/825	1951/885	1868/832	1954/868
Asp oocytes (*n*), mean/SD	9.9/6.8	10.2/6.1	10.1/5.7	10.1/5.7
IVF/ICSI (%)	85/15	68/32	48/52	37.2/62.8

DFI, DNA fragmentation index; IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection.

The study was approved by the ethical committee of Lund University and, following written information, the couples were given an option to be excluded from the study.

Semen collection and analysis

Semen samples were collected by masturbation. Conventional semen analysis including sperm concentration, motility, and morphology was performed according to the World Health Organization guidelines (WHO, 1999). Two hundred microliter of the raw semen was stored in Eppendorf snap-cap tubes in −80 °C ultra-cold freezer following the procedure described by Evenson (Evenson *et al.*, 2002) for subsequent SCSA analysis.

Sperm chromatin structure assay

The principles and procedure of SCSA are described in detail elsewhere (Evenson & Jost, 2000; Bungum, 2012). The technique is based on the phenomenon that a 30-sec treatment with pH 1.2-buffer denatures the fragments of DNA with single- or double-strand breaks, whereas normal double-stranded DNA remains intact. The sperm cells are then stained with the fluorescent DNA dye acridine orange, which stains differently intact and fragmented DNA. After blue light excitation in a flow cytometer, the intact DNA emits green fluorescence, whereas

denatured DNA emits red fluorescence. Sperm chromatin damage is quantified using the flow cytometry measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured DNA) fluorescence and displayed as red vs. green fluorescence intensity cytogram patterns. The extent of DNA denaturation is expressed as DFI, which is the ratio of red to total fluorescence intensity i. e. the level of denatured DNA over the total DNA. A total of 5–10,000 cells were analyzed by FACSort (Becton Dickinson, San Jose, CA, USA). Analysis of the flow cytometric data was carried out using dedicated software (SCSASoft; SCSA Diagnostics, Brookings, SD, USA), which imply that the DFI histogram is used to precisely determine the percentage of DFI. All SCSA measurements were performed on raw semen, which on the day of analysis was quickly thawed and analyzed immediately. For the flow cytometer setup and calibration, a reference sample was used from a normal donor ejaculate retrieved from the laboratory repository (Evenson & Jost, 2000). The same reference sample was used for the whole study period. A reference was run for every fifth sample. The intra-laboratory CV for DFI analysis was found to be 4.5%.

IVF and ICSI procedures

Controlled ovarian stimulation was achieved using a GnRH antagonist short protocol or a GnRH-agonist down-regulation long protocol. Ovarian stimulation was performed with recombinant FSH alternatively urine derived gonadotrophin. Patients were monitored with transvaginal ultrasound for a count and size of follicles and serum-estradiol level if necessary. Human chorionic gonadotropin (hCG) injection was administered with the presence of at least two >17 mm follicles. Oocyte retrieval was conducted 35 h later under conscious sedation.

Gamete handling as well as IVF/ICSI procedures, culturing and embryo transfer (ET) were performed as previously described (Bungum *et al.*, 2004).

Assessment of fertilization, embryo morphology classification, cryopreservation and embryo transfer

Fertilization was determined 18 ± 20 h after the IVF/ICSI procedure. The oocytes were considered as fertilized when two distinct pronuclei were visible.

Cleavage and classification of morphology was assessed on day 2 or 3 (Bungum *et al.*, 2006). On day 5, embryos were assessed according to scoring criteria for blastocysts (Gardner & Schoolcraft, 1999).

The term good quality embryo included embryos selected for embryo transfer in which on day 2 were 4–6 cells, grade 1 or 2, on day three 8–10 cells, grade 1 or 2, or on day 5 blastocysts with good expansion, inner cell mass and trophectoderm (A or B according to Gardner criteria).

One embryo with the best morphology was selected for embryo transfer on day 2, 3 or 5 after oocyte retrieval. In the 22 cases two embryos were transferred. All not transferred good quality embryos, were cryopreserved.

All embryo transfers were performed with a Cook Soft 5000 catheter (Cook, Brisbane, Qld, Australia).

