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Original Article

Hydrangeae Dulcis Folium Attenuates Physical Stress by Supressing ACTH-Induced Cortisol in Zebrafish*

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ABSTRACT **Objective:** To determine the effects of *Hydrangeae Dulcis Folium* (EHDF) on physical stress, changes in the whole-body cortisol level and behaviour in zebrafish (*Danio rerio*). **Methods:** One hundred and seventy-four fish were randomly divided into 4 [adrenocorticotropin hormone (ACTH) challenge test: 4 fish per group] or 6 groups (behavioural test: 10–12 fish per group, whole-body cortisol: 4 fish per group). Net handling stress (NHS) was used to induce physical stress. Fish were treated with vehicle or EHDF (5–20 mg/L) for 6 min before they were exposed to stress. And then, fish were sacrificed for collecting body fluid from whole-body or conducted behavioural tests, including novel tank test and open field test, and were evaluated to observe anxiety-like behaviours and locomotion. In addition, to elucidate the mode of action of the anti-stress effects of EHDF, ACTH (0.2 IU/g, i.p.) challenge test was performed. **Results:** The increased anxiety-like behaviours in novel tank test and open field test under stress were prevented by treatment with EHDF at 5–20 mg/L (P<0.05). Moreover, compared with the unstressed group, which was not treated with NHS, the whole-body cortisol level was significantly increased by treatment with NHS (P<0.05). Compared with the NHS-treated stressed control group, pre-treatment with EHDF at concentrations of 5–20 mg/L for 6 min significantly prevented the NHS-increased whole-body cortisol level (P<0.05). In addition, ACTH challenge test showed that EHDF completely blocked the effects of ACTH on cortisol secretion (P<0.05). **Conclusion:** EHDF may be a good anti-stress candidate and its mechanism of action may be related to its positive effects on cortisol release.

KEYWORDS net handling stress, zebrafish, *Hydrangeae Dulcis Folium*, whole-body cortisol, behavioural tests

Stress works as the cause of many diseases by being the origin of all diseases and causing both circulation disorders and disharmony in the human body.[1] When humans experience stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated and cortisol is secreted from the adrenal glands. In detail, the activation of the HPA axis induces the release of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH acts on the pituitary gland to stimulate adrenocorticotropin hormone (ACTH) synthesis. ACTH, in turn, stimulates cortisol secretion from the adrenal cortex, and then, the adrenal cortex secretes cortisol and glucocorticoid into the blood. Additionally, increased glucocorticoids regulate the action of the HPA axis by negative feedback.[2]

Cortisol has several significant functions and life-sustaining adrenal hormones essential to the maintenance of homeostasis.[3] Cortisol has advantageous effects such as supplying energy and improving immunity at a unstressed level.[4] However,

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when blood cortisol is maintained at a high level, it causes mental diseases including depression panic disorder, hippocampal damage in animals, memory loss and Cushing's syndrome.[5-7] The stress response transmitted by the HPA axis in mammals is similar to the situation of fish. The stress response is characterized by the activation of the hypothalamus-pituitary-interrenal (HPI) axis in fish.[8] Structures in fish homologous to the cortical tissue of the mammalian adrenal gland are in close proximity to or imbedded in the kidney. The zebrafish is a good model for the study of development genetics, the biology of human diseases, human diseases and behavioural pharmacology. Zebrafish have the advantages of easy handling as an experimental organism, cheap price, short generation length of approximately 3 months, and a high homogeny of genetic overlap of approximately 90% between zebrafish and humans.[9] Moreover, zebrafish are ideal for developing valid experimental models of stress, anxiety, and major depression and for discovering new therapeutics.[10]

Hydrangea serrata Seringe (Figure 1A) is a species of flowering plant in the family *Hydrangeaceae* that is widely cultivated in Asia, and it mainly grows on the southern coast including Jeju Island and Geoje Island in Korea. The total size is approximately 2–3 m, with colours ranging from white through pinks and violet. *Hydrangeae Dulcis Folium* (Figure 1B) is the dry and fermented leaf of *Hydrangea macrophylla or Hydrangea serrata* Seringe. It has antimicrobial and anti-allergic effects.[11] Extracts of *Hydrangeae Dulcis Folium* were examined for possible anti-malarial and anti-diabetic activities.[12,13] The principal chemical constituents of *Hydrangeae Dulcis Folium* and their derivatives are hydrangenol, phyllodulcin, thunberginols and halofuginones.[14,15] Hydrangenol, phyllodulcin and thunberginol A significantly lower the blood glucose and free fatty acid levels.[13] It has been newly demonstrated that halofuginone inhibits the development of autoimmunity in a mouse model of activating the amino acid response pathway.[16]

Figure 1. *Hydrangea serrata Seringe* (A) amd *Hydrangeae Dulcis Folium* (B)

Recently, people have been exposed to various types of stress, which may cause mental diseases such as depression and panic disorder. Therefore, a study that provides the theoretical background required for the development of new medicines and treatment methods by verifying the mechanism of the endocrine system hormones and stress is urgently required. However, until now, no attempts have been made to investigate the anti-stress activity of *Hydrangeae Dulcis Folium*. This study investigated the anti-stress activity of *Hydrangeae Dulcis Folium* in zebrafish using a method of behavioural pharmacology and molecular biology.

