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Sulphur dioxide and arsenic affect male reproduction *via* interfering with spermatogenesis in mice

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ABSTRACT

As two potential environmental hazards, sulphur dioxide (SO_2) and arsenic have adverse effects on male reproduction, but the mechanism of which and their combined toxicity are not clear. In this study, we investigate male reproductive toxicity with a focus on spermatogenesis by treating mice with 5 mg/m^3 SO_2 and/or 5 mg/L arsenic. Our results showed that arsenic exposure caused significant decreases in water and food consumption and body weight in mice, whereas these changes were not observed in the SO_2-only group. Both SO_2 and arsenic reduced sperm counts, increased the percentage of sperm malformation, and induced abnormal testicular pathological changes. Elevated H_2O_2 and MDA contents, declined T-SOD activity, decreased spermatogenic cell counts, enhanced caspase-3 activity, and increased TUNEL-positive cells were also observed in mice exposed to SO_2 and/or arsenic. Moreover, SO_2 and arsenic co-exposure changed the mRNA levels of Bax and Bcl-2, decreased serum testosterone levels, and downregulated the expression of steroidogenic-related genes (LHR, StAR, and ABP) in mice. These findings provide a new theoretical basis for understanding how SO_2 and arsenic interfere with spermatogenesis leading to infertility. These results also suggest that SO_2 and arsenic co-exposure likely result in an additive effect on male reproductive toxicity in mice.

1. Introduction

As a major air pollutant, sulphur dioxide (SO_2) is mainly produced from the burning of sulphur-containing fossil fuels (Li et al., 2016; Xue and Yi, 2018). SO_2 levels in many areas of the world are beyond the safe criteria because of the increased SO_2 emissions in countries undergoing rapid economic development (Zhao and Yi, 2014; Reno et al., 2015). It also presents in the workplace with high concentrations (Li et al., 2007). SO_2 is reported to be a systemic toxin and has been linked to many diseases, but is still widely used as a preservative in foods, beverages, and medicines because of its antimicrobial and antioxidant properties (Freedman, 1980; Cressey and Jones, 2009; Han et al., 2018). The toxicity of SO_2 is actually due to bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) generated when SO_2 is hydrated inside the body (Li et al., 2007). Although the respiratory tract is the primary target for SO_2 to exert its toxic effects, other organs and tissues can also be affected when the gas enters the systemic circulation *via* the bloodstream (Reno et al., 2015). Arsenic is a chemical element that exists ubiquitously in the environment. Mining, smelting of non-ferrous metals, burning of fossil fuels, and extensive use of arsenic in herbicides, pesticides, and wood preservatives are the major anthropogenic sources of arsenic contamination (Kim and Kim, 2015). Arsenic is commonly present in polluted water, air, or soil in mining or industrial areas (Kumagai and Sumi, 2007; Ganapathy et al., 2016). Hundreds of millions of people worldwide are affected by arsenic-contaminated drinking water (Lu et al., 2014). Some are also exposed to high levels of SO_2, especially in industrialised countries. High levels of SO_2 and arsenic co-exist in some occupational environments, and people living near certain contaminated areas are exposed to both SO_2 and arsenic through breathing polluted air and ingesting contaminated food and water. However, thus far few data are available on the combined toxicity and health effects of SO_2 and arsenic.

Male reproductive toxicity induced by environmental chemicals has become a major concern because of the declining sperm quality and increased incidence of male infertility in recent years (Hansen et al., 2010; Zhang et al., 2016a). Spermatogenesis is the process by which haploid spermatozoa develop from spermatogenic cells in the seminiferous tubules of the testes. Impairment of spermatogenesis is considered a common male fertility problem (Al-Maghrebi et al., 2016). Dejmek et al. (2000) reported that the declined fecundability was

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observed in couples who lived close to areas with serious SO_2 pollution. Zhang et al. (2016b) found that SO_2 lowered sperm quality by increasing the expression of spermatogenesis-related proteins in rat testes. Arsenic exposure can decrease semen quality and cause male infertility (Nie et al., 2006; Hsieh et al., 2008; Kim and Kim, 2015). Animal studies have demonstrated that arsenic induces the degeneration of spermatogenic cells and inhibits spermatogenesis in the testes (Sarkar et al., 2003; Sanghamitra et al., 2008; Li et al., 2012). These studies indicate that both SO_2 and arsenic interfere with spermatogenesis and affect male reproduction, but the underlying mechanism of which and the interaction between SO_2 and arsenic still need to be clarified.

The purpose of this study is to investigate the combined toxic effects of SO_2 and arsenic with special emphasis on the possible mechanism affecting spermatogenesis in the testes of mice. We measured water and food consumption, body weight, and testes coefficient in mice after exposure to SO_2 and/or arsenic. We then counted the sperm numbers, evaluated the sperm morphology, examined the testicular histopathological changes, analysed stage VII spermatogenesis, and determined the serum testosterone level in mice. The expression of Bax and Bcl-2, caspase-3 activity, TUNEL staining, oxidative damage indexes, and mRNA levels of steroidogenesis-related genes (LHR, StAR, P450scc, and ABP) were also investigated in this study. Our results elucidate a possible mechanism for how SO_2 and arsenic together severely affect male reproduction.

