DNA Damage and Decrease of Cellular Oxidase Activity in Piglet Sertoli Cells Exposed to Arsanilic Acid

Ming ZHANG^{1,2)}, Zuping HE³⁾, Hui YUAN^{1)*}, Li ZHU¹⁾, Chengzhi GUO¹⁾, Lu YIN¹⁾, Jing WU¹⁾, Sijun DENG¹⁾, Liyun YUAN¹⁾ and Lixin WEN¹⁾

¹⁾College of Veterinary Medicine, Hunan Agricultural University, Changsha, Hunan 410128, ²⁾Jiangxi Biotech Vocational College, Nanchang, Jiangxi 330200, P. R. China and ³⁾Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, 3900 Reservoir Road, NW, Washington, DC 20057, U.S.A.

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ABSTRACT. The study was designed to explore the toxic effects of arsanilic acid on piglet Sertoli cells. Sertoli cells were isolated from piglet testes using a two-step enzyme digestion followed by differential plating. Piglet Sertoli cells were cultured and classified into the following five groups: group A, the control without arsanilic acid treatment; group B, cultured with 5 μ M arsanilic acid; group C, cultured with 50 μ M arsanilic acid; group D, cultured with 0.5 mM arsanilic acid; and group E, cultured with 5 mM arsanilic acid. We found that Sertoli cell growth was inhibited by arsanilic acid at 0.5 mM compared with the control, group A. The oxidase activity of Sertoli cells was decreased by arsanilic acid at 0.5 mM as evidenced by the observations that arsanilic acid increased MDA content but decreased the SOD and GSH-Px activities of Sertoli cells. Moreover, 50 μ M of arsanilic acid was observed to cause DNA damage in Sertoli cells. The results of our study suggest that exposure of Sertoli cells to arsanilic acid leads to induction of oxidative stress and inhibition of cell growth at a high concentration, while arsanilic acid causes DNA damage in Sertoli cells at a low concentration.

KEY WORDS: arsanilic acid, DNA damage, oxidase activity, piglet Sertoli cells, toxicity.

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Arsanilic acid is an organoarsenic compound that is also referred to as p-aminophenylarsenic acid. It is a colorless solid and was used as a drug in the late 19th and early 20th centuries. Arsanilic acid has been employed as a livestock and poultry feed additive. It is a threat to animals as well as environment and food safety because of its improper use. Excessive amounts of arsanilic acid have been used in some nurseries, resulting in arsenic residue and environmental pollution. An *in vivo* study showed that lymphoma cells died in mice treated with the arsenic compound [12]. There are some clinical reports of animal poisoning by arsanilic acid, as evidenced by the findings that arsenic is toxic to reproduction [4, 6]. Notably, arsenic can significantly diminish the motility of sperm [22]. However, very little is known about the effects of arsanilic acid on somatic cells in the testes.

Spermatogenesis is a process by which spermatogonial stem cells divide and differentiate into sperm [7, 8]. Spermatogonial stem cells are maintained in a specialized microenvironment called the niche that is composed of male germ cells, somatic cells, and extracellular matrix [11]. Sertoli cells are the major somatic cells in mammalian testis, and more importantly, they can secrete a number of growth factors, such as glial cell line-derived neurotrophic factor [17], basic fibroblast growth factor, and epidermal growth factor [14], to support the proliferation and differentiation of spermatogonial stem cells. Significantly, Sertoli cells play a critical role in controlling all aspects of development of

male germ cells in the testis, thereby regulating spermatogenesis. This study was thus designed to probe the toxic effects of arsanilic acid on male reproduction in males using piglet Sertoli cells *in vitro* as an experimental model. Piglets were used in this study because pigs and humans have similar physiology, and thus, information on the toxic effects of arsanilic acid from piglet Sertoli cells may be applicable to humans.

MATERIALS AND METHODS

Piglet testes: Testes were obtained from 3- to 4-week-old healthy piglets (Changsha, Hunan, P.R. China), placed in ice-cold phosphate-buffered saline (PBS) with 600 IU/ml penicillin-streptomycin and sent to the laboratory within 2 hr. The testes were obtained as a by-product of routine castration, in accordance with the instructions of the local ethics committee.

