Effect of human oviductal *in vitro* secretion on human sperm DNA integrity

Celeste Robert · Adriana Caille · Carlos Zumoffen ·
Marcelo Cabada · Sergio Ghersevich

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Abstract

Purpose In the female genital tract spermatozoa interact with the oviductal secretion. The aim of the study was to evaluate the effect of conditioned media (CM) from cultures of human oviductal tissue, on sperm DNA integrity. The effect of H₂O₂ on sperm DNA integrity, before and after incubation under capacitating conditions, was also investigated.

Methods Motile sperm obtained from normozoospermic semen samples were incubated (4 h or 22 h) in the presence or absence of CM and further exposed to H₂O₂. DNA damage was detected by the comet assay.

Results The CM significantly reduced the DNA damage associated with sperm incubation, and also decreased the effect of H₂O₂ after 4 h incubation, compared to controls. The H₂O₂

caused a dose-dependent deleterious effect on sperm DNA integrity both before and following 22 h of capacitation.

Conclusion The oviductal tissue CM increased the stabilization of the sperm DNA structure under culture conditions.

Keywords Comet assay · DNA · Oviduct · Spermatozoa

Introduction

Defective sperm chromatin packaging, apoptosis and oxidative stress have been suggested as causes of DNA damage in human spermatozoa [1]. Human sperm are particularly sensitive to free radical assault because of their high content of polyunsaturated fatty acids and lack of DNA repair mechanisms [2]. Sperm are protected from oxidative damage by seminal plasma, which contains an abundance of antioxidant enzymes and scavengers such as albumin and taurine [3]. Morphologically abnormal spermatozoa (spermatozoa with cytoplasmic retention) and leukocytes have been established as the main sources of high reactive oxygen species (ROS) production in human ejaculates [2, 4, 5]. Separating spermatozoa from seminal plasma during semen processing results in a pro-oxidant state. In addition, repetitive washing of sperm by serial centrifugation increases production of ROS and impairs sperm function [6]. The generation of ROS could also result from the activation of a putative NADPH oxidase located in the sperm plasma membrane [7] or from a NADH-dependent oxido-reductase at the level of sperm mitochondria [8].

The excess of ROS attacks polyunsaturated fatty acids in the sperm plasma membrane [9] and has been associated with defective sperm function and infertility [2, 10]. In addition to cellular lipids, ROS affect proteins and DNA. One of the most dangerous DNA lesions is the

Capsule Oviductal tissue conditioned media contributes to sperm DNA stabilization.

C. Robert · A. Caille · C. Zumoffen · S. Ghersevich
Laboratory of Reproductive Studies,
Area of Clinical Biochemistry,
School of Biochemical and Pharmaceutical Sciences,
National University of Rosario,
Rosario, Argentina

M. Cabada
Instituto de Biología Molecular y Celular de Rosario
(IBR, CONICET), Area of Biology,
School of Biochemical and Pharmaceutical Sciences,
National University of Rosario,
Rosario, Argentina

S. Ghersevich (✉)
Laboratory of Reproductive Studies, Area of Clinical Biochemistry,
School of Biochemical and Pharmaceutical Sciences,
National University of Rosario,
Suipacha 531, (2000),
Rosario, Argentina
e-mail: sghersev@fbioyf.unr.edu.ar

double-strand break, which can cause apoptosis [11], inactivate key genes, or lead to serious chromosomal aberrations [10, 12].

In humans, intercourse may occur at any time during the menstrual cycle. During their ascent through the female reproductive tract, spermatozoa are separated from seminal plasma and can be more susceptible to oxidative damage. Spermatozoa can retain their physiological function within the female tract for several days after copulation [13] while they interact with different female fluids, such as the oviductal fluid. The female fluids can contribute to the redox status of the sperm environment. Exposure to semen elicits an inflammatory response in the female reproductive tract resulting in leukocyte recruitment and cytokine release [14–16]. In addition, it could be thought that any inflammatory process of the female reproductive tract, which induces the production of highly toxic ROS [17], could affect sperm DNA integrity.