METHODS

Materials

ACTH and tricaine were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). *Hydrangeae Dulcis Folium* was purchased at the Han-Nong-Won Co. (Daegu, Korea), and a voucher specimen (JJNUOPS 2014-01) was deposited at the Marine Biomedical Science of the College of Ocean Sciences, Jeju National University. L-theanine was purchased from Santa Cruz Biotech (Santa Cruz, CA). The material was authenticated by Prof. Yong Han Kim of the Department of Herbal Medicinal Pharmacology, College of Herbal Bio-industry, Daegu Haany University. All other materials were of the highest grade and were obtained from standard commercial sources.

Preparation of Extract

The pulverized *Hydrangeae Dulcis Folium* (EHDF, 63.3 g) was extracted with a 70% ethanolic solution (approximately 1 L) at 60 ℃ for 2 h twice. The ethanolic extract of EHDF was filtered, concentrated on a water bath under vacuum, frozen and lyophilized (Eyela, model FDU-1200, Japan, yield: 29.18%).

Animals

A total of zebrafish of 5–6 months of age were purchased from the World Fish Aquarium (Jeju, Korea). All fish were acclimated for at least 2 weeks in the experimental room and maintained in constant temperature (26 ± 1 ℃) tanks with aerated water. Fish were kept on a 14–10 h light/dark cycle (lights on from 07:00–21:00 h) and fed 2 times a day with TetraMin commercial flakes (Tetra, Germany). Animal treatment and maintenance were conducted in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, 8th edition) and approved

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IACUC animal protocol 08-19.

Drug Administration and Induction of Stress

Net handling stress (NHS) was induced using the method of Ramsay, et al.[17] To generate NHS, zebrafish were netted suspended in the air for 3 min, and then, fish were returned to the water for 3 min. Then, fish were suspended in the air for an additional 3 min again. Zebrafish was put into a medicated bath of 0.9% NaCl solution, EHDF (5–20 mg/L), or L-theanine (40 mg/L) for 6 min just before the test. The pilot studies were conducted to determine a valid concentration or time for treatment. In a pilot study, we observed that EHDF from the concentration of 5 mg/L exhibited anti-stress activity at least 6 min after immersion treatment. The fish were randomly divided into the unstressed group, stressed control group, stressed EHDF-treated group, stressed L-theanine-treated group, and positive control group. EHDF or L-theanine which were dissolved in a 0.9% NaCl solution, were filtered before immersion treatment.

High-Performance Liquid Chromatography Quantitative Analysis

High-performance liquid chromatography (HPLC)-diode array detection analysis was performed using HPLC instrument (Agilent, Waldbronn, Germany). Chromatographic separation was accomplished on an YMC-Triart C_{18} column at 25 ℃ and monitored at 213 nm. After ultracentrifugation at 9,000 r/min and filtration through a syringe membrane filter, 10 μL of sample was injected 3 times. The average retention time of thunberginol C was 19.8 min. The calibration curves of thunberginol C was as follows: y=59600x+28000; r^2=0.999724 for standard concentrations ranging from 0.5 to 50 μg/mL. Using this equation, the quantity of thunberginol C in the EHDF was calculated and determined as 290.0±37.5 μg/g (Figure 2).

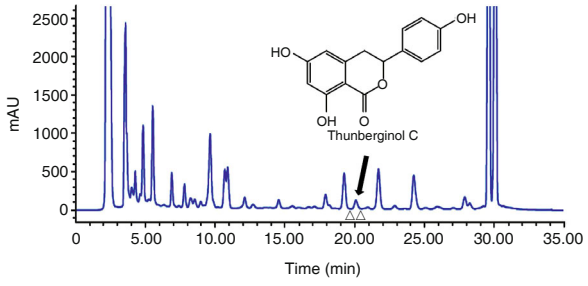

Figure 2. HPLC Chromatogram of Thunberginol C from 70% EHDF

Novel Tank Test

For the novel tank test (NTT) to assess zebrafish anxiety and locomotion,[18,19] a 1.5-L trapezoidal tank (15 cm in height × 28 cm in top × 23 cm in bottom × 7 cm in width) was filled with the maximum amount of water and divided into two equal virtual horizontal portions. In this experiment, each fish (n=10–12 zebrafish per group) were pre-treated with EHDF for 6 min. Zebrafish behaviours were recorded with subsequent automated analysis of generated traces by Ethovision XT 8.5 software (Noldus IT, Wageningen, Netherlands) from the side view for 6 min to calculate the duration in top, frequency into top, turn angle, distance moved, duration of not moving and velocity.

