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Paternal influence of sperm DNA integrity on early embryonic development

L. Simon[1], K. Murphy[1], M.B. Shamsi[1], L. Liu[1], B. Emery[1], K.I. Aston[1], J. Hotaling[1], and D.T. Carrell[1,2,3,*]

[1]Andrology and IVF Laboratory, Department of Surgery (Urology), University of Utah, Salt Lake City, UT 84108, USA [2]Department of Obstetrics and Gynecology, University of Utah, Salt Lake City, UT 84108, USA [3]Department of Human Genetics, University of Utah, Salt Lake City, UT 84108, USA

*Correspondence address. 675 Arapeen Drive, Suite 201, Salt Lake City, UT 84109, USA. Tel: +1-801-587-3772; Fax: +1-801-581-6127; E-mail: douglas.carrell@hsc.utah.edu

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STUDY QUESTION: Does sperm DNA damage affect early embryonic development?

SUMMARY ANSWER: Increased sperm DNA damage adversely affects embryo quality starting at Day 2 of early embryonic development and continuing after embryo transfer, resulting in reduced implantation rates and pregnancy outcomes.

WHAT IS KNOWN ALREADY: Abnormalities in the sperm DNA in the form of single and double strand breaks can be assessed by an alkaline Comet assay. Some prior studies have shown a strong paternal effect of sperm DNA damage on IVF outcome, including reduced fertilization, reduced embryo quality and cleavage rates, reduced numbers of embryos developing into blastocysts, increased percentage of embryos undergoing developmental arrest, and reduced implantation and pregnancy rates.

STUDY DESIGN, SIZE, DURATION: A cross-sectional study of 215 men from infertile couples undergoing assisted reproduction techniques at the University of Utah Center for Reproductive Medicine.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Sperm from men undergoing ART were analyzed for DNA damage using an alkaline Comet assay and classified into three groups: 'low damage' (0–30%), 'intermediate damage' (31–70%) and 'high damage' (71–100%). The cause of couples' infertility was categorized into one of the three types (male, female or unexplained). Each embryo was categorized as 'good', 'fair' or 'poor' quality, based on the number and grade of blastomeres. The influence of sperm DNA damage on early embryonic development was observed and classified into four stages: peri-fertilization effect (fertilization rate), early paternal effect (embryonic days 1–2), late paternal effect (embryonic days 3–5) and implantation stage effect.

MAIN RESULTS AND THE ROLE OF CHANCE: The paternal effect of sperm DNA damage was observed at each stage of early embryonic development. The peri-fertilization effect was higher in oocytes from patients with female infertility (20.85%) compared with male (8.22%; $P < 0.001$) and unexplained (7.30%; $P < 0.001$) infertility factors. In both the early and late paternal effect stages, the low DNA damage group had a higher percentage of good quality embryos ($P < 0.05$) and lower percentage of poor quality embryos ($P < 0.05$) compared with the high DNA damage group. Implantation was lower in the high DNA damage (33.33%) compared with intermediate DNA damage (55.26%; $P < 0.001$) and low DNA damage (65.00%; $P < 0.001$) groups. The implantation rate was higher following blastocyst transfer (58.33%), when compared with early stage blastocyst (53.85%; $P = 0.554$) and cavitating morula transfers (34.40%; $P < 0.001$). Implantation was higher when the female partner age was $\leq$35 years when compared with >35 year age group (52.75 versus 35.44%; $P = 0.008$).

LIMITATIONS, REASONS FOR CAUTION: A potential limitation of this study is that it is cross-sectional. Generally in such studies more than one variable could affect the outcome. Analyzing sperm is one part of the equation but a number of environmental and female factors also have the potential to influence embryo development and implantation. Furthermore, the selection of morphologically normal and physiologically motile sperm may result in isolation of sperm with reduced DNA damage. Therefore, selecting the best available sperm for ICSI may lead to experimental bias, as the selected sperm do not represent the overall sperm population in which the DNA damage is measured. Similar studies on selected sperm and with a larger sample size are now required.

WIDER IMPLICATIONS OF THE FINDINGS: The paternal influence of damaged chromatin is more prominent after zygotic transcriptional activation. A prolonged paternal effect on the developing embryo may be due to the active repair mechanism present in oocytes that tends to overcome the damaged paternal chromatin. The probability of eliminating an embryo fertilized by a sperm with damaged DNA is higher at the blastocyst stage than the cleavage stage; therefore blastocyst transfer could be recommended for better implantation success. Finally, we recommend ICSI treatment for patients with a higher percentage of sperm with DNA damage as well as additional studies with a larger sample size aimed at assessing DNA damage analysis as a diagnostic tool for IVF.

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Introduction

Assisted reproduction techniques (ART) have created the possibility of treating the majority of infertile couples; however, <50% of treatment cycles result in a pregnancy. Gametes and embryo quality are a major determinant of ART success (Scott, 2003; Terriou *et al.*, 2007; Ajduk and Zernicka-Goetz, 2013). It is well established that high-quality gametes are required to produce high-quality embryos, and that both the sperm and oocyte genomes contribute to the embryonic genome (Marteil *et al.*, 2009).

A number of studies have shown that abnormalities in the paternal DNA adversely impact embryo quality (Virro *et al.*, 2004; Benchaib *et al.*, 2007; Simon *et al.*, 2010), while poor sperm quality is also associated with delayed pronuclear formation and sluggish embryonic development (Ron-El *et al.*, 1991). In addition, Parinaud *et al.* (1993) showed an association between abnormal sperm morphology and poor embryo morphology suggesting that sperm is involved in embryonic quality and development. Such a finding in morphologically abnormal sperm is likely to be related to reduced sperm DNA integrity. Multiple studies have associated sperm DNA damage with abnormal sperm concentration (Nijs *et al.*, 2009), progressive motility, normal morphology (Tavalaee *et al.*, 2009), sperm viability (Ozmen *et al.*, 2007) and sperm maturity (Virro *et al.*, 2004). Lastly, recent studies have highlighted the role of epigenetic factors in embryogenesis (Carrell, 2012).

