Effects of *Dendropanax morbifera* Léveillé extract on hypothyroidism-induced oxidative stress in the rat hippocampus

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Abstract In this experiment, we verified the effects of *Dendropanax morbifera* Léveillé stem extract (DMS) on hypothyroidism-induced oxidative stress in the hippocampus of rats. Hypothyroidism was induced in rats by treating them with 0.03% 2-mercapto-1-methyl-imidazole dissolved in drinking water for 5 weeks. DMS (100 mg/kg) was also orally administered to the rats during the same period and the animals were sacrificed at 12 weeks of age. DMS administration tended to ameliorate these hypothyroidism-induced changes in serum triiodothyronine (T₃), thyroxine (T₄), and thyroid-stimulating hormone levels. DMS administration significantly reduced the hypothyroidism-induced increases in reactive oxygen species production as well as in lipid peroxidation in the hippocampus. In addition, DMS administration increased hippocampal Cu, Zn-superoxide dismutase (SOD1), catalase (CAT), and glutathione peroxidase (GPx) levels. These results suggest that DMS potentially ameliorates hypothyroidism-induced neuroendocrine phenotypes and oxidative stress in the hippocampus via the induction of antioxidant enzymes.

Keywords: antioxidants, *Dendropanax morbifera* Léveillé stem extract, hippocampus, hypothyroidism, oxidative stress

Introduction

Thyroid hormones such as triiodothyronine (T₃) and its prohormone thyroxine (T₄), regulate synaptic transmission in the central nervous system (1). In addition, thyroid hormones modify the sensitivity of their receptors (2) by regulating the turnover of neurotransmitters. Thyroid hormones are one of the major factors in the normal development of the brain, especially that of the hippocampus (3).

The hippocampus is a highly sensitive neural structure known to be involved in learning and memory. It has a high density of thyroid hormone receptors (4,5) and is highly vulnerable to the action of thyroid hormones (6–8). Abnormal levels of thyroid hormones, e.g., hyperthyroidism, and hypothyroidism, affect hippocampal functions as well as antioxidant enzyme capacity (9). Hyperthyroidism increases intracellular free radicals in the mitochondria by regulating energy

metabolism (10,11). Hypothyroidism by treatment with propylthiouracil for 4–5 weeks induces a significant reduction in cerebral volume (12). In addition, human studies have demonstrated that children with congenital hypothyroidism exhibit mild cognitive deficits in memory tasks involving the hippocampus (13,14). One important mechanism of tissue damage in the hippocampus due to hypothyroidism is oxidative stress mediated by reactive oxygen species (ROS) (15,16), although results of studies investigating oxidative damage due to hypothyroidism are controversial (17,18). Hypothyroidism causes oxidative damage because of low availability of antioxidants (9,19). In addition, thyroid hormones also directly regulate the expression of anti-oxidative enzymes (20).

Several lines of evidence in human studies have demonstrated that T₄ therapy improves the physiological phenotype of hypothyroidism, but does not completely restore neurocognitive function and

psychological well-being in patients (21,22). In previous studies, we have demonstrated *Dendropanax morbifera* Léveillé stem extract (DMS) in the hippocampus significantly ameliorates increases in ROS levels and decreases in antioxidants following cadmium- and mercury-induced oxidative stress (23,24). In addition, debarked DMS extract (25) and extract (26) from branches of *Dendropanax morbifera* Léveillé exhibits strong antioxidant effects. In the present study, we investigated the roles of DMS on hypothyroidism-induced neuroendocrine phenotypes in serum, ROS production and lipid peroxidation as well its effect on several antioxidant enzymes in the hippocampus.

Materials and Methods

Experimental animals Male Sprague-Dawley rats (6-week-old) were purchased from Orient Bio Inc. (Seongnam, Korea). Rats were housed in a conventional animal facility at 23°C and 60% humidity, with a 12 h/12 h light/dark cycle, and free access to food and tap water. Handling and care of the animals conformed to the guidelines established in order to comply with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (Approval number: SNU-130522-1). All of the experiments were conducted with an effort to minimize the number of animals used and the suffering caused by the procedures in the study.

