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The ubiquitination and acetylation of histones are associated with male reproductive disorders induced by chronic exposure to arsenite

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ABSTRACT

Exposure to arsenic, which occurs via various routes, can cause reproductive toxicity. However, the mechanism for arsenic-induced reproductive disorders in male mice has not been extensively investigated. Here, 6-week-old male mice were dosed to 0, 5, 10, or 20 ppm sodium arsenite ($NaAsO_2$), an active form of arsenic, in drinking water for six months. For male mice exposed to arsenite, fertility was lower compared to control mice. Moreover, for exposed mice, there were lower sperm counts, lower sperm motility, and higher sperm malformation ratios. Further, the mRNA and protein levels of the gonadotropin-regulated testicular RNA helicase (DDX25) and chromosome region maintenance-1 protein (CRM1), along with proteins associated with high mobility group box 2 (HMGB2), phosphoglycerate kinase 2 (PGK2), and testicular angiotensin-converting enzyme (tACE) were lower. Furthermore, chronic exposure to arsenite led to lower H2A ubiquitination (ubH2A); histone H3 acetylation K18 (H3AcK18); and histone H4 acetylations K5, K8, K12, and K16 (H4tetraAck) in haploid spermatids from testicular tissues. These alterations disrupted deposition of protamine 1 (Prm1) in testes. Overall, the present results indicate that the ubiquitination and acetylation of histones is involved in the spermiogenesis disorders caused by chronic exposure to arsenite, which points to a previously unknown connection between the modification of histones and arsenite-induced male reproductive toxicity.

1. Introduction

Arsenic is a naturally occurring geochemical metalloid element, found in abundance in nature and also in humans (Renu et al., 2018). Humans are exposed to arsenic via various routes, including drinking water, diet, and inhaled particulates (Winkel et al., 2008). Millions of people worldwide are exposed to arsenic in drinking water at concentrations exceeding the World Health Organization guideline value of 10 ppb (Bloom et al., 2014; George et al., 2014). For humans and other animals, arsenic induces adverse acute and chronic effects (Bibha Kumari et al., 2017). Chronic arsenic exposure affects multiple organs and systems, leading to skin diseases, cancers, diabetes, cardiovascular diseases, negative reproductive outcomes, and developmental problems (Ratnaike, 2003). Arsenic impairs the male reproductive system by affecting spermatogenesis and testosterone release, inhibiting testicular enzyme function, and reducing weights of the male sex organs (Chang et al., 2007; Jana et al., 2006). Although many studies report the toxic effects of arsenic, the data related to chronic, arsenic induced-

Abbreviations: $NaAsO_2$, Sodium arsenite; DDX25, Gonadotropin-regulated testicular RNA helicase; CRM1, Chromosome region maintenance-1 protein; HMGB2, High mobility group box 2; PGK2, Phosphoglycerate kinase 2; tACE, Testicular angiotensin-converting enzyme; ubH2A, Ubiquitinated H2A; Prm1, Protamine 1; IHC, Immunohistochemistry; H3AcK18, Histone H3 acetylation K18; H4tetraAck, Histone H4 acetylation K5, K8, K12, and K16; Uu, *Ureaplasma urealyticum*; AS3MT, Arsenic methyltransferase; DMEM, Dulbecco's Modified Eagle's Medium

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reproductive toxicity are limited.

The male infertility rate continues to climb worldwide. About 15% of couples in reproductive ages have difficulties with conception related to infertility; environmental factors may be involved (Otasevic et al., 2020). Arsenic, an endocrine disruptor, exerts various adverse effects on the male reproductive system (Wang et al., 2016). Epidemiological data from arsenic endemic areas of Mexico and Taiwan demonstrate that arsenic exposure induces sperm chromosomal aberrations and erectile dysfunction (Hsieh et al., 2008; Leke et al., 1993). High concentrations of arsenic (0.186 μg/mL in seminal plasma) are present in patients infected with *Ureaplasma urealyticum* (Uu), who have higher quantities of abnormal sperm than patients without Uu (Wang et al., 2005). Arsenic-exposed animals show effects on the male reproductive system, including impaired reproductive capacity, inhibition of spermatogenesis, and lower steroidogenesis (Jana et al., 2006; Pant et al., 2001). Exposure of adult mice to sodium arsenite (30 or 40 mg/L) in drinking water for 30, 45, or 60 days impairs, in a dose-dependent manner, the process of spermatogenesis at meiosis and post-meiotic stages, including interruption of spermatogenesis, via reduced seminiferous tubular diameters; reduced cell populations (gametogenic), including resting and pachytene spermatocytes; step 1–7 spermatid reductions, except for spermatogonia; along with an elevated degree of atrophy of Leydig cells (Sanghamitra et al., 2008). Sodium arsenite given to male mice (22-g body weight, 8 to 10 weeks old) for 365 days at a concentration 53.39 μmol/L in drinking water, alters testicular enzymatic activity, elevates abnormal sperm, and decreases sperm counts and motility of sperm; also, there is accumulation of arsenic in the epididymes, testicular tissue, prostate glands, and seminal vesicles (Pant et al., 2004). Sodium arsenite, 5 mg/kg for 12 weeks, reduces sperm counts, sperm motility, and luteinizing hormone and follicle-stimulating hormone in the serum of Teddy goat bucks (Zubair et al., 2016). For rats, sodium arsenite (8 mg/kg/day for 8 weeks) reduces sperm counts, sperm motility, viability of sperm, and sperm morphology (Momeni and Eskandari, 2012; Momeni et al., 2012). However, the mechanism of action for arsenic-induced suppression of reproductive health is still unclear. Various mechanisms have been considered as the mode of action for arsenic toxicity. According to the concentrations of sodium arsenite and exposure times used in prior studies 30 or 40 ppm for 30, 45, or 60 days (Sanghamitra et al., 2008); and 0, 0.2, 2, or 20 ppm for 6 months (Han et al., 2020); and the results of our preliminary experiments, we determined to use 0, 5, 10 and 20 ppm sodium arsenite to treat male mice for 6 months.

