RESEARCH ARTICLE

Prenatal exposure to atrazine induces cryptorchidism and hypospadias in F1 male mouse offspring

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Abstract

The main objective of the present study was to determine whether prenatal exposure to atrazine could affect testicle descent and penile masculinization. Atrazine has been demonstrated with a variety of endocrine disrupting activities and reproductive toxicities. However, the effects of prenatal atrazine exposure on male offspring's genital malformation, such as hypospadias and cryptorchidism, remain poorly understood. In this study, pregnant ICR mice were gavaged from gestational day 12.5–16.5 with different doses of atrazine. Although no sign of systemic toxicity was observed in F1 male pups, prenatal exposure to 100 mg/kg/day atrazine affected penile morphology, urethral meatus position and descent of testis, and reduced anogenital distance and penile size in postnatal day 21 F1 male pups. The comparative study with an androgen receptor (AR) antagonist vinclozolin suggested that these effects of atrazine on male genital development may not be through antagonism of AR. The results also revealed that atrazine exposure significantly reduced maternal serum testosterone levels, decreased AR nuclear translocation, and altered the expression levels of developmental gene networks in developing penis of mice. Atrazine exposure also affected the expression of insulin-like 3 (*Insl3*) and steroidogenic gene expression in developing reproductive tract. Therefore, our data indicate that prenatal atrazine exposure can induce hypospadias in F1 mice, likely through disruption of testosterone production, decreasing genomic androgen action, and then altering expression of developmental genes during sexual differentiation. Our data also suggest that prenatal atrazine exposure can induce cryptorchidism in F1 mice, possibly through down regulation of Insl3.

KEYWORDS
atrazine, cryptorchidism, hypospadias, penile development, prenatal exposure, undescended testis

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Hongli Tan, Guohui Wu, and Shanshan Wang have contributed equally to this study.

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1 | INTRODUCTION

Testis descent and masculinization of external genitalia are part of essential developmental processes during sexual differentiation in utero. Caused by genetic factors and/or extrinsic endocrine-disrupting compounds, cryptorchidism (i.e., failure of the testis to descend into the scrotal sac) and hypospadias (i.e., the urethral tube dysmorphogenesis with the ectopic ventral opening of the urethra along the penis, scrotum, or perineum) represent two most common congenital anomalies of human male genitalia (Bouty, Ayers, Pask, Heloury, & Sinclair, 2015). Cryptorchidism occurs in more than 1% of boys of 0–1 year old and hypospadias affects one out of 250 newborn boys (Bouty et al., 2015), but the etiology of cryptorchidism and hypospadias remains poorly understood. Babies with congenital urological or abdominal wall malformations, such as hypospadias, bladder exstrophy, prune–belly syndrome, gastroschisis, and omphalocele, are also more likely to have cryptorchidism (Braga & Lorenzo, 2017). A disruption in any phase of testicular descent and penile masculinization can be triggered by hereditary, hormonal, environmental, or social factors (Foresta, Zuccarello, Garolla, & Ferlin, 2008). In addition, although male infertility, which affects approximately 7% of all men, is commonly due to deficiencies in the semen, and semen quality, severe hypospadias, and/or cryptorchidism can also cause male infertility (Lotti & Maggi, 2015). Thus, studying early indicators of disturbed sexual development such as hypospadias and cryptorchidism might help us better understand the broader infertility issue.

Several genetic pathways have been identified in genital tubercle (GT) early stage patterning and growth, as well as later hormone-driven sexual dimorphic development. These pathways include fibroblast growth factor (Fgf), Hedgehog, Wnt, Bmp/Tgfβ signal pathway genes, and other "effector" genes (Murashima, Kishigami, Thomson, & Yamada, 2015). Mutation of key genes in these pathways causes severe genital malformations in later androgen-dependent penile masculinization stage, more evidence has been revealed that androgen interacts with *Wnt*, *Hedgehog*, *Fgf*, and other androgen responsive genes to control tubular urethra and penile formation (Miyagawa et al., 2009; Miyagawa et al., 2011; Wang, Lawless, & Zheng, 2020). Only several genes were discovered by mouse mutation research to play important roles in testicular descent (Virtanen et al., 2007). Testis descent in the second phase is androgen-dependent, genes involved in androgen biosynthesis and gonad development may also be involved in testis descent process (Emmen et al., 2000). Androgen receptor (*Ar*) gene mutations can result in androgen resistance and prevent the inguinoscrotal descent of the testes (Braga & Lorenzo, 2017). Disruption of AR in different developmental stages induces different penile anomalies (Zheng, Armfield, & Cohn, 2015). In addition, some environmental endocrine disruptors, such as AR antagonist vinclozolin (VCZ), could induce adverse effects on reproductive system in male rodents (Amato & McCoy, 2016; Hsieh, Breyer, Eisenberg, & Baskin, 2008; Vilela et al., 2007).

Atrazine (ATZ) is used to prevent pre- and post-emergent broadleaf weeds in crops and on turf grasses. The United States (US) Environmental Protection Agency (EPA) estimates that each year approximately 76.5 million pounds of ATZ are applied within the US, making it one of the most widely used herbicides in the country (Rinsky, Hopenhayn, Golla, Browning, & Bush, 2012). Due to its widespread applications and various toxicities, ATZ was banned in the European Union in 2003 (Sass & Colangelo, 2006). However, ATZ remains in use in more than 70 countries including the US, Brazil, Argentina, Mexico, and China (Chevrier et al., 2011). ATZ exhibits endocrine-disrupting activities in wildlife (Crain, Guillette Jr., Rooney, & Pickford, 1997; Suzawa & Ingraham, 2008) and laboratory rodents (Cooper, Stoker, Tyrey, Goldman, & McElroy, 2000; Cummings, Rhodes, & Cooper, 2000; Trentacoste, Friedmann, Youker, Breckenridge, & Zirkin, 2001). In mammals, ATZ disrupts normal development of reproductive organs (Trentacoste et al., 2001), thyroid gland (Rajkovic, Matavulj, & Johansson, 2010; Stoker, Guidici, Laws, & Cooper, 2002), and mammary glands (Enoch et al., 2007), as well as implantation and hypothalamic control (Cooper et al., 2000; Cummings et al., 2000). It could reduce prostate, seminal vesicle, and pituitary weights in rodents, decrease sperm count, viability, and motility (Stoker, Laws, Guidici, & Cooper, 2000; Trentacoste et al., 2001), and lead to reduced fertility in men living in agricultural areas (Swan, 2006). Studies also demonstrated that postnatal ATZ exposure could cause a significant decrease in serum and testicular testosterone levels (Friedmann, 2002; Stoker et al., 2000), while adult rats exposed to ATZ exhibited decreased levels of serum testosterone, follicle stimulating hormone, luteinizing hormone, and inhibin-B (Song, Jia, Chen, Hu, & Zhang, 2014). The effects of ATZ on the development of androgen-dependent reproductive organs in peripupertal (Friedmann, 2002; Trentacoste et al., 2001) and adult rats (Victor-Costa, Bandeira, Oliveira, Mahecha, & Oliveira, 2010) have been reported. However, literature about its effects on embryonic development, sexual differentiation, and male reproductive organ development remains very limited. To date, only two human studies evaluated the association of hypospadias with ATZ

exposure (Agopian, Lupo, Canfield, & Langlois, 2013; Winston et al., 2016). Of them, one study reported associations between medium-low levels of periconceptional maternal ATZ exposure and male genital malformations, including hypospadias and cryptorchidism (Agopian et al., 2013). Literature search revealed only two animal studies related to the effect of ATZ on hypospadias (Govers et al., 2020; Wu et al., 2007), while nothing has been done through animal models to evaluate cryptorchidism induction following ATZ exposure.

