Free Radical Research, July 2005; 39(7): 697–705

Accelerated impairment of spermatogenic cells in sod1-knockout mice under heat stress

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Accepted by Dr J. Yodi

(Received 17 November 2004; in revised form 4 April 2005)

Abstract

For normal spermatogenesis, the temperature of the scrotum is lower than that of the body. The mechanism by which mammalian testes undergoes cell death as the result of exposure to heat continues to be a matter of debate. Since generation of reactive oxygen species (ROS) during heat stress and involvement in spermatogenic cell damage are postulated, we induced experimental cryptorchidism in the testes of SOD1-knockout mice and examined effects of the gene deficiency. The cleavage of DNA in testicular cells, as judged by TUNEL staining, were elevated in SOD1-knockout mice at an earlier stage than in the wild-type mice. To confirm responsiveness of SOD1 for this high susceptibility to heat stress, spermatogenic cells were isolated from SOD1-knockout and wild-type mice and cultured at 32.5 and 37°C. The cells isolated from SOD1-knockout were more vulnerable at both temperatures than those from wild-type mice. The exposure of cultured rat spermatogenic cells to ROS induced the release of cytochrome c from mitochondria, while Sertoli cells were more resistant under the same conditions. Tiron, a superoxide scavenger, suppressed the heat-induced release of cytochrome c from mitochondria. Collectively, these data suggest that ROS are generated during heat stress and cause spermatogenic cell death. Alternatively, since even a short exposure triggers harmful damage to spermatogenic cells, generated ROS may function as a type of signal for cell death rather than directly causing oxidative damage to cells.

Keywords: *Reactive oxygen species, heat stress, cryptorchidism, super oxide, superoxide dismutase*

Abbreviations: *Cyt c, cytochrome c; DTT, dithiothreitol; FBS, fetal bovine serum; HE, hematoxylin and eosin; PBS, phosphate-buffered saline; PhGPx, phospholipid hydroperoxide glutathione peroxidase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; SOD1-KO, SOD1 knockout; TBST, tris-buffered saline containing 0.1% Tween-20; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling*

Introduction

A temperature lower than that of the body cavity is essential for normal spermatogenesis in the testes. Cryptorchidism is associated with male infertility due to an impairment of the spermatogenic process, leading to infertility. This pathogenesis is mainly attributed to high temperatures, since the *in situ* cooling of abdominal testes in the pig results in normal spermatogenesis [1]. Local heating of the lower abdomen to a temperature of 43°C for 15 min induces apoptosis in germ cells and renders the animal

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10715760500130517

transiently less fertile [2,3]. The surgical induction of cryptorchidism also causes a disruption in spermatogenesis [4–6]. Pachytene spermatocytes and early spermatids are primarily damaged in experimentally induced cryptorchid testes [7]. The damage is characterized by apoptosis within 2–4 days [7,8], eventually resulting in a germ cell deficiency [9].

However, the molecular basis of heat-induced spermatogenic cell damage is not fully understood, but the involvement of the Fas-ligand/Fas system and an apoptosis-related gene p53 is suspected [10,11]. Some pro- and anti-apoptotic proteins appear to be responsible for intracellular signal transduction in heat stress [12–14]. In addition to these protein factors, reactive oxygen species (ROS) also likely function as mediators of heat-induced germ cell damage. In fact, the generation of ROS [15] and elevations in the levels of lipid peroxidation products [16] have been reported. Thus, heat treatment may trigger oxidative stress which, in turn, has a harmful influence on testes.

A number of proteins play a protective role against ROS that are produced under conditions of oxidative stress. Among them, superoxide dismutase (SOD) is generally thought to play a central role because it scavenges superoxide anions, the initially generated ROS from molecular oxygen in cells [17]. Thus, that SOD plays a role in the male reproductive system is a distinct possibility [18,19]. Although CuZnSOD and MnSOD, encoded by the SOD1 and SOD2 genes, respectively, represent a major intracellular superoxide-scavenging system, the contribution of these proteins varies depending on the types of cells [20]. Contrary to the fact that a mutation in SOD1 is a cause of familial amyotrophic lateral sclerosis (FALS), SOD1-knockout mice grow normally and live healthily under conventional breeding conditions [21]. While female SOD1-KO mice are infertile, and no abnormality in the reproductive system has been reported for male SOD1-KO mice [22,23]. Intervention, however, frequently deteriorates the pathological conditions of SOD1-KO mice more severely than is the case for wild-type mice [24,25].

