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Imidacloprid disrupts the endocrine system by interacting with androgen receptor in male mice

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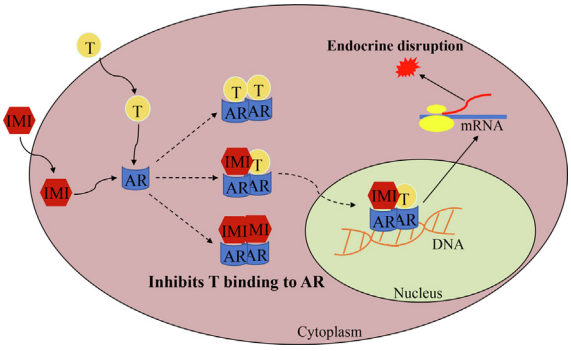
HIGHLIGHTS

- Subchronic imidacloprid exposure decreases the relative testis weight of mice.
- Imidacloprid reduces the serum T levels and the activities of aromatase.
- Imidacloprid can interact with AR and decreases AR expression in the testis.
- Imidacloprid exposure decreases the transcription of mRNA levels of T biosynthesis-related genes in the testis.

GRAPHICAL ABSTRACT



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ABSTRACT

In the current study, six-week-old male ICR mice were administered imidacloprid (IMI) at concentrations of 3, 10 and 30 mg/L for a duration of 10 weeks to investigate the toxicity of IMI on the endocrine system. We observed that testicular morphology was severely impaired and damaged, and the levels of serum testosterone (T) and the expression of androgen receptor (AR) decreased significantly. Molecular docking analysis suggested that IMI docks into the active site of AR successfully and that three key hydrogen bonds were formed with the active site residues Glu11, Gln41 and Lys138. The binding free energy value of the AR-IMI complex suggested a stable binding between IMI and AR. All these results indicated that IMI could interact with AR. In addition, major genes in the testis involved in the synthesis of cholesterol and T were generally inhibited, and the serum cholesterol sources were also reduced. Moreover, the aromatase in male mice was lacking after subchronic IMI exposure. The data acquired from the present study indicated that IMI could lead to endocrine disruption by interacting with AR and influence the expression of genes involved in the production of T in male mice.

1. Introduction

Insecticides are used to control insects to improve agricultural productivity (Wood and Goulson, 2017; Jin et al., 2017; Yuan et al., 2019). Imidacloprid (IMI), a neonicotinoid, is widely used due to its selective poisonousness to insects (Eddleston et al., 2004; Daisley et al., 2017). Since the insecticide entered the market in 1991, the use of IMI has been growing every year, accounting for more than 40 percent of the total usage of neonicotinoid insecticides (Mikolić and Karačonji, 2018). According to previous research, a high concentration of 120 ng/L was observed in groundwater around a solid waste treatment plant of Castellón (eastern

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Spain) (Jurado et al., 2019). The extensive use of IMI leads nontarget organisms to be highly exposed to its residues. IMI has been detected in the human body according to a number of studies (Chi-Hsuan et al., 2018; Zhang et al., 2019). Thus, it is necessary to evaluate the potential endocrine risk of IMI in animal models.

According to several previous studies, IMI has been shown to be associated with endocrine disruption (Bal et al., 2012; Hafez et al., 2016). For example, in female rats treated with 20 mg/kg/day IMI, ovarian weight decrease, significant pathomorphological changes in follicles, antral follicles and atretic follicles, together with hormonal imbalance were observed (Kapoor et al., 2011). The ratio of mating success and number of fetuses decreased significantly in the offspring of IMI-treated female Wistar rats (Nabiuni et al., 2015). Interestingly, studies have also observed that male rats were also subject to endocrine disruption by IMI exposure. For example, Najafi et al. (2010) found that oral gavage of male rats with a high dose of IMI led to adverse histological changes in testicular tissue and lower serum testosterone (T) levels compared with those of the control. However, the role of IMI in the function of androgen receptor (AR) and in the reduction in T expression has rarely been explored.

Androgen plays an essential role in the growth and reproductive function of male individuals, such as inducing sex differentiation, formation and maintenance of secondary sexual characteristics, spermatogenesis, and feedback regulation of pituitary hormone secretion (Schiffer et al., 2018). The AR, in response to the stimulation of circulating T and locally formed dihydrotestosterone, regulates the expression of a target gene (Rahmani et al., 2013). AR mutations lead to androgen insensitivity syndrome, which leads to abnormal development of internal and external male structures. Cholesterol can be recognized by membrane-bound low-density lipoprotein receptor (LDL-R) and scavenger receptor class B type I (SR-B1). The steroid-forming acute regulatory protein (StAR) and peripheral benzodiazepine receptor (PBR) transport cholesterol to the inner mitochondrial membrane (Martin et al., 2016), where it is metabolized to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc). Subsequently, pregnenolone is metabolized to T by enzymes in the smooth endoplasmic reticulum (Miller, 2017). The expression of these key enzymes and genes mentioned above could be influenced by endocrine disruption chemicals (EDCs), including insecticides (Jin et al., 2014; Jin et al., 2015).

