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Direct Visualization of Ascorbic Acid Inducing Double-Stranded Breaks in Single Nuclear Dna Using Singel-Cell Pulsed Field Gel Electrophoresis

KEYWORDS: single-cell pulsed-field gel electrophoresis, DNA fragmentation, ascorbic acid, pro-oxidant, Fenton reaction, edaravone

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ABSTRACT *To demonstrate the significance of instability analyses for single nuclear DNA, the action of hydroxyl radical generated by sodium ascorbate (SA)/transition metals was directly visualized using single-cell pulsed-field gel electrophoresis (SCPFGE). Human sperm were embedded in a thin film of agarose and digested with trypsin. SCPFGE pulled out several dozen long chain DNA fibers from the origin. After membrane extraction, DNA was still packed tightly with nuclear proteins. However, following the administration of SA, the DNA was cleaved into fibrous fragments and shortened to granular fragments in a dose-dependent manner. The lowest observed adverse effect level was visualized that large fibrous fragments were separated beyond the anterior end of the long chain fiber bundles. Swelling of DNA clusters after trypsin digestion facilitated the permeation of low-molecular weight agents and promoted the reaction; however, the presence of Cu or Ni were necessary as co-factors. Since the retention of hydroxyl radicals is very short, they must react at the proximal DNA. The reaction was suppressed by EDTA as well as edaravone, a hydroxyl radical scavenger. If some transition metals are bound to nuclear proteins, the above difference may be explicable that they diffused during trypsin digestion together with degraded peptides. SCPFGE is a sensitive analytical approach to assess not only naturally-occurring but also chemically induced DNA fragmentation in a single nucleus.*

INTRODUCTION

In regenerative medicine, it is essential to validate genomic homogeneity among cell popu-lations. Conventional genome sequencing methods require DNA extracted from a population of cells. However, genomic variations unique to individual cells are lost in a population average and *de novo* mutations in a cell may be concealed in the bulk signal. Single-nuclear analyses by means of high-throughput single-cell whole-genome amplification or ultra-high sensitive DNA sequencing [1-3] are proposed to be future tools for single-nuclear genomics. Hence, non-specific sequential damage, such as single- or double-strand breaks (SSB, DSB), confounds these pioneering technologies. SSB are produced daily in nuclei during DNA repair processes such as base excision repair [4]. If unrepaired, lesions on DNA threaten genetic integrity through their potential conversion into lethal DSB during DNA replication. The repair of DSB is intrinsically difficult compared to other DNA lesions [5, 6] as the critical threshold may be null or very low. Thus, verification of structural integrity, particularly detection during the early stages of DNA fragmentation, is a fundamental step in single nuclear DNA analyses.

Throughout the past decade, the neutral and alkaline comet assays have become recognized as the major tools for observing SSB and DSB in a single nucleus [7-10]. Cells are embedded in agarose and lysed with detergent under high salt conditions to remove histones and protamine. The alkaline pH conditions then result in the unwinding of the DNA double-strands [8, 10]. After electrophoresis, the degree of DNA damage is evaluated on the basis of the amount of granular fragments discharged from the origin, the so-called "comet tail". Our previous report [11] developed a single-cell pulsed-field gel electrophoresis (SCPFGE) to observe naturally occurring DNA damages, the embedded cells into agarose gel film were digested with trypsin prior to the electrophoresis [11]. SCPFGE revealed a different course of DNA fragmentation from those observed by the comet assay. First, several large fibrous fragments derived from a bundle of long-chain fibers, the cleavages in the DNA advanced until almost all the DNA was shredded into granular fragments [11, 12]. Certain nuclear DNA binding components were tolerant of high salt concentrations and high alkalinity, but these could be degraded by trypsin. Thus, a lack of trypsin digestion can lead to false-negative results in both the comet and SCPFGE assays. Further, the comet tail did not reflect the total amount of granular fragments, but rather, those that were released from the components [11, 12].

Mammalian sperm have a unique properties in terms of maintaining DNA integrity. Because their DNA repair capacity declines during late spermato-genesis, a portion of the male germ cells may accumulate DNA lesions [13, 14]. This property of the sperm makes it suitable as a test cell to evaluate chemically-induced DNA damage in individual cells: it is a free cell, the lack of a capacity for DNA repair allows for observations of net destabilizing effects, somatic cells are heterogeneous in terms of the cell cycle, and the sperm arrests the cycle.

