Effect on the dopaminergic metabolism induced by oral exposure to simazine during the prepubertal period in rats

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Abstract. The herbicide simazine is widely used in agricultural and non-agricultural fields. Studies have shown that simazine inhibits the proliferation of dopaminergic cells and affects the developmental differentiation of dopamine neurons. However, little is known about the effects of simazine on dopaminergic metabolism. Therefore, the present study examined the effects of simazine on Sprague-Dawley (SD) rats from weaning to puberty (40 days exposure). Simazine was administered orally to SD rats at doses of 0, 12.5, 50 and 200 mg/kg body weight. The contents of dopamine (DA), levodopa, dihydroxy-phenyl-acetic acid and homovanillic acid in the striatum were then examined by high-performance liquid chromatography with a fluorescence detector. Quantitative polymerase chain reaction and western blotting were used to analyze the mRNA and protein expression of aromatic amino acid decarboxylase (AADC), tyrosine hydroxylase, orphan nuclear hormone (Nurr1), dopamine transporter (DAT), vesicular monoamine transporter 2 (VMAT2), monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). The results indicated that simazine influenced the synthesis, transport and metabolism of DA and led to a reduction of DA levels in the striatum. One potential underlying mechanism is decreased levels of Nurr1, DAT and VMAT2 impacting upon the transport of DA; another is the decreased level of AADC and increased levels of MAO and COMT impacting upon the synthesis and metabolism of DA. These factors may eventually lead to neurological disorders of the dopaminergic system.

Introduction

Extensive application of herbicides not only pollutes the environment, but also endangers people's health. Herbicides are amongst the most harmful types of water-polluting agents, particularly the triazine derivative herbicides used worldwide as residual non-selective herbicides to control broad-leaved weeds and annual grasses (1). Owing to its high potency and the broad spectrum of weeds it kills, atrizine has been replaced gradually by simazine (6-chloro-*N*,*N*'-diethyl-1,3,5-triazine-2,4-diamine) since the 1960s (2) in agricultural and non-agricultural scenarios. Simazine, which is detectable in both surface and ground water (3), has multiple exposure pathways, including water and air, and particularly affects the food chain (4-7). The diversity of the means of exposure increases the risk to human health.

Previous studies on simazine have mainly focused on its mutagenicity, reproductive developmental toxicity and immunotoxicity (8-14). Lengthy exposure to a low dose of simazine has been demonstrated to influence the development of early life stages in mammals, where it acts as a neural endocrine disruptor and has an immunotoxic effect (14,15).

Dopamine (DA) is an important neurotransmitter in the mammalian brain and regulates movement, emotional, cognitive, memory and other physiological functions of the central nervous system. Dysfunction of the DA system may lead to Parkinson's disease (PD), schizophrenia, depression and other diseases (16-19). The aim of the present study was to investigate the effect of simazine on the DA system of rats that were exposed to simazine during the prepubertal period. The expression levels of the main metabolic factors in the dopaminergic system and the contents of the monoamines and their main metabolites were determined in order to evaluate the effects of simazine on DA metabolism.

Materials and methods

Materials. Simazine (98% pure) was obtained from Zhejiang Zhongshan Chemical Industry Group Co., Ltd. (Zhejiang, China). The solutions of simazine used for treatment at various levels (12.5, 50 and 200 mg/kg body weight) were prepared by dissolving simazine in 2% (w/v) starch. All standard materials used in this study were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Animals and treatment. All animal procedures in the present study were approved by the Medical Ethics Committee of Harbin Medical University (Harbin, China) and were conducted in accordance with the guidelines for animal experimenta-

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tion issued as standards for laboratory animal research by the National Institutes of Health (Bethesda, MD, USA). A group of 60 male Sprague-Dawley (SD) rats (21 days old; weighing 72-81 g) purchased from Vital River Laboratories Co., Ltd. (Beijing, China) was divided randomly into four groups (n=15) and body weight was taken into account when assigning the rats to each group.

All animals were kept in a feeding room under controlled environmental conditions (photoperiod, 12-h light/dark cycle; temperature, 22±2˚C; relative humidity, 50±15%) and supplied with a standard laboratory diet and purified water *ad libitum*.

The rats were allowed to acclimatize to these conditions for 1 week and no anesthetic was administered to avoid any interference with biochemical values. The four groups were fed separately in standard cages. A dosage of 0 (control group), 12.5, 50 or 200 mg/kg simazine (in 2% starch solution) was administered to the rats at the same time each morning by oral gavage for 40 days. The brains were then removed rapidly and placed into iced physiological saline. The striatum and midbrain from the whole brain were dissociated carefully and tissues were stored frozen at -80˚C.

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA separated from the midbrain tissues was isolated using TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's protocol. The concentration of RNA was measured with an ND-2000c spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). PrimeScript® RT with a gDNA Eraser reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to synthesize cDNA from 1 μg total RNA according to the manufacturer's protocol. The PCR primers were designed and synthesized by Generay Biotech Co., Ltd. (Shanghai, China) and the sequences are presented in Table I.

