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Generation of reactive oxygen species in sperms of rats as an earlier marker for evaluating the toxicity of endocrine-disrupting chemicals

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Abstract

Bisphenol A (BPA) and diethylstilbestrol (DES) have been reported to cause sperm toxicity. To identify an earlier marker of toxicity of environmental substances or food additives, this study determined whether the levels of reactive oxygen species (ROS) in sperms could serve as indices for the prediction of sperm toxicity and quality. Male Wistar rats were given drinking water containing various doses of BPA or DES for 8 weeks. Some rats were treated with 0.45% N-acetyl cysteine (NAC) for 2 days prior to the administration of DES or BPA. Administration of BPA or DES to rats for 1 week dose-dependently increased the production of ROS, even at doses and time points which had no effect on sperm motility. 4-Hydroxy-2-nonenal modified proteins increased in sperms 8 weeks after BPA or DES treatment. NAC reversed oxidative stress and prevented the loss of sperm function in the DES or BPA-treated group. During observation, changes in the sperm motility, sperm count and morphology were not correlated to the increase in ROS levels. These results suggest that ROS levels may be used as an early indicator of sperm count and quality decreases which result from chronic toxicity.

Keywords: *Sperm toxicity, xenobiotic agent, mitochondria, superoxide, biological marker*

Abbreviations: *BPA, bisphenol A; DES, diethylstilbestrol; ROS, reactive oxygen species; SOD, superoxide dismutase; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H) dione; DMEM, Dulbecco's modified Eagle's medium; MTR, MitoTracker Red; MTG, MitoTracker Green.*

Introduction

In the past 50 years, there has been a marked decrease of both normal and abnormal sperms among males in developed countries, possibly due to environmental substances. The cytotoxic effects of chemicals may have a greater influence on males than females because rapid and continuous cell cleavage is necessary for the formation of spermatozoa. Spermatogenesis is one of the most productive self-renewing systems in the body: the daily production of spermatozoa is on the order of 10^7 per gram in testis tissue across mammalian species. Therefore, the male reproductive system is more susceptible to injury, especially in the developmental stages. The role of reactive oxygen species (ROS) in the pathophysiology of human sperm function has been emphasized in the recent years [1–3]. ROS production in semen has been associated with loss of sperm motility, decreased ability of sperms to fuse with the oocyte and loss of fertility [4]. Spermatozoa are very sensitive to oxidative insults due to their high content of

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polyunsaturated fatty acids and lack of ability to repair DNA [5]. Therefore, we hypothesized that ROS production in sperms is a more sensitive toxicity index than other toxicity indices and it can be used to evaluate the safety of chemicals used in food additives, food preservatives and environmental endocrine disruptors.

Bisphenol-A (BPA) and Diethylstilbestrol (DES) are well known as oestrogenic chemicals. Exposure to BPA, a monomer in polycarbonate plastics and a constituent of resins used in food packaging and dentistry, is hazardous for humans [6]. Exposure to BPA (0.2 mg/mouse/day) or DES (50 μg/mouse/day) for 5 days caused a number of changes in testicular gene expression in adult mice [7]. Diethylstilbestrol (DES) was widely used to treat pregnant women until 1971. The reproductive tracts of female offspring exposed to DES *in utero* are characterized by anatomic abnormalities. The possibility that xeno-oestrogens may cause adverse effects in the reproductive tract was first highlighted by reports of adolescent sons born to women who had taken the highly potent synthetic oestrogen DES during pregnancy. These individuals developed a variety of testicular and epididymal abnormalities in adulthood [8].

The aim of this study was to investigate whether ROS production, when compared to the changes in routine sperm-function parameters such as sperm motility and sperm count, is an earlier and more sensitive index of toxicity. We experimented with BPA and DES, which are known to inflict testicular toxicity in rats.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were obtained from Gibco (Paisley, UK) and superoxide dismutase (SOD, 3000 U/mg) was obtained from Calbiochem (Nottingham, UK). 8-amino-5-chloro-7-phenylpyrido [3,4-*d*]pyridazine-1,4-(2*H*,3*H*) dione (L-012) and other reagents were obtained from Wako Chemical Company (Osaka, Japan).

BPA (purity, 99%) was obtained from Wako Chemical Co. and DES (purity, 99%) was obtained from Sigma Chemical Co. (St. Louis, MO). Appropriate quantities of BPA and DES were dissolved in tap water to achieve the desired concentrations. Fresh solutions of different concentrations were prepared every week and stored in glass containers.

Animals

Wistar rats (6 weeks of age) were purchased from SLC, Inc. (Shizuoka, Japan). Rats were exposed to a 12/12-h light–dark cycle with free access to standard rat chow and tap water. At 8 weeks of age, these rats were provided with drinking water containing 0, 1.0 or 10 mg/L BPA or 0.1 or 0.3 mg/L DES for 7 days a week for 8 weeks. The rats were divided into six groups of 10 each. To examine the effects of short-term administration of BPA and DES, we administered 1 mg/L of BPA or 0.1 mg/L of DES to rats (12 weeks of age) for 1 week.

All rats had free access to standard laboratory chow (CE-2; Clea, Tokyo, Japan) and tap water and were cared for according to the specifications outlined in the *Guiding Principles for the Care and Use of Laboratory Animals*, approved by the Authorities of the Local Committee on Experimental Animal Research.