Open Field Test

An open field test (OFT) was performed to observe the anti-stress effect of EHDF on the swimming pattern and locomotor activity.[20] A white plastic cylinder (21 cm in diameter, 24 cm in height) filled with water to a height of 10 cm was implemented for this test. Following drug pre-treatment for 6 min, the fish (n=10–12 zebrafish per group) were individually placed in the centre of the tank and recorded, with a subsequent automated analysis of the generated traces conducted using the Ethovision XT 8.5 software from the top view for 6 min to determine the distance moved, meandering movement, turn angle, duration of not moving and highly mobile movement.

Measurement of Whole-Body Cortisol Level

The whole-body cortisol level was measured using the previously described method.[21] Zebrafish were put into a medicated bath of EHDF for 6 min. Then, zebrafish were sacrificed by tricaine (Sigma-Aldrich, Mo) at a concentration of 150 mg/L to obtain the body fluid. After the skin moistness of the zebrafish was dried, it was put into a prepared cryo tube with 2 mL of 0.1 mol/L phosphate buffered saline (PBS) for homogenization. The ground mixture was added to 5 mL of diethyl ether and vortexed for 1 min. Then, it was centrifuged at 3,000 g for 10 min and then frozen for 30 s in liquid nitrogen. The supernatant was moved into a new test tube, and the diethyl ether was evaporated by a vacuum centrifugal concentrator (CVE-2000, Eyela, Japan). After the evaporation of diethyl ether, 1 mL of 0.1 mol/L PBS was added to the test tube, and the content was moved to a 1.7 mL tube. The tube was then stored at –20 ℃ until it was submitted for cortisol measurement. The whole-body cortisol level

was measured by a cortisol assay kit (R&D system, USA). Absorbance was measured at a wavelength of 450 nm by a microplate reader (Molecular Devices, USA) to analyze the ELISA plate. The absorbance value was converted into the cortisol concentration value based on the 4-parameter logistic (4-PL) curve. The whole-body cortisol levels were expressed as the ratio of concentration to the weight of each fish.

ACTH Challenge Test

Four treatment groups (n=4 zebrafish per group) were used to determine whether an ACTH challenge could short-circuit possible inhibitory effects at the level of the brain and pituitary exerted by 10 mg/L concentration of EHDF. The challenge dose of ACTH (freshly dissolved in sterile 0.1 mol/L PBS) was paired with EHDF or home tank water pre-treatment. The challenge dose of ACTH was determined from a pilot dose-response study, which examined 4 doses of ACTH in the range of 0.05–0.4 IU/g. The lowest dose examined was used because it produced a maximal stimulatory effect on cortisol secretion (data not shown). ACTH (40 μL, 0.2 IU/g) or vehicle (0.1 mol/L PBS, pH=7.4) were intraperitoneally injected after pre-treatment with EHDF for 6 min or 0.9% NaCl solution. Fish were anesthetized and sacrificed by tricaine (Sigma-Aldrich, Mo) at a concentration of 150 mg/L 15 min after ACTH injections for the measurement of the whole-body cortisol level.[22]

Statistical Analysis

Values are expressed as the means ± standard error of mean (S.E.M.). Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. For the challenge study, the interactions between the agonist and EHDF were analyzed separately with a two-way ANOVA; pairwise comparisons for the assessment of the influence of the drug on the ACTH effects were conducted by using the Student-Newman-Keuls test. The statistical significance was set at P<0.05.

RESULTS

Anti-stress Effect of EHDF on NTT

The duration in top, frequencies in top, distance moved, and velocity were reduced in the stressed control group by NHS induction (all P<0.05). Interestingly, in the EHDF-treated groups at concentrations of 5–20 mg/L, all the values were significantly recovered compared to the stressed control group (all P<0.05). In the L-theanine-treated group, the values mentioned above were significantly increased compared with the stressed control group (all P<0.05, Figure 3).

The turn angle and not moving duration were significantly increased by 88% and 180% in the stressed control group compared with the unstressed group (both P<0.05). A significant decrease was observed in the EHDF-treated groups at all concentrations compared to the stressed control group (all P<0.05). In the L-theanine-treated group, the turn angle and not moving duration were significantly decreased compared with the stressed control group (all P<0.05, Figure 3).

