Effects of pterostilbene on stress-related behaviors and their underlying neuroinflammatory and hormonal changes in mice

Byoungjin Park

Department of Medicine

The Graduate School, Yonsei University

Effects of pterostilbene on stress-related behaviors and their underlying neuroinflammatory and hormonal changes in mice

Byoungjin Park

Department of Medicine

The Graduate School, Yonsei University

Effects of pterostilbene on stress-related behaviors and their underlying neuroinflammatory and hormonal changes in mice

Directed by Professor Yong Jae Lee

The Doctoral Dissertation
submitted to the Department of Medicine
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Byoungjin Park

December 2019

This certifies that the Doctoral Dissertation of Byoungjin Park is approved.

Thesis Supervisor : Yong Jae Lee

Thesis Committee Member#1 : Duk-Chul Lee

Thesis Committee Member#2 : Kyong-Chol Kim

Thesis Committee Member#3: Jin Young Park

Thesis Committee Member#4: Jae Myun Lee

The Graduate School
Yonsei University

December 2019

ACKNOWLEDGEMENTS

First of all, I would like to thank God for having made everything possible. I am heartily thankful to my supervisor, Professor Yong Jae Lee, whose touching encouragement, inspiring guidance, and unwavering support from the initial to the final level enabled me to complete this doctoral dissertation.

Also, sincere thanks to Professor Duk-Chul Lee who generously offered guidance and mentoring for the academic research in the field of family medicine and health promotion. My deep appreciation extends to Professor Kyong-Chol Kim, Professor Jin Young Park, and Professor Jae Myun Lee for their providing indispensable advice throughout the coursework and academic research. In addition, I am very grateful to Chemon Nonclinical Research Institute in Gyeonggi Bio Center for superb laboratory technical help. Without their help, the outcome of this thesis could not be made.

At last, I am indebted to my family who all gave me courage and have prayed for me. Especially, I am so grateful to my wife, Jung Won Lee, who lovingly gave me sincere support. I’d like to dedicate this paper to all of you with all my heart.

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Effects of pterostilbene on stress-related behaviors and their underlying neuroinflammatory and hormonal changes in mice

Byoungjin Park

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Yong Jae Lee)

Background: Pterostilbene is a natural compound contained in various dietary sources that has received tremendous attention due to its antioxidant properties with promising benefits in cancers and vascular diseases. Currently, little is known about pterostilbene-associated neuroimmune-endocrine effects. We aimed to examine the efficacy of pterostilbene for improving stress-related behaviors, neuroinflammation, and hormonal changes in a mouse stress model.

Methods: To evaluate the efficacy of oral administration of pterostilbene or vehicle for 16 days for improving behavior, inflammation, and hypothalamic-pituitary-adrenal axis hyperactivity, mice were divided into a normal control group or one of five restraint stress groups—the vehicle group; the 20, 40, or 80 mg/kg/day pterostilbene treatment group; or the 20 mg/kg/day resveratrol treatment group. Open field and forced swimming tests were conducted. Hippocampal brain-derived neurotrophic factor (BDNF) levels, endocrine hormone levels, oxidative stress parameters, and histopathological features were assessed.

Results: Oral pterostilbene administration significantly increased the measured times in the open field and forced swimming tests, elevated the BDNF level, decreased the inducible nitric oxide synthase and superoxide dismutase levels in the brain, and reduced the plasma adrenocorticotropic hormone and corticosterone levels. Compared to vehicle treatment, pterostilbene dose-dependently increased the numbers of neurons and decreased the numbers of glial and tumor necrosis factor alpha-immunolabeled cells in the hypothalamus.

Conclusion: These findings suggest that pterostilbene may effectively modulate stress-related abnormal behaviors, neuroinflammation, and hyperactivity of the hypothalamic-pituitary-adrenal axis.

Key words: pterostilbene; stress; brain-derived neurotrophic factor; neuroinflammation; hypothalamic-pituitary-adrenal axis

Effects of pterostilbene on stress-related behaviors and their underlying neuroinflammatory and hormonal changes in mice

Byoungjin Park

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Yong Jae Lee)

I. INTRODUCTION

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a naturally derived active compound that is found in small quantities in blueberries, *Pterocarpus marsupium* heartwood, and several other types of berries.[1-3] Blueberries have been reported to contain approximately 150 ng of pterostilbene per gram and to be enriched in numerous bioactive ingredients, such as anthocyanins, phenolic acid, and vitamins.[3,4] Accumulating evidence suggests that blueberries have a wide range of potential beneficial effects on health that may be attributed collectively to all of those components.[5,6] Actually, blueberries contain amounts of anthocyanins and vitamin C approximately ten thousand times that of pterostilbene.[3,7]

Recently, the antioxidant activity of pterostilbene has been seen in both in vitro and in vivo studies, demonstrating preventative and therapeutic benefits.[8] In particular, pterostilbene has been reported to have various beneficial

antineoplastic effects in several common malignancies,[9] to attenuate cardiovascular disease,[10,11] and to ameliorate dysregulated glucose metabolism.[12] Pterostilbene has been reported to have pharmacological properties similar to those of resveratrol due to their structural similarities. Both pterostilbene and resveratrol are stilbenoids, but pterostilbene contains two methoxy groups and one hydroxyl group, whereas resveratrol has three hydroxyl groups. The presence of two methoxy groups makes pterostilbene more lipophilic than resveratrol, increasing its oral absorption and its potential for cellular uptake.[2,13] The half-life of pterostilbene is also longer than that of resveratrol,[14] because resveratrol is relatively more rapidly metabolized by phase II enzymes in the intestine and liver.[15] The metabolites of pterostilbene, like those of resveratrol, can cross the blood-brain barrier via UDP-glucuronosyltransferases; pterostilbene has advantages within the brain, along with better intestinal absorption than resveratrol.[16]

Stress can be induced by internal or external sources, such as psychological or physical stimuli, and is implicated in disrupting homeostasis, affecting both daily performance, and, more seriously, psychosocial functions.[17] Additionally, measuring biomarkers related to neuroinflammatory and neuroendocrinological characteristics may be considered seriously when assessing behavioral and affective changes.[18] The restraint stress model in rodents has been known to combine both the physical and emotional aspects of stress without the use of painful stimuli, causing a robust increase in basal cellular oxidative stress.[19] Oxidative stress has been observed to trigger neuroinflammation and hypothalamic-pituitary-adrenal (HPA) axis hyperactivity and may be a surrogate target for anti-stress interventions.[19] Restraint stress and immobilization models are among the most commonly adopted protocols to induce stress-related behavioral and biochemical changes.[20] Restraint stress protocols produce inescapable physical and mental stress for 7–21 days while avoiding habituation, with a low rate of adaptation.[17,21,22]

To date, little is known about the neuroinflammatory and neuroendocrine changes associated with the effects of pterostilbene on stress-related behaviors. This study was performed to evaluate the efficacy of repeated oral pterostilbene administration for improving stress-related behaviors, neuroinflammation, and HPA axis hyperactivity in a restraint stress mouse model.