There is much debate in the literature as to whether early embryo development is influenced exclusively by the maternal factors (Braude *et al.*, 1988) or whether there are also paternal controls at this early stage (Tesarik *et al.*, 2004; Baltaci *et al.*, 2006). It is well documented that the first two embryonic cell divisions are primarily controlled by the maternal genes (Braude *et al.*, 1988) and paternal effects commence at the 4-cell stage. Further, the detrimental effects of sperm DNA damage are more prominent during the later stages of embryo development (Dar *et al.*, 2013). The described paternal effects on pre- and post-implantation embryos include slower embryo cleavage, poor morphology, lower blastocyst formation and lower implantation rates (Parinaud *et al.*, 1993; Janny and Menezo, 1994). However, major discrepancies exist within the published data regarding the developmental timing and the extent of paternal effects on embryo quality.

IVF is a uniquely useful tool to compare the impact of male and female gametes on early embryo development, as the quality of sperm and oocyte, and embryo quality can be measured directly within in the clinical setting. To our knowledge, this is the first study to comprehensively evaluate the effect of sperm DNA damage on embryonic development and implantation in patient groups stratified by male, female and unexplained infertility factors. We assessed the influence of sperm DNA damage on embryo quality at several embryonic developmental stages, as described by Tesarik *et al.* (2004): (i) peri-fertilization effect—where oocytes fail to form a pronucleus following ICSI insemination, (ii) early paternal effect—embryo development until 4-cell stage prior to paternal genome activation, (iii) late paternal effect—embryo development from Day 2 to Day 5, during which time the paternal genome is known to contribute to embryo development and (iv) implantation stage effect—the percentage of blastocysts implanted in the uterus following Day 5 blastocyst transfer.

Materials and Methods

Subjects

Men presenting at the University of Utah IVF Andrology laboratory for ART between April 2011 and April 2013 were invited to participate in this study ($n = 215$). All subjects gave written informed consent for participation in this study, and the University of Utah Institutional Review Board approved the project. Semen samples were obtained after a recommended 2–5 days of sexual abstinence. All samples were subjected to a conventional light microscopic semen analysis to determine liquefaction, semen volume, sperm concentration, total sperm count and motility according to World Health Organization (WHO) recommendations (WHO, 1999).

ART procedures

Ovarian stimulation was performed using standard techniques. Oocytes were obtained using ultrasound-guided, transvaginal aspiration. Standard IVF involved insemination of metaphase II oocytes with 100 000–200 000 progressively motile sperm in a 100 μl drop of human tubal fluid medium. ICSI was performed using microtool sperm immobilization and injection. Resulting embryos were cultured for 3–5 days after oocyte retrieval in human tubal fluid medium, and then transferred to the uterus. All embryos were included in the determination of the patient's mean embryo score (Carrell *et al.*, 1999). Implantation was confirmed by fetal heartbeat 6 weeks after embryo transfer.

Alkaline Comet assay

Sperm DNA damage was assessed in the native semen using an alkaline single-cell gel electrophoresis (Comet) assay as modified previously by Hughes *et al.* (1997) and Donnelly *et al.* (1999). Sperm was considered

damaged or normal based on the presence or absence of a visible comet tail, respectively. Fifty to 100 Comets were scored per sample.

Blastocyst inclusion criteria for analysis

To identify blastocysts that successfully implanted following Day 5 transfers, the inclusion criteria required: (i) transfer of a single blastocyst, or (ii) more than one blastocyst was transferred but all transferred blastocysts were of the same stage and grade, or (iii) multiple blastocysts types were transferred but all or none of the blastocysts implanted.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS 18) for Windows (SPSS, Inc., Chicago, IL, USA). Sperm DNA damage was categorized into three groups: 'low damage' (0–30% damaged sperm), 'intermediate damage' (31–70% damaged sperm) and 'high damage' (71–100% damaged sperm). Embryo quality was scored on degree of cellular fragmentation and regularity of blastomere morphology. The study subjects were categorized into three groups based on the type of infertility (male, female and unexplained) determined by the physicians and based on the history of the patients. Patients were stratified based on the type of treatment (IVF or ICSI) separately and combined, and the age of female partners ($\leq$35 or >35 years). Each embryo was categorized into 'good', 'fair' or 'poor' quality embryo type, based on their blastomere number and grade (Carrell *et al.*, 1999). The chi-square statistic was used to test the paternal effect of sperm DNA damage between the patient infertility categories, type of insemination, embryo quality and level of DNA damage. Logistic regression was performed to determine the impact of sperm DNA damage and infertility factors simultaneously to predict the quality of developing embryos, implantation and or pregnancy success. Statistical power and sample size were calculated using the software SAS/STAT 9.22.

Results

Fertilization was achieved by standard IVF ($n = 81$), ICSI ($n = 115$), or a combination of both standard IVF and ICSI ($n = 19$). A total of 1259 oocytes were fertilized using ICSI and 951 oocytes were fertilized by IVF. The percentage of patients under each infertility type for IVF was male (27.4%), female (39.0%) and unexplained (33.6%), while for ICSI was male (28.9%), female (34.6%) and unexplained (36.5%).

Peri-fertilization effect

In order to eliminate variability in sperm penetration ability and fertilization timing, only ICSI cases were included in an analysis of pre-fertilization paternal effects of DNA damage. Of the 1409 oocytes inseminated by ICSI, 150 (10.65%) oocytes failed to perform normal pronuclear formation, resulting in 1259 fertilized embryos. When the cases were categorized into three infertility groups, couples with female factor infertility had a higher percentage of peri-fertilization defect (69 of 331 = 20.85%) when compared with couples with male (32 of 389 = 8.22%; $P < 0.001$), and unexplained (49 of 671 = 7.30%; $P < 0.001$) infertility issues. When each infertility category was categorized into high, intermediate or low levels of sperm DNA damage, the peri-fertilization defect was positively associated with sperm DNA damage in the male infertility group, but the observed difference was not statistically significant (Fig. 1).