The oral LD_{50} for IMI in mice is 131 mg kg^{-1} of body weight (bw) (Mikolić and Karačonji, 2018). Recently, it was reported that mammals and bees would be poisoned when IMI was consumed at doses below the LD_{50} (Arfat et al., 2014; Sun et al., 2016; Daisley et al., 2017). However, how IMI impacts the synthesis of steroid hormones and the action of androgen in mice remains unclear. Here, various low concentrations of IMI were subchronically administered through the drinking water to analyze the endocrine disruption effects in male mice. We hope that these results can bring new insight into the potential risks of IMI as an EDC.

2. Materials and methods

2.1. Chemicals

IMI [1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylide neamine] was purchased from YuanYe Company (CAS: 138261-41-3, purity: ≥98%).

2.2. Animals and experimental design

Thirty-two healthy male Institute of Cancer Research (ICR) mice, aged 6 weeks, were obtained from the China National Laboratory Animal Resource Center (Shanghai, China). All mice were maintained independently at 12 h of light: 12 h of dark and 24 ± 1 °C for 1 week, then the mice were divided into four groups (n = 8), randomly. Among of them, 3 groups of mice orally consumed 3 mg/L (IMI-3), 10 mg/L (IMI-10) or 30 mg/L (IMI-30) IMI in their drinking water for a duration of 10 weeks, and the control group (CON) was deionized water. Water and basic diet were available during the experiment.

All mice fasted at least for 12 h and were anesthetized by ether before being sacrificed. The blood was collected quickly, testis was removed and weighed. All of them were stored at −80 °C until further measurement. The experimental protocols were approved by the Ethical Committee of Zhejiang University of Technology.

2.3. Histopathological analysis of the testis

The paraformaldehyde-fixed testis was cut into 5 μm-thick sections embedded in paraffin wax at 56 °C, fixed in 0.1% (w/v) formaldehyde, and then stained with hematoxylin & eosin (H&E). Ability of spermatogenesis in the testis is analyzed according to a Johnsen score marked 1 to 10 (Johnsen, 1970; Tang et al., 2018; Jin et al., 2019).

2.4. Immunohistochemical analysis

The paraffin sections of the testis were dewaxed in xylene, recovered by heating in EDTA antigen repair buffer (pH 9.0) (G1203, Servicebio Biological Technology, Ltd., Wuhan, China) and treated with 3% hydrogen peroxide to block endogenous peroxidase. Then, the tissue was sealed with 3% BSA to prevent the antibody from flowing away. Rabbit anti-mouse AR (22089-1-AP, Proteintech) at a dilution of 1:200 was added to the sections and then incubated overnight at 4 °C. Next, the slices were incubated with HRP-conjugated secondary antibodies (GB23303, Servicebio Biological Technology, Ltd., Wuhan, China) for 50 min at room temperature and then stained with diaminobenzidine. The sections were finally counterstained with hematoxylin and observed with a microscope.

2.5. Determination of biochemical indexes

The total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) as well as low-density lipoprotein cholesterol (LDL-C) levels in serum and testis were measured by kits purchased from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). The levels of T and estradiol (E2) as well as the activity of aromatase in the serum of each mouse were determined using the corresponding mouse ELISA kits, which were purchased from the Shanghai Enzyme-linked Biotechnology Co. Ltd. (Shanghai, China).

2.6. Quantitative Real-time PCR

Testis was homogenized to extract total RNA using TRIzol reagent (Takara Biochemicals) and a reverse transcriptase kit (Toyobo) to synthesize cDNA. Real-time polymerase chain reaction (RT-qPCR) using the SYBR Green kit (Toyobo, Japan) was performed in a qTOWER3G (Analytik Jena AG, Germany). mRNA expression levels of 3-hydroxy-3-methyl-glutaryl *CoA (HMG-CoA) synthase*, *HMG-CoA reductase*, *LDL-R*, *SR-B1*, *PBR*, *StAR*, *P450scc*, cytochrome P450 17a-hydroxysteroid dehydrogenase *(P450 17α)*, 3β-hydroxysteroid dehydrogenase *(3β-HSD)* and 17β-hydroxysteroid dehydrogenase *(17β-HSD)* were analyzed. The sequences of the primers from a previous study were used (Jin et al., 2019). 18S rRNA transcript levels were determined as a housekeeping gene. The following cycling conditions were used: denaturation for 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

The qPCR of genes in all groups was performed as previously described (Xia et al., 2018; Pan et al., 2019).

2.7. Molecular docking and molecular dynamics (MD) simulations

The crystal structure of AR was obtained from the Protein Data Bank (PDB ID: 2QPY). Prior to molecular docking, all the water molecules were removed from the AR because none of them play any role in binding. AutoDock Tools have been used to generate the input files and identify the grid center. IMI was docked into the active site of AR via AutoDock Vina 1.1.2. The superior pose with the lowest docked energy was selected as the initial configuration for the molecular dynamic simulations. The MD simulations were performed with the sander module of AMBER 14 (Case et al., 2005). The AR-IMI complex was surrounded by a periodic TIP3P water box with a margin distance of 10 Å. After energy minimization and equilibration, the system was simulated for 5 ns with a time step of 2 fs. Generally, simulations on the nanosecond time scale are sufficient for a reliable evaluation of local conformational changes (Zhuang et al., 2016). The MD trajectories were saved every 1 ps and analyzed using CPPTRAJ (Roe and Cheatham, 2013).