Despite spermatozoa can remain several days in the oviduct, previous studies did not investigate the effect of the oviductal secretion on sperm DNA integrity. Due to ethical reasons and to methodological difficulties related with retrieving the fluids of the female genital tract, obtaining human tubal fluid, is not recommended. In order to evaluate the interaction between the human oviductal fluid and spermatozoa, previous studies have reported that coculture of sperm with oviduct epithelial cells or their conditioned media improved or maintained sperm survival and motility parameters and has shown beneficial effects on sperm membrane stabilization [18–21]. Although preservation of sperm membrane function and motility are important for best reproductive outcome, integrity of the sperm chromatin is a fundamental requisite [10]. It has been suggested that oxidative injury to sperm DNA can have profound implications for normal embryonic development and long-term health of progeny [2, 10].

Our laboratory has set up an *in vitro* system to obtain conditioned media (CM) from human oviductal explant cultures [21]. The CM was shown to modulate some sperm functions *in vitro* [21, 22]. Therefore, the aim of the present study was to investigate the effects of incubation in the presence of CM from human oviductal tissue cultures on sperm DNA integrity and on a further oxidative treatment. Hydrogen peroxide has been widely used to investigate the ROS effects on sperm structure and function [23–26]. Thus, in the present study H_2O_2 was used for the oxidative treatment of spermatozoa.

In addition, taking into account that the sperm capacitation process implies both structural and physiological changes [27, 28], the effect of an oxidative stress induced with H_2O_2 on sperm DNA fragmentation was evaluated before and after incubation under capacitating conditions.

Materials and methods

Study protocols were approved by the Institutional Bioethical Board of the School of Biochemical and Pharmaceutical Sciences, National University of Rosario, and written consent was obtained from all donors.

Chemicals and reagents

Unless mentioned in the text, all the chemicals and reagents were purchased from Sigma-Aldrich Inc. or from MP Biomedicals Inc. and were of the highest purity available.

Oviductal tissue cultures

Human oviductal tissues were obtained from pre-menopausal women ($n=6$, age: 43.4 ± 4.1) with no clinical history of infection or neoplastic disease, who had been scheduled for hysterectomies because of uterine fibromyoma. Patients gave written consent for removal of their oviducts. Tubal tissues were processed within 1 h following surgery, and were cleaned of connective and vascular tissue, sliced longitudinally and cut into 2–3 mm³ pieces. Tissue explants were cultured 24 h as described elsewhere [21], in DMEM/Ham's F-12 medium (GibcoBRL, Paisley, Scotland) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), fetal bovine serum (FBS, 10% v/v, Bioser, Buenos Aires, Argentina), L-glutamine (2 mM, Seromed, Biochrom KG, Berlin, Germany) and HEPES (15 mM, Imperial, UK). Explants were then thoroughly washed with protein-free medium and further cultured for 24 h. Following incubation, CM was collected and centrifuged 10 min at 700 g, to remove debris. The clarified media were then concentrated 6x using Centricon concentrators (10 kDa cut-off; Amicon, Millipore Corp., Bedford, MA, USA), and CM was stored at -70°C until use. All cultures and incubations were performed at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . The protein concentration of the CM was assessed using the BioRad Protein assay kit (Hercules, CA, USA).

Tissue DNA integrity

In order to evaluate tissue viability following culture, DNA integrity of oviductal explants was analyzed after incubation as described in Munuce *et al.* [22]. Briefly, explants were manually disaggregated with buffer B (NaCl 0.0875 mmol/l, EDTA 9 mmol/l, Tris 10 mmol/l) and supernatants were treated 20 h at 37°C with SDS (20% v/v) and proteinase K (10 mg/ml, Promega, MA, USA), after which suspensions were sequentially extracted with phenol, phenol/chloroform/isoamyl alcohol (25:24:1), followed by chloroform-isoamyl alcohol (24:1) [29]. DNA was precip-

itated by adding NaCl (5 mol/l) and absolute ethanol and incubating 2 h at -20°C . After centrifugation at 7000 g, DNA was washed with 70% (v/v) ethanol, air dried, resuspended in sterile water and stored at -20°C until use. Electrophoresis of DNA samples was carried out on 1% agarose gels (BioRad, Hercules, CA, USA) in TBE buffer (Tris base 0.089 mol/l, boric acid 0.089 mol/l, EDTA 0.02 mol/l, pH 8.3) for 2 h at 75 V. A commercial ϕ x 174 DNA/Hae III Marker (72–1353 bp; Promega, MA, USA) was used as molecular weight standard. Conditioned media obtained from tissues with altered DNA integrity were excluded from the study.