SOD2-KO mice, on the other hand, show dilated cardiomyopathy and die during the neonatal stage [26]. The overexpression or induction of SOD2 causes cells to become more resistant to various stimuli, such as inflammatory cytokines and toxins [20]. However, transgenic male mice that express higher levels of SOD2 exhibit a decreased fertility, for reasons that are not entirely clear [27].

We recently reported that heat stress at 42°C for 15 min triggers the release of cytochrome c from mitochondria, followed by apoptosis in spermatogenic cells and that this process can be inhibited by minocycline [28]. Here we present data that supports the involvement of ROS in heat-stress induced germ cell damage using SOD1-deficient mice.

Materials and methods

Animals

Male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). Three pairs of hetero B6 SOD1-KO mice, established by Matzuk *et al.* [23], were purchased through Jackson Laboratories (Bar Harbor, ME) and bred in our institute. The animal room climate was kept under specific pathogen free conditions at a constant temperature of 21–23°C with a 12 h alternating light-dark cycle. Animal experiments were performed in accordance with the Declaration of Helsinki under the protocol approved by the Animal Research Committee of this institution.

Preparation of testicular cells

Spermatogenic cells and Sertoli cells were isolated and cultivated by a previously described method [29]. All procedures were performed under sterile conditions. Premature male Wistar rats (40 days of age) or SOD1-KO mice were killed under diethylether anesthesia. The testes were removed and decapsulated mechanically. Seminiferous tubules were gently expressed and incubated in PBS containing 0.25% type I collagenase (Wako) for 15 min at 32.5°C with occasional shaking. The seminiferous tubules were then washed and again incubated in PBS containing 0.25% trypsin (Difco, Detroit, MI) for 15 min at 32.5°C with gentle shaking. The trypsin treatment was terminated by adding fetal bovine serum (FBS; Lifeteck Oriental, Tokyo, Japan) to a level of 10% (v/v). The resulting cell suspension was filtered through a metal mesh to remove cell aggregates and tissue debris. Cells were then collected by centrifugation and suspended in F12—L-15 medium, a 1:1 mixture of Ham's F12 and Lebovitz's L15 (ICN Biochemicals, Aurora) supplemented with 1 mg/ml sodium bicarbonate, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 15 mM Hepes (pH 7.3), and 10% FBS. The concentration of testicular cells in the medium was adjusted to 5×10^6/ml. The cells were incubated at 32.5°C, the optimal temperature for testicular spermatogenic cells, in a humidified atmosphere of 5% CO_2 in air. Cells that were attached to the plastic culture plates after 1 day were defined as Sertoli cells, and non-adhering cells were defined as the spermatogenic cell-rich fraction. For short heat-stressed conditions, cells were cultured at 42°C for 1 h followed by incubation at 32.5°C.

Preparation of cytosolic fraction from cultivated cells

This method is essentially the same as described previously [28]. Cells were incubated in media containing hypoxanthine and xanthine oxidase or exposed to heat at 42°C for 1 h and further

incubated at 32.5°C for 30 min in a CO_2 incubator. Cytosolic fractions were prepared as follows. Cells, 5×10^6, were washed in PBS and suspended in 250 μl of buffer (250 mM sucrose, 50 mM Pipes-KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). After standing for 30 min on ice, the cells were lysed with 10 strokes using pestle B in a Dounce homogenizer. After centrifugation at 14,000*g* for 15 min, the supernatant was regarded as the cytosolic fraction.