The animals were sacrificed at 12 weeks of age under anaesthesia with urethane (5 g/kg, i.p.). Blood was collected using heparinized syringes and epididymis and testis were dissected. The right epididymis of each rat was used to prepare sperm solutions and these solutions were used as separate samples. The epididymal fat was removed; subsequently, the epididymis was placed on a paper towel to remove any liquid and then weighed. For sperm sampling, the cauda epididymis was cut with surgical scissors at the side of the corpus epididymis, where the vas deference attaches to the epididymis.

Sperm concentration and motility

The sperm count in cauda epididymis was determined as follows. Briefly, the excised cauda epididymis was minced in 3 ml of TYH medium (Mitsubishi Chemical Medience Co. Tokyo, Japan) using anatomical scissors. The sperm suspension was filtered using a 70-μm cell strainer (BD Biosciences, Bedford, MA). A portion of the sperm suspension was diluted with TYH medium (first with 1:20 and then with 1:100) and then incubated at 37°C for 5 min in a CO_2 incubator. Then, 10 μl of the sperm solution (1:100) was immediately transferred to a slide chamber for the following measurements. For sperm motility analysis, we determined the sperm motility index (SMI), straight-line velocity, curvilinear velocity and amplitude and frequency of sperm head trajectory at 37°C using a Computer Aided Sperm Analysis system [9] (SMAS: Kaga Solnet, Tokyo, Japan) on a thermo plate (Tokai hit, Shizuoka, Japan) under a light microscope (Figure 1).

The supernatant fluid (1:100) was diluted with 4-fold dilution of 5% saline and an aliquot of the sperm/saline mixture was analysed under a haemocytometer; 10 μl of the diluted sperm suspension was introduced in each counting chamber.

Assay for ROS generation in sperm solutions

ROS generation in sperm solutions was determined by using L-012 [10]. L-012 is a luminol derivative

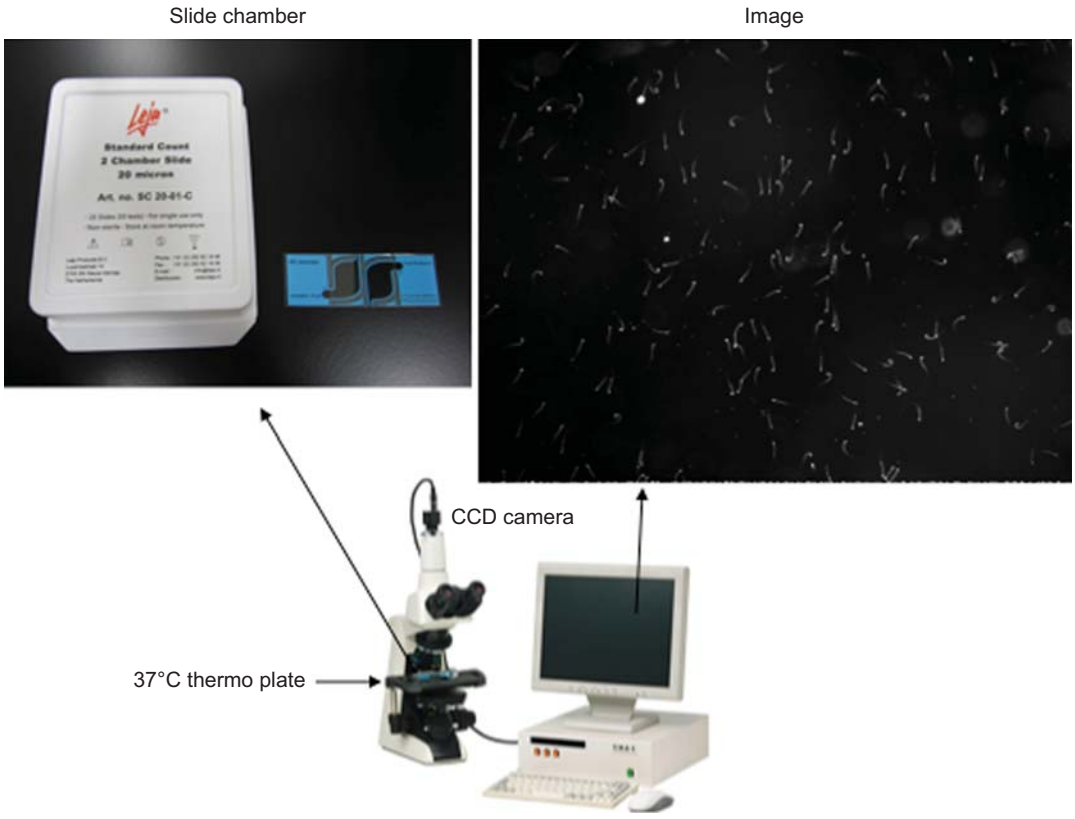

Figure 1. Sperm motility analysis system. Sperm motility parameters can be obtained with the use of computer-assisted sperm analysis.

that is highly sensitive for the detection of ROS such as $O_2^{\bullet-}$, H_2O_2, and OH•; L-012 itself does not exert redox cycling. Next, we added 5 µl of the aforementioned sperm suspensions (1:20) to 1 ml of DMEM (serum free) at 37°C and then recorded with a chemiluminescence reader (AccuFlex Lumi 400; Aloka, Tokyo). After 1 min of incubation, 50 µl of 2 mM L-012 solution was added through a reagent-dispensing unit and continuously recorded for 3 min. When SOD (50 U/ml) was added to the reaction mixture, ROS generation was almost completely abolished. Superoxide release from the spermatozoa was expressed as relative chemiluminescence that was calculated as the difference between the peak levels and the levels after the addition of SOD (50 U/ml).