Given the knowledge gaps identified above, our present study aimed to: (a) determine whether prenatal ATZ exposure could affect testicle descent and penile masculinization and induce hypospadias and/or cryptorchidism in F1 male mouse offspring and (b) explore potential molecular mechanisms associated with the effects. This study constitutes one of the first reports on the links between ATZ exposure and hypospadias/cryptorchidism. Our findings will contribute to a more comprehensive understanding of the endocrine disrupting activities of ATZ and its environmental and human health risks.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Reference standards of ATZ (CAS number 1912-24-9) and VCZ (CAS number 50471-44-8) were purchased from Fisher Scientific (Hanover Park, IL) and Sigma-Aldrich (St. Louis, MO), respectively. Reference standards of native testosterone and d_3-testosterone were obtained from Steraloids Inc. (Newport, RI). High performance liquid chromatography (HPLC) grade solvents and other chemicals were purchased from Fisher Scientific

2.2 | Animal treatments

The ICR mice were purchased from Envigo RMS Inc. (Indianapolis, IN). Mice were housed in a specific pathogen-free barrier facility on 12-hr light/dark cycles with access to food and water ad libitum. All experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals." The experimental protocol (17-021) was pre-approved by the Institutional Animal Care and Use Committee of Southern Illinois University Carbondale. Females were mated with males of the same strain and checked once daily at 9:00 a.m., and the discovering of a vaginal plug marked embryonic day (E) 0.5. The date of birth was designated as postnatal day (P) 0.

Due to limited solubilities of ATZ and VCZ in corn oil, we first suspended ATZ or VCZ in 100% ethanol and then diluted it with corn oil (final concentration of ethanol is 2.5%, v/v). The control group was gavaged with the same amount of corn oil (containing 2.5% ethanol, v/v). A minimum of five pregnant mice (for each dose/group) were gavaged with different daily doses (10 or 100 mg/kg) of ATZ, 150 mg/kg of VCZ, 100 mg/kg of ATZ plus 150 mg/kg of VCZ (referred to as ATZ + VCZ), or control solution once daily from E12.5 to E16.5. The treatment time was determined based on the sensitive time window of masculinization in external genital development, and this time window is also important in the transabdominal phase of testis descent (Hutson, 1985; Zheng et al., 2015). Therefore, in the present study the period of E12.5 to E16.5 was chosen as the exposure window.

The dose selection for ATZ was based on a previous study which suggested a prenatal dose of 100 mg/kg/day significantly delayed vaginal opening of female rat offspring (Rayner, Wood, & Fenton, 2004), and the EPA data which indicated that the acute developmental NOAEL (no-observed-adverse-effect level) and LOAEL (lowest-observed-adverse-effect level) of ATZ for rats are 6.25 and 12.5 mg/kg/day, respectively (U.S. EPA report, 2003, http://www.epa.gov/oppsrrd1/REDs/atrazine_ired.pdf).

Although our work focused on ATZ, we also included VCZ in the study for the exploration of ATZ's potential toxic modes of action. The effect of VCZ on penile masculinization and sexual differentiation resembles that of well-known AR antagonist flutamide (Gray Jr., Ostby, & Kelce, 1994). A dosage of 150 mg/kg was chosen based on previous studies which suggested that this dose of VCZ may interrupt male sexual differentiation, reproductive function and development without increasing mortality, or other adverse toxic effects (Amato & McCoy, 2016; Gray Jr. et al., 1994; O'Connor, Frame, & Ladics, 2002).

The F1 male mice from 5 to 8 litters per treatment group were collected on weaning day (P21) for measurement of body weight, anogenital distance (AGD), glans penis length, penile morphology, testis position, and testis weight.

In order to explore potential mechanism, additional pregnant mice (five per treatment group for each experiment) were gavaged daily with 100 mg/kg ATZ, 150 mg/kg VCZ, 100 mg/kg ATZ plus 150 mg/kg VCZ, or corn oil (control group) beginning from E12.5. Previous studies suggested that masculinization of male GTs was initiated at around E15 based on the findings that exposure to an anti-androgen at E14.5–E15.5 could induce penile malformations and that AR and estrogen receptor alpha (ERα) could undergo translocation to the nucleus at

E15.5 (Miyagawa et al., 2009; Zheng et al., 2015). Therefore, the E15.5 and E16.5 embryo tissues were saved for gene expression and immunohistochemistry analyses, respectively, sex was determined by microscopic observation of gonad and then genotyping using SMCX/SMCY primers later (Wang et al., 2020). Maternal sera were collected at E15.5 for testosterone measurement, and testis descent was also examined at this stage.

2.3 | Morphological, histological, and immunofluorescence measurements

The P21 mice were euthanized by CO_2 inhalation and then cervical dislocation. After measurement of AGD and body weight, the mice were dissected and photographed for testis position (Kaftanovskaya et al., 2012; Nef & Parada, 1999). Testes were taken out for weight measurement. The penis was then dissected and imaged for gross morphology. The testis position of E15.5 mice was measured using a calibrated eyepiece reticule on a Leica M80 stereo dissecting microscope equipped with a digital camera (Leica Microsystems Inc., Buffalo Grove, IL). The glans penis length was determined from the tip of the cartilage down to the glans/body penis curve. Testes from each pup were weighed on a Mettler Toledo balance (Mettler-Toledo, LLC, Columbus, OH) after removing residual epididymis and liquid on the surface.

Dissected penises were washed in phosphate-buffered saline, then fixed overnight in 4% paraformaldehyde at room temperature. After being dehydrated in graded ethanol and cleared in Histo-Clear II, they were embedded in paraffin wax. The 10 μm serial transverse sections from the proximal to the distal of all penises were cut and stained with eosin and hematoxylin. The sample size for histological analysis was 17 for the control group, $n = 18$ for ATZ at 10 mg/kg/day, $n = 17$ for ATZ at 100 mg/kg/day, $n = 16$ for VCZ at 150 mg/kg/day, and $n = 16$ for ATZ + VCZ (100 and 150 mg/kg/day, respectively).

Immunofluorescence of AR was performed as previously described with modifications (Wang, Shi, Zhu, Mathews, & Zheng, 2018). The slides were bleached in 3% hydrogen peroxide in methanol for 30 min before antigen retrieval and the rabbit anti-AR primary antibody (SC-816, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the slides at 1:50 dilution and incubated overnight at 4°C and detected using the Tyramide Signal Amplification fluorescence kit (Invitrogen) according to the manufacturer's protocol. Immunofluorescence samples were visualized on a Leica DM5500 confocal microscope. Stereology was used to determine the total (DAPI positive) and nuclear AR positive cell numbers as previously described (Seifert, Zheng, Ormerod, & Cohn, 2010;

Wang & Zheng, 2019). Six GTs were processed for each group. The detailed stereology and quantification method was summarized in supplementary materials.

2.4 | Measurement of maternal serum testosterone

Approximately 0.5 ml of serum sample collected from pregnant mice at E15.5 was spiked with 50 ng internal standard (d_3-testosterone) and extracted with 5 ml of a mixture of ethyl acetate and hexane (3:2, v/v) under shaking for 15 min. The mixture was centrifuged at 3,000 rpm for 5 min, and the organic phase was collected. The extraction was repeated twice and the resulting extracts were combined and concentrated to 200 μl. Final extract was analyzed on an Agilent 1,260 HPLC interfaced with a 3,200 Q Trap triple quadrupole mass spectrometry (MS) (AB Sciex; Toronto, Canada). The HPLC system was equipped with a Kinetex 2.6 μm C18 100 Å column (2.1×100 mm). The MS was equipped with a TurboIonSpray electrospray ionization (ESI) probe and operated in the multiple reaction monitoring (MRM) mode for quantitative measurement of testosterone (Table S1). Detailed information on the instrumental analysis was given in supplementary materials.