SDS-PAGE and immunoblot analysis

SDS-PAGE and immunoblot analyses of testicular proteins were performed as described previously [29]. Protein samples were subjected to 15% SDS-PAGE and then transferred to a Hybond-P membrane (Amersham Pharmacia) under semi-dry conditions by means of a Transfer-blot SD semi-dry transfer cell (Bio-Rad). The membrane was then blocked by incubation with 5% skimmed milk in TBS (150 mM NaCl and 20 mM Tris/HCl, pH 7.6) for 2 h at room temperature. The membranes were then incubated with a monoclonal anti-mouse cytochrome c Ig (Cyt c, 1:1,000 dilution) (Pharmingen, San Diego, USA), polyclonal antibodies to MnSOD [30], Prx1 [31], or β-actin overnight at 4°C. A polyclonal antibody to recombinant human CuZnSOD was raised in rabbit and also used. After washing with tris-buffered saline containing 0.1% Tween-20 (TBST), the membrane was incubated with 1:1,000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, USA) or goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature. After washing with TBST, the peroxidase activity on the membranes was detected by a chemiluminescence method using an ECL Plus kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to X-ray films (Kodak, Rochester, USA). The amounts of Cyt c protein were quantified by scanning X-ray films by Densitography (Atto, Tokyo, Japan).

Induction of experimental cryptorchidism

Experimental cryptorchidism was induced in mice as described previously [32]. A group of mature male mice were made cryptorchid surgically under sodium barbital anesthesia (4 mg/kg body weight). One testis of each animal was translocated and sutured to the lateral abdominal wall via the fat pad. The other testis was sham operated and used as a control. Care was taken not to injure blood vessels or the epididymis. The animals were killed at appropriate days after cryptorchidism. Mice were used for analyses only when the testes were located abdominally at post mortem and were atrophic.

Histological examination and detection of DNA fragmentation in situ

A half of each testis was fixed in Bouin solution for 16 h at 4°C and embedded in paraffin. Four micro meter thick sections were mounted on silan-coated glass slides, deparaffinized, and hydrated. They were digested with 20 μg/ml of proteinase K for 15 min at room temperature. Endogenous peroxidase was inactivated by incubation in a 3% hydrogen peroxide solution for 5 min at room temperature. A histological examination was performed on hematoxylin and eosin (HE)-stained sections. Apoptotic nuclei in tissue sections were identified by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique using an *in situ* apoptosis detection kit (Takara, Kyoto, Japan) according to the manufacture's instructions. Photographs were taken with a digital camera under microscopy (Olympus BX50, Tokyo, Japan). Cross sections of two hundred round, transversely cut seminiferous tubules from eight testes from each group were examined. The number of TUNEL-positive nuclei per tubule was calculated and are expressed as the mean ± SEM for each group, as described previously [28].

Statistical analysis

Statistical analyses of the data were carried out using the Mann–Whitney *U*-test. $P < 0.05$ was considered to be significant.

Results

Effects of experimental cryptorchidism on SOD1 KO and WT mice

To examine the possible involvement of ROS in spermatogenic cell damage *in vivo*, we employed SOD1-KO mice. If superoxide is generated during heat stress and is responsible for the damage, an enhanced testicular impairment would be expected in the SOD1-KO mice. As a result, although the testicular weight of SOD1-KO mice was not altered from that of WT mice at an early stage, it became significantly less at the late stage of cryptorchidism (Figure 1). Histological analysis indicated that vacuolar degeneration was more sever in the SOD1-KO than the WT mice (Figure 2). When DNA fragmentation was detected *in situ* using the TUNEL assay, more positive signals were found for SOD1-KO than for WT mice. The number of TUNEL-positive cells that were generally regarded as apoptotic cells was the highest at day 7 and then

