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Protective effect of mulberry crude extract against nonylphenol-induced thyroid disruption by inhibiting the activity of deiodinase in rats

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ABSTRACT

Nonylphenol (NP) is an endocrine-disrupting chemical (EDC) that can lead to thyroid disruption. We explored NP-induced toxicity in the rat thyroid and evaluated the mitigating effects of mulberry crude extract (MCE) on NP toxicity. First, we aimed to evaluate NP-induced thyroid disruption by dosing Sprague–Dawley (SD) rats with NP (0, 30, 90, or 270 mg/kg body weight) daily for 28 days. Second, we aimed to determine whether MCE had a detoxifying effect on NP-induced thyroid disruption by dosing SD rats with NP (270 mg/kg body weight) or/and MCE (30, 60, or 120 mg/kg body weight) daily for 28 days. We found that NP significantly inhibited free triiodothyronin (FT_3) and free thyroxine (FT_4) activity in rat serum ($P < 0.05$), but MCE intervention significantly increased FT_3 and FT_4 serum levels ($P < 0.05$). It is possible that changes in hormonal composition might trigger the TRH-TSH-TH automatic feedback loop. The activity of the three iodothyronine deiodinases increased significantly after NP-dosing ($P < 0.05$), but only deiodinase3 (D3) was downregulated after MCE treatment ($P < 0.05$). Therefore, MCE might be an effective NP-detoxification agent against thyroid disruption because it regulates D3 activity.

1. Introduction

Nonylphenol (NP), a lipophilic environmental chemical, is mainly generated by the biodegradation of nonylphenol ethoxylates (NPEOs). NPEOs are non-ionic surfactants widely used in products such as detergents, lubricants, and plastics (Cheng et al., 2014). NP has been shown to cause estrogenic responses in aquatic organisms and has thus been classified as an endocrine-disrupting chemical (EDC) (Scrimshaw and Lester, 2002). EDCs alter the endocrine systems of both wildlife and humans and pose a serious human and environmental health hazard (Mccormick et al., 2005; Xi et al., 2013). NP contamination has been found both in foods (including fish, meat and vegetables) and the plastics used in food processing and packing (Gyllenhammar et al., 2012). NP has been associated with reproductive difficulties (Lee et al., 1999; Liu et al., 2011), nerve impairment (Huang et al., 2009), immune system damage (Bennasroune et al., 2012), and obesity (Hao et al., 2012). NP may also have weak estrogenic activity and may increase the risk of cancer proliferation in estrogen-dependent cancers, such as ovarian cancer (Park and Choi, 2012). Increasing doses of NP decreased free triiodothyronin (FT_3) and free thyroxine (FT_4) and increased thyroid-stimulating hormone (TSH) in rat serum (Xi et al., 2013), suggesting that exposure to NP might induce thyroid toxicity. Indeed, a significant correlation was found between plasma TSH and thyroxine (T_4) in the serum of NP-exposed rats but not between plasma TSH and triiodothyronin (T_3) (Rognoni et al., 1984). Thyroid hormone (TH) plays critical roles in growth, differentiation, development, and maintenance of metabolic homeostasis (Skeaff, 2011). TH is degraded in the liver, primarily by deiodinases; reverse triiodothyronine (rT_3) is one of the intermediates formed (Fig. 1). Three different types of iodothyronine deiodinase (D1, D2 and D3) have been identified in mammalian. D2 catalyzes outer ring deiodination (ORD) and regulates TH in the brain, brown adipose tissue, and the pituitary gland (Arthur et al.,

Abbreviations: NP, nonylphenol; EDC, endocrine-disrupting chemical; MCE, mulberry crude extract; TH, thyroid hormone; rT_3, reverse triiodothyronine; D1, deiodinase1; D2, deiodinase2; D3, deiodinase3; FT_3, triiodothyronine; FT_4, free thyroxine; TSH, thyroid-stimulating hormone; T_4, thyroxine; T_3, triiodothyronine; ORD, outer ring deiodination; IRD, inner ring deiodination; TRs, thyroid hormone receptors