It is well known that reactive oxygen species (ROS) induce DNA strand breaks [15]. Ascorbic acid has a multiplicity of anti-oxidant properties, but it can also function as a conditional pro-oxidant *in vitro*, usually by reducing certain transition metals such as Cu, Ni, or Fe to generate free radicals through the Fenton reaction [16, 17]. It is still uncertain whether these pro-oxidant effects have any biological relevance toward intracellular DNA. Hence it is significant to analyze quantitatively the chemically-induced

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DNA damages in a single nucleus, SCPFGE directly visualized the lowest observed adverse effect level (LOAEL), the dose-effect relationship, and the mechanism of action of sodium ascorbate (SA)/transition metals for the purified human sperm without DNA fragmentation.

MATERIALS AND METHODS

Ethics Statement

All the authors submitted the written consent to the ethical committee, Ichikawa General Hospital, Tokyo Dental College that no patient and volunteer participated in this study, all the specimens (human semen/sperm) used for the experiments were provided by ourselves, and the authors were given the opportunity to opt out of donating these specimens if they so desired. The committee specifically approved this study.

Separation of human sperm with progressive motility

Sperm concentration and motility were observed with a computer-assisted image analyzer (C-Men, Compix Inc., PA, USA). Sperm with progressive motility were prepared as described in previous reports [11, 18-19]. Briefly, diluted semen was layered on 5 ml of a 98% isotonic Percoll (GE Healthcare, NJ, USA) density gradient and centrifuged in a swing-out rotor at 400 × *g* for 30 min. Hank's solution (2.0 mL) was overlaid onto the precipitate (200 µL) and the motile sperm that swam up into the upper layer was collected. The results were obtained using sperm that was prepared as follows: the ejaculate (vol. = 4.0 mL, conc. = 64 × 10^6 sperm/mL, motility = 54%), the precipitate of the density-gradient (200 µL, 370 × 10^6 sperm/mL, 78% motility), and the swim-up fraction (1.0 mL, 9 × 10^6 sperm/mL, 97% motility).

Single-cell pulsed-field gel electrophoresis

The methodology of SCPFGE has been described in the previous reports [11, 12]. An aliquot of the specimen (corresponding to 2 × 10^5 sperm) was adhered to a 7 × 7 mm area on agarose-coated glass slides by centrifugal auto-smear (Cyto-Tek, SAKURA, Tokyo, Japan). The melted 0.33% agarose (0.1 mol/L sodium acetate (pH 4.7) with 0.05% Triton X-100, 540 µL) was mixed with 60 µl purified bovine trypsin (200 µg/mL) at 40 °C. The sperm adhered on the glass slide was embedded in the agarose . After chilling, the gel was incubated in the cell-lytic reagent (30 mmol/L Tris-HCl, 8.2 mmol/L hexa-metaphosphate Na, 0.05% Triton X-100, 5.0 mmol/L dithiothreitol, pH 8.1, 37 °C for 30 min).

The SCPFGE apparatus had dual electrode pairs arranged at a 60 degree angle, the gel film on the glass slide was then positioned at the cross-point of the electric currents. The electrophoresis was performed at 1.5 V/cm with 3.0 sec switching intervals for 7 min in 30 mmol/L Tris-acetate, 8.2 mmol/L hexa-metaphosphate Na, pH 8.1. DNA was stained with ×10^4 diluted Cyber-Gold (Molecular Probes, Oregon, USA) and visualized using an epifluorescent microscope (excitation: 495 nm, emission: 537 nm).

The microscopic features of DNA were termed according to their sizes, the long-chain fiber was elongated without interruption from the origin, while the fibers and small particles being separated beyond the anterior end of the elongated long-chain fibers were termed as fibrous and granular fragments, respectively. Sperm observed microscopically to contain at least one fibrous fragment were evaluated as damaged.

RESULTS

Although human ejaculates contain a hetero-geneous sperm population in terms of the stages of DNA fragmentation, the density gradient centri-fugation and subsequent swim-up method excluded those in the advanced stages [11, 12]. The sperm with progressive motility in the resulting fraction discharged several dozen long-chain fibers from the point of origin (Fig. 1-C). Their electrophoretograms were assigned to either the group for which only the long-chain fibers were observed, or assigned to the group with fibrous fragments beyond the anterior end of the elongated fibers. The specimen shown in Fig. 1 was used for all subsequent experiments and the percentage of sperm that had at least one fragment was found to be 6.4%. Figures 1-A, B, and C compare the sizes of the embedded sperm, the swollen DNA after in-gel tryptic digestion, and the elongated DNA fibers, respectively. DNA fibers tightly packed in human sperm heads (Fig. 1-A) were in-gel digested with trypsin to degrade nuclear proteins. The DNA fibers were consequently swollen by negative charge repelling, which gave the appearance of a mass of tangled fibers with shaggy surface (Fig. 1-B).