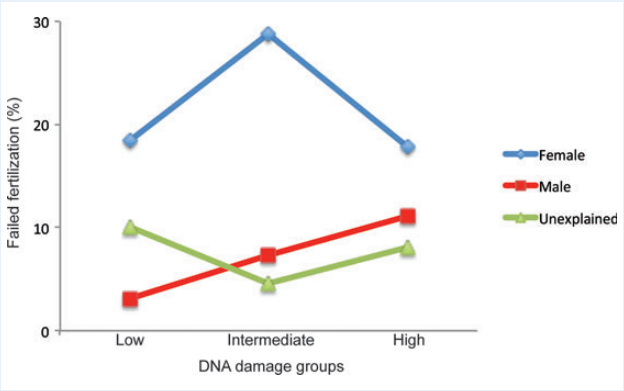

Figure 1 Differences in the percentage of human oocytes that failed to fertilize after ICSI, in the three DNA damage groups.

Early paternal effect

Early paternal effect was assessed on Day 2 of embryo development (4-cell stage) when the paternal genome is considered to be inactive. When both of the insemination groups were combined ($n = 2210$ embryos), there was a decrease ($P < 0.001$) in the percentage of good quality embryos and an increase ($P = 0.002$) in the percentage of poor quality embryos with increasing sperm DNA damage category. Likewise, when embryos were classified based on their infertility type [male ($n = 546$), female ($n = 898$) and unexplained ($n = 766$)], there was a decrease in the percentage of good quality embryos in the female ($P = 0.008$) and unexplained ($P = 0.033$) infertility groups and an increase in the poor quality embryos in the female ($P = 0.005$) and unexplained ($P = 0.002$) infertility group associated with increased sperm DNA damage (Tables I and II).

Late paternal effect

Late paternal effect was assessed on Day 3 cleavage stage embryos ($n = 2210$) and Day 5 blastocyst stage embryos ($n = 2019$), when the paternal genome is known to be actively involved in embryo development. Day 3 cleavage stage embryo transfer was performed in 33 patients. On Day 3 of culture, there was a decrease in the percentage of good quality embryos ($P = 0.006$) and an increase in the percentage of poor ($P = 0.002$) quality embryos with increasing sperm DNA damage (Table II). When all of the embryos on Day 3 were classified based on their infertility types, there was a significant decrease in the percentage of good quality embryos in the female ($P = 0.021$) and unexplained ($P = 0.002$) infertility groups and a significant increase in the poor quality embryos in the female ($P = 0.021$) and unexplained ($P < 0.001$) infertility group, with increase in sperm DNA damage. In couples with male infertility, there was an increase in the percentage of good quality embryos ($P = 0.013$) and a decrease in the fair quality embryos ($P < 0.001$) with increasing sperm DNA damage (Table I).

On Day 5, there was a decrease in the percentage of good quality embryos ($P = 0.014$) and an increase in the percentage of poor ($P = 0.001$) quality embryos with increase in sperm DNA damage (Table II). When all of the embryos on Day 5 were classified based on their infertility types, there was a significant decrease in the percentage of good quality embryos in the female ($P = 0.016$) infertility group and a significant increase in the poor quality embryos in the female

Table I Correlation between sperm DNA damage, determined by the Comet assay, and embryo quality in couples with different types of infertility.

	Unexplained factor infertility				Female factor infertility				Male factor infertility			
Embryo quality on Day 2 [percentage (*n*)]												
DNA damage (*n*)	Low (112)	Intermediate (229)	High (425)	*P*-value	Low (149)	Intermediate (342)	High (407)	*P*-value	Low (74)	Intermediate (267)	High (205)	*P*-value
Good	45.5 (51)	51.5 (118)	40.9 (174)	0.003	50.3 (75)	46.8 (160)	37.8 (154)	0.008	48.7 (36)	56.9 (152)	54.6 (112)	0.445
Fair	33.9 (38)	33.6 (77)	32.5 (138)	0.933	30.9 (46)	23.4 (80)	29.3 (119)	0.112	27.0 (20)	22.9 (61)	22.5 (46)	0.707
Poor	20.6 (23)	14.9 (34)	26.6 (113)	0.002	18.8 (28)	29.8 (102)	32.9 (134)	0.005	24.3 (18)	20.2 (54)	22.9 (47)	0.664
Embryo quality on Day 3												
DNA damage (*n*)	Low (112)	Intermediate (229)	High (425)	*P*-value	Low (149)	Intermediate (342)	High (407)	*P*-value	Low (74)	Intermediate (267)	High (205)	*P*-value
Good	52.7 (59)	48.9 (112)	37.6 (160)	0.002	48.3 (72)	40.9 (140)	35.6 (145)	0.021	33.8 (25)	51.7 (138)	52.7 (108)	0.013
Fair	15.2 (17)	25.8 (59)	15.8 (67)	0.004	15.5 (23)	14.1 (48)	15.0 (61)	0.899	33.8 (25)	13.9 (37)	17.6 (36)	<0.001
Poor	32.1 (36)	25.3 (58)	46.6 (198)	<0.001	36.2 (54)	45.0 (154)	49.4 (201)	0.021	32.4 (24)	34.4 (92)	29.7 (61)	0.557
Embryo quality on Day 5												
DNA damage (*n*)	Low (112)	Intermediate (212)	High (402)	*P*-value	Low (139)	Intermediate (315)	High (337)	*P*-value	Low (70)	Intermediate (246)	High (186)	*P*-value
Good	11.6 (13)	13.2 (28)	9.2 (37)	0.297	16.6 (23)	11.4 (36)	7.7 (26)	0.016	4.3 (3)	15.5 (38)	10.2 (19)	0.026*
Fair	28.6 (32)	26.9 (71)	26.9 (108)	0.226	30.9 (43)	28.3 (89)	24.3 (82)	0.278	37.1 (26)	30.9 (76)	30.1 (56)	0.537
Poor	59.8 (67)	63.9 (113)	63.9 (257)	0.037	42.5 (73)	60.3 (190)	68.0 (229)	0.004	58.6 (41)	53.6 (132)	59.7 (111)	0.428

Rows are % (*n*) values for embryo quality observed on Days 2, 3 and 5. The chi-square statistic was used to calculate *P*-values (shaded columns). The *P*-values compare the quality of embryos between the categories of sperm DNA damage.
*Although statistically significant, the small sample size in the good quality embryo/male infertility subgroup is underpowered to ascertain whether a statistically significant difference truly exists.

