Effect of Wnt1 and Wnt5a on the development of dopaminergic neurons, and toxicity induced by combined exposure to paraquat and maneb during gestation and lactation

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Abstract. Paraquat (PQ) and maneb (MB) are widely used herbicides. Wingless (Wnt) proteins serve a role in the development and differentiation of dopaminergic neurons. Previous studies demonstrated that combined exposure to PQ and MB damages dopaminergic neurons in the midbrain. Effects of PQ and MB exposure on midbrain Wnt proteins have also been previously reported. In the present study, from the 5th day of gestation to weaning of the offspring, pregnant Sprague-Dawley rats were administrated saline, or PQ and MB at two different doses: high, 15 mg/kg body weight PQ + 45 mg/kg body weight MB; or low, 10 mg/kg body weight PQ + 30 mg/kg body weight MB. Dopamine content in the striatum was examined by high performance liquid chromatography with a fluorescence detector and mRNA and protein expression of Wnt1, Wnt5a, nuclear receptor related factor 1 (Nurr1) and tyrosine hydroxylase (TH) in the midbrain was examined by reverse transcription-quantitative polymerase chain reaction and western blotting. Combined exposure to PQ and MB during development decreased mRNA and protein expression of Wnt1, TH and Nurr1 and increased expression of Wnt5a in the offspring.

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Abbreviations: PQ, paraquat; MB, maneb; TH, tyrosine hydroxylase; Wnt1, wingless 1; Wnt5a, wingless 5a; PD, Parkinson's disease; HPLC-FL, high performance liquid chromatography with a fluorescence detector; DA, dopamine; Nurr1, nuclear receptor related factor 1; MPP^+, 1-methyl-4-phenylpyridinium

Key words: Parkinson's disease, paraquat, maneb, neurotoxicity, Wnt1, Wnt5a

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder with high incidence among the elderly (1,2). Symptoms of PD include motor defects and other non-motor disabilities. The main motor impairments are resting tremor, postural instability and bradykinesia. Non-motor disabilities include depression, anxiety, cognitive impairment and sleep disorders (3). One of the symptoms of PD is pathological selective degeneration of dopaminergic neurons (4).

Epidemiological studies demonstrated that exposure to pesticides increases the risk of PD (5). Paraquat (PQ) is an organic herbicide that can affect energy metabolism by inhibiting mitochondrial respiratory chain reactions (6). PQ can control certain intracellular signaling cascades (7). Maneb (MB) is a fungicide widely used in crop production that can influence dopamine (DA) homeostasis and mitochondrial function. It has been proposed that PQ, in the presence of MB, alters the DA metabolism by enhancing the exposure of neurons to reactive oxygen species and reactive nitrogen clusters. A previous report, using rats as model organisms, demonstrated that combined exposure to PQ and MB is more toxic compared with exposure to PQ alone (8). MB can increase the content of PQ in the brain by changing the metabolic dynamics of PQ and therefore converting non-toxic PQ into a toxic form (9).

PQ and MB are lethal pesticides frequently used in combination for crop production in China, including potatoes, tomatoes and other vegetables. Residual PQ and MB are frequently detected in China (10,11).

The wingless (Wnt) protein family primarily includes secreted factors (12). Wnt1 and Wnt5a serve a role in the regulation of proliferation and differentiation of midbrain dopaminergic neurons (13). It has been demonstrated that Wnt1 and Wnt5a can interact to regulate the homeostasis of the nuclear receptor related factor 1 (Nurr1), and that Nurr1+ precursors promote formation of dopaminergic neurons in the midbrain (14,15).

Previous research has indicated that exposure to environmental toxins can cause permanent damages to biochemical homeostasis and behavior of adult rats (16). The present study investigated the hypothesis that combined exposure to PQ and

MB during the gestation and lactation period of rats can lead to alterations in Wnt1 and Wnt5a.