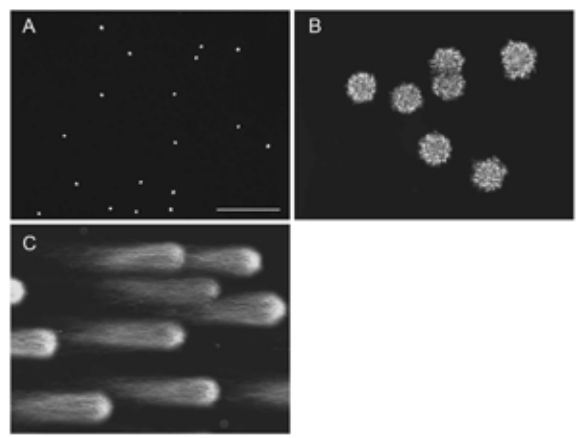

Figure 1: Comparison of the sizes of embedded sperm, swollen DNA after in-gel tryptic digestion and elongated of DNA fibers.

All the images in Fig. 1–5 except for Fig. 5-D have identical scale size. The scale bar in Fig. 1-A represents 40 µm. DNA was stained with Cyber-Gold as described in the methods section. A: embedded sperm, B: swollen DNA, C: elongated DNA fibers after SCPFGE.

A single administration of SA to the detergent-extracted sperm promoted DNA fragmentation in a dose-dependent manner. Various lengths of fibrous fragments was carved out from the long chain fibers by 1.0 µmol/L SA (Fig. 2-A). Along with the increasing concentration, the length of fibrous fragments were shortened and the amount of granular fragments were increased (10 µmol/L SA, Fig. 2-B). Ultimately, almost all the fibers degraded into granular fragments and the mass of the origin diminished after 100 µmol/L SA treatment (Fig. 2-C). SA did not influence motility or DNA in the living sperm regardless of the concentration used and the electrophoretic profiles were similar to those shown in Fig. 1-C. When the detergent-extracted sperm was incubated with 0.5 mmol/L EDTA for 5 min prior to the addition of 10 µmol/L SA, the action of SA was completely inhibited.

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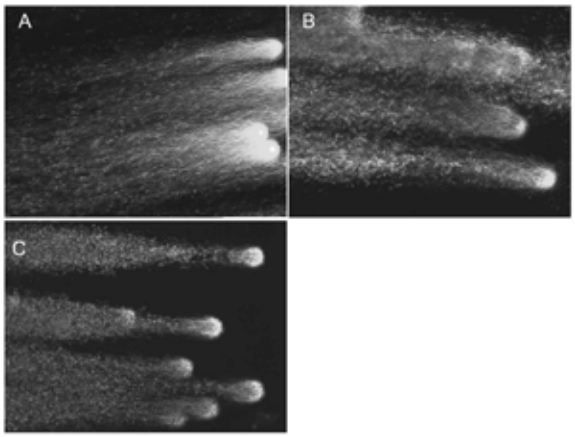

Figure 2: Fragmentation of DNA in detergent-extracted sperm by sodium ascorbate.

Suspensions of the sperm (2×10^6 cells/ml) were incubated with 1.0–100 µmol/L SA in 20 mmol/L Hepes-NaOH, pH 7.2, in the presence of 0.05% Triton X-100 at an ambient temperature for 20 min. Thereafter, an aliquot was adhered to agarose-coated glass slides to embed the sperm into the agarose for tryptic digestion. A: 1.0 µmol/L SA, B: 10 µmol/L SA, C: 100 µmol/L SA.

