Sperm DNA fragmentation induced by DNase I and hydrogen peroxide: an *in vitro* comparative study among different mammalian species

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Abstract

Sperm DNA damage may have adverse effects on reproductive outcome. Sperm DNA breaks can be detected by several tests, which evaluate DNA integrity from different and complementary perspectives and offer a new class of biomarkers of the male reproductive function and of its possible impairment after environmental exposure. The remodeling of sperm chromatin produces an extremely condensed nuclear structure protecting the nuclear genome from adverse environments. This nuclear remodeling is species specific, and differences in chromatin structure may lead to a dissimilar DNA susceptibility to mutagens among species. In this study, the capacity of the comet assay in its two variants (alkaline and neutral) to detect DNA/chromatin integrity has been evaluated in human, mouse, and bull sperm. The hypothesis that chromatin packaging might influence the amount of induced and detectable DNA damage was tested by treating sperm *in vitro* with DNase I, whose activity is strictly dependent upon its DNA accessibility. Furthermore, hydrogen peroxide (H₂O₂) was used to assess whether spermatozoa of the three species showed a different sensitivity to oxidative stress. DNase I-induced damage was also assessed by the sperm chromatin structure assay and the TUNEL assay, and the performances of these two assays were compared and correlated with the comet assay results. Results showed a different sensitivity to DNase I treatment among the species with human sperm resulting the most susceptible. On the contrary, no major differences among species were observed after H₂O₂ treatment. Furthermore, the three tests show a good correlation in revealing sperm with DNA strand breaks.

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Introduction

Sperm DNA integrity is considered pivotal for normal fertilization and transmission of paternal genetic information. Unspecific sperm DNA breaks can be conveniently detected by a variety of *in situ* tests, the most common being the single cell gel electrophoresis or comet assay (Singh *et al.* 1989, Speit *et al.* 2009), the TUNEL assay (Gorczyca *et al.* 1993), and the sperm chromatin structure assay (SCSA; Evenson *et al.* 1980, 2002). Their application has shown that sperm DNA (and chromatin) damage can be associated with reduced rates of fertilization *in vivo*, by natural and assisted conception (Spano *et al.* 2000, Bungum *et al.* 2007). Their predictive value for spontaneous abortions, malformations, developmental defects, or chromosomal damage in offspring is under active investigation (Lewis & Agbaje 2008, Sakkas & Alvarez 2010). These tests have been demonstrated to be also useful in epidemiology studies aimed at investigating the reproductive impact of environmental or occupational compounds and in

experimental toxicology trials to evaluate the reprotoxicity of physical–chemical noxae (Evenson & Wixon 2005, Rignell-Hydbom *et al.* 2005, Spano *et al.* 2005, Bennetts *et al.* 2008, Perry 2008, Delbès *et al.* 2010). In spite of the multiple applications of these tests in different fields and the efforts carried out to improve their sensitivity (Mitchell *et al.* 2010), further studies to fully understand their significance, sensitivity, and correlation seem necessary (Barratt *et al.* 2010).

The structural remodeling of sperm chromatin is species specific, and contributes to the protection of the nuclear genome of these DNA repair-deprived cells from the adverse environments to which they might be exposed before fertilization. During spermatogenesis, the chromatin of mammalian spermatozoa undergoes a dramatic reorganization, as histones are replaced by protamines. In mature sperm cells, this remodeled genomic architecture maintains the DNA in a highly compact and transcriptionally inactive state pending eventual fertilization of an oocyte (Braun 2001, Dadoune 2003, Oliva 2006). Protamines are cysteine-rich molecules

that locate to the minor groove of the sperm DNA and become extensively cross-linked by disulfide bridges during epididymal transit in order to confer stability upon such an extremely compressed nuclear structure. There are two protamine families, protamine 1 and protamine 2, the relative proportion of which is different among species. Within a species, both the total protamine concentration and their relative proportion are related to sperm DNA fragmentation and infertility (Aoki *et al.* 2005a, 2005b), an observation that highlights the significance of chromatin compaction as a key factor in its vulnerability to damage. Finally, several mammalian species retain a nucleohistone component within their sperm chromatin (Corzett *et al.* 2002, Dadoune 2003, Wykes & Krawetz 2003, Miller *et al.* 2010). The retained nucleosomes might be due to inefficient protamine replacement or, as recent research suggests, sperm histones could be associated with specific sequences important for embryo development (Zalenskaya *et al.* 2000, Nazarov *et al.* 2008, Arpanahi *et al.* 2009, Barroso *et al.* 2009, Hammoud *et al.* 2009, Miller *et al.* 2010). These differences in chromatin structure may determine differences in sperm DNA damage susceptibility to a given stressor (Bennetts & Aitken 2005) and, in turn, can also impact on the results obtained after using a particular sperm DNA fragmentation assay (Mitchell *et al.* 2010).

