A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Effects of the acute exposure oxytetracycline on the behavior and endocrine response in adult zebrafish

Eun Seong Ko

Department of Marine Life Science

GRADURATE SCHOOL

JEJU NATIONAL UNIVERSITY

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(Supervised by professor Seungheon Lee)

A thesis submitted in partial fulfillment of the requirement
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This thesis has been examined and approved.

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Abstract

Zebrafish (*Danio rerio*) has been more widely used to study pharmacology. Oxytectracycline (OTC) is a broad-spectrum antibiotic and works by interfering with the ability of bacteria to produce essential proteins. The aim of this study was to identify the effects of exposure to OTC on behavioral changes or endocrine response in zebrafish. The behavioral effects of exposure to OTC (50, 100 or 200 mg/L) were characterized in several novelty-based paradigms such as the novel tank or open field test in zebrafish. Moreover, to investigate effects of exposure to OTC on endocrine response, we measured whole-body cortisol level using cortisol ELISA assay kit. As results of novel tank test, duration in top and immobile duration were significantly increased by the exposure to OTC in a concentration-dependent manner ($p < 0.05$). On the other hand, distanced moved, highly mobile, velocity and zone transition were significantly decreased by the exposure to OTC in a concentration-dependent manner ($p < 0.05$). In addition, as results of open field test, the exposure to OTC increased immobile duration significantly ($p < 0.05$). However, distance moved, mobile duration and velocity were significantly decreased by the exposure to OTC in a concentration-dependent manner ($p < 0.05$). Besides, the exposure to OTC elevated whole-body cortisol levels in zebrafish. These results suggest that the exposure to OTC may induce chemical stress in zebrafish.

1. Introduction

Antibiotic substances are obtained from natural sources or synthetic materials. It has a function of killing microorganisms (bactericidal) or disturbing the growth of them (bacteriostatic). Antibiotics have been widely used in hospitals, conventional production systems on farms, or aquaculture environments for prevention or therapy [24].

Oxytetracycline (OTC) is broad-spectrum tetracycline antibiotic against Gram positive and Gram negative bacteria. It also effective against leptospira, rickettsia, chlamydia and mycoplasma strains [3]. OTC represents a bacteriostatic action by binding to 30S ribosome and restraining a protein synthesis. The dose is generally 75 mg/kg/day and it may be injected for 2-10 days depending on the species [22]. Typical side effects of OTC are gastrointestinal disorders, photosensitivity and may cause a damage to teeth and bones [10]. In addition, immunosuppression, renal toxicity, decline of growth, and resistant strain development were also reported in fish [6]. A large amount of OTC is frequently used especially in South Korea and the United Kingdom. It was found that such significant abuse might cause environmental contamination or develop resistant strains [3].

Zebrafish (Danio rerio) is one of the preferred animals which are extensively used in genetics, biology and pharmacological researches for various applications. This is because zebrafish and mammals have similar physiological responses even though they are each different species. For example, cortisol secretion for mammals occurs through the hypothalamic-pituitary-adrenal axis. Similarly to mammals, in zebrafish, cortisol is secreted in accordance with the signals of hypothalamic-pituitary-interenal axis [16, 21]. Actually, when a signal comes to the endocrine system of zebrafish, which was initially sent from the peripheral nervous system, It stimulates hypothalamus, and the received signal induces the secretion of corticotropin-releasing hormone (CRH). Adrenocorticotropic hormone (ACTH) is then secreted into the bloodstream from the pituitary gland by the action of CRH and the secreted ACTH reaches the head kidney which release cortisol.

It was shown from the study of mammals that secreted cortisol enhances the liver gluconeogenic capacity [12]. Released cortisol not only activates lipolysis of a fish body but also facilitate the activation of glycerol kinase and glyceraldehydes-3-phospate dehydrogenaesis in liver [2]. It was found that acute stress initially trigger the innate immune defenses and induces rapid elevation of white blood cell counts and Th1 cell reactivity [8, 25]. However, chronic stress suppresses innate immunity and distribution and differentiation of white blood cells therefore it causes a long-term immunosuppression by inducing an increase of Th2 reactivity [11, 26]. The aim of this study is therefore to investigate stress response by exposure to OTC on behavioral change and endocrine response of zebrafish.

