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SHORT COMMUNICATION

Shortening of alkaline DNA unwinding time does not interfere with detecting DNA damage to mouse and human spermatozoa in the comet assay

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The comet assay was performed on mouse and human spermatozoa to examine the effect of alkaline DNA unwinding time. The spermatozoa were treated *in vitro* with the DNA-damaging agents, methyl methanesulfonate (MMS) or hydrogen peroxide (H_2O_2), and then embedded in agarose gel on glass slides. The slides were immersed in alkaline solution (>pH 13) for 1, 5, 10 and 20 min, and then subjected to the electrophoresis under neutral conditions. In mouse spermatozoa, comet tails seen in solvent controls became brighter and longer as the alkaline DNA unwinding time increased. However, in the MMS-treated mouse spermatozoa, a smaller difference in the damage from that in the solvent control was seen with time within a dose. DNA damage induced by H_2O_2 could also be detected accurately after alkali treatment for 1–20 min. In human spermatozoa, DNA damage induced by MMS and H_2O_2 could be detected in a dose-dependent manner after alkali treatment for 1 min. The ability of the comet assay to detect DNA damage was not adversely affected by the short period (1 min) of the alkaline DNA unwinding time.
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INTRODUCTION

Single-cell gel electrophoresis assay (the comet assay) is a simple and high-throughput technique to detect the cellular DNA damage induced by various types of genotoxicants *in situ*. The comet assay is performed in one of two versions, alkaline and neutral. Alkaline comet assays visualize single- and double-strand breaks in cellular DNA, whereas the neutral comet assay reveals mainly double-strand breaks. Nowadays, the alkaline comet assay of reproductive cells has been recommended to screen genotoxic hazards. Furthermore, it is more practical in larger studies to evaluate the DNA integrity of cryopreserved mammalian spermatozoa.[1]

The standard alkaline comet assay includes a step of alkali treatment to unwind the DNA before electrophoresis under the alkaline condition. In another version of the assay, cells embedded in agarose gel are treated with an alkali (>pH 13) followed by electrophoresis under neutral conditions. This protocol is named the 'A/N protocol'.[2] Lower background levels of DNA damage and better dose responses are obtained by the A/N protocol.[2–4]

Recently, the comet assay A/N protocol has been reported for murine spermatozoa.[5–7] In most cases, the alkaline DNA unwinding time is set at 20 min or more. To our knowledge, there is little information on the optimal time of alkali treatment to detect sperm DNA damage with the A/N protocol. In the present study, the optimal time of the alkali treatment was determined for mouse spermatozoa by the comet assay with A/N protocol.

MATERIALS AND METHODS

Animals

Hybrid ($B6D2F_1$) male mice (7–12 weeks of age) were used in this study. The animals were maintained under a 14-h light/10-h dark photoperiod at a temperature of 22–24 °C. All experiments were performed according to the Guidelines for Animal Experiments of Asahikawa Medical University (Asahikawa, Japan).

Collection and treatment of mouse spermatozoa

Dense masses of mature spermatozoa were collected from the cauda epididymidis and placed at the bottom of a 1.5-ml polypropylene microcentrifuge tube containing 1.2 ml of Toyoda–Yokoyama–Hosi medium.[8] The tube was left standing for 10 min at 37 °C to allow the spermatozoa to disperse by swimming into the medium. The spermatozoa were treated with 12.5–100 μg ml^{-1} methyl methanesulfonate (MMS) or 25–100 μmol l^{-1} hydrogen peroxide (H_2O_2; Nacalai Tesque, Kyoto, Japan) for 2 h at 37 °C under 5% CO_2 in air. MMS or H_2O_2 were dissolved in distilled water and added to the sperm suspension at 1% of the volume of the suspension.