Preparation of DMS Fresh *Dendropanax morbifera* Léveillé was obtained from Hambakjae Farm (Jeju, Korea). The plant was authenticated by two practitioners of traditional Asian medicine and the voucher specimen was deposited in our laboratory (deposition number: 2012-030). Stems from the plant samples (100 g) were chopped, blended, soaked in 2 L of 80% ethanol, and refluxed three times at 20°C for 2 h as previously described (23,24). The insoluble materials were removed by centrifugation at 10,000×g for 30 min, and the resulting supernatant was concentrated and freeze-dried to yield a powder (yield: 3.3%). Before each experiment, the dried extract was dissolved in distilled and deionized water.

Experimental design Methimazole (2-mercapto-1-methyl-imidazole) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Animals were divided into three treatment groups ($n=10$ in each group): 1) a vehicle (distilled water)-treated group; 2) a group treated with methimazole (MMZ); and 3) a group treated with methimazole and DMS (MMZ-DMS). At 7 weeks of age, 0.03% methimazole was administered orally in drinking water for 5 weeks to prevent thyroid hormone synthesis by inhibiting coupling and iodination (27) as previously described (28). In addition, 100 mg/kg DMS was dissolved in distilled water and administered orally to 7-week-old rats, once a

day, for 5 weeks.

Serum levels of thyroid hormones and thyroid-stimulating hormones

(TSH) The animals from the vehicle, MMZ, and MMZ-DMS groups were sacrificed at the age of 12 weeks for the analysis of serum circulating TSH, free T_3 , and T_4 levels in the morning (09:00–11:00). Thyroid function was determined in these rats using AccuBind® Vast enzyme-linked immunosorbent assay (ELISA) kits (Monobind Inc., Lake Forest, CA, USA).

Measurement of ROS production in the hippocampus

The effects of hypothyroidism and/or DMS on hippocampal ROS production in the vehicle, MMZ, and MMZ-DMS groups ($n=5$ in each group) were assessed using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA) (29). Intracellular ROS oxidize DCFH-DA to dichlorofluorescein (DCF), an intense fluorescent chemical. The animals were deeply anesthetized and euthanized by decapitation. Hippocampi from both sides were collected using a surgical blade and the brain mitochondria from the left hippocampus were obtained as described previously (30). Mitochondrial protein quantification was determined using the Bradford method (31) with bovine serum albumin (BSA) as the standard. Mitochondria isolated from different groups (0.5 mg/mL) were incubated with 10 μ M DCFH-DA at 37°C for 60 min. The fluorescence intensity of DCF was measured at an excitation wavelength of 488 nm and an emission wavelength of 527 nm in a microplate reader (SpectraMax M5; Molecular Devices LLC, Sunnyvale, CA, USA).

Measurement of lipid peroxidation in hippocampal homogenates

The effects of hypothyroidism and/or DMS on lipid peroxidation levels in the vehicle, MMZ, and MMZ-DMS groups ($n=5$ in each group) were assessed by measuring malondialdehyde (MDA) formation using the Bioxytech MDA-586 kit (Oxis Research, Portland, OR, USA) as previously described (32). The right part of the hippocampus (matching the part used for measurement of ROS production) was homogenized in 20 mM PBS (pH 7.4) containing 5 mM butylated hydroxytoluene. After centrifugation of the homogenates at 3,000×g for 10 min at 4°C, the supernatants were collected. For each reaction, 10 μ L of probucol and 640 μ L of diluted R1 reagent (1:3 of methanol:*N*-methyl-2-phenylindole) were added and mixed with 150 μ L of 12 N HCl. Each reaction mixture was incubated at 45°C for 60 min and centrifuged at 10,000×g for 10 min. The supernatant was collected and MDA formation was determined by measuring the absorbance at 586 nm. MDA data were normalized to the protein concentration of each sample.

Measurement of antioxidant activity in hippocampal homogenates

To elucidate the effects of hypothyroidism and/or DMS on Cu, Zn-superoxide dismutase (SOD1), catalase (CAT), and glutathione peroxidase (GPx), the enzymatic activity was measured in the vehicle, MMZ, and MMZ-DMS groups ($n=5$ in each group) as previously described (23,24). Briefly, the hippocampi from both sides

were homogenized in a 10 mM Tris buffer containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at $600\times g$ for 10 min, and then at $13,000\times g$ for 20 min at 4°C .