II. MATERIALS AND METHODS

1. Animals

Male specific pathogen-free ICR mice (5 weeks old, 24.6–28.7 g) were obtained from OrientBio Inc. (Seongnam-si, Republic of Korea). After weighing and examination of their external appearance, the animals were acclimated to the laboratory conditions. Body weight and mortality were observed daily for the 6 days of the acclimation period. This study was performed in the behavior test room of the barrier animal facility area No. 3 of the Gyeonggi Bio Center, and the animals were housed in a room with a constant temperature of 23 ± 3 ℃, a relative humidity of 55 ± 15%, 12-h light/12-h dark cycle (from 08:00 to 20:00), 150–300 lx of luminous intensity, and 10–20 air changes per hour. Animals were offered Teklad Certified Irradiated Global 18% Protein Rodent Diet (2918C; ENVIGO, London, UK) purchased from Dooyeol Biotech Co., Ltd. (Seoul, Republic of Korea) ad libitum. Water disinfected by ultraviolet sterilization and ultrafiltration was provided ad libitum via a water bottle and examined by the authorized Gyeonggi-do Institute of Health & Environment (Suwon-si, Republic of Korea) to attest that the quality satisfied the standards for drinking water. All animals were housed in polycarbonate cages (W 170 × L 235 × H 125 mm) during the acclimation, dosing, and observation period. The present study was approved by the Institutional Animal Care and Use Committee (IACUC) of Chemon (approval no. 2019-04-007). The procedures were carried out in

accordance with the National Institute of Health Guide for the care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, the UK Animals (Scientific Procedures) Act of 1986 and associated guidelines or the European Communities Council Directive of 24 November 1986 (86/609/EEC). Furthermore, all efforts were made to minimize the number and suffering of animals used.

2. Reagents

Pterostilbene and resveratrol were obtained from Chemon Inc. (Suwon-si, Republic of Korea) and stored under refrigeration protected from moisture and light. For oral administration, pterostilbene and resveratrol were dissolved in a cremophor (a vehicle for poorly-water soluble reagents; Sigma Chemical Co., St. Louis, MO, USA) mixture (1:1:8 cremophor:ethanol:saline). Pterostilbene and resveratrol were administered once daily for 16 days by oral gavage using a 1 mL syringe. The administered volume was calculated based on the dose (10 mL/kg) and the body weight measured on the day of administration.

3. Experimental design

To evaluate the efficacy of repeated oral pterostilbene administration for improving stress-related behaviors, neuroinflammation, and HPA axis hyperactivity, the animals were divided into six groups, including the normal control group (G1) and five restraint stress groups (G2-G6). The restraint stress groups consisted of the vehicle group (G2); groups treated with the test article pterostilbene at 20, 40, and 80 mg/kg/day (G3, G4, and G5, respectively), and a group treated with the reference article resveratrol at 20 mg/kg/day (G6). Each group comprised seven mice (Figure 1).

To elicit chronic stress,[23,24] animals were restrained in a ventilated polypropylene 50 mL conical tube. One hour after the administration of either the vehicle only or the reagents, animals were restrained for 3 h. The restraint

procedure was performed daily for 14 days between 10:00 and 13:00. The protocols for the administration of each reagent and the restraint procedure were performed identically under an identical environment for all restraint stress groups (G2-G5).

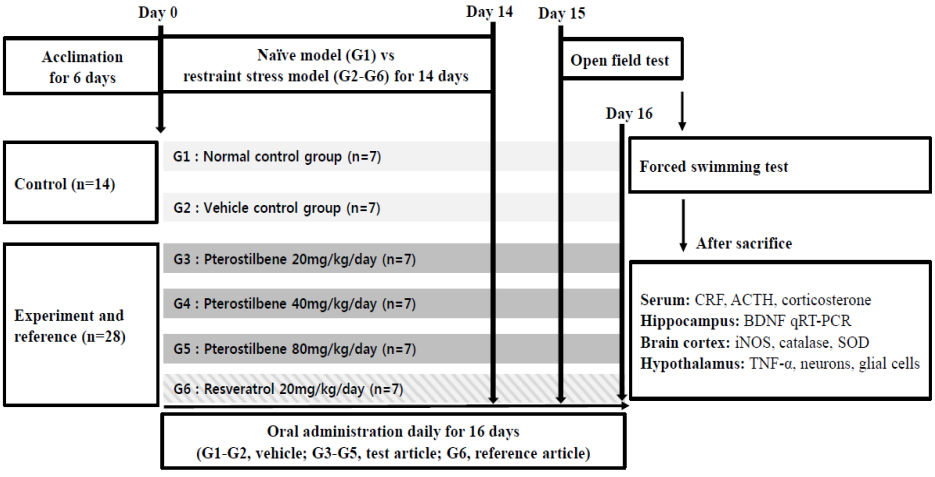


Figure 1. Flowchart of the study design. CRF, corticotropin-releasing factor; ACTH, adrenocorticotropic hormone; BDNF, brain-derived neurotrophic factor; iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase; TNF-α , tumor necrosis factor alpha.

4. Body weight measurement and behavioral tests

A. Clinical signs and body weight

The mortality of all animals were monitored once daily and individually recorded for 16 days. The day of the first drug administration was defined as day 1. The body weight was measured once weekly.

B. Open field test

On day 15, the open field test was conducted 1 h after the drug or vehicle administration. The test was conducted in an acrylic box (40 × 40 × 40 cm)

open to the air. The floor of the box was divided by lines into sixteen squares (10 × 10 cm). The animals were placed in the center of the box and allowed to explore the environment for 5 min.[25,26] Movements were recorded for later analysis using a video tracking system (SMART ver. 3.6.1, Harvard Apparatus, Holliston, MA, USA). The time spent in the central area ('time in center') of the apparatus was calculated.

C. Forced swimming test

On day 16, the forced swimming test was conducted for 10 min in a swimming pool with a diameter of 18 cm and a height of 50 cm filled to a depth of 20 cm with warm water (23 ± 2 ℃). One hour after drug or vehicle treatment, mice were placed individually in the pool. A weight (5% of the body weight) was attached to the tail root of the tested mouse. The swimming time, defined as the time until the mouse could not stay above the water surface for more than 5 s, was measured.[27,28]

5. Blood sampling and tissue collection

A. Endocrine hormone measurement

Mice were euthanized after the forced swimming test. After the mice were anesthetized with isoflurane, blood was drawn from the inferior vena cava. The collected blood was injected into a vacutainer tube containing a clot activator and allowed to stand at room temperature for 15–20 min. The coagulated blood was centrifuged for 10 min, and the serum was collected and stored in a freezer at - 80 ℃ for the measurement of endocrine hormone levels. Hormones were measured using enzyme-linked immunosorbent assay (ELISA) kits for corticotropin-releasing factor (CRF; Cusabio Biotech Co., Ltd., Houston, TX, USA), adrenocorticotropic hormone (ACTH; Cusabio Biotech Co., Ltd.), and corticosterone (Arbor Assays, Ann Arbor, MI, USA).

B. Brain-derived neurotrophic factor (BDNF) and oxidative stress measurements

After blood collection, brains were harvested. The hippocampus and cortex were frozen, and the hypothalamus was fixed in 10% neural buffered formalin solution. Hippocampal BDNF was quantitated by qRT-PCR using hippocampal samples. The primer sequences and product size were as follows:

Forward: 5'-GAC AAG GCA ACT TGG CCT AC-3'

Reverse: 5'-CCT GTC ACA CAC GCT CAG CTC-3'

Product size: 356 base pairs

The levels of inducible nitric oxide synthase (iNOS), catalase, and superoxide dismutase (SOD) in the brain cortex were measured using ELISA kits for iNOS, catalase, and SOD, respectively (all Cusabio Biotech Co., Ltd., Houston, TX, USA).

C. Histopathological assessment

Approximately equal regions of individual hypothalamus tissues were crossly trimmed. All hypothalamus tissue samples were fixed in 10% neutral buffered formalin solution for 24 h. Subsequently, paraffin blocks were prepared using an automated tissue processor (Shandon Citadel 2000; Thermo Scientific, Waltham, MA, USA) and embedding center (Shandon Histostar; Thermo Scientific, Waltham, MA, USA), and sections of 3–4 μm were prepared as two serial sections from each paraffin-embedded hypothalamus using an automated microtome (RM2255; Leica Biosystems, Nussloch, Germany). Representative sections were stained with 0.1% cresyl violet for general histopathology or with avidin-biotin-peroxidase complex-based immunohistochemical staining for tumor necrosis factor alpha (TNF-α) according to previously established methods. Samples were observed using a light microscope (Eclipse 80i; Nikon, Tokyo, Japan).

