Arsenic inhibits *in vitro* spermatogenesis and induces germ cell apoptosis in Japanese eel (*Anguilla japonica*)

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Abstract

The precise mechanism and direct effects of arsenic on fish, particularly in reproduction, are not well clarified. The aim of this study is to investigate the direct influence of arsenic on fish spermatogenesis using the Japanese eel (*Anguilla japonica*) *in vitro* testicular organ culture system. Eel testicular fragments were cultured *in vitro* with 0.1–100 μM arsenic with or without human chorionic gonadotropin (hCG) for 6 or 15 days at 20 °C. Arsenic treatment provoked a dose-dependent inhibition of hCG-induced germ cell proliferation as revealed by 5-bromo-2-deoxyuridine immunohistochemistry. Time-resolved fluorescent immunoassay showed that arsenic suppressed hCG-induced synthesis of 11-ketotestosterone (11-KT) in testicular fragments incubated with 0.0001–100 μM arsenic and hCG for 18 h. A 0.1 μM (7 $\mu\text{g/l}$) dose of arsenic which is lower than the World Health Organization drinking water quality guideline of 10 $\mu\text{g/l}$ most effectively reduced 11-KT production. The hCG-induced synthesis of progesterone from pregnenolone was significantly inhibited by low doses of arsenic (0.1–1 μM), implying an inhibition of 3 β -hydroxysteroid dehydrogenase activity. *In situ* TUNEL assays indicated that germ cells undergo apoptosis at the highest dose of arsenic (100 μM). An arsenic concentration-dependent increase in oxidative DNA damage was detected by 8-hydroxy-2'-deoxyguanosine (8-OHdG) immunohistochemistry. A peak in 8-OHdG index was observed in testicular fragments treated with 100 μM arsenic and hCG consistent with the TUNEL results. These data suggest that low doses of arsenic may inhibit spermatogenesis via steroidogenesis suppression, while high doses of arsenic induce oxidative stress-mediated germ cell apoptosis.

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Introduction

Arsenic, a trace element ubiquitously present in the environment and one of the most significant pollutants of aquatic systems, has been widely known to cause deleterious effects to humans and wildlife. Inorganic arsenic contamination is a serious environmental problem worldwide due to the large number of contaminated sites that have been identified, particularly in Asia. Arsenic contamination in drinking water has become a significant concern in Bangladesh, West Bengal, India, China, Mongolia, Nepal, Cambodia, Myanmar, Afghanistan, DPR Korea, and Pakistan (Mukherjee *et al.* 2006). Arsenic has also been reported to contaminate marine environments (Kubota *et al.* 2002), freshwater environments (Cullen & Reimer 1989), and groundwater (Alam *et al.* 2002).

Arsenic induces a broad range of effects including endocrine disruption (Kaltreider *et al.* 2001, Bodwell *et al.* 2006, Davey *et al.* 2007). Endocrine disruptors are defined as any substance that can interfere with normal hormone function. Chemicals that can act as endocrine disruptors, mimic or block function of

normal hormones and trigger an abnormal response in cells include a number of either industrial, municipal, and agricultural chemicals released into the environment or naturally occurring compounds. The toxicity of arsenic as a potent endocrine disruptor has been extensively studied in mammals. It was reported that arsenic alters gene regulation by disruption of the closely related steroid hormone receptors for glucocorticoids, mineralocorticoids, progesterone, and androgen (Kaltreider *et al.* 2001, Bodwell *et al.* 2004, 2006) functions. However, studies pertaining to the precise mechanism of direct influence of arsenic on fish reproduction are very limited.