SOD1 activity was measured by monitoring its capacity to inhibit the reduction of ferricytochrome *c* by xanthine/xanthine oxidase as described by McCord and Fridovich (33). Staining for assessment of SOD1 activity was performed using electrophoresis in 10% native polyacrylamide gels and visualized as described by Beauchamp and Fridovich (34). Briefly, the gel was soaked in 2.45 mM nitroblue tetrazolium solution for 15 min, followed by 30 min in 28 mM *N,N,N,N*-tetramethylethylenediamine and 28 μM riboflavin in 0.36 mM potassium phosphate buffer (pH 7.8). The gel was exposed to a fluorescent light source until the bands showed maximum resolution.

CAT activity was assayed at 25°C by determining the rate of H_2O_2 degradation in 10 mM of potassium phosphate buffer (pH 7.0) according to Aebi's method (35). An extinction coefficient of 43.6 mM/cm was used for the calculations. One unit was defined as 1 pmol of H_2O_2 consumed per min and the specific activity was recorded as units per mg of protein.

GPx activity was assayed by measuring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) with *t*-butylhydroperoxide as a substrate according to the method of Maral *et al.* (36). Briefly, the reaction was carried out at 25°C in 600 μL of a solution containing 100 mM potassium phosphate buffer (pH 7.7), 1 mM EDTA, 0.4 mM sodium azide, 2 mM glutathione, 0.1 mM NADPH, 0.62 U of glutathione reductase, and 50 μL of homogenate.

Statistical analysis All data are expressed as the mean $\pm$ standard error of the mean (SEM). To determine the effects of hypothyroidism and/or DMS, differences between the means were statistically analyzed using one-way repeated measures analysis of variance

(ANOVA) and Bonferroni's post hoc test using GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was considered at $p < 0.05$.

Results and Discussion

Effects of DMS on hypothyroidism-induced phenotypes *Dendropanax morbifera* is grown in the southwestern South Korea. In this study, we investigated the effects of DMS on hypothyroidism-induced neuroendocrine phenotypes and oxidative stress in the hippocampus. At 12 weeks of age, the serum T_3 and T_4 levels in the MMZ group were significantly decreased to 41.1% and 24.0% of the vehicle group, respectively. In contrast, TSH levels in the MMZ group were significantly increased to 8.1-fold that of the vehicle group. In the MMZ-DMS group, the serum T_3 and T_4 levels were increased to 1.4- and 1.5-fold that of the MMZ group, respectively, although the differences were not statistically significant. Similarly, in the MMZ-DMS group, the serum TSH level decreased to 81.4% of the MMZ group. However, there was no statistically significant difference between the MMZ and MMZ-DMS groups (Fig. 1).

At 12 weeks of age, body weight in the MMZ group was significantly decreased to 59.1% of the vehicle group. In the MMZ-DMS group, body weight was significantly increased to 1.4-fold that of the MMZ group. In the MMZ group, the weight of the thyroid gland was significantly higher (2.7-fold) compared with that in the vehicle group. In the MMZ-DMS group, the weight of the thyroid gland weight significantly decreased to 78.6% of the MMZ group (Fig. 1). DMS tends to ameliorate the phenotypes of MMZ-induced hypothyroidism such as increased body weight, serum T_3 and T_4 levels, weight of the thyroid glands, and serum TSH levels.

Fig. 1. Changes in circulating triiodothyronine (T_3), thyroxine (T_4), and thyroid stimulating hormone (TSH) levels, as well as body and thyroid gland weights in the vehicle-treated and methimazole-induced hypothyroid rats, with or without DMS. Differences between the means were analyzed using one-way ANOVA. * indicates a significant difference from vehicle group ($p < 0.05$); # indicates a significant difference from MMZ group ($n = 10$ per group). The bars indicate the mean $\pm$ standard error of the mean (SEM).

Fig. 2. Levels of intracellular reactive oxygen species production as determined by 2,7-dichlorofluorescein (DCF) and malondialdehyde (MDA) levels in the hippocampi of rats in the vehicle, methimazole (MMZ), and MMZ with *Dendropanax morbifera* Léveillé stem extract (MMZ-DMS) treatment groups. * indicates a significant difference from vehicle group ($p < 0.05$); # indicates a significant difference from MMZ group ($p < 0.05$; $n = 5$ per group). The data represent mean $\pm$ standard error of the mean (SEM).