The mean neuron and glial cell numbers (cells/mm^2) in the paraventricular nucleus (PVN) regions were calculated for histomorphometric analysis of the cresyl violet-stained samples using a computer-assisted image analysis program. Furthermore, cells with over 20% immunoreactivity for anti-TNF-α antibodies were considered immunopositive, and the mean numbers of immunopositive cells (cells/mm^2) in the PVN regions were determined by an automated image analyzer. One histological field per hypothalamus section, with a total of seven hypothalamus tissue samples per group, was considered for statistical analysis in the current study. The histopathologist was blinded to the experimental conditions when analyzing the samples.

6. Statistical analyses

All data were tested in normal distribution and homogeneity of variance, and abnormal data were eliminated. The results were expressed as the means ± standard errors of the mean (S.Es.). Comparison between the vehicle group and the normal control group were performed with Student's t-test. Then, one-way analysis of variance (ANOVA) followed by Dunnett's test was used for comparisons between the vehicle group and the experimental groups. Statistical analyses were conducted using SPSS 12.0K for Windows software (SPSS Inc., Chicago, IL, USA). Differences with *p*-value of less than 0.05 were considered significant.

III. RESULTS

1. Clinical signs and body weight

No mortality was observed in any of the six groups of mice. The vehicle group (G2) exhibited a significant decrease in body weight compared to the normal control group (G1) on day 8 and day 15 ($p < 0.01$ and $p < 0.05$, respectively).

The body weight changes in the test article groups (G3-G5) and the reference article group (G6) did not significantly differ from those in the vehicle group (G2; Table 1).

Table 1. Changes in body weights.

Group	Body weight (g)		
	Day 1	Day 8	Day 15
G1 (n=7)	31.71±0.45	33.15±0.63	34.30±0.84
G2 (n=7)	31.69±0.47	30.54±0.48^{++}	31.45±0.46^{+}
G3 (n=7)	32.06±0.78	31.34±0.73	32.04±1.05
G4 (n=7)	31.22±0.22	30.41±0.31	31.93±0.41
G5 (n=7)	31.74±0.45	30.07±0.92	31.50±0.97
G6 (n=7)	31.76±0.65	31.21±0.57	32.62±0.53

The data are expressed as the means ± S.Es. The results were statistically analyzed using Student's t-test and one-way ANOVA. ($^{+}$) Significantly different from G1, $p < 0.05$; ($^{++}$) significantly different from G1, $p < 0.01$. S.E., standard error of the mean.

2. Open field test

The vehicle group (G2) had a significantly decreased mean value for the parameter 'time in center' compared to the normal control group (G1) ($p < 0.01$). The group treated with 80 mg/kg/day pterostilbene (G5) spent significantly more time in the center than the vehicle group (G2, $p < 0.05$), whereas this parameter was not significantly different between the 20 mg/kg/day pterostilbene treatment group (G3) and the vehicle group (G2). The 40 mg/kg/day pterostilbene treatment group (G4), as well as the reference article group (G6), exhibited a tendency towards an increased 'time in center' compared to that in the vehicle group (G2; Figure 2).

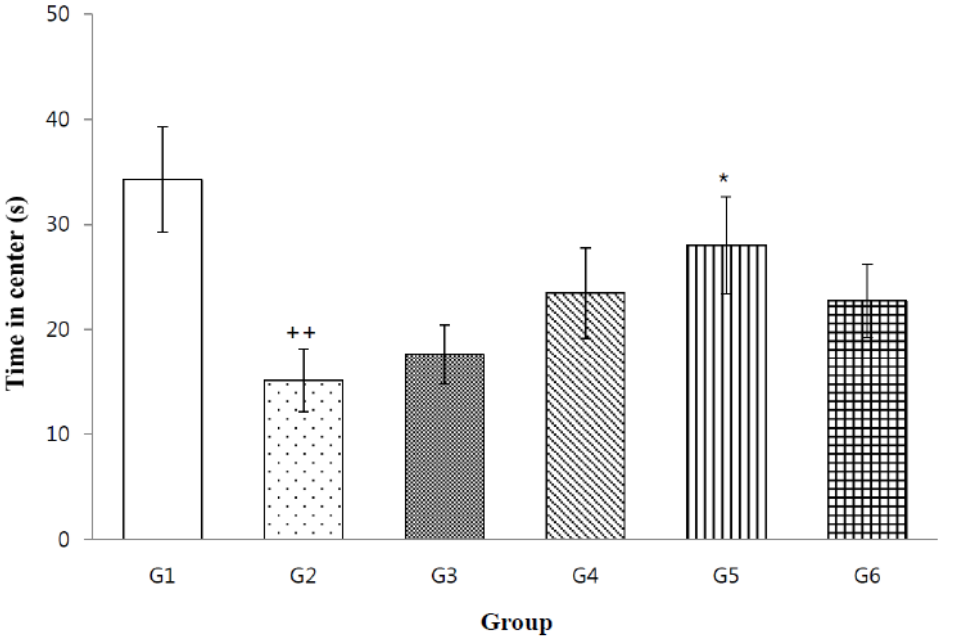


Figure 2. Open field test. The data are expressed as the means ± S.Es. The results were statistically analyzed using Student's t-test and one-way ANOVA. ($^{++}$) Significantly different from G1, $p < 0.01$; (*) significantly different from G2, $p < 0.05$. S.E., standard error of the mean.

3. Forced swimming test

The swimming time was significantly decreased in the vehicle group (G2) compared to the normal control group (G1) ($p < 0.01$). All test article (G3-G5) and reference article (G6) groups displayed a tendency towards an increased swimming time compared to that in the vehicle group (G2; Figure 3).

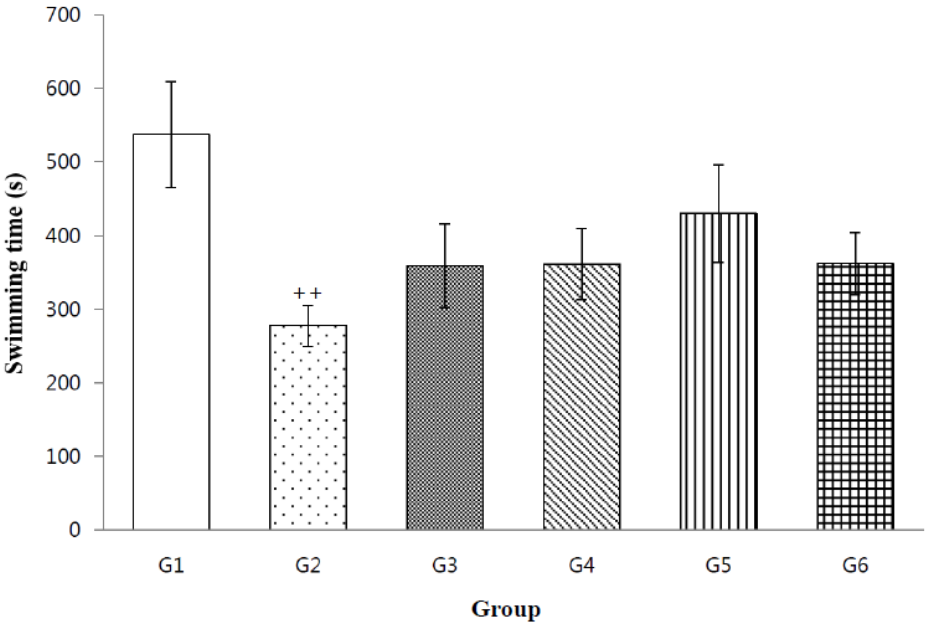


Figure 3. Forced swimming test. The data are expressed as the means ± S.Es. The results were statistically analyzed using Student's t-test and one-way ANOVA. ($^{++}$) Significantly different from G1, $p < 0.01$. S.E., standard error of the mean.