2.5 | Quantitative gene expression analysis

Total RNA was extracted from the GT and reproductive tract (including gubernaculum, testis, epididymis, and vas-defenses, GTEV) of E15.5 male mouse embryos of different groups, based on the TRIzol method according to the operation manual (Invitrogen, Cat: 10296010). RNA quality was assessed by a Nanodrop spectroscopy and the ratios of 28 s and 18 s ribosomal RNA after gel electrophoresis. The cDNA was synthesized from 500 ng total RNA using iScript Reverse Transcription Supermix (Bio-rad, Hercules, CA). Pathway-specific mRNA expression was determined by using the Hedgehog signaling PCR Array (PAMM-078Z, Qiagen) and the CFX96 Real-time PCR system (Bio-rad) according to the manufacturer's instructions. The Web-Based PCR Array Data Analysis system (Qiagen) was used to analyze PCR array results. The primers of hormone receptors, androgen biosynthesis pathway, and testicular descent related genes were designed using PrimerQuest Tool (Integrated DNA Technologies, Inc.) and validated, and primer sequences were listed in Table S2. The details for gene expression analyses and other information were summarized in supplementary materials.

2.6 | Statistical analysis

The one-way analysis of variance (ANOVA) with Dunnett's T3 post hoc analysis (Analytics Software & Solutions) was applied to determine if ATZ exposure produced significant effects on the evaluated parameters, including AGD, glans penis length, body, and testis weight. Comparisons between control and treatment groups in the incidences of male urogenital mating protuberance (MUMP) shape, urethral meatus position (hypospadias), and undescended testicle (cryptorchidism) were conducted via the Mann–Whitney tests (PASW Statistics 18.0). The effects on hormone levels and gene expression level (fold) changes were also determined using ANOVA except for the PCR array data. For each parameter, an average value per liter was included for statistical analysis. Data were logarithmically transformed to approximate a normal distribution prior to ANOVA analyses. The PCR array data analysis was done using Web-Based PCR Array Data Analysis system (Qiagen). The level of significance was set at $\alpha = 0.05$.

3 | RESULTS

3.1 | Prenatal ATZ exposure affected AGD and glans penis size

Our results indicated that prenatal ATZ (10 or 100 mg/kg) treatment exhibited no effect on litter sizes and sex ratios (Table 1). No pups died in either control or ATZ-treated groups from birth to weaning. ATZ-treated pups did not exhibit any significant changes in average body weight or testis weight compared with controls at the time of weaning. As a comparison, prenatal exposure to 150 mg/kg VCZ during E12.5–E16.5 did not affect litter size, sex ratio, body weight, or testis weight. However, exposure to VCZ + ATZ significantly reduced body weight ($p = .03$) and testis weight ($p = .01$), whereas no effect on litter size or sex ratio was observed (Table 1). The two important phenotypic parameters of external genitalia development in mice, AGD and glans penis length, were significantly reduced in F1 male pups following prenatal exposure to ATZ (100 mg/kg), VCZ (150 mg/kg), or ATZ + VCZ (Table 1 and Figure 1f). Compared with 100 mg/kg ATZ treatment, exposure to VCZ or ATZ + VCZ resulted in significantly reduced AGD ($p \leq .03$, Table 1) and penis size ($p \leq .04$, Figure 1f), whereas no remarkable difference was found between VCZ and ATZ + VCZ treatments (Table 1 and Figure 1f).

3.2 | Prenatal ATZ exposure induced cryptorchidism and hypospadias

Analysis of testis position in P21 mice revealed that prenatal ATZ exposure could induce cryptorchidism (undescended testicles). All 56 male pups in control group had testes located in the scrotum (Figure 1a). The unilateral

TABLE 1 The effect of prenatal atrazine treatment on litter size, sex ratio, body weight, testis weight, AGD, MUMP shape, urethral meatus position (hypospadias), and undescended testicle (cryptorchidism)

	Control	Atrazine (10 mg/kg)	Atrazine (100 mg/kg)	Vinclozolin (150 mg/kg)	Vinclozolin (150 mg/kg) atrazine (100 mg/kg)
Litter size	13.33 ± 1.03b	13.5 ± 1.05	12.55 ± 0.76	13.17 ± 1.17	12.17 ± 0.75
Male/female sex ratio	0.97 ± 0.17	1.03 ± 0.24	0.97 ± 0.18	1.05 ± 0.20	0.95 ± 0.22
Body weight (g)	16.49 ± 1.01	15.98 ± 0.92	15.65 ± 0.96	15.85 ± 1.05	15.03 ± 0.82*
Testis weight (mg)	78.85 ± 7.06	75.52 ± 7.38	69.38 ± 6.10	72.41 ± 5.37	68.05 ± 5.93*
Anogenital distance (AGD; mm)	10.29 ± 1.04	10.17 ± 0.87	8.91 ± 0.73*	7.92 ± 0.89**	7.51 ± 0.95**
Unilateral cryptorchidism	0	6 ± 8%	25 ± 13%**	3 ± 6%	16 ± 12%**
Bilateral cryptorchidism	0	0	0	0	0
Abnormal MUMP	10 ± 11%	66 ± 14%**	96 ± 7%***	97 ± 6%**	100%**
Distal tip hypospadias	0	6 ± 8%	47 ± 14%**	78 ± 7%**	60 ± 5%**
Middle or proximal glans-penis hypospadias	0	0	0	22 ± 8%**	40 ± 4%**

Note: (1) Each treatment included 5–8 litters of mice, and each litter contained 5–9 male pups. The treatment method was through oral gavage, once daily from E12.5 to E16.5. (2) The incidence rates of cryptorchidism, hypospadias, and abnormal MUMP were expressed as the percentage of the number of abnormal male pups to the total number of male pups in each litter in mean by litter ± *SD*. (3) Statistics was performed between each treatment group and control group, *p ≤ .05; **p ≤ .01; ***p ≤ .001.