2.8. Binding free energy calculation and energy decomposition analysis

The binding free energy of AR-IMI complexes was calculated using the Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) method encoded in the AMBER14. In order to explore hot-spot amino acids that play major contribution in the interaction between AR and IMI, the binding free energy was further decomposed into per-residue using MM/GBSA.py module of AMBER14 (Miller et al., 2012).

2.9. Statistical analysis

The data onto each IMI-treated group were compared with that of the control. Differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's protected least significant difference tests using SPSS 13.0 (SPSS, Chicago, Illinois, USA).

3. Results

3.1. Effects on the growth phenotypes and testes in mice

After subchronic exposure to 3, 10, or 30 mg/L IMI for 10 weeks, the body weights in all IMI-treated groups showed no significant change compared with the weights of the controls (Fig. 1A). However, the relative weights of the testis decreased significantly, especially in the 30 mg/L IMI-treated group (Fig. 1B). Subsequently, H&E staining was performed to assess the quantification of spermatogenesis in the testicular sections using the Johnsen score (Fig. 1C and D). In the control, the seminiferous tubules had a complete shape and contained regular spermatogenic cells and spermatozoa. However, exposure to 30 mg/L IMI resulted in histological damage to the testicular tissue. Most of the seminiferous tubules were thinner, the layers of spermatogenic cells were seriously decreased, and the seminiferous epithelium was irregularly arranged. In addition, the Johnsen score was also significantly decreased in the IMI-30 group compared to that of the control (Fig. 1D).

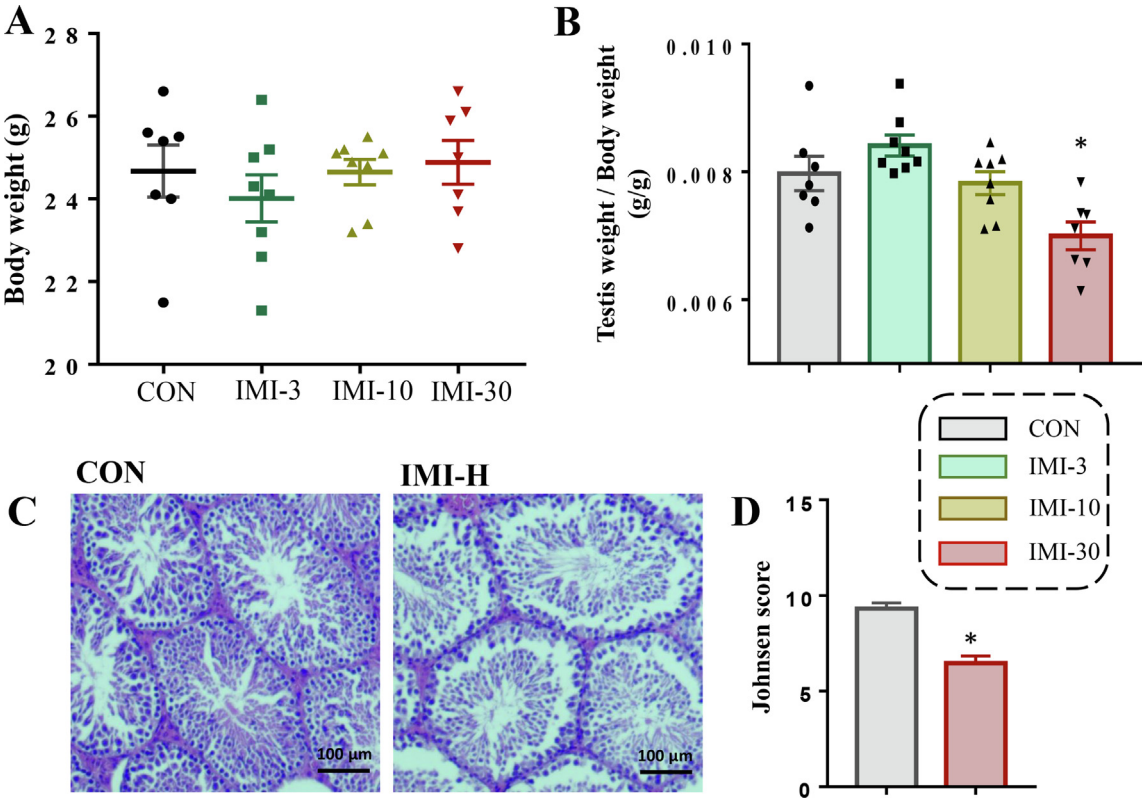

Fig. 1. Effects of IMI exposure on the growth phenotypes of mice. (A) Body weight after 12 h of fasting prior to sacrifice; (B) Testis weight relative to body weight; (C) Morphological alterations of H&E-stained sections in the testis of the control and IMI 30-treated mice; (D) Testicular damage as measured by the Johnsen score. As follows, 10, complete spermatogenesis; 9, irregularly arranged seminiferous epithelium; 8, for few sperm are present; 7, no late round sperm; 6, a small number of sperm cells exist; 5, no round sperm; 4, only a few spermatocytes exist; 3, only spermatogonia; 2, no germ cells; 1, no Sertoli cells. The presented values are the means ± SEM (n = 8). $^{*}p < 0.05$ versus control.