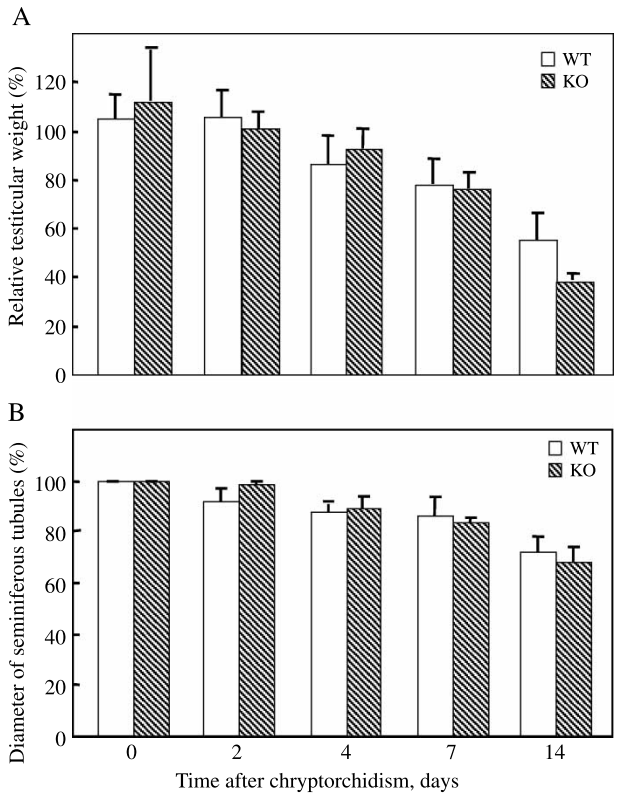

Figure 1. Changes in testicular weight of wild-type and SOD1-KO mice during experimental cryptorchidism. (A) After the induction of experimental cryptorchidism, testicular weight was measured over a 2-week period. Relative testicular weight to sham-operated control were shown (%). (B) Diameter of seminiferous tubules were also measured. (Numbers of animals were 3–6, except for 2 of 0-day control KO mice).

decreased (Figure 3). At day 14, positive signals were higher in the WT than the SOD1-KO mice. This can be explained by the exhaustion of spermatogenic cells in SOD1-KO mice at an early stage, due to their enhanced vulnerability. Thus, cell death by cryptorchidism is accelerated in SOD1-KO mice.

Expression of antioxidative/redox enzymes in cryptorchid mice

We then examined the levels of proteins that function as antioxidative and redox enzymes by immunoblotting (Figure 4). The levels of CuZnSOD were decreased slightly during cryptorchidism in WT mice while no CuZnSOD protein was detected in SOD1-KO mice. The levels of MnSOD, a mitochondrial SOD isoform, and Prx1, a thioredoxin-dependent peroxidase 1, were not altered significantly in either type of mice.

Effects of high temperature on cultured spermatogenic cells from SOD1-KO and WT mice

To gain an insight into the high vulnerability of SOD1-KO mice in cryptorchidism, spermatogenic cells were isolated from SOD1-KO and WT mice and cultivated at 32.5 and 37°C. While incubation, even at 32.5°C, slowly decreased the number of the cells isolated from WT mice, the number decreased at an accelerated rate in the cells from SOD1-KO mice (Figure 5A). The cells from SOD1-KO mice decreased more rapidly than those from WT mice when incubated at 37°C. The incidence of cell death was significantly higher in SOD1-KO than WT mice at day 3. Thus, a deficiency of SOD1 appears to be related to the high vulnerability of spermatogenic cells under the culture conditions used here. Spermatogenic cells were partially protected against heat by tiron, a superoxide-scavenging compound (Figure 5B). These data farther support the conclusion that ROS is involved in heat stress-induced cell damage.