Fish reproduction has been considered to be a reliable indicator of endocrine disruption in aquatic systems by chemical compounds including arsenic. In the freshwater fish *Colisa fasciatus* (striped gourami), high doses of arsenic caused testicular abnormalities and impaired spermatogenesis *in vivo* (Shukla & Pandey 1984). Arsenic is present in the environment as trivalent arsenite (As(III)) or pentavalent arsenate (As(V)). Of the two forms, As(V) typically dominates both in oxic sea and

freshwater (Smedley & Kinniburgh 2002, Duker *et al.* 2005); consequently, fish are likely to be exposed to As(V). However, few studies described the toxicity of the less toxic form, As(V), on fish reproduction. A recent study in Mekong Delta of Vietnam has reported that there is a significant correlation between the gonadal morphology and development abnormalities, and accumulation of trace elements including arsenic in the liver of catfish, *Pangasianodon hypophthalmus*. It was also demonstrated that *in vitro* treatment of As(V) inhibited spermatogenesis in Japanese eel (Yamaguchi *et al.* 2007). However, the mechanism involved in the direct influence of arsenic on fish spermatogenesis is not yet well clarified.

In this study, the direct effects and toxic mechanisms of arsenic, in the pentavalent form, on fish spermatogenesis were examined using the Japanese eel testicular organ culture system. In Japanese eel, spermatogenesis could be induced by human chorionic gonadotropin (hCG) injection *in vivo* or treatment with 11-ketotestosterone (11-KT, a fish-specific androgen derived from testosterone) or hCG *in vitro*. In vertebrates, this is the only system available for the induction of complete spermatogenesis *in vitro* from spermatogonial proliferation to spermiogenesis (Miura *et al.* 1991a). Various factors that play important roles in fish spermatogenesis, have been identified using this method (Miura *et al.* 2006, Ozaki *et al.* 2006). Moreover, this system has been used to study the effects of pollutants on fish spermatogenesis (Miura *et al.* 2005, Yamaguchi *et al.* 2007). Hence, this system is used for investigating the direct effects and toxic mechanisms of arsenic on fish spermatogenesis.

Results

Effects of arsenic on spermatogenesis

We analyzed the effects of arsenic on fish testis *in vitro* with or without hCG. Testicular fragments of the control group contained type A spermatogonia after 6 days of culture. The histological structure of testis of the control group and those cultured with hCG alone did not differ from each other (Fig. 1A and B). However, germ cells in testis treated with 100 μ M arsenic alone or with hCG exhibit cell death. Moreover, extensive cell death and fibrotic hypertrophy of interstitial tissue were observed in groups treated with 100 μ M arsenic and hCG compared with those treated with arsenic alone (Fig. 1C and D). There was no observed change in histological structure such as cell death, degeneration, or hypertrophy in testis exposed to 0.1–10 μ M of arsenic with or without hCG (data not shown).

The influence of arsenic on the progression of spermatogenesis analyzed by 5-bromo-2-deoxyuridine (BrdU) incorporation in germ cells is shown in Figs 2A–G and 3. After treatment with hCG, the number of proliferating germ cells increased after 6 days (Figs 2A and B

Figure 1 Representative light micrographs of Japanese eel testicular fragments cultured for 6 days. (A) Control (B) 0.05 IU/ml hCG. Normal morphology was observed with many spermatogonia. (C) 100 μ M arsenic. Some dead germ cells were observed. (D) 100 μ M arsenic with hCG. A notable increase in the number of dead germ cells was observed with fibrotic enlargement of interstitial tissue. dG, dead germ cells; SG, spermatogonia; *, enlarged interstitial tissue. Bars, 20 μ m.

and 3A) and 15 days of culture (Fig. 3B). However, addition of arsenic significantly inhibited hCG-induced spermatogenesis in a dose-dependent manner (Figs 2C–F and 3A and B). Even the lowest dose of arsenic (0.1 μ M) significantly inhibited hCG-induced germ cell proliferation ($P < 0.05$) in both 6 and 15 day-cultured testicular fragments (Figs 2C and 3A and B). In 15-day cultures treated with 100 μ M arsenic and hCG, not even a single BrdU-positive germ cell was detected due to extensive cell death (Figs 2G and 3B). Treatment with arsenic alone at lower doses (0.1–10 μ M) seemed to have no effect on germ cell proliferation both at 6 and 15 days of culture. However, at the highest dose of arsenic (100 μ M) alone, the number of BrdU-positive germ cell significantly decreased at 15 days of culture (data not shown).