Materials and methods

Chemicals and reagents. PQ was purchased from J&K Chemical Technology, Inc. (Beijing, China) and MB was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany; both 98% pure). Rabbit anti-Wnt1 (cat. no. ab85060; 1:1,000), anti-Nurr1 (cat. no. ab9332; 1:1,000), anti-Tyrosine hydroxylase (TH; cat. no. ab6221; 1:1,000) and anti-actin (cat. no. ab179467; 1:1,000) antibodies were purchased from Abcam (Cambridge, UK). An anti-Wnt5a antibody (cat. no. 55184-1-AP; 1:1,000) was purchased from Wuhan Sanying Biotechnology (Wuhan, China), and the goat anti-rabbit IgG antibody (cat. no. ZB-2305; 1:5,000) was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

Animals and treatments. The present study was approved by the Ethical Committee of the Harbin Medical University (Harbin, China). A total of 40 virgin female and 20 male Sprague-Dawley rats were purchased from Vital River Laboratories (Beijing, China). The animals were reared for1 week in barrier facilities and then female and male rats were mated at 2:1 proportion. Vaginal smear was examined the next day and when sperm was identified, rats were categorized as day 0 subjects of the study. Pregnant rats were randomly divided into three groups: i) The saline treatment group, 1 mg/kg body weight saline, (n=10); ii) the high dose treatment group, 15 mg/kg body weight PQ + 45 mg/kg body weight MB, (n=15); and iii) the low dose treatment group, 10 mg/kg body weight PQ + 30 mg/kg body weight MB, (n=15). Administration of saline, or PQ and MB was performed by gavaging twice a week from gestation to weaning. Rats were sacrificed following weaning.

Body weight and food consumption. Virgin female (230±20 g) and male Sprague-Dawley rats (310±20 g) used in the present study were 8 weeks old. The animals were provided water and food *ad libitum*. The animal cages were maintained at a constant 12 h light/dark cycle, temperature 22±2˚C and relative humidity at 50±15%. There was no significant difference in body weight and food consumption among females and offspring once a week during the experiment (data not shown).

High performance liquid chromatography with a fluorescence detector (HPLC-FL) determination of DA. The DA content was detected by HPLC-FL. The corpus striatum of the rats in each group were homogenized in 0.1 M perchloric acid and centrifuged at 2,000 x g for 20 min at 4˚C. The supernatant was then filtered through a 0.2 μm cellulose membrane. The following chromatography conditions were used: 45x150 mm VERIANODSCI8 column (5 μm; Nacalai Tesque, Inc., Kyoto, Japan); The mobile phase was composed of 20 mM trisodium citrate and 50 mM sodium hydrogen phosphate. The following chromatography conditions were used: Temperature, 35˚C; flow rate, 1.0 ml/min. A fluorescence detector (Agilent Technologies, Inc., Santa Clara, CA, USA) 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 peaks and external standards. The quantification was verified using calibration curves obtained from individual monoamine standards.

Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The midbrain tissue was dissolved in TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) reagent. Total RNA was extracted from the treated midbrain tissue according to the manufacturer's protocol. Primers for rat Wnt1, Wnt5a, Nurr1 and TH were designed using the Primer Premier software (version, 5.0; Premier Biosoft Internetional, Palo Alto, CA, USA; Table I), based on Gene Bank sequences of these genes. Synthesis of cDNA was performed using PrimeScript RT reagent kit and gDNA Eraser (both from Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. qPCR reactions were performed using SYBR® Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd.) in 20 μl reactions containing 2 μg cDNA template and 10 μM forward and reverse primers in an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 30 sec; 35 cycles of 94˚C for 5 sec; 57.4˚C for 20 sec and 72˚C for 20 sec; and final extension at 72˚C for 1 min. The threshold cycle was determined and the results are expressed as relative expression ratio. The relative expression ratio of a target gene was calculated as previously described (17).

Western blotting. Western blot analysis was used to quantify the protein amount of Wnt1, Wnt5a, Nurr1 and TH. Midbrain tissue was lysed in Radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) for 2 h on ice. The protein concentrations in the supernatants were determined with a Bicinchoninic Acid protein assay kit (Beyotime Institute of Biotechnology). Protein samples, 4 μl each protein per lane, were separated on 10% SDS-PAGE gels (80 V, 20 min). Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked in 5% skimmed dry milk in Tris-buffered saline (TBS) at room temperature for 30 min. Membranes were then incubated with primary antibodies at 4˚C overnight. The following day, PVDF membranes were washed with TBS containing Tween-20 four times for 5, 10, 10 and 15 min. PVDF membranes were then incubated with a goat anti-rat horseradish peroxidase secondary antibody at room temperature for 60 min. The PVDF membranes were then washed with TBS containing Tween-20 four times as previously. Antibody binding was detected using an enhanced chemiluminescence system (Tanon Science and Technology Co., Ltd., Shanghai, China). Density of the specific protein bands was standardized to β-actin with the Image J software (version 1.5; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. Data were analyzed using the SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). The difference between groups was analyzed using analysis of one way analysis of variance and Bonferroni's multiple comparison was

