A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Effect of the ethanolic extract of cactus pear (*Opuntia ficus-indica*) fruit on net handling stress in zebrafish

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Effect of the ethanolic extract of cactus pear (*Opuntia ficus-indica*) fruit on net handling stress in zebrafish

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Abstract in English

To determine the anti-stress effects of *Opuntia ficus-indica*(OF70E) and its fractions on physical stress, we monitored endocrine changes such as whole-body cortisol level and behavioral changes in zebrafish. To induce physical stress, we used net handling stress (NHS). In results, compared with normal group which did not induce by NHS, whole-body cortisol level were significantly increased by treatment with NHS in control group (p<0.05). To examine behavioral changes induced by NHS in zebrafish, we conducted open field test (OFT) or novel tank test (NTT) after the induction of NHS and effect of pretreatment of OF70E or its fractions. As results of OFT, compared with normal group which did not induce by NHS, distance moved were significantly decreased by treatment of NHS in control group (p<0.05). Interestingly, pretreatment with OF70E blocked decreases of distance moved increased by NHS (p<0.05). And meandering movement, immobility and turn angle were significantly increased by NHS as compared with normal group (p<0.05). However, pretreatment with OF70E prevented the increases of meandering movement immobility and turn angle (p<0.05). And pretreatment with OF70E at all concentrations for 6min significantly prevented the increase of whole-body cortisol levels induced by NHS. As compared with control group, pretreatment with EtOAc fraction, n-BuOH fraction or water fraction of OF70E significantly prevented the increase of whole-body cortisol levels induced by NHS except hexane fraction (p<0.05). As results of NTT, compared with normal group which were untreated by NHS, duration in top, distance moved, velocity and zone transition were significantly

decreased by treatment of NHS in control group (p<0.05). Interestingly, pretreatment with water fraction of OF70E blocked decreases of duration in top, distance moved, velocity and zone transition increased by NHS (p<0.05). In addition, meandering movement, immobility and turn angle were significantly increased by NHS as compared with normal group. And pretreatment with its H_2O fractions at concentrations of 15-60 mg/L for 6min significantly prevented the increase of whole-body cortisol levels induced by NHS. In conclusion these results suggest that the pretreatment of OF70E and its fraction may prevent stress responses, such as the behavioral and endocrine changes.

Abstract in Korean

백년초 에탄올 추출물이 net handling stress를 유도한 제브라피쉬에 미치는 영향

스트레스는 자율신경계 및 내분비 기능의 장애를 유발한다. 뿐만 아니라 만성 스트레스는 우울증 및 공황 장애과 같은 신경계 질환의 원인이 되기도 한다. 본 연구에서는 *Opuntia ficus-indica*(OF70E)를 70% EtOH 로 추출하고, 그 추출물을 용매분획하여 hexane, EtOAc, n-BuOH, 및 물분획물을 얻었다. OF70E 의 항스트레스 활성을 관찰하기 위하여 net handling stress(NHS) 유도 후 행동변화 및 whole-body cortisol level 을 측정하였다. OF70E 의 조추출물 또는 분획물로 전처리한 후 open field test 및 novel tank test 를 이용하여 행동 변화를 관찰한 결과, NHS 에 의하여 변화된 행동변화가 정상 수준으로 억제되는 것으로 나타내었다. Whole-body cortisol level, 또한 실험에 사용된 시료의 모든 농도에서 대조군에 비해 감소하는 결과가 관찰되었다. 이상의 결과는 OF70 가 항스트레스 효과를 가지고 있으며, OF70E 및 그 분획물은 항스트레스에 대한 치료제로 개발될 수 있음을 시사하는 것이라고 사료된다. 향후 분획 에서 분리된 유효성분으로부터 동일한 연구를 통하여 우수한 항스트레스 물질을 개발할 수 있을 것으로 보인다.

1. Introduction

Stress works as the cause of various diseases by being the origin of all diseases and causing disharmony and circulation disorder in human body [1]. When human body receives stress, the body protects itself by showing stress response through accelerating hypothalamus-pituitary gland-adrenal system and sympathetic nerve system, increasing epinephrine and norepinephrine, increasing the secretion of corticotrophin secretion factor at hypothalamus, increasing the secretion of adrenal cortex stimulating hormone at the pituitary gland adenohypophysis, increasing the secretion of cortisol and aldosterone at adrenal medulla [14]. Stress response is classified into short term responses and long term responses. Short term responses include increase of blood pressure, increase of heart rate, change of behavior and blood movement from gastrointestinal tract to skeletal muscle while long term responses involve decrease of spleen weight and immune suppression caused by the decrease in the number of leukocyte. If the intensity of stress is strong, if it continues for a long time or if human body has a defect in stress response system, the stress may cause a disease [8].

Zebrafish is widely used in recent studies such as genetics, biology and behavioral pharmacology. Zebrafish has the advantages that it is easy to handle as an experiment organism, it is cheap and it has short generation length of about 3 months [6]. According to what is known till today, the major genes of zebrafish are similar to the genomic structure of humankind or rodents while the amino acid sequence of zebrafish also has high homogeny of 90% with the amino acid sequence of

humankind [12, 25]. In addition, the stress-related endocrine system of zebrafish secrets cortisol in accordance with the sign of hypothalamus-pituitary gland-adrenal cortex which is similar to the hypothalamus-pituitary gland-adrenal axis of mammal [2]. Actually, when the stress signal sent from sensory system simulates hypothalamus, the hypothalamus secrets corticotropin-releasing hormone (CRH). And adrenocortical hormone (ACTH) is secreted into blood flow from pituitary gland by the action of the secreted CRH. The ACTH in blood flow reaches the interrenal gland of zebrafish and induces the secretion of cortisol [3]. Cortisol at normal concentration has advantageous effects such as energy supply and immunity improvement [10]. However, when blood cortisol of high concentration would be maintained, it causes mental disease such as depression and panic disorder [15].

Cactus fruit is the mature fruit of *O. ficus-indica var saboten*, of which the flower blooms in April/May while produces purple-colored fruit in November/December. *O. ficus-indica var saboten* is being cultivated in the south of Korea such as Jeju Island, Geoje Island and the southern coast. *O. ficus-indica var saboten* belongs to *O. lanceolata haw* family of *O. lanceolata haw* order in taxonomy [20]. *O. ficus-indica var saboten* has dark green color leaves in palm shape with thorns. Cactus fruit, the fruit of *O. ficus-indica var saboten*, has the shape of European pear. It has many seeds, viscous substance and high content of calcium [5]. Further, *O. ficus-indica var saboten* has about 5% of phenol substance and flavonoid, which have anti-oxidation and anti-cancer effect, various inorganic substances and amino acid, which function as nutritious tonic. The pectin substance separated from Cactus fruit is known to have high medicinal value by decreasing cholesterol level,

modulating blood glucose and preventing diabetes complication [11].

In modern times, people are exposed to many kinds of stress, which may causes mental diseases such as depression and panic disorder. Therefore, a study, which provides with theoretical background required for the development of new medicine and treatment method by verifying the mechanism of endocrine system hormone and stress, is urgently required. However, until now, no attempts have been made to investigate the anti-stress activity of Cactus fruit. This study investigated the anti-stress activity of Cactus fruit in zebrafish using method of behavioral pharmacology and molecular biology.