Collection and treatment of human spermatozoa

Semen samples provided by a healthy volunteer were allowed to liquefy at 37 °C for 30 min. A 0.5 ml aliquot was gently placed at the bottom of a small test tube containing 2 ml of Tris-buffered ethylene

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glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid solution[9] that had been prewarmed to 37 °C. The tube was left standing for 10 min at 37 °C to allow spermatozoa to disperse into the solution. In all, 1 ml of the upper layer containing the spermatozoa was transferred into another test tube. The spermatozoa were treated with 50–200 μg ml^{-1} MMS or 0.01–1 mmol l^{-1} H_2O_2 for 2 h at 37 °C under 5% CO_2 in air. MMS or H_2O_2 were dissolved in distilled water and added to the sperm suspension at 1% of the volume of the suspension.

Comet assay

Normal melting agarose (NMA; Agarose L03; Takara Bio, Otsu, Japan) was dissolved in phosphate-buffered saline without Ca^{2+} and Mg^{2+}, pH 6.8 at the concentration of 1.0% (w/v) and kept at 50 °C. The surface on each glass slide was smeared with the 1% NMA on a plate heated at 70 °C. The sperm suspension was mixed with the 1% NMA to the final concentration of 0.7% NMA and the mixture (100 μl) was applied on each presmeared glass slide warmed at 50 °C, and coverslips were placed on the slides and then stored at 4 °C for 10 min.

After removing the coverslips, the slides were incubated at 4 °C for 2 h, and then further for 1 h at 37 °C in lysis buffer composed of 2.5 mol l^{-1} NaCl, 50 mmol l^{-1} EDTA–Na, 10 mmol l^{-1} Tris–HCl (pH 10), 1% (v/v) Triton X-100 and 10 mmol l^{-1} DL-dithiothreitol (Sigma-Aldrich, Buchs, Switzerland).

The slides were washed three times (3 min each) with cold water (4 °C). Subsequently, the slides were immersed for exactly 1, 5, 10 and 20 min in 300 mmol l^{-1} NaOH supplemented with 1 mmol l^{-1} EDTA–Na (4°C), and then transferred to Tris-acetate–EDTA buffer, pH 8.3 (Sigma-Aldrich, St Louis, MO, USA) for neutralization. The slides were subjected to electrophoresis for 10 min (12 V, 10 mA) at room temperature in Tris-acetate–EDTA buffer. After electrophoresis, the slides were immersed in ethanol (100%) and air dried. Immediately or 1 day afterwards, the air-dried slides were stained by YOYO iodide (Invitrogen, Eugene, OR, USA).

In each assay, 50 comets per slide were analyzed using a fluorescent microscope (Olumpus, Tokyo, Japan). The percentage of DNA in the comet tail (% tail DNA) was measured with the software CometScore Freeware version 1.5 (TriTek, Sumerduck, VA, USA).

Statistical analysis

The mean % tail DNAs were compared using analysis of variance followed by Fisher's least significant difference for multiple comparisons. Significant differences were determined at $P<0.05$.

RESULTS AND DISCUSSION

At each instance of alkali treatment, MMS-induced DNA damage clearly showed a dose-dependent increase (Figure 1). Alkali treatment contributed to the higher % tail DNA in the solvent control. With regard to MMS-treated spermatozoa, the % tail DNA did not increase significantly with the time of alkali treatment (from 1 to 5 or 10 min, Figure 1). Comet tails appeared brighter at 20 min than at 1 min (Figure 2). The comet tails were fairly pale in alkali treatment for 1 min in the solvent control, representing a low background level of DNA damage (Figure 2a). Mouse spermatozoa were treated with MMS at doses of 12.5 and 25.0 μg ml^{-1} (Figure 3). Alkali treatment for 1 min induced considerable lower background DNA damage, resulting in a statistically significant difference ($P<0.05$) of damage between solvent control and the lowest dose of MMS (12.5 μg ml^{-1}). In contrast, no significant difference in the damage was seen at this dose when alkali treatment was carried out for 20 min (Figure 3).