During spermiogenesis, male germ cells undergo sequential changes in cell morphology and condensation of the nucleus, as represented by the stepwise emergence of round, elongating, condensing, and condensed spermatids (Pogany et al., 1981). Histones, which are basic nuclear proteins, are involved in maintaining active transcriptional states (Hecht, 1998; Steger, 2001). Upon commencement of nuclear elongation, both somatic and testis-specific histones are replaced, first with transition nuclear proteins and later with protamines, to achieve a spermatozoon-specific nuclear structure (Lewis et al., 2004; Meistrich et al., 2003). Histone acetylation regulates chromatin structure and promotes accessibility via condensation and relaxation of chromatin fibers (Shogren-Knaak et al., 2006). H2A and H2B ubiquitination, mediated, in elongating spermatids, by the E3 ubiquitin ligase, ring finger protein 8 (RNF8), is an initial step in H4 acetylation and histone removal (Gou et al., 2017; Lu et al., 2010). However, whether histone acetylation is involved in reproductive toxicity caused by sodium arsenite is unclear.

In the present study, male mice, chronically exposed to arsenite, had adverse fertility outcomes, lower sperm counts and sperm vitality, higher rates of sperm deformities, and abnormal acrosomal development. In addition, there were changes in the expression of mRNAs and proteins associated with sperm formation. Expressions of H3, H4, and other histone acetylation in the testes of chronic arsenite-exposed mice were also lower. These results contribute to an understanding of arsenite-induced male reproductive toxicity.

2. Material and methods

2.1. Mouse model for arsenite exposure

All procedures involving animals, including those exposed to arsenite, were approved by the Institutional Animal Care and Use Committee at Nanjing Medical University. Male C57BL/6 J mice (6–8 weeks old) and female C57BL/6 J mice (8 weeks old) were obtained from Nanjing Medical University and were maintained under controlled conditions (temperature, 22 ± 2 °C) and in a daily reversed light/dark cycle. Male mice were mated with female mice after male animals were exposed to 0, 5, 10, or 20 ppm sodium arsenite ($NaAsO_2$, purity: 99.0%, Sigma, St, Louis, MO) in their drinking water for 6 months. Before conducting the experiment, we tested the background levels of arsenic in the feed and drinking water; they all met international standards. The male fertility rates (number of males impregnating females/number of males mated × 100), the fetal resorption rates (number of resorptions/(number of viable fetuses + resorptions)), and litter weights (the weight of all offspring of each female mouse on the first day) were calculated. After exposure and mating, some pregnant female mice were euthanized on gestation day 18.5 to calculate the fetal resorption rates. After 6-months exposure to arsenite, male mice were sacrificed by CO_2 asphyxiation, and the epididymes and testes were removed and weighed. The right testes were frozen in liquid nitrogen. One part of the left testes was fixed in Bouin's solution for histological study; another part of the left testes was kept for electron microscopy observation.

2.2. Preparation of single-cell suspensions of testicular tissue and flow cytometry

After exposure to arsenite for 6 months, mice were humanely sacrificed, and their testes were removed. The tunica albuginea of each testis was excised and the seminiferous tubules isolated from interstitial tissue. Testicular preparations were first subjected to enzymatic digestion (5–10 min at 37 °C) with Collagenase Type IV (Invitrogen, Carlsbad, USA) in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, China). Seminiferous tubule fragments were then isolated by passage through a 40-μm nylon mesh to remove cell clumps and were centrifuged at 600 ×*g* for 5 min. The resulting pellet was placed in 0.25% Trypsin-EDTA (Gibco, USA) for 10 min to obtain single cell suspensions. The digestion was terminated with 10% fetal bovine serum. The suspension was centrifuged at 600 ×*g* for 5 min, and the resulting pellet was suspended in PBS. The single cell suspensions were stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, USA) for 30 min at 37 °C, centrifuged at 600 ×*g* for 5 min, and washed with PBS. The haploid spermatids were obtained by use of a flow cytometer (Becton Dickinson, USA).

2.3. Quantitative real-time polymerase chain reaction PCR (qRT-PCR)

The total RNA of testis tissues was extracted using the TRIzol agent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The UV absorption ratio (A260/A280) of total RNA in the range of 1.8–2.0 as the standard of RNA quality was determined by use of a NanoDrop 2000 (Thermo Fisher, USA), and the mRNA was reversed transcribed into cDNA using primeScript™ RT reagent Kits (Takara, Kusatsu, Japan) following the manufacturer's protocol. The primers used for qRT-PCR are listed in Table 1. The protocol was: 95 °C × 3 min, 95 °C × 3 s, 60 °C × 30 s × 40 cycles, which was performed with a 7900HT Fast PCR System (Applied Biosystems, Foster City, CA, USA). Data analysis was accomplished by the comparative Ct method with GAPDH as the normalization control.

Table 1
Primer sequences used.

DDX25	F: 5′-*ATGGCGTCGTTACTTTGGGG*-3′
	R: 5′-*AGAGCCGTCTATGTTTGGGAC*-3′
CRM1	F: 5′-*CTGCTTGATTTCAGCCAAAAACT*-3′
	R: 5′-*GTATTTCGTGTTCATGTTCTGCG*-3′
HMGB2	F: 5′-*AAGAGCGACAAAGCTCGTTATG*-3′
	R: 5′-*GCAGTATCTCCAATAGACAGGC*-3′
PGK2	F: 5′-*CCACCTCCAATGGCTGTATC*-3′
	R: 5′-*CTACCCCTGGAAGGGTTTTG*-3′
tACE	F: 5′-*AGTATGACCGGACAGCCAAG*-3′
	R: 5′-*CCAGGTGCCATATTTCAAGG*-3′
Prm1	F: 5′- *CCGTCGCAGACGAAGATGTC*-3′
	R: 5′-*CACCTTATGGTGTATGAGCGG*-3′
GAPDH	F: 5′-*GTCTTCACTACCATGGAGAAGG*-3′
	R: 5′-*TCATGGATGACCTTGGCCAG*-3′

2.4. Western blotting analysis

Total protein was extracted from testis tissues using RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations were determined with BCA kits (Beyotime Institute of Biotechnology, Shanghai, China), and 40 μg of protein was loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk for 1 h at room temperature, the membranes were incubated with a primary antibody, anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH, AF5009, Beyotime, China), DDX25 (sc-271,730, Santa Cruz Biotechnology, CA, USA), CRM1(ab191081, Abcam, USA), HMGB2 (ab124670, Abcam, USA), PGK2 (ab183031, Abcam, USA) or tACE (AF6147, Beyotime, China), H3AcK18 (ab40888, Abcam, USA), H3 (ab176842, Abcam, USA), H4tetraAck (ab177790, Abcam, USA), H4 (ab177840, Abcam, USA), Prm1 (ab66978, Abcam, USA), ubH2A (ab193203, Abcam, USA), or H2A (ab177308, Abcam, USA) at 4 °C overnight. The next day, after three washings in TBST (1% Tween diluted in PBS), the membranes were incubated with a secondary antibody at room temperature for 1 h. The immune complexes were detected by enhanced chemiluminescence (Cell Signaling Technology). For densitometric analyses, protein bands on the blots were measured by Image J software.