4. Hippocampal BDNF expression

The vehicle group (G2) exhibited a significantly lower relative BDNF expression than the normal control group (G1) ($p < 0.05$). Treatment with 20 and 80 mg/kg/day pterostilbene (G3 and G5, respectively) significantly increased the relative BDNF expression level compared to that in the vehicle group (G2, $p < 0.05$), whereas the group treated with 40 mg/kg/day pterostilbene (G4) showed a mean relative BDNF expression level similar to and not significantly different from that in the vehicle control group (G2). The reference article group (G6) also exhibited a significantly higher relative BDNF expression level than the vehicle group (G2, $p < 0.01$; Figure 4).

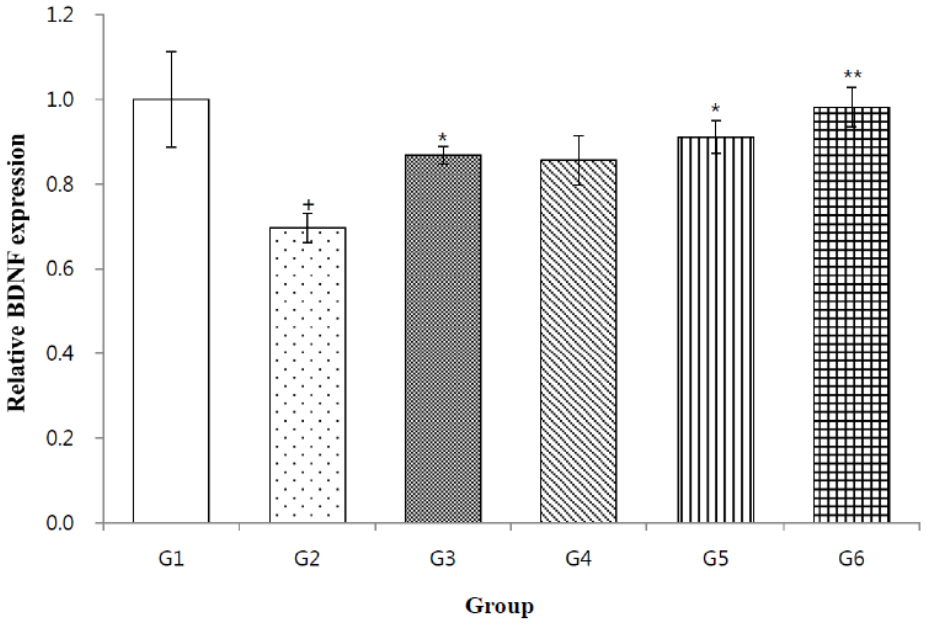


Figure 4. Relative BDNF expression in the hippocampus. The data are expressed as the means ± S.Es. The results were statistically analyzed using Student's t-test and one-way ANOVA. ($^{+}$) Significantly different from G1, $p < 0.05$; (*) significantly different from G2, $p < 0.05$; (**) significantly different from G2, $p < 0.01$. BDNF, brain-derived neurotrophic factor. S.E., standard error of the mean.

5. Endocrine hormonal assays

In the corticosterone measurements, the vehicle group (G2) had significantly higher corticosterone levels than the normal control group (G1, $p < 0.05$). The test article groups (G3-G5) exhibited a tendency towards decreased corticosterone levels compared to those in the vehicle group (G2), whereas the reference article group (G6) showed a statistically significant difference compared to the vehicle group (G2, $p < 0.05$; Figure 5a). Furthermore, we also measured the ACTH levels in the blood. The vehicle group (G2) exhibited significantly higher ACTH levels than the normal control group (G1, $p < 0.01$).

The group treated with 80 mg/kg/day pterostilbene (G5) exhibited significantly lower ACTH levels than the vehicle group (G2, $p < 0.05$), whereas for the other test article groups (G3-G4), a nonsignificant tendency towards reduced ACTH levels were observed with respect to the vehicle group. Moreover, the reference article group (G6) exhibited significantly lower ACTH levels than the vehicle group (G2, $p < 0.01$; Figure 5b). However, the CRF levels in the vehicle group (G2) were not significantly different from those in any other group (G1 and G3-G6; Table 2).

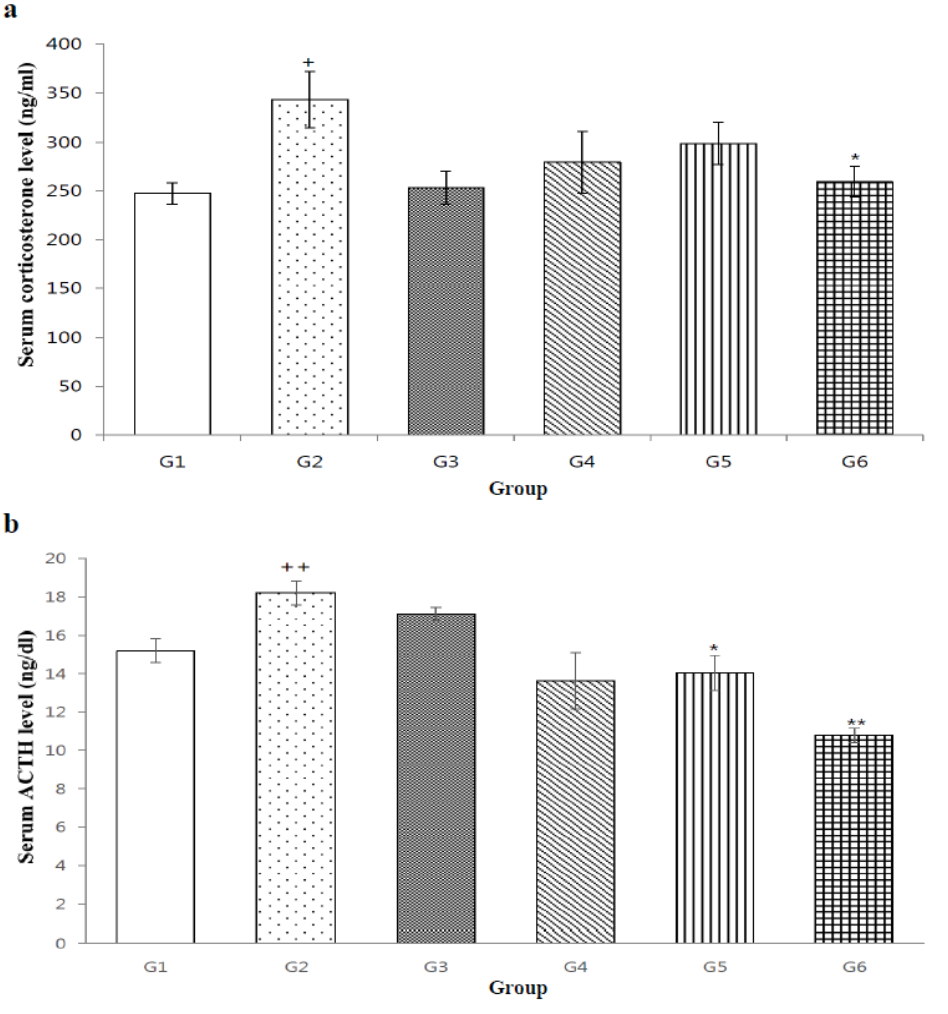


Figure 5. Corticosterone (a) and ACTH (b) levels. The data are expressed as the means ± S.Es. The results were statistically analyzed using Student's t-test and one-way ANOVA. ($^{+}$) Significantly different from G1, $p < 0.05$; ($^{++}$) significantly different from G1, $p < 0.01$; (*) significantly different from G2, $p < 0.05$; (**) significantly different from G2, $p < 0.01$. ACTH, adrenocorticotropic hormone. S.E., standard error of the mean.

Table 2. Endocrine hormone levels.