The evaluation of chromatin integrity is important not only in human clinical and toxicological studies but also in species used in laboratory toxicological studies, such as small rodents (Traina *et al.* 2003, Speit *et al.* 2009), and to assess sperm quality in animals selected for assisted reproduction practice in zootechny (Rybar *et al.* 2009).

In this study, the capacity of the alkaline and neutral comet assay to detect DNA/chromatin integrity has been evaluated in human, mouse, and bull spermatozoa. The relative amount of proteins associated with sperm chromatin is different in these species, bull sperm have only protamine 1 while the proportion of protamine 2 is 67 and 34% in human and mouse spermatozoa respectively. In addition, some nucleosomal structure is retained in human (up to 15%) and, in smaller quantity, in mouse spermatozoa (Pittoggi *et al.* 1999, Wykes & Krawetz 2003). DNA lesions were induced by treating spermatozoa *in vitro* with two compounds with different and well-known characteristics and mechanisms of action. DNase I is an endonuclease cleaving DNA phosphodiester bonds, thus inducing DNA strand breaks. The enzyme digests DNA with no sequence specificity, but its action in nuclei is dependent on the accessibility of chromatin and, for this reason, it has been used to study the organization of chromatin in somatic cells (Pan & Lazarus 1997, Staynov 2000, 2008). The effects of the DNase I treatment on the sperm from the three different species have also been assessed applying the SCSA and the TUNEL assay by flow

cytometry (FCM). The other compound tested was hydrogen peroxide (H_2O_2), a small molecule inducing DNA lesions through the generation of oxygen free radicals. Both the sugar and the base moieties are susceptible to oxidation, causing predominantly single-strand breaks (SSBs) and oxidative base damage, while double-strand breaks (DSBs) are considered to be rare events (Rueff *et al.* 1993).

Results

The amount of DNA lesions induced by different concentrations of DNase I has been evaluated by alkaline and neutral comet assay in human, bull, and mouse spermatozoa. Results, expressed as mean fraction of tail DNA and percentage of damaged cells, are reported in Fig. 1. Dose-response relationships obtained with alkaline and neutral comet assays were similar, suggesting a prevalence of DSBs induced by DNase I. Dose-effect relationships were different for the three species analyzed. Human spermatozoa, which showed a higher level of baseline lesions than the other species (in alkaline comet assay mean fraction tail DNA was 0.062 in humans and 0.023 and 0.019 in bull and mouse respectively), resulted the most sensitive, exhibiting a significant increase of comet parameters from the lowest dose tested. The curve reached a plateau and no further increase in DNA migration was observed from the concentration of 2 U/ml of DNase I onwards ($P < 0.001$ for all parameters). Bull spermatozoa showed a clear dose-dependent increase in comet parameters significant from the concentration of 100 U/ml of DNase I

Figure 1 Effects of DNase I treatment in human, bull, and mouse spermatozoa evaluated by alkaline (A and B) and neutral (C and D) comet assay. Columns represent the mean values of fraction of tail DNA (A and C) and the percentages of damaged cells (B and D) obtained in at least four independent experiments. Symbols show statistical difference from the relative controls (# $P \leq 0.05$; \$ $P \leq 0.01$; * $P \leq 0.001$).

that induced DNA damage in about 60% of spermatozoa ($P=0.01$ and $P=0.027$ for alkaline and neutral comet assay respectively). Enhancing the dose, the mean level of damage increased ($P<0.001$) and damaged cells reached 100% ($P<0.001$). Mouse spermatozoa were extremely resistant to the endonucleasic treatment not showing an increase in comet parameters but at the highest dose, which induced a high level of damage in 100% of spermatozoa ($P<0.001$ for all the parameter analyzed).