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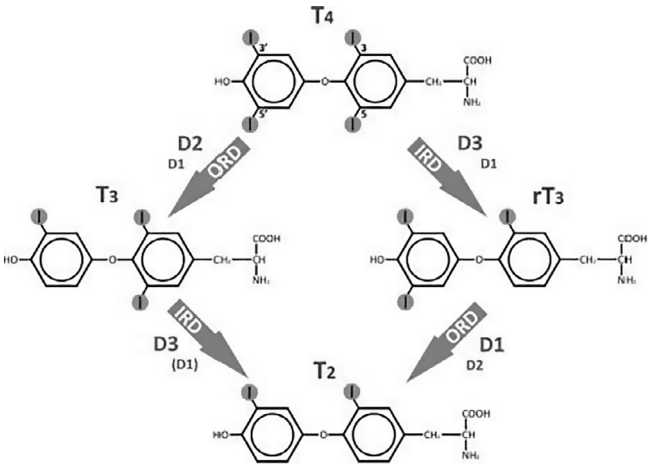

Fig. 1. Major pathways of thyroid hormone deiodination.

1991), while D3 catalyzes inner ring deiodination (IRD) and is found in the brain, skin, and eyes (Ogawa-Wong et al., 2016). D1 is non-selective, catalyzing both ORD and IRD (Darras and Van Herck, 2012); D1 is most active in the liver and kidney (Becker et al., 1995). All three deiodinases impact systemic T_3 levels and contribute directly or indirectly to intracellular T_3 availability in different tissues (Darras and Van Herck, 2012).

The mulberry tree is widely distributed throughout China. Although the mulberry tree is most commonly used as a food source for silkworms, mulberry fruit is also used in herbal medicines. In Chinese folklore, mulberries are said to protect against liver and kidney damage, strengthen joints, improve eyesight, and reduce the effects of aging. Mulberries are a good source of sugars, acids, and anthocyanin pigments; these are important constituents of juices, wines, and other beverages. Recent studies have suggested that mulberries are anti-diabetic (Asano et al., 2001), anti-oxidative (Bao et al., 2016), anti-inflammatory (Kim and Park, 2006), and anti-hyperlipidemic (Kim et al., 2001). It has also been suggested that mulberries protect against brain damage from diseases such as Parkinson's and from memory impairments induced by cerebral ischemia (Kang et al., 2006; Kim et al., 2010). Mulberry extracts help to induce an antioxidant defense system and to reduce memory deterioration in aging animals (Shih et al., 2010). Recently, mulberry juice has been shown to have the ability to reduce elevated plasma levels of lipid peroxides in mice exposed to water immersion restraint stress, and it may be an efficient detoxification against NP-induced toxic effects by activation of Nrf2 and Nrf2-related detoxifying enzymes in rat (Sakagami et al., 2007; Liu et al., 2017). In addition, mulberries potentially enhance cognition (Kaewkaen et al., 2012). Therefore MCE might be used as tool for the protection against EDCs exposures. To date, however, few studies have explored the effect of mulberry crude extract (MCE) on NP toxicity with respect to TH and deiodinase. We aimed to use rats as an animal model for NP exposure, and to test the detoxification effect of MCE on NP-induced thyroid toxicity.

2. Methods

2.1. Chemical

Nonylphenol/NP (CAS No. 84852-15-3, purity > 99%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Unless otherwise specified, all other reagents were analytical grade, and purchased from Tianjin Kermel reagent Co. Ltd (Tianjin, China).