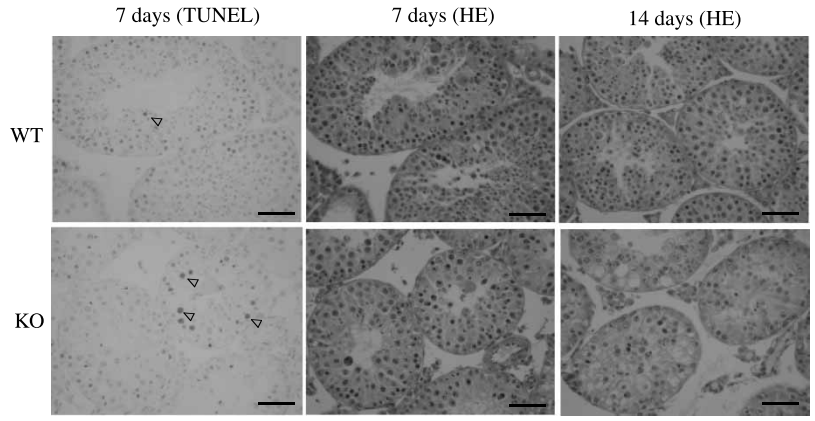

Figure 2. In situ detection of DNA fragmentation in WT and SOD1-KO mice. After the induction of experimental cryptorchidism, tissue sections were prepared from the isolated testes. A histological analysis was performed on HE-stained sections. Localization of apoptotic cells was detected by a TUNEL assay of a paraffin-embedded section from WT and SOD1-KO mice testes. Typical data at 7 and 14 days after cryptorchidism from a series of experiments were shown. Arrowheads; TUNEL-positive cells. (bar = 100 μm)

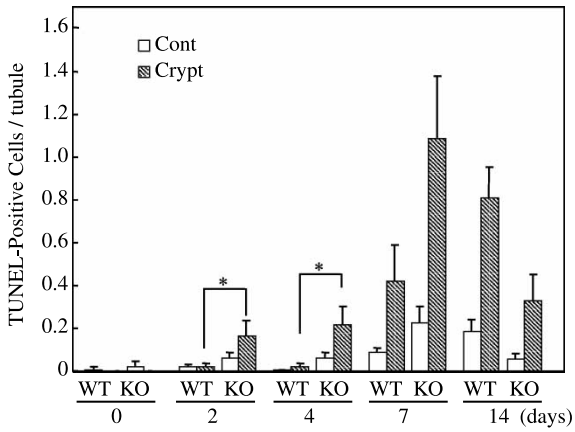

Figure 3. Comparison of the number of apoptotic cells between SOD1-KO and WT mice during experimental cryptorchidism. The TUNEL assay was performed for both SOD1-KO and WT mice at each time point. The number of TUNEL-positive cells per seminiferous tubules was counted for each mouse. (Numbers of animals were 3–8, *; $P < 0.05$ versus WT at the same time point).

Susceptibility of testicular cells to ROS

We examined the differential sensitivity of spermatogenic cells from Sertoli cells to ROS, the levels of which are assumed to be augmented by heat stress of the testes. Both spermatogenic cells and Sertoli cells were isolated from rats and cultivated separately at the normal testicular temperature of 32.5°C. These cells were incubated with 2 mM hypoxanthine and varying concentrations of xanthine oxidase, a ROS generator, that produces primarily superoxide, for 1 h. Cytosolic fractions were isolated and subjected to immunoblotting using the anti-Cyt c antibody (Figure 6). Cyt c was released into the cytoplasm from mitochondria in spermatogenic cells in a xanthine oxidase dose-dependent manner, while no change was observed in Sertoli cells. Thus, spermatogenic cells were found to be more susceptible to ROS than Sertoli cells. This profile of the two types of cells is consistent with the heat-induced Cyt c release in culture [28].

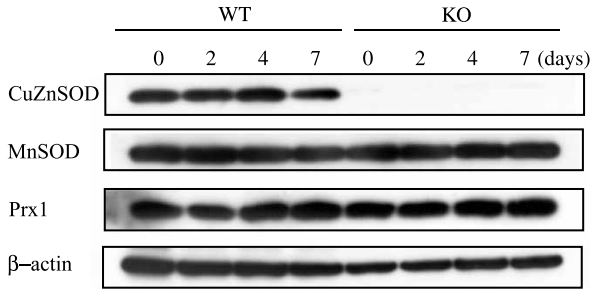

Figure 4. Immunoblot analysis of some antioxidative/redox proteins. Soluble proteins were prepared from testes at 0, 2, 4 and 7 days after the induction of cryptorchidism and subjected to immunoblot analysis using antibodies against SOD1, SOD2, Prx1, GR, and β-actin. Typical data of several experiments were shown.