Semen analysis

Semen samples were collected by masturbation from healthy donors ($n=13$) following 3–5 days of sexual abstinence. Donors were normozoospermic according to WHO guidelines and Kruger's strict morphology criteria [30, 31]. Proven fertile semen donors were recruited for this study. They had fathered at least one child within 2 years before the study.

Selection of motile spermatozoa was carried out by layering 1 ml of semen on the top of 2.5 ml of a discontinuous Percoll gradient (50%–70%–90%) in Ham's F10 medium (Hyclone, Road Logan, Utah, USA). Each tube was centrifuged 20 min at 275 g. The bottom gradient, containing the motile sperm fraction, was then washed 10 min with 2 ml of Ham's F10 medium, following which sperm

were assessed for viability and motility (WHO criteria). Viability was determined using 0.5% Eosin Y (Gibco, Grand Island, NY, USA) and reported as a percentage.

Effect of CM

Aliquots of motile spermatozoa were included in agarose, following Percoll isolation, mounted on slides and analyzed by the comet assay (C-0h, Fig. 1). A number of slides were pre-treated with $17.6\text{ }\mu\text{mol/l}$ of H_2O_2 (C-0h + H_2O_2).

The remaining spermatozoa were incubated under capacitating conditions for 4 h and 22 h (Fig. 1), in Ham's F-10 medium supplemented with BSA (3.5% w/v) at a concentration of 5×10^6 spermatozoa/ml, and in the presence (CM-4h or CM-22h, protein concentration of $0.2\text{ }\mu\text{g}/\mu\text{l}$) or the absence of CM (C-4h or C-22h). Sperm were then included in agarose and mounted on slides. A number of slides were further treated with $17.6\text{ }\mu\text{mol/l}$ H_2O_2 (CM-4h + H_2O_2 ; CM-22h + H_2O_2 ; C-4h + H_2O_2 ; C-22h + H_2O_2) previous to performing the comet assay.

Anti-oxidant properties of CM were evaluated in a similar manner to that proposed by Hughes *et al.* (1998). Percoll gradients with or without (control) $0.2\text{ }\mu\text{g}/\mu\text{l}$ CM proteins were used to assess whether the CM could reduce the oxidative damage to sperm DNA associated with the isolation of motile spermatozoa. Following separation, sperm samples were evaluated for viability and motility, embedded in agarose, mounted on slides, and analysed with the comet assay.

Fig. 1 Scheme of the experimental design used in the study. For explanation see "Materials and methods" section

Sperm capacitation and effect of H₂O₂

Spermatozoa before (0 h) or following an overnight incubation under capacitating conditions (22 h, Ham's F10 medium supplemented with 3.5% w/v BSA at 37°C in a humidified atmosphere of 95% air and 5% CO₂) were embedded in agarose and mounted on slides as described below for the comet assay. In order to evaluate the effect of an induced oxidative stress on sperm DNA, slides were incubated with or without (control) increasing concentrations of H₂O₂ (8.8 µmol/l to 280 µmol/l), after which spermatozoa were analysed by comet assay.