In vitro effects of arsenic on 11-KT synthesis and steroidogenic enzyme activity

Figure 4 shows the effects of arsenic on 11-KT synthesis in hCG-activated testicular fragments of Japanese eel. The results showed that hCG induced 11-KT production in testis. Interestingly, arsenic inhibited hCG-induced 11-KT synthesis in a dose-dependent manner. The most effective concentration of arsenic was 0.1 μ M. However, while 11-KT level was significantly lower than the positive control (hCG alone-treated group) at one low dose (0.1 μ M), at the other higher arsenic doses used (1–100 μ M) there was no significant difference

Figure 2 BrdU immunohistochemistry in sections of eel testicular tissue cultured for 6 days in 0.1–100 μM arsenic with hCG and 15 days in 100 μM arsenic with hCG. Brown-stained cells are BrdU-positive cells (arrowheads). (A) Control (B) 0.05 IU/ml hCG. An increase in the number of proliferating germ cells was observed. Addition of arsenic in an increasing concentration of 0.1 μM (C), 1 μM (D), 10 μM (E), and 100 μM (F) gradually decreased the number of proliferating germ cells induced by hCG and caused cell death at the highest dose. After 15 days of culture, no BrdU-positive germ cells were observed in group treated with 100 μM arsenic and hCG (G). dG, dead germ cells. Bars, 20 μm .

compared with the positive control. To clarify the mechanism involved in 11-KT synthesis inhibition by arsenic, we analyzed the low-dose effect of arsenic on steroidogenic enzymes' activity. A considerable effect was observed on progesterone synthesis using pregnenolone as a precursor. A similar trend was observed to that of the low-dose arsenic effect on 11-KT synthesis in which at 0.1 and 1 μM arsenic, progesterone level significantly decreased compared to the positive control (Fig. 5).

Apoptosis of germ cells

In order to determine whether arsenic-induced cell death is apoptosis or necrosis, TUNEL assay was conducted. The positive control group contained many TUNEL-positive cells, germ, and some somatic cells. In contrast, control group did not contain any

TUNEL-positive cell (Fig. 6A and B). In sections of testicular fragments treated with 100 μM arsenic alone, a few germ cells showed positive reaction. On the other hand, in those treated with 100 μM of arsenic and hCG, numerous germ cells exhibit positive signal compared with those treated with arsenic alone (Fig. 6C and D).

Induction of oxidative stress by arsenic

When cells are attacked by reactive oxygen species (ROS), DNA is oxidized, and deoxyguanosine, one of its constituents, is altered into 8-hydroxy-2'-deoxyguanosine (8-OHdG). Hence, this 8-OHdG is used as an indicator of oxidative damage in cells. Immunohistochemistry for 8-OHdG revealed that control and hCG-treated testicular fragment sections were not stained (Fig. 7A and B). Whereas, testicular fragments incubated with arsenic and hCG showed strong signal and many intensely stained germ cells compared with those treated with arsenic alone (Fig. 7C and D). In addition, the densitometric quantitation of 8-OHdG immunohistochemistry showed an increasing trend in 8-OHdG index with increasing arsenic concentration both in arsenic alone and arsenic with hCG-treated groups. At arsenic concentration of 10 μM with or without hCG, 8-OHdG index was already significantly different from control ($P < 0.05$) and from hCG alone-treated group ($P < 0.01$) respectively. A peak in 8-OHdG index ($7.5 \times 10^3 \pm 2.9 \times 10^3$ pixels/cell) was observed in the same treatment

Figure 3 Effects of arsenic on hCG-induced germ cell proliferation *in vitro* (A) after 6 and (B) 15 days culture. Control, without arsenic and hCG. The number of positively immunoreacted germ cells is expressed as a percentage of the total number of germ cells. Values are means $\pm$ S.E.M. ($n = 5$). Values with different letters are significantly different ($P < 0.05$). As, arsenic; N.D., BrdU-positive cells were not detected.

Figure 4 Effect of arsenic on hCG-induced 11-KT synthesis *in vitro*. Values are means $\pm$ S.E.M. Values with different letters are significantly different ($P < 0.05$). Control, without arsenic and hCG; As, arsenic.

