Double stranded sperm DNA damage is a cause of delay in embryo development and can impair implantation rates

Aida Casanovas, MSc [1,*]; Jordi Ribas-Maynou, PhD [2,*]; Sandra Lara-Cerrillo, MSc [2] ; Ana Raquel Jimenez-Macedo, PhD [1]; Olga Hortal MSc [1]; Jordi Benet, PhD [3]; Joan Carrera, MD [1]; Agustín García-Peiró, PhD [2]

1 UEG, Unitat d'Endocrinologia Ginecològica. 08500, Vic. Barcelona. Spain.
2 CIMAB, Centro de Infertilidad Masculina y Análisis de Barcelona. UAB Spin-off, 08192 Sant Quirze del Valles, Barcelona. Spain.
3 Unitat de Biologia Cel·lular i Genètica Mèdica. Departament de Biologia Cel·lular, Fisiologia i Immunologia. Facultat de Medicina. Universitat Autònoma de Barcelona. Campus de la UAB. Bellaterra, Barcelona. Spain.

*Authors should be considered similar in author order.

Corresponding Author: Agustín García Peiró

Centro de Infertilidad Masculina y Análisis de Barcelona (CIMAB)
C/Vallcorba 1-3 LOCAL
08192 Bellaterra. Spain.
Phone: +34 938587614
E-mail: info@cimab.es

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CAPSULE

The present work shows that double stranded sperm DNA damage may be an important male-factor parameter causing embryo delay and implantation failures.

ABSTRACT

Objective: To analyze the effect of single and double stranded sperm DNA fragmentation (ssSDF and dsSDF) on human embryo kinetics monitored under time-lapse system.

Design: Observational, double blind, prospective cohort study.

Setting: University, University spin-off and private center.

Patients: 196 embryos from 43 infertile couples included prospectively.

Interventions: None

Main outcome Measures: ssSDF and dsSDF were analyzed in the same semen sample used for ICSI. Embryo kinetics was then monitored using time-lapse technology, obtaining timings of each embryo division.

Results

When comparing embryos obtained from semen samples with low dsSDF and high dsSDF, splitting data using, a statistically significant delay in high dsSDF was observed in 2nd polar body extrusion, T4, T8, morula, and starting blastocyst ($p<0.05$) and embryo implantation rates were impaired ($p=0.037$). Embryo kinetics and implantation rates were not significantly affected when high values of ssSDF are present ($p>0.05$ and $p=0.102$). Different patterns of delay in embryo kinetics were observed for these different types of DNA damage: dsSDF caused a delay along all stages of embryo development, however, its major effect was observed at 2nd polar body extrusion and morula stages, coinciding with embryo DNA damage checkpoints activation described before; ssSDF caused its major effect at pronucleus stage, but embryo kinetics was then restored at all the following stages. Results show that dsSDF could be the main type of DNA damage present affecting embryo development in ICSI cycles, probably due motility-based sperm selection in this assisted reproduction procedure.

Conclusions

Double stranded sperm DNA damage caused a delay on embryo development and impaired implantation, while single stranded DNA damage did not significantly affect embryo kinetics and implantation.

INTRODUCTION

Standard methods to assess embryo quality are based on intermittent evaluation of parameters such as cell number, cell fragmentation, symmetry and embryo compaction (1). In a scenario where less than half of ICSI cycles show implantation (2), laboratories had done a technological effort in order to perform the best embryo selection to achieve implantation. Then, technologies such as time-lapse recording have been implemented to a vast number of ART centers. Thanks to time-lapse, embryo evaluation turned from discrete to dynamical observation, which allowed both clinicians and researchers to perform a better embryo evaluation along all the preimplantational development (1,3–5). Different studies analyzing embryo kinetics pointed out that times of embryo cleavage might be an important parameter defining the embryo implantation potential. This fact allowed to generate mathematic algorithms to predict the best embryo to transfer (4,6). However, the effectiveness of these models could be altered by multiple cofounding factors (7), evidencing the necessity of validating them in an independent set of samples to prove their utility (3,8). The research of biomarkers with high predictive power in ART success and new embryo selection criteria is a topic of high interest in clinical practice. Regarding to male-factor, studies in ICSI cycles show that the traditional semen analysis (sperm concentration, motility and morphology) is not predictive of ICSI implantation rates (9). The introduction of sperm DNA fragmentation techniques seemed promising as complementary parameter for the prediction of ART success. Different research groups have performed studies analyzing the predictive power of sperm DNA fragmentation (SDF) using different techniques (TUNEL, SCSA and SCD tests). Some studies showed a relation between DNA damage and implantation rates in ICSI (10–13) but others showed opposite results (14–18). These unclear results could be related to the bias between ejaculate analysis and the selection of a motile sperm cell prior to realization of ICSI (19,20), as it is known that a negative correlation is present between sperm motility and DNA fragmentation (21–23).