Suppression of cytochrome c release from mitochondria by Tiron

We next determined whether or not superoxide was actually involved in the release of Cyt c into the cytosol from mitochondria in spermatogenic cells. Cultivated spermatogenic cells were exposed to a temperature at 42°C for 1 h. A longer exposure than that used to induce heat stress in testis is required in a culture system because the temperature of the medium is elevated gradually in a CO_2 incubator compared with the use of hot water bath. Tiron at a concentration 10 μM significantly suppressed the release of Cyt c from mitochondria (Figure 7). This suggests that superoxide, generated during hyperthermic conditions, is responsible for the release of cytochrome c.

Discussion

Data is presented that supports the involvement of ROS in heat-induced spermatogenic cell damage in *ex vivo* and *in vivo* heat stress models. Spermatogenic cells in primary culture appeared to be highly susceptible to ROS and died (Figure 6). Since tiron, a superoxide scavenger, suppressed the release of cytochrome c from mitochondria (Figure 7), an elevation in superoxide levels under these conditions and its involvement in the cytotoxic process can be expected. The generation of ROS will be augmented in response to an elevated metabolism accompanied by oxygen consumption. The contents of the redox enzymes glutathione reductase and aldo-keto reductase, which function in the detoxification of ROS and resulting carbonyl compounds, are much lower in spermatogenic cells than in Sertoli cells [29,33,34]. If ROS are a direct cause of cell death, the higher susceptibility of spermatogenic cells to elevated temperature can be attributed to the lower levels of these antioxidative/redox enzymes.

Although we did not observe significant elevation of ROS in cultured cells under heat-stress (data not shown), we indirectly demonstrated involvement of ROS in spermatogenic cell death. The use of SOD1-KO mice supports the view that ROS are mediators of cytotoxicity. The expression of various genes, including some antioxidative enzyme genes, is altered in response to stimuli in rat testes and, hence, can be regarded as stress responsible genes [35]. A decrease in the levels of SOD1 activity and mRNA levels have been reported in experimental cryptorchid testes of genetically normal animals [36,37]. We have also found that the processing of Prx4, a multifunctional redox protein with thioredoxin-dependent peroxidase activity, presumably plays a role in the spermatogenic process [38,39] and is altered in the cryptorchid testes of mice [40]. However, the levels of MnSOD and Px1 were