2.2. Animals and treatment

All experiments performed in this study were approved by the Animal Care Committee of the Laboratory Animal Center of South China Agricultural University, Guangzhou, China. Many researches showed that low concentration NP exists in urine, blood and breast mike of human (Li et al., 2013; Kawaguchi et al., 2004; Azzouz et al., 2016). This toxicological study used high concentrations of NP to evaluate NP-induced thyroid disruption and determine whether MCE had a detoxifying effect on that disruption in rats. Our experiment was divided into two parts. In experiment I, we aimed to evaluate NP-induced thyroid disruption effects. In experiment II, we aimed to test the detoxification effects of MCE on NP-induced thyroid toxicity. For both experiments, we obtained 80 4–5 week old male Sprague–Dawley (SD) rats (body weight: 70–90 g) from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The qualified number of experimental animals was SCXK (Yue) 2013-0002. All rats were housed in controlled conditions (a 12 h light/dark cycle at 22 ± 0.5 °C and 50–60% relative humidity). Rats were fed commercial rodent chow and tap water ad libitum. After 7 days of adaptive feeding, 32 rats were randomly divided groups of eight for experiment I and 48 rats were randomly divided groups of eight for experiment II. For experiment I, we dosed the each group of rats orally with either 0, 30, 90, or 270 mg NP per kg body weight (bw) daily for 28 days (Table 1). In experiment II, we dosed six groups of rats with 270 mg NP per kg bp and/or 0, 30, 60 or 120 mg MCE per kg bw, daily for 28 days (Table 1).

All drugs were administered orally by gavage. Twenty-four hours after the last drug administration, all rats were weighed and euthanized by cervical dislocation. Blood samples were collected and centrifuged at 845*g* for 15 min at 4 °C to obtain serum. The liver of each rat was immediately excised, weighed, washed in buffer solution at 4 °C, then gently wiped and stored at −80 °C.

2.3. MCE preparation

We obtained mature mulberries from Guangzhou, Guangdong province, China. Mulberries were freeze-dried and stored at 4 °C. Freeze-dried mulberries were ultrasonically extracted in a 15 × volume of methanol (80%, w/v) for 90 min at room temperature. Extracts were filtered, evaporated, and concentrated at 45 °C using a rotary vacuum evaporator. We repeated the extraction twice. The MCE contained

Table 1
Experimental study design.

a. Experiment I

Groups	Gavage administration
	Every day 9:00 am
Control (C)	Corn oil
Low-dose nonylphenol (NPL)	30 mg/kg bw NP
Medium-dose nonylphenol (NPM)	90 mg/kg bw NP
High-dose nonylphenol (NPH)	270 mg/kg bw NP

b. Experiment II

Groups	Gavage administration	
	Every day 9:00 am	Every day 10:00 am
Control (C)	Corn oil	Physiological saline
Nonylphenol (NP)	270 mg/kg bw NP	Physiological saline
Mulberry crude extract (S)	Corn oil	120 mg/kg bw
NP + low-dose MCE (SL)	270 mg/kg bw NP	30 mg/kg bw
NP + middle-dose MCE (SM)	270 mg/kg bw NP	60 mg/kg bw
NP + high-dose MCE (SH)	270 mg/kg bw NP	120 mg/kg bw

Note: Gavage doses of mulberry group and intervention groups were calculated by polyphenol contents in MCE.

3.82 ± 0.02 mg/mL protein, 49.58 ± 0.22 mg/mL total sugars, 23.55 ± 0.10 mg/mL reducing sugars, 833.11 ± 3.70 mg/g water, 34.86 ± 0.09 mg/mL polyphenols, and 7.39 ± 0.05 mg/mL flavonoids. The mass of 100 mL MCE was 108.25 g (Liu et al., 2017).

2.4. Thyroid function and deiodinase activity indicators

Thyroid function and deiodinase activity were measured with corresponding kits (Shanghai Jiang Lai Biotechnology Co., Ltd., Shanghai, China), following the manufacturer's instructions. The thyroid function indicators we used were FT_3, FT_4, rT_3, and TSH in the serum, and the deiodinase activity indicators we used were D1, D2, and D3 in the liver homogenate. The lowest detectable quantity of the FT_3 kit, FT_4 kit and rT_3 kit is less than 0.1 pmol/L, 0.1 pmol/L and 5 pmol/L respectively. The lowest detectable quantity of the TSH kit is less than 0.1 mU/L. The lowest detectable quantity of the D1 kit, D2 kit and D3 kit is less than 80 ng/L, 25 ng/L and 20 ng/L respectively. The CVs of all kits in each sample batch and between sample batches were 9% and 11% respectively.