(100 μ M arsenic and hCG) that strongly caused apoptosis. There was no significant difference between doses other than the highest concentration of arsenic and arsenic with hCG-treated groups except for 10 μ M arsenic ($P < 0.05$). Moreover, the 8-OHdG index at the highest concentration of arsenic alone was significantly lower ($P < 0.01$) compared with those treated with arsenic and hCG (Fig. 8).

Discussion

Although previous data showed that there is a significant correlation between the alterations in histological structure and abnormalities in the development of testis, and accumulation of arsenic in collected fish (Yamaguchi *et al.* 2007), the biochemical basis of arsenic toxicity and the mechanism involved in this process are not yet fully understood. In the present study, we demonstrate that arsenic at high concentrations (100 μ M), alone or with hCG, causes degenerative

changes in eel testis and induces germ cell death *in vitro* in eel testis cultures. In groups cultured in arsenic with hCG, almost all testicular fragments died and exhibited severe enlargement of interstitial tissue. In previous studies, similar effects of arsenic have been reported. Alterations in testicular morphology, such as degenerative changes in the lobules, reduction in diameter of interstitial Leydig cells (steroid-secreting cells), necrosis, and pyknosis, were observed in the freshwater fish, *C. fasciatus*, following exposure to high doses of arsenic (Shukla & Pandey 1984). Moreover, in an *in vitro* study using Japanese eel (Yamaguchi *et al.* 2007), treatment with high doses of arsenic and 11-KT, a major active androgen in teleosts (Borg 1994), induced cell death. To further analyze the influence of arsenic on spermatogenesis, we investigated the effects of arsenic on germ cell proliferation. Similar to previous studies (Miura *et al.* 1991a, 1991b), our study showed that hCG induced germ cell proliferation via 11-KT, as assessed by the BrdU index. Moreover, treatment with arsenic alone has no effect on germ cell proliferation except at the highest dose (100 μ M), which induced cell death, while treatment with arsenic and hCG significantly inhibited germ cell proliferation dose dependently. Furthermore, a low dose of arsenic (0.1 μ M), in the presence of hCG, already significantly suppressed germ cell proliferation. In mammals, reduced sperm count and motility after oral administration of sodium arsenite in mice (Pant *et al.* 2001) and early arrest of spermatogenesis after i.p. administration of sodium arsenite in rats (Ahmad *et al.* 2008) have been observed. Thus, previous published literature using *in vivo* experiments indicates that arsenic inhibits spermatogenesis similar to the conclusion of this *in vitro* work on the Japanese eel.

Previous *in vitro* studies by Miura *et al.* (1991b) and Ozaki *et al.* (2006) have revealed that eel testicular fragments produce 11-KT in response to hCG. Since 11-KT is a major active androgen in teleost fish that initiates and maintains the progression of spermatogenesis through the action of Sertoli cells (Miura & Miura 2003), we assumed that arsenic may exert its effect on spermatogenesis via 11-KT. To confirm the influence of arsenic on 11-KT production, we investigated the effects of arsenic on hCG-induced 11-KT synthesis *in vitro*. Our results showed that 11-KT synthesis was inhibited by treatment with arsenic, particularly at a low dose of 0.1 μ M (7 μ g/l) – a dose environmentally relevant and relatively lower than the World Health Organization guideline for drinking water quality (10 μ g/l; WHO 1996). This result suggested that low-level exposure to arsenic may inhibit spermatogenesis via inhibition of 11-KT synthesis. Sarkar *et al.* (1991) reported that injection of As(III) in rats resulted in the inhibition of β 3-hydroxysteroid dehydrogenase (HSD3B) and HSD17B activities in the testis. In our study, we examined whether inhibition of 11-KT synthesis by low-dose arsenic is mediated by inhibition of

Figure 5 Effect of low-dose arsenic on hCG-induced progesterone (P4) synthesis from pregnenolone (P5) *in vitro*. Values are means $\pm$ S.E.M. Values with different letters are significantly different ($P < 0.05$). Control, without arsenic and hCG; As, arsenic.