Table I. Primer sequences for the reverse transcription-quantitative polymerase chain reaction.

Target	Forward 5'→3'	Reverse 5'→3'	Length (bp)	Annealing temperature (˚C)
Wnt1	TTTTCTCTCCGTGTCCCT	GCTCCCCAACCTTATTTC	227	60
Wnt5a	GACTTACCTCGGGACTGG	GACCTGCTTCATTGTTGTG	166	58
Nurr1	CCAATCCGGCAATGACCAG	TGATGATCTCCATAGAGCCAGTCAG	129	60
TH	AGCTGTGCAGCCCTACCAAGA	GTGTGTACGGGTCAAACTTCACAGA	140	62
β-actin	GGAAATCGTGCGTGACATTAAAG	CGGCAGTGGCCATCTCTT	74	60

Wnt, wingless; Nurr1, nuclear receptor related hormone 1; TH, tyrosine hydroxylase.

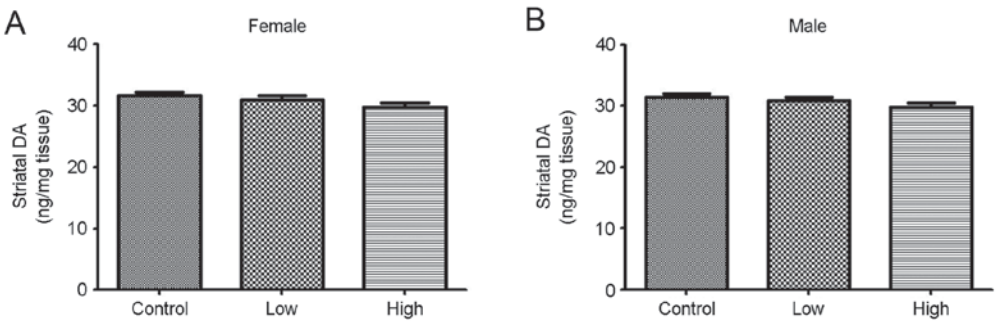

Figure 1. Effect of exposure to PQ and MB on striatal DA levels for (A) female and (B) male during gestation and lactation. Data are presented as the mean ± standard deviation. PQ, paraquat; MB, maneb; DA, dopamine.

used as a post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of combined exposure to PQ and MB during development on DA levels. DA levels were measured following combined exposure to PQ and MB during gestation and lactation periods. The DA levels decreased upon exposure to PQ and MB, but the differences were not statistically significant (Fig. 1).

Effects of combined exposure to PQ and MB during development on Wnt1 mRNA and protein expression levels. The effects of combined exposure to PQ and MB during development on midbrain Wnt1 protein and mRNA expression levels were studied in female and male offspring. mRNA and protein expression levels of Wnt1 decreased significantly upon combined exposure to PQ and MB at both high and low doses, compared with the unexposed control group. Among female offspring, mRNA expression levels decreased by 77.40 and 81.02%, and protein expression levels decreased by 21.69 and 32.75% in the low and high dose groups, respectively. Among male offspring, mRNA expression levels decreased by 74.00 and 87.62%, and protein expression levels decreased by 28.23 and 33.00% in the low and high dose groups, respectively (Fig. 2).

