ReProComet: A New In Vitro Method to Assess DNA Damage in Mammalian Sperm

Eugenia Cordelli, Anna Maria Fresegna, Alessia D’Alessio, Patrizia Eleuteri, Marcello Spanò, Francesca Pacchierotti and Paola Villani

BAS—Section of Toxicology and Biomedical Sciences, ENEA CR Casaccia, Via Anguillarese 301-00123, Rome, Italy

Received May 23, 2007; accepted July 23, 2007

The increasing request of chemical safety assessment demands for the validation of alternative methods to reduce the resort to animal experimentation. Methods that evaluate reproductive toxicity are among those requiring the largest use of animals. Presently, no validated in vitro alternative exists for the assessment of reproductive toxicity. Mammalian sperm are sensitive targets of DNA-reactive chemicals, which form premutagenic adducts. Here, we propose a new method based on comet assay to detect DNA damage induced by potential germ cell mutagens in bull sperm available from assisted reproduction practices. In somatic cells, chemical-induced adducts can be revealed by comet assay that detects DNA breaks produced during adduct repair. Mature sperm, however, are devoid of repair enzymes, and adducts are processed only after fertilization. For this reason, comet assay is not sensitive to detect DNA lesions induced in sperm by most chemicals. To overcome such limitation, we developed a modified comet assay based on the addition of a protein extract from HeLa cells to agarose-embedded sperm on microscopic slides. To test the method, sperm were treated in vitro with methyl methanesulfonate (MMS) or melphalan (MLP) and comet assay was conducted both with and without protein supplementation. No effect of MMS or MLP was detected without protein supplementation; on the contrary, a clear-cut dose-dependent effect was measured after addition of the cell extract. These results represent a proof of concept of a novel in vitro mutagenicity test on sperm that could offer a promising approach to complement previously validated in vivo germ cell genotoxicity assays.

Key Words: comet assay; reproductive toxicology; bull sperm; ReProTect; in vitro toxicity; DNA damage.

Economic and ethical issues associated with use of laboratory animals for chemical toxicity evaluation provide strong motivation for the development of alternative tests. Furthermore, the recently approved European legislation (http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm) increases the number of chemicals for which an in-depth risk assessment is required. The achievement of this aim could strongly impact on the use of animal resources. The methods to evaluate reproductive toxicity are among those requiring the largest use of animals. Presently, no validated in vitro alternative exists for the assessment of reproductive toxicity (Bremer et al., 2005). Epidemiological evidence links paternal exposure to chemical agents with an increased risk of pregnancy loss, developmental and morphological defects, infant mortality, infertility, and genetic diseases in the offspring, including cancer (Aitken et al., 2004). Furthermore, exposure of rodents to germ cell mutagens can have markedly adverse consequences on fertility and pregnancy outcomes, presumably by altering chromosome number or structure or through unrepaired DNA damage (Marchetti and Wyrobek, 2005). The sensitivity to mutation induction may be influenced by different factors such as the accessibility of DNA to mutagenic compounds, the interval between DNA damage and the next round of DNA synthesis, as well as the capability of DNA repair (Favor, 1999). The final stages of gamete differentiation in male mammals are sensitive targets of DNA-reactive chemicals (Sega and Owens, 1983); in fact, once germinal cells, deprived of the cytoplasm that houses protective enzymes, including those implicated in DNA repair, are released from germinal tissue they can no longer rely on the protection of Sertoli cells and are vulnerable to DNA damage by a variety of xenobiotics. Only the extremely condensed chromatin remains to represent a protective factor (Aitken et al., 2004; Marchetti and Wyrobek, 2005).

In the last decade, methods such as FISH, TUNEL, SCSA, and comet assay for sperm chromatin/DNA quality assessment have been improved and applied in different fields such as diagnosis of male infertility, occupational, pharmacological, epidemiological, and toxicological studies (Cordelli et al., 2005). Nevertheless, their application in in vitro detection of male germ cell mutagens is rather scanty and, in our knowledge, only the comet assay has been applied to detect oxidative DNA damage induced on spermatozoa by chemical agents (Anderson et al., 1997, 2003; Arabi, 2005). Another method to investigate the effect of mutagens on sperm is the interspecific in vitro fertilization, which allows the detection of cytogenetic damage on the first metaphase after fertilization (Kamiguchi and Tateno, 2002; Kamiguchi et al., 1995). This...
method, although informative, is difficult and expensive to be performed and only a few reports have been published.