Isolation, culture and identification of testicular Sertoli cells of piglets: The testicular capsule was removed under sterile conditions, and seminiferous tubules were isolated from the piglet testis using mechanical dissociation and a one-step enzymatic digestion with 1 g/l collagenase and 2.5 g/l trypsin solution, according to the procedure described previously [7, 15]. Sertoli cells were further separated from male germ cells using a second enzymatic digestion followed by differential plating [3, 9]. For differential plating, germ cells and Sertoli cells were placed into a tissue culture dish in the Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium supplemented with 10% fetal calf serum (FCS) for 3 hr at 34°C. Sertoli cells attached to the culture plates, whereas male germ cells remained in suspension and were

* CORRESPONDENCE TO: YUAN, H., College of Veterinary Medicine, Hunan Agricultural University, Changsha, Hunan 421008, P.R. China.
e-mail: yuanhui7269@yahoo.com.cn

removed. Cell viability of Sertoli cells was determined with 0.4% trypan blue. The freshly isolated Sertoli cells were plated at a density of 2×10^6 cells/cm² in DMEM supplemented with 10% FCS in a humidified incubator with 5% CO₂. Sertoli cells were identified by Oil red O staining when 80–90% of the dish was confluent with cells.

Experimental groups and MTT assay: Piglet Sertoli cells were classified into the following five groups: group A, the control without arsanilic acid treatment; group B, cultured with 5 μ M arsanilic acid; group C, cultured with 50 μ M arsanilic acid; group D, cultured with 0.5 mmol (mM) arsanilic acid; and group E, cultured with 5 mM arsanilic acid. After 24 hr of culture with different concentrations of arsanilic acid, Sertoli cells were collected for MTT assay in quadruplicate, according to the procedure previously described [18].

Determination of SOD, MDA, and GSH-Px activities of piglet Sertoli cells: The activity of SOD in sonicated Sertoli cells was measured by the xanthine oxidase method [16] and expressed as units per mg of protein. The activity of GSH-Px in sonicated Sertoli cells was measured by the DTNB reaction test [5] and expressed as units per mg of protein. The TMDA level in sonicated Sertoli cells was measured by the thiobarbituric acid method [10] and expressed as nmol per mg of protein. All the specific steps were in accordance with the instructions provided with kits from Nanjing Jiancheng Company (Nanjing, P.R. China).

Determination of DNA damage of Sertoli cells caused by arsanilic acid: The DNA damage of Sertoli cells was measured according to a previously reported procedure [20] with some modification: the duration of lysis, denaturation, and electrophoresis were changed to 2 hr, 1 hr and 40 min, respectively. Sertoli cells were observed for epifluorescence under Fluophot microscopy, and 400 cells were counted in each group (4 parallel glass slides per group).

Statistical analysis: All values were presented as means $\pm$ SEM, and statistically significant differences ($P < 0.05$) and extremely statistically significant differences ($P < 0.01$) were determined among the various groups by ANOVA and Tukey post-test using the SPSS 12.0 statistical software.

RESULTS

Isolation, identification and culture of piglet Sertoli cells: Piglet Sertoli cells were isolated by a two-step enzymatic digestion with trypsin and collagenase followed by differential plating. Cell viability of Sertoli cells was up to 90% as assayed by trypan blue exclusion. After 24 hr of culture, Sertoli cells attached to the culture dish and assumed a large columnar or irregular appearance with an elongated cellular body. Sertoli cells were connected and showed a mosaic-like arrangement and intercellular irregular protrusions. On both sides of cells, there were several prominences and strong refraction. Phagocytosed objects with various sizes of vacuoles and the cell nucleus were observed in the cytoplasm of the cells. Oil red O staining showed that red lipid droplets occurred near the nucleus or at the two poles of the

Fig. 1. Identification of piglet Sertoli cells by Oil red O staining. After staining with Oil red O, lipid droplets were observed near the nucleus or at the two poles of cytoplasm in piglet Sertoli cells. Magnification: $\times 400$.

cytoplasm (Fig. 1).

Effects of arsanilic acid on proliferation of piglet Sertoli cells: Piglet Sertoli cells were treated with different concentrations of arsanilic acid for 24 hr, and the effect of arsanilic acid on the proliferation of Sertoli cells was assessed by MTT assay. As shown in Table 1, with the increase of arsanilic acid concentration, Sertoli cell proliferation decreased gradually. Cell growth inhibition in groups B and C was not significantly different from that of the control, group A. However, group D showed a statistically significant difference ($P < 0.05$), while group E showed extremely significant difference ($P < 0.01$) compared to the group A. The results suggest that arsanilic acid had a dose-dependent effect on growth inhibition of Sertoli cells.

Effects of arsanilic acid on antioxidant enzyme-activities: SOD and GSH-Px are two important enzymes in the antioxidant defense system [24], and MDA is regarded as a major marker of lipid peroxidation in tissue [13]. After exposure to arsanilic acid, the MDA content and SOD and GSH-Px activities of Sertoli cells were measured, and the data are shown in Table 2. Compared with the control, group A, the increase of MDA content in groups B and C was not significantly different. In contrast, the increase of the MDA content in group D was statistically significant ($P < 0.05$) compared with group A, and the increase of the MDA content in group E was extremely significant ($P < 0.01$). These results indicate that the increase of MDA content of Sertoli cells caused by arsanilic acid occurs also in a dose-dependent manner.