Abbreviation: MUMP, male urogenital mating protuberance.
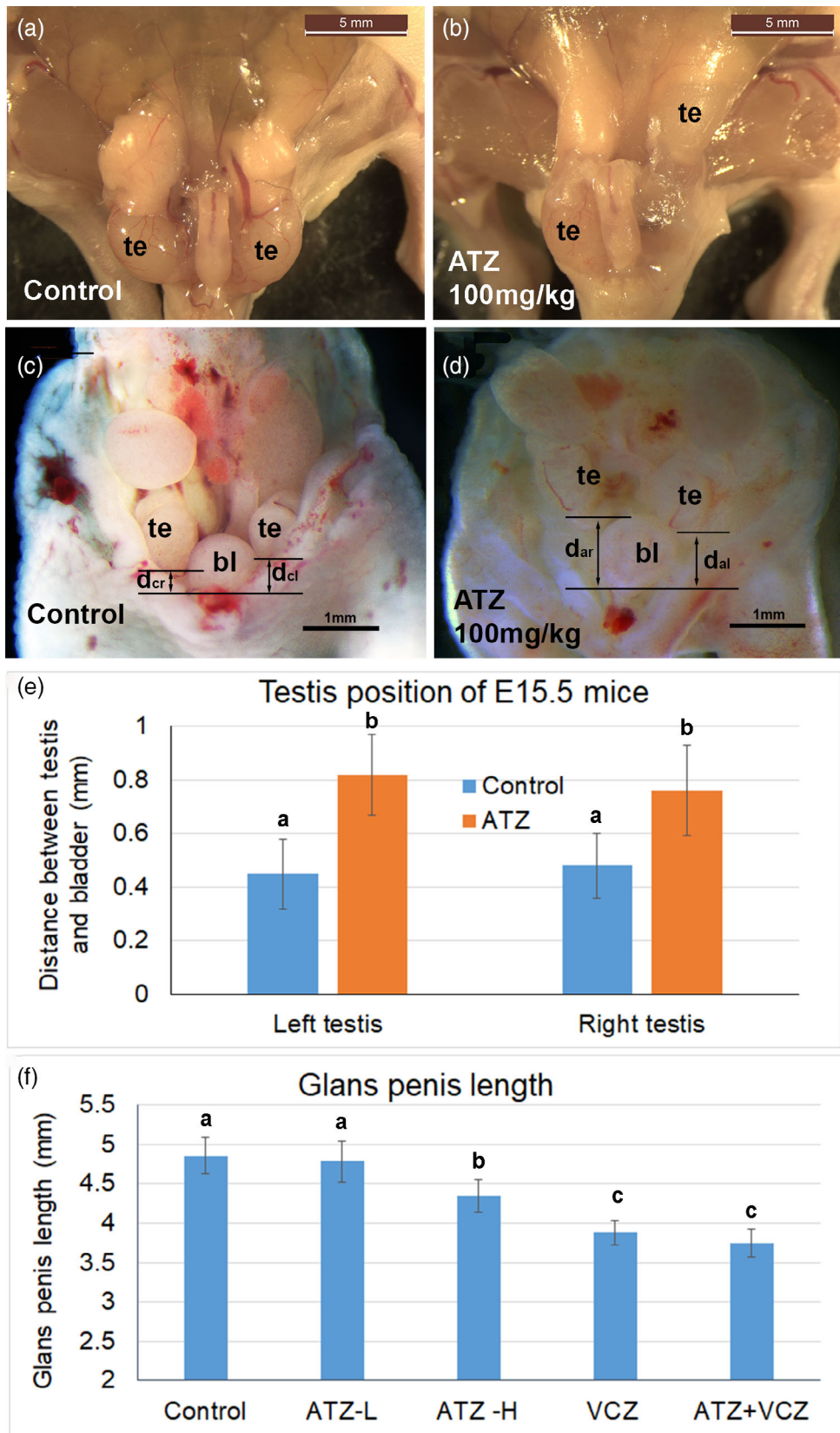
FIGURE 1 Effects of prenatal atrazine (ATZ) exposure on glans penis size and undescended testicles in F1 mouse offspring. Ventral views of the abdominal cavity of (a) control and (b) 100 mg/kg ATZ-treated mice at weaning (P21), showing ATZ-induced undescended testis. Ventral views of abdominal cavity showing testis position in E15.5 (c) control and (d) 100 mg/kg ATZ-treated mice. (e) Comparison of the distance between testis and bladder in E15.5 male mouse embryos between control group ($n = 4$ litters, 25 embryos) and 100 mg/kg ATZ group ($n = 4$ litters, 22 embryos). (f) Comparison of glans penis size among control group and groups treated with 10 mg/kg ATZ (ATZ-L for low dose), 100 mg/kg ATZ (ATZ-H for high dose), 150 mg/kg vinclozolin (VCZ), and 100 mg/kg ATZ plus 150 mg/kg VCZ (ATZ + VCZ). Sample size in "f" are no <5 litters, at least 32 male pups. The data in "e" and "f" are represented as mean ± SE. Letters (A–C) indicate significant differences between groups; between A B, and B C: $p \leq .04$; between A C: $p \leq .01$; Lines in "c" and "d" show testis and bladder position, double arrow lines indicate the distance between testis and bladder. *bl*, bladder; *dcl/dcr*, distance between left/right testis and bladder, respectively, in control mice; *dal/dar*, distance between left/right testis and bladder, respectively, in ATZ-treated mice; *te*, testis. Scale bars in "a and b": 5 mm, in "c and d": 1 mm

cryptorchidism rate (the percentage of the number of abnormal male pups to the total number of male pups in each litter) was determined to be $6 \pm 8\%$ (mean by litter $\pm SD$) in the 10 mg/kg ATZ groups, and the rate increased to $25 \pm 13\%$ in the 100 mg/kg ATZ group (Figure 1b, Table 1). The majority of the affected

individuals exhibited left side cryptorchidism. Exposure to VCZ alone resulted in a unilateral cryptorchidism rate of $3 \pm 6\%$, whereas the rate increased to $16 \pm 12\%$ in the ATZ + VCZ group (Table 1). To reveal which phase of testis descent was affected, we measured the testis position (related to bladder) in control and 100 mg/kg ATZ-treated mice at E15.5. The results showed that the distance between testis and bladder in ATZ-treated group was significantly larger than that in control group (Table 1 and Figure 1c and d).
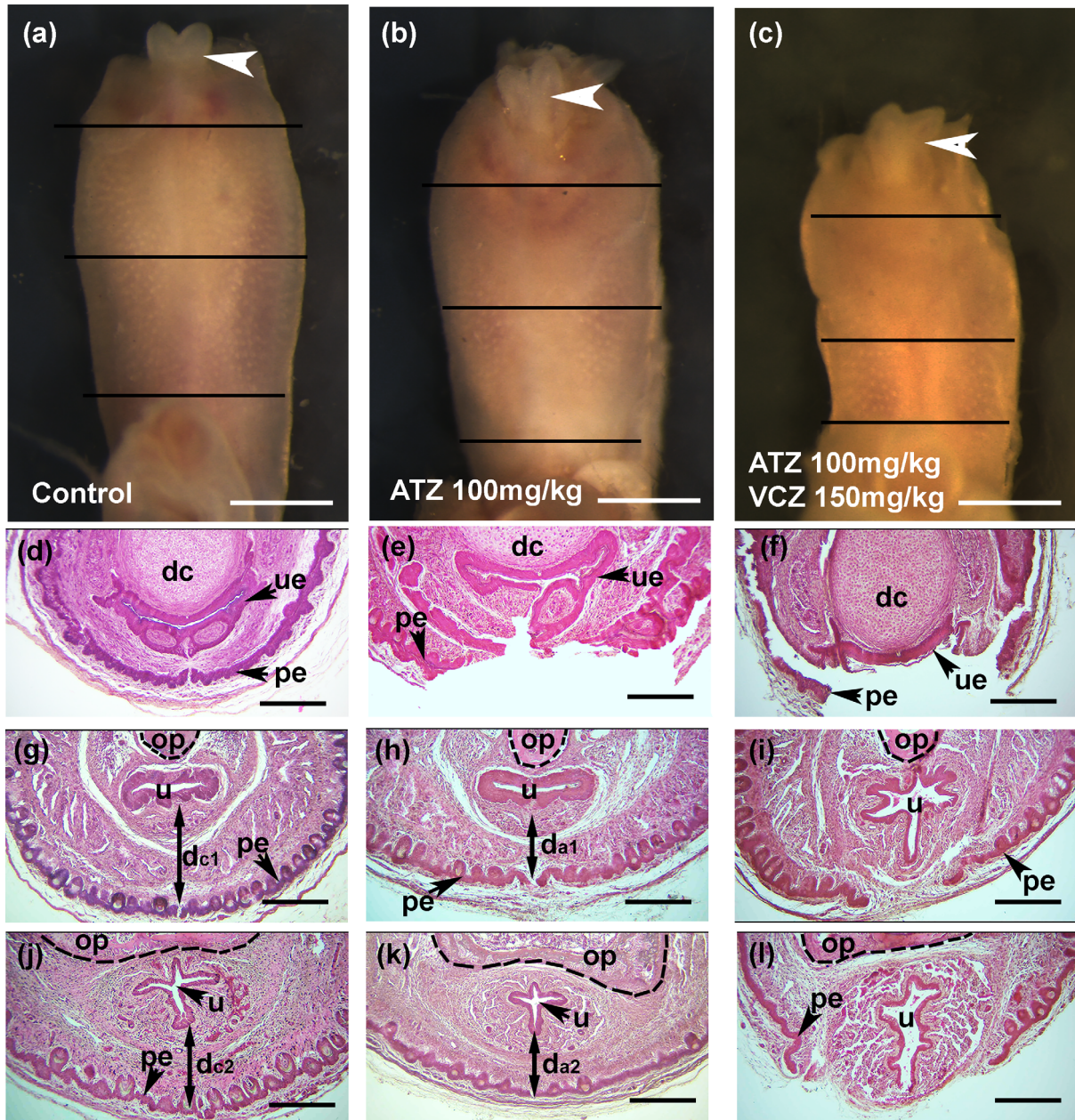
FIGURE 2 Effects of prenatal atrazine (ATZ) exposure on the morphology and urethral opening of mouse penis. (a–c) Gross morphology of representative penises of P21 mouse pups prenatally treated with corn oil (control) (a), 100 mg/kg ATZ (b) and 100 mg/kg ATZ plus 150 mg/kgVCZ (c). Images in "a–c" are ventral views of mouse penises with distal at the top. All section images are transverse through penis with dorsal at the top. "d, g, j", "e, h, k", and "f, i, l" are sections of a, b, and c, respectively, the black lines in "a–c" indicate the levels of the transverse sections. "d–f" are distal sections; "g–i" and "j–l" are middle and proximal sections, respectively. White arrow heads in "a–c" indicate the male urogenital mating protuberance (MUMP). Double arrow lines in "g, h, j, and k" indicate the distance (dc1 and dc2 indicate distance in control penis, and da1 and da2 indicate distance in ATZ-treated penis) between the urethra and ventral penile epithelium. dc, distal cartilage; op, os-penis; pe, penis epithelium; u, urethra; ue, urethral epithelium. Scale bars in images "a–c" and "d–l" are 1 mm and 200 μm, respectively

