

Testicular Toxicity of Arsenic on Spermatogenesis in Fish

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Abstract—Arsenic (As), a well-known water pollutant, has been associated with various human health problems, however, studies pertaining to its reproductive toxicity on fish are very limited. In this study, we investigated the direct influence of As on fish spermatogenesis using Japanese eel (*Anguilla japonica*) *in vitro* testicular organ culture system. Eel testicular fragments were cultured *in vitro* with increasing concentrations of As with or without human chorionic gonadotropin (hCG). Thereafter, steroid synthesis, steroidogenic enzyme activity, testicular morphology and proliferation of germ cells were analyzed. As inhibits hCG-induced germ cell proliferation in a dose-dependent manner. Highest dose of As (100 μ M) with hCG strongly induced germ cell apoptosis. Moreover, a dose of As (0.1 μ M) lower than the WHO drinking water quality guideline most effectively suppressed 11-ketotestosterone (11-KT) synthesis. At the same As concentration, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) expression and activity were inhibited. A high oxidative DNA damage was observed in testicular fragments exposed to the same treatment (100 μ M As + hCG) which caused apoptosis. These results suggest that low dose of As inhibits spermatogenesis via suppression of steroidogenic enzyme activity and expression while high dose of this compound induces oxidative stress-mediated germ cell apoptosis.

Keywords: *Anguilla japonica*, apoptosis, DNA damage, oxidative stress, testis

INTRODUCTION

Arsenic (As) contamination in water has long been a global concern. In Asia, groundwater and sediments in Vietnam and in Bengal Delta have been contaminated by inorganic arsenic (Berg *et al.*, 2001; Agusa *et al.*, 2006). A previous monitoring research in Mekong Delta of Vietnam revealed a negative correlation between gonadal development and accumulation of arsenic in catfish, *Pangasianodon hypophthalmus* (Yamaguchi *et al.*, 2007). Thus, in this study, we clarify the direct effects of As on fish spermatogenesis.

In Japanese eel (*Anguilla japonica*), spermatogenesis could be induced by human chorionic gonadotropin (hCG) injection *in vivo* or treatment with 11-ketotestosterone (11-KT) or hCG *in vitro*. In vertebrates, this is the only available

system for induction of complete spermatogenesis *in vitro* from spermatogonial proliferation to spermiogenesis and has been used in previous studies to demonstrate the mechanisms of toxic effects of chemical pollutants on spermatogenesis in fish (Miura *et al.*, 2005; Yamaguchi *et al.*, 2007). Hence, we investigated the direct effects of As on testis in fish using this system.

MATERIALS AND METHODS

Animals

Cultivated male Japanese eel, *A. japonica* (BW: 180–200 g) were purchased from a commercial eel supplier and kept in circulating freshwater tanks at 23°C before use.

Testicular organ culture

Testicular organ culture techniques were carried out following Miura *et al.* (1991) with minor modifications. Freshly removed eel testes were cut into 1 mm × 1 mm × 0.5 mm pieces and placed on 1.5% cylindrical agarose (Sigma, Germany) gels covered with a nitrocellulose membrane in 24-well plastic tissue culture dishes. Testicular fragments were then cultured in 1 ml of Leibovitz' L-15 medium (Invitrogen, Ltd., CA, USA) for eel with 0, 0.1, 1, 10 and 100 μM of As (Na₂HAsO₄) and/or 0.05 U/ml of hCG, for 6 days and 15 days at 20°C in humidified air. For 15-day culture, the medium was changed on day 7. After culture, the testicular fragments were incubated with 5-bromo-2-deoxyuridine (BrdU) to analyze germ cell proliferation. These fragments were then fixed and processed for histological analysis. Immunohistochemistry for BrdU was done following the methods of Miura *et al.* (2005).

In vitro 11-ketotestosterone (11-KT) synthesis assay

Testes removed from the eel's body cavity after the anesthetization were placed into eel ringer solution and cut into small pieces. Afterwards, these fragments were incubated with 1 U/ml of hCG with or without 0.0001 to 100 μM of As in Ringer's solution for 18 hrs at 20°C with shaking. 11-KT in serum 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 (TR-FIA).

3β-hydroxysteroid dehydrogenase (3β-HSD) activity and expression analysis

In order to clarify the effects of As on 3β-HSD activity, testicular fragments of eel were incubated with pregnenolone at 100 ng/ml with or without As (0.1–100 μM) in eel ringer for 18 hrs at 20°C. The progesterone levels were then measured similarly with the method described above for 11-KT. Moreover, to analyze the influence of As on 3β-HSD expression, testicular fragments were cultured in As of the same concentration as above with hCG for 24 hrs at 20°C, total RNA was then extracted then RT-PCR was performed. The DNA were run

in 2% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

Analysis of germ cell death

To determine whether As induces apoptosis, testicular fragments were cultured as above for 3 days in 2 ml of Leibovitz' L-15 medium. After culture, the fragments were fixed and analyzed for histology. TdT-mediated nick-end labeling (TUNEL) assay was then conducted on 5 μm thick testicular tissue sections following the manufacturer's protocol (In Situ Cell Death Detection Kit, POD; Roche, Germany). For positive control, sections were treated with DNase I (Takara Bio Inc., Shiga, Japan).