2. Materials and Methods

2.1 Preparation of extract and fractionation

Cactus fruit was purchased at the Jeju Agricultural Research Services (Jeju, Korea), and a voucher specimen (JJNUOPS 2013-05) was deposited at the Marine Biomedical Science of the College of Ocean Sciences, Jeju National University. The pulverized Cactus fruit (63.3 g) were extracted with 70% ethalonic solution (about 1 L) at 60 °C for 2 h twice. The ethalonic extract of Cactus fruit (OF70E) was filtered, concentrated on a water bath under vacuum, frozen and lyophilized (Eyela, model FDU-1200, Japan) (yield: 79.18%). The dried OF70E (50 g) was suspended in distilled water and partitioned with hexane (1 g), EtOAc (0.4 g), n-BuOH (8.42 g) and H_2O (39.59 g), successively (Fig. 1).

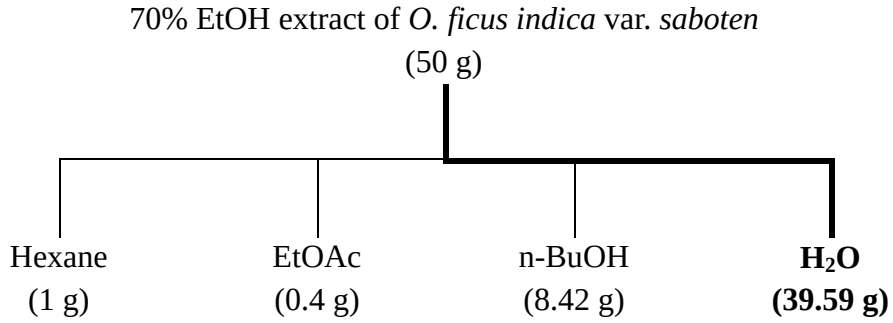

Figure 1. The scheme of fractionation of *Opuntia ficus indica* var. *saboten*

2.2 Animals

Zebrafish were purchased from the World fish aquarium (Jeju, Korea). All fish were acclimated for at least two weeks in the experimental room and maintained under constact temperature (26 ± 1 ℃) tanks with aerated water. Fish were kept on a 14–10 h day/night cycle and fed two times a day with commercial flakes, TetraMin (Tetra, Germany). All protocols were approved by the Institutional Animal Care Committee [8].

2.3 Drug administration and induction of stress

Net handling stress (NHS) was induced using method of Ramsay et al [9]. **S**ubjected to NHS, zebrafish were netted suspended in the air for 3 min. And then, fish were returned to water for 3 min. Afterward fish were suspended in the air for an additional 3 min again. Fish were induced to NHS after treatment with OF70E or its fraction for 6 min [10]. The fish were randomly divided in unstressed normal, stressed control, and stressed OF70E-treated groups. OF70E or its fractions were dissolved in 0.9% NaCl solution. And zebrafish was put into a medicated bath of 0.9%

NaCl solution, OF70E (25-100 mg/L), or fractions (hexane: 2 mg/L, EtOAc: 1 mg/L, n-BuOH: 10 mg/L. H_2O fraction: 15-60 mg/L), drugs for 6 min just before test.

2.4 Behavioral test

Behavioral testing was performed between 10:00 and 15:00 h using tanks with water adjusted to the holding room temperature. The present study used several different behavioral tests, including the novel tank test (NTT) and open field test (OFT) [17]. Prior to testing, fish were pre-treated with OF70E or its fractions in a 500 ml plastic beaker for 6 min. During testing, zebrafish behavior was recorded with subsequent automated analysis of generated traces by Ethovision XT 8.5 software (Noldus IT, Wageningen, Netherlands).

2.4.1 Open field test

Open field test was performed to observe the effect of OF70E or its fraction on the swimming pattern and locomotor activity [26]. A white plastic cylinder (21 cm diameter, 24 cm height) filled with water to a height of 10 cm was implemented for this test. Following drug pretreatment, the fish (n=10–12 in each group) were individually placed in the center of the tank, and video-recorded from the top view for 6 min, using Ethovision XT 8.5 to calculate duration in the center zone, velocity, meandering movement, turn angle, Immobility, zone transition and distance moved.

2.4.2 Novel tank test

For the novel tank test to assess zebrafish anxiety and locomotion [4, 13, 22],

a 1.5-L trapezoidal tank (15 cm height×28 cm top×23 cm bottom×7 cm width) was maximally filled with water and divided into two equal virtual horizontal portions. In this experiment, fish (n = 10–12 in each group) were pre-treated with OF70E for 6 min. Zebrafish behaviors were video-recorded from the top view for 6 min by using Ethovision XT 8.5 to calculate duration in the top of the tank, velocity, meandering movement, turn angle, immobility, zone transition and distance moved.

2.5 Measurement of whole-body cortisol level

The level of whole-body cortisol was measured using the method of Grossman et al. [16]. Zebrafish was put into a medicated bath of OF70E or its fraction for 6 min. Zebrafish were sacrificed by tricaine (Sigma-Aldrich, Mo) at the concentration of 150 mg/L to obtain body fluid. After skin moistness of zebrafish was dried, it was put into a prepared cryo tube with 2 ml of 1 x phosphate buffer saline (PBS) for grinding. The ground mixture was added of 5 ml of diethyl ether and vortexed for 1 min. Then it was centrifuged at 3000 g for 10 min and then, frozen for 30 seconds in liquid nitrogen. The supernatant was moved into a new test tube and diethyl ether was evaporated by a vacuum centrifugal concentrator (CVE-2000, Eyela, Japan). After the evaporation of diethyl ether, 1 ml of 0.5M 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 ELISA plate. Absorbance value was converted into the cortisol

concentration value based on the 4-parameter sigmoid minus curve. The whole-body cortisol levels were expressed as the ratio of concentration to weight of each fish [16].

2.6 Statistics

Values are expressed as means ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-keuls test for multiple comparisons. Statistical significance was set at $p<0.05$.

3. Result

3.1 Drug screening

To verify the anti-stress activity of native plants in Jeju island, zebrafish was given a treatment bath in the extract solution of each plant. Then the change in whole-body cortisol level by NHS induction was observed. The normal group without NHS induction had the whole-body cortisol level of 294.2 ± 72.8 ng/g while the control group which was not treated any extracts before NHS induction had the whole-body cortisol level of 517.0 ± 43.0 ng/g; which was significant increase. Among the plant extract treated groups used in screen, the OF70E – treated group had the lowest whole-body cortisol level of 195.2 ± 30.6 ng/g (Fig. 2).

