Xanthine oxidase inhibitors suppress testicular germ cell apoptosis induced by experimental cryptorchidism

Akiko Kumagai¹, Hideya Kodama²,³, Jin Kumagai¹, Jun Fukuda¹, Kazuhiro Kawamura¹, Hideo Tanikawa¹, Naoki Sato¹ and Toshinobu Tanaka¹

¹Department of Obstetrics and Gynecology, Akita University School of Medicine and ²Akita University College of Allied Medical Science, 1-1-1 Hondo, Akita City, 010-0041 Japan
³To whom correspondence should be addressed. E-mail: kodamah@ams.akita-u.ac.jp

Apoptotic degeneration of germ cells in cryptorchid testis is associated with an increased level of reactive oxygen species, and may be prevented by antioxidant treatment. The objective of this study was to investigate whether xanthine oxidase inhibitors could confer such protection. Unilateral cryptorchidism was surgically induced in the immature rats, which were then left untreated or treated with xanthine oxidase inhibitors, and the testes were evaluated 7 days after the operation. In the control group, the weight of the cryptorchid testis was decreased to 47% of that of the contralateral scrotal testis. However, administration of a xanthine oxidase inhibitor allopurinol (≥1 mg/kg/day) significantly attenuated weight reduction of the cryptorchid testis (68–77% of the contralateral scrotal testis, P < 0.01 versus control). Another highly specific xanthine oxidase inhibitor, BOF-4272, also attenuated cryptorchidism-induced testis regression. The effects of allopurinol were associated with decreased apoptosis in germ cells as evaluated by in-situ staining of fragmented DNA. In testicular cells cultured at 37°C, either allopurinol or BOF-4272 prevented DNA cleavage characteristic of apoptosis. In conclusion, xanthine oxidase inhibitors can inhibit germ cell apoptosis induced by experimental cryptorchidism, and may be considered for treatment of male infertility associated with heat stress.

Key words: apoptosis/cryptorchidism/male infertility/testis/xanthine oxidase

Introduction

Surgical induction of cryptorchidism in experimental animals causes rapid degeneration of testicular germ cells and infertility (Moor, 1924; Nelson, 1951; Davis and Firlit, 1966). The mechanisms whereby the germ cell degeneration is induced in the cryptorchid testis, have been attributed to testicular exposure to the suprascrotal abdominal temperature (Martti, 1967; Chowdhury and Steinberger, 1970; Blackshaw et al., 1973). The mechanisms underlying the process of germ cell demise in response to heat stress are not completely elucidated, but recent studies have shown the involvement of apoptosis, a form of programmed cell death (Shikone et al., 1994; Henriksen et al., 1995; Heiskanen et al., 1996; Yin et al., 1997; Ikeda et al., 1999).

The generation of reactive oxygen species (ROS) occurs constantly during normal cell metabolism in all living cells (Yu, 1994). Free radical generation that exceeds the capacity of antioxidant defences results in oxidative stress, which possibly elicits irreversible degenerative responses, including apoptosis or necrosis, in living cells (Buttke and Sandstrom, 1994). Experimental cryptorchidism in adult rats leads to increased peroxidation of cellular lipids, a sign of oxidative stress (Ahotupa and Huhtaniemi, 1992). The levels of lipid peroxidation are significantly increased either in untreated immature rats before normal testicular descent or in experimentally induced cryptorchidism (Peltola et al., 1995). These data indicate that there is an increased level of ROS in the cryptorchid testis, and this may contribute to apoptosis induction in testicular germ cells.

Although the level of ROS in the testis has been shown to be elevated in the cryptorchid testis, the origin of the free radicals is largely unknown. Xanthine oxidase is a widely distributed enzyme, especially in the microvascular endothelium. It converts hypoxanthine to xanthine and also xanthine to uric acid, with concomitant production of the superoxide anion. This endothelial-derived enzyme has received considerable attention as a source of ROS in post-ischaemic reperfusion injury (McCord, 1985; Granger et al., 1986; Kinuta et al., 1989). Allopurinol, a competitive xanthine oxidase inhibitor, is known to reduce such injury in several organ systems (Stewart et al., 1985; Werns et al., 1986). However, in the testis, the effects of allopurinol on the ischaemic damage of testis after acute spermatic cord torsion have been inconsistent (Akgur et al., 1994; Prillaman and Turner, 1997).
We hypothesized that apoptotic death of germ cells could be prevented by reducing the level of ROS in the cryptorchid testis, and, if the xanthine oxidase reaction is associated with the generation of ROS in the testis, inhibition of this enzyme could be effective in attenuating testicular cell apoptosis. In this study, we sought to determine whether administration of xanthine oxidase inhibitors could confer protection against apoptotic degeneration of testicular germ cells in experimentally induced cryptorchidism in rats. Our data have suggested possible use of xanthine oxidase inhibitors for the treatment of male infertility associated with heat stress.