Oxidative stress detection

Immunohistochemistry for 8-hydroxy-2'-deoxyguanosine (8-OHdG) using anti-8-OHdG antibody (JalCa, Shizuoka, Japan) was performed on serial section of testicular tissue used for TUNEL following the manufacturer's protocol with minor modifications. Deoxyguanosine (dG) is one of the constituents of DNA and when it is oxidized, it is altered into 8-hydroxy-2'-deoxyguanosine (8-OHdG). Antibody for 8-OHdG binds to 8-OHdG and hence could be detected by immunohistochemistry.

Statistical analysis

A one-way analysis of variance followed by Tukey's multicomparison test was used to analyze the differences of means. Significant difference was accepted at $p < 0.05$ in all cases.

RESULTS AND DISCUSSION

Our study showed that hCG induced germ cell proliferation via 11-KT synthesis similarly with the previous studies (Miura *et al.*, 1991; Yamaguchi *et al.*, 2006) as assessed by BrdU index, i.e., percentage of BrdU positive germ cells. However, addition of As inhibits spermatogenesis in a dose dependent manner. At low dose (0.1 μM), As already inhibited germ cell proliferation. This is true for both 6 and 15 day-cultured testicular fragments. Concurrently, histological analysis showed that control and hCG-treated sections have normal morphology, containing spermatogonia. However, treatment with As at 100 μM with or without hCG induced germ cell death. As and hCG-treated testicular tissue section showed a severe enlargement of interstitial tissue compared to those treated with As alone. Since spermatogenesis is a complex process governed by various factors and enzymes (Miura and Miura, 2003; Miura *et al.*, 2006), it may be possible that As affects spermatogenesis through these factors. In fish, 11-KT, a major androgen, initiates and maintains the progression of spermatogenesis through the action of Sertoli cells (Miura and Miura, 2003). Thus, As may exert its effect on spermatogenesis via 11-KT. Our results showed that indeed 11-KT synthesis was inhibited by treatment with As, particularly at low dose of 0.1 μM

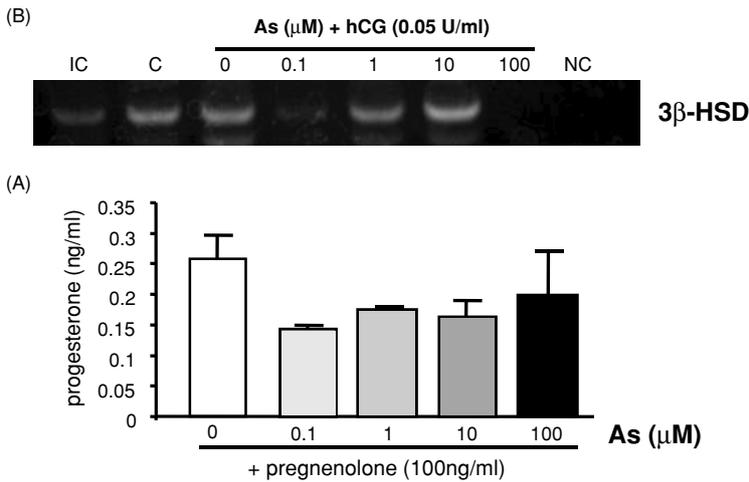


Fig. 1. Effects of As on 3β -HSD activity and expression *in vitro*. (A) Progesterone levels in eel testicular fragments cultured with pregnenolone and increasing concentrations of As. (B) RT-PCR using specific primers for 3β -HSD gene of eel testicular fragments cultured with increasing concentrations of As with hCG. IC, initial control; C, control; NC, negative control.

(approx. $7 \mu\text{g/L}$), a dose relatively lower than the WHO guideline for drinking water quality ($10 \mu\text{g/L}$).