Comet assay

Alkaline single-cell gel electrophoresis (comet) assay was based on existing methods [32, 33]. A total of three replicate microscope slides were used for each sample. Slides were dipped in a solution of 1% multipurpose agarose (BioRad, Hercules, CA, USA) dissolved in distilled water, and air dried overnight at room temperature. Ten microliters of sperm suspension (5×10^6 spermatozoa/ml) in Ham's F-10 medium were then mixed with 90 µl of 0.5% w/v low-melt agarose solution (BioRad, Hercules, CA, USA) in PBS. This mix was layered onto the slides and allowed to solidify at 4°C a few minutes. A final layer of 0.5% w/v low-melt agarose solution was added onto the slide, which was then overlaid with a coverslip, and allowed to solidify at 4°C for at least 1 h. After removal of the coverslip, slides were immersed 1 h in lysis buffer (2.5 mol/l NaCl, 100 mmol/l EDTA, 10 mmol/l Tris-HCl, 10% v/v dimethylsulfoxide, and 1% v/v Triton X-100, pH=10) at 4°C. The lysis solution was drained from the slides and replaced with a proteinase K solution (60 µg/ml proteinase K, Promega, MA, USA, 2.5 mol/l NaCl, 10 mmol/l Tris-HCl and 100 mmol/l EDTA, pH=10), and incubated overnight at 37°C. Slides were then immersed 20 min in a horizontal gel tank filled with alkaline buffer (300 mmol/l NaOH, 1 mmol/l EDTA, pH=12.5) to allow DNA to unwind. Electrophoresis was carried out for 5 min at 20 V. Slides were then washed 5 min with 0.4 mol/l Tris-HCl (pH=7.4), followed by three washes with fresh buffer. After rinsing, slides were air dried overnight. Sperm DNA was detected by silver stain (Silver Stain Plus, BioRad, Hercules, CA, USA), and comet analysis was carried out under a light microscope (Carl Zeiss, Germany). A total of 100 randomly selected cells were scored for each replicate slide. The total length of DNA migration (DM) for comets from each treatment group was assessed using a micrometer [34]. Comets were defined based on their DM, according to the following types: 0 (DM≤35 mm), 1 (35<DM≤45 mm), 2 (45<DM≤55 mm), 3 (55<DM≤60 mm) and 4 (DM>60 mm). The type averages (TA) of the spermatozoa

belonging to each treatment group was considered for comparative purposes, with a higher TA reflecting higher sperm DNA damage.

Statistics

The significance of the differences between mean results from different treatment groups was analysed by ANOVA test and Dunnett T3 test for multiple comparisons. A $p < 0.05$ was considered statistically significant. Results are presented as the means ± SEM, and every experiment was conducted on at least 3 semen samples from different donors.

Results

Tissue DNA integrity and CM protein concentration

In order to determine tissue viability after culture, DNA integrity was evaluated following incubation. DNA was extracted as described in M & M, and analyzed by agarose gel electrophoresis. None of the samples ($n=6$) presented an altered DNA pattern, as determined by the absence of low molecular weight fragments.

In order to perform comparable experiments total protein concentrations in each CM was measured. Mean protein concentration in CM was 7.3 ± 1.3 µg/µl.

Sperm viability and motility

Both, sperm viability and progressive motility at the different incubation times and in the presence or absence of CM were similar to those in controls at 0 h for each treatment. Viabilities and progressive motilities were always higher than 90% and 62%, respectively. Considering that the presence of dead cells affects the results of the comet assay, samples which viability is lower than 90% should not be used for this method.

CM and sperm DNA integrity

Taking into account that spermatozoa may stay in the oviduct during long periods, the potential protective effect of CM on DNA integrity of spermatozoa incubated under capacitating conditions was analysed.

Representative images for comets type 2 and 4 stained with silver are shown in Figs. 2A and B, respectively. There was a significant increase in DNA damage after 4 h incubation, even in the presence of CM ($p < 0.01$) compared with C-0h (Fig. 3). However, the TA of spermatozoa incubated in the presence of CM (CM-4h) was significantly lower than in its absence (C-4h) ($p < 0.001$), indicating a

Fig. 2 Photomicrograph of two different types of comets detected with silver staining. (A) type 2, (B) type 4. Bar: 20 μ m

Fig. 3 Effect of CM on type average of spermatozoa incubated 4 h in the presence (CM-4h) or absence (C-4h) of CM. A number of slides were further treated with H₂O₂ (C-4h + H₂O₂ and CM-4h + H₂O₂). The figure shows a representative result from 5 independent experiments. Line inside box, median; and limits of box, 75th and 25th percentiles. ^a $p < 0.01$; ^b $p < 0.001$; ^c $p < 0.001$

Fig. 4 Effect of CM on type average of spermatozoa incubated 22 h in the presence (CM-22h) or absence of CM (C-22h). A number of slides were further treated with H₂O₂ (C-22h + H₂O₂ and CM-22h + H₂O₂). The figure shows a representative result from three independent experiments. Line inside box, median; and limits of box, 75th and 25th percentiles. ^a $p < 0.001$; ^b $p < 0.05$

higher DNA integrity in the presence of the conditioned media. This protective effect of CM was also evident when spermatozoa were exposed to H₂O₂, as indicated by the fact that the TA of CM-4h + H₂O₂ spermatozoa was significantly lower than in C-4h + H₂O₂ ($p < 0.001$). These experiments were performed with spermatozoa from five different donors.