2.5. Statistical analysis

We calculated single factor analysis of variance (one-way ANOVA) in SPSS v19.0 (XX) to determine the significance of differences among groups. If variances were homogeneous, the least significant difference (LSD) test was applied. If not, we used the Games-Howell test. We considered $P < 0.05$ statistically significant.

3. Results

3.1. Effects of different NP doses on thyroid-related hormones

The concentrations of FT_3 and FT_4 in the serum were significantly lower in the NPH group than in the C group ($P < 0.05$), while the concentration of rT_3 in the serum was significantly higher ($P < 0.05$; Fig. 2). The concentration of TSH in the NPL, NPM, and NPH groups was significantly higher than the concentration of TSH in the C group ($P < 0.05$).

3.2. Effects of different NP doses on deiodinase activity

D1 activity in the rat liver homogenate was significantly higher in the NPH group than in the C group ($P < 0.05$; Table 2). The activity of D3 in the rat liver homogenate increased significantly with increased NP dose, as compared to the C group ($P < 0.01$; Table 2). D2 activities in the NPL and NPM groups were higher than in the NPH group, but only the activity of D2 in the NPH group had significant difference compared with the C group ($P < 0.05$; Table 2). In post-hoc analysis such as LSD test, it would inevitably lead to the tendency of inflating Type I error (false positive) when variances were highly inhomogeneous and sample size was very small. Thus, it was necessary to obtain a higher power to prove whether the activity of D2 was really affected by NP in the future.

3.3. Effects of MCE on thyroid-related hormones

The serum concentrations of FT_3 and FT_4 were significantly lower in the NP, SL, and SM groups than in the C and S groups ($P < 0.05$ for all comparisons; Fig. 3). The serum concentration of FT_3 increased with dose in the three intervention groups, but only in the SH group was the FT_3 concentration significantly higher than in the NP group ($P < 0.05$ for all comparisons; Fig. 3). The serum concentration of FT_4 also increased with dose in the three MCE-dosed groups; the FT_4 levels in these three groups were significantly higher than in the NP group ($P < 0.05$ for all comparisons; Fig. 3). The serum concentration of rT_3 was significantly higher in the NP, SL, and SM groups, as compared to the C and S groups ($P < 0.05$ for all comparisons; Fig. 3). The serum concentration of rT_3 decreased with NP dose in the three intervention groups, but only in the SH group was the decrease significant compared