In the present study, we propose a new method, based on a modification of the comet assay, to detect chemically induced DNA damage in bull sperm. Bovine sperm, available from assisted reproduction practices, has the advantage to be easily obtained through noninvasive means in sufficiently large amount to produce several aliquots from the same ejaculate. Furthermore, it can be stored frozen in liquid nitrogen for long period without losing its functional/structural properties. In somatic cells, chemically induced adducts can be revealed by comet assay. This assay detects breaks produced in the double helix during adduct repair by differential electrophoretic migration of broken versus unbroken DNA of single cells (Collins, 2004). Mature sperm, however, are essentially devoid of repair enzymes, and adducts are processed only after fertilization by oocyte enzymes that produce DNA breaks of repair enzymes, and adducts are processed only after fertilization by the oocyte enzymes that produce DNA breaks of repair enzymes, and adducts are processed only after fertilization by the oocyte enzymes.

Eventually leading to heritable genetic defects (Aitken et al., 2002), chemically induced adducts can be revealed by comet assay. This assay detects breaks produced in the double helix during adduct repair by differential electrophoretic migration of broken versus unbroken DNA of single cells (Collins, 2004). Mature sperm, however, are essentially devoid of repair enzymes, and adducts are processed only after fertilization by oocyte enzymes that produce DNA breaks of repair enzymes, and adducts are processed only after fertilization by the oocyte enzymes that produce DNA breaks of repair enzymes, and adducts are processed only after fertilization by the oocyte enzymes.

To overcome such limitation, we sought to develop a modified comet assay based on the addition of a crude protein extract from somatic cells (Repair Proficient Comet [ReProComet]). The use of cell extracts in comet assay has been previously proposed by Collins et al. (2001) and used to measure the capacity of human lymphocyte extracts to perform the initial steps of base and nucleotide excision repair (Langie et al., 2000) with the aim to assess the interindividual differences in DNA repair capability. Here, we propose the addition of HeLa cells protein extract to allow the detection of DNA damage induced in vitro on spermatozoa by DNA-reactive chemicals.

To test our working hypothesis, the sperm samples were treated in vitro with methyl methanesulfonate (MMS), a mono-functional alkylating agent, or melphalan (MLP), a bifunctional alkylating compound, and comet assay was conducted both with and without protein supplementation. Both chemicals are known to induce chromosomal aberrations, dominant lethal mutations, and heritable translocations in postmeiotic germ cells in mice (Ashby et al., 1996; Generoso et al., 1995; Russell et al., 1992; Vogel and Nivard, 1997).

**MATERIALS AND METHODS**

**Chemical treatment and slides preparation.** Bull semen, stored in paillels in liquid nitrogen, was purchased from Semen Italy (Modena, Italy). Samples were thawed at 37°C, diluted in PBS, divided in aliquots, and centrifuged to remove the cryoprotective medium. The pellets of spermatozoa were resuspended in PBS or PBS-containing chemicals and maintained for 120 min at room temperature in the dark. MMS (CAS 66-27-3) concentrations were 0.09, 0.22, 0.45, 0.9, and 2.2mM. MLP (CAS 148-82-3) concentrations were 0.01, 0.1, and 1mM. The chemicals were purchased from Sigma Aldrich (St Louis, MO).

**Preparation of cell extracts.** Cell extracts were prepared according to Frosina et al. (1999). Briefly, exponentially growing cells were harvested adding 10mM Na2EDTA in PBS and centrifuged. Pellets were resuspended (1 × 10⁹ cells/20 μl) in extraction buffer 1 (10mM Tris-HCl [pH 7.8], 200mM KCl, proteinase inhibitor [Roche, Basel, Switzerland]) and immediately extraction buffer 2 (10mM Tris-HCl [pH 7.8], 200mM KCl, 2mM Na2EDTA, 40% glycerol, 0.2% Nonidet P40, 2mM DTT, and proteinase inhibitor) was added.

Sperm viability. Sperm viability was assessed immediately after recovery from liquid nitrogen and after 2 h of treatment with solvent or tested chemicals at different concentrations by the SYBR-14/propidium iodide test (Molecular Probes, Eugene, OR) followed by flow cytometric analysis (Garner et al., 1997). Staining was performed according to the instructions provided by the supplier. Flow cytometric analysis was carried out using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Living spermatozoa are defined as those staining with SYBR-14.