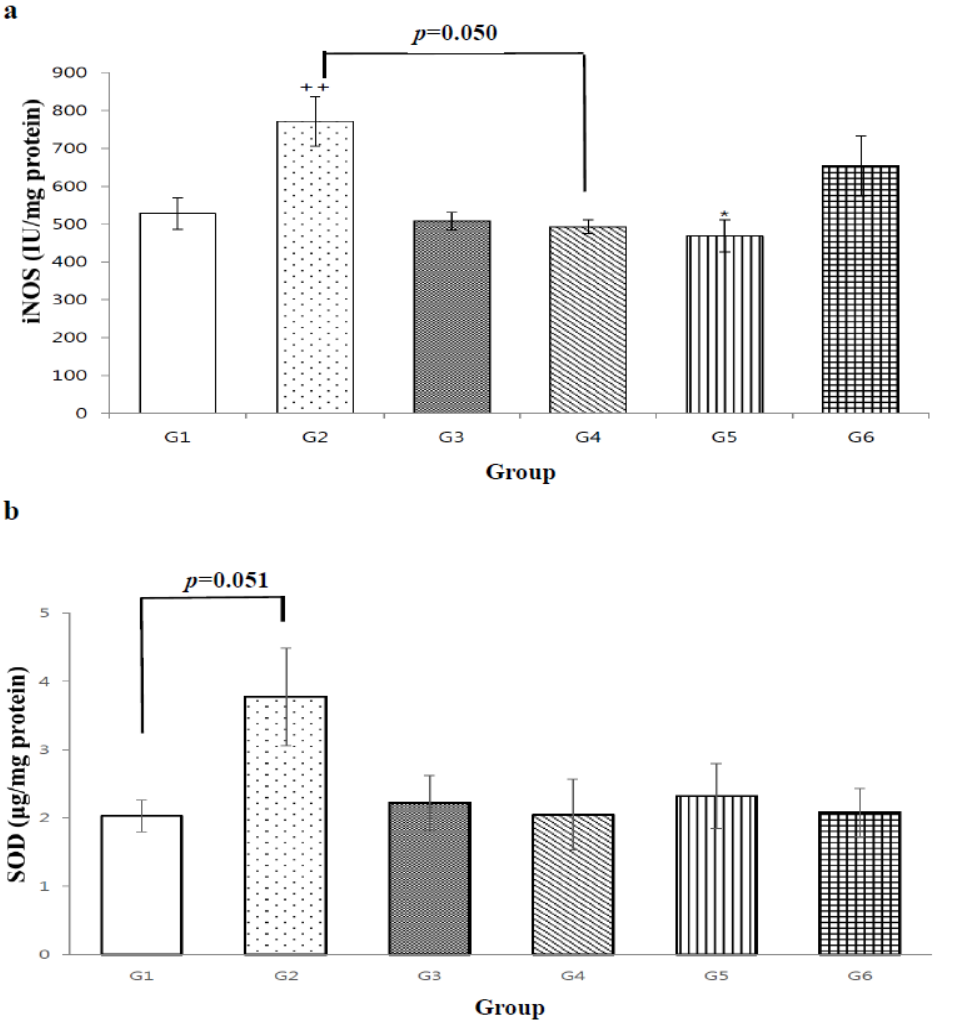
Group	Corticosterone (ng/mL)	ACTH (ng/dL)	CRF (pg/mL)
G1 (n=7)	247.37±10.86	15.19±0.63	6.68±0.44
G2 (n=7)	343.56±28.83^{+}	18.21±0.64^{++}	6.70±0.49
G3 (n=7)	253.29±16.81	17.11±0.35	9.03±0.64
G4 (n=7)	279.00±31.70	13.64±1.47	8.26±0.87
G5 (n=7)	298.67±21.69	14.03±0.91*	6.75±0.30
G6 (n=7)	259.15±15.56*	10.77±0.39**	7.51±0.29

The data are expressed as the means ± S.Es and were statistically analyzed using Student's t-test and one-way ANOVA. ($^{+}$) Significantly different from G1, $p < 0.05$; ($^{++}$) significantly different from G1, $p < 0.01$; (*) significantly different from G2, $p < 0.05$; (**) significantly different from G2, $p < 0.01$. ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor. S.E., standard error of the mean.

6. Measurement of oxidative stress in the cerebral cortex

Cerebral cortex samples from the vehicle group (G2) exhibited significantly higher iNOS levels than the normal control group (G1, $p < 0.01$). The iNOS levels in the 80 mg/kg/day pterostilbene group (G5) were significantly lower than those in the vehicle group (G2, $p < 0.05$). All other test article groups (G3 and G4) and the reference article group (G6) exhibited a trend towards

decreased iNOS levels compared to those in the vehicle group (G2; Figure 6a). In further measurements of the SOD levels, the vehicle group (G2) tended to increased levels in comparison to the normal control group (G1). By contrast, the test article (G3-G5) and reference article (G6) groups presented the tendency for a decrease in SOD levels compared to the vehicle group (G2; Figure 6b). The catalase levels of the vehicle group (G2) were not significantly different from all other groups (G1 and G3-G6; Figure 6c).



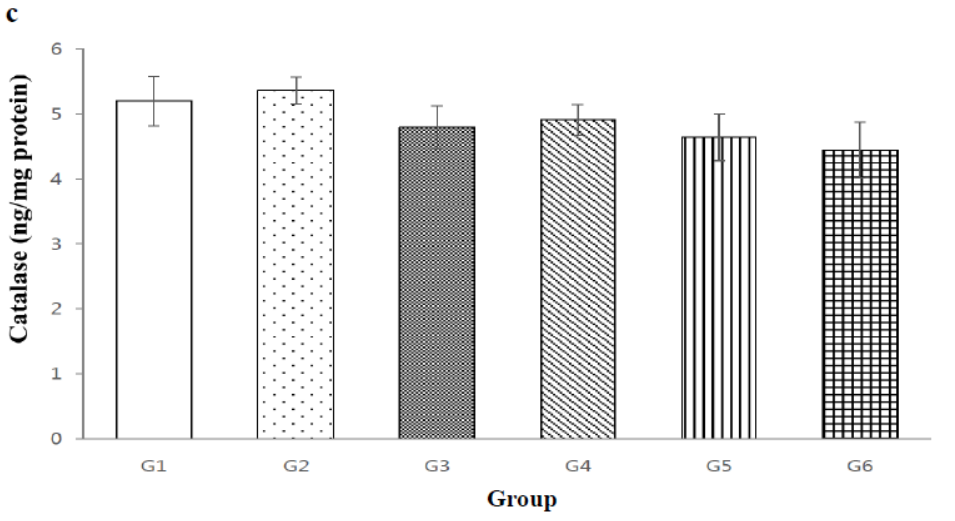


Figure 6. iNOS (a), SOD (b), and catalase (c) levels. The data are expressed as the means ± S.Es. The results were statistically analyzed using Student's t-test and one-way ANOVA. ($^{++}$) Significantly different from G1, $p < 0.01$; (*) significantly different from G2, $p < 0.05$. iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase. S.E., standard error of the mean.

7. Histopathological analysis of hypothalamus tissue

We also determined the numbers of neurons in brain slices containing hypothalamus tissue. These numbers were significantly lower in the vehicle group (G2) than in the normal control group (G1, $p < 0.01$). This effect was reversed in the test article (G3-G5) and reference article (G6) groups with significantly increased numbers of neurons compared to the vehicle group (G2, $p < 0.01$). By contrast, the numbers of glial cells were significantly increased in the vehicle group (G2) than in the normal control group (G1, $p < 0.01$). The groups treated with 40 and 80 mg/kg/day pterostilbene (G4 and G5) had significantly fewer glial cells than the vehicle group (G2, $p < 0.01$), whereas the group treated with 20 mg/kg/day pterostilbene (G3) showed only a tendency towards a decrease. Similar to the higher pterostilbene

concentrations, the reference article group (G6) also presented significantly decreased numbers of glial cells compared to the vehicle group (G2, $p < 0.05$). Regarding the numbers of TNF-α immunopositive cells, the vehicle group (G2) had significantly increased numbers of immunolabeled cells compared to the normal control group (G1, $p < 0.01$). All test article groups (G3-G5) exhibited statistically significant decreases in the numbers of TNF-α immunolabeled cells compared to the vehicle group (G2, $p < 0.05$ or $p < 0.01$). Similarly, the reference article group (G6) exhibited significantly fewer TNF-α immunolabeled cells than the vehicle group (G2, $p < 0.01$; Table 3, Figure 7).