Effects of developmental exposure to PQ and MB on Wnt5a mRNA and protein expression levels. The effect of combined exposure to PQ and MB during development on Wnt5a protein and mRNA expression levels in the midbrain, was investigated. mRNA and protein levels of Wnt5a were significantly increased following exposure to a high dose of PQ and MB, compared with the control group (all, P<0.05). Only protein expression of Wnt5a in both males and females was significantly increased upon exposure to low doses of PQ and MB, compared with the control group (P<0.05). Among female offspring, mRNA expression levels increased by 18.94 and 86.68% and protein expression levels increased by 22.67 and 66.94%, in the low and high dose groups, respectively. Among male offspring, mRNA expression levels increased by 30.61 and 117.03% and protein expression levels increased by 70.70 and 95.87%, in the low and high dose groups, respectively (Fig. 3).

Effects of developmental exposure to PQ and MB on Nurr1 mRNA and protein expression levels. As presented in Fig. 4, the effect of combined exposure to PQ and MB during development on midbrain Nurr1 protein and mRNA expression levels was studied in female and male offspring. mRNA and protein expression levels of Nurr1 were significantly decreased following exposure to high doses of PQ and MB, compared with the control group (P<0.05). Among female offspring, mRNA expression levels decreased by 32.29 and 49.84%, and protein expression levels decreased by 12.93 and 23.90%, in the low and high dose groups, respectively. Among male offspring, mRNA expression levels decreased by 55.57 and 62.50%, and protein expression levels decreased by 16.55 and 28.47%, in the low and high dose groups, respectively.

Effects of exposure to PQ and MB during development on TH mRNA and protein expression levels. The effects of

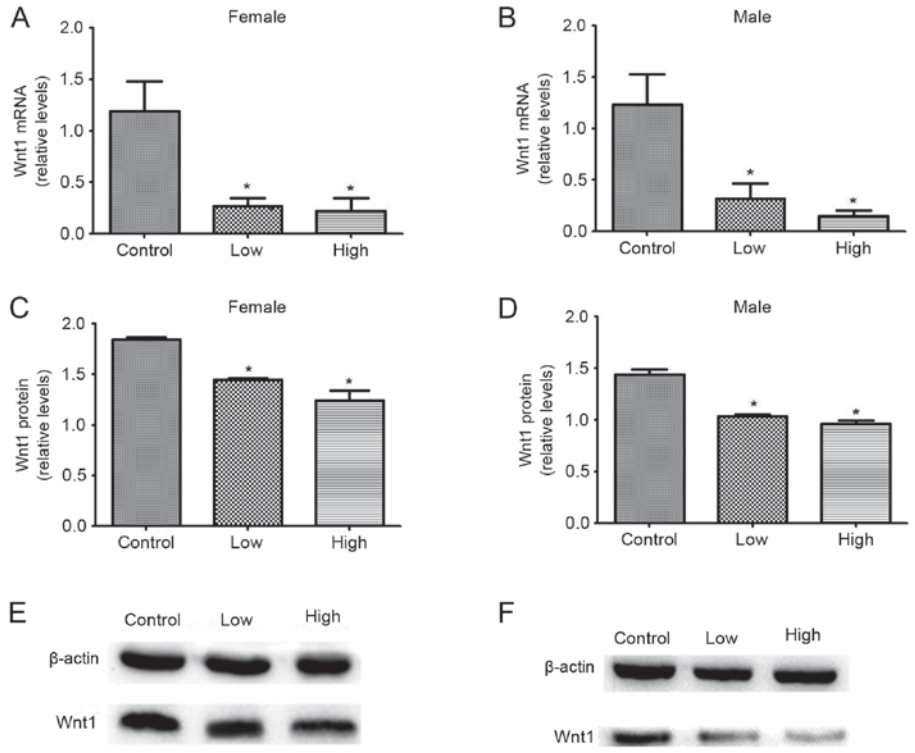

Figure 2. Wnt1 mRNA and protein expression levels in midbrain of offspring following combined exposure to PQ and MB during gestation and lactation. Alterations in Wnt1 mRNA levels in (A) female and (B) male offspring. Alterations in Wnt1 protein levels in (C) female and (D) male offspring. Western blot presenting relative expression of Wnt1 in (E) females and (F) males. Data are presented as the mean ± standard deviation. *P<0.05 vs. control. Wnt1, wingless; PQ, paraquat; MB, maneb; high, 15 mg/kg body weight PQ + 45 mg/kg body weight MB; low, 10 mg/kg body weight PQ + 30 mg/kg body weight MB.