The outcome of DNase I treatment on sperm DNA was also evaluated by TUNEL assay and by SCSA, and results, expressed as percentages of damaged cells, are reported in Figs 2 and 3 respectively. Also processed by these techniques, human spermatozoa resulted more sensitive to DNase I treatment than spermatozoa from the other two species, showing a significant increase in percentage of damaged cells at all the doses tested. In mouse, only the highest concentration of DNase I induced a significant increase in damaged spermatozoa. As for comet assay at 1000 U/ml, TUNEL assay revealed that almost all cells were damaged ($P<0.001$), while only 9% of cells were positive analyzed by SCSA ($P=0.011$).

Percentages of damaged cells observed with the different techniques were compared and are reported in Fig. 4. A significant correlation was observed between the four methods as shown by the high values of Pearson's correlation coefficients (R). The slope of the linear regressions suggests a similar sensitivity of the methods in detecting damaged cells; only in mouse, comparing SCSA with the other techniques, the slope of regression lines indicated that only a fraction of damaged sperm could be revealed by this method.

Alkaline and neutral comet assays were used to assess DNA damage induced by different concentrations of H_2O_2 , and data obtained are reported in Fig. 5. As shown

Figure 2 Effects of DNase I treatment in human, bull, and mouse spermatozoa evaluated by TUNEL assay. Columns represent the percentages of positive cells obtained in at least four independent experiments. Symbols show statistical difference from the relative controls ($^{\#}P\leq 0.05$; $^*P\leq 0.001$).

Figure 3 Effects of DNase I treatment in human, bull, and mouse spermatozoa evaluated by SCSA. Columns represent the percentages of DFI obtained in at least four independent experiments. Symbols show statistical difference from the relative controls ($^{\#}P\leq 0.05$; $^{\$}P\leq 0.01$; $^*P\leq 0.001$).

by the histograms, differently from DNase I treatment, the amount of DNA lesions induced by H_2O_2 was similar in the different species. The alkaline comet assay gave a dose-dependent increase in the mean values of fraction tail DNA, while this parameter was not enhanced using the neutral protocol, reinforcing the notion that H_2O_2 prevalently induces DNA SSBs.

Discussion

The tightly packaged structure of chromatin confers to mammalian sperm a protection from the effect of genotoxic factors. Nevertheless, in humans, a fraction of spermatozoa has a relative high degree of DNA fragmentation. This damage can derive from aberrant chromatin packaging during spermatogenesis, defective apoptosis, or excessive production of reactive oxygen species in the ejaculate. Furthermore, DNA damage can be induced by environmental exposure to genotoxic agents whose efficiency depends also on the accessibility of DNA (Bennetts & Aitken 2005, Chohan *et al.* 2006, Perry 2008, Sakkas & Alvarez 2010). The proposed mechanisms are obviously not mutually exclusive and, recently, a two-step hypothesis has been put forward where faulty spermatogenesis can easily lead to defective chromatin remodeling where regions of underprotaminated DNA are more susceptible and vulnerable to a variety of stressors (Aitken *et al.* 2009, Aitken & De Iuliis 2010).

Recently, DNase I digestion was used to characterize the sequences associated with nucleosomal structures in sperm chromatin, exploiting its ability to preferentially attack chromatin that is in an open conformation (Arpanahi *et al.* 2009). In our paper, the hypothesis that chromatin packaging might influence the amount of induced DNA damage was tested by treating sperm

Figure 4 Relationships between the percentages of sperm with damaged DNA obtained with the different assays in human, bull, and mouse spermatozoa.

in vitro with DNase I. Our results with comet assay showed that although in all species at the highest dose tested all sperm were damaged, the amount of migrated DNA did not exceed 60%. This finding could be in accordance with a model for sperm chromatin structure, in which DNA is organized with protamines into toroids connected by more relaxed toroid linker regions. These regions could be more accessible to DNase I. At higher concentrations, DNase I cleaves also the chromatin fibers that are on the surface of the protamine toroid while the chromatin fibers inside the toroid are completely covered by neighboring protamine–DNA strands and are totally inaccessible to any exogenous protein (Sotolongo *et al.* 2003, 2005, Ward 2010). This could explain that not all the DNA is digested by the enzyme, and not all the DNA can exit from the nucleoid during electrophoresis. Furthermore, results revealed a different sensitivity among the three species tested, with human spermatozoa being the most sensitive to DNase I treatment suggesting that the bulky molecule of DNase I could better reach and digest the human sperm DNA. In sperm, the ratio between histones and protamines differs among species, and the characteristics of the protamines also vary from species to species (Wykes & Krawetz 2003, Miller *et al.* 2010, Ward 2010). It has been demonstrated that