**Cell culture.** HeLa cells were cultured in flasks in DMEM (Gibco, Milan, Italy) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 15 μg/ml Penicillin/Streptomycin (Sigma Aldrich). Cells were maintained at 37°C in a 5% CO₂ atmosphere.

**Standard comet assay.** The assay was performed essentially according to Cordelli et al. (2003). The slides were immersed in a lysis solution (2.5M NaCl, 100mM Na2EDTA, 10mM Tris, pH 10) containing 10% DMSO (Carlo Erba) and 1% Triton X-100 (Sigma Aldrich), overnight at 4°C. Slides were then immersed 30 min in 10mM DTT in lysis solution. The slides were placed in a horizontal gel electrophoresis tank with fresh alkaline electrophoresis buffer (300mM NaOH, 1mM Na2EDTA; HCl was added to reach pH 12.1) and left in the solution for 10 min at 4°C to allow the DNA to unwind. Electrophoresis was carried out at 4°C for 3 min, 25 V (0.8 V/cm) and 300 mA, using a Hoefer power supply (Hoefer Pharmacia Biotech Inc., San Francisco, CA). After electrophoresis, the slides were immersed in 0.3M sodium acetate in ethanol for 30 min. Microwells were then dehydrated in absolute ethanol for 2 h and immersed for 5 min in 70% ethanol. Slides were air-dried at room temperature. Immediately before scoring, slides were stained with 12 μg/ml ethidium bromide (Sigma Aldrich) and examined, at 200× magnification, with an Olympus fluorescent microscope. Slides were analyzed by a computerized image analysis system (Delta Sistemi, Rome, Italy). To evaluate the amount of DNA damage, computer-generated fraction of tail DNA, tail length, and tail moment values were used.

**Modified comet assay (ReProComet).** Slides were processed using the method described for the standard assay but after DTT treatment 100 μl of HeLa extract at the concentration of 5 mg/ml in nicking buffer (70mM HEPES pH 7.8, 10mM DTT, 3mM MgCl₂, 1mM EDTA) were added to each slide. Slides were incubated overnight at 37°C. At the end of incubation, slides were washed and underwent the same procedure of the standard assay.

To evaluate possible effects of the buffer, in one experiment, slides from samples treated with different concentrations of MMS or MLP were overnight exposed to the nicking buffer alone under the same conditions as those used for cell extracts.
To estimate the influence of preparation of cell extracts on the variability of results, the effects of three independently obtained cellular extracts on samples of sperm treated with 0.22mM MMS were compared.

**Statistical analysis.** Significance of differences was determined using one-tailed Student’s t-test for unpaired samples. Differences between control and treated samples were considered to be statistically significant if the probability of the difference being due to chance was less than 5% (\( p < 0.05 \)).

## RESULTS

Sperm viability, assessed by the SYBR-14/propidium iodide flow cytometric analysis, of thawed samples was about 30% and was not affected by 2 h MMS or MLP treatment (Table 1).

To verify if the overnight incubation of slides with the cellular extract could damage sperm DNA, because of the long stay at 37°C or the possible presence of endonucleases in the cellular extract, control values obtained with standard and modified comet assay were compared. Data expressed as fraction of tail DNA, tail length, and tail moment, reported in Figure 1, do not show a significant difference between the two methods.

In Figure 2, results obtained after treatment with different concentrations of MMS are reported. Standard comet assay did not detect any increase of DNA damage parameters. In contrast, a clear-cut MMS dose–dependent increase in DNA damage was measured after the addition of HeLa cell extract. The increase of tail length was statistically significant at all the MMS concentrations tested and significant increases of fraction of tail DNA and tail moment were observed from the concentration of 0.22mM. ReProComet data could not be obtained at the highest MMS concentration because sperm DNA was so damaged that the image analysis software was unable to calculate comet parameters. Results obtained after MLP treatment are reported in Figure 3. As expected, no effect of MLP was detected without protein supplementation, while the mean values of fraction of tail DNA, tail length, and tail moment obtained with ReProComet assay showed a statistical significant increase for all MLP concentrations tested.