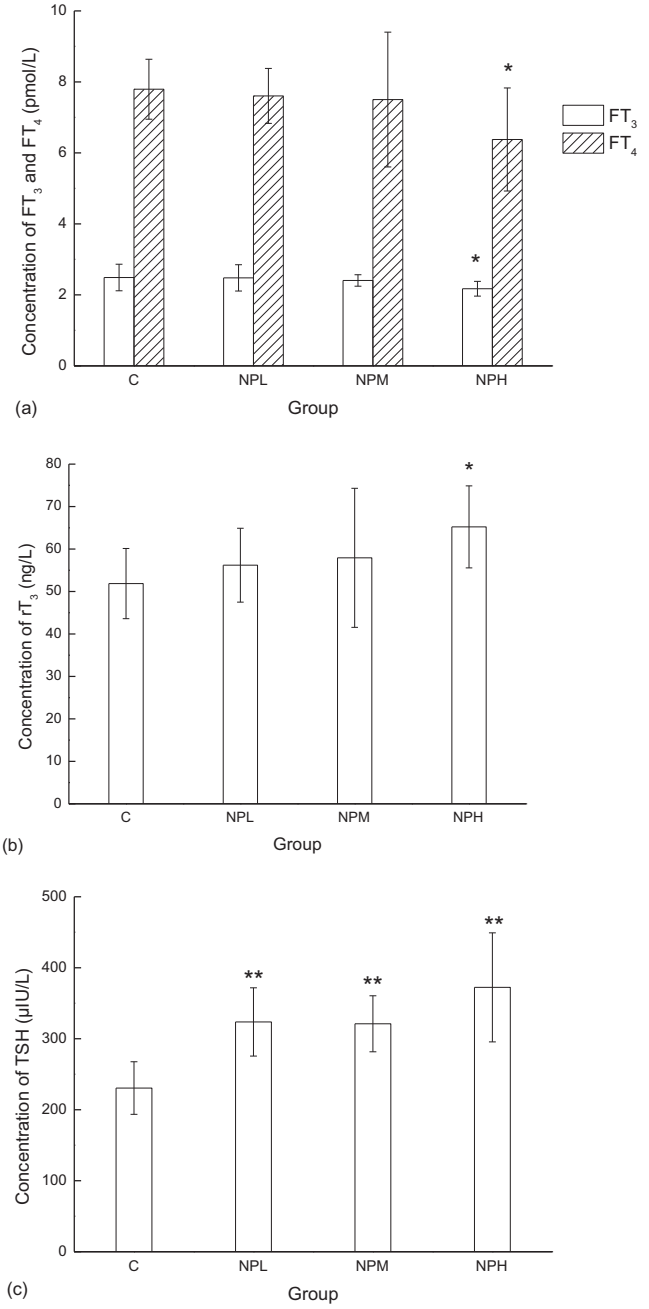

Fig. 2. Serum concentrations of TH and TSH in rats exposed to different doses of NP (experiment I). (a) Serum concentrations of FT_3 and FT_4. (b) Serum concentration of rT_3. (c) Serum concentration of TSH. *, $P < 0.05$ compared to group C; **, $P < 0.01$ compared to group C.

Table 2
Effect of NP on deiodinase activity in rat livers (data are means ± SD; n = 8).

Groups	D1 (ng/g prot)	D2 (ng/g prot)	D3 (ng/g prot)
C	3.79 ± 1.18	2.91 ± 0.54	1.80 ± 0.48
NPL	4.11 ± 1.37	4.75 ± 1.61	3.11 ± 0.91**
NPM	4.72 ± 0.92	4.37 ± 1.57	3.16 ± 0.97**
NPH	5.31 ± 1.24*	4.21 ± 0.94*	3.54 ± 0.83**

*, $P < 0.05$ compared to group C; **, $P < 0.01$ compared to group C.