Table II Comparison of embryo quality between the DNA damage groups and insemination methods.

	ICSI insemination				IVF insemination				Combining ICSI + IVF			
Combining low, intermediate and high DNA damage groups [percentage (*n*)]												
	Day 2 (1259)	Day 3 (1259)	Day 5 (1142)	*P-value	Day 2 (951)	Day 3 (951)	Day 5 (877)	*P-value	Day 2 (2210)	Day 3 (2210)	Day 5 (2019)	*P-value
Good	51.3 (646)	48.2 (607)	12.2 (139)	<0.001	40.6 (386)	37.0 (352)	9.6 (84)	<0.001	46.7 (1032)	43.4 (959)	11.0 (223)	<0.001
Fair	30.4 (383)	17.6 (222)	33.3 (380)	<0.001	25.4 (242)	15.9 (151)	23.1 (203)	<0.001	28.3 (625)	16.9 (373)	28.9 (583)	< 0.001
Poor	18.3 (230)	34.2 (430)	54.5 (623)	<0.001	34.0 (323)	47.1 (448)	67.3 (590)	<0.001	25.0 (553)	39.7 (878)	60.1 (1213)	<0.001
In the low DNA damage group												
	Day 2 (196)	Day 3 (196)	Day 5 (196)	*P-value	Day 2 (139)	Day 3 (139)	Day 5 (125)	*P-value	Day 2 (335)	Day 3 (335)	Day 5 (321)	*P-value
Good	49.0 (96)	50.5 (99)	12.8 (25)	<0.001	47.5 (66)	41.0 (57)	11.2 (14)	<0.001	48.4 (162)	46.6 (156)	12.2 (39)	<0.001
Fair	36.2 (71)	19.9 (39)	39.8 (78)	<0.001	23.7 (33)	18.7 (26)	18.4 (23)	0.469	31.0 (104)	19.4 (65)	31.5 (101)	<0.001
Poor	14.8 (29)	29.6 (58)	47.4 (93)	<0.001	28.8 (40)	40.3 (56)	70.4 (88)	<0.001	20.6 (69)	34.0 (114)	56.3 (181)	<0.001
In the intermediate DNA damage group												
	Day 2 (472)	Day 3 (472)	Day 5 (427)	*P-value	Day 2 (366)	Day 3 (366)	Day 5 (346)	*P-value	Day 2 (838)	Day 3 (838)	Day 5 (773)	*P-value
Good	59.1 (279)	55.5 (262)	15.2 (65)	<0.001	41.3 (151)	35.0 (128)	10.7 (37)	<0.001	51.3 (430)	46.5 (390)	13.2 (102)	<0.001
Fair	26.9 (127)	18.4 (87)	36.3 (155)	<0.001	24.9 (91)	15.6 (57)	23.4 (81)	0.004	26.0 (218)	17.2 (144)	30.5 (236)	<0.001
Poor	14.0 (66)	26.1 (123)	48.5 (207)	< 0.001	33.8 (124)	49.4 (181)	65.9 (228)	<0.001	22.7 (190)	36.3 (304)	56.3 (435)	<0.001
In the high DNA damage group												
	Day 2 (591)	Day 3 (591)	Day 5 (519)	*P-value	Day 2 (446)	Day 3 (446)	Day 5 (406)	*P-value	Day 2 (1037)	Day 3 (1037)	Day 5 (925)	*P-value
Good	45.9 (271)	41.6 (246)	9.4 (49)	<0.001	37.9 (169)	37.4 (167)	8.1 (33)	<0.001	42.4 (440)	39.8 (413)	8.9 (82)	<0.001
Fair	31.3 (185)	16.2 (96)	28.3 (147)	<0.001	26.5 (118)	15.3 (68)	24.4 (99)	<0.001	29.2 (303)	15.8 (164)	26.6 (246)	<0.001
Poor	22.8 (135)	42.1 (249)	62.3 (323)	<0.001	35.6 (159)	47.3 (211)	67.5 (274)	<0.001	28.4 (294)	44.4 (460)	64.5 (597)	<0.001
**P-values comparing embryos between low DNA damage and high DNA damage group												
Good	0.447	0.029	0.194	–	0.044	0.450	0.290	–	0.057	0.029	0.086	–
Fair	0.202	0.239	0.003	–	0.522	0.332	0.164	–	0.424	0.125	0.093	–
Poor	0.016	0.001	<0.001	–	0.135	0.146	0.541	–	0.005	<0.001	0.009	–

Rows are % (*n*) values for embryo quality observed on Days 2, 3 and 5, categorized into the levels of sperm DNA damage. The chi-square statistic was used to calculate *P*-values (shaded columns).

**P*-values compare the quality of embryos between different time points of development—Days 2, 3 and 5 (shaded columns).

***P*-values compare the quality of embryos (Good, Fair or Poor) between the low DNA damage and high DNA damage categories at each time point—Days 2, 3 and 5 (shaded rows).

($P = 0.004$) and unexplained ($P = 0.037$) infertility group, with increasing sperm DNA damage. In contrast to this, couples with male infertility type had a significantly higher percentage of good quality embryos ($P = 0.026$) with increased sperm DNA damage (Table I).