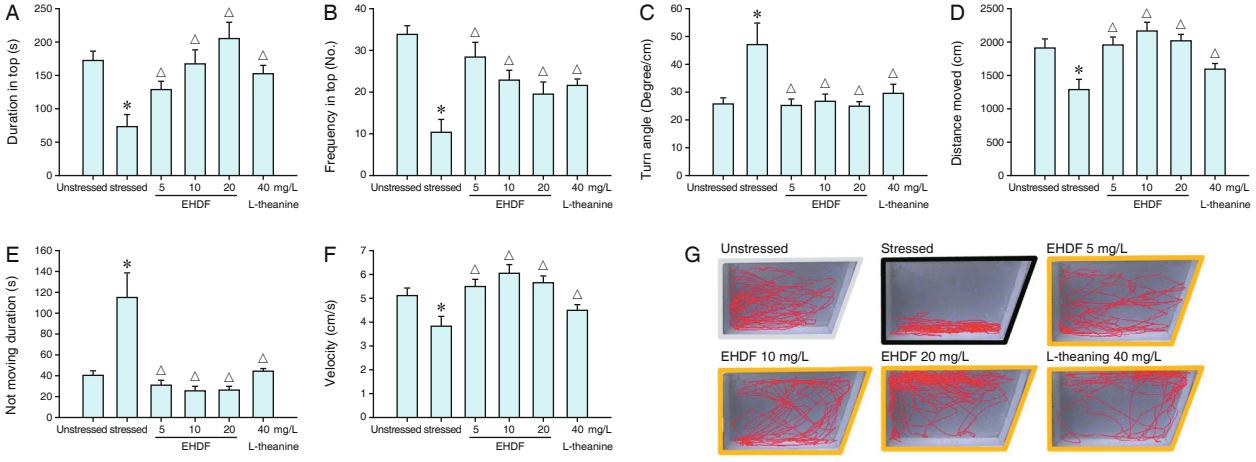

Figure 3. Behavioral Effect of 70% Ethanolic EHDF (5–20 mg/L) and L-theanine (40 mg/L) on Novel Tank Test in Adult Zebrafish ($\bar{x}$ ± S.E.M.)

Notes: The graph shows duration in top (A), frequency in top (B), turn angle (C), distance moved (D), not moving duration (E), velocity (F) and visual data (G). $^{*}P$<0.05 *vs*. unstressed group; $^{\triangle}P$<0.05 *vs*. stressed control group; n=10–12 in each group.

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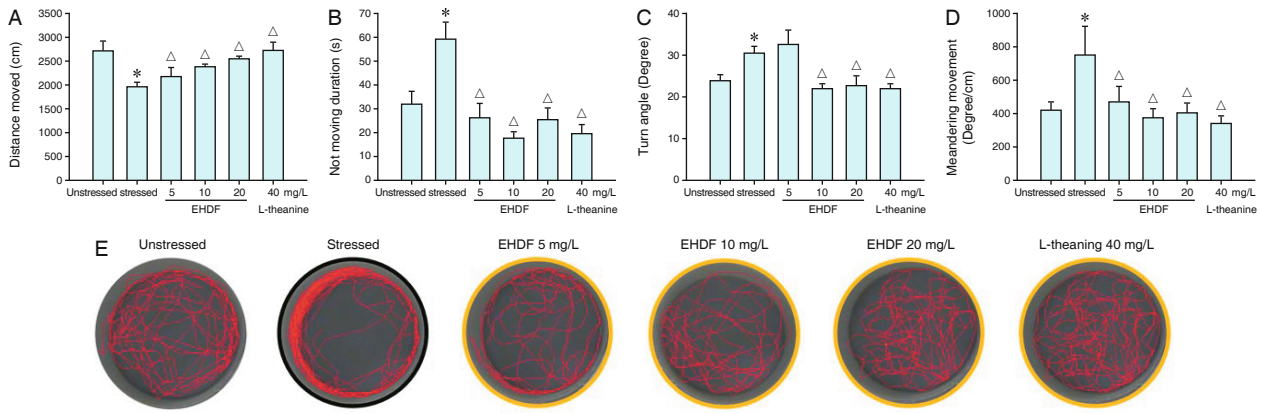

Figure 4. Behavioral Effect of 70% Ethanolic EHDF (5–20 mg/L) and L-theanine (40 mg/L) on Open Field Test in Adult Zebrafish ($\bar{x} \pm$ S.E.M.)