3.2. Effects on the sources of cholesterol

Subchronic IMI exposure tended to significantly reduce the serum LDL-C levels without affecting the serum TC and HDL-C levels (Fig. 2A and Table S1). Interestingly, the testicular TC levels were significantly reduced in the 30 mg/L IMI-treated group, while no marked difference was observed in the testicular LDL-C between the IMI-treated and the control groups (Fig. 2B and Table S1). In addition, in de novo testis synthesis, the expression of *HMG-CoA synthase* was also significantly inhibited (Fig. 2C).

3.3. Effects on the serum T and E2 levels and the activity of aromatase

Subchronic exposure to 30 mg/L IMI resulted in a significant decrease in the serum T and E2 concentrations (Fig. 3A and B). However, the ratio of E2/T did not change after IMI exposure for 10 weeks (Fig. 3C). Interestingly, the serum activity of aromatase in the 30 mg/L IMI-treated group decreased significantly (Fig. 3D). These results indicated that subchronic exposure to IMI disturbed the homeostasis of T and E2 in male mice.

3.4. Effects of IMI exposure on the AR expression

To determine the molecular mechanism of endocrine disruption caused by IMI exposure, the mRNA levels of *AR* in the testis were studied (Fig. 4B). IMI exposure markedly inhibited the mRNA levels of *AR* in the testes of mice. AR expression in the testes was immunohistochemically examined. Hematoxylin stained the nucleus blue, and the positive expression of diaminobenzidine was brownish-yellow. AR was always present in Leydig cells, Sertoli cells, and peritubular myoid cells. AR expression was decreased in IMI-H-treated testes (Fig. 4A).

3.5. IMI had binding characteristics with the AR protein

The interaction between IMI and AR was explored at the atomic level. The AR-IMI complex obtained by molecular docking was used to perform MD simulations. The dynamic stability of the complex was monitored and confirmed by the analysis of the root mean square deviation (RMSD) as a function of time. The AR-IMI system reached equilibrium after 3 ns in the simulation (Fig. 5B). The representative conformation of IMI was extracted from the last 2 ns of simulations (Fig. 5A). IMI docks into the active site of AR successfully and forms three hydrogen bonds with the active site residues Glu11, Gln41 and Lys138.

3.6. Binding free energy and free energy decomposition

To quantitatively evaluate the molecular interaction between AR and IMI, the binding free energy value of the AR-IMI complex was calculated using the MM-GBSA method (Table S2). The binging free energy (△G bind) value of −36.59 kcal/mol suggested a stable binding between IMI and AR. The electrostatic and van der Waals components in the binding free energy are two predominant contributors driving the binding of IMI to AR. The binding free energy was then decomposed into per-residue to identify the hot-spot