In situ determination of ROS generation

Loading of sections with MitoTracker Green (MTG) or MitoTracker Red (MTR), which are mitochondrial fluorescence probes, showed that perigranular, sub-plasmalemmal and perinuclear mitochondria were predominant. MTR (CM-H2XRos, reduced form of the probe) is non-fluorescent. When the probe is oxidized by ROS, it becomes fluorescent. The details have been described previously [11]. The oxidized product binds to thiol groups and proteins within the mitochondria. MTR is excited at 579 nm with an emission spectrum of 599 nm. MTG (excitation wavelength, 490 nm; emission wavelength, 516 nm) is a mitochondrion-selective fluorescent label that covalently binds to the inner mitochondrial membrane and fluoresces independently of membrane potential ψ and $[Ca^{2+}]_{mt}$. Next, we incubated 100-µl sperm suspensions on 8-chamber glass slides and added MTR (2 µM) to each group of sperm suspensions. Subsequently, the slides were incubated in a light-protected humidified chamber at 37°C for 15 min and then 200 nM MTG was added. After incubation, the suspensions were centrifuged at 2000 rpm for 5 min and washed twice with phosphate-buffered saline (PBS). Images were obtained using a Biozero fluorescence microscope (BZ-8000; Keyence, Osaka, Japan) that was coupled to an upright microscope with a Nikon Plan-Apo objective (×20; N.A., 0.75; Nikon, Tokyo) at the Central Research Laboratory, Osaka City University Medical School, Japan.

The MTG (green) and CM-H2XRos (red) fluorescence patterns of the cells were processed as one-colour images or two-colour overlays, as indicated. Neither signals exhibited significant photo-bleaching with Prolong Gold Antifade reagent (Invitrogen, Tokyo, Japan) during the time frame of analysis. The intracellular distribution of ROS generation was examined by comparing the MTG and MTR staining patterns with those of the mitochondrion-specific probes. MTR fluorescence was not observed in some SOD-treated samples (50 U/ml).

Lipid peroxidation of spermatozoa

4-Hydroxy-2-nonenal (HNE)-modified proteins and its protein adducts have been demonstrated to be good markers for lipid peroxidation induced by reactive oxygen species. Spermatozoa were homogenized with ice-cold 20 mM phosphate buffer (pH 7.4) and centrifuged at 3000 g for 10 min. The collected supernatants (20 μg) were analysed by immunoblotting for anti-HNE-j2 antibody (Japan Institute of the Control Age, Shizuoka, Japan) using 12.5% gels, as described above. Antibodies were diluted with Can Get Signal™ solution (TOYOBO).

Evaluation of sperm mitochondrial membrane potential

Sperm solution (300 μl of 20-fold dilution of the original solution) was incubated with 10 μg/ml of the lipophilic cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) according to the instructions of Mitochondrial Membrane Potential Detection Kit (Cell Technology, CA) for 10 min and finally analysed in a flow cytometer (BD FACS Aria™ II, Becton Dickinson, San Jose, CA) using an argon laser (488 nm) for excitation and emissions at 530 nm (green) and 575 nm (red/orange) were quantified using the threshold signal for intact cells. JC-1 has advantages over other cationic dyes in that it can selectively enter into mitochondria and reversibly change colour from green to red as the membrane potential increases. In healthy cells with high mitochondrial $\Delta \Psi m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low $\Delta \Psi m$, JC-1 remains in the monomeric form, which only shows green fluorescence. Briefly, harvest sperms with functional mitochondria contain red JC-1 J-aggregates and are detectable in the FL2 channel (PE-A). Apoptotic or unhealthy cells with collapsed mitochondria contain mainly green JC-1 monomers and are detectable in the FL1 channel (FITC-A). To measure the change of membrane potential by the inhibition of mitochondria electron transport complex I, 5 μM rotenone was treated to normal sperm for 10 min at 37°C.

Statistics

We performed statistical analyses using the StatView v 5.0 software package (SAS Institute, Cary, NC). The difference was considered to be statistically significant if $p < 0.05$.

The effect of oral administration of BPA or DES on the sperm parameters (for sperms obtained from the cauda epididymal specimens) of Wistar rats was evaluated using ANOVA and post-hoc multiple comparisons.

Results

Test articles dose verification

The concentrations of BPA and DES in drinking water solutions were analysed each week prior to their use in the experiment. The average concentrations over the 4-week dosing period ranged from 99–103% of the nominal values.

Animal health status evaluations

No deaths or clinical alterations were observed in the animals of any study group. There were no consistent differences in terms of food consumption between the BPA-treated animals and the control animals. However, food consumption in the DES-treated animals was slightly lower than that in the control animals. The animals in the 0.1 mg/L-DES-treated group exhibited reduced body weight.

Water consumption between time intervals varied slightly across all groups. No apparent treatment-related effects were observed in any rats of the BPA-treated group, but the water consumption of the 0.1 mg/L- and 0.3 mg/L-DES-treated groups decreased slightly by 13.8% and 14.5%, respectively.