histone-bound DNA of somatic cells is much more sensitive to DNase I than protamine-bound chromatin (Sotolongo *et al.* 2003, Arpanahi *et al.* 2009). Taking into account that in human sperm, about 15% of the DNA remains associated with histones as compared with ~1% in mouse and bull, which show a similar total protamine mass to DNA mass ratio (Palmer *et al.* 1991, Bench *et al.* 1996, Miller *et al.* 2010), this could explain the higher sensitivity of this species with respect to the others.

It has been proposed that oxidative stress constitutes one of the mechanisms for creation of DNA damage in sperm, and that in humans, defective chromatin remodeling renders sperm particularly susceptible to oxidative attack (Aitken & De Iuliis 2010). To investigate whether sperm with dissimilar histone to protamine ratio had a different sensitivity, in this study, alkaline and neutral comet assays were applied to detect DNA lesions induced by H₂O₂ in human, bull and mouse spermatozoa. No major differences among species were observed after H₂O₂ treatment indicating that the differences in chromatin compactness do not impact on the capacity of this small molecule to reach and interact with DNA. A previous study (Bennetts & Aitken 2005) found a marked vulnerability to oxidative stress in marsupial spermatozoa, attributable to the extremely relaxed chromatin due to the lack of disulfide cross-linking in

Figure 5 Effects of H_2O_2 treatment in human, bull, and mouse spermatozoa evaluated by alkaline (A) and neutral (B) comet assay. Columns represent the mean values of fraction of tail DNA obtained in at least four independent experiments. Symbols show statistical difference from the relative controls (# $P \leq 0.05$; * $P \leq 0.001$).

their sperm chromatin with respect to sperm of eutherian species. They also found a slight higher sensitivity to H_2O_2 of human than mouse spermatozoa. The discrepancies between these and our results could be attributable to the different techniques used and to the different range of concentrations used.

Our results show that only the alkaline electrophoretic conditions could detect H_2O_2 -induced DNA damage. Recently, some controversies arose regarding the kind of DNA breaks the neutral and alkaline version of comet assay could detect (Collins *et al.* 2008, Speit *et al.* 2009). Since H_2O_2 -induced DSBs are negligible with respect to SSB, our results showing no DNA migration under neutral electrophoretic conditions even after extremely high concentrations, adding further evidence to the hypothesis that SSBs are not detected under non-denaturing conditions. Our alkaline comet assay, conducted under pH 12.1 unwinding and electrophoretic conditions, showed a lower sensitivity than that reported by other authors at $\text{pH} \geq 13$ (Hughes *et al.* 1996, Bennetts *et al.* 2008) reflecting the contribution of

H_2O_2 -induced oxidized bases, which could be revealed as alkaline labile sites only at $\text{pH} \geq 13$.

In the present work, further to neutral and alkaline comet assay, we applied TUNEL assay and SCSA to detect DNase I-induced DNA lesions. Comet assay detects DNA strand breaks exploiting the electrophoretic mobilization of DNA fragments from nuclei after chromatin decondensation obtained by high salt and disulfide-reducing agents treatment (Speit *et al.* 2009). TUNEL assay relies on the enzymatic addition of labeled nucleotides to an end of a break. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3'-hydroxyl ends of double- or single-stranded DNA generates DNA strands with exposed 3'-hydroxyl ends. The amount of SSB- and DSBs is analyzed on single cells by FCM (Sakkas *et al.* 2002). SCSA detects the susceptibility of sperm chromatin to acid denaturation. In this technique, sperm are incubated in suspension with a mild acid solution under the hypothesis that only the fragmented DNA is denatured. Cells are stained with acridine orange, which emit a red fluorescence when bound to single-stranded DNA and a green fluorescence when intercalated into the double helix, and analyzed by FCM (Spano & Evenson 1993). The comparisons of results obtained with the different techniques show a good correlation among the tests in revealing sperm with DNA strand breaks induced by DNase I. Furthermore, the comparable results obtained with alkaline and neutral comet assay were in agreement with a prevalence of DSBs induced by DNase I. The slope of linear regressions indicates that the same amount of damaged cells is revealed by methods detecting DNA strand breaks (alkaline and neutral comet assay and TUNEL assay). On the contrary, when comparing comet and TUNEL assays with SCSA, especially in the case of mouse sperm, only a small percentage of damaged sperm were also SCSA positive. It must be considered that SCSA does not directly measure DNA breaks but the susceptibility of sperm to acid-induced DNA denaturation. It is likely that the susceptibility to acidic denaturation does not rely only on the amount of DNA strand breaks but also on their relative position within the chromatin: it could be speculated that mouse chromatin has such a structure that its DNase I-accessible sites are distributed in a way that breaks do not produce enough chromatin relaxation to make DNA prone to acidic denaturation.