ATZ treatments also affected penile morphology. The MUMP in control mice was usually symmetric (Figure 2a, white arrowhead indicating MUMP). By contrast, the majority of male mice prenatally exposed to low dose ATZ (10 mg/kg) and all males exposed to high dose ATZ (100 mg/kg) and ATZ + VCZ displayed asymmetric MUMP (Figure 2b,c and Table 1). Distal tip hypospadias was observed in only two out of 36 male mice in the 10 mg/kg ATZ group, whereas the rate increased to 47% in the 100 mg/kg ATZ group (Figure 2 and Table 1). Exposure
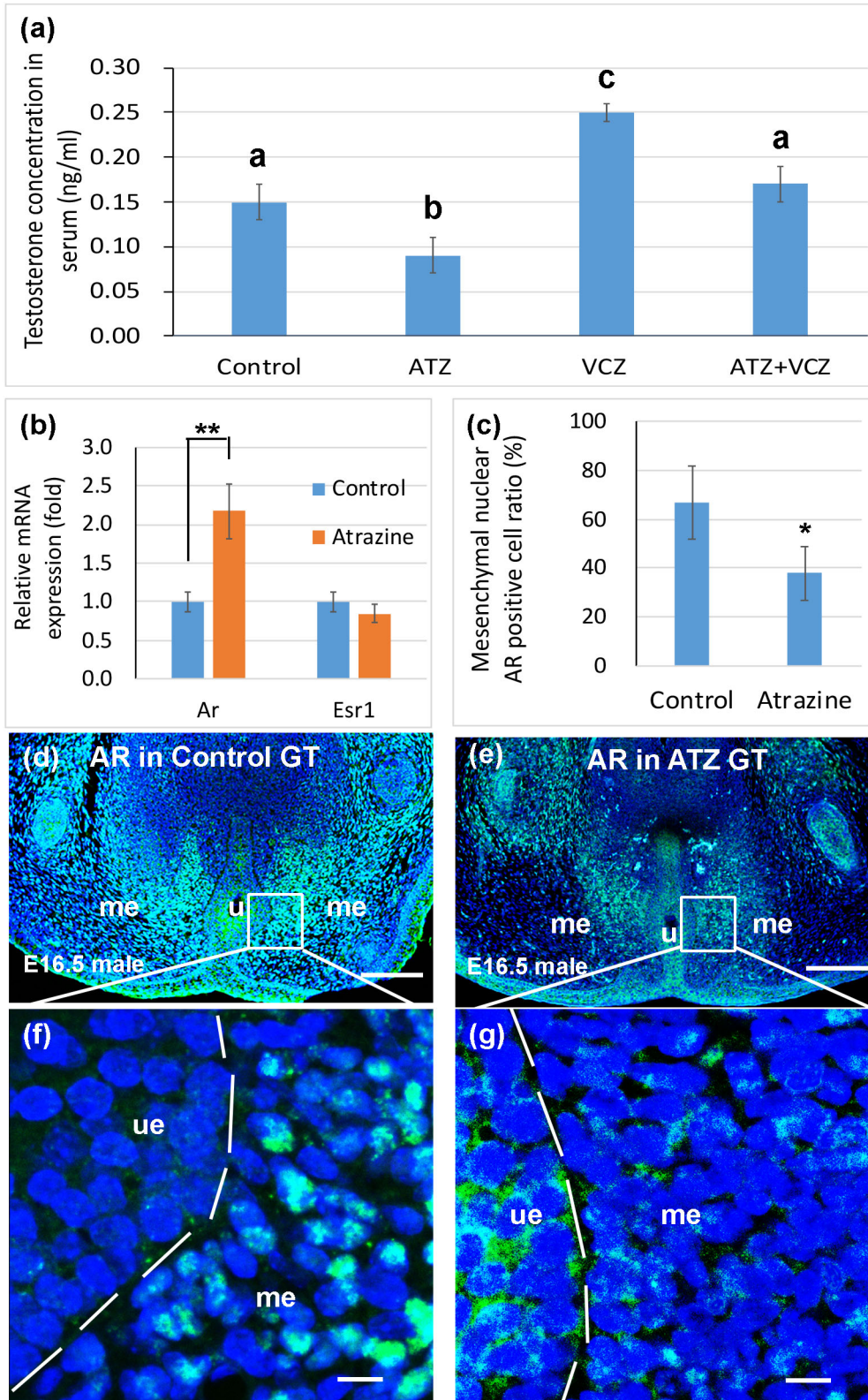
FIGURE 3 Effects of prenatal atrazine (ATZ) exposure on testosterone production and androgen receptor (AR) expression. (a) Serum testosterone in dams treated with corn oil (control), 100 mg/kg ATZ, 150 mg/kg VCZ, and 100 mg/kg ATZ plus 150 mg/kg VCZ. The data are presented as mean ($n = 5$) ± SE. (b) Relative transcript levels of *Ar* and *Esr1* in E15.5 genital tubercles (GTs) of male mice prenatally exposed to 100 mg/kg ATZ compared with those in control group. Control is assigned a value of 1. The data are presented as mean ($n = 5$) ± SE. (c) Nuclear localized AR ratio (nuclear AR positive cells/total cells in urethra closing area) between 100 mg/kg ATZ-treated and control GTs at E16.5. The data are presented as mean ($n = 6$) ± SE. (d–g) Section images are transverse to the E16.5 GTs with dorsal at the top. "f, g" are amplified areas of "d, e", respectively. Green staining is AR signal and blue represents DAPI. Dashed white lines show the boundary of urethral epithelium and glans mesenchyme. Letters "A–C" in "a" indicate significant differences between groups; between a b, $p \le .031$; between a c and b c, $p \le .01$. In "b and c", *$p \le .05$, **$p \le .01$. *me*, mesenchyme; *u*, urethra; *ue*, urethral epithelium. Scale bars in "d, e" and "f, g" are 100 and 10 μm, respectively

to VCZ or ATZ + VCZ resulted in hypospadias in 100% of male mice, and the distal tip hypospadias was about 78% (VCZ group) and 60% (ATZ + VCZ group; Table 1 and Figure 2). It was also observed that exposure to VCZ or ATZ + VCZ could induce proximal and middle glans hypospadias in 22% and 40% of male mice, respectively. By contrast, no proximal or middle glans hypospadias was observed in mice exposed to ATZ alone (Figure 2g–l and Table 1). The comparison of histological sections of penises between control and 100 mg/kg ATZ groups revealed that urethra was closer to penile epithelium in ventral side of the exposure groups (Figure 2g,h,j, and k).