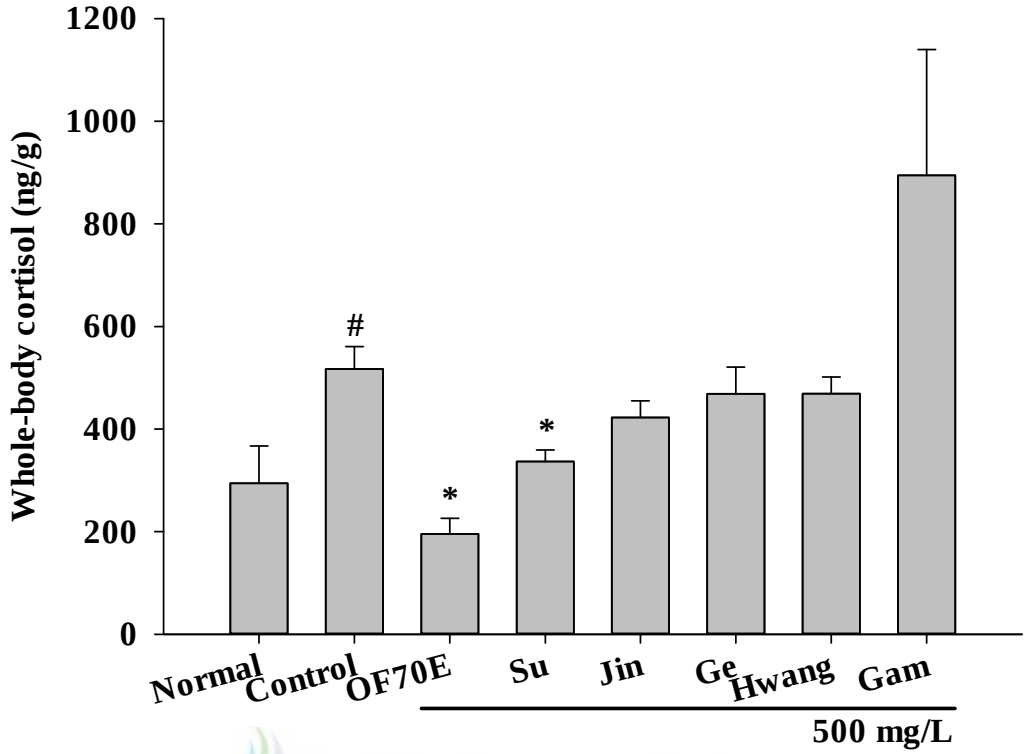

Figure 2. Level of whol-body cortisol after treatment with natural products for 6 min. (n=4, # $p < 0.05$ vs normal, * $p < 0.05$ vs control, one-way ANOVA)

3.2 Anti-stress effect of OF70E on NTT

3.2.1 Duration in top

Duration in top was measured for 6 min in NTT. The duration in top was 133 ± 10.7 sec in the normal group while it was 3.1 ± 2 sec in the control group which was about 97 % decreased by NHS induction ($p<0.05$). Interestingly, the duration in top was 129.1 ± 31.7 sec at concentration of 25 mg/L of OF70E ($p<0.05$), 78.4 ± 18.8 sec at concentration of 50 mg/L of OF70E ($p<0.05$) and 62.3 ± 16.6sec at concentration of 100 mg/L of OF70E ($p<0.05$). In the OF70E-treated groups at 50-100mg/L concentrations, duration in top significantly increased compared to the

control group (Fig. 3A).

3.2.2 Zone transition

Zone transition was measured for 6 min in NTT. The zone transition was 34.8 ± 5.3 times in the normal group while it was 0.8 ± 0.5 times in the control group which was about 85 % decreased by NHS induction (p<0.05). Interestingly, the zone transition were 4.3 ± 1.3 times at concentration of 25 mg/L of OF70E (p<0.05), 20.6 ± 4.2 times at concentration of 50 mg/L of OF70E (p<0.05) and 20.6 ± 5.9 times at concentration of 100 mg/L of OF70E (p<0.05). In the OF70E-treated groups at 50-100mg/L concentrations, zone transition significantly increased compared to the control group (Fig. 3B).

3.2.3 Turn angle

The turn angle was 23.3 ± 1.4 deg/s in the normal group however, it increased by 95% to 45.9 ± 3.2 deg/s in the control group (p<0.05). It was 23.7 ± 2.3 deg/s in OF70E 25 mg/L (p<0.05), 23.5 ± 1.6 deg/s in OF70E 50 mg/L (p<0.05) and 25.7 ± 1.3 deg/s in OF70E 100 mg/L (p<0.05). Significant decrease was observed in the OF70E-treated groups at all concentrations compared to the control group (Fig. 3C).

3.2.4 Immobility

Immobile duration was measured for 6 min in NTT. The immobile duration was 150.4 ± 13.9 sec in the normal group while it was 295.6 ± 11.4 sec in the control group which was about 96 % increased by NHS induction (p<0.05). Interestingly, the

immobile duration were 271.4 ± 16.5 sec at concentration of 25 mg/L of OF70E (p<0.05), 184.6 ± 15.3 sec at concentration of 50 mg/L of OF70E (p<0.05) and 224.4 ± 15.7 sec at concentration of 100 mg/L of OF70E (p<0.05). In the OF70E-treated groups at 50-100mg/L concentrations, immobile duration significantly decreased compared to the control group (Fig. 3D)

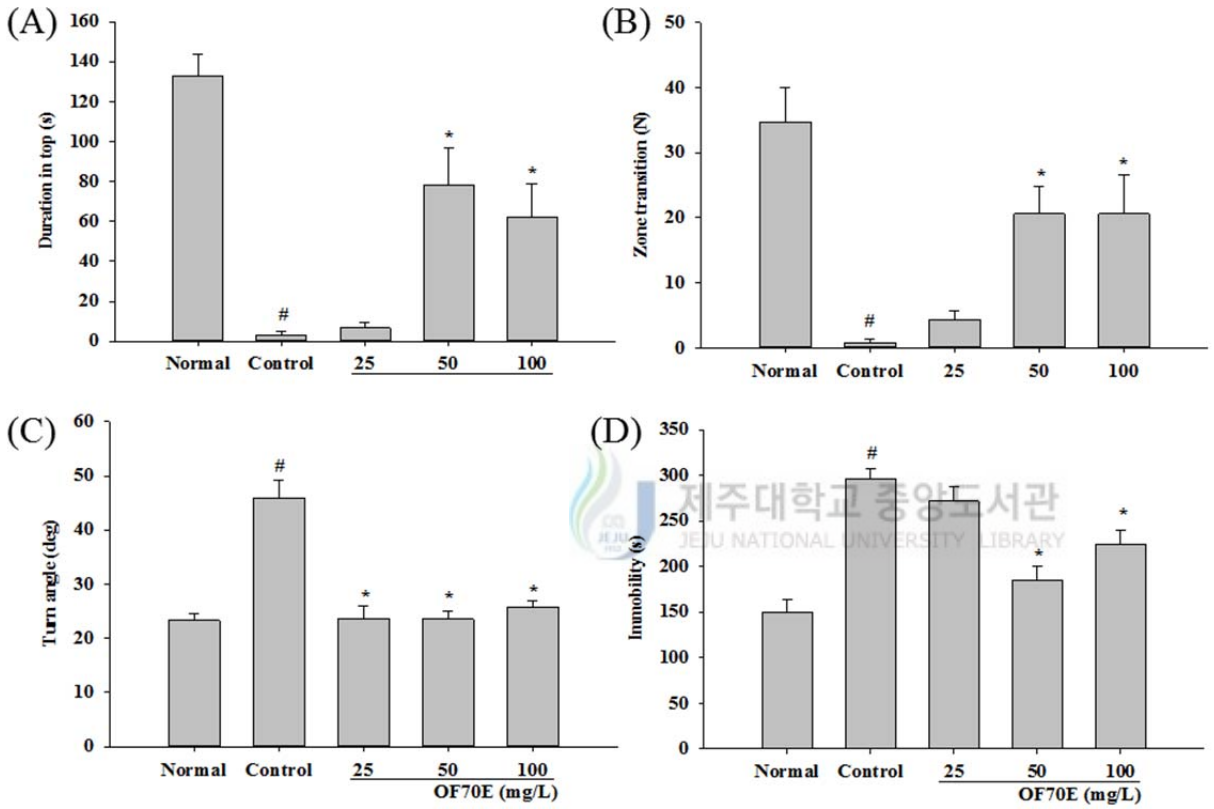

Figure 3. Summary of behavioral effects of OF70E (25-100 mg/L) on zebrafish tested in the 6 min novel tank test (n=10-12) A) duration in top, B) zone transition, C) turn angle, D) immobility. Each bar represents mean ± S.E.M. of zebrafish. P values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test (n=10-12, # $P < 0.05$ vs normal, *$P < 0.05$ vs control, one-way ANOVA).