Materials and methods

**Xanthine oxidase inhibitors and other chemicals**

Allopurinol was purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium-8-(3-methoxy-4-phenylsulphonylphenyl) pyrazolo [1,5-a]-1,3,5-triazine-4-olate monohydrate (BOF-4272) was obtained from Otsuka Pharmaceutical Factory, Inc. (Naruto, Japan). Other chemicals used were purchased from Sigma.

**Unilateral experimental cryptorchidism**

Male Wistar rats (40 days of age) were purchased from Nihon SLC Co. (Shizuoka, Japan). Unilateral cryptorchidism was experimentally induced in the rats under pentobarbital anaesthesia according to previously described methods (Shikone et al., 1994). Briefly, a midline abdominal incision was made, and the left testis was displaced from the scrotum to the abdomen. After the gubernaculum was cut to prevent testis descent, the testis was sutured to the abdominal wall. The right testis remained in the scrotum as an euthermic control. The testis weight over time within each group was determined using one-way analysis of variance (ANOVA) followed by the Scheffé’s F-test for multiple comparisons. Differences in the testis weight over time within each group were determined using ANOVA with repeated measures. Statistical significance was determined at the 5% level.

**Detection of apoptotic cells by in-situ analysis**

After measurement of testis weight, the testicular tissue was fixed in 20% formalin solution, embedded in paraffin, and cut into 3µm sections. The sections were stained by the haematoxylin–eosin method. Apoptotic cells were identified by in-situ analysis using the ApopTag™ kit (Oncor, Gaithersburg, USA), according to the manufacturer’s instructions. After in-situ staining, the number of apoptotic cells in 20 cross-sections of the seminiferous tubules was counted, and the mean values of apoptotic cells per testis section were determined.

**Culture of testicular germ cells**

Rats were killed by ether anaesthesia, and testicular cells were isolated by a published method (Nagao, 1989) with slight modifications. Briefly, testes were removed and decapsulated mechanically. Seminiferous tubules were gently excised and incubated in phosphate-buffered saline (PBS) containing 0.25% collagenase (Type 1) for 15 min at 32.5°C with occasional shaking. Then, the seminiferous tubules were washed, and incubated again in PBS containing 0.25% trypsin (Difco, Detroit, USA) for 10 min at 32.5°C with occasional shaking. After incubation, the trypsin treatment was terminated by adding fetal bovine serum (FBS; Lifeteck Oriental Co., Tokyo, Japan) to 10% (vol/vol). The resulting cell suspension was filtered through a 106µm nylon mesh to remove cell aggregates and tissue debris, and then the cells were collected by centrifugation. The cells recovered were resuspended in F12-L15 medium (1:1 mixture of Ham’s F12 and Lebovitz’s L15; Lifeteck Oriental Co.) supplemented with 1mg/ml of sodium bicarbonate, 100 IU/ml of penicillin-G, 100 mg/ml of streptomycin sulphate, 14 mg/ml of Phenol Red, and 10% FBS. The final concentration of testicular cells in the medium was adjusted to ~5×10⁶/ml. Two ml of the cell suspension was plated in each well of a 6-well C-1 plate (Sumitomo Verkleit Co., Tokyo, Japan). The cells were incubated in a humidified atmosphere of 5% CO₂ in air at 32.5°C, an optimal temperature for testicular germ cells, for 2 days prior to the experiments described below.

**DNA electrophoresis**

For analysis of DNA fragmentation by agarose gel electrophoresis, total DNA was isolated from testicular cells using a commercial DNA extraction kit (Apopt Ladder EXTM Kit; Takara Syuzou Co., Tokyo, Japan). The isolated DNA was suspended in TE buffer (10 mmol/l Tris–HCl, 1 mmol/l EDTA, pH 7.5) and quantified by absorbance at 260 nm. The DNA samples, 2µg per each lane, were loaded onto 2.0% agarose gel (agarose LE; Nacalaitesque, Kyoto, Japan) containing TAE buffer (40 mmol/l Tris-acetate, 2 mmol/l EDTA, pH 8.0), separated by electrophoresis for 30 min at 100V and stained by ethidium bromide. DNA bands were visualized by a UV transilluminator (TM-15; Funakoshi Co.) before being photographed with a Polaroid camera (Cambridge, MA, USA).

**Data analysis**

The data were expressed as means ± SEM. Differences of the tests weight between groups were determined using one-way analysis of variance (ANOVA) followed by the Scheffé’s F-test for multiple comparisons. Differences in the testis weight over time within each group were determined using ANOVA with repeated measures. Statistical significance was determined at the 5% level.