Comparison of embryo quality between IVF and ICSI insemination

The total number of embryos analyzed in this study on Day 2 and 3 was [IVF ($n = 951$) and ICSI ($n = 1259$)] and on Day 5 was [IVF ($n = 877$) and ICSI (1142)]. The percentage of poor quality embryos was significantly lower and the percentage of good quality embryos was significantly higher after ICSI insemination method compared with microdrop insemination (Table II). In addition, when compared between the DNA damage groups, the quality of embryos was statistically different between IVF and ICSI insemination (Table III). Following ICSI insemination, there was a significant decrease in the percentage of good quality embryos and an increase in the percentage of poor quality embryos with increasing sperm DNA damage. However, no such correlations were observed after IVF insemination.

Progression of embryo development

In ICSI, IVF or both insemination types combined, we observe an increase in the percentage of poor quality embryos and a decrease in the percentage of good quality embryos from Day 2 to Day 5 (Table II). In order to more precisely evaluate the effect of sperm DNA damage on the developing embryo over time, we sought to track the progression of individual embryos from Day 2 through Day 5. For this analysis, the number of embryos in some groups was not sufficient to generate statistical power when IVF and ICSI were considered separately, due to lack of power. Supplementary Tables SI–SIV are included, with stratification by IVF or ICSI, but any statistically significant values with sample sizes <40 in each group are underpowered to detect any difference <10%. Therefore, we combined IVF and ICSI insemination types and compared the percentage of good, fair and poor quality embryos between low DNA damage and high DNA damage groups as they progressed from Day 2 to Day 3 (Fig. 2), and from Day 3 to Day 5 (Fig. 3). Although we see a trend, the progression of embryo development is not statistically different between the DNA damage groups from Day 2 to Day 3 ($P > 0.05$; Fig. 2). However, a significant difference is seen between the DNA damage groups when the embryo progression was analyzed from Day 3 to Day 5 (Fig. 3).

Table III *P*-values comparing embryo quality between IVF and ICSI insemination methods in the three DNA damage groups.

		Low	Intermediate	High
Embryo quality on Day 2	Good	0.786	<0.001	0.010
	Fair	0.793	0.503	0.089
	Poor	0.002	<0.001	<0.001
Embryo quality on Day 3	Good	0.085	<0.001	0.173
	Fair	0.785	0.276	0.663
	Poor	0.041	<0.001	0.096
Embryo quality on Day 5	Good	0.677	0.001	0.485
	Fair	<0.001	<0.001	0.178
	Poor	<0.001	<0.001	0.097

The percentage of good, fair and poor quality embryos used for the comparison between the IVF and ICSI insemination methods are presented in Table II. The chi-square statistic was used to calculate the *P*-value, between the two types of insemination.

Implantation stage

An implantation rate of 49.58% was observed following transfer of 355 blastocysts in 182 patients (excluding patients with Day 3 embryo transfers). The implantation rate was lower in the high DNA damage group compared with intermediate ($P < 0.001$) and low DNA damage groups ($P < 0.001$; Table IV). Implantation was higher when the female partner age was ≤35 years when compared with >35 age group (52.75 versus 35.44%; $P = 0.008$). The high DNA damage group had a lower implantation rate when the female partner age was >35 years when compared with ≤35 years age group ($P < 0.001$). However, no difference in implantation rate was observed between the age groups in the low ($P = 0.136$) and intermediate ($P = 0.631$) DNA damage categories (Table IV). Couples with unexplained infertility had a lower implantation rate (32.7%) when compared with couples having female (54.0%; $P = 0.001$) and male (60.0%; $P < 0.001$) infertility.

To be able to track the individual blastocyst that successfully implanted following Day 5 transfers, a blastocyst inclusion criterion was followed as described above. A total of 297 blastocysts were included from 151 patients having an implantation rate of 48.15% (143/297). Predominantly, four grades of embryos were transferred in the study: grade 2 blastocyst (B2, 48.48%), grade 2 early blastocyst (EB2, 8.42%), grade 2 cavitating morula (M2cav, 35.02%), grade 2 morula (M2, 5.05%) and others (3.03%). The number of M2 and other (B1, B3, EB3, M3Cav and M3) grade blastocysts was too low for any statistical analysis and hence they were not included in the following statistics. B2 grade blastocysts resulted in 58.33% implantation, EB2 grade blastocysts resulted in 52.00% implantation and M2Cav stage blastocysts resulted in 35.58% implantation. When the implantation rate of each embryonic stage and grade was analyzed between the three DNA damage groups, all three blastocyst types showed higher implantation rate in the low DNA damage group when compared with intermediate and high DNA damage groups (Fig. 4).

Pregnancy outcome

Clinical pregnancies were lower in the high DNA damage group (44.8%) when compared with low DNA damage (69.7%; $P = 0.013$) and intermediate DNA damage (68.6%; $P < 0.001$) groups. Couples with unexplained infertility had lower clinical pregnancy rates (48.4%) compared with couples with female (60.2%; $P = 0.137$) and male (70.9%; $P = 0.013$) infertility. ICSI had a higher pregnancy rate (60.7%; 82/135) compared with the IVF (52.5%; 42/80) insemination method ($P = 0.237$). There was a statistically significant difference between IVF and ICSI pregnancies in the low DNA damage group (54.6 versus 88.2%; P 0.044). However, no statistical significance difference was observed in the clinical pregnancies between IVF and ICSI insemination methods in the intermediate (72.7 versus 62.9%; $P = 0.335$) and high (33.3 versus 50.0%; $P = 0.116$) DNA damage groups. Our results for obtaining a successful clinical pregnancy are also supported by the receiver operating curve analysis, as the area under the curve is 0.672 cm^2 ($P = 0.008$) for IVF insemination and 0.697 cm^2 ($P < 0.001$) for ICSI insemination method.