3.3 Anti-stress effect of OF70E on OFT

3.3.1 Distance moved

Distance moved was measured for 6 min in OFT. The distance moved was 2983.9 ± 294 cm in the normal group while it was 2004.3 ± 179.6 cm in the control group which was about 32 % decreased by NHS induction (p<0.05). Interestingly, the distance moved were 2372.6 ± 83.4 cm at concentration of 25 mg/L of OF70E (p<0.05), 2432.8 ± 163.3 cm at concentration of 50 mg/L of OF70E (p<0.05) and 2567.0 ± 89.2 cm at concentration of 100 mg/L of OF70E (p<0.05). In the OF70E-treated groups at 50-100 mg/L concentrations, distance moved significantly increased compared to the control group (Fig. 4A).

3.3.2 Meandering movement

Meandering movement was measured for 6 min in OFT. The meandering movement was 352.6 ± 101.6 deg/s in the normal group while it was 939.7 ± 344.9 deg/s in the control group which was about 166 % increased by NHS induction (p<0.05). Interestingly, the meandering movements were 223.2 ± 18.1 deg/s at concentration of 25 mg/L of OF70E (p<0.05369.3 ± 113.6 deg/s at concentration of 50 mg/L of OF70E (p<0.05) and 190.7 ± 28.1 deg/s at concentration of 100 mg/L of OF70E (p<0.05). In the OF70E-treated groups at all concentrations, meandering movement significantly decreased compared to the control group (Fig. 4B).

3.3.3 Turn angle

The turn angle was 21.3 ± 2.2 deg/s in the normal group however, it increased

by 61% to 34.9 ± 4.8 deg.s in the control group (p<0.05). It was 20.0 ± 1.2 deg/s in OF70E 25 mg/L (p<0.05), 23.1 ± 1.6 deg/s in OF70E 50 mg/L (p<0.05) and 18.0 ± 1.6 deg/s in OF70E 100 mg/L (p<0.05). Significant decrease was observed in the OF70E-treated groups at all concentrations compared to the control group (Fig. 4C).

3.3.4 Immobility

Immobile duration was measured for 6 min in OFT. The immobile duration was 31.1 ± 9.2 sec in the normal group while it was 72.1 ± 19.3 sec in the control group which was about 132% increased by NHS induction (p<0.05). Interestingly, the immobile duration were 22.9 ± 2.1 sec at concentration of 25 mg/L of OF70E (p<0.05), 30.8 ± 8.2 sec at concentration of 50 mg/L of OF70E (p<0.05) and 16.8 ± 2.5 sec at concentration of 100 mg/L of OF70E (p<0.05). In the OF70E-treated groups at all concentrations, immobile duration significantly decreased compared to the control group (Fig. 4D).

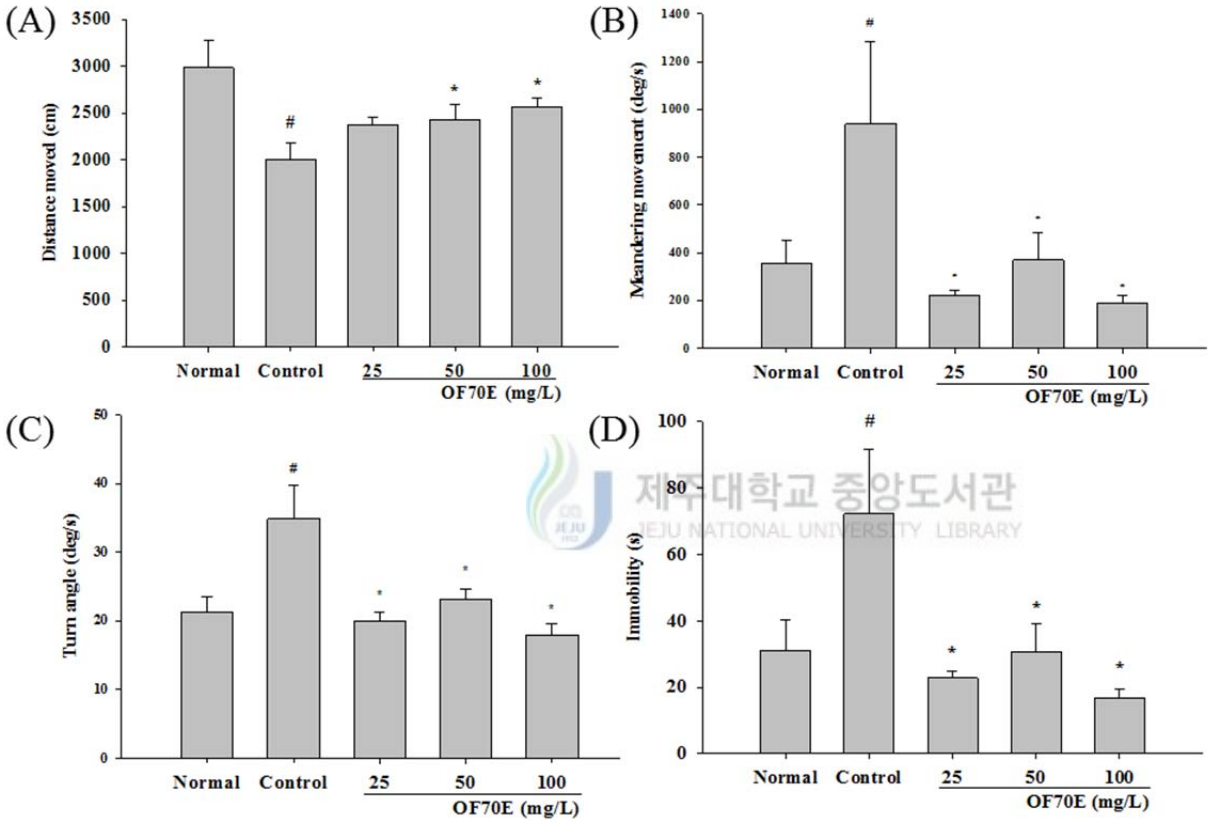

Figure 4. Summary of behavioral effects of OF70E (25-100 mg/L) on zebrafish tested in the 6-min open field test (n=10-12) A) distance moved, B) meandering movement, C) turn angle, D) immobility. Each bar represents mean ± S.E.M. of zebrafish. P values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test (n=10-12, # $P < 0.05$ vs normal, *$P < 0.05$ vs control, one-way ANOVA).

3.4 Effect of OF70E on whole-body cortisol level

To investigate whether changes in biochemical parameters such as whole-body cortisol after stress are normalized or prevented by OF70E treatment, we measured whole-body cortisol level in zebrafish treated with OF70E (Fig. 5). In the unstressed normal group, the mean whole-body cortisol level was 373.3 ± 55.2 ng/g, and this increased significantly in stressed control group(722.3 ± 77.8 ng/g, p<0.05). Increases whole-body cortisol levels after stress were significantly reduced by OF70E at concentratios of 25, 50, and 100 mg/L (409 ± 27.4, 360.9 ± 78.3, 394.9 ± 82.8 ng/g, respectively) versus the stressed control group.