The cDNA was amplified using the SYBR-Green method (SYBR® Premix Ex Taq™ II; Takara Biotechnology Co., Ltd.) in an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions comprised denaturation at 95˚C for 5 sec, and 40 cycles of annealing at 58˚C for 34 sec with extension at 72˚C for 30 sec. The cycle at which sample fluorescence reached the threshold was defined as the threshold cycle, also known as quantification cycle (C_q). The results are expressed as the relative expression ratio calculated upon the basis of the qPCR efficiency (E) and ΔC_q. The ΔC_q value for each gene (target or reference) was calculated by subtracting the C_q value of the target sample from that of the control sample. As shown in equation 1, the ratio of target gene expression in treatment versus control samples was derived from the ratio between target gene efficiency (E target) to the power of target ΔC_q (the ΔC_q target value) and reference gene efficiency (E reference) to the power of the reference ΔC_q (ΔC_q reference) (20).

$$\text{Ratio} = \frac{(E_{target})^{\Delta C_{q_{target}}}}{(E_{reference})^{\Delta C_{q_{reference}}}} \qquad \text{equation 1}$$

Immunoblotting. Protein was extracted from the midbrain tissues using radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) on ice for 2 h and then centrifuged at 12,000 x g for 15 min at 4˚C. The supernatant was collected and a BCA protein assay kit (Beyotime Institute of Biotechnology) was used to measure the protein concentration. Equal amounts of total protein (60 μg) were subjected to 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 0.5% (w/v) bovine serum albumin (Promega Corporation, Madison, WI, USA) for 0.5 h at room temperature. The membranes were then stripped and incubated overnight at 4˚C with rabbit polyclonal orphan nuclear hormone (Nurr1) antibody (1:500 dilution in blocking buffer; sc-990; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-tyrosine hydroxylase (TH) antibody (1:1,000 dilution in blocking buffer; AB152; EMD Millipore, Billerica, MA, USA), anti-DA transporter (DAT) antibody (1:500 dilution in blocking buffer; sc-14002), anti-catechol-O-methyltransferase (COMT) antibody (1:500 dilution in blocking buffer; sc-25844), anti-monoamine oxidase (MAO) antibody (1:500 dilution in blocking buffer; sc-18401), anti-AADC antibody (1:200 dilution in blocking buffer; sc-46909) and anti-vesicular monoamine transporter 2 (VMAT2) antibody (1:500 dilution in blocking buffer; sc-15314) (all from Santa Cruz Biotechnology, Inc.), and β-actin (1:500 dilution in blocking buffer; YT0099; ImmunoWay Biotechnology Co., Plano, TX, USA). After washing three times with Tris-buffered saline containing 0.1% (v/v) Tween-20 at room temperature, membranes were incubated at room temperature for 1.5 h with alkaline phosphatase (ALP) goat anti-rabbit secondary antibody (AP-1000; Vector Labs, Burlingame, CA, USA) at a 1:1,000 dilution in blocking buffer. Membranes were washed with Tris-buffered saline three times at room temperature and then incubated with Western Blue® Stabilized Substrate for ALP (Promega Corporation) for 3 min at room temperature (26-28˚C). Relative expression (%) was calculated based upon protein density using the ChemiQ3650 Analysis System (Bioshine, Shanghai, China). The density was calculated using Quantity One v4.6.2 software. Sample blot density was normalized to β-actin.

Quantification of monoamines and their main metabolites. The concentrations of monoamines and their main metabolites in the striatum were assessed using high-performance liquid chromatography with a fluorescence detector (HPLC-FLD). The corpus striatum tissues of the rats in each group were homogenized in 0.1 M perchloric acid and centrifuged at 12,000 x g for 15 min at 4˚C, then filtered through a 0.2-μm cellulose membrane. The supernatants were collected to measure the contents of monoamines and main metabolites. Three samples per group were injected into a Waters chromatograph equipped with a fluorescence detector (G1321C, 1260 FLD; Agilent Technologies, Inc., Santa Clara, CA, USA) and a Cosmosil C18 column (5 μm, 4.6x250 mm; Nacalai Tesque, Inc., Kyoto, Japan).

The mobile phase consisted of 20 mM trisodium citrate and 50 mM sodium hydrogen phosphate. Samples were separated at 28˚C and a flow rate of 1.2 ml/min. The detector was set at an excitation wavelength of 285 nm and an emission wavelength of 333 nm. The data were quantified using the area under the peak technique and external standards. Quantification was verified using calibration curves obtained from individual monoamine standards.

Table I. Primers used for polymerase chain reaction amplification.

Targets	Primer sequences	Size (bp)	Accession no. (GenBank)
DAT	F: 5'-tcaccaataactgctatagagacgc-3' R: 5'-gaagacgacgaagccagaggag-3'	83	NM_012694.2
TH	F: 5'-agcctgtgtactttgtgtccgaga-3' R: 5'-tgtgagggctgtccagtacgtc-3'	138	NM_012740.3
Nurr1	F: 5'-ccaatccggcaatgaccag-3' R: 5'-tgatgatctccatagagccagtcag-3'	129	NM_019328.3
VMAT2	F: 5'-actcttccaggagggcagtcac-3' R: 5'-tatgaatgggttagtgaggagctgg-3'	174	NM_013031.1
AADC	F: 5'-attccttcagatggcaactactc-3' R: 5'-gcagcaagatgtggttccta-3'	123	NM_001270853.1
MAO	F: 5'-ttcgccagccagtaggtaggat-3' R: 5'-attcaacacctctctagctgctcg-3'	116	NM_033653.1
COMT	F: 5'-ctgacttcctggcgtatgtgagag-3' R: 5'-gattgccttctccaagccgtc-3'	104	NM_012531.2
β-actin	F: 5'-ccgtaaagacctctatgccaaca-3' R: 5'-ctaggagccagggcagtaatctc-3'	102	NM_031144.2

F, forward; R, reverse; DAT, dopamine transporter; TH, tyrosine hydroxylase; Nurr1, orphan nuclear hormone; VMAT2, vesicular monoamine transporter 2; AADC, aromatic amino acid decarboxylase; MAO, monoamine oxidase; COMT, catechol-O-methyltransferase.