### TABLE 1

<table>
<thead>
<tr>
<th>% SYBR-14 positive sperm (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Solvent</td>
</tr>
<tr>
<td>MMS 0.09mM</td>
</tr>
<tr>
<td>MMS 0.45mM</td>
</tr>
<tr>
<td>MMS 0.9mM</td>
</tr>
<tr>
<td>MMS 2.2mM</td>
</tr>
<tr>
<td>MLP 0.01mM</td>
</tr>
<tr>
<td>MLP 0.1mM</td>
</tr>
<tr>
<td>MLP 1mM</td>
</tr>
</tbody>
</table>

**FIG. 1.** (A) Fraction of tail DNA, (B) tail length, and (C) tail moment obtained with standard and ReProComet assay in untreated bull spermatozoa. Columns represent the mean values from at least three experiments.
show that a clear-cut, comparable effect of MMS could be detected with respect to the solvent-matched controls with all the extracts.

**DISCUSSION**

The elevated number of laboratory rodents used for chemical safety assessment, especially for reproductive toxicology, demands for the urgent development of alternative *in vitro* tests. In this context, the integrated project ReProTect, a consortium set up by the European Center for the Validation of Alternative Methods, aims at the development of a testing strategy in the area of reproductive toxicity (Hareng *et al.*, 2005). Due to the reproduction cycle complexity, it is not possible to model the whole cycle in one *in vitro* system capable of detecting effects of a chemical on all aspects of mammalian reproduction. For this reason, in the ReProTect project, the cycle was broken down into its biological components which were studied individually or in combination. In this context, our study aimed at developing an *in vitro* method to detect the impact of potential germ cell mutagens on sperm...
DNA. Experiments were conducted on bovine sperm, available from assisted reproduction practices and easily obtainable without invasive procedures for animals. A modification of comet assay was proposed and challenged by measuring DNA damage after in vitro treatment of spermatozoa with MMS and MLP, two well-known genotoxic agents.

Comet assay is a widely applied method to measure DNA damage in a variety of cell types, sperm included (Singh and Stephens, 1998; Tice et al., 2000). It has been applied in reproductive toxicological studies to evaluate the effects of physical and chemical agents on reproductive system on experimental animals (Cordelli et al., 2005) as well as in epidemiological studies to examine the effects of environmental chemicals, professional exposure, or lifestyle factors such as cigarette smoke on DNA integrity (Belcheva et al., 2004; Duty et al., 2003; Hauser et al., 2003; Migliore et al., 2002; Xu et al., 2003). Concerning its application in in vitro reproductive toxicology, free radical–mediated DNA lesions were detected on mammalian sperm after treatment with different chemical and physical agents (Anderson et al., 1997, 2003; Arabi, 2005; Dobrzynska et al., 2004; Hughes et al., 1999; Singh and Stephens, 1998). However, most chemicals do not interact with DNA through the production of free radicals but bind covalently to nucleophilic sites on bases. These alterations are then removed by DNA repair machinery which produces transient abasic sites and breaks on DNA. Due to the absence of repair capabilities of spermatozoa (Marchetti and Wyrobek, 2005), these lesions are not produced and the damage remains undetectable by comet assay in these cells. To overcome this limitation, we have modified the comet assay by adding, to agarose-embedded sperm, HeLa crude cellular extract with the assumption that repair enzymes contained in the extract could initiate the incision process and produce DNA-strand breaks. Purified repair enzymes can be included in the comet assay and are used to detect specific DNA lesions such as oxidized or alkylated bases (Collins, 2004; Gedik and Collins, 2005). Nevertheless, the specificity toward a particular kind of lesion makes these enzymes unfit for the screening of compounds with unknown mechanisms of action. The capacity of a crude cellular extract to recognize and incise chemically modified DNA of agarose-embedded nuclei had been previously demonstrated and applied to evaluate interindividual differences in DNA repair (Collins et al., 2001; Langie et al., 2006). Nevertheless, its use to supplement sperm repair enzymes deficiency in the assessment of chemically induced DNA lesions has not been described yet.
Most repair genes are evolutionary conserved (Resnick and Cox, 2000), thus it was to be expected that human proteins could supplement the bull sperm repair deficiency, but the capacity of repair enzymes to nick DNA associated with protamines and not organized in nucleosomes was not obvious. In the present study, we showed that a crude cellular extract from human HeLa cells, when added to the comet assay, resulted in detection of DNA breaks presumably produced at sites of MMS or MLP alkylation of DNA. These results suggest that enzymes, presumably DNA repair enzymes, in the HeLa cell extract recognized the adducts and initiated repair, with the result that DNA-strand breaks were produced and then measured by the comet assay.