2.5. Testis histology

Three mice of each group were selected randomly for testicular histopathology. The right-side testes were fixed in 4% neutral buffered formalin overnight and then washed with a Li_2CO_3-saturated solution of 70% ethanol. Following standard procedures for paraffin-embedded tissues, testes were cut into 3-μm sections and stained with hematoxylin and eosin (H&E) according to a standard protocol for histopathologic analysis (Cai et al., 2000) and periodic acid Schiff staining. The histopathology slides were scanned with a Panoramic Scan (3DHISTECH Ltd., Hungary), at 40 × magnification. Three testicular photomicrographs per mouse were compiled and analyzed using Adobe Photoshop and ImageJ.

2.6. Immunohistochemistry (IHC) assay

Right-side testes were embedded in paraffin and cut into 3-μm thick sections. For IHC staining, the slices were dewaxed and rehydrated, and, after an antigen retrieval process and blocking, they were incubated with primary antibodies against protamine 1 (Prm1) or ubH2A overnight. The histopathology slides were scanned with Pannoramic Scan (3DHISTECH Ltd., Hungary) at 40 × magnification.

2.7. Semen analysis for mice

For each sacrificed mouse, the right-side cauda epididymis was isolated, transferred into 200 μL of warmed (37 °C) modified human tubal fluid (mHTF; InVitroCare), and excised with scissors. After 10 min of incubation, spermatozoa were allowed to swim out, and undissolved tissue was removed. Portions of suspended sperm were placed in warmed chambers (2 × -CEL Chamber, 20 μL, Hamilton Thorne). From two chambers covering 20 different fields, sperm counts and sperm motility were measured by the CASA system, IVOS (Hamilton-Thorne Research, Inc.).

2.8. Transmission electron microscopy (TEM)

Testes and cauda epididymes were trimmed and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4 °C, fixed in 2% osmium tetroxide for 1 h, dehydrated in ascending graded ethanol, and embedded in EPON 812 at 60 °C for 24 h. Ultrathin (60-nm) sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7700 transmission electron microscope.

2.9. Assay of hormones

The collected blood samples were centrifuged, and serum was separated. The serum levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), and androgen were measured using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Hengyuan Biological Technology, Shanghai, China) according to the manufacturer's instructions.

2.10. Statistical analysis

The data presented are means ± SD of independent replicates ($n \geq 3$). One-way analysis of variance (ANOVA), followed by least significant difference (LSD) for multi-group comparison. p values < 0.05 were considered statistically significant. All statistical analyses were performed with SPSS 19.2.

3. Results

3.1. Chronic exposure to arsenite reduces fertility and increases fetal resorption rate

Compared with male mice in the control group, the physiological activities of male mice in the chronic arsenite-exposed groups showed normal growth, and all survived until adulthood. After co-housing of male and female mice, the numbers of female mice with vaginal plugs (in the present study, all were pregnant), the fetal resorption rate, the litter sizes of female mice, and the average weight per litter of mice in the group exposed to 20 ppm $NaAsO_2$ were statistically different compared with the control group; however, there was no difference for the group exposed to 5 ppm $NaAsO_2$ and the controls ($p < 0.05$) (Table 2). Also, there was no significant difference, relative to controls, in fertility, fetal resorption rate, or litter size for the group exposed to 10 ppm arsenite. There was also no difference in the sex ratio of offspring between groups (Table 2). However, in the sera, the levels of LH, FSH, and androgen were lower in arsenite-exposed male mice (Fig. S1). Thus, although male mice exposed chronically to arsenite had normal mating behavior, their fertility was diminished.

3.2. Chronic exposure to arsenite induces abnormal testicular development, decreases sperm counts and motility, and increases sperm malformations in mice

After exposure to arsenite for 6 months, the testis sizes of arsenite-exposed mice were smaller than controls, especially for mice dosed with

Table 2
The fertility of control and arsenite-exposed mice.

Groups	0 ppm ($NaAsO_2$)	5 ppm ($NaAsO_2$)	10 ppm ($NaAsO_2$)	20 ppm ($NaAsO_2$)
Fertility (%)	100 (15/15)	93.33% (14/15)	86.67% (13/15)	73.33% (11/15)*
Fetal resorption rate (%)	8.59 ± 5.98	12.81 ± 3.61	13.58 ± 3.66	22.06 ± 11.99*
Litter size (n = 15)	8.13 ± 0.92	7.53 ± 1.19	7.40 ± 1.24	6.33 ± 1.50*
Litter weight (n = 15)	10.82 ± 0.75	10.18 ± 0.95	9.64 ± 0.94*	8.42 ± 0.74*
Male mice ratio (%)	0.52 ± 0.13	0.53 ± 0.13	0.48 ± 0.11	0.49 ± 0.13

Values are expressed as means ± SD.
* Indicates a significant difference from the control group, $p < 0.05$.