Notes: The graph shows distance moved (A), meandering movement (B), turn angle (C), not moving duration (D) and visual data (E) . *P<0.05 *vs*. unstressed group; △P<0.05 *vs*. stressed control group; n=10–12 in each group.

Anti-stress Effect of EHDF on OFT

The distance moved was significantly decreased in the stressed control group compared with the unstressed group by NHS induction (P<0.05). Interestingly, in the EHDF-treated groups at 5–20 mg/L concentrations and the L-theanine-treated groups, the distance moved was significantly recovered compared to the stressed control group (all P<0.05, Figure 4).

The not moving duration, turn angle and meandering movement were increased by NHS induction compared with the unstressed group, significantly (all P<0.05). In the EHDF-treated groups at 5–20 mg/L concentrations (except for 5 mg/L concentration of EHDF on turn angle), the values were significantly decreased compared to the stressed control group (all P<0.05). Moreover, in the L-theanine treated group, all the values mentioned above were significantly decreased compared with the stressed control group (all P<0.05, Figure 4).

Effect of EHDF on Whole-body Cortisol Level

In the unstressed group, the whole-body cortisol level was significantly increased in the stressed control group (P<0.05). Increases in the whole-body cortisol levels after stress were significantly reduced by EHDF at concentrations of 5–20 mg/L compared with the stressed control group (all P<0.05). Additionally, in the L-theanine-treated group, the whole-body cortisol level decreased significantly compared with the stressed control group (P<0.05, Figure 5).

ACTH Challenge Test

An ACTH challenge was administered after pre-

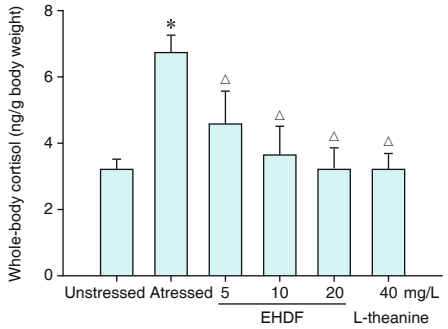

Figure 5. Effect of 70% Ethanolic EHDF (5–20 mg/L) and L-theanine (40 mg/L) on Whole-Body Cortisol in Adult Zebrafish ($\bar{x} \pm$ S.E.M.)

Notes: *P<0.05 *vs*. unstressed group; △P<0.05 *vs*. stressed control group; n=4 per group.

treatment with either vehicle or EHDF (10 mg/L) to determine if EHDF exerted an inhibitory effect on the cortisol secretion at the level of the interrenal glands (Figure 6). The 0.2 IU/g dose of ACTH stimulated cortisol release comparable to typical stress response levels (P<0.05). EHDF completely blocked the ACTH-

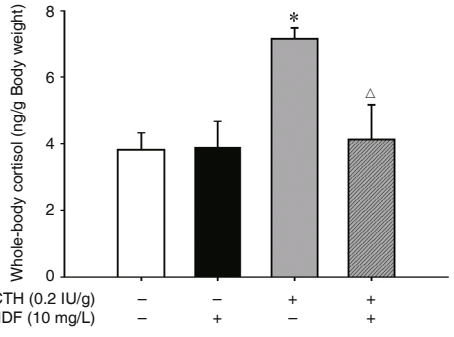

Figure 6. Increase of Cortisol Induced by ACTH Was Blocked by 70% Ethanolic EHDF (10 mg/L) in ACTH Challenge Test ($\bar{x} \pm$ S.E.M.)

Notes: *P<0.05 *vs*. PBS-treated control; △P<0.05 *vs*. ACTH-treated group; n=4 per group.

induced cortisol secretion (P<0.05).

DISCUSSION

Stress has provided major information in explaining the behavioural changes and physiological factors that affect physical health.[23] Stress is all the responses that a living organism shows to protect itself when it receives harmful stimulations from the external environment.[24,25] Activation of the HPA by stressors, resulting in increasing plasma levels of ACTH and cortisol, is significantly involved in the responses that advance the protection of mammals.[26] The cortisol-related endocrine system of zebrafish acts similarly to the endocrine system of mammals.[27] The stress response is characterized by the activation of the HPI axis in zebrafish.[8] In other words, stress-induced synthesis and secretion of cortisol involves the activation of the hypothalamus, pituitary and interrenal glands in fish.[28]