Statistical analysis. Experimental data were analyzed using SPSS statistical analysis software, version 18.0 (SPSS, Inc., Chicago, IL, USA) and expressed the data as mean ± standard error of the mean. Intergroup differences in body weight and food consumption were analyzed using one-way analysis of variance (ANOVA) and significant differences among groups were detected using Bonferroni's multiple comparison tests when significant differences among the groups were identified by ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Body weight and food consumption. No statistically significant difference in body weight or food consumption was detected among the groups during the 40 days of treatment (data not shown).

Changes in monoamine and main metabolite contents. The contents of levodopa (L-DOPA), DA, dihydroxy-phenyl-acetic acid (DOPAC) and homovanillic acid (HVA) in the striatum were measured following 40 days exposure to simazine and are presented in Fig. 1. The levels of L-DOPA (Fig. 1A), DOPAC (Fig. 1C) and HVA (Fig. 1D) exhibited a slight reduction in the simazine treatment groups compared with the control group; however, the difference was not significant ($P>0.05$). The levels of DA (Fig. 1B) in the simazine treatment groups were lower compared with that in the control, and the DA level in the 12.5 mg/kg group was decreased significantly compared with the control ($P<0.05$).

Changes in the levels of Nurr1, DAT, VMAT2 and TH mRNA following exposure to simazine. Fig. 2 shows the changes in the levels of Nurr1, DAT, VMAT2 and TH mRNA induced by exposure to simazine. The Nurr1 (Fig. 2A) mRNA levels of the simazine treatment groups were all lower compared with that of the control and the differences were statistically significant ($P<0.05$). In addition, the Nurr1 mRNA level of 50 mg/kg group was increased significantly compared with that of the 12.5 mg/kg group ($P<0.05$). The levels of TH mRNA (Fig. 2B) in the simazine treatment groups were all slightly lower compared with that of the control group, but the differences were not significant ($P>0.05$). DAT mRNA levels (Fig. 2C) of the simazine treatment groups were all significantly lower compared with that of the control group ($P<0.05$). The VMAT2 (Fig. 2D) mRNA levels of the 12.5 and 200 mg/kg groups were significantly lower compared with that of the control group ($P<0.05$). However, the DAT and VMAT2 mRNA levels of the 50 and 200 mg/kg groups were significantly increased compared with that of the 12.5 mg/kg group ($P<0.05$).

Changes in the levels of MAO, COMT and aromatic amino acid decarboxylase (AADC) mRNA induced by exposure to simazine. MAO (Fig. 3A) mRNA levels of the simazine treatment groups exhibited an upward trend, and the MAO mRNA level of the 12.5 mg/kg group was significantly decreased compared with that of the control group ($P<0.05$), while the levels of the 50 and 200 mg/kg groups were increased compared with that of the 12.5 mg/kg group and the differences were significant statistically ($P<0.05$). The COMT (Fig. 3B) mRNA levels of the simazine treatment groups also exhibited an uptrend, but only the level of the 500 mg/kg group was increased significantly compared with those of the control and 12.5 mg/kg groups ($P<0.05$). The AADC (Fig. 3C) levels of the 12.5 and 200 mg/kg groups