In contrast to the results shown in Fig. 2, a single administration of SA could not cleave the swollen DNA fibers (Fig. 1-B) and the electro-phoretic profiles were similar to those shown in Fig. 1-C. This action required the presence of $CuSO_4$, whereas the metal itself had no cleavage activity. When the specimens were incubated with various concentrations of SA in the presence of 0.5 mmol/L $CuSO_4$ (Fig. 3), the electrophoretic profiles were different from those of corresponding doses shown in Fig. 2. The elongated long-chain fibers have already been disappeared at 0.1 µmol/L SA. Smaller granular fragments with faster electrophoretic mobilities increased, and the mass of the origin diminished in a dose-dependent manner (Fig.3-B, C and D). The same experiment was performed with 0.5 mmol/L $NiCl_2$ and $ZnCl_2$. $NiCl_2$ resulted in similar cleavage profiles as $CuSO_4$, whereas $ZnCl_2$ had no activity.

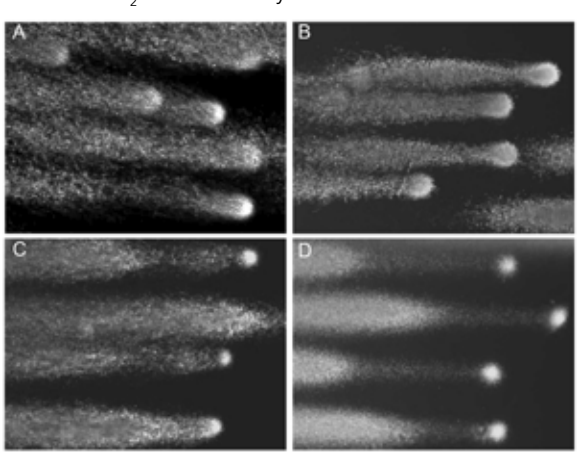

Figure 3: Effect of sodium ascorbate on swollen DNA fibers.

Embedded sperm was digested with trypsin as described in the methods section. The swollen DNA fibers were pre-incubated for 5 min with 0.5 mmol/L $CuSO_4$ in 20 mmol/L Hepes-NaOH, pH 7.2, and treated with various concentrations of SA at an ambient temperature for 20 min. A: 0.1 µmol/L SA, B: 1.0 µmol/L SA, C: 10 µmol/L SA, D: 100 µmol/L SA.

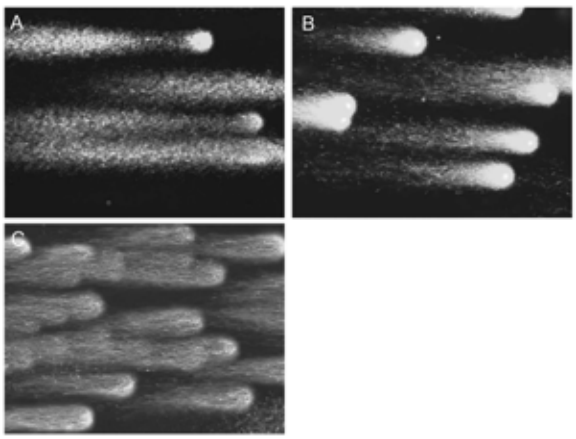

Figure 4: Competitive inhibitory effect of the hydroxyl radical scavenger, edaravone, against the pro-oxidant activity of sodium ascorbate/$CuSO_4$.

Swollen DNA fibers were pre-incubated for 5 min with 0.5 mmol/L $CuSO_4$ and various concentrations of edaravone (ED) in 20 mmol/L Hepes-NaOH, pH 7.2. SA was then added to obtain a final concentration of 10 µmol/L and incubated at an ambient temperature for 20 min. A: 10 µmol/L ED, B: 100 µmol/L ED, C: 1.0 mmol/L ED.

Figure 4 examined the inhibitory effect of the hydroxyl radical scavenger, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; ED) [20, 21], against the pro-oxidant activity of 10 µmol/L SA/0.5 mmol/L $CuSO_4$. ED competitively inhibited the reaction in a dose-dependent manner. Ten µmol/L ED were unable to protect DNA fibers, whereas a portion of fibers were protected in the presence of 100 µmol/L ED. At 1.0 mmol/L ED, DNA cleavage was completely inhibited and the electrophoretic profiles were similar to those shown in Fig. 1-C.