HNE-modified protein

HNE-modified proteins and their protein adducts have been demonstrated to be good markers for lipid peroxidation induced by reactive oxygen species. In rats treated with BPA or DES for 8 weeks, HNE-modified proteins in the epididymis were measured. BPA or DES increased HNE-modified protein at 30 kDa dose-dependently (Figure 2). NAC decreased the HNE-modified protein in BPA (1 mg/L) or DES (0.1 mg/L)-treated rats (Figure 2).

Sperm concentration and seminal parameters

In rats treated with BPA for 8 weeks, the sperm concentrations were unchanged (Figure 3) but sperm

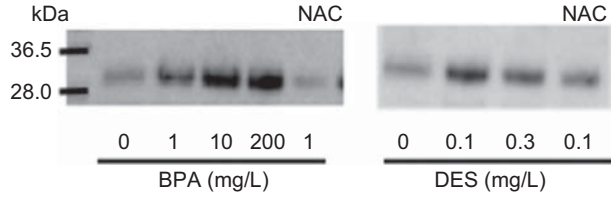

Figure 2. Lipid peroxidation of spermatozoa after BPA or DES treatment. Control, BPA (1 mg/L, 10 mg/L and 200 mg/L) and DES (0.1 mg/L and 0.3 mg/L) solutions were administered to Wistar rats for 8 weeks. Some animals were fed by 0.45% N-acetyl cysteine (NAC) diet just 2 days before BPA (1 mg/L) or DES (0.1 mg/L) drinking water. Spermatozoa were homogenized with ice-cold 20 mM phosphate buffer (pH 7.4) and centrifuged at 3000 g for 10 min. The supernatants (20 μg) collected were analysed by immunoblotting for anti-HNE-j2 antibody.

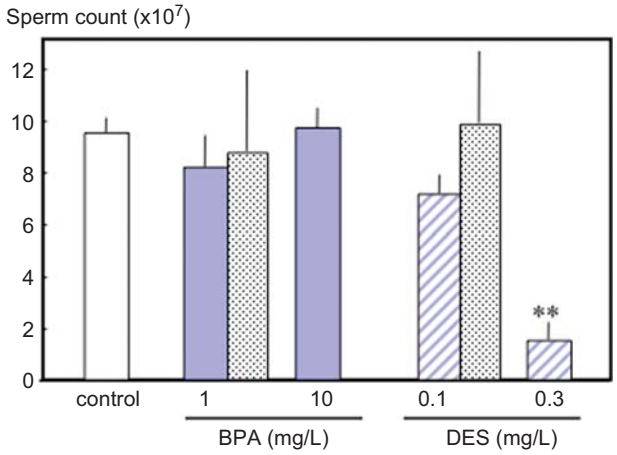

Figure 3. Effect of BPA or DES on sperm count. The control, BPA (1 mg/L and 10 mg/L) and DES (0.1 mg/L and 0.3 mg/L) solutions were administered to Wistar rats for 8 weeks. Some animals were treated with 0.45% N-acetyl cysteine (NAC) for 2 days prior to the administration of drinking water containing BPA (1 mg/L) or DES (0.1 mg/L). Sperm count in the right cauda epididymis was determined for each rat by diluting the sperm suspension with 3 ml of TYH medium. Values are represented as mean ± SE (n = 6–7). $^{**}p < 0.01$ as compared with that of the control group. Dot column, NAC-treated group.

motility was reduced markedly (Figure 4). DES-treated rats also exhibited marked decrease in both sperm concentrations and motility. Total number of sperms with reduced motility is shown in Figure 4. Treatment with both BPA and DES markedly decreased the overall sperm motility but did not significantly affect other parameters (straight-line velocity, curvilinear velocity and amplitude and frequency of sperm head trajectory) in any group. NAC prevented both the decrease of sperm concentration and motility (Figures 3 and 4). Morphological analysis revealed no significant changes in the sperm structure in BPA-treated rats (results not shown), while in DES-treated rats the sperm structure exhibited a wide range of abnormalities, such as defects in the head (microcepphatic, bicepharous, amorphous and acephalic), neck and tail (results not shown).

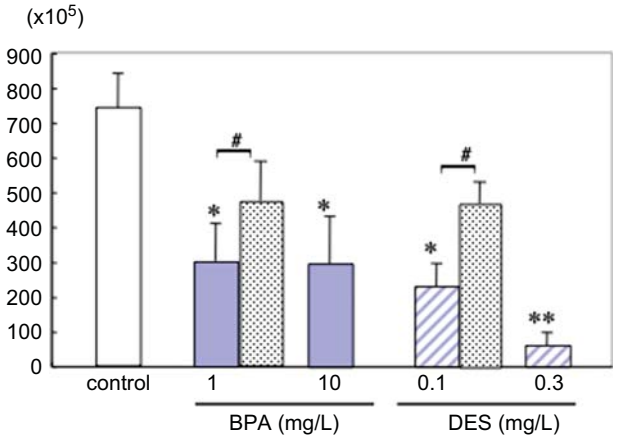

Figure 4. Effect of BPA or DES on the total number of motility in the sperm. Animals were treated as described in Sperm motility was determined at 37°C as described in the Methods section. The sperm motility and sperm count of each rat was determined individually as described in The total value of motility of each rat was calculated. Values are represented as mean ± SE (n = 6–7). $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with that of the control group. $^{\#}p < 0.05$ as compared with that of BPA or DES-treated rats. Dot column, NAC-treated group.