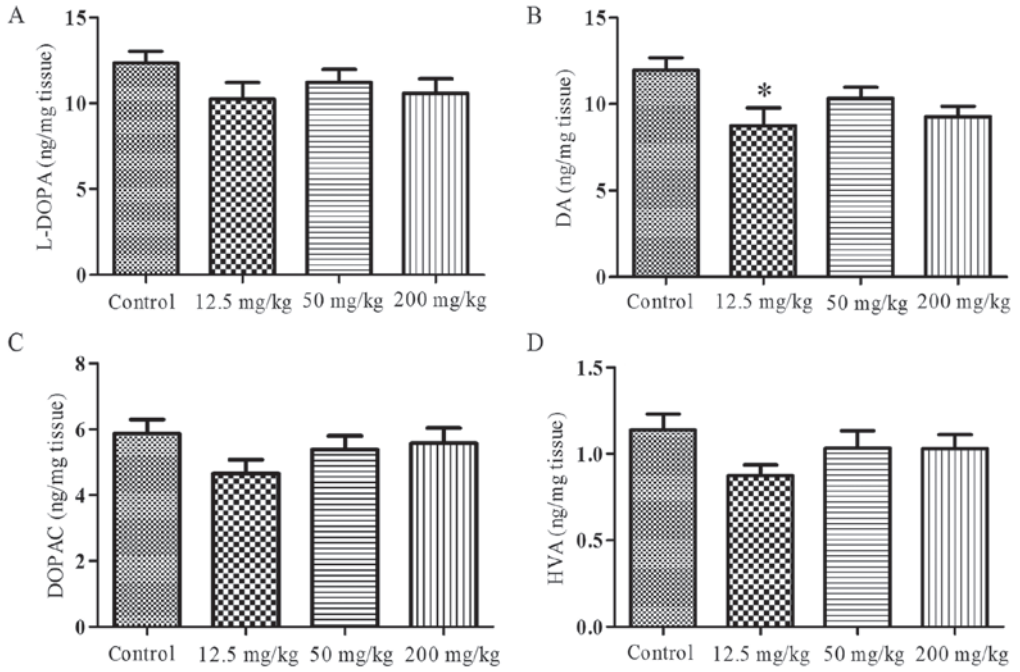

Figure 1. Analysis of the levels of (A) L-DOPA, (B) DA, (C) DOPAC and (D) HVA in the striatum after 40 days simazine treatment by high-performance liquid chromatography with a fluorescence detector. The x-axis shows the different dosages of simazine, and the y-axis shows the contents of monoamines and main metabolites. Data are presented as mean ± standard error of the mean (ng/mg). *P<0.05 vs. the control. (B) The DA content of the 12.5 mg/kg group was decreased significantly compared with the control (P<0.05). All monoamine and metabolite contents in the simazine treatment groups in (A-D) exhibited a tendency to decrease compared with the control. L-DOPA, levodopa; DA, dopamine; DOPAC, dihydroxy-phenyl-acetic acid; HVA, homovanillic acid.

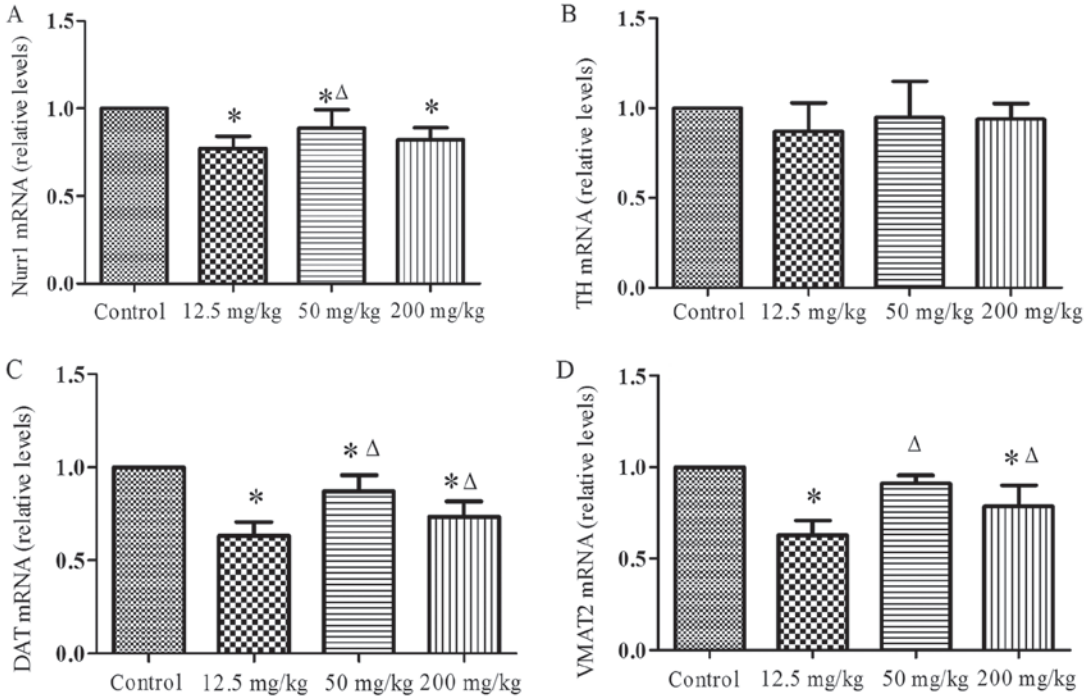

Figure 2. Analysis of the mRNA levels of (A) Nurr1, (B) TH, (C) DAT and (D) VMAT2 in the ventral midbrain exposed to simazine for 40 days by reverse transcription-quantitative polymerase chain reaction. The x-axis shows the different dosages of simazine, and the y-axis shows the mRNA levels. Data are presented as mean ± standard error of the mean. *P<0.05 vs. the control; ΔP<0.05 vs. the 12.5 mg/kg group. (A, C and D) The mRNA levels of the simazine treatment groups were all significantly decreased compared with the control group (P<0.05), with the exception of the 50 mg/kg group in (C). The mRNAs of all genes in the simazine treatment groups in (A-D) exhibited a tendency to decrease compared with the control. Nurr1, orphan nuclear hormone; TH, tyrosine hydroxylase; DAT, dopamine transporter; VMAT2, vesicular monoamine transporter 2.

were significant decreased compared with that of the control group (P<0.05), while the levels of the 50 and 200 mg/kg groups were increased compared with that of the 12.5 mg/kg group and the differences were statistically significant (P<0.05).