To clarify the mechanism of 11-KT synthesis inhibition by As, we analyzed the activity and expression of 3β -HSD, a key enzyme for conversion of 3β -hydroxysteroid such as pregnenolone to 3-ketosteroid such as progesterone which is an essential step in the biosynthesis of 11-KT. Our results showed that progesterone level tended to decrease at $0.1 \mu\text{M}$ of As which may imply that decrease in 11-KT synthesis at low dose of As may be due to the inhibition of activity of 3β -HSD (Fig. 1A). Moreover, RT-PCR using specific primers for 3β -HSD showed that 3β -HSD expression also decreased at $0.1 \mu\text{M}$ of As treatment and almost undetectable at $100 \mu\text{M}$ (Fig. 1B). A previous *in vivo* study by Sarkar *et al.* (1991) on rats also showed that As inhibits 3β -HSD activity. In our study, however, hCG, in some eels, did not induced 3β -HSD expression, and showed either a decreased or no changes in this expression in all treatments in some eels. This may indicate that changes in steroidogenic activity do not always correlate with change in 3β -HSD expression. These results suggest that 11-KT synthesis in testis was inhibited by low dose of As. Moreover, As may inhibit steroidogenic enzyme gene expression and/or their activities such as that of 3β -HSD.

Furthermore, in order to determine whether As induced apoptotic cell death, TUNEL assay was conducted. Positive control group contains many TUNEL positive cells, both germ and somatic cells; while control group did not contain TUNEL positive cell. In sections of testicular fragments treated with $100 \mu\text{M}$ of As alone, a few germ cells showed positive reaction. On the other hand, in those

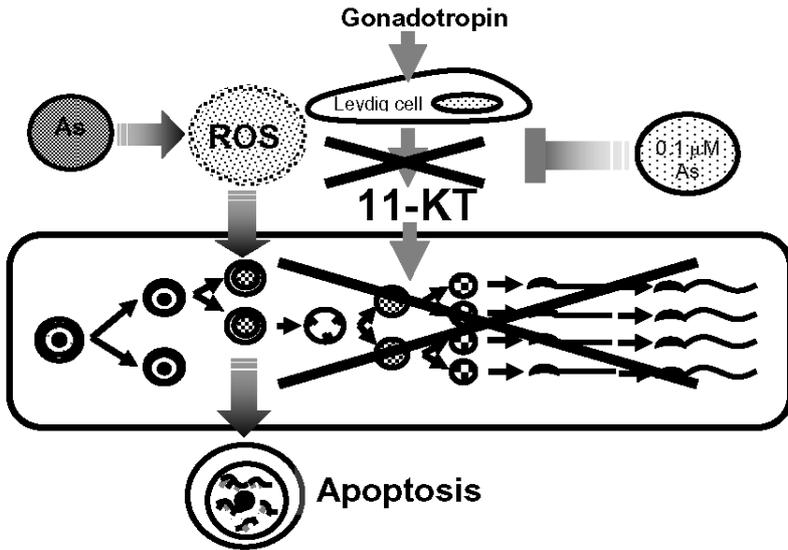


Fig. 2. Possible mode of toxic action of As on fish spermatogenesis.

treated with $100 \mu\text{M}$ of As with hCG, many germ cells exhibit TUNEL-positive signal. These data indicate that high dose of As caused apoptosis of germ cells, and As combined with hCG strongly induced germ cell death compared to As alone.

It is known that apoptosis could be induced by oxidative stress (Fiers *et al.*, 1999), and reactive oxygen species (ROS) responsible for this stress could induce DNA damage (Takeuchi *et al.*, 1994). Among oxidative DNA damage, 8-OHdG is typical, hence we performed immunohistochemistry for 8-OHdG. Immunohistochemistry for 8-OHdG revealed that control and hCG-treated testicular fragment sections were not stained. Whereas, testicular fragments incubated with As and hCG showed strong signal and many intensely stained germ cells than those treated with As alone. This is consistent with the results in TUNEL assay. In addition, 8-OHdG index showed that highest DNA damage brought by oxidative attack was observed in the same treatment which strongly caused apoptosis. Thus, this result suggests that ROS mediates As-induced germ cell apoptosis.

Hence, collectively, our study demonstrated that low dose, $0.1 \mu\text{M}$, of As may inhibit 11-KT synthesis via suppression of steroidogenic enzyme activities such as 3β -HSD, which may continuously inhibit spermatogenesis. Furthermore, exposure of testis to high concentration of As produced ROS in testis which consequently cause apoptosis of germ cells, especially after induction of spermatogenesis by hCG via 11-KT synthesis (Fig. 2). The present study showed that oxidative stress-mediated As-induced apoptosis is strongly manifested only

after hormonal induction of spermatogenesis. This may imply that sensitivity to ROS may vary among different germ cell stages. Moreover, previous studies have implicated that As induces a number of stress proteins (Roy and Bhattacharya, 2006) and antioxidant enzymes, such as superoxide dismutase (SOD) (Bhattacharya and Bhattacharya, 2007), which protect cells and tissues from superoxide anions by catalyzing the conversion of strong ROS (superoxide anions) to weaker forms (hydrogen peroxide). Hence, further studies are to be conducted to clarify the role of ROS and antioxidant enzyme, such as SOD, in the antioxidant system response against As in eel testis.

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