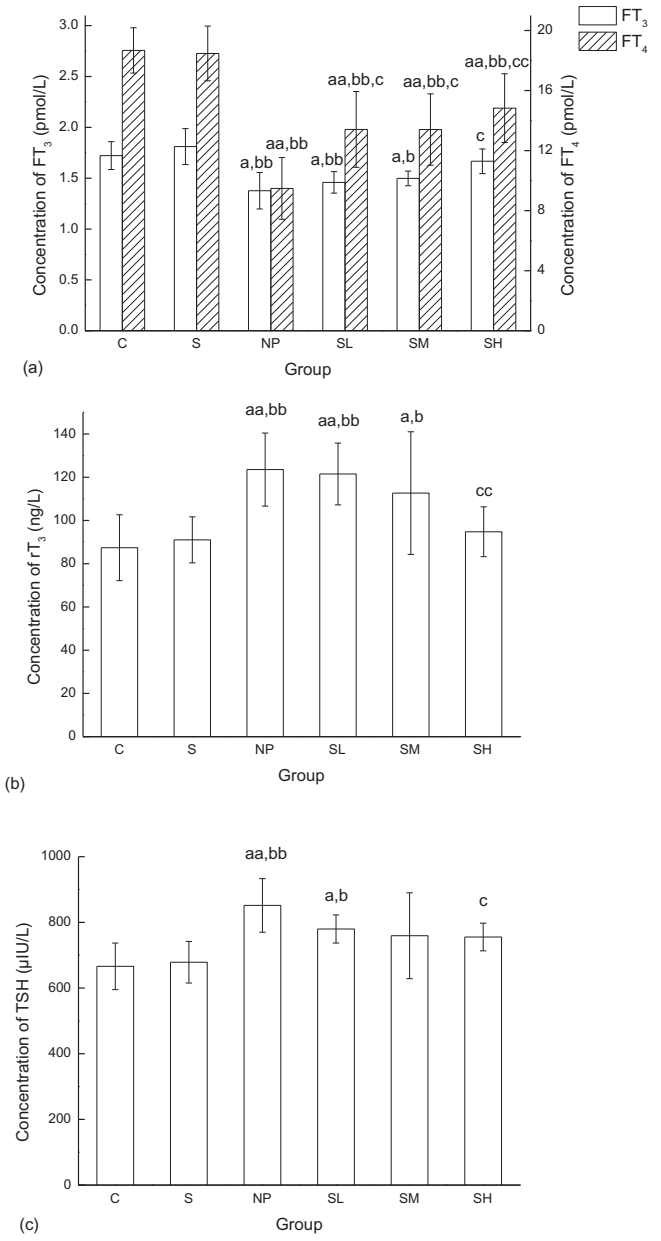

Fig. 3. Serum TH and TSH levels in different groups of rats exposed to different combinations of NP and MCE (experiment II). (a) Effects of MCE on FT_3 and FT_4 serum levels. (b) Effects of MCE on rT_3 serum levels. (c) Effects of MCE on TSH serum levels. [a], $P < 0.05$ compared to group C; [aa], $P < 0.01$ compared to group C; [b], $P < 0.05$ compared to group S; [bb], $P < 0.01$ compared to group S; [c], $P < 0.05$ compared to group NP; [cc], $P < 0.01$ compared to group NP.

to the NP group ($P < 0.01$; Fig. 3). The serum concentration of TSH was significantly higher in the NP and SL groups than in the C and S groups ($P < 0.05$ for all comparisons; Fig. 3). Finally, the serum concentration of TSH decreased with increased NP dose in the three intervention groups, but only in the SH group was the decrease significant compared to the NP group ($P < 0.05$; Fig. 3).

3.4. Effect of MCE on deiodinase activities

The activity of D1 and D2 in the rat liver homogenate was significantly higher in the NP, SL, SM, and SH groups, as compared to the C and S groups ($P < 0.05$ for all comparisons; Table 3). The activity of D3 in rat liver homogenate was significantly higher in the NP, SL, and SM groups, as compared to the C and S groups ($P < 0.05$ for all comparisons; Table 3). The activity of D3 decreased with increased MCE dose; in the SH group, the activity of D3 was significantly lower than in the NP group ($P < 0.05$).

Table 3
The effects of MCE on deiodinase activity in the liver of rats given various doses of NP and MCE (data are means ± SD; n = 8).

Groups	D1 (ng/g prot)	D2 (ng/g prot)	D3 (ng/g prot)
C	15.38 ± 1.42	13.00 ± 1.11	9.05 ± 1.08
S	15.88 ± 1.01	13.08 ± 1.30	9.15 ± 0.41
NP	18.99 ± 1.68[a,b]	14.71 ± 1.37[a,b]	12.02 ± 0.62[aa,bb]
SL	18.96 ± 1.96[a,b]	14.83 ± 1.25[a,b]	11.09 ± 0.55[a,b]
SM	19.07 ± 1.81[a,b]	14.95 ± 1.19[a,b]	10.58 ± 0.55[a,b]
SH	18.86 ± 1.68[a,b]	14.94 ± 1.77[a,b]	9.44 ± 1.52[c]

[a], $P < 0.05$ compared to group C; [aa], $P < 0.01$ compared to group C; [b], $P < 0.05$ compared to group S; [bb], $P < 0.01$ compared to group S; [c], $P < 0.05$ compared to group NP; [cc], $P < 0.01$ compared to group NP.