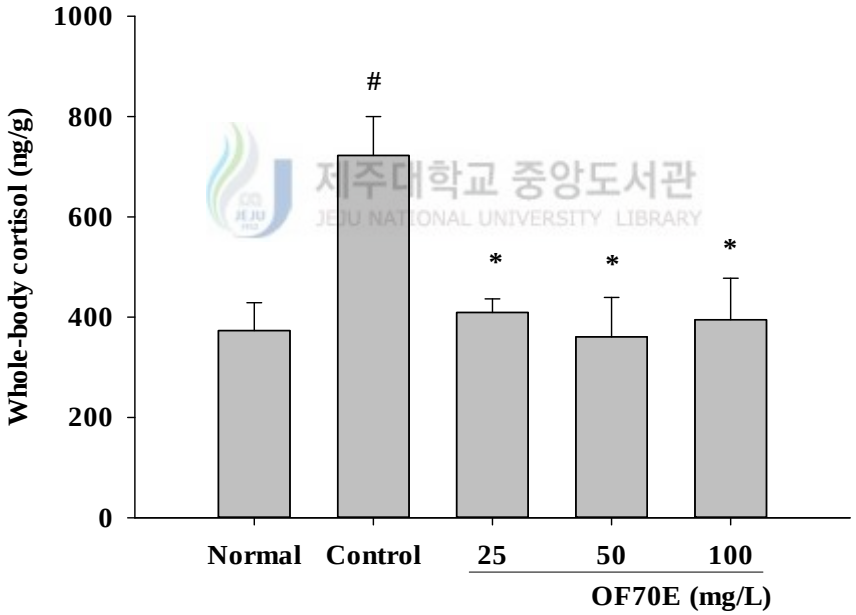

Figure 5. Whole-body cortisol level after treatment with OF70E for 6 min. (n=4, # P < 0.05 vs normal, *p < 0.05 vs control, one-way ANOVA)

3.5 Effect of OF70E fractions on whole-body cortisol level

We measured whole-body cortisol level in zebrafish treated with OF70E fraction, In the unstressed normal group, the mean whole-body cortisol level was

204.2 ± 37.3 ng/g, and this increased significantly in stressed control group (529.6 ± 57.3 ng/g, $p<0.05$). whole-body cortisol level increased by NHS were significantly reduced by treatment of at EtOAc fraction, n-BuOH fraction or H_2O fraction (270 ± 49.2 ng/g, 259.9 ± 21.2 ng/g, 214.6 ± 41.3 ng/g, respectively) versus the stressed control group ($p<0.05$, Fig. 6). Thereafter, we adopted H_2O fraction for further studies.

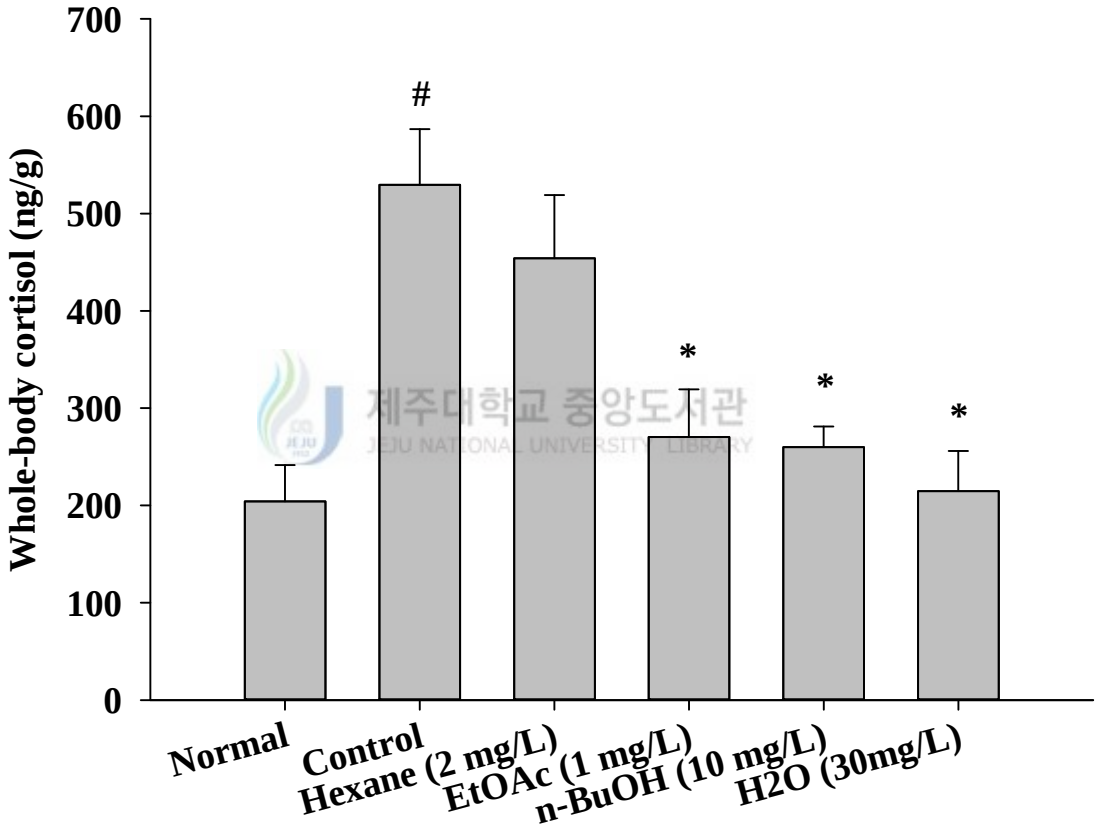

Figure 6. Whole-body cortisol level after treatment with OF70E fraction for 6 min. (n=4, # P < 0.05 vs normal, *p < 0.05 vs control, one-way ANOVA)

3.6 Anti-stress effect of OF70E water-fractions (OFWF) on NTT

3.6.1 Duration in top

Duration in top was measured for 6 min in NTT. The duration in top was 92.1 ± 23.1 sec in the normal group while it was 7.9 ± 2.9 sec in the control group which was about 90% decreased by NHS induction (p<0.05). Interestingly, the duration in top were 129.1 ± 31.7 sec at concentration of 15 mg/L of OFWF (p<0.05), 81.7 ± 17.4 sec at concentration of 30 mg/L of OFWE (p<0.05) and 39.3 ± 13.8 sec at concentration of 60 mg/L of OFWF (p<0.05). In the OFWF-treated group at 15 mg/L concentrations, duration in top significantly increased compared to the control group (Fig. 7A).

3.6.2 Zone transition

Zone transition was measured for 6 min in NTT. The zone transition was 24.2 ± 3.9 times in the normal group while it was 3.1 ± 1.1 times in the control group which was about 83 % decreased by NHS induction (p<0.05). Interestingly, the zone transition were 13.3 ± 2.5 times at concentration of 15 mg/L of OFWF (p<0.05), 11.8 ± 1.7 times at concentration of 30 mg/L of OFWF (p<0.05) and 11.5 ± 3.5 times at concentration of 60 mg/L of OFWF (p<0.05). In the OFWF-treated group at 15 mg/L, zone transition significantly increased compared to the control group (Fig. 7B).