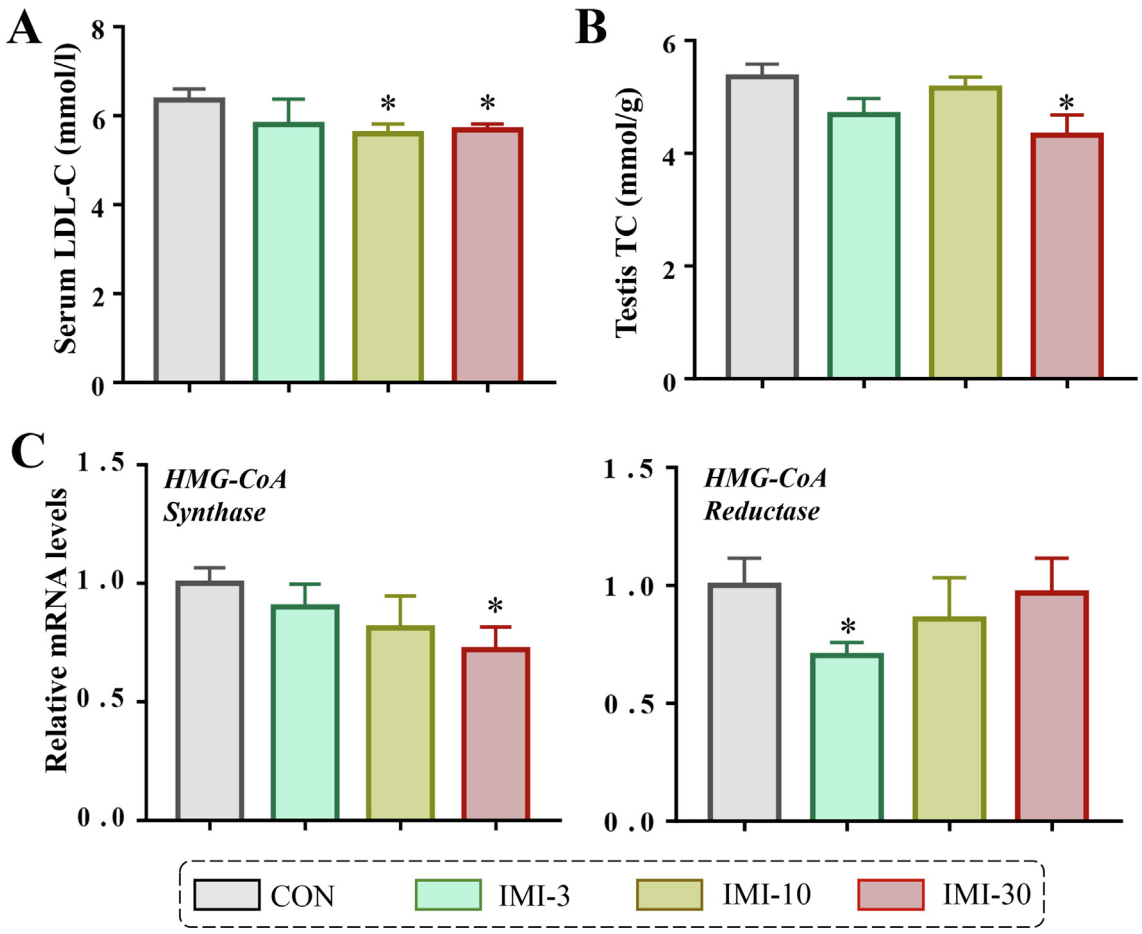

Fig. 2. Effects of IMI exposure on the cholesterol source in mice. Effects of IMI exposure on the serum LDL-C (A) or testicular TC (B). The mRNA levels of cholesterol synthesis-related genes (C), including *HMG-CoA reductase* and *HMG-CoA synthase*, in male mice. The presented values are the means ± SEM (n = 8). *$p < 0.05$ versus control.

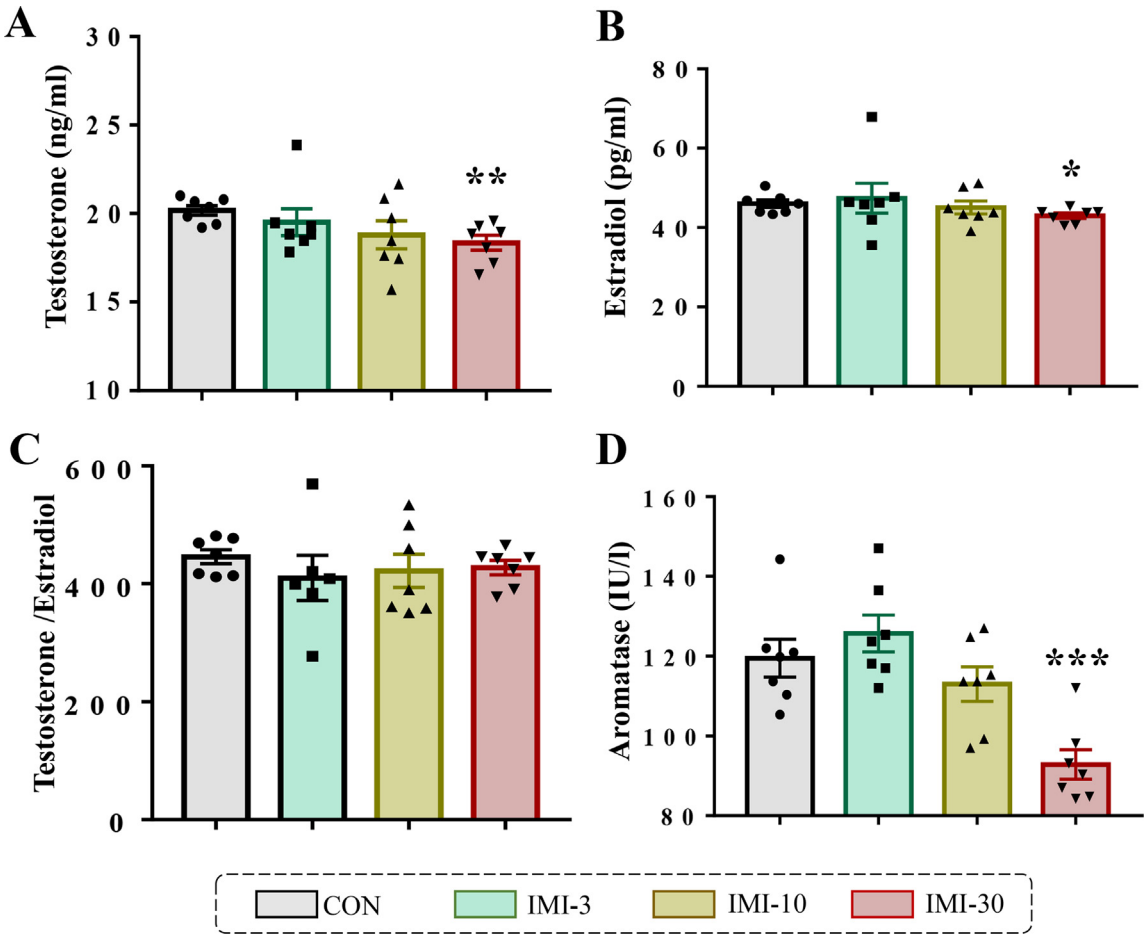

Fig. 3. Effects of IMI on the serum hormone levels and the activity of aromatase in male mice. (A) ELISA results of estradiol in male mice; (B) ELISA results of testosterone in male mice; (C) ELISA results of T/E2 in male mice; (D) ELISA results of aromatase in male mice. The presented values are the means ± SEM (n = 7). $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

amino acid residues of AR for the binding of IMI. The top ten favorable contributors were Lys138, Glu11, Phe134, Hie44, Trp81, Val45, Pro12, Leu135, Trp48 and Ala78 (Fig. 5C).