To verify whether the overnight incubation of agar-embedded sperm with protein extract could alter comet parameters, possibly due to a nonspecific endonuclease activity in cellular extract, fraction of tail DNA, tail length, and tail moment obtained in control samples with standard comet and ReProComet protocols were compared. Results obtained on control samples with the two protocols did not differ significantly. This suggests also that the level of endogenously damaged bases in the experimental sperm samples was small and not detectable. On the contrary, the incubation of MMS- or MLP-treated sperm with protein extracts produced DNA breaks clearly detected by the increase of comet parameters. DNA lesions produced by the same concentrations of chemicals were not detectable either by the standard comet assay or by the addition of the nicking buffer alone.

In particular, MMS treatment induced a dose-dependent increase of all comet parameters. A comparable sensitivity had been found in somatic cells as shown by the increase of comet parameters after in vitro treatment with 0.05mM MMS in human lymphocytes (Baohong et al., 2005) and 0.1mM in L5178Y mouse lymphoma cells (Miyamae et al., 1998). The genotoxicity of MMS on germ cells is well known and described in several in vivo studies reporting the induction of chromosomal aberrations and chromatin alterations in sperm as well as dominant lethal mutations, specific-locus mutations, and heritable translocations (Ehling and Neuhauer-Klaus, 1990; Evenson et al., 1993; Marchetti and Wyrobek, 2005). Clastogenic effects of MMS were also demonstrated on human and bull sperm after in vitro treatment by interspecific fertilization with zona-free hamster oocytes and cytogentic analysis. Applying this method, difficult and expensive to perform, a clastogenic effect could be detected only at a MMS concentration of 100 µg/ml (corresponding to 0.9mM) (Kamiguchi and Tateno, 2002; Kamiguchi et al., 1995). We could detect the induction of DNA lesions at 10 times lower MMS concentration. Although comet assay and interspecific fertilization are not directly comparable because the first method detects premutagenic lesions which could be repaired after fertilization, while the other method reveals irreversible chromosomal alterations, our results suggest that ReProComet could be a sensitive screening assay to demonstrate and quantify the capacity of chemicals to interact with and modify sperm DNA. At the moment, no data are available in the literature to evaluate ReProComet sensitivity in relation to other methods, such as SCSA or TUNEL, detecting DNA/chromatin alterations induced in sperm after in vitro treatment with MMS. A relatively modest effect was shown by SCSA (Evenson et al., 1993) 1 day after treatment of mice with MMS, while a much larger response was shown between 3 and 13 days after treatment, suggesting that this method could be more suitable to detect alterations induced in maturing than in mature sperm.

MLP is a direct-acting alkylating substance used as a therapeutic agent. Its mutagenicity is related to its ability to

![FIG. 5. (A) Fraction of tail DNA, (B) tail length, and (C) tail moment obtained with three different cellular extracts after treatment with solvent or 0.22mM MMS.](image-url)
produce monoadducts and to form DNA intra- and interstrand cross-links via the two chloroethyl groups of the molecule (Cordelli et al., 2004; Povirk and Shuker, 1994). It is a potent inducer of chromosomal aberrations and micronuclei in bone marrow cells and it is known to be carcinogenic in rats, mice, and humans (IARC, 1987; Shelby et al., 1989). It has been shown that in vivo treatment of male rodents with MLP induces dominant lethal mutations, heritable translocations, and specific-locus mutations (Generoso et al., 1995). No studies regarding the in vitro genotoxicity on spermatozoa have been reported yet. Results obtained applying ReProComet showed a significant increase of comet parameters at all the concentrations tested.

The results described in the paper represent a proof of concept that cellular extracts added to spermatozoa can produce comet assay–detectable DNA-strand breaks presumably resulting from an enzymatic incision of DNA at sites of MMS or MPL alkylation. Although we did not show the actual formation of adducts on sperm DNA, literature (Sega and Owens, 1983) reports that treatment of mice with MMS induces DNA alkylation in mature sperm. To further develop this approach into an in vitro test for reproductive toxicity assessment, potentially useful to reduce the resort to animal experimentation, several issues must be addressed. In particular, the reproducibility of cellular extract is critical for the test standardization. To start exploring this issue, we showed that independently obtained cellular extracts yielded comparable results. A biochemical characterization of the extracts and a comparison between the performances of extracts from cell lines proficient and deficient for specific repair pathways could support the biological bases of the assay. Also, the test will have to be characterized in terms of sensitivity by testing weak and strong mutagens and specificity by testing toxicants with weak genotoxicity on spermatozoa using the comet assay in vitro. Mutat. Res. 578, 149–157.


FUNDING


REFERENCES