Figure 6 TUNEL assay in eel testicular tissue cultured for 3 days with 100 µM arsenic alone or 100 µM arsenic with hCG. Positively stained germ cells are indicated by arrowheads. (A) Positive control treated with DNase before TUNEL. (B) Control. TUNEL-positive germ cells were not observed. (C) 100 µM arsenic-treated group. Some TUNEL-positive germ cells were observed. (D) 100 µM arsenic with hCG-treated group. Note the increase in number of germ cells with positive TUNEL labeling. Bars, 20 µm.

steroidogenic enzyme activity. Our findings demonstrate that low doses of arsenic significantly inhibit progesterone synthesis from pregnenolone, which is a similar trend to that observed for the effects of low doses of arsenic on 11-KT synthesis, except that a significant difference was also found at 1 µM of arsenic. It is known that HSD3B is the enzyme that catalyzes the conversion of pregnenolone to progesterone, and other Δ^5 -3 β -hydroxysteroids to Δ^4 -3 ketosteroids, an essential step in the biosynthesis of all biologically active steroids (Keeney *et al.* 1993) including 11-KT, implying that HSD3B activity may be affected by low doses of arsenic.

In contrast to the lowest dose, moderately higher doses (1–10 µM) of arsenic seemed to have no significant effect on 11-KT production unlike their significant effect on germ cells proliferation. The reason for these discrepancies is unclear. It has been demonstrated that treatment of 1.25–10 µM arsenite induces an increased expression of TP53, a checkpoint protein in cell cycle progression, in human fibroblast (Yih & Lee 2000). Activation of TP53 can result in cell cycle arrest (Lane 1998). Also, in human skin fibroblast, moderate doses (1.25–5 µM) of arsenic caused chromosomal aberrations and chromosomal loss, and G2 arrest (particularly at a 5 µM concentration) via altering levels of the mitotic regulatory proteins, cyclin B1 and CDC2 (Yih *et al.* 1997). Hence, moderately high sublethal doses of arsenic may directly inhibit mitotic germ cell proliferation via these signal transduction pathways.

Moreover, arsenic at the highest dose induced a considerable decrease in hCG-induced germ cell proliferation. Although hypertrophy of interstitial tissue was observed with the highest dose of arsenic, 11-KT production was not significantly affected. In teleost, 11-KT is synthesized by interstitial Leydig cells in testis, which leads to induction of spermatogenesis (Miura *et al.* 1991a). Hence, our data imply that the effects of the highest dose of arsenic on germ cell proliferation are not via altering steroid production by Leydig cells, but rather may have direct detrimental action on germ cells.

Furthermore, significant germ cell death was also induced at the highest dose of arsenic. TUNEL assay results revealed a positive staining in germ cells of testis exposed to the highest dose of arsenic alone or with hCG. Moreover, numerous germ cells exhibit positive signal in testis exposed to the highest dose of arsenic combined with hCG. These data imply that high doses of arsenic induce germ cell apoptosis especially after induction of spermatogenesis by hCG. Although there is no such report of arsenic-induced germ cell apoptosis in any other teleosts, in the freshwater fish, *C. fasciatus*, exposure to high dose of arsenic resulted in altered spermatogenesis associated with pyknosis (Shukla & Pandey 1984). Pyknosis or irreversible condensation of chromatin in the nucleus of a cell is one of the typical and important features of apoptosis (Zamzami & Kroemer 1999). Previous studies have shown arsenic to induce

Figure 7 Immunohistochemistry for 8-OHdG in testicular tissue cultured for 3 days in 100 µM arsenic alone or 100 µM arsenic with hCG. Positively stained germ cells are indicated by arrows. (A) Control. (B) hCG-treated group. No germ cells were stained. (C) 100 µM arsenic-treated group. Few germ cells were weakly stained. (D) 100 µM arsenic with hCG-treated group. Numerous germ cells showed strong 8-OHdG positive staining. Bars, 20 µm.