ROS production

Chemiluminescence assays revealed that the ROS levels were increased in the sperms of both BPA- and DES-treated groups (Figure 5); further, the increase in the ROS levels was lower in the BPA-treated group than in the DES-treated group. Moreover, the ROS levels in the DES-treated group increased in a dose-dependent manner. NAC pre-treatment significantly inhibited ROS production in BPA (1 mg/L) or DES (0.1 mg/L). Treatment of rats with rotenone (5 μM), which is an inhibitor of complex I of the mitochondrial respiratory chain, increased ROS production (data not shown).

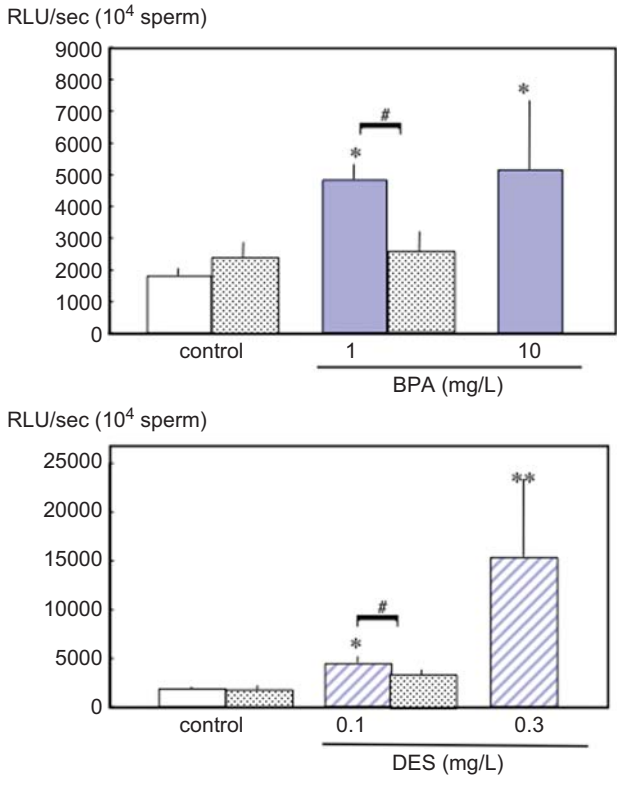

Figure 5. Effect of BPA or DES on the ROS production of sperms. Animals were treated as described in Five microlitres of sperm suspensions (1:20) were diluted with 1 ml of DMEM medium (albumin free), incubated at 37°C, and the fluorescence was then recorded using a chemiluminescence reader. After 1 min of incubation, 50 μl of 2 mM L-012 solution was added and the ROS production was continuously recorded for 3 min. When SOD (50 U/ml) was added to the reaction mixture, ROS generation was abolished. Values (per 10^4 sperms) are represented as mean ± SE (n = 6–7). $^{*}p < 0.05$, $^{**}p < 0.01$, as compared with that of the control group. Dot column, NAC-treated group. RLU: relative light units.