Exposure to sudden excessive stress induces the secretion of the stress hormone, which works on the central nervous system (CNS) or endocrine system. It results in the changes in the behaviour, immunity function or body weight.[29,30] The previous study reported that zebrafish tend to show lack of movement, sinking down to the bottom or are observed as being overexcited so that they perform abnormal swimming patterns, erratic movement or jumps out of water under states of anxiety, stress or panic.[31] Therefore, in this study, we observed behavioural parameters including the total distance moved, duration of not moving, duration at the top, turn angle and meandering movement utilizing the NTT or OFT. In the NTT results, because of the exploration response to the stimuli of NHS, the duration at the top, frequency at the top, distance moved and velocity were decreased. Further, the turn angle and duration of not moving were significantly increased. In addition, in the OFT results, the distance moved was decreased. Conversely, the meandering movement, turn angle and duration of not moving were increased. Accordingly, we verified that anxiety-like behaviours were increased and locomotive activity was reduced in zebrafish under stress. In the present study, we observed that EHDF recovered the anxiety-like behaviours or reduction of locomotor activity by NHS in the NTT and OFT comparable to that observed for L-theanine, an anti-stress functional supplement. The results of the present study provide evidence for the potential role of EHDF as an anti-stress material with some significant findings.

Exposure to stress also causes behavioural changes including increases in anxiety-like behaviours or decreases in locomotor activity as well as anxiety or depression.[32] In addition to anxiety-like behaviours, which are related to the increase of cortisol levels,[33] it was demonstrated that the plasma corticosterone levels were elevated by obestatin, a chemical stressor, which induced anxiety-like effects in mice in the elevated plus-maze and OFT, thus exhibiting correlation between the activation of the stress axis and anxiety.[1] Changes in the whole-body cortisol level are useful biomarkers of the primary stress responses.[25]

In our results, the whole-body cortisol was increased by NHS in the same manner as the previous study.[18] Therefore, we speculate that behavioural changes induced by NHS result from the increase in the whole-body cortisol level. Interestingly, increases in the whole-body cortisol levels induced by NHS were blocked by pre-treatment with EHDF. These results suggest that the anti-stress effect of pre-treatment with EHDF, which prevents behavioural changes in the NTT and OFT, could be dependent upon the blockage of increases in the whole-body cortisol level.

The HPA axis is well known for many mammals, with CRF and ACTH being the most important hormones and corticosterone or cortisol as the main end product of the HPA axis.[34] This general mammalian mode also applies to teleost.[35] However, the secretion of corticosteroid occurs from the interrenal cells in fish, as ACTH is mainly responsible for cortisol secretion in mammals and fish.[36] Our results suggest that EHDF may effectively block NHS-induced cortisol secretion. Thereby, we hypothesized that EHDF might exhibit its effects through altering the HPI axis because it is a major part of the endocrine system that controls the secretion of cortisol. We investigated this hypothesis whether the secretion of cortisol by ACTH would be antagonized by the anti-stress properties of EHDF.[22] In the results, EHDF completely blocked the induction of cortisol secretion by ACTH. In the present study, these results suggest that the anti-stress effects of EHDF are mediated through the melanocortin receptor.

In previous research, phyllodulcin, a constituent of *Hydrangeae Dulcis Folium*, has been found to be a potential phosphodiesterase (PDE) inhibitor.[35] It

is well-known that cyclic adenosine monophosphate (AMP) is biotransformed to 5'-AMP, a biologically inactive compound, by intracellular PDE.[35] Kawamura, et al[37] reported that phyllodulcin exhibited a PDE inhibitory effect due to enhanced cyclic AMP-induced steroidogenesis in a concentration-dependent manner in bovine adrenocortical cells. Although the authors did not perform a measurement of the concentration of cortisol in the bovine adrenocortical cells after treatment with phyllodulcin for reasons mentioned above, phyllodulcin might potentially produce or secrete cortisol. Nevertheless, in this study, the pre-treatment with EHDF reduced the whole-body cortisol level.

As shown in the Methods, EHDF was standardized based on the amount of thunberginol C. Based on the amount of thunberginol C contained in EHDF (approximately 5.8 μg in 20 mg of EHDF), it would not be enough to induce anti-stress activity because the effective concentration. Unfortunately, we did not know of any active compound of EHDF yet. However, we could speculate as to the possible mechanism of EHDF action on cortisol secretion through research using bovine adrenal fasciculata cells. ACTH stimulates cytosolic calcium movement, which was biphasically enhanced by its concentrations, and also induces cortisol production mainly via phospholipase A_2-dependent processes. In addition, guanosine-5'-triphosphate (GTP) may play an important physiological role in the regulation of cortisol synthesis.[38] Simultaneous activation of Ca^{2+} messenger systems by GTP could cooperatively enhance cortisol production.[28] In this way, cortisol secretion is closely related with Ca^{2+} signalling. Thunberginols, isolated from *Hydrangeae Dulcis Folium*, inhibited the rise in intracellular Ca^{2+} concentrations in RBL-2H3 cells induced by antigen.[38] Our present results and these previous reports suggest that the anti-stress mechanism of EHDF could be dependent upon the inhibition of Ca^{2+} signalling in the interrenal gland of zebrafish.