Table 3. Histomorphometric analysis of the hypothalamic PVN regions.

Group	Neurons (cells/mm^2)	Glial cells (cells/mm^2)	TNF-α positive cells (cells/mm^2)
G1 (n=7)	287.43±8.49	147.43±9.67	12.57±3.54
G2 (n=7)	163.71±12.18^{++}	339.43±28.57^{++}	106.86±13.54^{++}
G3 (n=7)	235.43±12.74**	219.43±14.60	34.29±5.91*
G4 (n=7)	244.00±16.17**	208.57±17.88**	29.14±5.36**
G5 (n=7)	267.14±7.24**	193.14±21.08**	22.86±4.60**
G6 (n=7)	244.57±15.80**	223.14±23.52*	27.43±6.20**

The data are expressed as the means ± S.Es and were statistically analyzed using Student's t-test and one-way ANOVA. ($^{++}$) Significantly different from G1, $p < 0.01$; (*) significantly different from G2, $p < 0.05$; (**) significantly different from G2, $p < 0.01$. PVN, paraventricular nucleus; TNF, tumor necrosis factor.

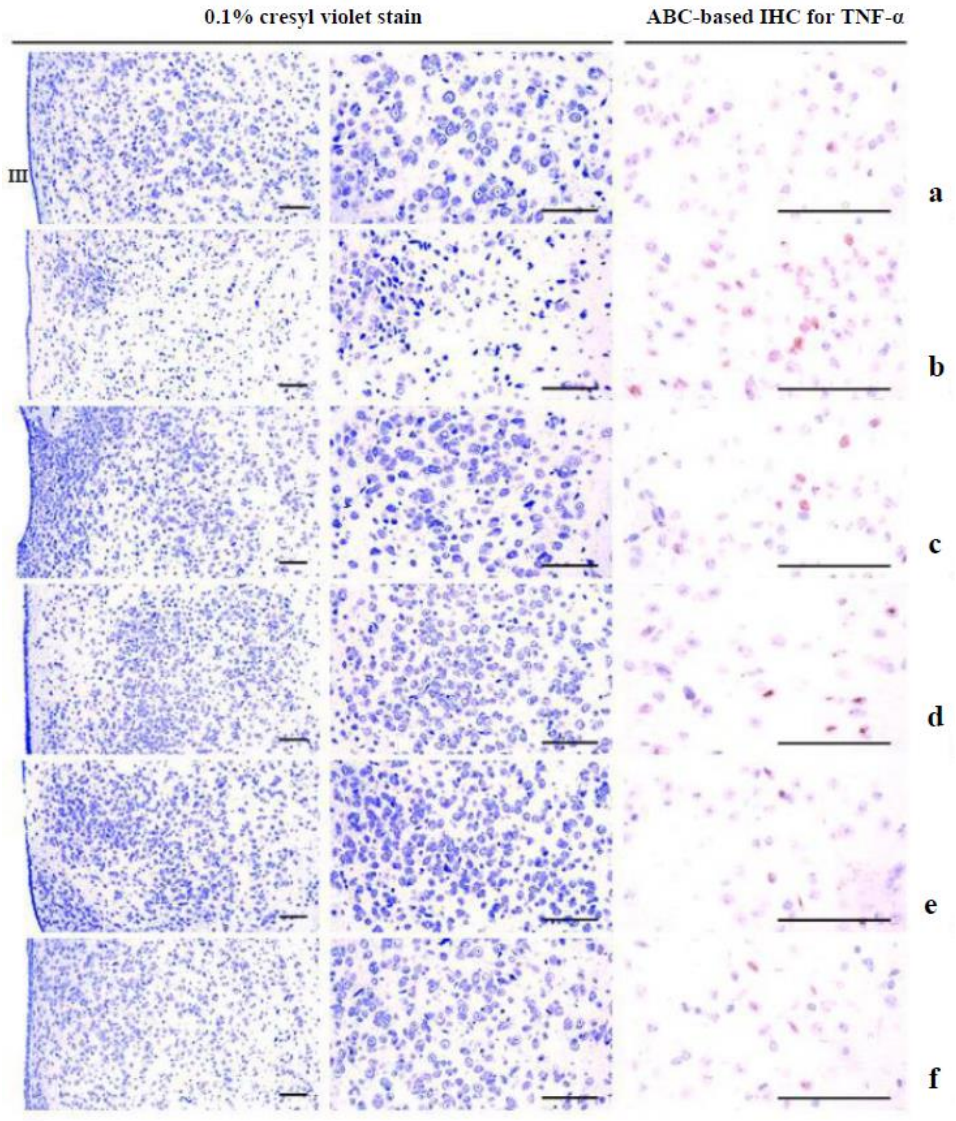


Figure 7. Representative histopathological profiles of the hypothalamic PVN regions. a = Normal control (G1), b = Vehicle control (G2), c = 20 mg/kg/day pterostilbene (G3), d = 40 mg/kg/day pterostilbene (G4), e = 80 mg/kg/day pterostilbene (G5), f = 20 mg/kg/day resveratrol (G6). All scale bars represent 80 μm. TNF-α, tumor necrosis factor alpha; ABC, avidin-biotin-peroxidase complex; IHC, immunohistochemistry; III, third ventricle.

IV. DISCUSSION

We found that in a mouse stress model, oral pterostilbene administration ameliorated stress-related behavioral anxiety and despair, which were associated with neuroinflammatory modulations, including restoration of BDNF levels, reduction in the oxidative stress burden, and inhibition of HPA axis hyperactivity.

The stress model comprises the response of the animal to various harmful factors, the corresponding defense reactions, and the general maladaptation syndrome. The restraint stress model in rodents simultaneously induces physical and mental stress and is usually performed to study behaviors associated with anxiety and depression in humans, thus helping to assess pathogenic mechanisms and potential clinical drug effects.[29]

This study was performed to evaluate the efficacy of repeated oral pterostilbene administration for 16 days on stress-related behaviors, neuroinflammation, and HPA axis hyperactivity in a mouse stress model. Compared to the normal control group, the stress-exposed vehicle group had a significantly decreased body weight on days 8 and 15, which may have been induced by prolonged physical and emotional stress. In the open field and forced swimming tests, the vehicle group spent significantly less time in the central area than the normal control group, and the swimming time was similarly reduced. Pterostilbene treatment partially reversed these effects; we observed a tendency towards increased times in the pterostilbene-treated groups compared to those in the vehicle group. The open field and forced swimming tests are used to observe changes in behavioral patterns related to anxiety and depression.[30] The open field test is usually performed to evaluate general locomotor and exploratory behaviors, which are associated with anxiety-like behaviors in mouse models.[31] The forced swimming test has been extensively adopted because the immobility time during stressful conditions may reflect the

tendency for behavioral despair or helplessness in animals.[32] Based on our study results, pterostilbene effectively improves stress-induced abnormal behaviors.