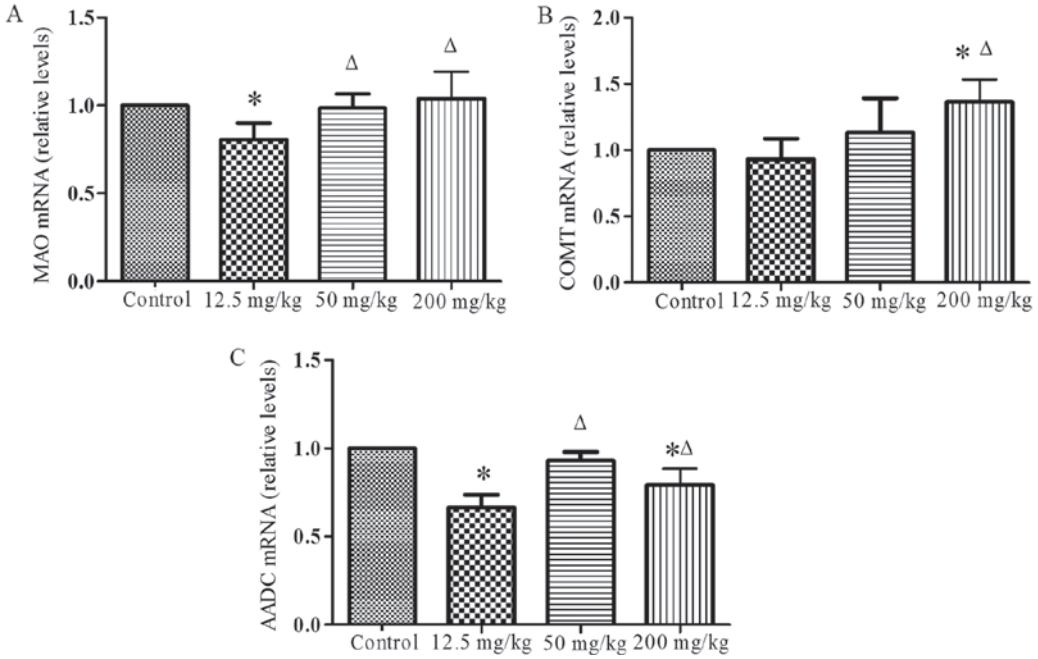

Figure 3. Analysis of the mRNA levels of (A) MAO, (B) COMT and (C) AADC in the ventral midbrain exposed to simazine for 40 days using reverse transcription-quantitative polymerase chain reaction. The x-axis shows the different dosages of simazine and the y-axis shows the mRNA levels. Data are presented as mean ± standard error of the mean. *P<0.05 vs. the control; ΔP<0.05 vs. the 12.5 mg/kg group. (A and B) The simazine treatment groups exhibited rising trends. MAO, monoamine oxidase; COMT, catechol-O-methyltransferase; AADC, aromatic amino acid decarboxylase.

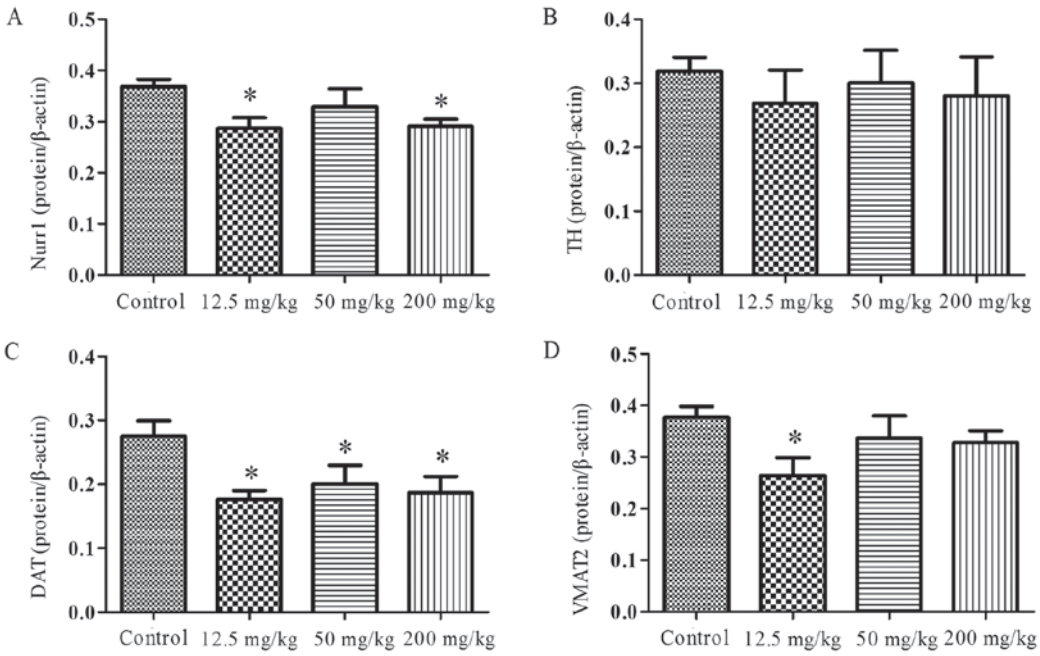

Figure 4. Analysis of the expression of (A) Nurr1, (B) TH, (C) DAT and (D) VMAT2 proteins in the ventral midbrain exposed to simazine for 40 days by western blotting. The x-axis shows the different dosages of simazine and the y-axis shows the protein levels. Data are presented as mean ± standard error of the mean. *P<0.05 vs. the control. In the four graphs, the protein levels of all simazine dose groups were lower than the control. Nurr1, orphan nuclear hormone; TH, tyrosine hydroxylase; DAT, dopamine transporter; VMAT2, vesicular monoamine transporter 2.