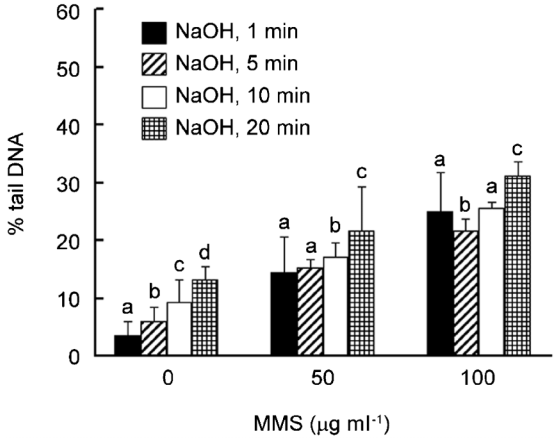

Figure 1 Results of comet assay using mouse spermatozoa treated *in vitro* with 50 and 100 μg ml^{-1} MMS. At each dose of MMS, three independent experiments were performed with one mouse per experiment. Treatment with NaOH was carried out to unwind the spermatozoal DNA for 1, 5, 10 and 20 min before electrophoresis. Data are expressed as mean±SD. Bars not sharing the common letters (a–d) differ significantly ($P<0.05$) within a dose of MMS. MMS, methyl methanesulfonate.

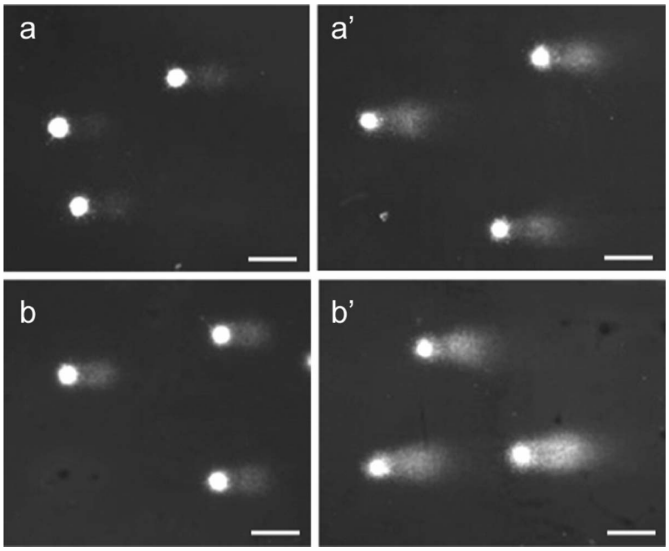

Figure 2 Comets of mouse spermatozoa assayed according to the A/N protocol. Alkaline DNA unwinding time was set at 1 min (**a**, solvent control; **a'**, 100 μg ml^{-1} MMS) and 20 min (**b**, solvent control; **b'**, 100 μg ml^{-1} MMS). Scale bars=50 μm. MMS, methyl methanesulfonate.

In the standard alkaline comet assay on mouse skin keratinocytes, long-term treatment (8 h or more) with alkali caused an increase in the background damage in the control cells.[10] DNA damage induced by genotoxins may be concealed by overlap with the background damage level.

DNA damage induced in mouse spermatozoa by H_2O_2 could be detected at the doses of 50 μmol l^{-1} or more (Figure 4). Under this assay condition, both background DNA damage and H_2O_2-induced DNA damage showed a little difference among alkaline DNA unwinding times. It was found that shortening the alkaline DNA unwinding time did not interfere with the detection of DNA damage induced in mouse spermatozoa by H_2O_2.

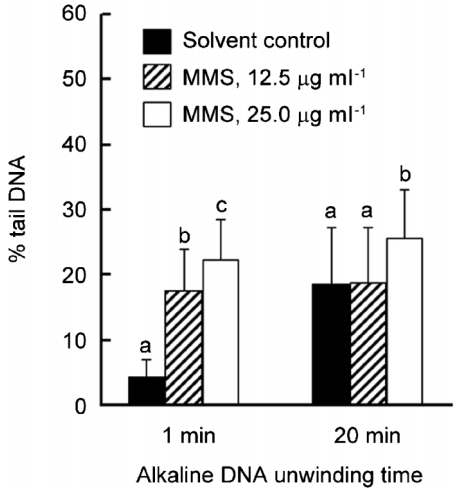

Figure 3 Results of comet assay using mouse spermatozoa treated *in vitro* with 12.5 and 25.0 μg ml^{-1} MMS. At each dose of MMS, four independent experiments were performed with one mouse in each experiment. Treatment with NaOH was carried out to unwind the spermatozoal DNA for 1 and 20 min before electrophoresis. Data are expressed as mean±SD. Bars not sharing the common letters (a–c) differ significantly ($P<0.05$) within the alkaline DNA unwinding time. MMS, methyl methanesulfonate.