The results showed that the anti-stress effects of EHDF mediated the secretion of cortisol and may be partly attributable to endocrine. The present findings may also provide important scientific evidence for the application and development of relaxation functional foods to conquer stress, and EHDF can be a potential candidate for treating stress-related disorders such as anxiety and depression. As diseases caused by stress have rapidly increased recently, a current trend in studies is finding and studying natural substance materials such as *Schizandra chinensis* and *Scutellaria baicalensis*, which have less adverse effects in the efficient treatment of stress-related diseases.[33]

The results of this study suggests that EHDF can be developed as a medicine, which restricts stress, as a material for functional foods. In further studies, we need to clarify the mechanism and active components in *Hydrangeae Dulcis Folium* that inhibit stress response. We are currently working on the isolation of active components in EHDF using an activity-guided fractionation approach. In this study, functional properties and its possible mechanism of the EHDF were evaluated in terms of anti-stress activities. By the blockade of the effects of ACTH on cortisol secretion, EHDF decreased anxiety-like behaviours and whole-body cortisol levels and showed the anti-stress effect in NHS model. EHFD might contribute to the well-being of people or fish and improved quality of life suffering from physiological and psychological stresses.

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

Author Contributions

Conception and design: Oh J, Kim DH and Lee S. Acquisition of data: Oh J and Kim GW. Analysis and interpretation of data: Kim GY and Jung JW. Drafting the article: Oh J and Lee S. Critically revising the article: Kim GY, Park EJ, Ryu JH and Park SJ. Statistical analysis: Oh J. Study supervision: Lee S.

REFERENCES

1. Guo JY, Huo HR, Li LF, Guo SY, Jiang TL. Sini Tang prevents depression-like behavior in rats exposed to chronic unpredictable stress. Am J Chin Med 2009;37:261-272.
2. Malek H, Ebadzadeh MM, Safabakhsh R, Razavi A, Zaringhalam J. Dynamics of the HPA axis and inflammatory cytokines: Insights from Mathematical modeling. Comput Biol Med 2015;67:1-12.
3. O'Connor DB, Ferguson E, Green JA, O'Carroll RE, O'Connor RC. Cortisol levels and suicidal behavior: a meta-analysis. Psychoneuroendocrinology 2015;63:370-379.
4. Dhabhar FS. Effects of stress on immune function: the good, the bad, and the beautiful. Immunol Res 2014;58:193-210.
5. Gerlai R. Zebrafish antipredatory responses: a future for translational research? Behav Brain Res 2010;207:223-231.
6. Starkman MN, Gebarski SS, Berent S, Schteingart DE. Hippocampal formation volume, memory dysfunction, and cortisol levels in patients with Cushing's syndrome. Biol Psychiatry 1992;32:756-765.
7. Ito T, Maeda T, Goto K, Miura T, Wakame K, Nishioka H, et