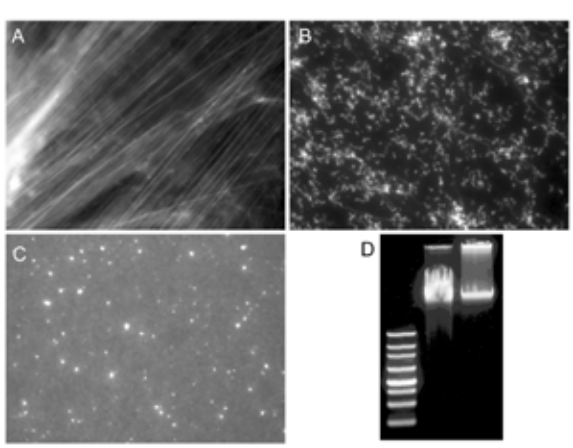

Figure 5: Features of DNA fibers in free solution and their cleavage by sodium ascorbate/$CuSO_4$.

Sperm was suspended in the cell-lytic reagent solution containing Cyber-Gold ($\times 10^4$ diluted), and an aliquot (10 µL, corresponding to 4×10^4 sperm) was mounted on a glass slide and covered with a coverslip. The slide was then incubated at 37 °C for 20 min (A). The same experiments were performed in the presence of 1.0 µmol/L

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SA, 0.5 mmol/L $CuSO_4$ (B), or 1.0 mmol/L SA/1.0 mmol/L $CuSO_4$ (C), respectively. *Lambda DNA and* a set of known DNA size markers (250 to 2000 base pairs) *were* stained with Cyber-Gold and electrophoresed in a 0.5% agarose gel (30 mmol/L Tris-acetate, 0.5 mmol/L EDTA, pH 8.2). The electrophoretic profiles were visualized under blue-ray illumination (D).

Sperm digestion in free solution yielded extremely long chain fibers which were spread over the microscopic field of view (Fig.5-A). Lysis of sperm in the presence of 1.0 µmol/L SA/0.5 mmol/L $CuSO_4$ yielded short fibers of similar sizes (Fig.5-B), which oscillated by Brownian movement. To deter-mine the sizes of the fragments using conventional submarine gel electrophoresis, the short fibers were further shredded using a high concentration of 1.0 mmol/L SA/0.5 mmol/L $CuSO_4$. All of the short fibers were then degraded into fine granules (Fig. 5-C). Figure 5-D shows the electrophoretic profiles of the specimen in Fig.5-C (10 µL), *Lambda DNA* (*5.0 µg,* 48, 502 base pairs) and the DNA ladder markers. A band with similar mobility to *Lambda DNA was found,* while the other part remained in the well. Cyber-Gold-stained *Lambda DNA was not observed as granules by fluorescence* microscopy. It should be noted that the size of the fine granules shown under the microscope (Fig. 5-C) are substantially larger compared to those commonly observed in molecular biology.

DISCUSSION

The neutral and alkaline comet assays are widely employed to examine the anti-oxidant [22, 23] and pro-oxidant [24, 25] actions of ascorbic acid in single nuclear DNA. Our previous reports [11, 12] described some technical issues associated with the comet assays in that the lack of trypsin digestion led to false-negative results and that the high alkalinity of the assay yielded artifactual DNA fragments. To analyze chemically-induced DNA cleavage, it is essential to measure the lowest observed adverse effect level (LOAEL), the dose-effect relationship, and the mechanism of action. SCPFGE directly visualized the LOAEL, wherein the large fibrous fragments appeared beyond the anterior end of a bundle of long chain fibers, the lengths of fibrous fragments were shortened and the granular fragments inversely increased in a dose-dependent manner (Fig. 2 and 3). Single administration of SA could cleave the DNA fibers which were packed tightly in the membrane-extracted sperm, and the elongated long chain fibers were still observed after treatment with 1.0 µmol/L SA (Fig. 2-A). The packing state of DNA fibers and the positional relationship between the metal ions and DNA affected the pro-oxidant action of SA. Tryptic digestion swelled the cluster of DNA fibers (Fig. 1-B). As summarized in Fig. 2 and 3, the enlarged void volume facilitated the permeation of low-molecular weight agents and affected the dose-effect relationship. The long chain fibers have already been cleaved to the short fibrous fragments after treatment with 0.1. µmol/L SA (Fig. 3-A), whereas the action required the presence of the metal ions.