In spermatozoa incubated for 22 h, there was also a significant increase in TA even in the presence of CM ($p < 0.001$), when compared with C-0h (Fig. 4). Nevertheless, DNA damage was lower in the presence of CM than in its absence ($p < 0.05$). Despite this, when the oxidative treatment was applied, a similar TA was detected for both sperm incubated with CM (CM-22h + H₂O₂) or without CM (C-22h + H₂O₂).

To further evaluate whether CM could protect sperm DNA from the oxidative damage associated with semen processing, motile spermatozoa were isolated using Percoll with or without CM (0.2 μ g/ μ l). The presence of CM in the Percoll gradient neither affected sperm viability nor motility, which were similar to those in control spermatozoa. The results of the comet analysis indicated that the presence of CM did not decrease the DNA damage as compared with controls (2.35 ± 0.03 vs. 2.13 ± 0.02).

Sperm capacitation and effect of H₂O₂

Following Percoll isolation, motile spermatozoa ($t = 0$ h) were analyzed by the comet assay. A sample number of slides was treated with increasing doses of H₂O₂ before analysis. The results of the comet assays revealed signifi-

cant dose-dependent increases in TA for spermatozoa treated with H_2O_2 , with respect to controls (Fig. 5A), except with the lowest dose of H_2O_2 (8.8 $\mu\text{mol/l}$) that caused a significant decrease in TA.

In order to assess whether the incubation under capacitating conditions modulated the effects of H_2O_2 on sperm DNA, overnight capacitated spermatozoa were further exposed to increasing doses of H_2O_2 . In contrast to that observed at 0 h, the TA of spermatozoa incubated for 22 h under capacitating conditions and further treated with 17.6 $\mu\text{mol/l}$ of H_2O_2 did not differ from that in controls. However, higher doses of H_2O_2 increased DNA damage as observed in sperm at $t=0$ h (Fig. 5B).

Discussion

Sperm DNA is complexed with basic proteins, enabling a high degree of packing which minimizes damage by exogenous agents [35]. Previous studies have shown that certain levels of ROS favour the sperm capacitation process [25, 26,

36], but its excess could result in structural and functional sperm damage [2, 36, 37]. The risk of oxidative stress affecting sperm DNA integrity may come from the presence of leukocytes or morphologically abnormal spermatozoa in the ejaculated, from factors arising from semen processing, from inflammatory processes of the female genital tract or from external agents such as ionizing radiations.

In the present study primary DNA damage at the individual cell level was detected by single-cell gel electrophoresis assay (SCGE, comet assay), which usefulness for evaluation of DNA damage in human sperm has been well established [32, 35, 38].

The results showed that long term incubations of spermatozoa in the presence of CM did not affect sperm survival or motility, both of which were similar to those found for post-Percoll controls. These findings are also in agreement with results from a recent study from our laboratory indicating that a two-hour incubation of spermatozoa in the presence of the CM did not affect sperm motility or survival [21].

The results indicated that sperm DNA integrity decreased after a four-hour incubation under capacitating conditions. This is in agreement with previous studies showing the effect of *in vitro* manipulation of sperm in the absence of seminal plasma, which protects sperm from oxidative damage [6, 24, 39]. After spermatozoa are deposited in the female tract, they can remain in contact with the female tissue secretions, such as the oviductal secretion, for hours and even days. Taking into account that conditioned media (CM) obtained from oviductal tissue cultures contains several *de novo* produced proteins [21] and that a number of these could possess antioxidant properties, the present study examined whether CM could reduce the effect of the incubation on sperm DNA integrity. Despite the fact that DNA damage was higher than in controls at 0 h, the TA in the presence of CM was significantly lower than in its absence, after both 4 h and 22 h incubation periods, supporting the idea of a protective effect of CM on sperm DNA. In addition, exposure to H_2O_2 resulted in less DNA damage in sperm after 4 h-incubation in the presence of CM, than in its absence. It can be hypothesized that the ability of CM to reduce the effect of an oxidative agent could be due to a further stabilization of the sperm DNA structure. However, this effect was not observed after the 22 h incubation, where the oxidative agent significantly increased the TA even in the presence of CM, suggesting that the protective effect of CM can be overwhelmed after a long-term incubation. Previous studies have shown that the coincubation of spermatozoa with conditioned media from oviductal cell cultures had beneficial effects on sperm viability, motility and membrane stabilization [18–21], but the effect on sperm DNA integrity was not investigated.