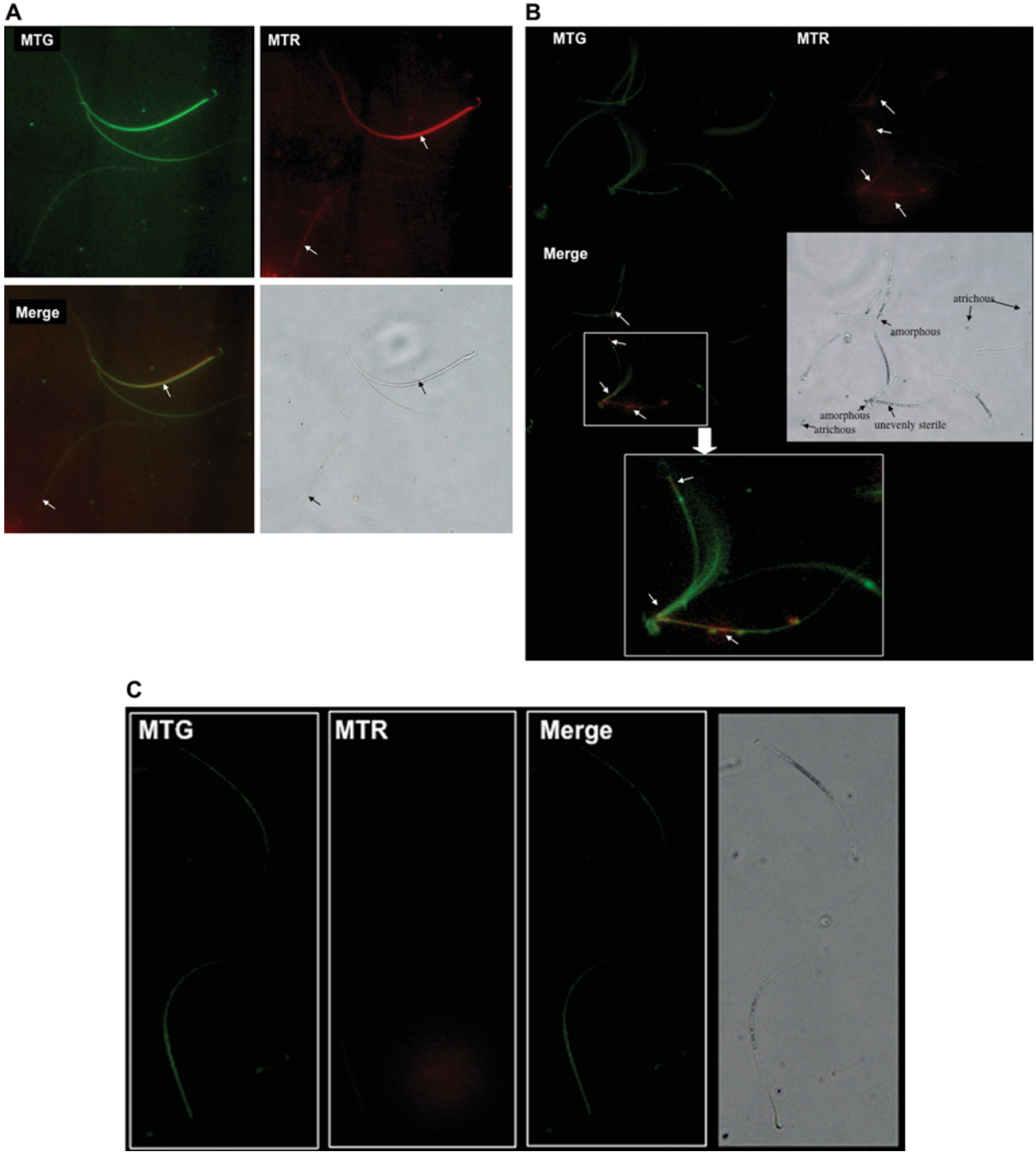

Figure 6. Representative image of ROS production from seminal mitochondria in BPA- and DES-treated rats. MTR is non-fluorescent. The probe becomes fluorescent upon oxidization by ROS. MTG is a mitochondrion-selective fluorescent label that covalently binds to the inner mitochondrial membrane. Each photograph is an image of the phase lag. (A) BPA (10 mg/L) was administered as described in the Methods section. BPA-treated sperms did not exhibit any morphological changes, but some sperms generated ROS from mitochondria. Arrows are representative sperms generating ROS. (B) DES (0.3 mg/L) was administered as described in the Methods section. Some sperms in an abnormal form were observed and ROS generation was also detected. (C) Normal form of sperms in control rats. There was almost no increase in the signal intensity.

In situ ROS production

Superoxide production by sperms was assessed on the basis of fluorescence of MitoTracker probes (Figure 6). Some morphologically normal sperms in BPA groups generated ROS in BPA or DES-treated group. A representative picture is shown in Figure 6A (sperms of 10 mg/L-BPA treated rats). Superoxide production by abnormal sperms (indicated by an arrow) in the 0.3 mg/L-DES-treated group is shown in Figure 6B. Furthermore, MTG and MTR fluorescent probes were used to detect the *in situ* production of mitochondrial ROS. The normal sperms in the control group did not generate superoxides (Figure 6C).

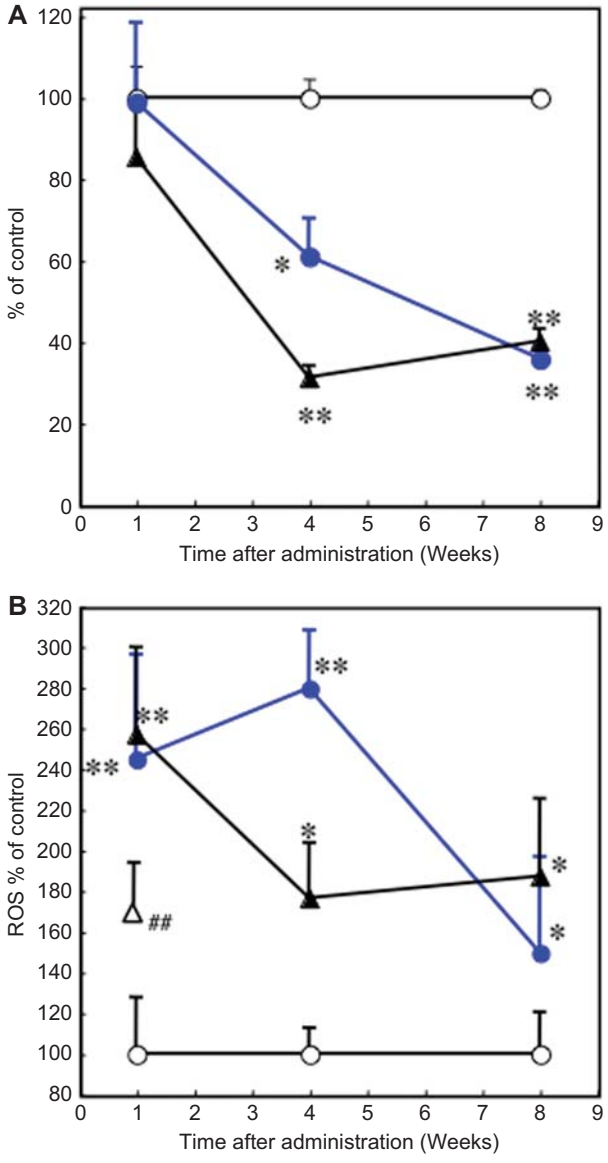

Figure 7. Time-dependent effect of BPA or DES on sperm motility and ROS production. Control, BPA (1 mg/L) or DES (0.1 mg/L) solutions were administered as described in the Methods section. The results of sperm motility of BPA- or DES-treated rats are represented as percentages of that of control animals. Values are represented as mean ± SE (n = 6–7). $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with that of the control group. Open circle, control; closed circle, BPA; closed triangle, DES; open triangle, NAC. (A) % control of total sperm motility. (B) ROS production was measured as described in Figure 5.