Figure 8 Densitometric quantitation of 8-OHdG immunostaining in arsenic alone and arsenic with hCG-treated testicular fragments. Values are means $\pm$ s.e.m. ($n=5$). As, arsenic; -, without hCG; +, with hCG. †Significantly different from control without hCG ($P<0.05$). *Significantly different from control with hCG ($P<0.01$).

apoptosis in hepatocyte cancer cell lines *in vitro* (Zhang *et al.* 2003) and in *Caenorhabditis elegans* germline *in vivo* (Pei *et al.* 2008). Moreover, it has also been reported that As(III) causes the release of an apoptosis-inducing factor (AIFM1), a flavoprotein that induces apoptosis of isolated nuclei *in vitro* (Susin *et al.* 1999), from the mitochondrial intermembrane space that triggers apoptosis (Larochette *et al.* 1999). In normal human fibroblast, toxic (50 μ M) concentration of arsenic increased TP53 levels (Vogt & Rossman 2001), which could initiate apoptosis if the DNA damage is irreparable. Arsenic compounds also have apoptogenic effects on normal cells, e.g. hematopoietic precursors (Wang *et al.* 1998) and embryonic cells (Mirkes & Little 1998). Although evidences of arsenic-induced germ cell apoptosis in fish *in vivo* are limited, our data clearly suggested that high levels of As(V) considered to be less toxic than As(III) could induce apoptosis in germ cells.

It is known that apoptosis could be induced by oxidative stress (Fiers *et al.* 1999), and ROS responsible for this stress could induce DNA damage (Takeuchi *et al.* 1994). 8-OHdG is the most typical among oxidative DNA damage (Kasai & Nishimura 1986), and has recently been mostly used in mammals as a marker to study DNA damage caused by oxidative stress (Matsui *et al.* 1999, Toyokuni 1999, Nomoto *et al.* 2002). To clarify whether oxidative stress is involved in arsenic-induced apoptosis *in vitro*, we performed immunohistochemistry for 8-OHdG. Our data showed that high-dose arsenic with or without hCG induced DNA damage caused by oxidative stress in germ cells as assessed by 8-OHdG immunostaining. In previous studies in hamster (Liu *et al.* 2001), human (Pineda-Zavaleta *et al.* 2004), and fish (Bhattacharya & Bhattacharya 2007), it is apparent that arsenic induced ROS production, which may consequently cause oxidative damage to DNA, RNA, and proteins. Wang *et al.* (1996) reported an induction of apoptosis in

Chinese hamster ovary cells concomitant with the increase in intracellular peroxide level. It has also been proposed that oxidative stress induced by arsenic could be responsible for apoptosis (Ercal *et al.* 2001, Gupta *et al.* 2003). Interestingly, intense oxidative DNA damage was induced by high doses of arsenic after induction of spermatogenesis by hCG treatment compared to treatment with arsenic alone, which coincide with the TUNEL assay results. Thus, high doses of arsenic induced oxidative stress and caused apoptosis; and proliferating germ cells may be more prone to ROS-induced damage than spermatogonial stem cell. Considering these results, the present study implies that sensitivity to arsenic toxicity and/or ROS induced by arsenic may vary between the different germ cell stages. Although not yet clarified, this variation may be a result of differences in the degree of antioxidant system responses brought by antioxidant enzymes expressed in germ cells. Previous studies have implicated that arsenic modulates the expression of a number of stress proteins such as heat shock proteins in liver and kidney of freshwater teleost, *Channa punctatus* (Roy & Bhattacharya 2006), and in human keratinocytes (Deaton *et al.* 1990), and antioxidant enzymes such as catalase, superoxide dismutase, glutathione-S-transferase, and glutathione peroxidase in liver and kidney of the freshwater teleosts (Allen & Rana 2003, Bhattacharya & Bhattacharya 2007). Many of these proteins may play a role in the antioxidant system response against arsenic in eel testis. Further studies are needed to unfold the antioxidant system in testis.

It is concluded that a low dose of arsenic inhibits 11-KT production via suppression of HSD3B activity, which may consequently inhibit spermatogenesis. Moreover, a high dose of arsenic induces germ cell apoptosis mediated by oxidative stress, which induces DNA damage in cells especially during the onset of spermatogenesis induced by hCG. However, the precise mechanism by which moderately high levels (1–10 μ M) of arsenic inhibit germ cell proliferation remains to be determined. Also, further studies are still needed to elucidate the antioxidant system response of germ cells against arsenic-induced toxicity, which would likely serve as a key to understanding the underlying mechanism of overall arsenic toxicity and provide significant information for establishing a hazard assessment tool for arsenic in fish.