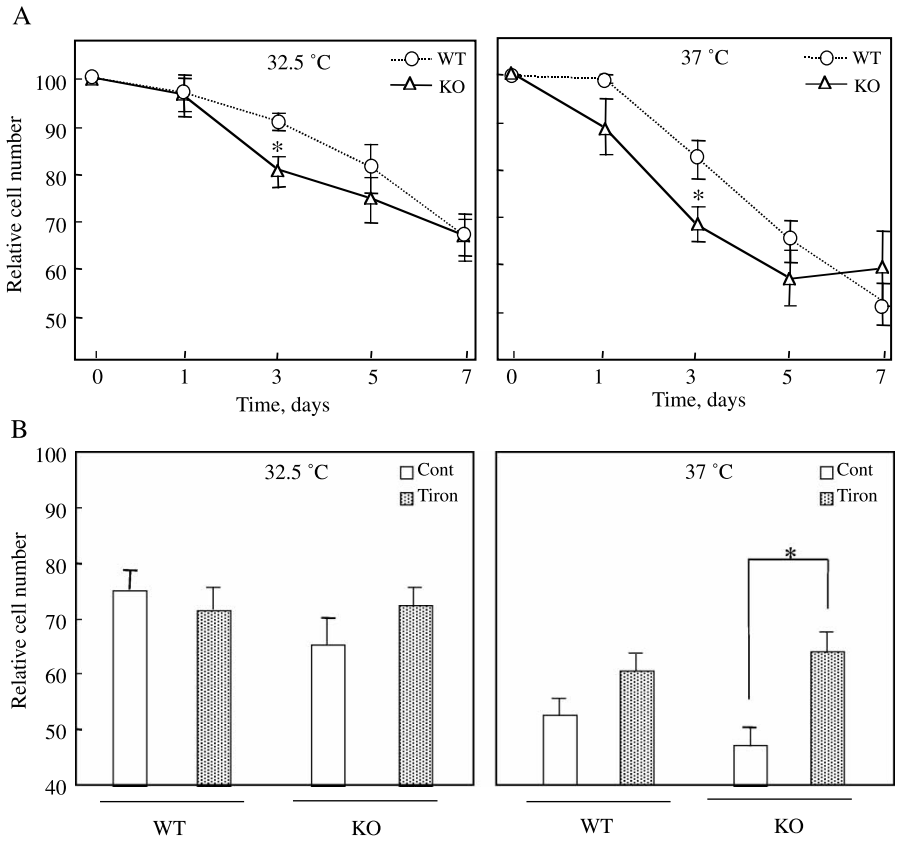

Figure 5. Comparison of the effects of heat stress on the vulnerability of spermatogenic cells between SOD1-KO and WT mice in culture. (A) After isolation from testes, the cultured cells were incubated at either 32.5 or 37°C for up to 7 days. ($n = 3$, *; $P < 0.05$ versus WT at the same time point). (B) Tiron was added to 10 μM into culture media at day 0 and further incubated at 32.5 or 37°C for 3 days. Number of cells was counted at each time point. ($n = 3$, *; $P < 0.05$ versus control).

unchanged during this period of cryptorchidism (Figure 4), suggesting a minimal role of these antioxidative/redox proteins in the heat-induced damage of spermatogenic cells.

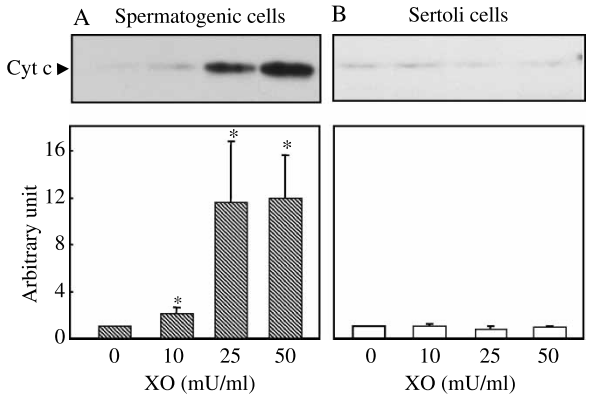

Figure 6. Effects of ROS produced from hypoxanthine/xanthine oxidase on the release of cytochrome c in spermatogenic and Sertoli cells. Spermatogenic cells (A) and Sertoli cells (B) were isolated from rat testis and cultivated at 32.5°C. After incubation with 2 mM hypoxanthine and varying concentrations of xanthine oxidase for 1 h, the cytosolic fraction was prepared from the cells. Proteins were analyzed by immunoblotting with an anti-cytochrome c monoclonal antibody. Cytochrome c bands on blots were quantified by densitometry. ($n = 3$, *; $P < 0.05$ versus without XO).

An elevation in lipid peroxidation products has been reported in experimental cryptorchidism in the case of mouse and rat testes [16]. Since testes are rich in unsaturated fatty acids, which are essential for sperm motility and highly susceptible to peroxidation, protection against oxidative modification by phospholipid hydroperoxide glutathione peroxidase (PhGPx) is of major importance [41]. Dysfunction in PhGPx and male infertility are highly correlated [42,43]. Thus, protection against oxidative stress, including lipid peroxidation, appears to be a central mechanism in the maintenance of male fertility. Although a linkage analysis between male infertility and genetic variations of PhGPx has been systematically carried out in men, the results were inconclusive [44]. Similarly, no correlation between a genetic defect of SOD1 and infertility has been reported. Further study will be required to clarify this point.