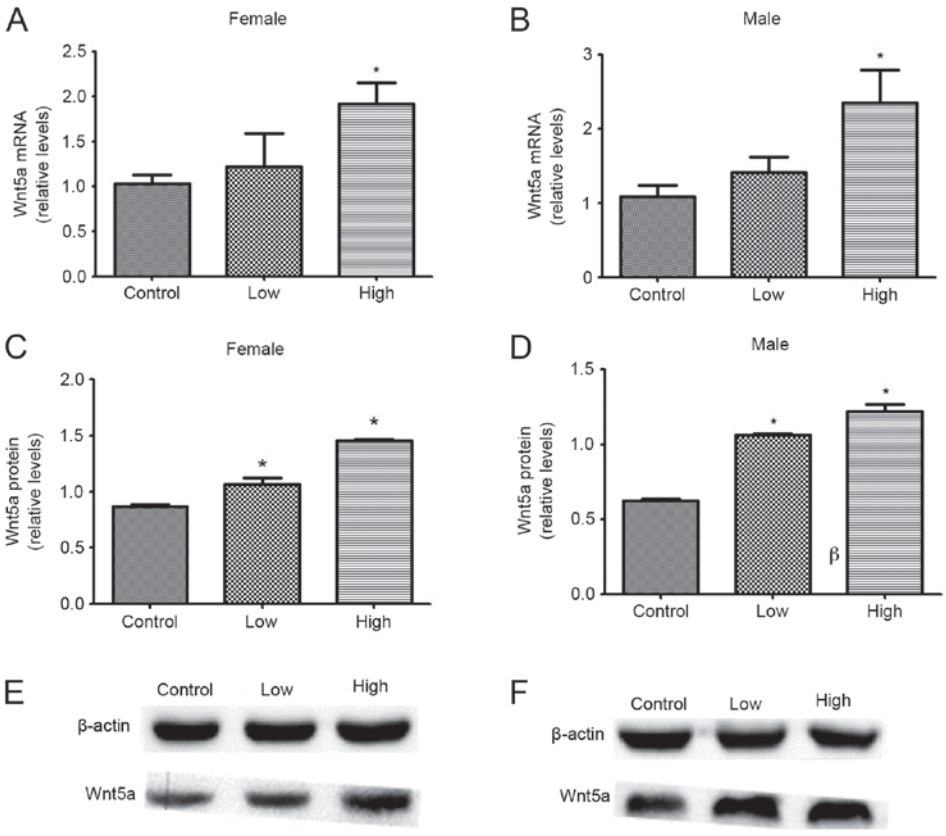

Figure 3. Wnt5A mRNA and protein expression levels in midbrain of offspring following combined exposure to PQ and MB during gestation and lactation. Alterations in Wnt5A mRNA levels in (A) female and (B) male offspring. Alterations in Wnt5A protein levels in (C) female and (D) male offspring. Western blot presenting relative expression of Wnt5A in (E) females and (F) males. Data are presented as the mean ± standard deviation. *P<0.05 vs. control. Wnt5A, wingless 5A; PQ, paraquat; MB, maneb; high, 15 mg/kg body weight PQ + 45 mg/kg body weight MB; low, 10 mg/kg body weight PQ + 30 mg/kg body weight MB.