4. Discussion

TH plays a critical role in growth, differentiation, development, and maintenance of metabolic homeostasis via thyroid hormone receptors (TRs) (Buchholz et al., 2006); TRs control the expression of TR target genes. There are two primary forms of TH: T_3 and T_4. T_3 is more active, while T_4 is the main form of TH in blood. T_4 is secreted into the blood by the thyroid (Zhang et al., 2013). In addition, TH can also regulate the sex steroid endocrine system (Sharma et al., 2016).

Here, the decreases in FT_3 and FT_4 and the increase in TSH in the NP-treated groups were statistically significant compared to the control group ($P < 0.05$ for all comparisons; Fig. 2). Consistent with this, serum levels of FT_3 and FT_4 increased in rats exposed to the EDC bisphenol-A (Saied and Hassan, 2014). TH-induced modulation of AMP-activated protein kinase activity and lipid metabolism in the hypothalamus is critical for whole-body energy homeostasis regulation (López et al., 2010). One of the primary role of TH is the stimulation of the hypothalamus-pituitary-thyroid axis, by regulating the central nervous system (CNS) via negative feedback. In the hypothalamus-pituitary-thyroid axis, thyrotrophin-releasing hormone (TRH) released by the hypothalamus stimulates the pituitary to secrete TSH, which in turn enhances the synthesis and secretion of TH. Negative feedback is triggered when FT_3 and FT_4 levels reach a given threshold and inhibit the secretion of TRH and TSH, closing the TRH-TSH-TH feedback loop (Kamble et al., 2013).

The serum TSH concentrations in the three NP-treated groups were all significantly higher than in control group ($P < 0.01$; Fig. 2), but the serum concentrations of FT_3 and FT_4 in the NPH group were significantly lower than in the control group ($P < 0.05$; Fig. 2). In the other NP-treated groups, FT_3 and FT_4 serum concentrations decreased in a dose-dependent manner (Fig. 2). These results suggested that, because of its estrogenic effects, NP might enhance the sensitivity of the pituitary to TRH and stimulate the secretion of TSH. In addition, the structure of the thyroid might have been damaged by increased NP-exposure duration, decreasing TH synthesis, increasing TRH levels, and stimulating further secretion of TSH.

Many of the physiological functions of tissue cells depend on the dynamic regulation of TH (Power et al., 2001); iodothyronine deiodinase is critical to this regulation. D1 and D2 concentrations in the NPH group were significantly greater than in the control group ($P < 0.05$); D3 concentrations in all three NP-treated groups were significantly greater than in the control group ($P < 0.01$; Table 2). This result is consistent with a previous study, where bisphenol-A significantly increased the activity of D2 and D3 in the goldfish liver (Qu et al., 2007). It might be that environmental estrogens enhance the metabolic rate of TH in vivo by inhibiting the binding of THs and TRs, thus causing a decrease in TH activity and an increase in iodothyronine deiodinase

activity. It is possible that inhibition of TH-binding by NP damaged the TRH-TSH-TH regulatory pathway. Alternatively, NP might accelerate the TH metabolic rate by enhancing the activity of iodothyronine deiodinase, leading to a decrease in FT_3 and FT_4 activity. And it has been suggested that NP could act on the thyroid through different mechanisms like interference with binding of T_3 to transthyretin, or antagonism to T_3 binding to the TH receptors (Moriyama et al., 2002; Fukazawa, 2003).