The need of a further validation and standardization of methods to assess sperm DNA integrity before their application in clinical, epidemiological, and toxicological research has been recently stressed (Barratt *et al.* 2010). In particular, reference control samples seem essential to estimate intra- and inter-laboratory variability and to understand the findings obtained with the different techniques. Our results suggest that DNase I

could be useful for evaluating and comparing the performance of different techniques in their capacity to pick up sperm DNA breaks. Furthermore, the different responses obtained in the tested species underline that species-specific differences in sperm chromatin structure should be considered when extrapolating results from experimental animal studies to human risk assessment, especially in the case of bulky molecules. Finally, as the histone to protamine ratio seems to be associated with fertility potential in humans (Carrell *et al.* 2007), further investigation could address the inter-individual sensitivity to DNase I digestion in sperm from normal donors and infertile patients, and evaluate the predictive power of this feature for infertility diagnosis.

Materials and Methods

Samples

Human sperm was collected, after informed consent, from ten healthy, normospermic volunteers, pooled, aliquoted, and, without further manipulation, immediately frozen in cryotubes, and placed at -80°C (Spano *et al.* 2000). Bull sperm: cryopreserved aliquotes were purchased from Semenitaly (Modena, Italy) and maintained in liquid nitrogen. Mouse epididymal spermatozoa were sampled from 3-month-old C57/Bl6 mice. After killing, sperm collected from cauda epididymis were suspended in TNE buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM Na_2EDTA , pH 7.4), centrifuged, resuspended in TNE buffer plus 10% glycerol, aliquoted, and frozen at -80°C (Cordelli *et al.* 2003). The experiments with mice were approved by the Institutional Animal Care and Use Committee and officially authorized by the National Ministry of Health.

Treatment

Semen samples were thawed at room temperature, washed in PBS, and treated as follows.

DNase I treatment

Pellets were resuspended for 2 min in a permeabilizing solution (0.1% sodium citrate, 0.1% Triton X-100) and treated for 30 min at 37°C with different concentrations of DNase I (2000 U/mg, Roche Diagnostics) in PBS + 5 mM MgCl_2 . DNase I concentrations were 0, 1, 2, 10, 100, and 1000 U/ml.

H_2O_2 treatment

Samples were treated for 1 h at 37°C with different concentrations of H_2O_2 (Sigma-Aldrich). H_2O_2 concentrations were 0, 0.1, 1, 10, and 100 mM in PBS. At the end of the treatment, samples were washed in PBS and analyzed by the different techniques.

Comet assay

Four slides were prepared from each sample, two for the alkaline comet assay (pH 12.1) and the other for the neutral

comet assay (pH 8.0). The assay was performed essentially according to Cordelli *et al.* (2003). The slides were immersed in a lysing solution (2.5 M NaCl, 100 mM Na_2EDTA , 10 mM Tris, pH 10) containing 10% DMSO (Carlo Erba, Milan, Italy) and 1% Triton X-100 (Sigma), overnight at 4°C . Slides were then immersed 30 min in 10 mM dithiothreitol (Sigma) in lysis solution. Slides were placed in a horizontal gel electrophoresis tank (Starlab, Milan, Italy) with fresh electrophoresis buffer. Conditions for alkaline comet assay were as follows: DNA denaturation: 10 min at 4°C in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA ; HCl was added to reach pH 12.1). Electrophoresis: 5 min, 27 V (0.8 V/cm), 300 mA at 4°C . Conditions for neutral comet assay were: equilibration: 20 min in TBE buffer (2 mM Na_2EDTA , 90 mM Tris, and 90 mM boric acid; pH 8) at 4°C . Electrophoresis: 5 min, 27 V (0.8 V/cm), 10 mA at 4°C .