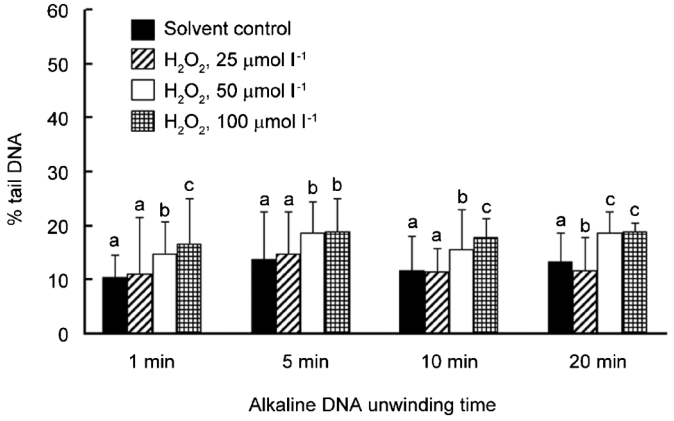

Figure 4 Results of Comet assay using mouse spermatozoa treated *in vitro* with 25–100 μmol l^{-1} H_2O_2. At each dose of H_2O_2, three independent experiments were performed with one mouse in each experiment. Treatment with NaOH was carried out to unwind the spermatozoal DNA for 1, 5, 10 and 20 min before electrophoresis. Data are expressed as mean±SD. Bars not sharing the common letters (a–c) differ significantly ($P<0.05$) within the alkaline DNA unwinding time. H_2O_2, hydrogen peroxide.

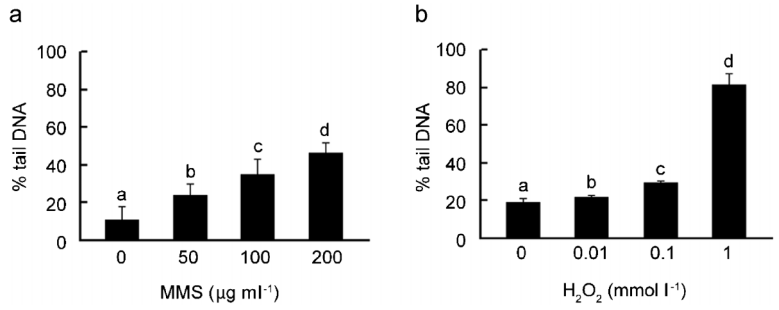

Figure 5 Results of comet assay using human spermatozoa treated *in vitro* with (**a**) 50–200 μg ml^{-1} MMS and (**b**) 0.01–1 mmol l^{-1} H_2O_2. Three independent experiments were performed using a fresh semen sample for each experiment. Treatment with NaOH was carried out to unwind the spermatozoal DNA for 1 min before electrophoresis. Data are expressed as mean±SD. Bars not sharing the common letters (a–d) differ significantly ($P<0.05$). H_2O_2, hydrogen peroxide; MMS, methyl methanesulfonate.

We demonstrated that short-term (1 min) treatment with alkali is adequate to detect accurately the DNA damage induced in human spermatozoa by MMS and H_2O_2 (Figure 3). As previously reported by Hughes *et al.*,[11] background DNA damage in human spermatozoa is higher (11.2%, Figure 5a; 19.1%, Figure 5b) than that in mouse spermatozoa (3.53%, Figure 1; 4.40%, Figure 3; 10.5%, Figure 4), being similar to the baseline in somatic cells.

According to the present A/N protocol, alkali treatment carried out for 1 min is sufficient for unwinding the DNA in mouse, and probably, human spermatozoa.

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