EDCs are a chemically diverse class of substances deriving either from natural plant sources or from industrial products. The effects of EDCs on reproductive and endocrine system regulation have been well studied (Toppari, 2002). In particular, EDCs affect the thyroid axis (Schmutzler et al., 2007). Previous studies have indicated that active endocrine compounds seem to disrupt the feedback regulation of the thyroid axis, but each compound tested has elicited its own spectrum of alterations, suggesting that EDCs interfere in various ways with TH metabolism and the complex network of TH interactions (Schmutzler et al., 2004). Generally, EDCs impair thyroid endocrine systems probably by affecting iodine uptake or TH synthesis (Zhou et al., 2004; Cooper, 2003). For instance, perchlorate inhibits iodine uptake, which is dependent on the presence of other environmental sodium/iodide symporter inhibitors and iodine intake itself (De Groef et al., 2006). Some types of EDCs interfere with TH synthesis by blocking thyroid peroxidase, an enzyme that is critical for iodide ion oxidation and TH production (Cooper, 2003). Polychlorinated biphenyls and bisphenol-A may disrupt the functions of various types of nuclear hormone receptors and their cofactors, disturbing the internal hormonal environment (Miyazaki et al., 2004; Moriyama et al., 2002). In addition, many EDCs decrease the activity of iodothyronine deiodinases. As NP is an EDC, it might influence TH activity via the regulation of multiple targets within the complex regulatory network of TH metabolism and activity, including TRs, which mediate gene regulation in response to T_3 (Marchand et al., 2001); deiodinase, which catalyzes the outer ring deiodination of T_4 to the biologically active T_3 and the hypothalamus-pituitary-thyroid axis, which contains the TRH-TSH-TH feedback loop (Kamble et al., 2013; Orozco and Valverde-R, 2005).

Mulberries are commonly used in traditional Chinese medicine because of their low toxicity and good therapeutic performance (Li, 1998). The entire mulberry plant is thought to have medicinal value, including the roots, bark, branch, leaves, and fruit (Thabti et al., 2012). We prepared the MCE used in this study with fresh mulberries. Our MCE contained 34.86 mg/mL polyphenols and 7.39 mg/mL flavonoids (Liu et al., 2017). It has been shown that polyphenols and flavonoids affect serum TH and TSH concentrations, and thus influence metabolic control in humans (Da-Silva et al., 2007; Kohrle et al., 1989). After dosing NP-treated rats with MCE, levels of FT_3 and FT_4 recovered to varying degrees, and D3 decreased to normal levels. MCE had no effect on D1 and D2. In vivo, most T_3 is generated from T_4 catalyzed by D2; the degradation of T_3 is catalyzed by D3. In the NP-treated groups, D2 and D3 levels in the rat livers were significantly higher than in the control group. The FT_3 concentration was also lower. It is possible that the T_3 generation rate is slower than the T_3 degradation rate. Alternatively, MCE might significantly inhibit D3 activity compared to the control group without affecting D2. In this case, MCE would not affect the conversion of T_4 to T_3 but would decrease the degradation rate of T_3, resulting in an overall increase in serum FT_3 concentration.

We therefore speculate that, in NP-dosed rats, high doses of MCE significantly inhibited the increase in D3 concentration, promoted the production of FT_4, and induced the increase in FT_3 and FT_4 concentrations. This speculation is consistent with the results of Huang et al. (2000), who found that D3 activity was closely related to TH levels. The decrease in rT_3 serum concentration we observed in the intervention group might have been because the T_4 to rT_3 conversion was significantly inhibited by D3 activity in the rat livers. The consequent increase in serum FT_3 and FT_4 concentrations would inhibit TSH secretion via negative feedback regulation in the high-dose MCE group. Therefore, it is possible that MCE decreases D3 activity in the liver, increasing the TH concentration, restoring thyroid function, and leading to a reduction in the toxic effects of NP on the thyroid. Notably, the main active compounds in MCE might be polyphenols and flavonoids (Liu et al., 2017).

5. Conclusion

We found that NP might cause hypothyroidism in rats because it damages the TRH-TSH-TH regulatory pathway or because it accelerates the metabolism of TH by enhancing the activity of iodothyronine deiodinase. MCE not only inhibited the activity of D3 in the liver, increasing serum T_3 and T_4 concentrations and decreasing serum rT_3 concentrations, but it also reduced TSH secretion through negative feedback regulation, reducing NP toxicity in the rat thyroid.

Acknowledgments

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Ethics approval and consent to participate

All experimental procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All animal tests were approved by the Animal Care Committee of the Laboratory Animal Center of South China Agricultural University, Guangzhou.

Conflicts of interest

All authors declare that they do not have potential conflicts of interest.

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