BDNF regulates the density of inhibitory synapses in organotypic hippocampal cultures and is important for modulating synaptic transmission and neuronal survival.[33] Animal studies have demonstrated that the pathophysiological changes in repeated stress situations or mood disorders include decreased BDNF levels, which may lead to decreased neurogenesis and increased remodeling of dendrites, thus inducing vulnerability to subsequent episodes of depression.[34,35] In our experiments, the vehicle group had significantly lower BDNF levels than the normal control group due to the chronic stress conditions, whereas the pterostilbene treatment groups generally exhibited higher BDNF levels than the vehicle group. Although the mechanisms underlying anxiety and depression are incompletely understood, stress is an important inducing factor and is usually accompanied by dysfunction of the neuroimmune-endocrine connection.[25] Indeed, HPA axis hyperactivity can be a crucial biological mechanisms underlying maladaptive behavioral changes. In our study, the vehicle group exhibited significantly higher ACTH and corticosterone levels than the normal control group. These stress-related effects were reversed by pterostilbene treatment, with significant decreases—or at least trends towards a decrease—in ACTH and corticosterone levels compared to those in the vehicle group. Therefore, pterostilbene is effective in reversing stress-induced abnormalities in BDNF, ACTH, and corticosterone levels.

The HPA axis can be affected by many factors, such as cytokines and nitric oxide (NO). Cytokines, which are generally secreted by immune cells, can also be produced and secreted by nonimmune cells to signal to neuroimmune cells. Proinflammatory cytokines, including interleukin-1β, interleukin-6, and TNF-α, are secreted by all components of the HPA axis.[36,37] NO, acting as a neurotransmitter in the brain, is generated by neuronal nitric oxide synthase (nNOS) and iNOS in the brain and is involved in the regulation of the HPA axis,

but overexpression of nNOS and iNOS can lead to HPA hyperactivity with stress-related behavioral changes.[38] In our measurements of iNOS, SOD, and catalase levels, the vehicle group presented significant increases—or trends towards increases—in these levels compared to those in the normal control group. Pterostilbene treatment either significantly decreased or caused a trend towards a decrease in the levels of oxidative stress markers compared to those in the vehicle group. Our data suggest that administration of pterostilbene reduced reactive oxygen species generation and macrophage activation induced by restraint stress.

Finally, we analyzed histopathological changes in the hypothalamus. Compared to the normal control group, the vehicle group had significantly decreased numbers of neurons and, simultaneously, increased numbers of glial cells and TNF-α immunolabeled cells in the hypothalamic PVN regions. This pattern suggests that restraint stress can induce neuroinflammation-related gliosis and neuronal loss in the hypothalamic PVN. However, these effects, stress-induced gliosis and neuronal loss in the hypothalamic PVN, were significantly inhibited by pterostilbene in a dose-dependent manner. These results suggest that the oral administration of pterostilbene exerts beneficial, dose-dependent, protective effects against stress-induced damage to the hypothalamic PVN, potentially indicating anti-inflammatory activity via the inhibition of TNF-α, a proinflammatory cytokine and a marker of gliosis. In addition, pterostilbene exerts favorable protective effects against neuroinflammation in a dose-dependent manner, while additionally exhibiting beneficial properties that reduce oxidative stress in the brain. Based on the good safety profile of pterostilbene,[39] higher dosages may be considered in a future study. Taken together, these observations indicate that pterostilbene reverses stress-related upregulation of oxidative stress markers and hypothalamic hormones. Additionally, pterostilbene administration prevented stress-induced upregulation of proinflammatory markers and neuronal loss in the

hypothalamus. In addition, the effects of pterostilbene effects on mitochondria may be, in part, linked to an increase in the oxidative stress-related threshold for mitochondrial permeability transition, thereby preventing the potential apoptosis of vulnerable cells.[8]

This study provided the first convincing data that induced stress alters neuroinflammatory and neuroendocrinological parameters and that these changes are, at least partially, reversed by oral administration of pterostilbene, a substance that can be found in natural products such as blueberries. This finding may lead to novel insights into the pathophysiology of depression and anxiety, ultimately leading to new therapeutic approaches. However, we should acknowledge the limitations of our study. To evaluate stress-related hormonal changes, blood samples were obtained from the inferior vena cava. The pterostilbene treatment groups had lower levels of ACTH and corticosterone but not CRF than the vehicle group. To evaluate CRF activity more accurately, examination of CRF receptors or CRF gene transcription in the brain are better assessment approaches.

V. CONCLUSION

The oral administration of pterostilbene for 16 days in restraint-induced stress mice increased significantly the times in the open field and forced swimming tests, elevated the BDNF levels, decreased iNOS and SOD levels in the cerebral cortex, and reduced plasma levels of ACTH and corticosterone. Additionally, pterostilbene was shown to dose-dependently increase the numbers of neurons and decrease the numbers of glial cells and TNF-α immunolabeled cells in hypothalamic PVN regions in comparison to the vehicle group. These findings suggest that pterostilbene could be an effective treatment option in stress-related abnormal behaviors that are based on the modulation of neuroinflammation and the HPA axis hyperactivity.

REFERENCES

1. Roupe KA, Remsberg CM, Yáñez JA, Davies NM. Pharmacometrics of stilbenes: seguing towards the clinic. Curr Clin Pharmacol 2006;1:81-101.
2. Lin HS, Yue BD, Ho PC. Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in pre-clinical pharmacokinetic study. Biomed Chromatogr 2009;23:1308-15.
3. Rimando AM, Kalt W, Magee JB, Dewey J, Ballington JR. Resveratrol, pterostilbene, and piceatannol in vaccinium berries. J Agric Food Chem 2004;52:4713-9.
4. Patel S. Blueberry as functional food and dietary supplement: The natural way to ensure holistic health. Med J Nutrition Metab 2014;7:133-43.
5. Kalt W, Joseph JA, Shukitt-Hale B. Blueberries and human health: a review of current research. J Am Pomological Soc 2007;61:151-60.
6. Ma L, Sun Z, Zeng Y, Luo M, Yang J. Molecular mechanism and health role of functional ingredients in blueberry for chronic disease in human beings. Int J Mol Sci 2018;19:E2785.
7. Shi M, Loftus H, McAinch AJ, Su XQ. Blueberry as a source of bioactive compounds for the treatment of obesity, type 2 diabetes and chronic inflammation. J Funct Foods 2017;30:16-29.
8. McCormack D, McFadden D. A review of pterostilbene antioxidant activity and disease modification. Oxid Med Cell Longev 2013;2013:575482.
9. McCormack D, McFadden D. Pterostilbene and cancer: current review. J Surg Res 2012;173:e53-e61.
10. Park ES, Lim Y, Hong JT, Yoo HS, Lee CK, Pyo MY, et al. Pterostilbene, a natural dimethylated analog of resveratrol, inhibits rat

aortic vascular smooth muscle cell proliferation by blocking Akt-dependent pathway. Vascul Pharmacol 2010;53:61-7.

11. Zhang L, Zhou G, Song W, Tan X, Guo Y, Zhou B, et al. Pterostilbene protects vascular endothelial cells against oxidized low-density lipoprotein-induced apoptosis in vitro and in vivo. Apoptosis 2012;17:25-36.

12. Pari L, Satheesh MA. Effect of pterostilbene on hepatic key enzymes of glucose metabolism in streptozotocin-and nicotinamide-induced diabetic rats. Life Sci 2006;79:641-5.

13. Kapetanovic IM, Muzzio M, Huang Z, Thompson TN, McCormick DL. Pharmacokinetics, oral bioavailability, and metabolic profile of resveratrol and its dimethylether analog, pterostilbene, in rats. Cancer Chemother Pharmacol 2011;68:593-601.

14. Remsberg CM, Yáñez JA, Ohgami Y, Vega-Villa KR, Rimando AM, Davies NM. Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. Phyto Res 2008;22:169-79.

15. Rotches-Ribalta M, Andres-Lacueva C, Estruch R, Escribano E, Urpi-Sarda M. Pharmacokinetics of resveratrol metabolic profile in healthy humans after moderate consumption of red wine and grape extract tablets. Pharmacol Res 2012;66:375-82.