Luteal phase support, pregnancy test and miscarriage

All the patients received luteal phase support in the form of daily vaginal administration of micronized progesterone, 90 mg once a day starting on the day following oocyte retrieval and continuing until the day of the pregnancy test (i.e., day 12 after embryo transfer). A positive pregnancy test was defined by a plasma βHCG concentration >15 IU/L. A clinical pregnancy was defined as ultrasound detected intrauterine gestational sac with a heart activity 3 weeks after a positive HCG test. Miscarriage was defined as spontaneous expulsion of gestational sac up to 18th week of gestation which is verified by gynecological examination/ultrasound.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 22 software (SPSS Inc., Chicago, IL, USA). The couples were categorized into four groups, according to the DFI value: DFI ≤ 10% (reference group), 10% < DFI ≤ 20%, 20% < DFI ≤ 30%, DFI > 30%. All the calculations were done separately for standard IVF and ICSI and after merging both procedures. All the results were adjusted for female age as a covariate. Following calculations were performed:

- Fertilization rate expressed as number of fertilized oocytes as percentage of the number used for IVF/ICSI procedures [(100 × Fertilized eggs/total number of injected oocytes) and (100 × Fertilized eggs/total number of oocytes inseminated)]. Univariate analysis of variance was applied.
- Embryo quality rate, calculated as number of good quality embryos (GQE) as a percentage of the number of successful fertilizations. To do this calculation additional 158 cases where none oocyte was fertilized were excluded. Univariate analysis of variance was done on 1475 residual procedures.
- Since GQE is a pre-requisition for performing ET, the cases with at least one GQE were identified and odds ratio (OR) for at least one GQE in each DFI group were calculated using binary logistic regression.
- Pregnancy rate defined as the number of pregnancies as a percentage of the number of ET with GQE. Pregnancy was defined as serum hCG ≥ 15 IU/L on day 12 post ET. For this analysis the cases with no GQE as well as those in which ET was not performed for other reasons (e. g. ovarian hyperstimulation syndrome) were excluded. Totally 526 cases were excluded and 1107 used for analysis. Binary logistic regression was applied for calculation of OR.
- Miscarriage rate defined as a number of spontaneous abortions as a percentage of all pregnancies. Only the 471 cases where the pregnancy was achieved were included in this calculation. Odds ratio was calculated using binary logistic regression. For this end point, additional calculations were done for DFI > 40%.
- Successful pregnancy outcome defined as OR for live births in those having done ovum pick-up (OPU). In order to obtain higher statistical power, for this calculation the two highest DFI groups were merged. Apart from comparing the groups with DFI higher than 10% with the reference group (≤10%) for each DFI group the OR for live birth by ICSI was calculated with standard IVF as reference.

RESULTS

Fertilization rate

Mean fertilization rate according to DFI group is shown in Table 3. No significant statistical difference in fertilization rate in respective DFI groups were seen when results of IVF and ICSI

Table 3 Fertilization rate according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	Mean % (SE)	*p*-value	*n*	Mean % (SE)	*p*-value	*n*	Mean % (SE)	*p*-value
0-10	501	51.4 (1.32)	–	89	61.3 (2.71)	–	590	52.8 (1.2)	–
>10-20	445	47.6 (1.4)	0.05	208	61.6 (1.78)	0.94	653	52.1 (1.14)	0.68
>20-30	117	45.6 (2.73)	0.056	128	61.1 (2.27)	0.95	245	53.8 (1.86)	0.66
>30	54	38.1 (4.0)	0.02	91	61 (2.68)	0.92	145	52.6 (2.41)	0.94

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Fertilization rate = fertilized oocytes/total number of injections or inseminations. Univariate analysis of variance. Reference = the '0–10%' DFI category. Results adjusted for female age.

Table 4 Good quality embryo rate according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	Mean% (SE)	*p*-value	*n*	Mean% (SE)	*p*-value	*n*	Mean% (SE)	*p*-value
0-10	453	46.6 (1.73)	–	85	42.9 (4.36)	–	538	45.9 (1.64)	–
>10-20	386	46.3 (1.87)	0.91	201	44.2 (2.84)	0.80	587	45.7 (1.57)	0.91
>20-30	98	43.5 (3.72)	0.45	124	40.7 (3.63)	0.69	222	41.8 (2.55)	0.17
>30	40	37.2 (5.81)	0.12	88	43.6 (4.29)	0.91	128	41.6 (3.36)	0.25

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Good quality embryo rate = number of good quality embryo/number of successful fertilizations. Univariate analysis of variance. Reference = the '0–10%' DFI category. Results adjusted for female age.

were merged. However, when standard IVF and ICSI were calculated separately, in the standard IVF group fertilization rate, as compared to the reference group, was lower for all DFI groups, this difference reaching statistical significance for DFI > 10–20% and DFI > 30% and borderline statistical significance for those with DFI > 20–30%. No such differences were seen in the ICSI group.