Changes in the expression levels of Nurr1, TH, DAT and VMAT2 protein induced by simazine. The expression levels of Nurr1, TH, DAT and VMAT2 proteins induced by exposure to simazine are shown in Fig. 4. The Nurr1 protein level (Fig. 4A) was decreased in all simazine treatment groups, but the levels in the 12.5 and 200 mg/kg groups were significantly different compared with that in the control group (P<0.05). The expression levels of TH protein (Fig. 4B) in all simazine treatment groups were slightly lower compared with that of the control group, but the differences were not significant (P>0.05). The expression levels of DAT protein (Fig. 4C) of all simazine treatment groups were lower compared with that of the control

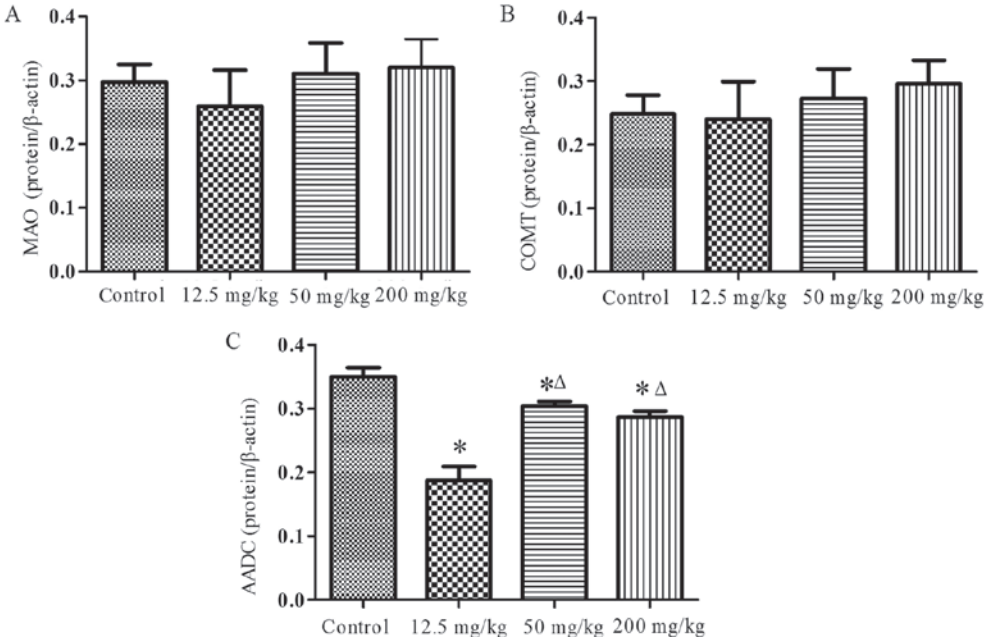

Figure 5. Analysis of the expression of (A) MAO, (B) COMT and (C) AADC proteins in the ventral midbrain exposed to simazine for 40 days by western blotting. The x-axis shows the different dosages of simazine and the y-axis shows the protein levels. Data are presented as mean ± standard error of the mean. *P<0.05 vs. the control; ΔP<0.05 vs. the 12.5 mg/kg group. The AADC protein level of all simazine treatment groups was lower than that of the control. (A and B) Protein levels of all simazine treatment groups exhibited a tendency to increase. MAO, monoamine oxidase; COMT, catechol-O-methyltransferase; AADC, aromatic amino acid decarboxylase.

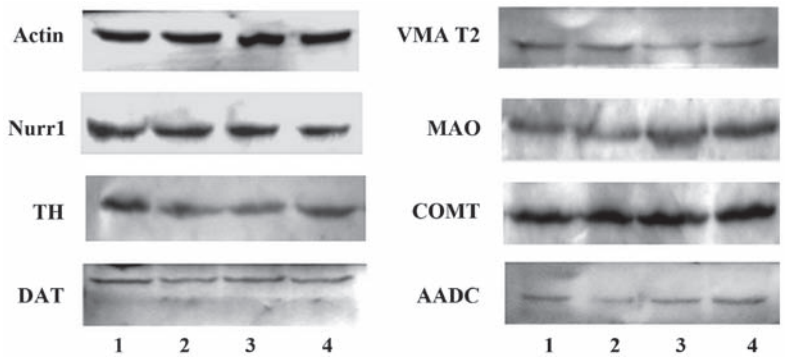

Figure 6. Representative images of protein expression detected via western blotting. Lane 1, control group; lane 2, 12.5 mg/kg simazine group; lane 3, 50 mg/kg simazine group; and lane 4, 200 mg/kg simazine group. Notably, lanes 2-4 for MAO and COMT appeared darker than lane 1. Nurr1, orphan nuclear hormone; TH, tyrosine hydroxylase; DAT, dopamine transporter; VMAT2, vesicular monoamine transporter 2; MAO, monoamine oxidase; COMT, catechol-O-methyltransferase.

and the differences were statistically significant (P<0.05). The VMAT2 protein level (Fig. 4D) was decreased in all dose groups; however, only the 12.5 mg/kg group exhibited a significant reduction in VMAT2 expression compared with the control group (P<0.05).