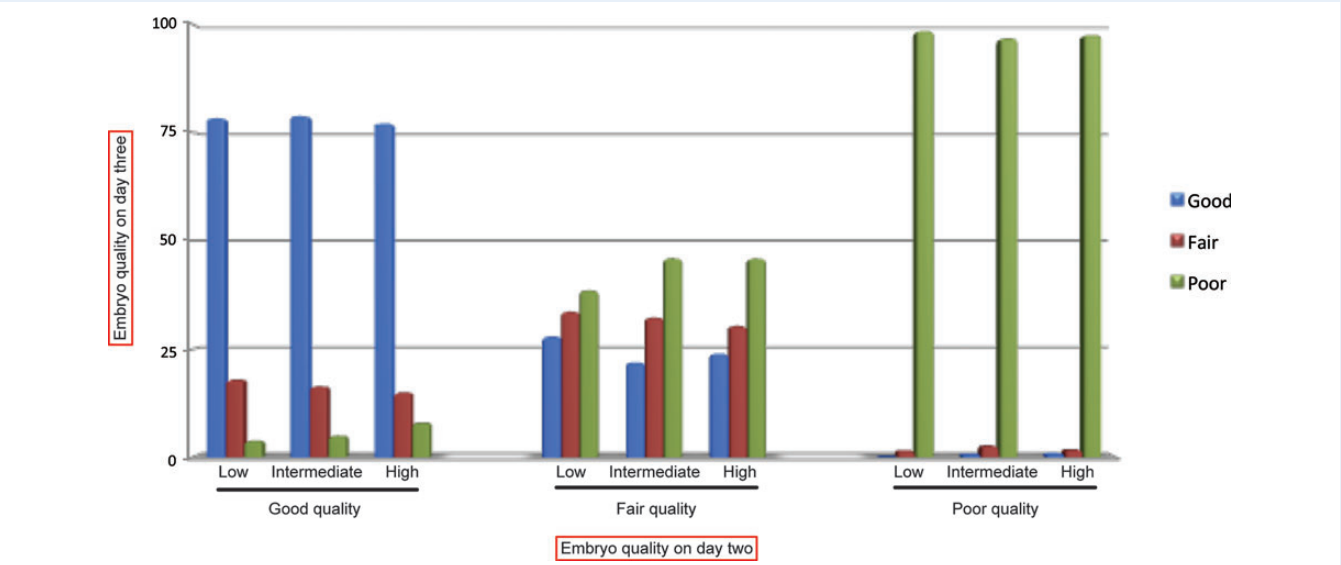

Figure 2 Progression of human embryo development from Day 2 to Day 3 stratified by DNA damage levels (low, intermediate and high). Values expressed as percentage of embryo quality on Day 3 in each strata of DNA damage. Results are stratified by Good, Fair and Poor embryo quality on Day 2 (*x*-axis) as denoted by three groups of results to visually display the % of embryos of a given quality that progress from Day 2 to Day 3 (*y*-axis). Thus, each set of blue, red and green bars sum to 100, representing 100% of the proportion of embryos moving from Day 2 to Day 3. The impact of DNA damage is demonstrated by substratifying results by sperm DNA damage levels. None of these differences were found to be significant.

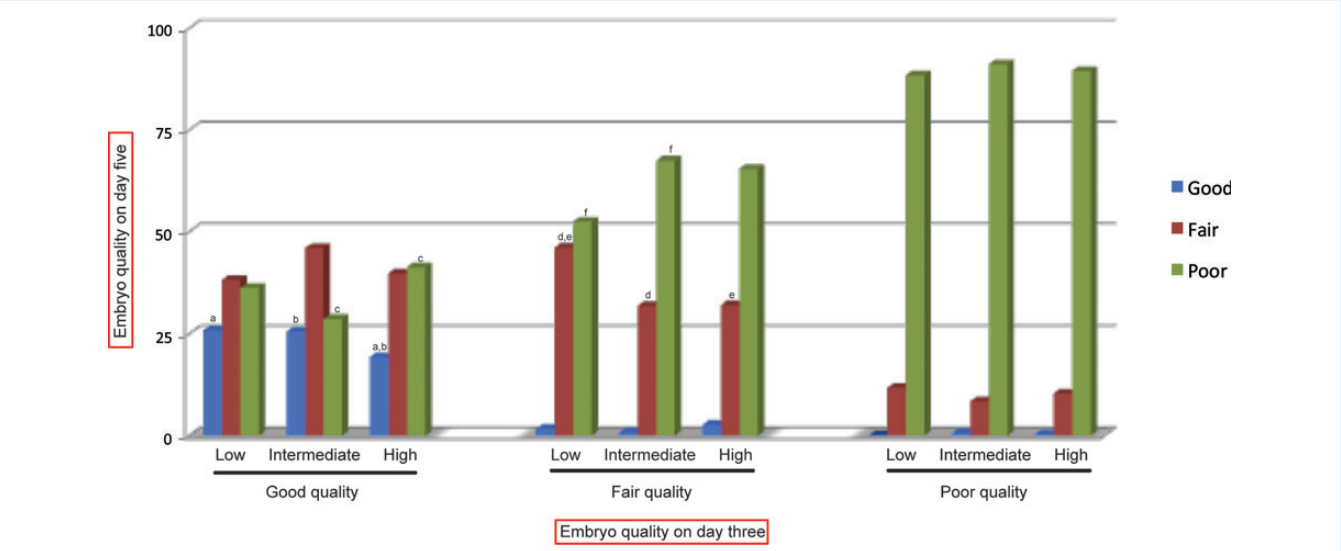

Figure 3 Progression of human embryo development from Day 3 to Day 5 stratified by DNA damage levels (low, intermediate and high). Values expressed as percentage of embryo quality on Day 3 in each strata of DNA damage. Columns with similar letters are statistically significant $P < 0.05$. Results are stratified by Good, Fair and Poor embryo quality on Day 3 (*x*-axis) as denoted by three groups of results to visually display the percentage of embryos of a given quality that progress from Day 3 to Day 5 (*y*-axis). Thus, each set of blue, red and green bars sum to 100, representing 100% of the proportion of embryos moving from Day 3 to Day 5. The impact of DNA damage is demonstrated by substratifying results by sperm DNA damage levels.