Good quality embryo

Good quality embryo rate according to DFI group is shown in Table 4. When expressed in relation to successful fertilizations, no statistically significant association between DFI level and the GQE was observed. The results in the standard IVF group show a trend toward a decreasing GQE rate with increasing DFI.

The data regarding OR for achieving at least one GQE are shown in Table 5. Whilst the groups with DFI above 10% did not differ from the reference group when IVF and ICSI were merged, in standard IVF group the ORs for GQE were significantly lower for 20% < DFI ≤ 30% and for DFI > 30%. In ICSI group, ORs for GQE were higher in all DFI intervals reaching the significance for 20% < DFI ≤ 30%.

Pregnancy and risk of miscarriage

Table 6 presents the OR for pregnancy rate in those receiving ET with GQE according to DFI intervals. No statistically significant differences between the DFI groups were seen, neither when IVF and ICSI were treated separately nor for the merged group.

Odds ratios for miscarriage are presented in Table 7. No statistically significant differences between the DFI groups were seen, when IVF and ICSI were treated separately. If the additional group with DFI > 40% was extracted the OR for miscarriage was significantly increased for the merged group (OR 3.8; 95% CI: 1.2–12; $p = 0.02$).

Live births

Table 8 presents OR for live birth for couples who underwent OPU. For DFI > 20%, statistically significantly lower OR was seen for IVF but not ICSI. When comparing ICSI to IVF the OR for live birth by ICSI were statistically significantly higher for DFI > 20% (OR 1.7; 95% CI: 1.0–2.9; $p = 0.05$), whereas for DFI ≤ 10% and 10% < DFI ≤ 20%, no such difference was seen.

DISCUSSION

The main clinically applicable finding of this study was significantly decreased chance of live birth in standard IVF treatments performed with ejaculates with DFI above 20%. For this DFI subgroup the live birth rates were also significantly higher for ICSI as compared to IVF. These findings were paralleled by negative association between DFI and fertilization rate as well as the chance of obtaining at least one GQE- a prerequisite for performing embryo transfer- in standard IVF treatments but not in ICSI. Our results are in agreement with some previous studies reporting negative association between DFI level, fertilization rate and embryo quality after IVF/ICSI procedure (Virro *et al.*,

Table 5 Odds ratio for at least one good quality embryo following oocyte pick-up, according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value
0-10	501	Ref	–	89	Ref	–	590	Ref	–
>10-20	445	0.86 (0.64-1.15)	0.32	208	1.51 (0.87-2.61)	0.14	653	0.97 (0.75-1.25)	0.83
>20-30	117	0.61 (0.40-0.94)	0.025	128	1.93 (1.04-3.59)	0.04	245	0.94 (0.67-1.31)	0.69
>30	54	0.36 (0.2-0.63)	0.000	91	1.72 (0.88-3.35)	0.12	145	0.75 (0.51-1.12)	0.16

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Logistic regression. Reference = the '0–10%' DFI category. Results adjusted for female age.

Table 6 Odds ratio for pregnancy for couples who have undergone embryo transfer, according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value
0-10	345	Ref	–	52	Ref	–	397	Ref	–
>10-20	302	0.98 (0.71-1.34)	0.89	149	0.92 (0.49-1.73)	0.79	451	1.02 (0.77-1.33)	0.92
>20-30	71	0.79 (0.46-1.34)	0.37	95	0.78 (0.4-1.54)	0.48	166	0.90 (0.62-1.3)	0.58
>30	26	1.04 (0.47-2.34)	0.92	67	0.79 (0.38-1.65)	0.54	93	1.02 (0.64-1.61)	0.95

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Logistic regression. Reference = the '0–10%' DFI category. Results adjusted for female age.