Changes in the expression levels of MAO, COMT and AADC protein induced by simazine. The expression levels of MAO, COMT and AADC protein induced by exposure to simazine are shown in Fig. 5. The MAO (Fig. 5A) and COMT (Fig. 5B) protein levels in all simazine treatment groups exhibited an upward trend, but the differences were not significant (P>0.05). The AADC (Fig. 5C) protein levels in all simazine treatment groups were lower compared with that in the control and the differences were statistically significant (P<0.05). In addition, the expression levels of AADC in the 50 and 200 mg/kg groups were increased significantly compared with that in the 12.5 mg/kg group (P<0.05). Representative western blotting results for the MAO, COMT and AADC proteins are presented in Fig. 6.

Discussion

Due to its widespread use, simazine has been detected in samples of soil and ground water and the toxicity of simazine to mammals urgently requires investigation. The present study attempted to research the effects of simazine on the metabolism of dopaminergic neurons. DA synthesis and transfer disorders may lead to the onset of PD, Alzheimer's disease or other common neurological disorders (21-23). Since puberty is a crucial period of dopaminergic neuron development (24), 60 male SD rats were treated with different doses of simazine throughout puberty in order to detect changes of

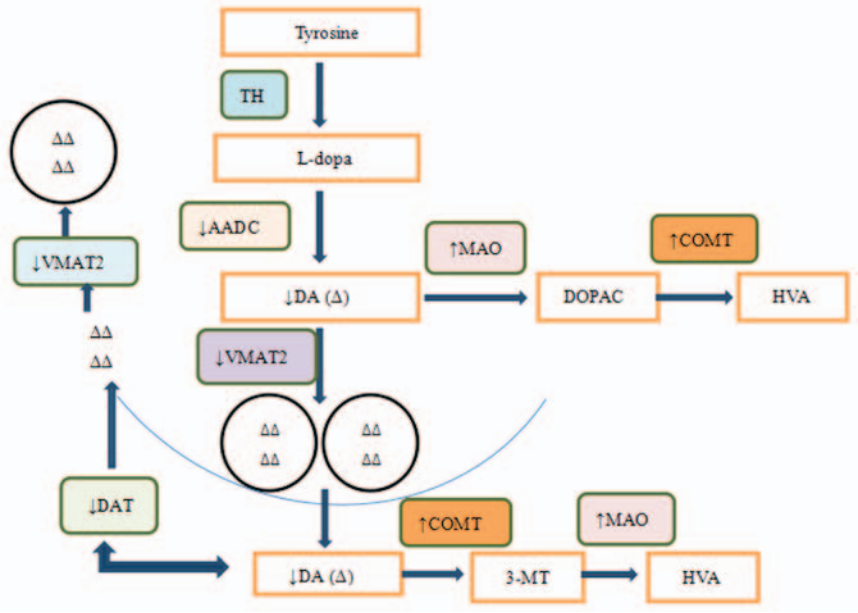

Figure 7. Effects on DA synthesis and metabolism induced by exposed to simazine. Δ represents a certain amount of DA; ↑ indicates upregulation; ↓ indicates downregulation; the black circles which contain dopamine indicate vesicles. Analysis indicated that MAO and COMT were upregulated and other main genes in the pathway were downregulated. These changes affected the content of DA in the midbrain. AADC, aromatic amino acid decarboxylase; COMT, catechol-O-methyltransferase; DA, dopamine; DAT, dopamine transporter; DOPAC, dihydroxy-phenyl-acetic acid; HVA, homovanillic acid; MAO, monoamine oxidase; 3-MT, 3-methoxytyramine; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter 2.

the main factors involved in the process of DA synthesis and metabolism.

The process of DA metabolism in the brain includes synthesis, storage, release, reuptake and inactivation. The tyrosine in catecholamine neurons is converted to L-DOPA under the catalytic action of TH. The L-DOPA is then converted to DA by AADC (25). Following the synthesis of DA in dopaminergic neurons, DA is transported to and stored in vesicles by VMAT2. When an action potential reaches a presynaptic terminal, DA is released to the synaptic cleft and functions through binding with the postsynaptic receptors. Reuptake of DA includes two steps. In the first step, DA is transported from the synaptic cleft back into the presynaptic membranes by DAT. In the second step, DA is stored in vesicles by VMAT2 (26,27). DA in the synaptic cleft is transformed to the final product, HVA, under the enzymolysis of COMT and MAO, while intracellular DA is transformed to DOPAC by MAO (Fig. 6). In normal circumstances, each factor acts to maintain the steady-state condition of the DA system. When any of the steps or factors is affected, it may disrupt the balance of the DA system and cause a series of physiological effects (28,29).

TH is a key rate-limiting enzyme, and TH^+ neurons are able to produce either L-DOPA or DA in target areas of ventral midbrain dopaminergic neurons. In addition, Keber *et al* (29) suggested that striatal TH^+ cells may synthesize DA cooperatively and subsequently contribute to supraphysiological concentrations of synaptic DA. In the present study, the mRNA and protein expression levels of TH in the ventral mesencephalon were detected following exposure to simazine for 40 days during the prepubertal period, and the results demonstrated that TH exhibited no significant changes. Since TH is required for the synthesis of L-DOPA, the content of DA is not likely to change via this mechanism. Expression of the TH gene may involve interaction with numerous other genes, particularly Nurr1, and previously reported data indicate that mRNA and protein expression of TH exhibited a high consistency with that of Nurr1 (30,31).