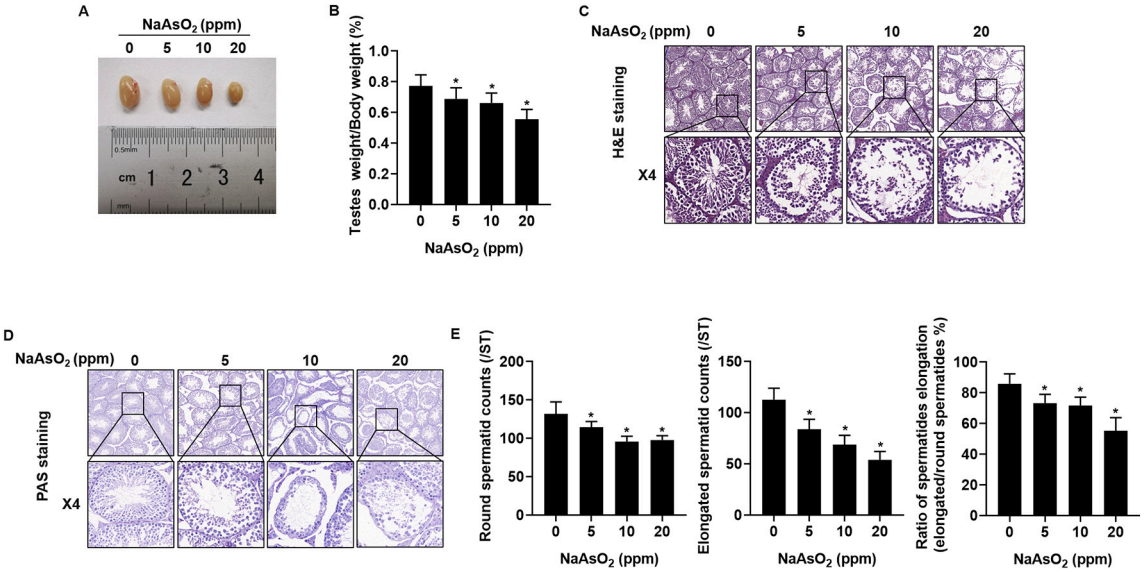

Fig. 1. Chronic exposure to arsenite induces abnormal testicular development in mice.
Male mice were exposed to 0, 5, 10, or 20 ppm $NaAsO_2$ in their drinking water for 6 months. (A) Gross morphology of the testes and (B) testis-to-body weight ratios for mice, n = 15, mean ± SD, $*p < 0.05$, compared to the control group. Testicular histology was assessed by H&E staining (C) and by periodic acid-Schiff staining (D), Scale bar = 100 μm. (E) Round spermatid counts, elongated spermatid counts, and ratios of spermatid elongation, as determined by PAS staining of testes, n = 10, mean ± SD, $*p < 0.05$, compared to the control group.

20 ppm $NaAsO_2$ (Fig. 1A). The relative weights for testes were lower for arsenite-exposed mice (Fig. 1B). The testicular tissue and seminiferous tubule structure were intact and clear, and, in the control group, spermatogenic cells in various stages were distributed in the lumen. Compared to controls, exposure altered the structures of seminiferous tubules and the seminiferous epithelium, increased the gaps between seminiferous tubules, and reduced the spermatogenic cells at various stages (Fig. 1C and D). In arsenite-exposed groups, the counts of round spermatids, the counts of elongated spermatids, and the spermatid elongation ratios were lower (Fig. 1E). In addition, sperm counts and sperm motility, parameters of sperm quality, were evaluated for control and arsenite-exposed groups. For the arsenite-exposed groups, sperm counts (Fig. 2A), sperm motility (Fig. 2B), and the percentages of motile sperm (Fig. 2C) were lower, with a concomitant increase in the sperm malformation ratios (Fig. 2D and E). Furthermore, analysis of the sperm ultrastructure revealed that their acrosomal integrity was defective (Fig. 2F). These results indicate that chronic exposure to arsenite impairs spermatogenesis.

3.3. Chronic exposure to arsenite reduces expression of genes related to sperm formation in testes of mice

DDX25, which is active in the haploid periods of spermatogenesis, interacts with *CRM1* to regulate the expression of proteins, including H4, HMG2, TP2, and PGK2 (Dufau and Kavarthapu, 2019; Sheng et al., 2006; Yang et al., 2015). Haploid spermatids from testicular tissues were obtained by flow cytometry (Fig. S2), the mRNA expressions of *DDX25*, *CRM1*, *HMGB2*, *PGK2*, and *tACE* were evaluated in the control mice and those exposed to arsenite. mRNA expression of *DDX25*, *CRM1*, *HMGB2*, *PGK2* and *tACE* were lower in the arsenite-exposed groups (Fig. 3A). In these groups, the protein expressions of DDX25, CRM1, PGK2, and tACE were also decreased (Fig. 3B and C). These results suggest that DDX25, CRM1, HMGB2, PGK2, and tACE are involved in the changes of spermatids induced by chronic exposure to arsenite.

3.4. Chronic exposure to arsenite induces histone modifications and disrupts Prm1 deposition in testes of mice

To determine whether acetylation of core histones is altered in mice exposed to arsenite, proteins from the testes of the mice were examined. Acetylation levels of H3 at K18 (H3AcK18) and H4 at K5, K8, K12, and K16 (H4tetraAcK) were lower in the haploid spermatids from testes of mice chronically dosed with arsenite as compared with control mice (Fig. 4A and B). Protamines, small and highly-basic proteins with an arginine-rich core, are sperm-specific nuclear proteins with high DNA affinity. In eutherian mammals, including mice and humans, protamines are characterized by arginine and cysteine residues (Oliva, 2006). During spermiogenesis, arginine residues mediate formation of highly stable DNA-protamine complexes that condense chromatin into toroidal structures (Carrell et al., 2007). Therefore, we examined Prm1 expression and found that Prm1 in sperm was lower in groups exposed to 10 or 20 ppm $NaAsO_2$ (Fig. 4C). Furthermore, protein expression was