Time course of sperm motility and ROS production after administration of BPA or DES

Administration of 1 mg/L of BPA or 0.1 mg/L of DES for 1 week had almost no effect on the total numbers of sperm motility compared with the control (100%) (Figure 7A). However, ROS generation was significantly high in both groups (Figure 7B). NAC significantly inhibited ROS production. The seminal ROS production and sperm motility after 4 weeks or 8 weeks of DES- or BPA-exposure were not observed to be inversely correlated.

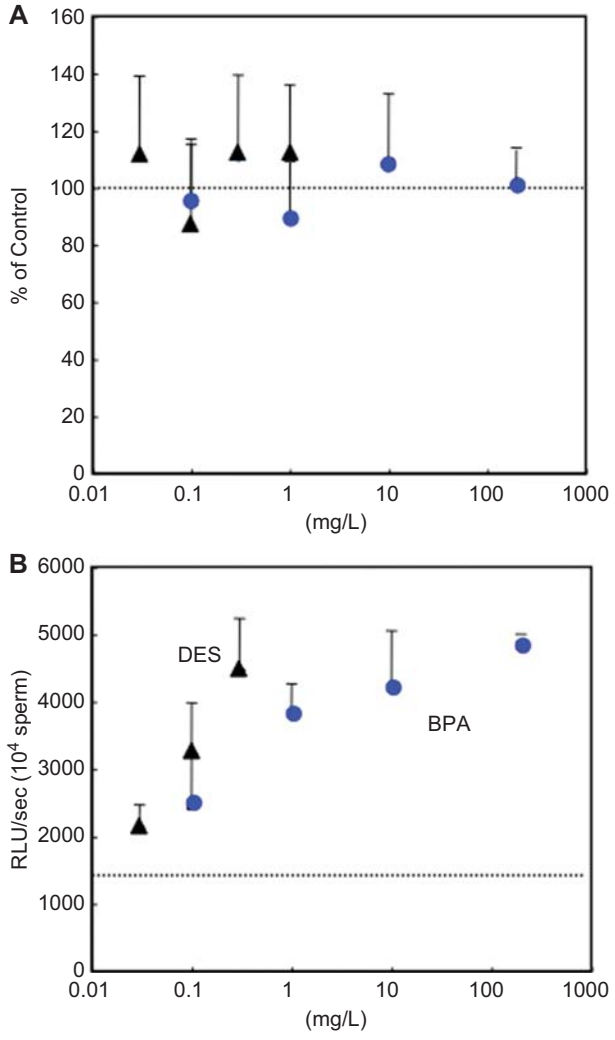

Figure 8. Dose-dependent effect of BPA or DES on sperm motility and ROS production. Animals were treated with various doses of BPA or DES for 1 week. Closed circle, BPA; closed triangle, DES; Dotted line, normal levels. (A) % control of total sperm motility. (B) ROS production.

Dose dependency of BPA or DES on sperm motility and ROS production

Animals were treated with various doses of BPA or DES for 1 week. Sperm motility was unaffected by any dosage (Figure 8A). However, ROS production of sperm was dose-dependent on BPA or DES treatment (Figure 8B).

Sperm mitochondrial membrane potential

Rotenone treatment changed mitochondrial membrane potential (Figure 9). Treatment with BPA or DES at all dosages reduced high mitochondrial inner transmembrane potential $\Delta\psi(m)$.

Discussion

The results of this study suggest that the measurement of ROS production in rat sperms is an effective method for evaluating and predicting sperm function