The changes of the SOD and GSH-Px activities in groups B and C were not significantly different when compared with the control, group A. However, significant decreases in the SOD and GSH-Px activities were observed in group D ($P < 0.05$), and an extremely significant decrease was observed in group E ($P < 0.01$). Notably, the decreases of the SOD and GSH-Px activities were related to the concentration of arsanilic acid.

Fig. 2. DNA damage of piglet Sertoli cells by arsanilic acid. Comet assay showed the DNA image of the control group did not have a tail (A). Compared with the control group, there was DNA image in the piglet Sertoli cells treated with 5 μ M arsanilic acid as evidenced by the presence of tail (B). Arsanilic acid induced DNA damage and migration of piglet Sertoli cells.

Table 1. Cell proliferation of piglet Sertoli cells as assessed by MTT assay

Groups	OD Value at 490 nm	Inhibition of proliferation (%)
Group A	0.82 ± 0.04	0
Group B	0.72 ± 0.03	11.51
Group C	0.70 ± 0.02	14.08
Group D	$0.59 \pm 0.04^*$	28.15
Group E	$0.35 \pm 0.02^{**}$	56.67

Compared with group A, an asterisk (*) indicate a significant difference ($P < 0.05$), and two asterisks (**) indicate an extremely significant difference ($P < 0.01$). Inhibition of proliferation of Group E (5 mM) was 56.67%. In the test dose range, the inhibition of proliferation increased when the dose of arsanilic acid was enhanced.

Table 2. Effects of arsanilic acid on MDA content and SOD and GSH-Px activities

Groups	MDA (nmol/mg prot)	SOD (U/mg prot)	GSH-Px (U/mg prot)
Group A	3.43 ± 0.25	250.33 ± 29.63	20.34 ± 5.48
Group B	3.83 ± 0.47	241.79 ± 21.87	18.67 ± 3.22
Group C	4.15 ± 1.04	229.82 ± 22.65	17.93 ± 4.77
Group D	$7.57 \pm 0.87^*$	$197.65 \pm 22.58^*$	$15.08 \pm 3.14^*$
Group E	$10.29 \pm 1.79^{**}$	$117.14 \pm 17.48^{**}$	$11.37 \pm 3.23^{**}$

Compared with group A, an asterisk (*) indicates a significant difference ($P < 0.05$), and two asterisks (**) indicate an extremely significant difference ($P < 0.01$). As the dose of arsanilic acid increased, the MDA content enhanced whereas the activities of SOD and GSH-Px decreased. Group E (5 mM) showed a significant difference.

DNA damage of piglet Sertoli cells caused by arsanilic acid: Comet tail length is an important parameter in evaluating DNA damage. After UV excitation, Sertoli cell DNA from the arsanilic acid-treated groups showed an orange-red color. Typical and clear images of nuclear DNA and migration of DNA were observed in arsanilic acid-treated groups (Fig. 2B), whereas no tail was observed in control group (Fig. 2A), suggesting that arsanilic acid induces DNA damage in Sertoli cells. The rate of DNA tail was used to show the degree of DNA damage which was classified into grades 0 to 4 [2, 21]. Table 3 shows that the degree of DNA damage in the control group did not reach grades 3 and 4 and that

91.56% of DNA damage was classified into grade 0. The degree and amount of Sertoli cells damage in the arsanilic acid-treated groups were enhanced when arsanilic acid concentration was increased. An increase of DNA tailing rate was observed with the increase with arsanilic acid concentration. These data indicate that the overall level of DNA damage gradually increased in an obvious dose response to arsanilic acid.

DISCUSSION

Arsanilic acid plays an important role in promoting

Table 3. Effect of arsanilic acid on DNA damage of Sertoli cells

Groups	Total cell number	Cell number according to the different degree of damage					
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Rate of DNA tail (%)
Group A	100	91.56 ± 1.00	5.32 ± 1.15	3.12 ± 1.13	0.00 ± 0.00	0.00 ± 0.00	8.44 ± 0.21
Group B	100	85.33 ± 2.51	7.01 ± 0.08	3.67 ± 1.75	2.67 ± 1.15	1.32 ± 0.53	14.67 ± 1.46
Group C	100	76.58 ± 4.73*	10.64 ± 1.79*	5.39 ± 0.97*	4.82 ± 1.13*	2.57 ± 0.46*	23.42 ± 3.13*
Group D	100	54.67 ± 0.48*	19.15 ± 0.57**	12.47 ± 0.55**	7.33 ± 1.53**	6.83 ± 3.73**	43.33 ± 1.51**
Group E	100	39.33 ± 2.511**	23.47 ± 1.89**	13.00 ± 2.65**	14.13 ± 1.73**	10.07 ± 2.52**	60.67 ± 2.39**