3.6.3 Turn angle

The turn angle was 30.9 ± 0.6 deg/s in the normal group however, it increased by 60% to 48.3 ± 4.1 deg/s in the control group (p<0.05). It was 36.2 ± 3.1 deg/s in

OFWF 15 mg/L (p<0.05), 30.9 ± 2.5 deg/s in OFWF 30 mg/L (p<0.05) and 39.3 ± 6.9 deg/s in OFWF 60 mg/L (p<0.05). Significant decrease was observed in the OFWF-treated groups at 30 mg/L concentrations compared to the control group (Fig. 7C).

3.6.4 Immobility

Immobile duration was measured for 6 min in NTT. The immobile duration was 38.9 ± 4.7 sec in the normal group while it was 113.3 ± 21.9 sec in the control group which was about 200 % increased by NHS induction (p<0.05). Interestingly, the immobile duration were 42.2 ± 5.2 sec at concentration of 15 mg/L of OFWF (p<0.05), 53.8 ± 8.4 sec at concentration of 30 mg/L of OFWF (p<0.05) and 57.2 ± 10.1 sec at concentration of 60 mg/L of OFWF (p<0.05). In the OFWF-treated group at all concentrations, immobile duration significantly decreased compared to the control group (Fig. 7D).

3.6.5 Distance moved

Distance moved was measured for 6 min in NTT. The distance moved was 2530.5 ± 232.5 cm in the normal group while it was 1421.9 ± 196.5 cm in the control group which was about 43 % decreased by NHS induction (p<0.05). Interestingly, the distance moved were 2119.6 ± 98.4 cm at concentration of 15 mg/L of OFWF (p<0.05), 1945.3 ± 82.9 cm at concentration of 30 mg/L of OFWF (p<0.05) and 1975.7 ± 129.1 cm at concentration of 60 mg/L of OFWF (p<0.05). In the OFWF-treated group at 15 mg/L concentrations, distance moved significantly increased

compared to the control group (Fig. 7E)

3.6.6 Velocity

Velocity was measured for 6 min in NTT. The velocity was 6.9 ± 0.7 cm/s in the normal group while it was 4.1 ± 0.6 cm/s in the control group which was about 43% decreased by NHS induction ($p<0.05$). Interestingly, the velocity were 6.1 ± 0.3 cm/s at concentration of 15 mg/L of OFWF ($p<0.05$), 5.2 ± 0.3 cm/s at concentration of 30 mg/L of OFWF ($p<0.05$) and 5.6 ± 0.3 cm/s at concentration of 60 mg/L of OFWF ($p<0.05$). In the OFWF-treated group at 15 mg/L concentrations, velocity significantly increased compared to the control group (Fig. 7F)

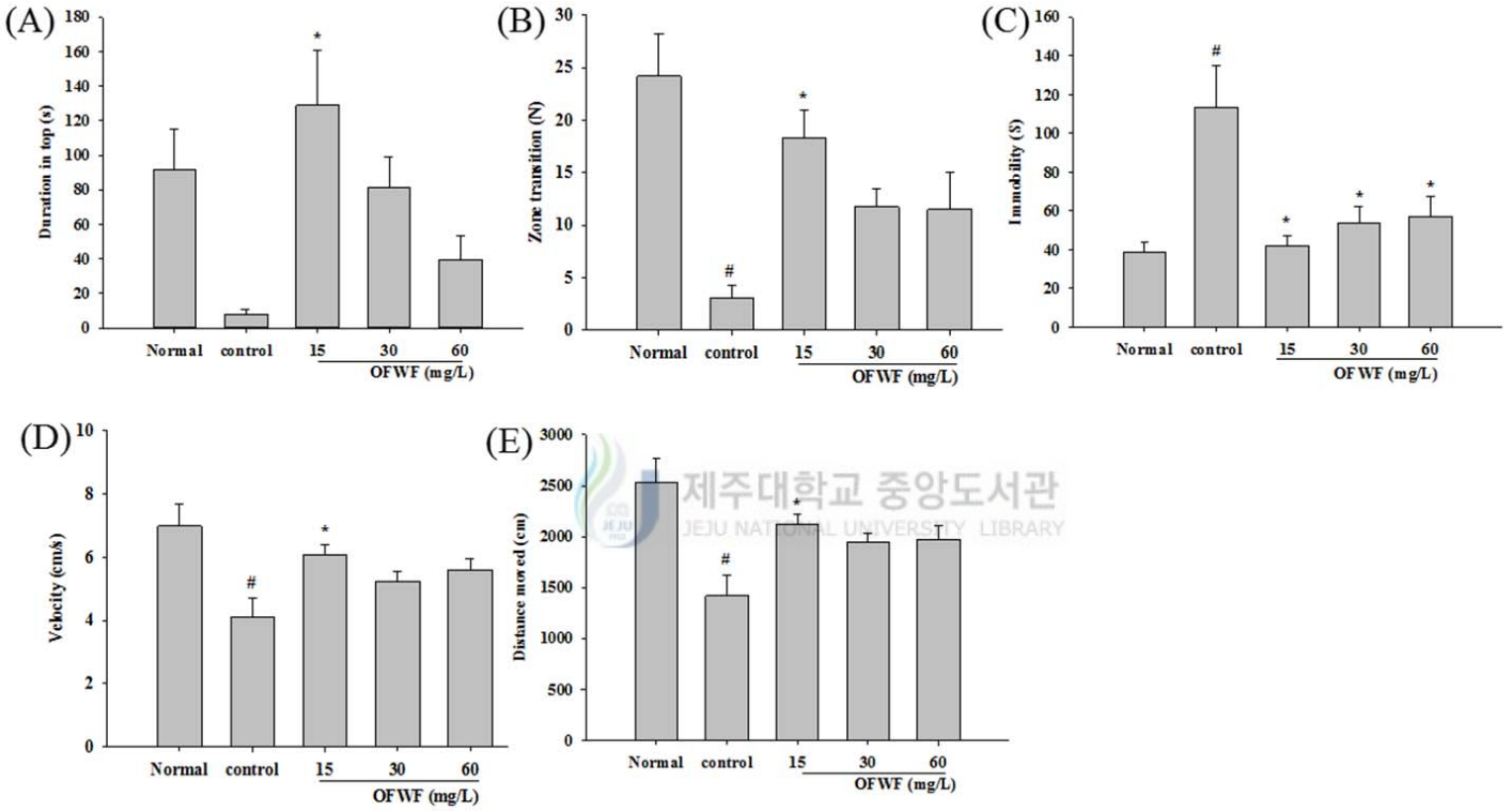

Figure 7. Behavioral effects of OF70E water fraction (15-60 mg/L) on zebrafish tested in the 6-min novel tank test (n=10-12) A) duration in top, B) zone transition, C) immobility, D) velocity, E) distance moved. Each bar represents mean ± S.E.M. of zebrafish. P valu values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test (n=10-12, # $P < 0.05$ vs normal, *$P < 0.05$ vs control, one-way ANOVA).