In the recent years, studies analyzing the clinical effect of different types of sperm DNA damage showed that ssSDF detected by alkaline Comet is an extensive DNA damage related to natural pregnancy achievement (24,25), showing good correlation with TUNEL, SCSA and SCD tests (26). Alternatively, DNA breaks detected using neutral Comet correspond to matrix attachment region (MAR) specific double stranded DNA damage (24–26) related to higher miscarriage risk. These highly localized DNA breaks do not show a correlation to the prior techniques neither to sperm motility. In animals, physiological studies showed that induced double strand breaks in mouse sperm cells cause complex paternal chromosomal reorganizations at male pronucleous, showing a delay in first embryo DNA replication (27). Other studies inducing sperm double strand breaks through radiation identified embryo checkpoints related to p53 and p21 in response to paternal double stranded DNA damage, and less number of fetuses were found when an irradiated sperm sample was used for fertilization (28,29). Studies analyzing the effect of double stranded sperm DNA breaks in human ICSI treatments are still emerging (10), but is a topic that has not been extensively studied.

Until our knowledge, only two studies have analyzed the relation between sperm DNA fragmentation and embryo kinetics during preimplantational development, showing a slower embryo development when high SDF is detected using sperm chromatin dispersion test (30) and TUNEL assay (31). As single and double strand DNA fragmentation show different clinical implications in natural pregnancies, the aim of the present study is to analyze the relation of both types of sperm DNA damage and embryo kinetics in ICSI cycles.

MATERIAL AND METHODS

Study design and participants

The present prospective and double blind study included data for 196 embryos from 43 infertile couples that attended to our center seeking for assisted reproductive treatments. The patients included in the study were not under antioxidant treatment. The Parc Taulí Hospital Ethics Committee approved the study (Ref. 2017902).

From the patients included in the study, a semen sample with three or less days of abstinence was obtained the day of the ICSI, a 250 μl aliquot was cryopreserved, and the rest of the sample was used for ICSI procedure. Sperm concentration and motility was also determined and single and double stranded sperm DNA fragmentation analysis was performed using Comet assay. All the embryos were cultured under time-lapse monitoring system (Primo Vision^{TM}, Vitrolife, Sweden).

Comet assay: Single and double strand sperm DNA fragmentation analysis

Particular conditions of Comet assay methodology allow the discrimination of single and double strand DNA damage. The alkaline Comet protocol described previously (24) and used in the present work mainly evidences the presence of single strand DNA breaks, while the neutral Comet shows double strand DNA breaks. Briefly, semen sample was thawed, washed in PBS and sperm concentration was adjusted to $1{\cdot}10^6$ spermatozoa/mL. 25 μL of sample was mixed in 50 μL of melted 1% agarose in distilled water. The mixture was allowed to jellify on two slides at 4ºC, and slides were treated in two consecutive lysis solutions (0.8M Tris-HCl, 0.8M DTT, 1% SDS, pH 7.5 and 0.4M Tris-HCl, 0.4M DTT, 50 mM EDTA, 2M NaCl, pH 7.5) for 30 minutes each in order to remove proteins and unwind sperm DNA. After lysis step, the slide designated to dsSDF analysis was electrophoresed at 20 volts for 12.5 minutes in TBE buffer at pH 8, and washed in 0.9% NaCl solution. Meanwhile, the slide designated to ssSDF analysis was denatured in NaOH solution at 4ºC and electrophoresed at 20 volts for 4 minutes in NaOH at pH 13. Both slides were washed in TBE and dehydrated in ethanol series (70%, 90% and 100%) for two minutes each. Finally, samples

were stained using DAPI (Thermo Fisher Scientific, Waltham, MA, USA) and spermatozoa were scored under epifluorescence microscope (Nikon E200, Tokyo, Japan) following the criteria reported before (24) and depicted in figure 1. Results were expressed as a percentage of fragmented sperm cells. An internal control sample was repeatedly included in each experiment in order to control technique variability.