Table 7 Odds ratio for spontaneous abortion according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value
0-10	144	Ref	–	26	Ref	–	170	Ref	–
>10-20	122	1.04 (0.6-1.81)	0.9	72	1.4 (0.53-3.71)	0.5	194	1.27 (0.8-2.01)	0.31
>20-30	25	0.95 (0.36-2.51)	0.91	41	0.78 (0.26-2.33)	0.66	66	0.99 (0.52-1.89)	0.97
>30-40	9	1.99 (0.49-8.0)	0.34	18	0.92 (0.24-3.53)	0.09	27	1.45 (0.6-3.51)	0.42
>40	2	2.32 (0.14-38.2)	0.56	12	3.08 (0.72-13.1)	0.12	14	3.75 (1.2-11.7)	0.02

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Spontaneous abortions/total number pregnancies. Logistic regression. Reference = the '0–10%' DFI category. Results adjusted for female age.

Table 8 Odds ratio for live birth following ovum pick-up, according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value
0-10	501	Ref	–	89	Ref	–	590	Ref	–
>10-20	445	0.95 (0.70-1.29)	0.76	208	1.28 (0.69-2.36)	0.43	653	1.01 (0.77-1.31)	0.97
>20	171	0.61 (0.38-0.97)	0.04	219	1.29 (0.70-2.37)	0.42	390	0.85 (0.62-1.16)	0.30

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Logistic regression. Reference = the '0–10%' DFI category. Results adjusted for female age.

2004; Check *et al.*, 2005; Zini *et al.*, 2005; Jiang *et al.*, 2011), also confirmed by some meta-analysis data (Evenson & Wixon, 2006). In contrast, some other studies have not been able to show such association (Larson-Cook *et al.*, 2003; Bungum *et al.*, 2007; Lin *et al.*, 2008; Speyer *et al.*, 2010; Dar *et al.*, 2013). This study represents, so far, the largest IVF/ICSI single center study in which the outcome of the treatment was related to the level of DFI.

A meta-analysis made by Collins (Collins *et al.*, 2008) has shown a statistically significant negative association between DFI and pregnancy in IVF and ICSI cycles. However, it was concluded that the magnitude of the effect of high DFI was not sufficiently high to provide a clinical indication for routine use of these tests in male infertility evaluation.

Our data show that both as considers the OR for live birth as well as for obtaining a GQE, the alteration in OR for the high DFI group, as compared to the reference group (DFI ≤ 10%), was of a magnitude which may have profound implications for the clinical outcome of ART. Our results do also indicate that the decreased fertilization rate was the major biological mechanism leading to the negative association between DFI and the lower birth rate as well as chance of obtaining a GQE.

Numerous of studies demonstrate that a significant part of men in infertile couples has remarkably high degrees of fragmented sperm DNA (Erenpreiss *et al.*, 2008; Oleszczuk *et al.*, 2013) and also men with high DFI have lower chance to cause pregnancy (Giwercman *et al.*, 2003; Sakkas & Alvarez, 2010). This problem can be overcome by using ART, especially by the use of ICSI (Bungum *et al.*, 2007). The results of our study which shows a significant difference of fertilization rate in the standard IVF group and does not show this difference in ICSI group are in agreement with previous observation regarding pregnancy in vivo (Spano *et al.*, 2000; Bungum *et al.*, 2007). Thus, our findings suggest that the cause and effect link between fertilization rate and sperm chromatin integrity is placed on the early stage of fertilization process based on a fusion between an oocyte and a spermatozoon. This can theoretically be bypassed by ICSI which can be confirmed by our observation that the fertilization rate is generally higher in the ICSI group. However, it must be noted that results in standard IVF and ICSI group are not entirely comparable, because in the ICSI but not the IVF group, the immature oocytes are excluded prior to assessment of the fertilization rate. On the other hand, significantly higher live birth rates in the ICSI group as compared to IVF for DFI > 20% might indicate that the former method is more efficient in this group of patients.

The biological explanation of superiority of ICSI over the IVF technique in case of increased DFI is not directly documented. Two possible explanations were suggested by Bungum (Bungum *et al.*, 2007). In the ICSI group, infertility is mainly caused by male factor which means that women in this group might be more fertile, e.g., as a result of younger age, and possibly produce oocytes with a better DNA repair capacity. In our material there was 1 year difference in the mean age of ICSI and IVF women. Another possible explanation was based on two completely different culture environments used for IVF and ICSI. While IVF oocytes were exposed to spermatozoa for 90 min, in ICSI, the spermatozoon are injected directly into the oocyte and