3.7. Effects on the genes involved in the synthesis of T in the testis

To test whether IMI exposure would affect cholesterol transport in the testis, we further examined the mRNA levels of *SRB1*, *LDL-R*, *PBR*, and *StAR* in the testes of mice exposed to IMI for 10 weeks. The mRNA levels of *StAR* and *PBR* decreased significantly in the testes of mice when subchronically exposed to 10 and 30 mg/L IMI for 10 weeks (Fig. 6A). In addition, except for dramatically improving the expression of *17β-HSD*, subchronic IMI exposure greatly decreased the mRNA levels of *P450scc*, *3β-HSD*, *P450 17α* and *17β-HSD* compared with levels in the control (Fig. 6B).

4. Discussion

It was reported that a great number of environmental pollutants, including pesticides, posed an increasing risk to humans and animals (Jin et al., 2013, 2014; Defarge et al., 2016; Wu et al., 2018; Wang et al., 2019). These chemicals are generally named EDCs, which are defined as exogenous agents that interfere with the synthesis, transport, or action of natural hormones that maintain homeostasis (Gray et al., 2001; Ahmad et al., 2017). The widespread use of IMI has increased the potential risk of exposure to residues for humans and untargeted animals (Morrison et al., 2018). According to several previous studies, the toxicity of IMI has been linked to hepatotoxicity, nephrotoxicity, and neurotoxicity in several experimental models (Arfat, et al., 2014; Lonare et al., 2014). Some previous studies have also clearly demonstrated that IMI could disrupt the endocrine system (Gu et al., 2013; Mohamed et al., 2016; Mzid et al., 2017). Interestingly, IMI was also confirmed to disrupt the endocrine system in male animals. For example, Bagri et al. (2015) found that mice exposed to 5.5 mg/kg/day IMI for 14 days induced significant sperm head abnormalities. Developing male rats (7 days of age) that received 0.5, 2 and 8 mg/kg IMI for 90 days had lower concentrations of epididymal sperm and higher rates of abnormal sperm (Bal et al., 2012). All these results proved that IMI exposure could influence the male endocrine system; however, the mechanism still remains unclear. Our current study showed that male mice demonstrated some adverse effects on their endocrine system after oral administration of a concentration of 3, 10 and 30 mg/L IMI for 10 weeks, including disruption of related gene transcription and reduction in serum T concentration.

T is an important hormone in male animals that regulates various physiological activities by binding to AR and activating the expression of downstream genes. Studies have shown that some pesticides can bind to AR as an analog of T and interfere with the endocrine system of animals. A great number of pesticides, including dieldrin, endosulfan, methiocarb, and fenarimol, all acted as androgen antagonists (Andersen et al., 2002). As a pesticide, IMI

was also shown to interact with AR, but the mechanism is not clear (Mikolić and Karačonji, 2018). Here, we observed that subchronic exposure to IMI could significantly reduce the expression of AR in testicular tissue, indicating that IMI could inhibit the function of AR. In addition, molecular docking analysis also suggested that IMI docks into the active site of AR successfully, and three key hydrogen bonds were formed with the active site residues Glu11, Gln41 and Lys138 (Fig. 5A). The binding free energy value of the AR-IMI complex suggested a stable binding between IMI and AR. All these results indicated that IMI had the potential to interact with AR directly.

The interaction of IMI with AR results in a decrease in T levels. As demonstrated by previous studies, male rats that received 0.5, 2 or 8 mg/kg bw/day IMI by oral gavage for three months had reduced T levels (Bal et al., 2013). Rats treated with IMI at 1 mg/mL for 65 days exhibited downregulation of the activity of steroidogenic genes, including *3β-HSD* (Mohamed et al., 2016). We speculated that IMI exposure might influence the expression of the main genes in the pathways regulating the steroid to decrease T levels in adult male mice. Cholesterol, supplied in the form of LDL and HDL, is the foundation for synthesizing all steroid hormones. Here, we observed that serum LDL-C and testicular TC decreased markedly after IMI exposure (Fig. 2A and B). In addition, in de novo testis synthesis, the expression of *HMG-CoA synthase* was also significantly inhibited (Fig. 2C). LDL-R and SR-B1 are responsible for the transport of LDL and HDL, respectively, from blood to testis. PBR and StAR play key regulatory roles in cholesterol transport to the inner mitochondrial membrane (Miller, 2017). IMI-30 greatly decreased the expression of *PBR* and *StAR*, while *SR-B1* and *LDL-R* were not obviously affected (Fig. 6A). IMI can not only affect the transport of cholesterol but can also affect the mRNA of the key genes involved in T synthesis, except for