Materials and Methods

Animals

Cultivated male Japanese eel, *Anguilla japonica* (BW: 180–200 g), were purchased from a commercial eel supplier and kept in circulating freshwater tanks at 23 °C until use. The experimental procedures complied with the Guide for Care and Use of Animals at Ehime University.

Testicular organ culture

All fish were anesthetized using 0.05% (v/v) ethyl-*p*-amino benzoate before dissection. Testicular organ culture techniques were carried out following Miura *et al.* (1991a) with minor modifications. Testes from five eels were used in *in vitro* experiments representing five replicates per treatment. Freshly removed testes were cut into 1 × 1 × 0.5 mm pieces and placed on 1.5% (w/v) agarose (Agarose S; Wako Inc., Osaka, Japan) cylinders covered with a nitrocellulose membrane in 24-well plastic tissue culture dishes. Testicular fragments ($n=2-4$) from each fish were randomly assigned to each control and treatment group and then cultured in 1 ml of Leibovitz's L-15 medium (Invitrogen, Ltd) containing 10 mM HEPES, 1.7 mM L-proline, 0.1 mM L-aspartic acid, 0.1 mM L-glutamic acid, 0.5% (w/v) BSA, and 1 mg/l bovine insulin with 0, 0.1, 1, 10, and 100 μ M of arsenic (Na_2HAsO_4) and/or 0.05 IU/ml of hCG for 6 or 15 days at 20 °C in humidified air. The concentrations of arsenic used were based on the previous data from monitoring research in Vietnam (Yamaguchi *et al.* 2007). For 15 day cultures, the medium was changed on day 7. Testicular fragments were then fixed in Bouin's solution and cut into 4 μ m serial paraffin sections and stained with Delafield's hematoxylin–eosin for histological analysis. Two to three slides per fragment and four to six sections per treatment in each slide were analyzed.

Cell proliferation assay

To analyze proliferation of germ cells, immunohistochemistry for BrdU (Amersham Pharmacia Biotech) incorporated into replicating DNA was performed. After 6 and 15 days of culture, testicular fragments were labeled with a 1:1000 dilution of BrdU for 18 h and fixed in Bouin's solution. Fixed testicular fragments were embedded in paraffin, cut into 4 μ m sections, and stained immunohistochemically by anti-BrdU antibody. The sections were counterstained by Delafield's hematoxylin. The number of BrdU-positive germ cells was counted and expressed as percentage of the total number of germ cells in an area comprising of 100–250 germ cells.

In vitro 11-KT synthesis and steroidogenic enzyme activity assay

Testes removed from the eel's ($n=5$) body cavity were placed into eel Ringer's solution and cut into small pieces. Afterwards, these fragments (25–50 mg per treatment) were incubated with 1 IU/ml of hCG with or without 0.0001–100 μ M of arsenic in Ringer's solution in 24-well plastic plates for 18 h at 20 °C with shaking. Five eels used in this experiment represent five replicates per treatment. This incubation experiment was then repeated at least three times on separate occasions. 11-KT in Ringer's solution was extracted with diethyl ether and dissolved in assay buffer. The concentrations of 11-KT in Ringer's solution were then measured by time-resolved fluorescent immunoassay following Yamada *et al.* (1999) with minor modifications. In brief, 0.05 μ g/l of KT-3-(*O*-carboxymethyl) oxime–bovine serum albumin (KT-3-CMO–BSA, Steraloids Inc., Wilton,

NH, USA) in sodium carbonate buffer (pH 9.3) was immobilized to the wells of 96-well plate and allowed to stand at 4 °C overnight. After three washes with 0.1% (w/v) saline containing 0.05% (w/v) NaN_3 , the plate was blocked with 0.1% (w/v) BSA in 0.05 M Na_2HPO_4 containing 3% (w/v) sucrose and 0.05% (w/v) NaN_3 for 2–3 h at room temperature. The blocking solution was discarded before 50 μ l standard or sample, and 150 μ l anti-11-KT antibody in the assay buffer were dispensed to the wells, followed by shaking at 4 °C overnight. After three washes, 200 μ l europium (Eu)-labeled goat anti-rabbit IgG was added, and the plate was shaken for 2 h at room temperature, followed by three washes to remove the excess amount of Eu-labeled IgG. Eu was dissociated from the Eu-labeled IgG bound to the antigen–antibody complex by the addition of 200 μ l DELFIA enhancement solution (Perkin Elmer Life Sciences, Turku, Finland). The plate was shaken for 5 min at room temperature, and Eu fluorescence was measured with a time-resolved fluorometer (ARVO MX 1420, Perkin Elmer).