3.3 | ATZ-induced male genital malformation through disrupting testosterone production and AR nuclear translocation

We hypothesized that prenatal ATZ exposure could affect male genital development in offspring through the disruption of androgen production in pregnant dams. To test this hypothesis, we measured the serum testosterone levels in different treatment and control groups. Compared with control group, ATZ treatment (100 mg/kg) significantly reduced maternal serum testosterone levels (mean 0.09 vs. 0.15 ng/ml, $p = .02$), on the contrary, VCZ significantly increased serum testosterone levels (mean 0.25 ng/ml, $p = .01$, Figure 3a). Interestingly, ATZ + VCZ-treated group showed similar testosterone levels (mean 0.17 ng/ml) to those of controls ($p = .37$, Figure 3a). As androgens control genital development through AR and testosterone can be converted to estrogen through aromatase, we also compared *Ar* and *Esr1* mRNA levels in developing male GTs at E15.5 (Figure 3b). The results indicated that *Ar* mRNA was upregulated in ATZ-treated GTs ($p = .008$, Figure 3b), but no significant change was observed for *Esr1* expression ($p = .08$). We further analyzed AR protein localization (Figure 3d–g) and quantified the nuclear AR positive cells in mesenchyme of E16.5 male GTs (Figure 3c). The average AR positive cell ratio in control group was 67.4%, significantly greater than that (37.8%) determined in ATZ-treated GTs ($p = .02$, Figure 3c).

3.4 | Prenatal ATZ-induced penile malformation through disrupting the key genes expression of external genital developmental gene networks

In addition to the direct disruption of androgen levels in pregnant dams, we also hypothesized that ATZ could

affect penile malformation through the disruption of genes expression involved in penile development and masculinization. To test this hypothesis, in the present study, we performed pathway-focused gene expression analysis using laboratory-verified Hedgehog signaling PCR array, which includes most key genes in hedgehog, Bmp and Wnt pathways, and several Fgf pathway genes, and compared the relative expression levels of totally 88 genes in E15.5 GTs between control and ATZ-treated (100 mg/kg) males. We found that the expression of 44 genes were significantly changed in ATZ-treated groups ($p \leq .05$, Table S3), and 17 genes from those changed genes showed at least two-fold down/up regulation, including Wnt/β-catenin pathway gene *Wnt2b* (Figure 4a), Bmp pathway genes *Bmp4* and *Bmp7* (Figure 4b and c), Hedgehog pathway genes *Gli3*, *Hhip* (Figure 4d and e) and genes with interaction with these three pathway genes *Boc*, *Lats2*, *Lrp2*, *Mapk1*, *Npc1*, *Csnk1a1*, *Nf2*, *Numb*, *Stk36*, *Trp53*, and *Frmd6* (Figure 4f–p). In addition, some other important genes, such as *Bmp2*, *Ptch1*, *Ptch2*, *Shh*, *Smo*, *Wnt5a*, and *Wnt7a* in Bmp, Hedgehog, and Wnt pathways were also significantly downregulated in ATZ-treated GTs (Table S3), while Wnt signaling pathway inhibitor *Dkk2* and a stress response gene *Atf3* were significantly upregulated after ATZ treatment (Figure 4q and Table S3).

3.5 | Prenatal ATZ-affected testis descent possibly through disruption the expression of Insl3 and steroidogenesis related genes

To reveal how ATZ induces undescended testis in mice, we compared the expression of known cryptorchidism-related genes between control and ATZ-treated internal reproductive organs GTEV in E15.5 mouse embryos. The results showed *Insl3* (Figure 5a) and *Wt1* (Figure 5e) in ATZ-treated GTEV were downregulated up to 4- and 2.5-fold, respectively ($p \leq .01$). Several other cryptorchidism related genes, such as *Rxfp2*, *Hoxa10*, and *Hoxa11*, exhibited no significant changes ($p \geq .08$, Figure 5b–d). To test whether prenatal ATZ treatment affected steroid biosynthesis in fetal testes, the mRNA expression of several key enzymes was compared (Figure 5f–i). Prenatal ATZ exposure (100 mg/kg) significantly downregulated the expression of *Cyp17a1* ($p = .02$, Figure 5g) and *Hsd17b4* ($p = .02$, Figure 5i). *Star* mRNA expression was also marginally downregulated ($p = .05$) following ATZ exposure (Figure 5f). Aromatase (*Cyp19a1*) mRNA was detected in fetal GTEV, but no difference ($p = .14$) was found between control and ATZ-treated male reproductive organs (Figure 5h).
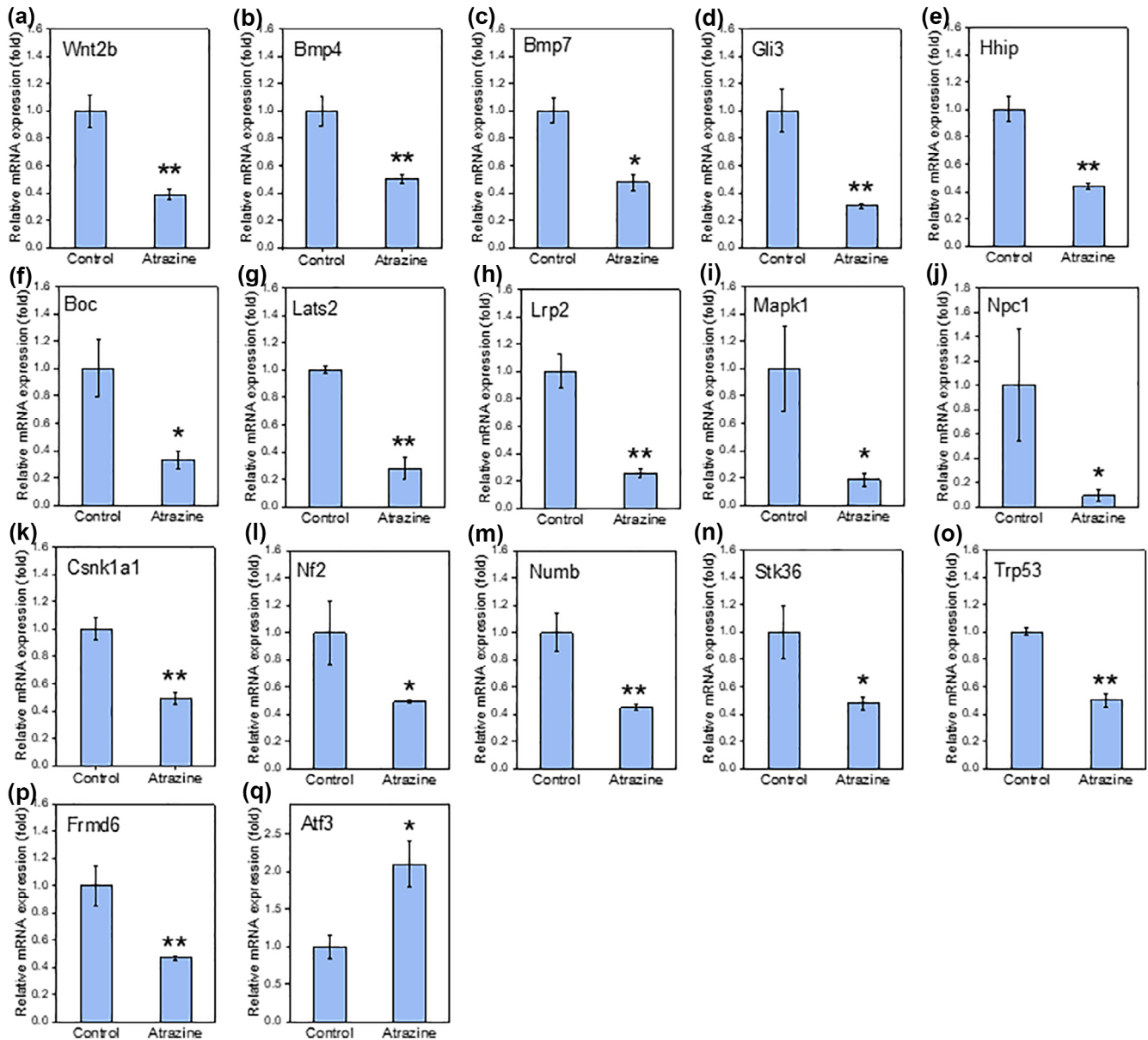
FIGURE 4 Comparison of the quantitative gene expressions in E15.5 genital tubercles between control and 100 mg/kg atrazine treatment groups. Relative transcript levels of pathway specific penile developmental genes showed at least two-fold down/up regulation. Control is assigned a value of 1. Error bars represent mean ($n = 5$) $\pm$ SE. $*p \leq .05$; $**p \leq .01$

4 | DISCUSSION

In this study, our results clearly indicate that prenatal ATZ exposure could disrupt reproductive organ development in F1 male offspring. Among the different phenotype changes observed, hypospadias and cryptorchidism are intriguing as these effects have rarely been reported for ATZ. Although the dose (i.e., 100 mg/kg/day) leading to a relatively high occurrence rate of hypospadias and cryptorchidism is much greater than environmental exposure levels in most cases, it does relate to a commonly used reference dose for reproductive effect endpoints in rodents (Rayner et al., 2004). That low dose of ATZ could induce distal tip hypospadias was also consistent with the

recently reported findings (Govers et al., 2020). The results suggest that such a low reference dose should be re-considered for future investigation of ATZ's reproductive developmental effects via animal models.