Effects of DMS on hypothyroidism-induced oxidative stress We tried to observe several markers for oxidative stress and antioxidant enzyme activity in the hippocampus. At 12 weeks of age, DCF fluorescence levels in the MMZ group were significantly increased to 1.8-fold that of the vehicle group. In the MMZ-DMS group, DCF fluorescence was significantly decreased to 53.7% of the MMZ group and similar to that in the vehicle group (Fig. 2). In the MMZ group, MDA levels were significantly increased to 2.8-fold that of the vehicle group. In the MMZ-DMS group, MDA levels were significantly decreased to 63.7% of the MMZ group, but MDA levels were significantly higher than in the vehicle group (Fig. 2). It was reported that *Dendropanax* extract showed a similar antioxidant capacity

based on DPPH radical scavenging activity with butylated hydroxytoluene, a synthetic antioxidant, in low concentration and vitamin C in high concentration (26).

Hypothyroidism demonstrates low total antioxidant capacity levels in serum in the human patients (37) and increases lipid peroxidation in the serum, amygdala, and hippocampus in rats (38,39). In the MMZ group, SOD1 was significantly increased to 1.26-fold that of the vehicle group, while CAT and GPx activities were significantly decreased to 65.5 and 67.9% of the vehicle group, respectively. In the MMZ-DMS group, in contrast, SOD1, CAT, and GPx activities were increased compared to those in the MMZ group. In particular, CAT and GPx were significantly increased compared with the MMZ group (Fig. 3).

Dendropanax extract ameliorates high fat diet induced reduction in SOD activity and increase in lipid peroxidation and oxidized glutathione ratio (40). Thyroid hormones have antioxidant properties (41) and directly modulate the genes of antioxidant enzymes (20). MMZ-induced hypothyroidism significantly increases lipid peroxidation, ROS, and nitric oxide synthase levels in the rat hippocampus and amygdala (38). In the human study, hypothyroid and subclinical hypothyroid patients show elevated serum MDA levels compared with controls (16). These results suggest that hypothyroidism induces oxidative stress in the hippocampus. In the present study, the administration of DMS significantly reduced ROS formation and lipid peroxidation in the hippocampus. This result was supported by our study as well as other studies that reported prevention of cisplatin-induced nephrotoxicity *Dendropanax morbifera* extracts through the preservation of mitochondrial function (42) and potential reduction of cadmium- or mercury-induced ROS formation and lipid peroxidation in the hippocampus (23,24).

Hypothyroidism significantly increased SOD1 activity and decreased CAT and GPx activities in hippocampal homogenates. This result was consistent with previous studies indicating that hypothyroidism increases manganese dependent superoxide activity and decreases CAT activity in the liver cells. This conditions consequently increases peroxide activity in the mitochondria from liver cells (17). In addition, hypothyroidism significantly increases total SOD activity (43). In the previous study, our colleagues observed increases in the SOD1 level in the CA1 of hypothyroid gerbils after transient forebrain ischemia

Fig. 3. Cu, Zn-Superoxide dismutase 1 (SOD1), catalase (CAT), and glutathione peroxidase (GPx) activity in the hippocampi of rats from the vehicle, methimazole (MMZ), and MMZ with *Dendropanax morbifera* Léveillé stem extract (MMZ-DMS) treatment groups. * indicates a significant difference from the vehicle group ($p < 0.05$); # indicates a significant difference from the MMZ group ($p < 0.05$; $n = 5$ per group). The data represent mean $\pm$ standard error of the mean (SEM).

(44). The administration of DMS significantly increased the SOD1, CAT, and GPx activities in the hippocampal homogenates. This result is supported by previous studies that found that *Dendropanax* extracts significantly increase antioxidant enzymes in cadmium- or mercury-induced oxidative stress.

In conclusion, adult-onset hypothyroidism significantly increases oxidative stress and the administration of DMS significantly reduces oxidative stress by increasing antioxidant activity in the hippocampus. This result suggests that DMS could have therapeutic potential for treatment of tissue damage induced by hypothyroidism.

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Disclosure The authors declare no conflict of interest.

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