Ovarian stimulation, oocyte retrieval and ICSI

Patients were stimulated following a personalized protocol that included an initial ovarian stimulation with 150 – 300 UI FSH (Menopur, Ferring, Switterland). Doses were calculated considering patient's age, body mass index, ovarian reserve and the response if the patient had previous cycles. LH and/or urine gonadotropins were added to the stimulation protocol considering patient's age and LH basal values. In most cases, short protocols with GnRH antagonists, triggering with an agonist 34-36h before egg collection to initiate ovarian maturation were used. Cumulus oocyte complexes (COC) were retrieved 34-36h post-GnRH trigger injection. COCs were washed using HEPES medium (LifeGlobal, Canada) and cultured in Global Fertilization medium (LifeGlobal, Canada) in a Labotect C60 incubator (Labotect, Germany) at 7.2% CO_2, atmospheric O_2 and 37ºC for 2-3h before denudation. ICSI was performed 4h after egg collection using HEPES medium (LifeGlobal, Canada) and injected eggs were placed in a pre-equilibrated Primo Vision^{TM} slide (Vitrolife, Sweden) containing 80µl of Global medium (LifeGlobal, Canada) and 4 ml overlay of mineral oil (Ovoil, Vitrolife, Sweden).

Embryo incubation and time-lapse imaging and data acquisition

Embryos were cultured during 5/6 days after sperm injection in the Primo Vision culture dish at 7.2% CO_2, atmospheric O_2 and 37º. Embryos were cultured uninterruptedly through the whole development until blastocyst stage using Global medium (LifeGlobal, Canada).

Time-lapse imaging provided images every 10 minutes for all embryos analyzed. These images were compiled in videos, and one researcher annotated time points corresponding to the following stages of embryo development: 2nd polar body extrusion, pronuclei appearance, pronuclei disappearance, starting first cell division (Starting T2), two cells (T2), three cells (T3), four cells (T4), five cells (T5), six cells (T6), seven cells (T7), eight cells (T8), nine cells (T9), morula stage, starting blastocyst and blastocyst stage. Data obtained is expressed in hours post ICSI.

Embryo scoring and selection

Embryo morphology was evaluated using Gardner's blastocyst classification (32), taking into account the blastocel expansion, the number of trophectoderm cells and the inner cell mass. Embryos were vitrified and one or two best quality embryos were transferred a month later following a natural cycle that was determined by the LH peak.

Statistics

Data distribution was evaluated using Kolmogrov-Smirnov Test. Comparisons of quantitative variables were performed using Mann-Whitney U test, since non-parametric analysis was required due to lack of normality. The 95% of confidence interval was chosen for statistical significance ($p<0.05$) in all statistical tests.

RESULTS

General data about patients

Patients' data for low or high ssSDF and dsSDF is displayed in Table 1. Median values were taken as the cut value to discriminate low or high ssSDF and dsSDF. No differences were found in any parameter when comparing couples with high ssSDF and couples with low ssSDF. However, when patients were classified by double strand DNA damage, implantation rate showed significantly higher rate in low dsSDF compared to high dsSDF. None of the other parameters showed statistical differences (Table 1).

Single and double stranded DNA damage and progressive motility

A negative correlation between progressive motility and single stranded DNA fragmentation has been found ($r=-0.390$; $p=0.037$), while no correlation was found between progressive motility and double stranded DNA damage ($p=-0.092$; $p=0.642$).

Single strand sperm DNA fragmentation and embryo kinetics

Embryo kinetics results were classified according low or high ssSDF (Table 2), expressed in median (range) of hours after fertilization. None of the timings showed statistically significant results when comparing low ssSDF to high ssSDF samples ($p>0.05$) (Table 2).

Double strand sperm DNA fragmentation and embryo kinetics

Embryo development timings were also classified according low or high values for dsSDF. Median (range) for every stage is displayed in Table 2. Statistically significant results were found in different stages ($p<0.05$): 2nd polar body extrusion, T4, T8, morula, and starting blastocyst.

Single and double strand sperm DNA damage caused different patterns of delay in embryo kinetics