2. Materials and methods

2.1. Animals and treatment protocols

Specific pathogen-free male C57BL/6 mice (6 weeks old), supplied by the Academy of Military Medical Sciences (Beijing, China), were used in this study. They were housed under standard conditions of 23 ± 1 °C, 55 ± 10% humidity, and a 12 light:12 dark cycle in the animal centre at the China Institute For Radiation Protection (Taiyuan, China). Animal care and all of the animal experiments were performed according to the Approved Animal Use Protocol of Shanxi University (Number: HZ20140503). After a one-week acclimatisation period, the mice were randomly allocated into 4 equal groups (n = 10): control, SO_2, sodium arsenite ($NaAsO_2$), and SO_2 + $NaAsO_2$. In the $NaAsO_2$ group and the SO_2 + $NaAsO_2$ group, the mice were given double-distilled water containing 5 mg/L arsenic (approximately 8.67 mg/L $NaAsO_2$) orally for 60 consecutive days. The other two groups of mice were received only double-distilled water. Arsenic-containing water was freshly prepared and provided to the mice every Monday morning and Thursday afternoon. From days 31 to 60, the mice treated with SO_2 alone or with SO_2 plus arsenic were inhaled 5 mg/m^3 (approximately 1.75 ppm) SO_2 continuously for 6 h/day in fumigation chambers according to the method of Li et al. (2018). The mice in the control group and the $NaAsO_2$ group were inhaled filtered air in other identical chambers on these days. During the SO_2 exposure process, none of the mice were allowed to eat or drink. The water and food consumption of every cage was recorded daily, and the mouse body weight was recorded weekly during the treatment period.

At the end of the treatments, the mice fasted for 12 h and were then weighed and sacrificed. The serum was isolated from whole blood to determine testosterone level. The testes were carefully removed and weighed. The left testes were fixed in 4% buffered formaldehyde for histological assay and the right testes were quickly frozen in liquid nitrogen and then stored at − 80 °C for future study. The epididymides were collected for counting sperm numbers and evaluating sperm morphology.

2.2. Sperm count and sperm morphology analysis

Sperm suspension was prepared using the method of Zhang et al. (2016a, 2016b). Sperm numbers were counted using a haemocytometer and eosin stained slides were used to evaluate sperm morphology. Approximately 1000 normal and malformed sperms were counted from different fields of the mouse semen smear to calculate the sperm malformation percentage in each group.

2.3. Histological assay

The fixed testes tissues were removed, dehydrated, cleared, embedded in paraffin wax, and then sliced into 4–5 μm-thick sections. The sections were subsequently stained with haematoxylin-eosin (HE) for histopathological analysis or with periodic acid Schiff (PAS)-haematoxylin for evaluation of spermatogenesis. The HE-stained sections were examined at 400× and 1000× magnifications using light microscopy (Olympus, Tokyo, Japan). The sections stained with PAS-haematoxylin were used to identify the different stages of the spermatogenic cycles. Spermatogenesis at stage VII was quantitatively analysed using the method of Li et al. (2012). Various types of spermatogenic cells were counted in round or nearly round seminiferous tubules, including type-A spermatogonia (Asg), preleptotene spermatocytes (pLSc), mid-pachytene spermatocytes (mPSc), and stage 7 spermatids (7Sd). The tubular diameter and epithelium height were also measured.

2.4. Determination of serum testosterone levels

Serum testosterone levels were determined using an ELISA Kit (Elabscience, Wuhan, China) according to the manufacturer's instructions. All of the samples were determined at the same time to avoid inter-assay variation.

2.5. qPCR analysis

Total RNA was extracted from 50 mg testes using the TransZol reagent (TransGen, Beijing, China). RNA concentration was measured using a spectrophotometer (Eppendorf, Germany). The purity and integrity were further confirmed by 1% agarose gel electrophoresis and by A260 nm/A280 nm ratios. Extracted RNA was reverse-transcribed to complementary DNA (cDNA) using a Prime Script RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). All of the cDNA samples were diluted by 32 times as templates. qPCR was performed using SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 system. Each reaction (20 μL) was carried out in duplicate. The relative mRNA expression of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). β-actin was used as an internal control. The sequences of the primers are listed in Table S1.

2.6. Caspase-3 activity assay

The caspase-3 activity was measured by detecting the cleavage of caspase-3 substrate (Ac-DEVD-pNA) according to the instructions of Caspase-3 Activity Assay Kit (Beyotime Biotech, Shanghai, China). The absorbance was measured at 405 nm in a microplate reader. One unit of enzyme activity is defined as the amount of enzyme that cleaves 1.0 nmol Ac-DEVD-pNA per hour at 37 °C under saturated substrate concentrations. The protein content was determined using the Bradford method (Bradford, 1976).

2.7. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay

TUNEL assay was performed using *In Situ* Cell Death Detection Kit (Roche, Basel, Switzerland). Paraffin-embedded tissue blocks were cut into 4-μm thick sections. After dewaxing and rehydration, the tissues were treated with proteinase K solution, successively incubated with TUNEL reaction mixture and converter POD, and then covered with 3,3′-diaminobenzidine (DAB) solution. Finally, the sections were

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