Fig. 5 (A) Dose-dependent effect of H_2O_2 on TA of spermatozoa after Percoll separation (0 h). The figure shows a representative result from three independent experiments. (B) Effect of H_2O_2 on TA of spermatozoa after 22 h incubation under capacitating conditions. The figure shows a representative result from three independent experiments. Concentrations are expressed as square roots. ^a $p < 0.001$ with respect to control (0 $\mu\text{mol/l}$ H_2O_2)

It has been reported that sperm *in vitro* manipulation, such as centrifuging, affects DNA integrity [6]. In the present study, motile sperm were separated by Percoll density gradient, which has been shown to cause oxidative damage to sperm DNA [6]. In a previous study, Hughes *et al.* [40] demonstrated that supplementation of Percoll with an antioxidant compound reduces the damage to sperm DNA. Taking into consideration the potential antioxidant effect of CM on sperm DNA, motile spermatozoa were separated by Percoll gradients in the presence or absence of CM. Separation procedure in the presence of CM did not reduce sperm DNA damage, suggesting that the protective effect of CM on sperm DNA did not result from a high antioxidant activity and, perhaps, it may require longer incubations times.

The present results indicate that CM was able to reduce the DNA damage associated with incubations of human spermatozoa, and also reduce the effect of an oxidant agent after 4 h incubation. The lack of effect of CM when added to Percoll gradients, together with the fact that the oxidative stress was applied in the absence of CM, suggests that CM could contribute to the stabilization of the sperm DNA structure rather than act as only a radical scavenger.

In another series of experiments, the effects of H₂O₂ on sperm DNA integrity before and after 22 h incubation under capacitating conditions was investigated. In non-capacitated sperm, treatment with increasing doses of H₂O₂ enhanced DNA damage in a dose-dependent manner. However, in agreement with previous studies, the treatment with the lowest dose of H₂O₂ (8.8 µmol/l) resulted in a lower DNA fragmentation than in control sperm [24, 36]. It was suggested that this effect is the result of protamine cross linkage, which completes the chromatin compaction process and thus, protects the sperm DNA from fragmentation [41].

The capacitation process, which occurs during sperm transit through the female genital tract, consists of a series of physiological changes that lead to the acquisition of sperm fertilizing ability [27]. This process is also carried out during human sperm *in vitro* incubation under specific conditions [28]. Thus, the effect of an oxidative agent on sperm DNA integrity after an overnight incubation under capacitating conditions was investigated. Because ROS are able to modulate the sperm capacitation process, the effect of H₂O₂ was investigated after sperm incubations. While the highest doses of H₂O₂ increased the TA, the TA of spermatozoa treated with a dose of 17.8 µmol/l of H₂O₂ was similar to controls, in contrast to that observed at *t*=0 h. It is likely that after 22 h capacitation, the sperm chromatin structure can be more compact, and thus, less likely to be affected by the oxidant treatment. Previous studies have described a H₂O₂ dose-dependent increase in sperm DNA fragmentation, but have used only short

periods of incubation (no more than 2.5 h) under capacitating conditions and, in addition, sperm were incubated in the presence of the oxidative agent during capacitation [24, 36].

Taking into account that the oviductal tissue CM increased the stabilization of the sperm DNA structure under culture conditions and considering that spermatozoa may stay in the oviduct from hours to days before they interact with the oocyte, it could be hypothesized that the oviductal secretion could contribute to the preservation of sperm DNA integrity until fertilization takes place.

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