In order to clarify the mechanism involved in low-dose arsenic effects on 11-KT synthesis, testicular fragments (25–50 mg per treatment) of five eels (as five replicates) were incubated with different steroid hormone precursors, i.e. pregnenolone, progesterone, 17 α -hydroxyprogesterone, 17 α -hydroxypregnenolone, androstenedione, testosterone, and 11 β -hydroxytestosterone at 100 ng/ml and 1 IU/ml hCG with or without arsenic (0.1–1 μ M) in eel ringer for 18 h at 20 °C. The steroid levels were then measured similarly with the method described above for 11-KT. The inter- and intra-assay coefficient of variation (CV) were determined using serum samples from eels ($n=10$). The intra- and inter-assay CV were 9.4 and 10.3% respectively for 11-KT, and 9.55 and 17.4% respectively for progesterone.

Analysis of germ cell death

To determine whether arsenic induces apoptosis, testicular fragments from five eels were cultured *in vitro* as described above for 3 days in 1 ml of Leibovitz's L-15 medium. After culture, the fragments were fixed in Bouin's solution and embedded in paraffin. TUNEL assay was then conducted on 5- μ m-thick serial sections following the manufacturer's protocol (*In Situ* Cell Death Detection Kit, POD; Roche Diagnostics). For positive control, sections were treated with 600 U/ml DNase I (Takara Bio Inc., Shiga, Japan) prior to TUNEL assay.

Immunohistochemistry for 8-OHdG

Immunohistochemistry for 8-OHdG using anti-8-OHdG MAB (Japan Institute for the Control of Aging, Shizuoka, Japan) was performed on serial sections of testicular tissue used for TUNEL to investigate oxidative stress in testis following the manufacturer's protocol with some modifications. After deparaffinization and rehydration, the sections were microwave heated in 10 mM citric acid buffer (pH 6.0). The sections were then blocked in 1% (w/v) DIG-blocking reagent (Roche Diagnostics) in PBS pH 7.4 for 30 min at room temperature, and the primary antibody was added and incubated at

4 °C overnight. Biotin-labeled rabbit anti-mouse IgG was used as the secondary antibody, followed by a streptavidin–alkaline phosphatase complex (Nichirei Biosciences Inc., Tokyo, Japan).

Quantitation of 8-OHdG immunostaining

The following formula was used for the densitometric quantitation of 8-OHdG immunohistochemistry (8-OHdG index), as previously described (Toyokuni 1999):

$$8\text{-OHdG index} = \Sigma((X - \text{threshold})$$

$$\times \text{area (pixels)})/\text{total cell number}$$

where X is the staining density indicated by a number between 0 and 256 in grayscale, and X is more than the threshold. Cell numbers were determined using the Analyze Particle command after setting a proper threshold. To determine the integrated density of each file, a density slice between 100 and 256 pixels was selected for the measurement command. Mean values of the integrated density per cells, which were obtained from two independently obtained files, were used as the representative value for '8-OHdG index'. The brightness of each image file was uniformly enhanced by Adobe Photoshop software (Adobe Systems Inc.), followed by analysis using National Institutes of Health (NIH) image freeware, which is available on the Internet via file transfer protocol from zippy.nimh.nih.gov.

Statistical analysis

All values were expressed as mean $\pm$ S.E.M. A one-way ANOVA followed by Tukey's multicomparison test was used to analyze the differences of means using KaleidaGraph statistical software (Synergy Software, Reading, PA, USA). Significant difference was accepted at $P < 0.05$ in all cases unless indicated.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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