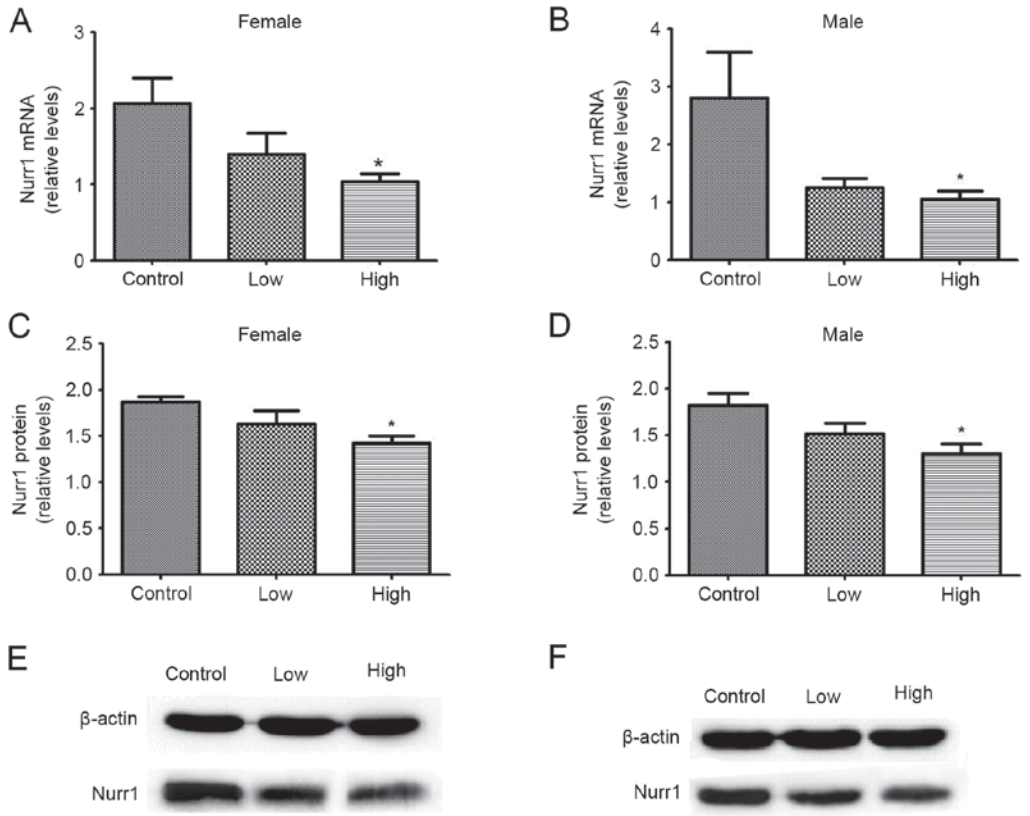

Figure 4. Nurr1 mRNA and protein expression levels in midbrain of offspring following combined exposure to PQ and MB during gestation and lactation. Alterations in Nurr1 mRNA levels in (A) female and (B) male offspring. Alterations in Nurr1 protein levels in (C) female and (D) male offspring. Western blot presenting relative expression of Nurr1 in (E) females and (F) males. Data are presented as the mean ± standard deviation. *P<0.05 vs. control. Nurr1, nuclear receptor related hormone 1; PQ, paraquat; MB, maneb; high, 15 mg/kg body weight PQ + 45 mg/kg body weight MB; low, 10 mg/kg body weight PQ + 30 mg/kg body weight MB.

combined exposure to PQ and MB during development on midbrain TH protein and mRNA expression levels in female and male offspring are summarized in Fig. 5. mRNA and protein expression levels of TH were significantly decreased following exposure to PQ and MB compared with the control group, but only when administrered at a high dose (P<0.05). Among female offspring, mRNA levels decreased by 19.90 and 52.43% and protein levels decreased by 13.68 and 28.79%, in the low and high dose groups, respectively. Among male offspring, mRNA expression levels decreased by 14.21 and 48.14% and protein expression levels decreased by 7.86 and 33.35%, in the low and high dose groups, respectively.

Discussion

PQ can cross the brain blood barrier directly as it has a similar structure to the toxic 1-methyl-4-phenylpyridinium (MPP^+), but the process is less efficient. MPP^+ can be bound by a transport protein of the dopaminergic neurons and transported to mitochondria. This process promotes excessive free oxygen radical secretion and induction of oxidative stress responses, which inhibit the activity of mitochondrial respiratory chain complex I and the synthesis of ATP, leading to denaturation and death of dopaminergic neurons (18). A number of studies investigated the effects of PQ on the Wnt signaling pathway. L'Episcopo *et al* (19) demonstrated that exposure to MPP^+ decreased protein expression of β-catenin and led to overexpression of phosphorylated glycogen synthase kinase 3β in mice, affecting the Wnt signaling pathway. According to the results obtained by Gollamudi *et al* (20), combined treatment with PQ and MB affects Wnt pathways more significantly compared with the effect of PQ alone. MB selectively inhibits mitochondrial complex III which may lead to a reduction in DA release, and therefore an increase in DA concentration in synaptic vesicles (21). Therefore, combined treatment with MB and PQ can induce oxidative stress, and dopaminergic neurons can be more susceptible to injury caused by the oxidative stress (22). PQ can induce the injury of PC-12 cells in dopaminergic neurons through Wnt signaling pathways (23). Investigation of the combined effect of PQ and MB can have toxicological implications.