3.7 Anti-stress effect of OF70E water-fractions (OFWF) on OFT

3.7.1 Distance moved

Distance moved was measured for 6 min in OFT. The distance moved was 2724.8 ± 150.9 cm in the normal group while it was 1015.9 ± 86.5 cm in the control group which was about 62% decreased by NHS induction ($p<0.05$). Interestingly, the distance moved were 2436.9 ± 111.6 cm at concentration of 15 mg/L of OFWF ($p<0.05$), 2140.1 ± 218.2 cm at concentration of 30 mg/L of OFWF ($p<0.05$) and 2274.8 ± 165.6 cm at concentration of 60 mg/L of OFWF ($p<0.05$). In the OFWF-treated group at all concentrations, distance moved significantly increased compared to the control group (Fig. 8A).

3.7.2 Velocity

Velocity was measured for 6 min in OFT. The velocity was 7.7 ± 0.4 cm/s in the normal group while it was 3.3 ± 0.3 cm/s in the control group which was about 57% decreased by NHS induction ($p<0.05$). Interestingly, the velocity were 6.4 ± 0.5 cm/s at concentration of 15 mg/L of OFWF ($p<0.05$), 6.7 ± 0.4 cm/s at concentration of 30 mg/L of OFWF ($p<0.05$) and 6.3 ± 0.5 cm/s at concentration of 60 mg/L of OFWF ($p<0.05$). In the OFWF-treated group at all concentrations, velocity significantly increased compared to the control group (Fig. 8B).

3.7.3 Duration in the center zone

Duration in the center zone was measured for 6 min in OFT. The duration in the center zone was 93.9 ± 15.1 sec in the normal group while it was 19.2 ± 6.7 sec

in the control group which was about 79 % decreased by NHS induction (p<0.05). Interestingly, the duration in the center zone were 86.4 ± 7.3 sec at concentration of 15 mg/L of OFWF (p<0.05), 70 ± 15.2 sec at concentration of 30 mg/L of OFWF (p<0.05) and 70 ± 10.7 sec at concentration of 60 mg/L of OFWF (p<0.05). In the OFWF-treated group at all concentrations, duration in the center zone significantly increased compared to the control group (Fig. 8C).

3.7.4 Meandering movement

Meandering movement was measured for 6 min in OFT. The meandering movement was 917.1 ± 389.1 deg/s in the normal group while it was 3706.9 ± 632.9 deg/s in the control group which was about 304% increased by NHS induction (p<0.05). Interestingly, the meandering movements were 716.9 ± 150.1 deg/s at concentration of 15 mg/L of OFWF (p<0.05). 1148.4 ± 416.6 deg/s at concentration of 30 mg/L of OFWF (p<0.05) and 1135.1 ± 288.4 deg/s at concentration of 60 mg/L of OFWF (p<0.05). In the OFWF-treated group at all concentrations, meandering movement significantly decreased compared to the control group (Fig. 8D).

3.7.5 Turn angle

The turn angle was 27.1 ± 1.8 deg/s in the normal group however, it increased by 140% to 65.2 ± 5.4 deg/s in the control group (p<0.05). It was 31.2 ± 2.1 deg/s in OFWF 15 mg/L (p<0.05), 33.9 ± 4.4 deg/s in OFWF 30 mg/L (p<0.05) and 35.8 ± 4.6 deg/s in OFWF 60 mg/L (p<0.05). Significant decrease was observed in the OFWF-treated groups at all concentrations compared to the control group (Fig. 8E).

3.7.6 Immobility

Immobile duration was measured for 6 min in OFT. The immobile duration was 103.9 ± 18.4 sec in the normal group while it was 317.7 ± 9.5 sec in the control group which was about 207% increased by NHS induction (p<0.05). Interestingly, the immobile duration were 164.9 ± 19.2 sec at concentration of 15 mg/L of OFWF (p<0.05), 164.6 ± 28.7 sec at concentration of 30 mg/L of OFWF (p<0.05) and 176.8 sec ± 19.6 sec at concentration of 60 mg/L of OFWF (p<0.05). In the OFWF-treated group at all concentrations, immobile duration significantly decreased compared to the control group (Fig. 8F)

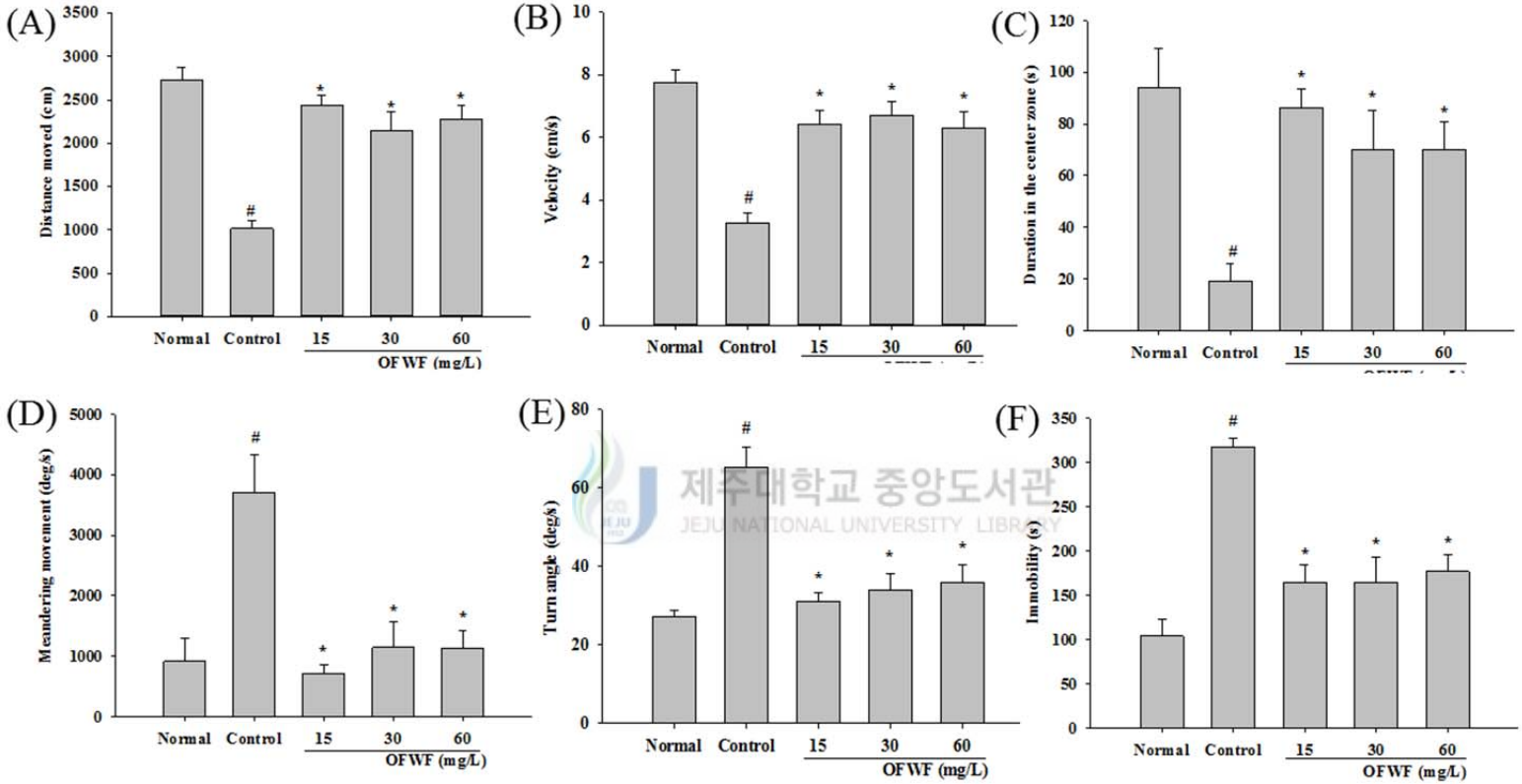

Figure 8. Summary of behavioral effects of OF70E water fraction (15-60 mg/L) on zebrafish tested in the 6-min open field test (n=10-12) A) distance moved, B) velocity, C) duration in the center zone, D) meandering movement, E) turn angle, F) immobility. Each bar represents mean ± S.E.M. of zebrafish. P value values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test (n=10-12, # $p < 0.05$ vs normal, *$p < 0.05$ vs control, one-way ANOVA).