Discussion

This study associates the paternal effect of sperm DNA damage with early embryonic development through implantation in a cohort of 215 couples. Although, the paternal influence of damaged chromatin was observed at all embryonic developmental stages, we found a more prominent paternal effect after embryonic genome activation had occurred. While the peri-fertilization stage showed a stronger maternal effect than a paternal influence, more significant differences in the quality of embryos with respect to sperm DNA damage were observed as

Table IV Comparison between sperm DNA damage and implantation rate.

	Low	Intermediate	High	*P*-value
Implantation rate % (*n*)	65.0 (60)	55.3 (114)	33.3 (123)	<0.001
Female age ≤35 years % (*n*)	60.4 (48)	56.6 (83)	44.8 (87)	<0.001
Female age >35 years % (*n*)	83.3 (12)	51.6 (31)	5.6 (36)	<0.001

The chi-square statistic was used to calculate the *P*-value. *P*-values compare the implantation rate between the sperm DNA damage categories.

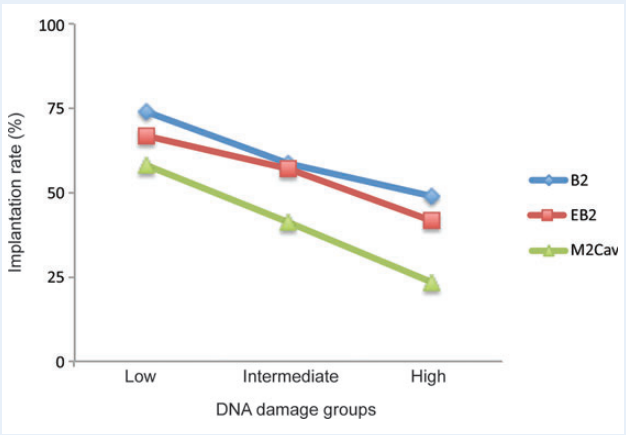

Figure 4 Implantation rate for the three major types of human blastocyst transferred in the three DNA damage groups. Values expressed as a percentage. B2, blastocyst; EB2, early-stage blastocyst; M2Cav, cavitating morula.

embryos were cultured *in vitro* to later stages. Moreover, a strong paternal influence on implantation and pregnancy rates was observed following Day 5 transfers.

Failure to fertilize following ICSI may be related to defective sperm and/or oocyte. The quality of both oocytes and sperm has previously been associated with diminished fertilization (Nasr-Esfahani *et al.*, 2001, 2004, 2005; Emery *et al.*, 2005; Greco *et al.*, 2006; Neri *et al.*, 2013). In the present study, ~11% of the oocytes after ICSI did not fertilize normally. We did not observe any association between sperm DNA damage and a peri-fertilization defect. These results indicate that sperm with DNA fragmentation can undergo successful fertilization, pronuclear formation and syngamy. Fatehi *et al.* (2006) and Chen *et al.* (2012) showed experimentally that DNA damage induced in sperm by irradiation does not block fertilization. These studies are in agreement with our finding that sperm DNA damage has minimal effects at the pronuclear stages. We also observed that couples with female factor infertility had a higher incidence of pronuclear stage defect, which is consistent with a previous report (Navot *et al.*, 1991).

Development of the embryo post-fertilization depends on a reserve of maternal mRNA accumulated during oocyte growth and maturation (Telford *et al.*, 1990). Previous studies have reported that significant transcription of the embryonic genome only takes place after the 4-cell stage in humans (Braude *et al.*, 1988). In our study, we observe a decrease (6%) in good quality embryos and an increase (8%) in the poor quality embryos between the low and high DNA damage groups on Day 2. Although the effect of sperm DNA damage is more apparent in later stages of embryogenesis, this small but significant effect observed during the first couple of embryonic cell divisions is not likely to be due to paternal gene expression abnormalities, as the embryonic genome is not yet activated. Harrouk *et al.* (2000) reported that paternal DNA damage might be translated into chromosome aberrations after the first metaphase. Consistent with this, our study supports an effect of sperm DNA damage during stages prior to embryonic genome activation. This novel finding is intriguing and highlights the need for further studies on the effects of DNA damage on chromatin remodeling post-fertilization.

We showed that on Day 3 and Day 5 of culture, there is a significant decrease in the percentage of good quality embryos and an increase in the percentage of poor quality embryos with increased sperm DNA damage. Fatehi *et al.* (2006) reported that when oocytes are fertilized with sperm containing induced DNA damage, embryonic development completely arrests after the second or third cleavage, and only sporadic blastocyst formation occurs. Jackson and Bartek (2009) reported that DNA damage can affect a wide variety of cellular processes, including DNA repair mechanisms, transcription and cell cycle control (Jackson and Bartek 2009). Thus, it is possible that extensive paternal DNA damage could affect these normal embryonic cell processes (Bordignon and Smith, 1999). While the oocyte can repair some damaged DNA (Bazrgar *et al.*, 2014), if the level of DNA damage exceeds the oocyte's capacity to completely repair the damage, then the cell might undergo apoptosis. In the male infertility group, we observed an increase in embryo quality with increased sperm DNA damage. This result was in contrast to female and unexplained infertility group and can likely be attributed to the very low sample size in this category; 3, 38 and 19 embryos in the low, intermediate and high DNA damage groups, respectively (Table I).