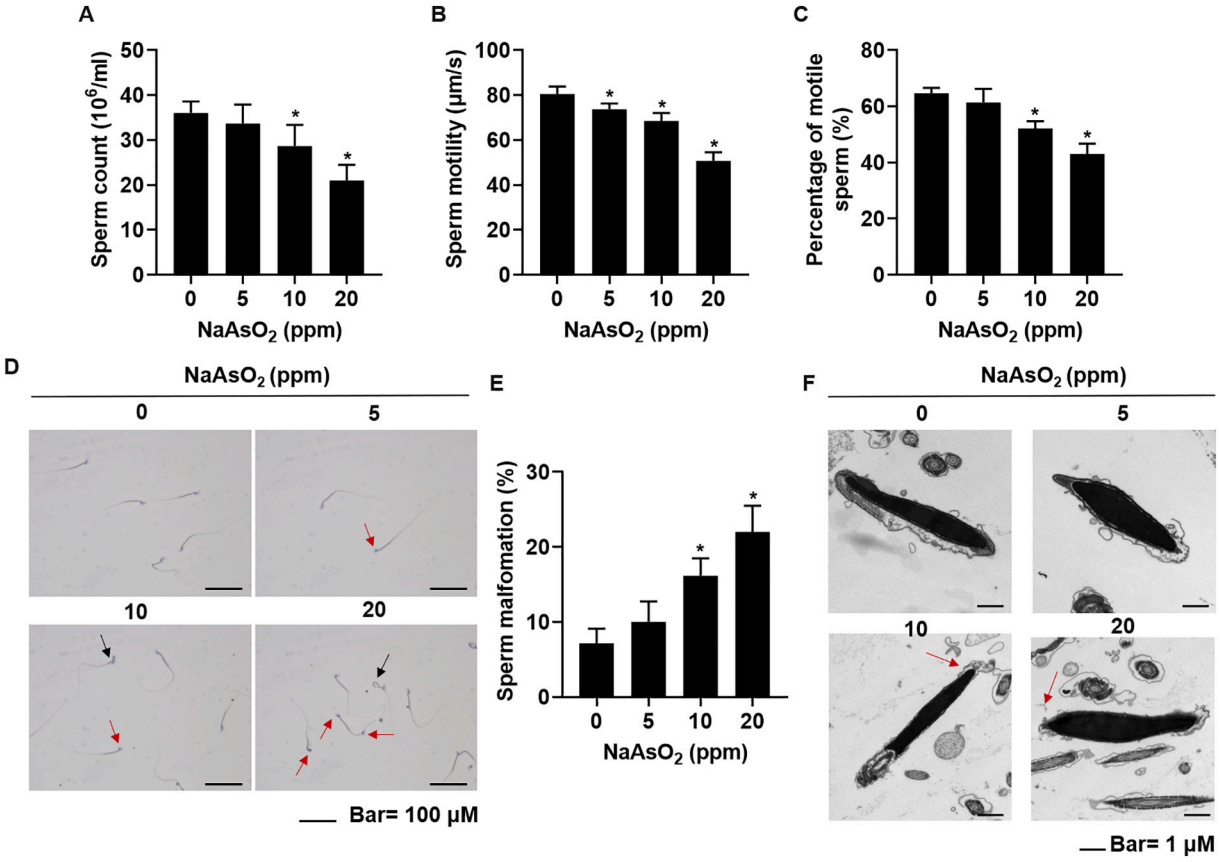

Fig. 2. Chronic exposure to arsenite lowers sperm counts and sperm motility and increases sperm malformations in mice.
Male mice were exposed to 0, 5, 10, or 20 ppm $NaAsO_2$ in their drinking water for 6 months. Sperm counts (A), sperm motility (B), and percentages of motile sperm (C) in semen of mice, n = 6, mean ± SD. *p < 0.05 compared to the control group. (D) The morphology of sperm as shown by H&E staining. The red arrows indicate abnormal sperm head morphology. The black arrows indicate abnormal sperm neck morphology. Scale bar = 100 μm. (E) Malformed sperm in mice were counted, n = 6, mean ± SD, *p < 0.05, compared to the control group. (F) Typical electron microscopic images of sperm heads, with the red arrows indicating abnormal acrosomes, Scale bar = 1 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

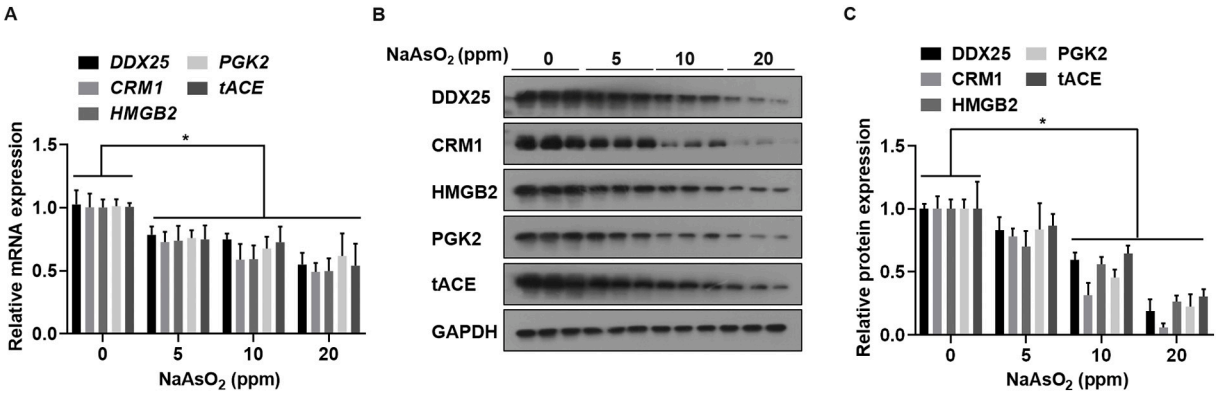

Fig. 3. Chronic exposure to arsenite decreases expression of genes related to sperm formation in testes of mice.
Male mice were exposed to 0, 5, 10, or 20 ppm $NaAsO_2$ in their drinking water for 6 months. (A) mRNA levels of *DDX25*, *CRM1*, *HMGB2*, *PGK2,* and *tACE* in haploid spermatids isolated from testes by FACS as determined by qRT-PCR, n = 6, mean ± SD, *p < 0.05, compared to the control group. (B) Western blots were performed, and (C) relative protein levels of DDX25, CRM1, HMGB2, PGK2, and tACE in haploid spermatids isolated from testes were determined, n = 3, mean ± SD, *p < 0.05, compared to the control group.

lower in sperm isolated from caudal epididymes of mice dosed with arsenite (Fig. 4D and E). Immunochemical evaluation of testes also revealed lower levels of Prm1 in mice exposed to arsenite (Fig. 4F). These results indicate that, during spermiogenesis, arsenite exposure results in less histone acetylation and protamine deposition.

3.5. Chronic exposure to arsenite leads to lower ubH2A levels in elongating spermatids

Ubiquitinated H2A (ubH2A) and H2B (ubH2B) control H4 acetylation through a trans-histone modification mechanism (Gou et al., 2017; Lu et al., 2010). To evaluate whether ubH2A is involved in the impaired histone-to-protamine exchange during spermiogenesis in arsenite-