After electrophoresis, the slides were immersed in 0.3 M sodium acetate in ethanol for 30 min. Microgels 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 $\mu\text{g}/\text{ml}$ ethidium bromide (Sigma) and examined, at 200 $\times$ 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, 100 cells were analysed from two different slides, and computer-generated fraction of tail DNA values were used. To quantify the percentage of sperm with abnormal DNA, a cutoff of 10% tail DNA was used to discriminate undamaged and damaged cells.

Flow cytometric SCSA

Sperm cells were prepared and stained according to the procedure described in Spano *et al.* (2005). Briefly, aliquots (0.2 ml) containing $(1-2) \times 10^6$ cells were mixed with 0.4 ml acid-detergent solution (0.1% Triton X-100, 0.15 M NaCl, and 0.08 M HCl, pH 1.2). After 30 s, the cells were stained by adding 1.2 ml of a solution containing 6 $\mu\text{g}/\text{ml}$ of chromatographically purified acridine orange (AO; Molecular Probes, Eugene, OR, USA) in staining buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 , 1 mM EDTA, 0.15 M NaCl, pH 6.0). When excited with blue light, AO intercalated into double-stranded DNA fluoresces green. AO associated with single-stranded nucleic acids emits red fluorescence. Sperm abnormal chromatin structure, here defined as an increased susceptibility to induced denaturation, is FCM measured, on cell-by-cell basis, in terms of green (native DNA) to red (denatured, single-stranded DNA) shift. AO fluorescence intensity shift is described using the index called DNA fragmentation index (DFI) representing the ratio of red to total (red+green) fluorescence. Normal, native chromatin remains structurally sound and produces a narrow DFI distribution. DNA in sperm with abnormal chromatin structure has increased red fluorescence, which yields an altered DFI distribution. Cells were analysed by BD FACSCalibur flow cytometer (Becton Dickinson, San José, CA, USA). Green fluorescence was collected after a 530 ± 30 nm band pass filter and red fluorescence after a LP620 long pass filter. The two colors were splitted by a 560 nm dichroic filter. Data were stored in list-mode at 10-bit

resolution. A total of 1×10^4 events were accumulated for each measurement. For uniformity, recorded measurements were begun 3 min after staining. Measurement rate was about 200 cells/s. Cytogram and DFI analyses were carried out on the list-mode data by using the SCSASoft software (SCSA Diagnostics, Brooking, SD, USA).

Flow cytometric in situ nick end labeling (TUNEL) assay

TUNEL labeling was carried out using the *In Situ* Cell Death Detection Kit Fluorescein (Roche Diagnostics) and was performed according to the manufacturer's instructions. Briefly, samples were centrifuged (500 g for 6 min), and the pellet was resuspended in PBS with 1% (w/v) BSA (pH 7.4) to a final concentration of 1.5×10^6 cells/100 μ l. One hundred microlitres of 1% paraformaldehyde was added, and samples were shaken for 1 h at 15–25 °C. After fixation, the cells were washed once, resuspended in 1 ml PBS/1% BSA, and divided into two aliquots (negative control and test sample). After centrifugation, test samples were resuspended in 50- μ l reaction mix containing 45- μ l labeling solution (supplied with the *in situ* Cell Death Detection Kit) and 5- μ l TdT. The negative controls were suspended in the labeling solution without TdT. Samples were incubated for 1 h at 37 °C in the darkness. At the end of incubation, cells were washed twice and resuspended in PBS containing 5 μ g/ml propidium iodide. Cells were analysed by BD FACSCalibur flow cytometer (Becton Dickinson). Green fluorescence was collected after a 530 ± 30 nm band pass filter and red fluorescence after a LP620 long pass filter. A total of 1×10^4 events were accumulated for each measurement. A marker was set in the TUNEL dot plot at the borderline of background signals, as determined from the negative sample; the same marker was maintained in all plots to quantify the percentage of positive cells.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Inc., Chicago, IL, USA). Comparison between group means was performed by one-way ANOVA, and Dunnett's test was used for *post-hoc* comparison of treatments to controls. Comparison between the percentages of damaged cells observed with the different techniques was performed by two-tailed Pearson's correlation. For all experiments, a *P* value of < 0.05 was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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