Compared with group A, an asterisk (*) indicates a significant difference ($P < 0.05$), two asterisks and (**) indicate an extremely significant difference ($P < 0.01$). When the dose of arsanilic acid increased, the numbers of cells classified in the different degrees of damage and the tail rate increased. Group E (5 mM) showed the highest damage.

aquaculture as a feed additive. On the other hand, it also leads to accumulation of arsenic in animals and the environment and causes deleterious effects, and thus, it attracts scientific attention. In the current study, we found that arsanilic acid inhibited the proliferation of Sertoli cells, increased the MDA content and DNA damage, but decreased SOD and GSH-Px activities of Sertoli cells, in a dose-dependent manner. This study thus provides new insight into the mechanisms of arsanilic acid's toxicity on male reproduction.

Very few reports are available on the toxicity of organic arsenic compounds. Recently, we have demonstrated that arsanilic acid can induce cell apoptosis in primary rat hepatocytes [23]. We found that arsanilic acid results in decrease of the activities of GSH-Px and SOD and an increase of the activity of catalase in rat primary hepatocytes *in vitro* [23]. Arsanilic acid also induces cell apoptosis of rat primary hepatocytes [23]. Taken together, arsanilic acid decreases the activities of SOD and GSH-Px and leads to cell apoptosis of both Sertoli cells and hepatocytes, suggesting that Sertoli cells and hepatocytes are toxic targets of arsanilic acid.

There is some evidence showing that arsenic is also toxic to male reproduction [4, 6], and arsenic has adverse effects on male germ cell function because arsenic can significantly decrease the motility of sperm [22]. However, the effects of arsanilic acid on somatic cells in the testis need to be defined. In this study, we found that arsanilic acid had a toxic effect on male somatic Sertoli cells, further confirming the toxicity of arsanilic acid on male reproduction.

SOD and GSH-Px, two major parts of the oxidase defense system, are natural scavengers of active oxygen and superoxide anion radicals. SOD and GSH-Px act synergistically in preventing cell membrane lipid peroxidation and destroying metabolite formation. They directly capture and eliminate free radicals, prevent the action of free radicals, suppress cell membrane lipid peroxidation during pathological lesion and protect biological macromolecules and membranes from damage [1]. The SOD, GSH-Px and MDA levels of the oxidative metabolites obtained in the present study indicate that arsanilic acid can inhibit the activity of antioxidant enzymes in piglet Sertoli cells *in vitro*. The SOD and GSH-Px activities were decreased in the arsanilic

acid-treated groups. However, arsanilic acid exposure caused an increase in MDA in groups B-E, while it apparently affected the activities of SOD and GSH-Px in groups D and E (0.5 and 5 mM arsanilic acid). In addition, we also observed a decrease in the activity of antioxidative enzymes, which indicates a decrease in the cellular ability to repair the oxidative damage caused by oxygen species formation, membrane lipid peroxidation and other related harmful factors. The inability to cope with the oxidative damage may lead to cell necrosis or apoptosis.

MDA is often considered to be the major hallmark of lipid peroxidation damage, which causes secondary damage to cell functions, genotoxicity, and carcinogenesis [13, 19]. The tail ratio in arsanilic acid-treated groups with 50 μ M was remarkably different from that of the control, as detected by single cell gel electrophoresis (SCGE). This suggests that arsanilic acid can cause DNA damage in piglet Sertoli cells at a low concentration. The activities of SOD, GSH-Px and MDA in arsanilic acid-treated groups D and E (arsanilic acid at 0.5 and 5 mM) were significantly different from those of the control, whereas DNA damage was observed in groups exposed to arsanilic acid at concentrations above 50 μ M.

In summary, we observed that arsanilic acid is toxic to piglet Sertoli cells at concentrations between 5 and 50 μ M, which indicates that the minimum pathogenic concentration of arsanilic acid is in this range. Thus, we believe that arsanilic acid at a concentration of 50 μ M can cause DNA damage. This level is lower than the limits approved by the Ministry of Agriculture of China (45–90 mg/kg) and much lower than the amount of arsanilic acid added daily in feed-stuff (above 150 mg/kg). We conclude that even the accepted levels of arsanilic acid to feed may possibly cause adverse health effects in animals, decrease reproductive efficiency and increase an individual's susceptibility to external pathogenic factors. Moreover, arsenic can be accumulated in the organism of livestock, thereby affecting human health through the food chain.

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