The significance of Zn for male reproductive health has been well-studied [26]. Zinc is abundant in human seminal plasma [27] as well as in sperm nuclei [28]. P2 protamine in human sperm are Zn-finger proteins with one Cys2/His2 motif [29]. However, the Zn in P2 protamine is not involved in the reaction. The N-terminal amino acid sequence of human P2 protamine contains Cu and Ni binding domains [30, 31]. It is well known that the half-life of hydroxyl radicals are extremely short; thus, SA and transition metals must encounter and react at the immediate proximity of DNA fibers. Since the reaction in Fig. 2 was suppressed by EDTA, some intra-nuclear transition metals might act as a counterpart to SA at the proximity of DNA, whereas addition of $CuSO_4$ or $NiCl_2$ were necessary to cleave the swollen DNA fibers (Fig. 3). These results indicate the possibility that some metals were bound to nuclear proteins and diffused during tryptic digestion together with the degraded peptides. As shown in Fig. 4, the dose-dependent scavenging action of ED demonstrated that hydroxyl radicals, through the Fenton reaction [16, 17], directly cleaved the double-stranded DNA structure. The concentration of ascorbic acid in body fluids has often been determined by means of reverse-phase high-performance liquid chromatography coupled with UV detection [32] and total ascorbic acid concentration in human seminal plasma has been reported to be approximately 400 µmol/L [33]. The motile sperm with intact plasma membrane blocked permeation of SA, whereas LOAEL for the membrane extracted sperm was found to be extremely low when compared to physiological concentrations (Fig. 2). Human ejaculates contain sperm with various levels of DNA fragmentation [11, 12]. Various publications have emphasized that enzymatic strand breaks with intracellular Ca/Mg-dependent endonucleases [34, 35] in the apoptotic processes, abnormal influx of extracellular ascorbic acid through impaired membrane or transmembrane channels might be caused by non-apoptotic DNA fragmen-tation. Our present results at least indicated that extracellular ascorbic acid should be washed out prior to detergent treatment of cells for DNA extraction.

CONCLUSIONS

SCPFGE directly visualized a bundle of long-chain fibers in a single nucleus and serpentine-curved tracks by means of the pulse-field voltage impression efficiently fixed the fibers in the porous structure of the agarose. The present results suggested that it was a sensitive analytical approach to assess not only naturally-occurring but also chemically induced DNA fragmentation. Gene mapping on a set of stretched DNA fibers in a single nucleus is proposed as a future tool for discrete validation of cytogenetic polymorphisms. The continuity of DNA fibers have to be confirmed in order to distinguish that the lack of signals are due to deletion of the target sequence rather than loss by DNA fragmentation. SCPFGE may play important roles in advanced verification of single-cell genomics.