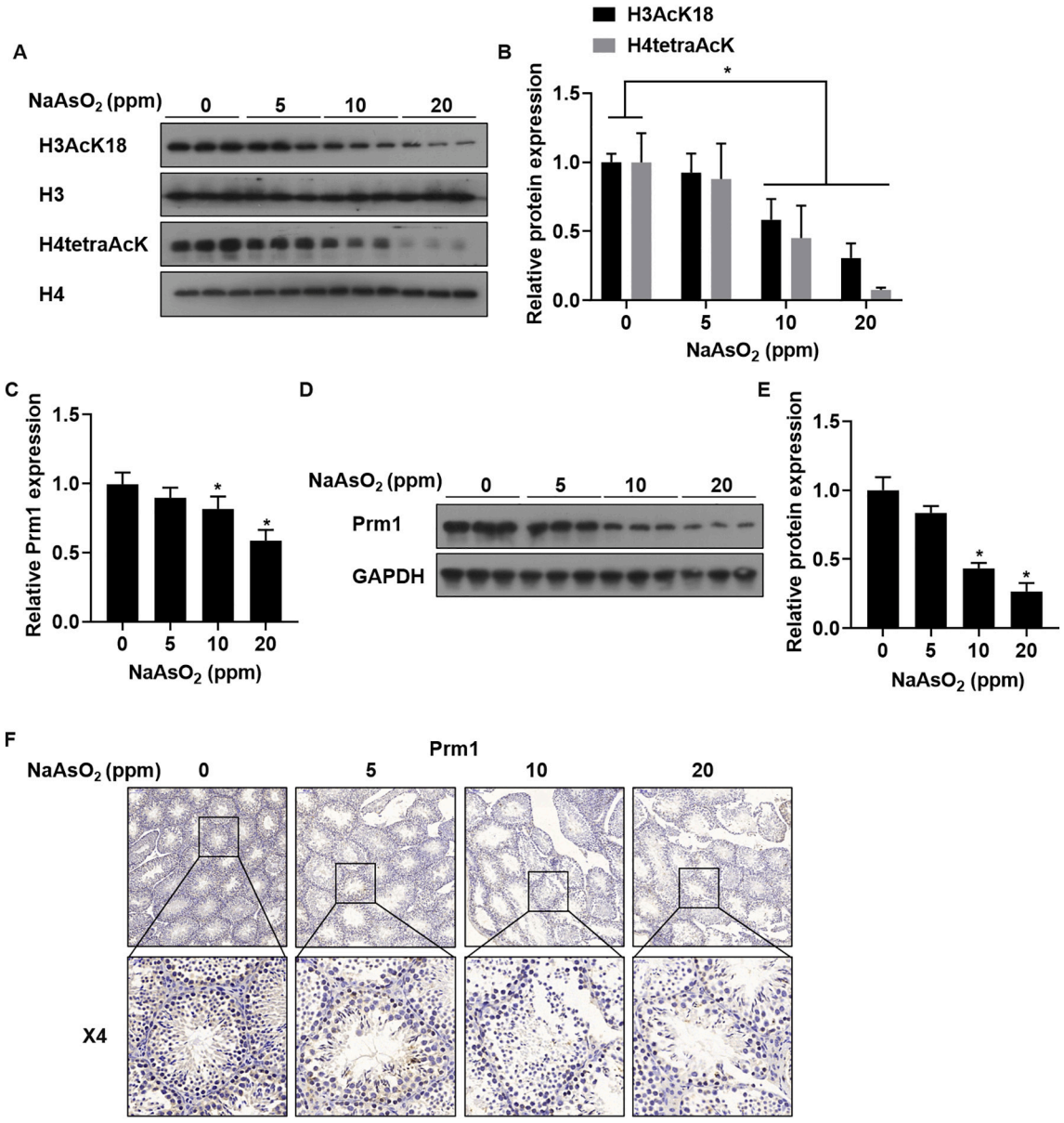

Fig. 4. Chronic exposure to arsenite alters histone modifications and disrupts Prm1 deposition in testes of mice.
Male mice were exposed to 0, 5, 10, or 20 ppm $NaAsO_2$ in their drinking water for 6 months. (A) Western blots were performed, and (B) relative protein levels of H3AcK18, H3, H4tetraAck, and H4 in haploid spermatids isolated from testes were determined, n = 3, mean ± SD, $^*p < 0.05$ compared to the control group. (C) The levels of *Prm1* in sperm were determined by qRT-PCR, n = 6, mean ± SD, $^*p < 0.05$ compared to the control group. (D) Western blots were performed, and (E) relative protein levels of Prm1 were determined, n = 3, mean ± SD, $^*p < 0.05$ compared to the control group. (F) Immunohistochemical staining of Prm1 in the testes of mice, Scale bar = 100 μm.

exposed mice, ubH2A levels were examined. In the haploid spermatids from testes of mice exposed to arsenite, ubH2A levels were lower and H2A levels were higher than those for control mice (Fig. 5A and B). As shown by immunohistochemistry, ubH2A signals were weaker in arsenite-exposed mice than in control mice (Fig. 5C). The results suggest that chronic exposure to arsenite suppresses ubH2A in spermatids.

4. Discussion

The aim of this study was to assess the reproductive toxicity of arsenite for male mice. We found that chronic exposure to arsenite affected their fertility, lowered the litter weights, and decreased litter sizes. Further, arsenite impaired the process of spermatogenesis, resulting in testicular damage, decreased sperm motility, increased sperm malformations, and destruction of acrosome structures. Also, arsenite decreased the expression of factors involved in spermatogenesis, including DDX25, CRM1, HMGB2, Prm1, PGK2, and tACE, and it blocked normal histone acetylation and ubiquitination modifications.

Various studies have demonstrated that arsenic causes a variety of diseases, including skin cancer (Chayapong et al., 2017), cardiovascular disease, bladder cancer, lung disease, diabetes (Renu et al., 2018), and reproductive toxicity (Huang et al., 2016). In humans and most other mammals, arsenic methyltransferase (AS3MT) converts inorganic arsenic (iAs) into monomethyl as (MAS) and dimethyl as (DMAS) to detoxify arsenic (Lin et al., 2002; Thomas et al., 2007). Studies of the mechanistic basis of iAs-related diseases have been hindered by differences between experimental animals and humans in their abilities to metabolize and detoxify iAs (Lu et al., 2007). Different from humans, mice show a high arsenic methylation rate. This species difference is related to faster rates of urinary clearance of methylated metabolites