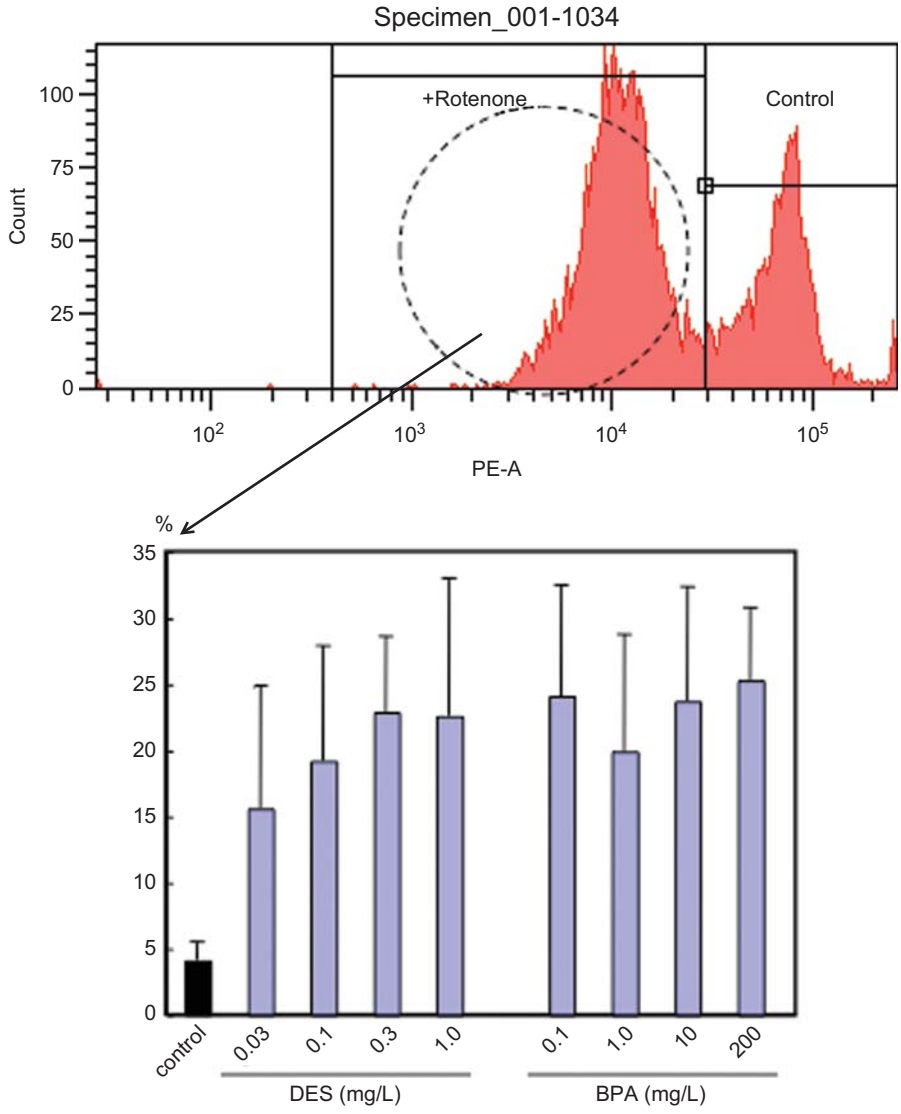

Figure 9. Changes in mitochondrial membrane potential after BPA or DES treatment. Sperm solution (300 µl) was incubated with 10 ug/ml JC-1 according to the instruction of Mitochondrial Membrane Potential Detection Kit (Cell Technology, CA) for 10 min and analysed in a flow cytometer using an argon laser (488 nm) for excitation and emsissions at 530 nm (green) and 575 nm (red/orange) were quantified using the threshold signal for intact cells. Upper panels. normal sperm (control) moved to the left (decrease of red fluorescence) by 5 µM of rotenone treated-sperm. Bottom panel, % changes in mitochondrial membrane potential.

even when the sperm numbers and morphology are not affected by the exposure to toxic drugs. NAC, which is an antioxidant, inhibited ROS production and prevented the loss of sperm function.

One of the principal mechanisms by which oxidative stress induces infertility is by causing sperm damage, either through direct oxidation of DNA and proteins by ROS or by the initiation of apoptosis. It has been reported that antioxidant therapy significantly improves DNA integrity and protamine packaging and is accompanied by a reduction in seminal ROS production and apoptosis [12]. Various experimental data suggest that high level of ROS can induce sperm damage and can be responsible for some forms of unexplained infertility. Cells possess various defense mechanisms against oxidative insults, primarily the production of antioxidant molecules. Hence, treatment with antioxidants can improve the seminal parameters and sperm function *in vitro*, thereby representing a possible form of treatment for selected forms of infertility [13].

The process by which spermatozoa produce ROS is not fully understood. Although it has been suggested that it is less likely that mature human sperms may possess significant NADPH oxidase activity, some reports indicate that they produce ROS by an unknown mechanism. There is strong evidence showing that ROS are produced in animal sperms, although these ROS may primarily be of mitochondrial origin [14]. Defects in mitochondrial respiration might result in meiotic arrest of cells and cause abnormalities in sperm morphology, thereby implying that mitochondrial respiratory function plays an important role in mammalian spermatogenesis. We found that ROS was generated from the mitochondria in both morphologically

normal and abnormal sperms of DES- and BPA-treated rats (Figure 7).

In humans, sperm count and progressive motility were found to be significantly lower in the infertile group than in the fertile controls. Semen malondialdehyde and ROS levels of the infertile group were significantly higher ($p < 0.0001$), due to the mutation of mitochondrial DNA, compared to those of the control group [15]. In this study, BPA did not affect sperm count significantly during observation, but increased the ROS levels. These results were confirmed by the data obtained from the short-term administration of BPA or DES (Figure 8). Administration of BPA or DES for 1 week significantly and dose-dependently increased ROS production without decreasing sperm counts. Subsequently, the number of spermatozoa decreased but ROS production increased at 4 weeks. These results suggest that the measurement of ROS is a more sensitive indicator of sperm toxicity than routine sperm measures. Since no ROS production is observed once the sperms die due to toxicity, measurement of ROS levels may indicate mild damage of the spermatozoa.

It has been reported that spermatogenic cycle length of the meiotic cells or young spermatids of rats is 8.6 days [16]. The use of ROS measurement for the estimation of cell toxicity of food additives, preservatives, endocrine-disrupting chemicals and new medicines is considered effective, because the sensitivity of spermatozoa to the toxicity of these chemicals is higher than that of other cells. Furthermore, the evaluation of sperm ROS generation might be a more feasible and quantifiable method than the determination of the toxicity indices of the spermatozoa using a microscope. Whether other chemicals such as endocrine disturbing chemicals, food additives or post-harvest agents are similar index markers of sperm toxicity is presently being studied.

In conclusion, ROS production from the sperm is a sensitive surrogate marker of sperm toxicity.

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