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REFERENCE [1] Kalisky T, Quake SR (2011) Single-cell genomics. Nature Methods 8:311-314. [2] Varadarajan N, Julg B, Y.J. Yamanaka YJ, et al. (2011) A high-throughput single-cell analysis of human CD8+ T cell functions reveals discordance for cytokine secretion and cytolysis. J Clin Invest 121:4322-4331. [3] Zong C, Lu S, A.R. Chapman AR, Xie XS (2012) Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. Science 338: 1622-1626. [4] Fromme JC, Verdine GL (2004) Base excision repair. Advances in Protein Chemistry 69: 1-41. [5] Khanna KK, Jackson SP (2001) DNA double-strand breaks: signaling, repair and the cancer connection. Nature Genet 27: 247-254. [6] Hefferin ML, Tomkinson AE (2005) Mechanism of DNA double-strand break repair by non-homologous end joining. DNA Repair 4: 639-648. [7] Tice RR, Agurell E, Anderson D, et al. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35: 206 -222. [8] Hartmann A, Agurell E, Beevers C, et al. (2003) Recommendations for conducting the in vivo alkaline comet assay. Mutagenesis 18: 45-51. [9] Olive PL, Banáth JP (2006) The comet assay : a method to measure DNA damage in individual cells. Nature Protocols 1:23 - 29. [10] Fairbairn DW, Olive PL, O'Neill KL (1995) The comet assay: a comprehensive review. Mutation Research/Reviews in Genetic Toxicology, 339: 37-59. [11] Kaneko S, Yoshida J, Ishikawa H, Takamatsu K (2012) Single-cell pulsed-field gel electrophoresis to detect the early stage of DNA fragmentation in human sperm nuclei. PLoS ONE 7: e42257. [12] Kaneko S, Yoshida J, Ishikawa H, Takamatsu K (2013) Single-nuclear DNA instability analyses by means of single-cell pulsed-field gel electrophoresis - Technical problems of the comet assay and their solutions for quantitative measurements. J Mol Biomark Diagn S5: 005. [13] Jansen J, Olsen AK, Wiger R, et al. (2001) Nucleotide excision repair in rat male germ cells: low level of repair in intact cells contrasts with high dual incision activity in vitro. Nucleic Acids Res 29: 1791-1800. [14] Olsen AK, Duale N, Bjoras M, et al. (2003) Limited repair of 8-hydroxy-7,8-dihydroguanine residues in human testicular cells. Nucleic Acids Res 31: 1351-1363. [15] Cooke MS, Evans MD, Dizdaroglu M (2003) Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J 17: 1195-1214. [16] Carr A, Frei B (1999) Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J 13: 1007-1024. [17] Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. Free Radical Biol Med 18: 321–336. [18] Kaneko S, Oshio S, Kobanawa K, et al. (1986) Purification of human sperm by a discontinuous Percoll density gradient with an innercolumn. Biol Reprod 3: 1059-1063. [19] Kaneko S, Sato H, Kobanawa K (1987) Continuous-step density gradient centrifugation for the selective concentration of progressively motile sperm for insemination with husband's semen. Arch Androl 19: 75-84. [20] Pérez-González A, Annia Galano A (2011) OH Radical Scavenging Activity of Edaravone: Mechanism and Kinetics. J Phys Chem B 115: 1306–1314. [21] Tomatsuri N, Yoshida N, Takagi T, et al. (2004) Edaravone, a newly developed radical scavenger, protects against ischemia-reperfusion injury of the small intestine in rats. Int J Mol Med 13: 105-109. [22] Nishi EE, Campos RR, Bergamaschi CT (2010) Vitamin C prevents DNA damage induced by renovascular hypertension in multiple organs of Wistar rats. Hum Exp Toxicol 29: 593-599. [23] Sram RJ, Blanka Binkova B, Pavel Rossner P (2012) Vitamin C for DNA damage prevention. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 733: 39–49. [24] Turkez H (2011) The role of ascorbic acid on titanium dioxide-induced genetic damage assessed by the comet assay and cytogenetic tests. Exp Toxicol Pathol 63: 453–457. [25] Ullah MF, Khan HY, Zubair H,et al. (2011) The antioxidant ascorbic acid mobilizes nuclear copper leading to a prooxidant breakage of cellular DNA: implications for chemotherapeutic action against cancer. Cancer Chemother Pharmacol 67: 103-110. [26] Yamaguchi S, Miura C, Kikuchi K, et al. (2009) Zinc is an essential trace element for spermatogenesis. PNAS 106: 10859-10864. [27] Katayama M, Kaneko S, Takamatsu K, et al. (2013) Sensitive and simultaneous determination of trace metals in human seminal plasma by means of inductively coupled plasma mass spectrometry and multivariate statistical analyses of relationships between their concentrations and sperm parameters. J Mol Biomark Diagn 4: e147. [28] Bench G, Corzett MH, Kramer CE, et al. (2000) Zinc is sufficiently abundant within mammalian sperm nuclei to bind stoichiometrically with protamine 2. Mol Reprod Dev 56: 512-519. [29] Bianchi F, Rousseaux-Prevost R, Sautiere P,et al. (1992) P2 protamines from human sperm are zinc -finger proteins with one Cys2/His2 motif. Biochem Biophys Res Commun 182: 540–547. [30] Bal W, Kasprzak KS (2002) Induction of oxidative DNA damage by carcinogenic metals. Toxicol Lett 127: 55–62. [31] Bal W, Lukszo J, Kasprzak KS (1997) Mediation of oxidative DNA damage by nickel(II) and copper(II) complexes with the N-terminal sequence of human protamine HP2. Chem Res Toxicol 10: 915–921. [32] Liau LS, Lee BL, New AL, et al. (1993) Determination of plasma ascorbic acid by high-performance liquid chromatography with ultraviolet and electrochemical detection. J Chromatogr 612: 63–70. [33] Colagar AC, Marzony ET (2009) Ascorbic acid in human seminal plasma: Determination and its relationship to sperm quality. J Clin Biochem Nutr 45: 144–149. [34] Widlak P, Garrard WT (2009) Roles of the major apoptotic nuclease-DNA fragmentation factor-in biology and disease. Cell Mol Life Sci 66: 263-274. [35] Arends MJ, Morris RG, Wyllie AH (1990) Apoptosis. The role of the endonuclease. Am J Pathol 136: 593-608.