Here, for the first time, we show that the progression of development from good quality Day 3 embryos to blastocysts is highly influenced by the level of sperm DNA damage. It was previously observed that faster dividing cleavage stage embryos more often develop into blastocysts (McKiernan and Bavister, 1994; Lonergan *et al.*, 1999; Lee *et al.*, 2012; Knez *et al.*, 2013), and are associated with higher pregnancy rates (van Montfoort *et al.*, 2004). We speculate that sperm DNA damage levels are directly related to embryonic cleavage rates, such that the activation of additional DNA repair pathways forces delays in cell division (Su *et al.*, 2000; Wells *et al.*, 2005; Bazrgar *et al.*, 2014), resulting in a poor quality blastocyst (Ivec *et al.*, 2011). Because these differences in embryo quality are often not apparent before Day 5, our results suggest that patients with high sperm DNA damage could benefit from Day 5 blastocyst transfers, as opposed to Day 3 transfers.

In recent years, the high success rate achieved by ICSI has led some to suggest that ICSI should be performed for all ART cases (Fishel *et al.*, 2000; Oehninger and Gosden, 2002). Yoeli *et al.* (2008) reported that the embryo quality depends on intrinsic factors of the gametes, rather than the fertilization process. When we compared the development of embryos between the DNA damage groups after IVF and ICSI insemination, we observed a significant increase in the percentage of good quality embryos and decrease in the percentage of poor quality embryos in the

intermediate DNA damage groups after ICSI. We hypothesize that the selection of physiologically motile and morphologically normal sperm for ICSI insemination (Sakkas, 2013) increases the probability of picking sperm with low DNA damage. This hypothesis is supported by the fact that sperm DNA damage is negatively associated with both normal morphology (Said *et al.*, 2005) and progressive motility (Simon and Lewis, 2011); however, this hypothesis merits further study.

Our results are in agreement with a number of studies demonstrating that low implantation and spontaneous miscarriages may involve a paternal effect (Carrell and Liu, 2003; Carrell *et al.*, 2003; Virro *et al.*, 2004; Hjollund *et al.*, 2005; Slama *et al.*, 2005; Simon *et al.*, 2014). Here for the first time, we show that the paternal effect of sperm DNA damage is more prominent in the higher maternal age group. We hypothesize that in this group of patients, the oocyte's competence to repair could be a contributing factor for the observed lower implantation rate (Wang *et al.*, 2009). It is established that transfer of good quality blastocysts results in higher pregnancy rates compared with early stage blastocysts and cavitating morula (Kovacic *et al.*, 2004; Goto *et al.*, 2011; van den Abbeel *et al.*, 2013). We show that sperm DNA damage also has a negative impact on implantation of each blastocyst type. Importantly, these results suggest that patients with lower sperm DNA damage levels have a higher likelihood of implantation, and would benefit from single embryo transfer.

In this study, we used a Comet alkaline assay to quantify sperm DNA damage. A number of other studies have previously used alternative methods, including sperm chromatin dispersion assay (Muriel *et al.*, 2006; Velez de la Calle *et al.*, 2008; Rama Raju *et al.*, 2012), terminal deoxyuridine nick end labeling (TUNEL) assay (Seli *et al.*, 2004; Benchaib *et al.*, 2007; Avendano *et al.*, 2010), and sperm chromatin structure assay (Saleh *et al.*, 2003; Virro *et al.*, 2004; Check *et al.*, 2005; Zini *et al.*, 2005; Lazaros *et al.*, 2013), to measure DNA damage in sperm and correlate the levels with embryo quality. Importantly, the Comet assay results confirm the findings using other assays that higher sperm DNA damage is associated with lower embryo quality, increased embryo development arrest and decreased pregnancy rates, while lower sperm DNA damage is associated with higher embryo quality, better embryo development and increased pregnancy rates (Seli *et al.*, 2004; Virro *et al.*, 2004; Benchaib *et al.*, 2007; Velez de la Calle *et al.*, 2008; Simon *et al.*, 2011, 2014). Moreover, the results presented here agree with the conclusion put forth by Virro *et al.* (2004) that DNA fragmentation analysis can be utilized as a predictor for IVF/ICSI outcomes. These results are also supported by an *in vivo* study (Evenson *et al.*, 1999) where DNA fragmentation correlates with fertility status, time to pregnancies and spontaneous miscarriages.

In conclusion, this study reveals a strong paternal effect of sperm DNA damage in all stages of early embryonic development following fertilization as well as pregnancy rates. Such a prolonged paternal effect may be due to chromosomal aberrations, defective cellular processes, delayed cell cleavage or a combination of these factors. If the paternal damage is beyond repair, the blastomeres may undergo apoptosis, leading to reduced embryo quality or embryonic arrest. Interestingly, our results show that ICSI-derived embryos are of better quality than IVF derived embryos when the sperm DNA damage is higher. Although the literature is conflicting, we propose that measurement of sperm DNA fragmentation has the potential to become an important prognostic tool for various natural conception and IVF practices. For patients with high sperm DNA damage, we recommend selection of physiologically motile and morphologically normal sperm by ICSI for patients with a high percentage of sperm with DNA damage, Day 5 embryo transfer, and a possible justification for multiple embryo transfer. We conclude that the paternal effect of sperm DNA damage on embryonic development was significant, and we recommend additional studies with large sample size aimed at assessing DNA damage analysis as a diagnostic tool for IVF.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

L.S. is a postdoctoral fellow and was responsible for performing sperm DNA damage assay, statistical analysis and writing the manuscript. K.M. was responsible for collecting ART data from patients enrolled in the study and writing the manuscript. M.B.S. and L.L. were responsible for collecting ART data from patients enrolled in the study. B.E. was responsible for consenting the study subjects and generating the figures for the manuscript. K.I.A. and J.H. contributed to the writing of the manuscript and analysis of results. D.T.C. is the principle investigator for this study and is responsible for design of the study. D.T.C. is the head of ART unit and is the corresponding author for this manuscript.

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Conflict of interest

D.T.C. has received no personal financial support for this work. L.S. has no conflict of interest. K.M. has no conflict of interest. M.B.S has no conflict of interest. L.L. has no conflict of interest. K.I.A. has no conflict of interest. B.E. has no conflict of interest. J.H. has no conflict of interest.

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