Nurr1, which is crucial for homeostasis and the development of DA neurons, is an essential transcription factor for the differentiation, maturation and maintenance of midbrain dopaminergic neurons (32,33). Disruption of Nurr1 function contributes to dopaminergic neuron dysfunction as indicated by considerable clinical and experimental data (34-37). Studies have shown that while the midbrain structure is complete in Nurr1-deficient mice, these mice have a lack of dopaminergic neurons and the expression of certain genes, including AADC, VMAT and DAT, which are associated with dopaminergic synthesis, transport, storage and release (35,38,39). Therefore, during dopaminergic neuron development, Nurr1 influences the expression of a number of other genes, including TH, DAT and VMAT2 (40). In the present study, simazine-induced reductions in Nurr1 mRNA and protein expression in the ventral mesencephalon were observed in all dose groups. The reduction of Nurr1 gene expression influences the expression of DAT and VMAT2, and affects the content of DA as a result (41,42).

DAT is synthesized and expressed by the soma, dendrites and axons of dopaminergic neurons and is distributed on the membranes of dendrites and axons. The main function of DAT is to take up DA in the synaptic cleft. Due to its specificity for DA, the content of DAT reflects dopaminergic system function. Therefore, numerous studies have evaluated the presynaptic function of dopaminergic neurons via DAT, and some have reported the complex regulatory effects of DAT and its effects on the regulation of other proteins (43-47). The main function of VMAT2 is to store DA in vesicles. The inhibition of VMAT2 contributes to dopaminergic neuron death

and recent evidence suggests that the vesicular storage of DA may contribute to the demise of the nigral neurons in PD (49). Therefore, VMAT2 serves a key role in the storage of DA in vesicles to avoid its degradation by MAO. Approximately 80% of monoamine neurotransmitters are reabsorbed by nerve terminals and reuptake is the main method of suspending the physiological effects of monoamine neurotransmitters; therefore, DAT and VMAT2 are crucial (48-50). The present study demonstrated that exposure to simazine during the prepubertal period decreased DAT and VMAT2 expression in the ventral mesencephalon. A previous study revealed that the mRNA and protein levels of DAT in the blood leukocytes of patients with PD were significantly reduced (51). Lower expression levels of DAT and VMAT2 is likely to reduce the transport and storage capacity of DA and inhibit the reuptake of DA into vesicles. This may increase the accumulation of DA in the cytoplasm and induce oxidative stress and toxicity leading to the death of dopaminergic neurons. Thus, it may be speculated that affecting the transport and reuptake of DA by influencing Nurr1, DAT and VMAT2 expression is one pathway by which the content and activity of DA are decreased by simazine.

AADC is a homodimeric pyridoxal phosphate-dependent enzyme responsible for the synthesis of DA. AADC converts L-DOPA to DA and is considered the rate-limiting enzyme for the synthesis of biogenic amines. Studies have shown that AADC deficiency is a rare pediatric neuro-metabolic disease in children and indicate that defects in the AADC gene result in neurotransmitter deficiencies (52-56). COMT and MAO are important DA-degrading enzymes. The COMT gene has attracted strong neuroscientific interest due to its implication in dopaminergic neurotransmission (57). These two genes have been observed to have an association with cognitive function (58,59). MAO and COMT inhibitors are the present optimal PD treatments and they maintain the monoamine balance (60). The effects of COMT and MAO on DA differ according to their site of action (Fig. 7). In the present study, it was observed that the expression of COMT and MAO mRNA tended to increase following exposure to simazine, and the expression levels in the 50 and 200 mg/kg groups for the MAO gene, and the 200 mg/kg group for the COMT gene were significantly higher compared with those of the 12.5 mg/kg group. However, no significant difference in the corresponding protein expression was observed. The mRNA and protein expression of AADC in the simazine treatment groups was lower than that in the control group, but exhibited a rising trend as the simazine dose increased. The reduced expression of AADC is likely to lead to a reduction in the concentration of DA, while the increasing levels of COMT and MAO would accelerate the degradation of DA, but cause no significant changes in its metabolic products, DOPAC and HVA. Therefore, it may be speculated that affecting the synthesis and metabolism of DA by influencing AADC, MAO and COMT expression is another pathway involved in the reduction of the content and activity of DA by simazine.

In conclusion, the present study observed the neurotoxic effect of simazine on the dopaminergic metabolism system. Simazine affected the synthesis, transport and metabolism of DA and led to dysfunction in the natural balance of the brain dopaminergic system. One possible underlying mechanism is a reduction in the levels of Nurr1, DAT and VMAT2 impacting upon the transport of DA; another is the decreased level of AADC and increased levels of MAO and COMT impacting upon the synthesis and metabolism of DA. The above factors resulted in a reduction in the level of DA and may lead to dopaminergic system-associated neurological disorders. The understanding of the neurotoxicity of simazine remains incomplete and further research is required to elucidate it further.

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