206 Taken the differences from Table 2, we expressed the relative percentage of embryo delay of high
207 ssSDF and dsSDF groups in relation to low ssSDF and dsSDF groups, respectively. Figure 2 displays
208 these differences along the embryo development. Single stranded DNA damage shows its major
209 effect at pronuclei stage, but no increase of delay is shown among other stages. Alternatively,
210 double stranded DNA damage cause a delay at the extrusion of second polar body, but the
211 embryo development delay shows a progressive increase as embryo development proceeds. The
212 major effect of double stranded DNA damage is shown to be at second polar body and between
213 T9 and morula stages.
214
215 *Implantation and embryo kinetics*
216 We afterwards obtained results for those embryos that were known to achieve an implantation
217 with positive heart beat (n=16) and those that were transferred and did not succeed as an
218 implantation (n=28). Timings showed by this small subgroup of embryos are displayed in Table 3.
219 After obtaining these embryo development timings, they were compared to those embryo timings
220 from patients with low or high dsSDF displayed in Table 2. Embryo kinetics of those embryos that
221 achieved implantation was similar to low dsSDF kinetics (mean of 0.4% of difference; p=0.975)
222 and different to high dsSDF kinetics (mean of 3.8% of difference; p=0.001). Embryo kinetics from
223 those embryos that did not achieve implantation was similar to high dsSDF kinetics (mean of 1.3%
224 of difference; p=0.670) and different to low dsSDF kinetics (mean of 5.7% of difference; p=0.001).
225

DISCUSSION

In the present study, the effect of single and double stranded DNA damage on embryo kinetics, monitored using time-lapse was evaluated. Results suggest that double stranded DNA fragmentation could be the main type of DNA damage affecting embryo kinetics. Available data showed a delay in embryo kinetics and worse implantation rates when double stranded DNA damage is increased in the semen sample used for ICSI (Table 1). In contrast, an increase of single stranded sperm DNA damage did not cause any significant effect neither on embryo kinetics nor in implantation rates (Table 1). These results may bring light about the effect of different types of sperm DNA damage in embryo development. Until our knowledge, this is the first study analyzing different types of sperm DNA breaks and human embryo development.

The effect of sperm DNA fragmentation in embryo quality and ICSI outcomes has been a controversial topic in the last years, existing some studies which found an association between these parameters (10–13), and others showing opposite conclusions (14–18). However, studies analyzing the paternal effect on embryo development may show different cofounding factors based on oocyte quality (13) or the technique used for fertilization (33). The consensus point is that oocyte may have the ability to repair some of the paternal DNA breaks, depending on its quality (19,34–39). In this sense, a vast study of female factors would be desirable in order to elucidate the effect of male factor, and this may be a limitation for any male factor study. In fact, in the present study we confirmed that no differences were present between DNA fragmentation groups regarding oocyte age, years of infertility, previous pregnancies and MII oocytes retrieved (Table 1), however, more parameters such as body mass index or FSH levels would have been interesting to be included.

Results showed that single stranded DNA breaks were not associated to a delay of embryo kinetics in any stage (Table 2). This fact may be explained by the negative correlation found between progressive motility and ssSDF measured by alkaline Comet assay and other techniques measuring SDF (21–23). Taking into account this correlation, and knowing that all sperm cells

252 selected for ICSI show good motility, one would expect that most ICSI selected sperm cells would
253 not show a high amount of single stranded DNA breaks regardless of ejaculate ssSDF. In fact, this
254 strong bias introduced by the sperm selection in ICSI cycles was previously described in studies
255 trying to find associations between DNA integrity and embryo quality (19,20). More research is
256 necessary in order to clarify the effect of oxidative DNA breaks in embryo development.
257 Regarding double stranded DNA breaks, an association to a delay has been found at different
258 stages of embryo development, including 2nd polar body extrusion, T4, T8, morula and starting
259 blastocyst (Table 2). Following the hypothesis explained above, dsSDF do not show a correlation
260 to progressive motility, therefore, one would expect that the proportion of positive dsSDF in ICSI-
261 selected sperm cells and the ejaculate should be similar. Then, dsSDF could be the predominant
262 DNA damage in ICSI-selected sperm cells and this could explain the associations found between
263 dsSDF and embryo kinetics. From studies in somatic cells, it is well known that double stranded
264 DNA breaks trigger the initiation of DNA repair machinery and/or apoptosis (40,41) and a
265 misrepair of a double strand break is the previous step to chromosome reorganizations, loss of
266 chromosomal fragments and/or complex reorganizations (42–46). In reproduction, these
267 processes may be happening in a similar way during gametogenesis and embryo development
268 (47–49), as it is known that chromosome reorganizations may be present in germ cells, causing
269 higher risk of miscarriage and infertility (24,50–52). Thus, we previously observed that alterations
270 of dsSDF are related to a higher risk of recurrent pregnancy loss in couples experiencing natural
271 pregnancy (25). During embryo development in ICSI treatments, embryos may accumulate
272 structural chromosomal alterations from paternal origin, leading to a slower development to
273 blastocyst. In fact, aneuploidy has been described as one factor causing delay in embryo
274 development (6,53–55). Moreover, the presence of blastomere multinucleation, related to
275 implantation rates (56), supports the presence of chromosomal alterations. This multinucleation
276 may resemble micronuclei appearing when chromosomal aberrations due to double strand breaks
277 occur in somatic cells (57–59).