Results

Effects of xanthine oxidase inhibitors on testicular weight after experimental cryptorchidism in rats

Allopurinol at different concentrations (0.1–100 mg/kg) or 0.1 mol/l NaHCO₃ (control) was administered i.p. on a daily basis for 7 days to rats after surgical induction of unilateral cryptorchidism. The testes from each animal were then removed on day 7 to determine the testis weight (Figure 1). The weight of the cryptorchid testis in the control group was decreased to 47 ± 2% of that of the contralateral scrotal testis. Allopurinol at a concentration of 1 mg/kg/day conferred significant protection against such weight reduction of the cryptorchid testis. The maximum inhibition was obtained when 10 mg/kg/day of allopurinol was administered daily as the cryptorchid testis weight was only 77 ± 4% of the contralateral scrotal testis weight (P < 0.01 versus control).

Histological findings of the cryptorchid testis with or without allopurinol administration for 7 days are shown in Figure 2. In the control cryptorchid testis, most germ cells have disappeared from the seminiferous tubules, whereas in the cryptorchid testis after treatment with allopurinol (10 mg/kg/day), a considerable number of germ cells were still present in most seminiferous tubules.

Administration of BOF-4272 (300 µg/kg), a more specific xanthine oxidase inhibitor, also provided a significant inhibitory effect on weight reduction induced by experimental cryptorchidism, as the cryptorchid testis weight was 83 ± 2% of the contralateral scrotal testis weight, compared with 47 ± 2% in the control group (P < 0.01 versus control). This degree of inhibition was comparable to that caused by allopurinol (50 mg/kg) treatment (Figure 3).

Effects of allopurinol on the timing of testicular weight changes and the occurrence of apoptosis after experimental cryptorchidism

After induction of unilateral cryptorchidism, 10 mg/kg of allopurinol or 0.1 mol/l NaHCO₃ (control) was administered daily, and testes were removed on days 1, 3, 5 and 7. The weight of the testis was determined before histological staining for apoptotic cells (Figures 4 and 5). In both groups, the weights of the cryptorchid testis slightly increased on day 1, and then decreased progressively. However, the rate of reduction on and after day 5 was significantly inhibited in the group treated with allopurinol (Figure 4b). As for the histological staining of the cryptorchid testis, in the control group (Figures 4c and 5a) the number of apoptotic cells per section of seminiferous tubules was remarkably increased from day 3 to day 5, and reached 37.5 ± 2.1 cells per tubule section on day 5. Positive staining was observed most frequently in primary spermatocytes, recognizable by their large size and location near the epithelium. After that, most of the degenerating germ cells disappeared on day 7. In the group treated with allopurinol, the number of apoptotic cells was only gradually increased from day 1 to day 7. The rate of increase was much slower than in the control group and a great increase of apoptotic cells on day 5 was not observed (Figure 5b).

Effects of allopurinol and BOF-4272 on heat stress-induced DNA fragmentation cultured testicular germ cells

In order to confirm that allopurinol and BOF-4272 could inhibit heat stress-induced apoptosis of testicular germ cells, 2 mmol/l allopurinol or 300 µmol/l BOF-4272 was added to isolated testicular germ cells, and incubated at 32.0 or 37.0°C. Appearance of fragmented DNA was determined by agarose gel electrophoresis after 24 h of culture (Figure 6). The control cells cultured at 37.0°C demonstrated ladders of fragmented DNA (lane 1), whereas, cells cultured at 37.0°C and treated with allopurinol (lane 2) or BOF-4272 (lane 3) demonstrated minimal DNA fragmentation, consistent with low levels of apoptosis through the study period. DNA fragmentation was not detected in control cells incubated at 32.0°C (lane 4).

Discussion

The present data support the hypothesis that apoptotic death of germ cells in experimental cryptorchidism can be prevented by administration of xanthine oxidase inhibitors. Although allopurinol inhibits the xanthine oxidase-catalysed production of uric acid and the superoxide anion, allopurinol may also...
Xanthine oxidase inhibitors suppress testicular apoptosis

Figure 3. Effects of BOF-4272 administration on weight reduction of cryptorchid testis on day 7 after induction of unilateral cryptorchidism. The weight of the cryptorchid testis \((n = 4\) for each experimental group) on day 7 after induction of cryptorchidism and daily administration of BOF-4272 \((300 \mu mol/l/kg)\), allopurinol \((50 mg/kg)\) or \(0.1 mol/l NaHCO_3\) (control) is presented as the percentage of the weight of the contralateral scrotal testis. There were no significant differences in the absolute scrotal testis weight between the control and treatment groups \((0.60 \pm 0.04, 0.57 \pm 0.05\) and \(0.58 \pm 0.04\) g). Data are presented as means \(\pm\) SEM. **\(P < 0.01\) versus control.