therefore probably less exposed to reactive oxygen species (ROS) than in IVF. The general knowledge about the negative influence or ROS and oxidative stress on sperm chromatin integrity can also support our observation of the difference in success rates between ICSI and IVF. It is observed that the high level of estrogenic compounds causes oxidative stress, which leads to DNA damage in human spermatozoa (Bennetts *et al.*, 2008). In the IVF environment, not only the oocyte and the sperm are present, but also the cumulus complex consisting of a high number of corona cells is a natural part of the culture. In contrast, in the ICSI environment, all corona cells are chemically and mechanically removed. It may be speculated that sperms with high DFI are more vulnerable to the adverse effects of ROS because of release of estradiol from corona cells surrounding the oocyte during standard IVF procedure (Kattera & Chen, 2003), which has also been shown to have a direct toxic effect on the embryo (Valbuena *et al.*, 2001).

Our study has several strengths one of them being the large sample size, giving the study sufficient statistical power and making it possible to defining multiple DFI subgroups and, thereby, defining a DFI-threshold for impairment of fertilization and higher miscarriage risk. We have also been able to perform a separate analysis for standard IVF and ICSI treatments and were, thereby, able to conclude that the impact of DFI on ART outcome differs in those two scenarios. Furthermore, we have been able to focus on one method for assessment of DFI, some of the previous studies mixing both different types of ART and methods of DFI analysis (Zini *et al.*, 2008). Also, by collecting large numbers of treatments from a single center and having almost 100% SET, we excluded the potentially confounding effect of heterogeneous patient cohorts, diverging treatment protocols and differences in methodology used for assessment of DFI (Collins *et al.*, 2008). This may be the reason why we, in contrast to a recent published study (Simon *et al.*, 2014) found even SCSA to be predictive for the outcome of IVF treatment. Although the SCSA data were available for only 62% of eligible couples, apart from exclusion of those with sperm concentration below 1×10^6/mL no selection bias is expected. For those cases excluded because of very low sperm counts ICSI is, anyhow, the only feasible method of treatment and a comparison with IVF is not relevant.

Although it is common for infertility studies that a distinction between presences of male and/or female factor is made, we have omitted to include this classification in this study. The reason is that we find such categorization as quite inaccurate and highly dependent on the number of investigations included in the work up of the couple. Thus, in a recent paper (Oleszczuk *et al.*, 2013) we have shown, that in 25% of cases of 'unexplained infertility' the DFI is above the level of 20%, which indicates that impairment of sperm DNA integrity might be one of the explanations of the couple's infertility problem. The fact that DFI seems to have a predictive value in relation to the IVF outcome, without discriminating between possible causes of infertility, makes this marker even more valuable in the daily clinical practice.

The retrospective design of the study represents its major weakness. Thus, ideally, the patients with high DFI fulfilling the criteria for standard IVF should be randomized to this treatment or to ICSI. Such a study are not yet available but our results indicating impairment of the outcome of standard IVF for DFI exceeding the level of 20%, facilitates a design of such study. Our study has profound clinical implications. Thus, the DFI as measured by SCSA above the level of 20–30% may be an indication for switching from standard IVF treatment to ICSI, in order to increase the chance of embryo transfer. Owing to a certain level of intra-individual variation in DFI (Oleszczuk *et al.*, 2011), the analysis should, ideally, be performed on the semen sample to be used for IVF or ICSI. Furthermore, since a recent Cochrane analysis (Showell *et al.*, 2014) has indicated increased pregnancy rates following antioxidant treatment of males in couples seeking fertility assistance, there is an urgent need of clarifying the effects of this treatment in management of men with high DFI.

In conclusion, we found that DFI-SCSA levels of 20% or higher, as seen in almost 25% of men entering IVF or ICSI treatment are associated with significant lowering of live birth rate when, using standard IVF but not ICSI treatment. Furthermore, the miscarriage rate was significantly increased for those having DFI of 40% or higher. These results point to sperm DNA testing as a useful tool in selection of the most effective ART-method in a given couple and also encourage to testing new treatments modalities which might improve sperm DNA integrity.

AUTHOR CONTRIBUTIONS

K. O, A. G, and M. B have all given substantial contributions to conception and design of the present study. All authors have contributed to acquisition of data, analysis as well as interpretation of data. K. O has drafted the manuscript and A. G and M. B have revised the content critically. All authors have made final approval of the version to be published.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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