Previous studies have demonstrated that PQ and MB can permeate into embryos through the placental barrier during the early stage of brain development, affecting the development of dopaminergic neurons (24). Wnt1 and Wnt5a are secreted glycoproteins that serve a role on the formation and differentiation of midbrain dopaminergic neurons (25). Wnt1 activates the Wnt signaling pathway to promote the formation of midbrain dopaminergic neurons (26). Knockdown of Wnt1 impairs proliferation of dopaminergic precursors and leads to the death of dopaminergic neurons in the midbrain (27). Wnt5a promotes differentiation of neural cells into dopaminergic precursors (28). The knockdown of Wnt5a leads to enhanced proliferation of progenitor cells (29).

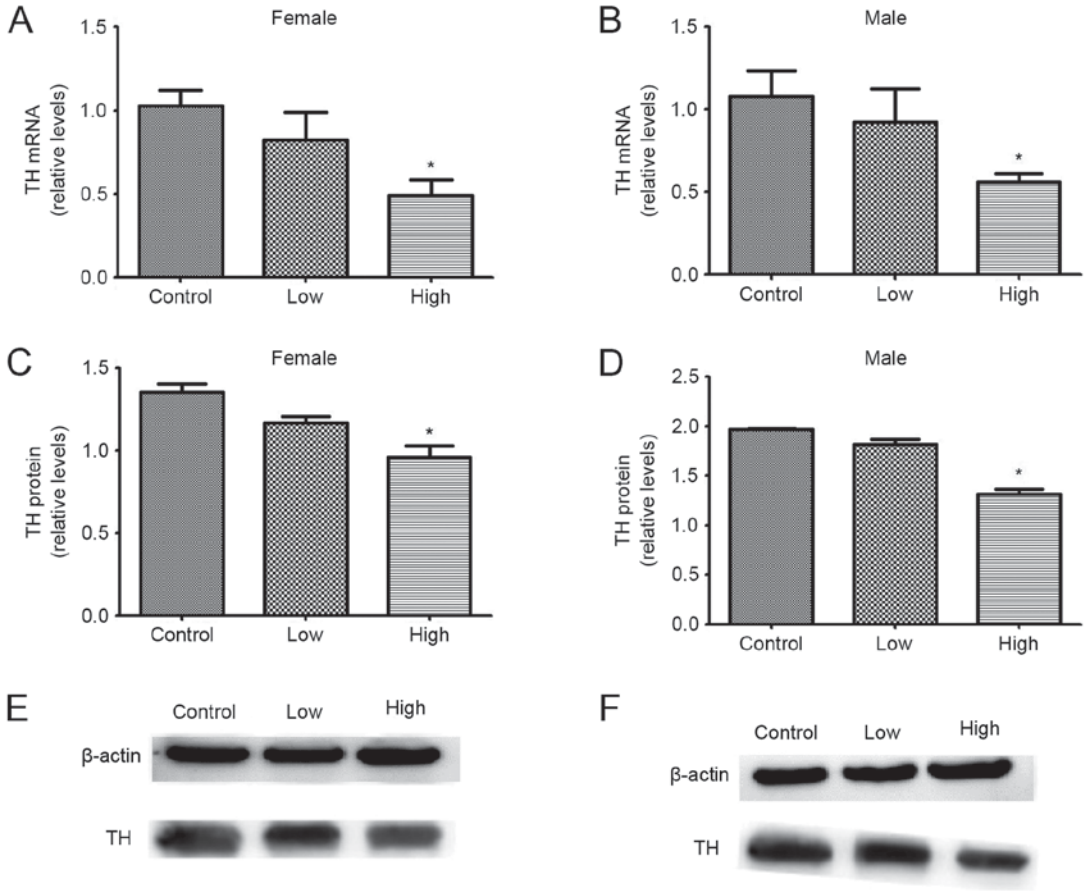

Figure 5. TH mRNA and protein expression levels in midbrain of offspring following combined exposure to PQ and MB during gestation and lactation. Alterations in TH mRNA levels in (A) female and (B) male offspring. Alterations in TH protein levels in (C) female and (D) male offspring. Western blot presenting relative expression of TH in (E) females and (F) males. Data are presented as the mean ± standard deviation. *P<0.05 vs. control. TH, tyrosine hydroxylase; PQ, paraquat; MB, maneb; high, 15 mg/kg body weight PQ + 45 mg/kg body weight MB; low, 10 mg/kg body weight PQ + 30 mg/kg body weight MB.

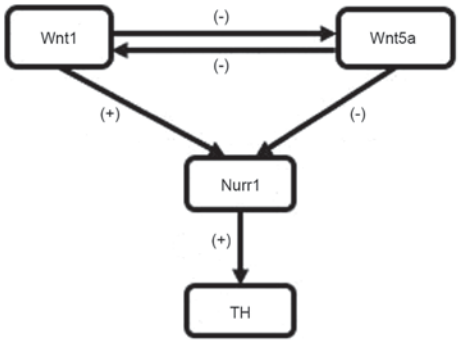

Figure 6. Effects of exposure to paraquat and maneb on Wnt proteins, Nurr1 and TH. Wnt, wingless; Nurr1, nuclear receptor related hormone 1; TH, tyrosine hydroxylase; (-), downregulated; (+), upregulated.

In the present study, pregnant rats were exposed to PQ and MB from the 5th day of gestation to weaning, a period during which Wnt proteins begin to be expressed (30). In the present study, it has been observed that exposure to PQ and MB during gestation and lactation leads to reduced and increased expression of Wnt1 and Wnt5a mRNA and protein expression, respectively, in both female and male offspring. Wnt1 and Wnt5a coordinate to promote the formation and proliferation of midbrain dopaminergic precursors (31).