al. Enzyme-treated asparagus extract promotes expression of heat shock protein and exerts antistress effects. J Food Sci 2014;79:H413-H419.
8. Pijanowski L, Jurecka P, Irnazarow I, Kepka M, Szwejser E, Verburg-van Kemenade BM, et al. Activity of the hypothalamus-pituitary-interrenal axis (HPI axis) and immune response in carp lines with different susceptibility to disease. Cell Physiol Biochem 2015;41:1261-1278.
9. Brittijn SA, Duivesteijn SJ, Belmamoune M, Bertens LF, Bitter W, de Bruijn JD, et al. Zebrafish development and regeneration: new tools for biomedical research. Int J Dev Biol 2009;53:835-850.
10. Fonseka TM, Wen XY, Foster JA, Kennedy SH. Zebrafish models of major depressive disorders. J Neurosci Res 2016;94:3-14.
11. Zhang H, Matsuda H, Yamashita C, Nakamura S, Yoshikawa M. Hydrangeic acid from the processed leaves of *Hydrangea macrophylla* var. thunbergii as a new type of anti-diabetic compound. Eur J Pharmacol 2009;606:255-261.
12. Kamei K, Matsuoka H, Furuhata SI, Fujisaki RI, Kawakami T, Mogi S, et al. Anti-malarial activity of leaf-extract of *Hydrangea macrophylla*, a common Japanese plant. Acta Med Okayama 2000;54:227-232.
13. Zhang H, Matsuda H, Kumahara A, Ito Y, Nakamura S, Yoshikawa M. New type of anti-diabetic compounds from the processed leaves of *Hydrangea macrophylla var*. thunbergii (*Hydrangeae Dulcis Folium*). Bioorg Med Chem Lett 2007;17:4972-4976.
14. Matsuda H, Shimoda H, Yamahara J, Yoshikawa M. Immunomodulatory activity of thunberginol A and related compounds isolated from *Hydrangeae Dulcis Folium* on splenocyte proliferation activated by mitogens. Bioorg Med Chem Lett 1998;8:215-220.
15. Guantai E, Chibale K. How can natural products serve as a viable source of lead compounds for the development of new/novel anti-malarials? Malar J 2011;10 Suppl 1:1-8.
16. Keller TL, Zocco D, Sundrud MS, Hendrick M, Edenius M, Yum J, et al. Halofuginone and other febrifugine derivatives inhibit prolyl-tRNA synthetase. Nat Chem Biol 2012;8:311-317.
17. Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, Schreck CB. Whole-body cortisol response of zebrafish to acute net handling stress. Aquaculture 2009;297:157-162.
18. Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, et al. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 2009;205:38-44.
19. Levin ED, Bencan Z, Cerutti DT. Anxiolytic effects of nicotine in zebrafish. Physiol Behav 2007;90:54-58.
20. Stewart A, Cachat J, Wong K, Gaikwad S, Gilder T, DiLeo J, et al. Homebase behavior of zebrafish in novelty-based paradigms. Behav Processes 2010;85:198-203.
21. Canavello PR, Cachat JM, Beeson EC, eds. Neuromethods. New York: Humana Press;2011:135-142.
22. Felix AS, Faustino AI, Cabral EM, Oliveira RF. Noninvasive measurement of steroid hormones in zebrafish holding-water. Zebrafish 2013;10:110-115.
23. Jaggi AS, Bhatia N, Kumar N, Singh N, Anand P, Dhawan R. A review on animal models for screening potential anti-stress agents. J Neurol Sci 2011;32:993-1005.
24. Gronquist D, Berges JA. Effects of aquarium-related stressors on the zebrafish: a comparison of behavioral, physiological, and biochemical indicators. J Aquatic Anim Health 2013;25:53-65.
25. Schacter DL, Gilbert DT, Wegner DM, eds. Psychology. 2nd ed. New York: Worth Publishers; 2011:702-710.
26. Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr Rev 1984;5:25-44.
27. Alderman SL, Bernier NJ. Ontogeny of the corticotropin-releasing factor system in zebrafish. Gen Comp Endocrinol 2009;164:61-69.
28. Schoonheim PJ, Chatzopoulou A, Schaaf MJ. The zebrafish as an *in vivo* model system for glucocorticoid resistance. Steroids 2010;75:918-925.
29. Harris RB, Mitchell TD, Simpson J, Redmann SM Jr., Youngblood BD, Ryan DH. Weight loss in rats exposed to repeated acute restraint stress is independent of energy or leptin status. Am J Physiol Regul Integr Comp Physiol 2002;282:R77-R88.
30. Smagin GN, Howell LA, Redmann S Jr., Ryan DH, Harris RB. Prevention of stress-induced weight loss by third ventricle CRF receptor antagonist. Am J Physiol 1999;276:R1461-R1468.
31. Champagne DL, Hoefnagels CC, de Kloet RE, Richardson MK. Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): relevance for stress research. Behav Brain Res 2010;214:332-342.
32. Jung JM, Park SJ, Lee YW, Lee HE, Hong SI, Lew JH, et al. The effects of a standardized *Acanthopanax koreanum* extract on stress-induced behavioral alterations in mice. J Ethnopharmacol 2013;148:826-834.
33. Lee S, Kim DH, Jung JW, Oh JH, Park HJ, Park C, et al. *Schizandra chinensis* and *Scutellaria baicalensis* counter stress behaviors in mice. Phytother Res 2007;21:1187-1192.
34. Bencan Z, Sledge D, Levin ED. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. Pharmacol Biochem Behav 2009;94:75-80.
35. Serezani CH, Ballinger MN, Aronoff DM, Peters-Golden M. Cyclic AMP: master regulator of innate immune cell function. Am J Respir Cell Mol Biol 2008;39:127-132.
36. Speedie N, Gerlai R. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). Behav Brain Res 2008;188:168-177.
37. Kawamura M, Kagata M, Masaki E, Nishi H. Phyllodulcin, a constituent of "Amacha", inhibits phosphodiesterase in bovine adrenocortical cells. Pharmacol Toxicol 2002;90:106-108.
38. Omura M, Suematsu S, Nishikawa T. Role of calcium messenger systems in ACTH-induced cortisol production in bovine adrenal fasciculo-reticularis cells. Endocr J 2007;54:585-592.

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