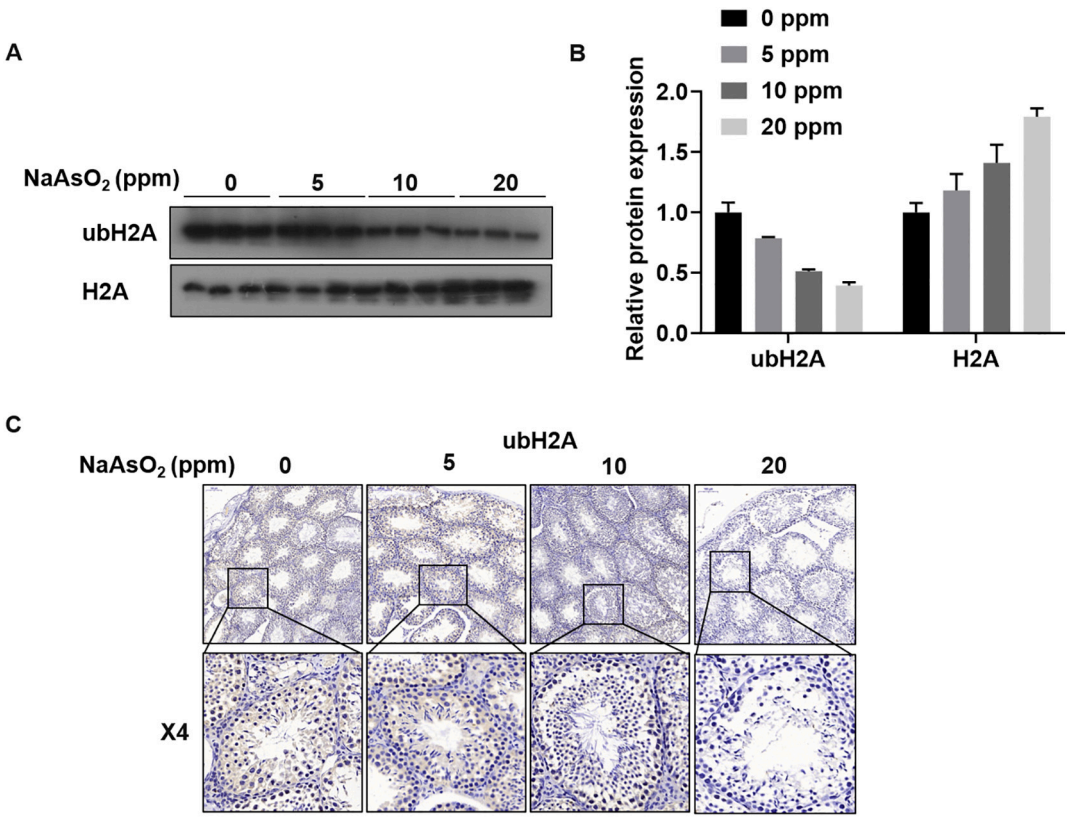

Fig. 5. Chronic exposure to arsenite reduces ubH2A levels in elongating spermatids.
Male mice were exposed to 0, 5, 10, or 20 ppm $NaAsO_2$ in their drinking water for 6 months. (A) Western blots were performed, and (B) relative protein levels of ubH2A and H2A in haploid spermatids isolated from testes were determined, n = 3, mean ± SD, $*p < 0.05$, compared to the control group. (C) Immunohistochemical staining of ubH2A in the elongating spermatids of mice, Scale bar = 100 μm.

and the advantage of DMAs as the main urine metabolites of iAs in mice (Vahter, 1999).

Human epidemiological studies show that arsenic exposure is associated with male infertility and that arsenic increases the risk of erectile dysfunction and lowers testosterone levels (Hsieh et al., 2008). In a cohort study, arsenic reduced sperm concentrations and reduced sperm quality (Xu et al., 2012). In experimental studies, arsenic impaired spermatogenesis, including decreasing seminiferous tubule diameters, abnormal sperm counts, sperm motility, and sperm morphology (Chang et al., 2007). Male mice exposed to arsenic trioxide showed lower sperm counts and degeneration of the seminiferous epithelium (Chang et al., 2007; Pant et al., 2001). In the present study, we found that the fertility of male mice chronically exposed to arsenite was lower, and the litter weights and litter sizes were also affected. Further, in exploring the effect of chronic exposure to arsenite on the reproductive system of male mice, we assessed the spermatogenesis process in the testes. The results showed that the weights and volumes of testes of mice chronically exposed to arsenite were reduced, the interstitial spaces of seminiferous tubules were enlarged, and the spermatogenic cells were disordered. Chronic exposure to arsenite decreased the number and motility of mature spermatozoa and increased the rate of sperm malformation. In male mice chronically exposed to arsenite, the acrosome structure of spermatozoa was damaged, suggesting that arsenite caused a decrease in the quality of mature spermatozoa by affecting the spermatogenesis process, leading to male reproductive toxicity. For females, arsenite also induces reproductive toxicity through ovarian dysfunction, and arsenic trioxide (As_2O_3) negatively affects mean body weights, litter sizes, organ coefficients, and stereological indices (Ommati et al., 2020), results that are consistent with those of the present study.

To determine which stage of spermatogenesis was affected by arsenite, we measured the levels of mRNA and protein expression of genes involved during spermatogenesis. *DDX25* is closely related to the process of sperm elongation, for the spermatogenesis of DDX25-null mice stops at step 8, and the mice are sterile (Tsai-Morris et al., 2004). Arsenite exposure changes the ultrastructure of chromatoid bodies and reduces the molecular components of round spermatids. Further, DDX25 regulates the expressions of *HMGB2*, *PGK2,* and *tACE*, which are in the CRM1-dependent nuclear export pathway; this process is involved in the arsenite-induced damage to male reproduction during the haploid period (Han et al., 2020). In the present study, our results indicated that arsenite exposure reduced the levels of mRNA and protein expression of DDX25, CRM1, HMGB2, PGK2 and tACE, suggesting that arsenite impaired sperm elongation, resulting in a decrease in sperm quality during spermatogenesis. In addition, a difference from Han's research is that we found that histone modifications have a regulatory role in abnormal sperm formation caused by arsenite. Further, in Han's study, unlike the lower testicular weights in our study, the weights of testes increased, and the pathological changes in testes were less extensive. Some possible factors involved in the differences between the two studies may include following reasons. On one hand, these differences may relate to different effects of As_2O_3 and $NaAsO_2$ on testes. On the other hand, Malki et al. found that an adverse environment produces stress-related differences at a genomic level, which are more prevalent in certain mouse strains (Malki et al., 2015). Mouri et al. found that the reactions caused by phencyclidine (PCP) in C57BL/6 J were greater than in ICR, which indicated that there was the mouse strain difference in the damages induced by chemicals (Mouri et al., 2012). Therefore, C57BL/6 J and ICR mice may have different sensitivity to arsenic, which will lead to differences in the reproductive toxicity. More experiments are required to explore these results.