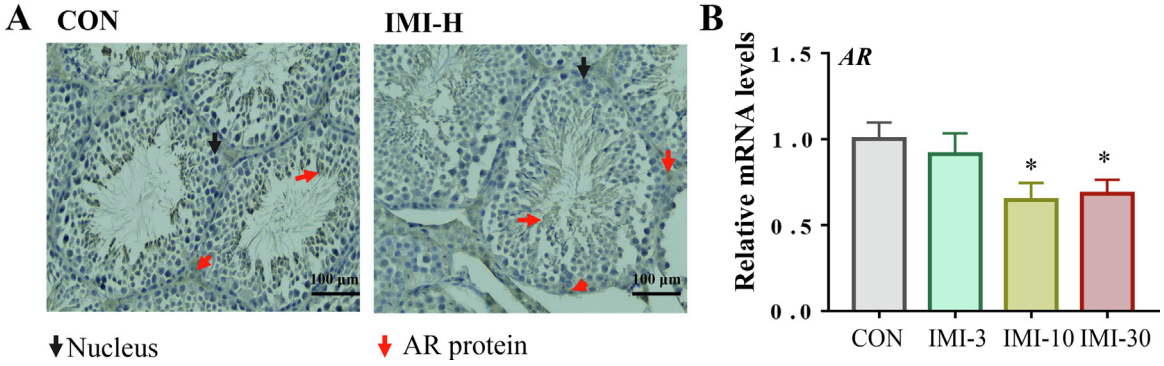

Fig. 4. Effects of IMI exposure on the expression of androgen receptor in mice. (A) Immunohistochemistry of AR in the testis; (B) The mRNA levels of testicular *AR* in male mice. The presented values are the means ± SEM (n = 8). *p < 0.05 versus control.

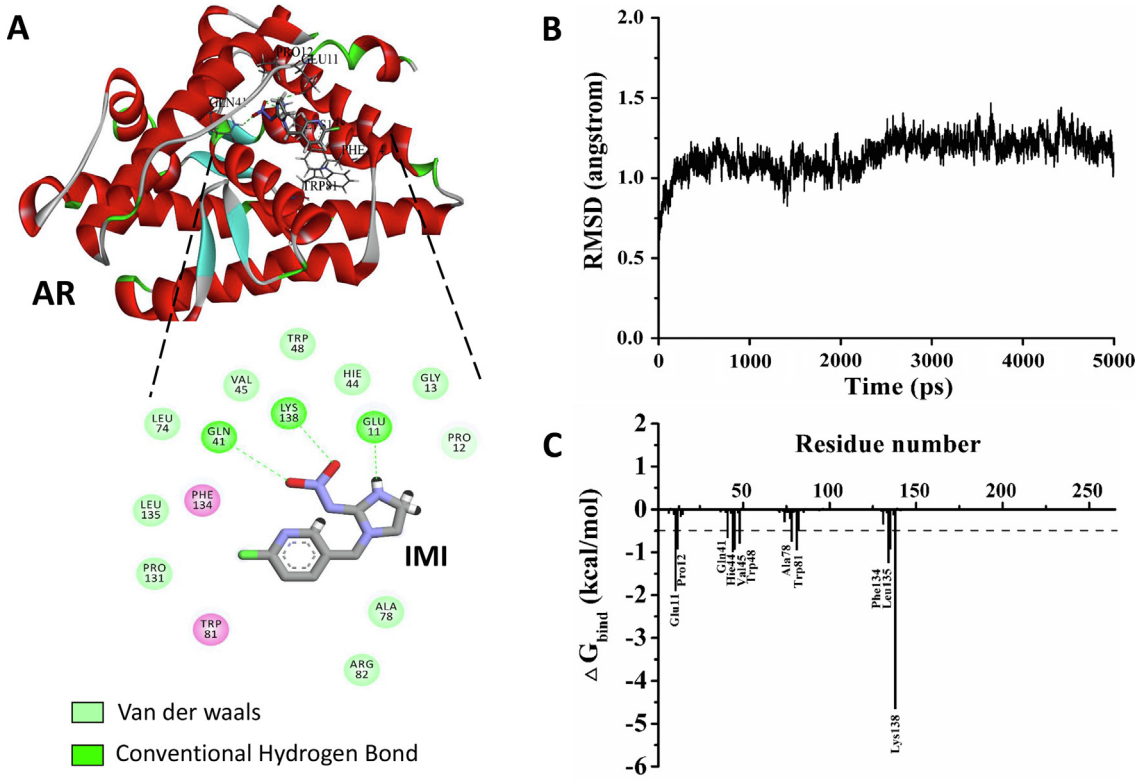

Fig. 5. Molecular docking models. (A) The root mean square deviation (RMSD) of the AR-IMI complex as a function of time; (B) The major amino acid residues of AR that contribute to the binding of IMI to AR. (C) The binding mode between IMI and AR at the atomic level.