4.1 | Prenatal ATZ induces penile malformation possibly through inhibition of testosterone production and genomic androgen signaling

The reproductive tissues derived from "anlagen" or precursor structures which are identical in both males and females during early development. Subsequent male
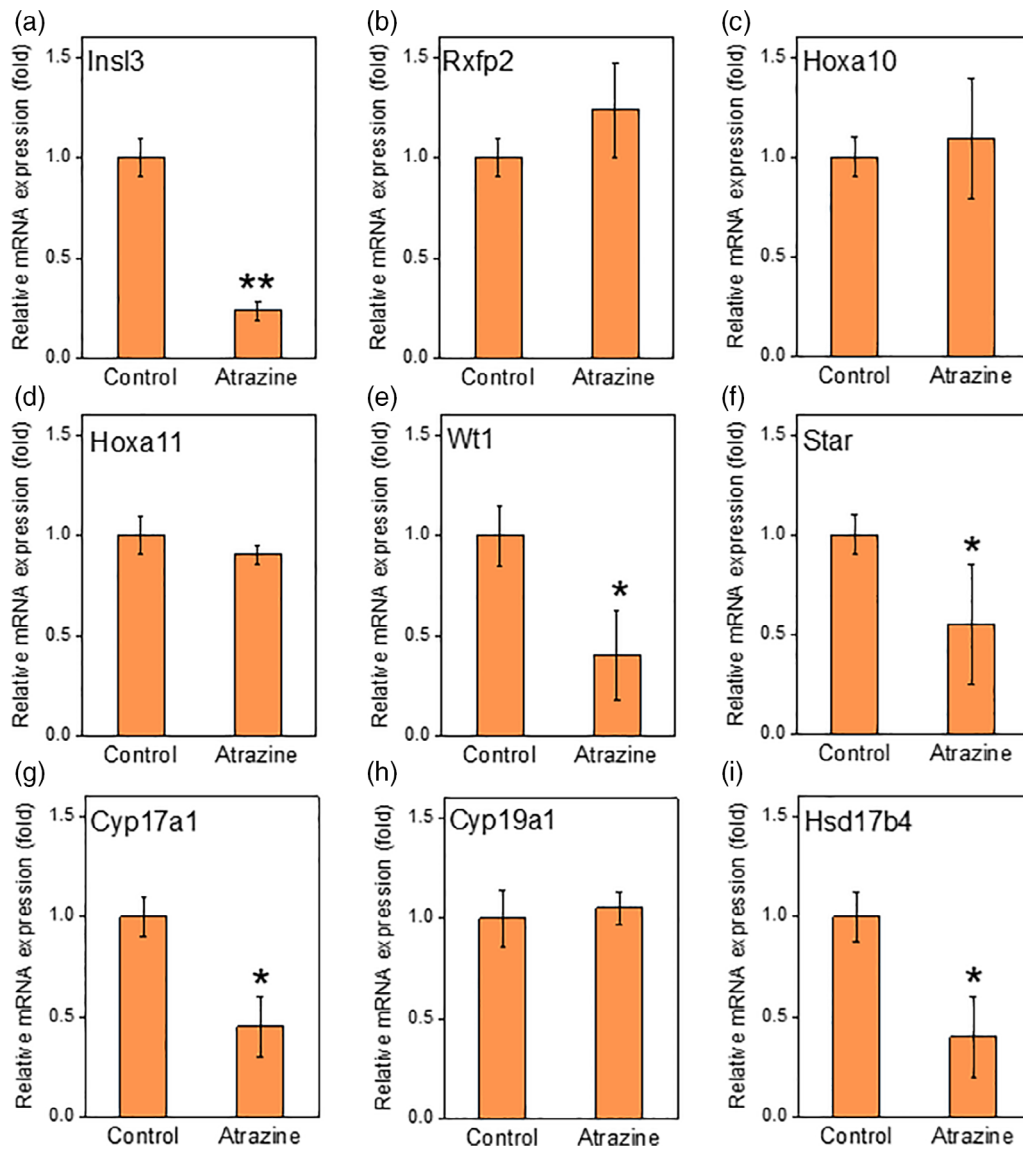
FIGURE 5 Comparison of the quantitative gene expressions in E15.5 internal reproductive organs (GTEV: gubernaculum, testis, epididymis, and vas-deferens) between control and 100 mg/kg atrazine treatment groups. Relative transcript levels of selected genes are expressed as fold change (y-axis). Control is assigned a value of 1. Error bars represent mean ($n = 5$) $\pm$ SE. *$p \leq .05$; **$p \leq .01$

sexual differentiation of reproductive tract anlage starts after testicular differentiation and androgen production. Male and female gonads are morphologically indistinguishable until E11.5 and androgen production begins rapidly after Leydig cell differentiation on E12.5-13 (O'Shaughnessy et al., 1998). The transabdominal phase of testis descent in mice is from E14.5 to E17.5 (Hutson, 1985). Although the sexually dimorphism of AGD shows at E15.5 (Zheng & Cohn, 2011), the morphological difference of male and female GT can only be observed after E16.5 (Perriton, Powles, Chiang, Maconochie, & Cohn, 2002). The androgen sensitive time window and key sexual differentiation stage for mouse external genital development during prenatal stage is E12.5 to E16.5 (Zheng et al., 2015). The phenotypes we

observed, that is, stunted growth of glans penis and induction of hypospadias in prenatally ATZ-exposed male mice, and the finding of reduced AR nuclear translocation in developing male GT, consistently suggest that prenatal ATZ exposure disrupts genomic androgen activation during male reproductive organ development. Inhibition of steroidogenesis by ATZ has been reported in peripubertal males and male fetuses in rats (Fang et al., 2018; Trentacoste et al., 2001). However, little is known about the effect of prenatal ATZ exposure on androgen levels in pregnant dams. Our finding that prenatal ATZ exposure reduced testosterone levels in pregnant dams was contrary to the result of VCZ. As a strong AR antagonist in penile development, VCZ increased maternal testosterone levels following prenatal exposure,

a phenomenon resembling the effect of flutamide on rats and men (Kerrigan, Veldhuis, & Rogol, 1994; Kubota et al., 2003). These findings suggest that ATZ may induce penile malformation not directly through AR antagonism, but via a different mechanism, such as disruption of androgen production. Although VCZ is not a pure AR antagonist and has been reported to exhibit weak estrogenic effect as well (Lemaire, Mnif, Mauvais, Balaguer, & Rahmani, 2006), but low dose of prenatal estrogen had little effect on penile development during sexual differentiation (Zheng et al., 2015).