Normal spermatogenesis often involves chromatin remodeling, in

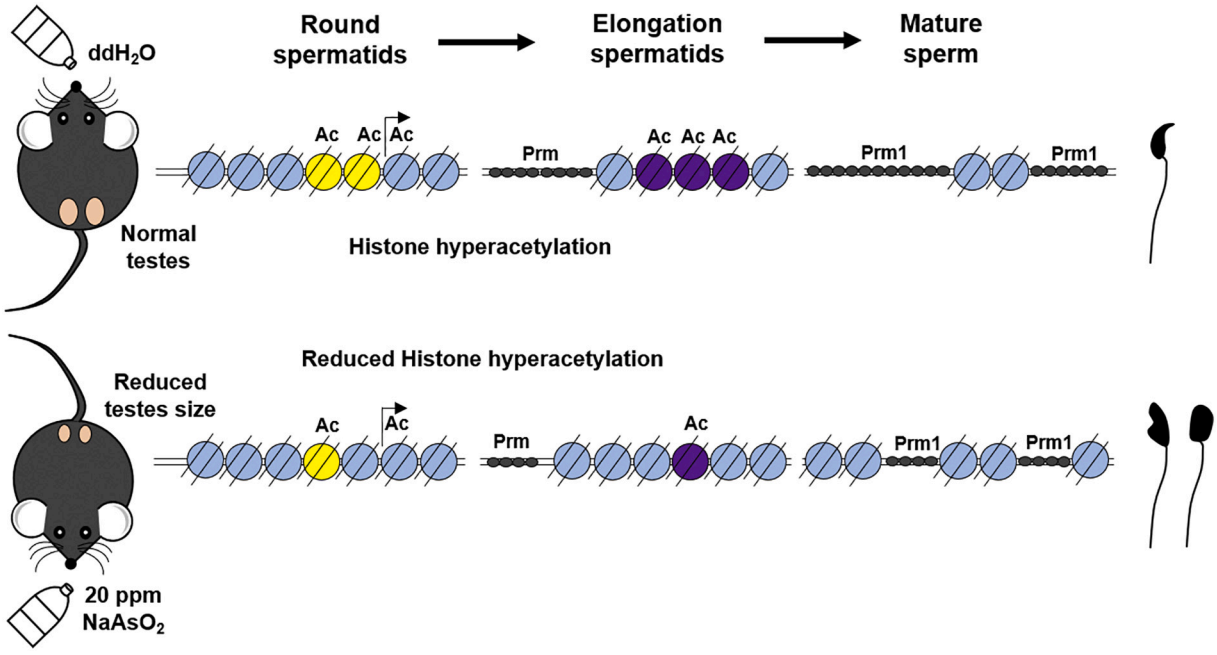

Fig. 6. Schematic diagram showing that chronic exposure to arsenite disturbs spermatogenesis by reducing histone acetylation.
In the testes of control mice, histone hyperacetylation occurs in round spermatids (yellow) or elongation spermatids (purple), which is later replaced by Prm1. However, in mice chronically exposed to arsenite, the lack of acetylation leads to elevated histone retention in sperm and lower levels of Prm1, resulting in more sperm malformations and lower sperm quality, which in turn affects the fertility of male mice. ddH_2O, double-distilled water. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which a large number of histones in spermatocytes are replaced by protamine. In this manner, the DNA of sperm cells is protected from external damage (Jenkins and Carrell, 2011). In the process of protamine replacement of histones, histones undergo an acetylation modification to facilitate the opening of DNA double strands and thereby promote protamine replacement of histones (Francis et al., 2014; Gaucher et al., 2010; Jenkins and Carrell, 2011). As determined in the present study, chronic exposure to arsenite reduced the acetylation modification of H3 and H4, and the expression of Prm1 in spermatozoa was lower than that in the control group, indicating that arsenite affected the process of histone replacement by protamine through reducing the acetylation modification of histone, thus making the DNA structure in spermatozoa more vulnerable to external damage and reducing their quality.

During spermiogenesis, ubiquitination of histones is necessary prior to the histone-to-protamine exchange (Wang et al., 2019). Ubiquitination of histones, especially H2A and H2B in developing male germ cells (Baarends et al., 1999; Jason et al., 2002), is an epigenetic mark involved in chromatin remodeling during spermatogenesis (Govin et al., 2004; Rathke et al., 2014; Sheng et al., 2014). Our results revealed that ubH2A levels were lower in mice exposed to arsenite than in control mice. Moreover, after the inhibition of histone deacetylase by trichostatin A, sperm exhibit high acetylation during sperm maturation, and their quantity is lower (Awe and Renkawitz-Pohl, 2010; Fenic et al., 2004; Hazzouri et al., 2000), effects that are related to higher DNA damage and breakage of DNA strands (Marcon and Boissonneault, 2004). The acetylation of H4K16, H3K14, and H3K9 and methylation of H3K9, H3K4me, and H3K27 amino acid residues occur during exposure to arsenite (Chervona et al., 2012; Jo et al., 2009).

During arsenic-induced cancer development, there are changes in histone acetylation and methylation; arsenite exposure caused a time-dependent increase in histone H3 acetylation of lysines K4, K9, K14, K18, K23, and K27, but a decrease in acetylation of lysines K5, K8, K12, and K16 of histone H4 (Ge et al., 2018). As demonstrated here, for mice chronically exposed to arsenite, H4tetraAcK was lower, which is consistent with the above results. In the present study, however, the levels of H3AcK18 were inconsistent with previous studies, which may be caused by the different functions of H3AcK18 in different organisms. Nevertheless, H3AcK18 and H4tetraAcK were lower with Prm1 deposition in sperm, indicating that ubiquination of histones is associated with the defective histone-to-protamine transition observed in mice chronically exposed to arsenite.

Although exposure to arsenite at 5 and 10 ppm also affected expression of some indicators for histone remodeling and had a dose-response relationship, male fertility and fetal loss were not affected by arsenite at these concentrations. Arsenite apparently impairs the male reproductive system via other pathways; for example, arsenite causes DNA damage in rat germinal cells and inhibits androgen receptor transcriptional activity (Nava-Hernandez et al., 2009; Rosenblatt and Burnstein, 2009). Although we considered that histone modifications are involved in the reproductive disorders induced by exposure to arsenite, this result suggested that arsenite has to reach a certain dose to decrease male fertility and increase fetal loss though this pathway.

5. Conclusions

In summary, arsenite blocks sperm elongation by reducing the expression of genes involved in spermatogenesis, and it affects the process of protamine substitution for histones by reducing ubiquitination and acetylation modification of histones, which makes sperm DNA more susceptible to the external environment. The combined effect of these factors leads to a decline in sperm quantity and quality, which is manifested as low male fertility (Fig. 6).

Credit author statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other

publication before its appearance in the Special Issue of (SI) of Toxicology and Applied Pharmacology (TAAP) with a theme of "Arsenic as an Environmental Toxicant and a Therapeutic Agent: Foe and Friend".

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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