Isolated spermatogenic cells spontaneously die, even at the scrotum temperature 32.5°C, while Sertoli cells grow. A lack of support by Sertoli cells would be a major cause for this cell death. In addition, the hyperoxic environment in a CO_2 incubator, compared to the testis, could also be another factor. Hyperoxic conditions may enhance the production of ROS in cells during the metabolic process and impair cell

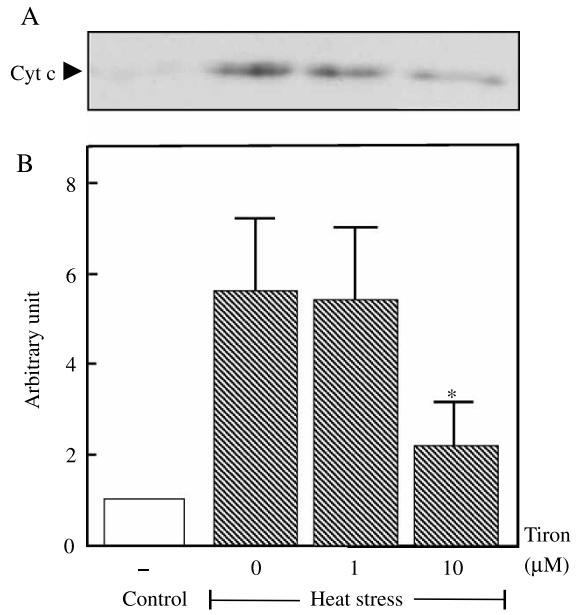

Figure 7. Effects of tiron on the heat stress-induced release of cytochrome c from spermatogenic cells. Spermatogenic cells were incubated at 42°C for 1 h in the presence or absence of tiron. After 30 min, the cytosolic fraction was prepared from the cells. Proteins were analyzed by immunoblotting with an anti-cytochrome c monoclonal antibody. Cytochrome c bands on blots were quantified by a densitometry. ($n = 3$, *; $P < 0.05$ versus the heat-stress group without tiron).

viability. An increase in ROS production by heat stress has been reported in spermatogenic cell cultures [15]. The origin of ROS may be xanthine oxidase activity because the use of allopurinol, a xanthine oxidase inhibitor, resulted in a lower number of TUNEL-positive cells and ameliorated spermatogenic cell damage in cryptorchid testes of rats [45]. Although vulnerable cells, such as pachytene spermatocytes and early spermatids die quickly, the remaining cells appear to be rather resistant [7]. This explains why the cell number under cultural conditions was significantly lower at day 3 only in SOD1-KO, compared to WT mice (Figure 5).

There is another possible explanation for the involvement of ROS in spermatogenic cell death by heat stress. Even a short period of heat exposure, e.g. 43°C for 15 min, triggers damage in spermatogenic cells without any apparent increase in ROS levels. This suggests that ROS generated under these conditions may function as a type of signal rather than a direct oxidant. In the intrinsic apoptotic pathway, ROS typically triggers the release of cytochrome c from mitochondria into the cytosol and leads to the activation of the caspase cascade [46]. ROS is known to modulate the function of signaling molecules, such as protein-tyrosine phosphatase [47] and G proteins [48]. Since an SOD1 deficiency would prolong the ROS signal, the acceleration in the testicular damage of SOD1-KO mice by heat stress might be induced by the production of a sustained signal for cell death.

In conclusion, since SOD1 deficiency accelerated testicular impairment and tiron suppressed cytotoxicity under heat stress, ROS are likely cytotoxic mediators in spermatogenic cells during heat stress. There are two possible pathways for the involvement of ROS. One is the oxidation of specific important molecules, such as unsaturated fatty acids [16], resulting in the direct impairment of cells. The other is signal mediation and the activation of the death pathway in response to heat stress. Even weak signal that is not harmful for somatic cells would cause a detrimental consequence in spermatogenic cells because of quality control of sperm. Further analysis of death signaling pathway in germ cells by heat stress would clarify this issue.

Acknowledgements

We thank Ms Masako Seki for maintenance of laboratory equipments and secretarial services. This work was supported, in part, by Grant-in-Aid for Scientific Research (C) (No. 16590238), JSPS Fellowship (No. 13007321), and 21st Century COE Program from Japan Society for the Promotion of Science (JSPS).

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