In previous studies, deletion of *Ar* at different times using tamoxifen induced Rosa-Cre led to different phenotypes, among which the most severe one is ambiguous genitalia (Zheng et al., 2015). When ambiguous genitalia occured, <20% nuclear AR positive cells were remained in the mesenchyme of GT of mutant males compared with that of wildtype males, whereas more than 45% nuclear AR positive cells were detected in a less severe hypospadias mutant (Zheng et al., 2015), suggesting that the proportion of nuclear AR positive cells can be used as an index of androgen activation. Our results indicate that prenatal ATZ exposure reduced nuclear AR positive cells in mesenchyme of developing male GT, suggesting the reduction of AR activation in ATZ-treated tubercles. As AR nuclear translocation requires androgen binding, it is very likely that the reduced testosterone production in mother and/or maybe fetuses also constitutes the main cause of the demasculinized genital phenotypes, such as smaller glans penis, abnormal MUMP, and hypospadias.

Although androgen function is exerted upon AR, the mRNA and protein levels of AR may not be consistent with androgen action. Upregulated *Ar* mRNA in penises of hypospadias patients and mice has been reported (Agras, Willingham, Liu, & Baskin, 2006; Pichler et al., 2013). We also found the upregulation of *Ar* mRNA levels with a more than two-fold change in ATZ-treated GTs (Figure 3b), but nuclear AR positive cells were reduced. Decreased AR DNA binding and functional capability may result in a compensatory upregulation of *Ar* mRNA or even proteins. As downregulation of *Ar* mRNA was also reported in the urethral mucosa of patients with middle idiopathic hypospadias (Silva et al., 2013), thus, using *Ar* mRNA and/or protein levels to determine AR signal is insufficient.

4.2 | ATZ can alter some Wnt, Hedgehog, and Bmp pathway genes expression in developing penis

Androgen was found to interact with the Wnt/β-catenin pathway for the masculinization of external genitalia (Miyagawa et al., 2009). Prenatal ATZ exposure

downregulated several Wnt family member genes (*Wnt2b*, *Wnt5a*, and *Wnt7a*) and upregulated the expression of Wnt inhibitor *Dkk2* and stress response gene *Atf3*, which is also a direct target of Wnt/β-catenin pathway gene (Inoue et al., 2018), suggesting that ATZ may disrupt the interaction between androgen and Wnt/β-catenin pathway. Several Bmp pathway genes including *Bmp2*, *Bmp4*, and *Bmp7* are strongly expressed in developing GT (Kojima, Kohri, & Hayashi, 2010). *Bmp2*, *Bmp4*, and *Bmp7* play important roles in GT outgrowth (Kojima et al., 2010). *Bmp7* also exerts dose-dependent effects on ureteric bud or collecting duct cell proliferation and apoptosis (Piscione, Phan, & Rosenblum, 2001). *Shh* is strongly expressed in developing external genitalia at early stages (until E 15.5 in mice) and *Ihh* in later stages, *Shh* is required for GT outgrowth and tubular urethral formation, and *Ihh* also play important roles in penile growth at later stage (Perriton et al., 2002; Zheng et al., 2015). Disruption of these genes will affect cell proliferation and/or cell death (Kojima et al., 2010). Urethral epithelial cell death and mesenchymal cell proliferation play key roles in tubular urethral formation (Wang & Zheng, 2019). ATZ may disrupt these Bmp and hedgehog pathway genes expression and then alter the cell proliferation and apoptosis directly or indirectly through androgen signaling to induce abnormal penile masculinization and development. Fgf pathway genes also play important roles in external genital development (Kojima et al., 2010). We compared several Fgf pathway genes (*Fgf9*, *Fgfr2*, and *Fgfr3*) between prenatal ATZ-treated male GTs and controls, but none of them showed significant differences. One possible reason is the Fgf pathway genes we tested mainly contribute to early stages of external genital development, ATZ treatment during sexual differentiation may have little effect on their expression during penile masculinization. Further studies are needed to better elucidate how ATZ affects Wnt, Bmp, Hedgehog, and Fgf pathway and other penile developmental genes and proteins expression and whether it alters cell proliferation and apoptosis.

4.3 | ATZ induces cryptorchidism likely through downregulation of Insl3 in transabdominal phase

Testicular descent, can be generally divided into two phases (Hutson, 1985). The transabdominal phase occurs in humans between week 10 and 15 of gestation and in mice between E 14.5 and E16.5. The descent of the gonad in this stage is from the original para-renal location into a low abdominal position. Experimental data revealed that this phase of testis descent is androgen independent and controlled by the peptide hormone insulin-like 3 (INSL3),

produced in testicular Leydig cells (Bogatcheva & Agoulnik, 2005; Nef & Parada, 1999). The second, inguinoscrotal phase of testicular descent is androgen dependent; it is characterized by the movement of the testes from an intraabdominal position, through the inguinal canal, and across the pubic region to the scrotum.

The distance between the undescended testis and the bladder in ATZ-treated group was large, which suggested that ATZ-affected testis decent at least in transabdominal phase. Our data also suggested prenatal ATZ exposure induced cryptorchidism in mice likely through downregulation of *Insl3*, but not the expression of its receptor *Rxfp2*. ATZ mainly induced unilateral cryptorchidism, suggesting dose-dependent control of testicular descent by *Insl3*. ATZ also disrupted the expression of steroidogenesis-related genes in fetal testis (Figure 5f, g, and i), which was consistent with the reduction of testosterone levels. *Insl3* expression reflects the differentiation status of the Leydig cells (Ivell, Wade, & Anand-Ivell, 2013), therefore, ATZ may affect *Insl3* expression through disruption of steroid biosynthesis, but the mechanism needs better elucidation. Reduction of *Wt1* expression also suggests a complicated effect of ATZ. This study was only focused on the effect of ATZ on prenatal sexual differentiation stage. Because ATZ could inhibit androgen production, we assume that ATZ in the postnatal stage may also affect testis decent in inguinoscrotal phase; a long-term exposure (from prenatal to puberty) may cause a higher rate of cryptorchidism. The effect of ATZ (especially long term of low dose exposure) on cryptorchidism need further study.

Overall, our data demonstrated that prenatal ATZ exposure can induce hypospadias and cryptorchidism in mice, likely through disruption of testosterone production, decreasing genomic AR signaling, and then altering genital developmental gene expression (hypospadias), and downregulation of Insl3 (cryptorchidism). Although ATZ was banned in the European Union in 2003, it continues to be used quite extensively in the majority of the world, especially in the US and Australia (Chevrier et al., 2011; Gore et al., 2015). Previous publications have reported the association between endocrine-disrupting chemicals (EDCs) and increasing incidences of cryptorchidism and hypospadias in humans (Nelson et al., 2005; Virtanen & Toppari, 2008), as well as the high rates in agricultural regions and boys with farmer mothers (Carbone et al., 2007; Weidner, Moller, Jensen, & Skakkebaek, 1998). Along with these epidemiological studies, our data indicate that the role of ATZ as one of the possible causative agents in the etiology of cryptorchidism and hypospadias warrants further studies.

It is noted that as one of the main limitations of this study, our experiments employed doses greater than environmental levels. However, the occurrence of many different types of herbicides and pesticides, among which many are estrogenic or anti-androgenic agents, may create a cocktail with doses far greater than that of individual chemicals. Nevertheless, future studies should include exploring the effects under environmentally realistic scenarios by using environmentally relevant doses of ATZ, alone or together with other EDCs.

5 | CONCLUSION

Our study demonstrated that prenatal ATZ exposure could induce cryptorchidism and hypospadias in F1 male mouse offspring. We also explored potential mechanisms through which prenatal ATZ exposure induces such penile developmental defects and from the perspective of genomic androgen signaling and gene expressions involved in penile development during sexual differentiation. The induction of ATZ on undescended testis may be through the disruption of *Insl3* expression. This study advances the current understanding of the toxicity of ATZ to male reproductive system, and provides a new insight into the mechanisms affecting genital development, which is crucial for promoting protective measures against environmental pollutants induced health problems. Additional studies are needed to better elucidate underlying mechanisms and pinpoint the responsive signaling pathways leading to the observed effects on male genital development.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.