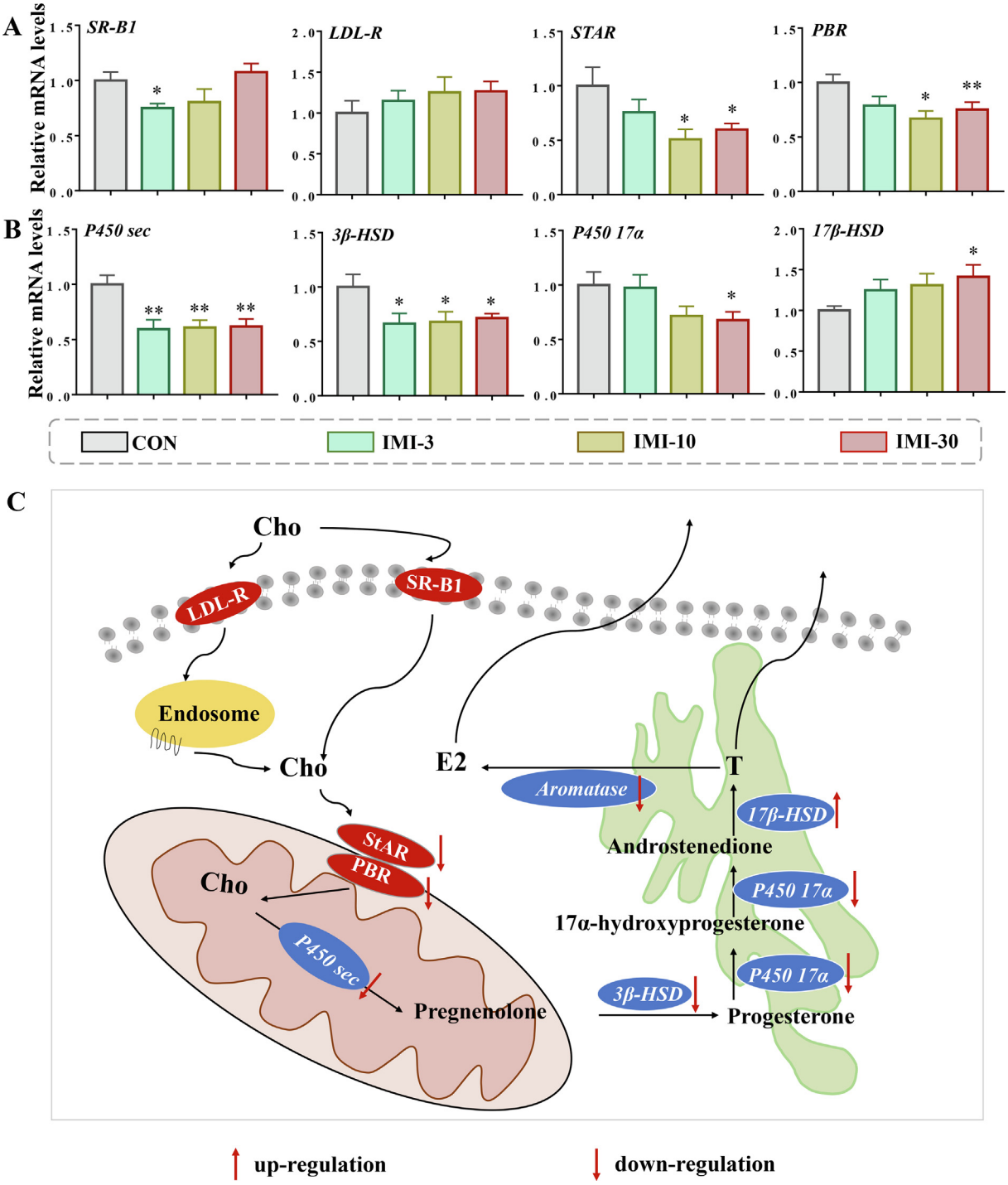

Fig. 6. Effects of IMI exposure on the mRNA levels of genes related to estradiol and testosterone biosynthesis in mice. (A) The mRNA levels of testicular cholesterol transport-related genes; (B) The mRNA levels of T biosynthesis-related genes; (C) Exposure to IMI influences some key genes in the steroidogenic pathway, and the arrows refer to changes in the mRNA levels. The presented values are the means ± SEM (n = 8). $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

17β-HSD, which was inhibited (Fig. 6B). In accordance with these results, 30 mg/L IMI treatment resulted in testicular histopathological damage and a decrease in testicular weight and the serum T concentration. In addition, low T levels may affect the cardiovascular system and induce type 2 diabetes in men (Hwang and Miner, 2015; Goodale et al., 2017). Some studies have suggested that IMI could potentiate high-fat diet-induced adiposity and insulin resistance in male C57BL/6 mice by influencing the AMPKα-mediated pathway (Sun et al., 2016).

Furthermore, our studies showed that IMI-30 significantly inhibited AR expression. Exposure to IMI inhibited *AR* transcriptional activity and protein levels, suggesting that unintended exposure to IMI may disrupt the endocrine system via influencing nuclear hormone receptors such as AR. The reduction in AR influences the effects of T, which in turn leads to androgen insensitivity syndrome (Komori et al., 1999; Wang et al., 2009; Mongan et al., 2015; Döhnert et al., 2017). However, excessive levels of AR can lead to a range of diseases, such as bladder cancer and pancreatic

cancer (Kanda et al., 2014; Rodriguez-Vida et al., 2015; Schweizer and Yu, 2017). In our experiments, the decrease in AR and the IMI interaction with AR may lead to androgen insensitivity, which may further induce infertility, hypogonadism, and even testicular dysgenesis syndrome in mice.

The content of aromatase decreases due to lower T levels. Aromatase can produce estrogen from androgen (Fig. 6C). The deficiency of aromatase explains the decrease in E2 in IMI-treated mice. Aromatase deficiency could also be associated with several other features (Takeda et al., 2003; Honda et al., 1998), such as the impairment of spermatogenesis and germ cell development and thus fertility (Simpson, 1998; Robertson et al., 1999; Carreau et al., 2003; Jones et al., 2006). These impairments include reduced sperm motility, poor hypospermatogenesis, and germ cell arrest (Carani et al., 1997). There are also reported cases in which men lack aromatase and experience infertility (Herrmann et al., 2002; Dostalova et al., 2017). In accordance with these results, damage was observed in the testicular histopathological analysis after IMI exposure (Fig. 1B and C). In summary, we speculated that IMI exposure might influence the expression of genes and pathways producing T and affect the AR to disrupt the endocrine system in male mice.

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.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135163.

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