The embryo kinetics shown in Figure 2 present different patterns of delay: while the sperm cells from samples with high single stranded DNA damage cause their major effect at pronuclei appearance, double stranded DNA damage causes an initial delay after fertilization which is restored and then a progressive increase of delay is observed until reaching morula stage (Figure 2). On one hand, ssSDF may cause defects of DNA replication at pronuclear stage because its extensive nature affects the whole genome (25). If a spermatozoa containing single stranded DNA breaks is used for fertilization, the zygote may induce single stranded DNA repair before DNA replication in an efficient manner, since the complementary DNA strand is present (29,34,60,61). Therefore, DNA repair might be the reason why embryos do not present delays after first cleavage, and ssSDF may not have a more severe implication in embryo development.

On the other hand, double stranded DNA breaks in sperm cells are localized mainly at MAR regions (25) and both DNA ends have been demonstrated to be attached at the nuclear matrix (62), which is inherited at male pronucleus until first mitotic division (63,64). One of the first steps carried out at male pronucleus is the replacement of protamines by histones. Due to the attachment of both ends of a DNA break to protamines and to the nuclear matrix, the DNA repair must be produced during this phase, where two DNA ends remain tightly attached (62). In fact, DNA repair and paternal pronucleus replication mechanisms have hypothesized to be linked to the nuclear matrix (60,65–68). The results obtained here are similar to those obtained by (27) in a mouse model with induced double stranded DNA breaks, where a delay was observed in paternal pronucleus compared to maternal pronucleus before the first embryo cleavage, with the presence of H2AX phosphorylation and chromosome aberrations (27). If DNA damage cannot be repaired during this short stage, embryo divisions can be performed but double strand breaks may remain on the embryo, causing split of chromosome fragments, chromosome reorganizations, or complex chromatin reorganizations to maintain chromosomal integrity (29,48,49,69). These chromosomal aberrations may remain in some blastomeres causing mosaicism, which is in fact an observation described in PGS embryos (70–73). It is not until morula stage, where chromosome fragments can

activate G1/S and G2/M checkpoints, triggering DNA damage apoptotic mechanisms on affected cells (29). In fact, when mouse sperm cells with induced dsSDF are used to fertilize p21 and p53 knockout embryos, where apoptosis is continuously suppressed even on day 3.5, embryos accumulate chromosomal aberrations and failed implantation (28). In relation to that, a higher implantation rate was observed when patients were classified regarding dsSDF (Table 1).

We finally selected the small subgroup of transferred embryos (n=44) and analyzed the coincidence of embryo kinetics between embryos with and without successful implantation and low or high dsSDF groups (Tables 2 and 3). Results obtained were that embryos with successful implantation showed higher similarity to low dsSDF group, whereas embryos without successful implantation showed higher similarity to high dsSDF group. Therefore, embryos from high dsSDF patients that achieved implantation might either have been successful on dsSDF repair or they do come from a spermatozoa lacking of dsSDF. Different works related a delay of embryo kinetics at different stages to a lower implantation, and some authors propose decision algorithms to select the best embryo to transfer (1,3,4,74,75). However, achieving a reduction of sperm double strand breaks incidence could also be an important factor to improve implantation rates.

Conclusion

Double stranded DNA damage, and not single stranded DNA damage, has an effect in embryo kinetics and is related to implantation rates. The analysis of dsSDF in ICSI patients could be a relevant prognostic value of male-factor in ICSI cycles.

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FIGURES AND TABLE LEGENDS

Figure 1. Composition of non-fragmented and fragmented sperm cells visualized after alkaline or neutral Comet. Fragmented sperm cells show migrated DNA towards the cathode.

Figure 2. Percentage of delay between low and high ssSDF and dsSDF. Single stranded DNA damage caused a delay at pronuclei appearance, but kinetics was recovered at next stages. Alternatively, double stranded caused its major delay at 2^{nd} polar body extrusion and morula stages, however, the delay is present along all the preimplantational embryo development.

Table 1. General data of couples included in the study. Data was split into groups regarding low or high single and double stranded sperm DNA fragmentation.
** statistical differences ($p<0.05$)

Table 2. Embryo kinetics classified into low or high single and double stranded sperm DNA fragmentation. ** statistical differences ($p<0.05$)

Table 3. Embryo kinetics for transferred embryos according if they achieved implantation or not.