3.8 Whole-body cortisol

To investigate whether changes in biochemical parameters such as whole-body cortisol after stress are normalized or prevented by OFWE treatment, we measured whole-body cortisol level in zebrafish treated with OFWE (Fig. 9). In the unstressed normal group, the mean whole-body cortisol level was 107.9 ± 34.7 ng/g, and this increased significantly in stressed control group(661.2 ± 72.5 ng/g, $p<0.05$). Increases whole-body cortisol levels after stress were significantly reduced by OFWE at concentrations of 15, 30, and 60 mg/L (224.2 ± 52.8, 343.7 ± 21.1 and 350.8 ± 46.7 ng/g, respectively) versus the stressed control group.

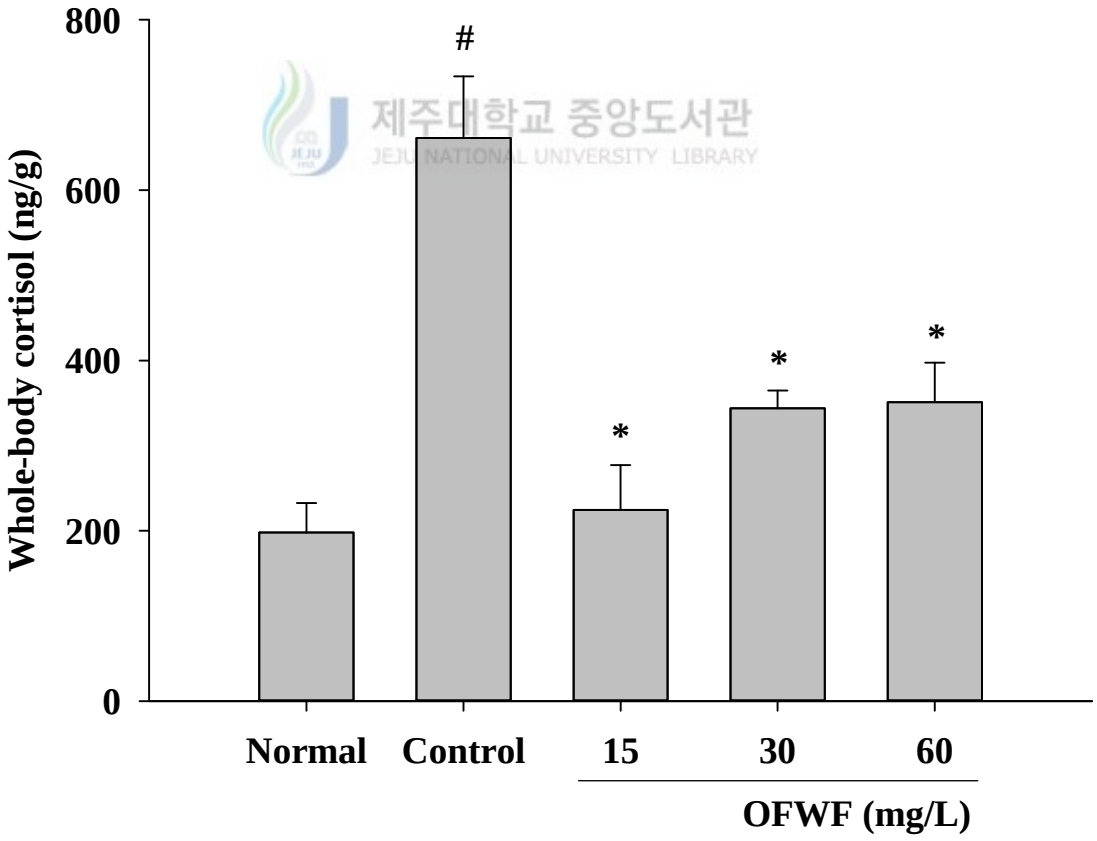

Figure 9. Whole-body cortisol level after treatment with OF70E water fraction for 6 min. (n=4, #$p < 0.05$ vs normal, *$p < 0.05$ vs control, one-way ANOVA)

4. Discussion

Stress is all the responses, which a living organism shows to protect itself when it receives harmful stimulations from external environment. Stress stimulates hypothalamus by the signal from the peripheral nervous system and CRH secretes ACTH to bloodstream. The secreted ACTH stimulates adrenal cortex and then, promotes the secretion of cortisol in mammals [9]. Zebrafish with similar gene with humankind also secretes cortisol through its HPI axis. The endocrine system of zebrafish works similarly to the cortisol-related endocrine system of mammals [2].

When excessive stress is retained, stress hormone works on central nervous system and hormone system strongly. It results in the change in behavior or decrease in immunity function [18, 23]. The previous literatures reported that zebrafish under stress becomes immobile as adaptation to the new environment, sinks down to bottom and stays immobile, becomes overexcited and performs abnormal swimming patterns with its head directed to bottom or shows erratic movement and jumps out of water [7, 24]. Therefore, in this study, we measured total distance moved, immobility duration, duration in top, turn angle, meandering movement and zone transition utilizing NTT or OFT. In the present study, we observed that locomotor activity was reduced and anxiety-like behavior increased in stressed zebrafish. In addition to these behavioral parameters, whole-body cortisol levels were increased. Stress-induced anxiety behaviors, which in the present were characterized by a reduced duration in top, increased meandering movement or increased turn angle, were reduced by OF70E treatment. Furthermore, increases in whole-body cortisol levels induced NHS

were reversed by OF70E treatment in a dose-dependent manner. Our results suggest that OF70E may effectively block stress-induced cortisol release. The results showed that anti-stress effects of OF70E 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 food to conquer the stress.

The major constituents of *O. ficus indica* var. *saboten* are flavonoid, such as, quercetin, (+)-dihydroquercetin, and quercetin 3-methyl ether, which have antioxidant and anti-inflammatory activities [11]. Quercetin also exhibited anxiolytic activity via positive allosteric modulation of the GABA receptor complex by interacting with the benzodiazepine site [19]. Modifications of side chains on flavones have been found to be selective for a number of receptor systems, including the opiate receptor and the GABA receptor [19, 27] Therefore, although It was not possible to verify anti-stress compound of *O. ficus indica* var. *saboten* , we speculated that the anti-stress effect of OF70E is due to the actions of flavonoids, and that it is mediated by their antioxidative activities and by interactions with GABA receptor.

As diseases caused by stress rapidly increase recently, current study trend is the finding and studying natural substance materials such as *Scutellaria baicalensis* and *Schizandra chinensis*, which have less adverse effect in the efficient treatment of stress-related diseases [21]. The result of this study suggests that OF70E can be developed to a medicine, which restricts stress, as material for functional food. In further study, we need to clarify mechanism and active components in *O. ficus indica*

var. *saboten* which inhibits stress response.

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