Nurr1 is expressed in the central nervous system and promotes the differentiation of neural precursors into dopaminergic neurons (32-34). Nurr1 expression begins at day 10 of embryo development, reaches peak 1-2 days following birth, and then gradually decreases, but its expression remains high in the midbrain (35). Knock out of Nurr1 during embryonic development can result in incomplete development of dopaminergic neurons (36). In the present study, exposure to PQ and MB during gestation and lactation led to reduced expression of Nurr1 mRNA and protein expression, in both female and male offspring.

Previous studies have demonstrated that Nurr1 directly regulates the promoter of TH gene (37). Upon activation, Nurr1 translocates to the nucleus, where it binds to the promoter region and activates the transcription of TH (38). TH is a marker for dopaminergic neurons and a rate limiting enzyme in the synthesis of dopamine (39-41). TH is only expressed in mature dopaminergic neurons, when enzymes and transporters specific for dopaminergic neurons are fully expressed and functional. Combined exposure to PQ and MB during gestation and lactation reduced TH mRNA and protein expression in both female and male offspring.

The effects of combined exposure to PQ and MB on DA levels in the striatum of offspring were investigated in the present study. DA levels in the striatum of offspring were not significantly decreased, even though TH expression was

downregulated. It has been previously hypothesized that in order to maintain homeostasis, the synthesis, release, degradation and re-uptake of DA are regulated by multiple mechanisms (42). A plausible explanation of the results of the present study is that the transportation and re-uptake of DA were altered in order to compensate for the lack of DA synthesis and to maintain the function of the DA system. In theory, it is possible that the DA content in the striatum decreases with age and this decrease can cause symptoms of PD.

In conclusion, the present study demonstrated that combined exposure to PQ and MB during gestation and lactation alters the expression of proteins associated with the formation and development of dopaminergic neurons in offspring. It has been demonstrated that exposure to PQ and MB during gestation and lactation leads to upregulation of Wnt5a and down-regulation of Wnt1, Nurr1 and TH (Fig. 6). Future studies should investigate whether exposure to PQ and MB during gestation and lactation followed by a subsequent re-exposure during adulthood would enhance susceptibility of dopaminergic neurons to environmental risk factors.

Acknowledgements

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