Xanthine oxidase inhibitors exert protective effects through another mechanism. For example, both allopurinol and its metabolite, oxypurinol, have been reported to function as direct free radical scavengers (Moorhouse et al., 1987). In order to confirm that the effect of allopurinol is actually attributed to inhibition of xanthine oxidase, a more specific xanthine oxidase inhibitor, BOF-4272, was tested (Okamoto and Nishino, 1995). Both xanthine oxidase inhibitors, allopurinol and BOF-4272, were found to attenuate testicular weight reduction induced by cryptorchidism in the rats. The effect of allopurinol appears to be mediated by an inhibition of germ cell apoptosis, since the remarkable increase in the number of apoptotic germ cells in the cryptorchid testis on day 5 after operation was not observed in the group treated with allopurinol. In addition, formation of the DNA ladder characteristic of apoptosis in cultured testicular cells under heat stress was inhibited by addition of the xanthine oxidase inhibitors.

The present data also indicate the importance of xanthine oxidase as a source of ROS in the rat testis. Xanthine oxidase is known to be abundant within the microvascular endothelium of all organs (Ahotupa and Muhtaniemi, 1992; Buttke and Sandstrom, 1994; Peltola et al., 1995). Xanthine oxidase activity has been detected in the testis of humans (Yaman et al., 1999) and rats (Koizumi and Li, 1992), and has been implicated in the mechanisms of testicular damage induced by reperfusion or toxic chemicals. Detorsion after experimental induction of testicular torsion in rats has been shown to significantly increase the level of lipid peroxidation in the testis, and pretreatment with allopurinol before detorsion prevents such reperfusion injury (Akgur et al., 1994). With severe haemorrhagic damage in the testis induced by a single carcinogenic dose of cadmium chloride, xanthine oxidase activity in the Leydig cells, the target cell population for cadmium carcinogenesis, is elevated (Koizumi and Li, 1992), and testicular damage induced by cadmium chloride can be prevented by oxypurinol or superoxide dismutase (Agarwal et al., 1997). The localization of xanthine oxidase in the testis has not been investigated, and whether xanthine oxidase activity is present in the testicular germ cells remains to be determined. A highly specialized membrane-associated NADPH oxidase, which is located on the cell membrane of

Figure 4. Time-course of changes in testis weight and the number of apoptotic cells in the cryptorchid testis after surgical induction of cryptorchidism and daily administration of allopurinol. After induction of unilateral cryptorchidism in the rats, 50 mg/kg of allopurinol or \(0.1 mol/l NaHCO_3\) (control) was administered daily, and the testes were removed on days 1, 3, 5 or 7, then weighed, and subjected to in-situ analysis for staining of apoptotic cells. The absolute weights of the scrotal testis in each group are shown in (a) (control, □; allopurinol treatment, ■). A time-course of the cryptorchid testis weight changes was presented as the percentage of the contralateral scrotal testis weight in (b) (control, ---; allopurinol treatment, ■). A time-course of changes in the number of apoptotic cells per each seminiferous tube section in each day group is presented in (c) (control, ---; allopurinol treatment, —-). Data are presented as mean \(\pm\) SEM. **\(P < 0.01\) versus control.
neutrophils and macrophages, may be another important source of ROS in vivo (Babior, 1978). Recently, this enzyme has been implicated in the changes of redox status during maturation of mammalian spermatozoa when they pass through the epididymis (Aitken and Vernet, 1998).

Although levels of free radicals have been shown to increase in the cryptorchid testis, whether elevation of testicular temperature induces xanthine oxidase in the testis remains to be determined. Under normal circumstances, xanthine dehydrogenase is the predominant enzyme that metabolizes hypoxanthine and xanthine, but xanthine dehydrogenase is converted to xanthine oxidase in the ischaemic tissues (Battelli et al., 1973). In addition, sulphydryl oxidation of xanthine dehydrogenase may also lead to formation of xanthine oxidase in various rat tissues (McKelvey et al., 1988). Whether oxidative stress in the cryptorchid testis involves xanthine dehydrogenase or is simply due to inactivation of antioxidant enzymes (Ahotupa and Huhtaniemi, 1992) remains to be determined.

From the clinical perspective, this study has supported the possible use of xanthine oxidase inhibitors for treatment of cryptorchidism and other types of male infertility associated with the elevation of testicular temperature, such as varicocele. The possible use of xanthine oxidase inhibitors for the treatment of these types of male infertility should be investigated in the future.

Acknowledgement
The authors thank professor Aaron J.Hsueh at division of Reproductive Biology of Stanford University School of Medicine for his help in preparation of this manuscript.

References
Xanthine oxidase inhibitors suppress testicular apoptosis


Received on April 17, 2001; accepted on October 18, 2001