16. Temsamani H, Krisa S, Mérillon J-M, Richard T. Promising neuroprotective effects of oligostilbenes. Nutr Aging (Amst) 2015;3:49-54.

17. Campos AC, Fogaça MV, Aguiar DC, Guimarães FS. Animal models of anxiety disorders and stress. Brazilian J Psychiatry 2013;35:S101-S11.

18. Lopresti AL, Hood SD, Drummond PD. A review of lifestyle factors

that contribute to important pathways associated with major depression: diet, sleep and exercise. J Affect Disord 2013;148:12-27.

19. Zafir A, Banu N. Induction of oxidative stress by restraint stress and corticosterone treatments in rats. Indian J Biochem Biophys 2009;46:53-8.

20. Kvetňanský R, Mikulaj L. Adrenal and urinary catecholamines in rats during adaptation to repeated immobilization stress. Endocrinology 1970;87:738-43.

21. Kasuga S, Ushijima M, Morihara N, Itakura Y, Nakata Y. Effect of aged garlic extract (AGE) on hyperglycemia induced by immobilization stress in mice. Nihon Yakurigaku Zasshi 1999;114:191-7.

22. Rai D, Bhatia G, Sen T, Palit G. Comparative study of perturbations of peripheral markers in different stressors in rats. Can J Physiol Pharmacol 2003;81:1139-46.

23. Choi YJ, Lee HY, Kim Y, Cho SH. Scolopendra pharmacopuncture ameliorates behavioral despair in mice stressed by chronic restraint. J Pharmacopuncture 2017;20:257-64.

24. Son SW, Lee JS, Kim HG, Kim DW, Ahn YC, Son CG. Testosterone depletion increases the susceptibility of brain tissue to oxidative damage in a restraint stress mouse model. J Neurochem 2016;136:106-17.

25. Wang S, Wang C, Yu Z, Wu C, Peng D, Liu X, et al. Agarwood essential oil ameliorates restrain stress-induced anxiety and depression by inhibiting HPA axis hyperactivity. Int J Mol Sci 2018;19:E3468.

26. Yan MZ, Chang Q, Zhong Y, Xiao BX, Feng L, Cao FR, et al. Lotus leaf alkaloid extract displays sedative-hypnotic and anxiolytic effects through GABAA receptor. J Agric Food Chem 2015;63:9277-85.

27. Yankelevitch-Yahav R, Franko M, Huly A, Doron R. The forced swim test as a model of depressive-like behavior. J Vis Exp 2015;97:e52587.

28. Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. Arch Int Pharmacodyn Ther 1977;229:327-36.

29. Bourin M, Petit-Demoulière B, Nic Dhonnchadha B, Hascöet M. Animal models of anxiety in mice. Fundam Clin Pharmacol 2007;21:567-74.

30. Lee EH, Jung IC. Anti-depressive effects of OnDam-Tang with addition of Linderae Radix (ODT-L) after chronic Immobilization Stress in C57BL/6 mice. J Physiol & Pathol Korean Med 2014;28:403-10.

31. Moore A, Beidler J, Hong M. Resveratrol and depression in animal models: a systematic review of the biological mechanisms. Molecules 2018;23:E2197.

32. Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology (Berl) 1985;85:367-70.

33. Ivanova T, Beyer C. Pre-and postnatal expression of brain-derived neurotrophic factor mRNA/protein and tyrosine protein kinase receptor B mRNA in the mouse hippocampus. Neurosci Lett 2001;307:21-4.

34. Li S, Wang C, Wang M, Li W, Matsumoto K, Tang Y. Antidepressant like effects of piperine in chronic mild stress treated mice and its possible mechanisms. Life Sci 2007;80:1373-81.

35. Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. Neurobiology of depression. Neuron 2002;34:13-25.

36. Gądek-Michalska A, Tadeusz J, Rachwalska P, Bugajski J. Cytokines, prostaglandins and nitric oxide in the regulation of stress-response systems. Pharmacol Rep 2013;65:1655-62.

37. Berthold-Losleben M, Himmerich H. The TNF-α system: functional

aspects in depression, narcolepsy and psychopharmacology. Curr Neuropharmacol 2008;6:193- 202.

38. Zhou QG, Zhu LJ, Chen C, Wu HY, Luo CX, Chang L, et al. Hippocampal neuronal nitric oxide synthase mediates the stress-related depressive behaviors of glucocorticoids by downregulating glucocorticoid receptor. J Neurosci 2011;31:7579- 90.

39. Riche DM, McEwen CL, Riche KD, Sherman JJ, Wofford MR, Deschamp D, et al. Analysis of safety from a human clinical trial with pterostilbene. J Toxicol 2013;2013:463595.

ABSTRACT (IN KOREAN)

마우스에서 pterostilbene이 스트레스 관련 행동변화 및 기저 신경염증과 호르몬 변화에 미치는 영향

<지도교수 이 용 제>

연세대학교 대학원 의학과

박 병 진

배경: pterostilbene은 베리류 등에 소량 함유되어 있는 식물성 영양소로 최근 강력한 항산화 기능이 알려지면서 주목을 받기 시작했다. 이전의 동물실험에서 여러 암 종류와 대사 이상에 대한 연구들이 이루어진 바 있으나 신경면역 및 신경내분비 관련 연구는 이루어진 바 없다. 본 연구에서는 마우스에서 pterostilbene이 스트레스 관련 행동변화 및 기저 신경염증과 호르몬 변화에 미치는 영향을 규명해 보고자 하였다.

방법: 모두 여섯 개의 그룹으로 나누고 (각 그룹당 마우스 수=7) 첫 번째 그룹은 정상 생활군, 나머지는 스트레스군으로 설정하였다. 스트레스군은 각각 위약군, pterostilbene kg당 20mg, 40mg, 80mg 투여군, 참고군으로 레스베라트롤 kg당 20mg 투여군으로 분류하였다. 14일간 구속스트레스 프로토콜을 동일하게 진행하였고, 16일간 실험물질을 경구 투여하였다. 행동평가를 위해 open field test, forced

swimming test를 시행하였고, 혈액에서 시상하부-뇌하수체-부신 축 관련 호르몬을 측정하였고, 뇌의 해마에서 뇌유래신경영양인자(brain-derived neurotrophic factor)를, 뇌 피질에서 산화스트레스 지표 (inducible nitric oxide synthase, superoxide dismutase, catalase)를 평가하였다. 끝으로 시상하부 뇌실곁핵에서 종양괴사인자 알파 (tumor necrosis factor alpha)와 신경세포 및 신경아교세포 수를 측정하였다.

결과: pterostilbene은 open field test와 forced swimming test에서 스트레스 유발 행동변화에 대해 최소 부분적 호전 이상의 효과를 보였고, 스트레스로 인해 저하된 뇌 해마의 뇌유래신경영양인자의 하향조절을 개선하였다. 더불어, 스트레스로 인해 증가된 산화 스트레스와 시상하부-뇌하수체-부신 축 활성화에 대한 조절 효과를 보였다. 특히, 시상하부 뇌실곁핵에서 종양괴사인자 알파와 신경아교세포 수는 감소시키고, 신경세포 손실을 예방하였으며 pterostilbene 용량 증가에 따른 의미 있는 반응 효과를 보였다.

결론: 이 연구를 통해 스트레스에 의해 신경염증 및 신경호르몬의 생체지표 변화를 관찰하였고, pterostilbene의 투여에 의해 스트레스 관련 행동변화 및 기저 신경염증과 호르몬 변화의 개선을 확인하였다. 이는 스트레스 관련 행동변화에 대한 병태생리적 이해의 지평을 넓히고 치료적 접근에 있어 추가적 단초를 제시할 것으로 기대한다.

핵심되는 말: pterostilbene; 스트레스; 뇌유래신경영양인자; 신경염증; 시상하부-뇌하수체-부신 축