2. Materials & methods

2.1 Animal housing

Total 116 adult male and female wild type (short fin) zebrafish were obtained from a local commercial distributer (World fish, Korea). All fish were given at least 14 days to acclimate to the laboratory environment and housed in groups of 30–40 fish per 60 L tank. The tanks were filled with filtered water and maintained at 25–27 ℃. Illumination (1000–1100 lx) was provided by light on a 16-8 hour cycle (on: 09:00 hour, off: 18:00 hour) according to the standards of zebrafish care [27]. All fish used in this study were experimentally naive, and fed Tetramin Tropical Flakes (Tetra, Germany) twice a day.

2.2 Behavioral test

Behavioral testing took place between 10:00 and 15:00 hours using tanks with water adjusted to 25–27 ℃. Several behavioral tests including the novel tank test (NTT) and open field test (OFT) were performed in this study [13]. Prior to testing, fish were pre-exposed to OTC in a 1000 ml plastic beaker for 6 minutes. Zebrafish behaviors were recorded by Ethovision XT 8.5 software (Noldus , Wageningen, Netherlands).

2.2.1 Novel tank test

For the novel tank test to assess anxiety and locomotion in zebrafish [4, 9, 18], 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-exposed to OTC for 6minutes. Zebrafish behaviors were video-recorded from the top view for 6 minutes by using Ethovision XT 8.5 to calculate duration in the top of the tank, velocity, highly mobile duration, immobile duration, latency to first in top, zone transition and distance moved.

2.2.2 Open field test

Open field test was performed to observe the effect of OTC on the behavior change and the extent of locomotor activity [5]. 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 pre-exposure, 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 minutes, using Ethovision XT 8.5 to calculate the distance moved, duration in the center zone, duration in outter zone, immobile duration, mobile duration, turn angle, and velocity.

2.3 Measurement of whole-body cortisol

The level of whole-body cortisol was measured using the method of Grossman et al. [13]. Zebrafish was put into a medicated bath of distilled water or OTC for 6 minutes. zebrafish were sacrified 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 minute. Then it was centrifuged at 3000 g for 10 minutes 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 0.5 M 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.

2.4 Statistical analysis

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 Effect of OTC on novel tank test

NTT is an experimental method to determine state of anxiety or stress of zebrafish, in which the degree of anxiety is verified by duration in top, total distance, velocity, and etc. After 6 minutes exposure to OTC at the concentration of 50, 100 or 200 mg/L, distance moved of control group was 2124.5 ± 191.6 cm. In OTC-exposed group, distance moved, which were 1543.5 ± 82.5 cm (50 mg/L), 1168.5 ± 136.1 cm (100 mg/L) and 1098.9 ± 109.9 (200 mg/L), respectively, were significantly reduced in a concentration-dependent manner (p <0.05, Fig. 1A). In latency to first in the top and duration in top, increased tendencies were observed by exposure to OTC, but significant difference was not found. The immobile duration were significantly reduced by 279.6 ± 9.0 seconds (50 mg/L), 306.5 ± 7.6 seconds (100 mg/L), and 327.9 ± 5.9 seconds (200 mg/L), respectively, in a concentration-dependent manner, versus the 203.1 ± 13.5 seconds control group by the exposure to OTC ($p<0.05$, Fig. 1D). By the exposure to OTC, the highly mobile duration in NTT were significantly reduced by 3.0 ± 0.4 seconds (50 mg/L), 1.7 ± 0.4 seconds (100 mg/L), and 1.9 ± 0.4 seconds (200 mg/L), respectively, in a concentration-dependent manner, versus 5.6 ± 1.1 seconds the control group .(p <0.05, Fig. 1E). With regards to the velocity, control value was 6.4 ± 0.3 cm/s. When processing at concentration of 50, 100 and 200 mg/L, the result for each concentration was 4.1 ± 0.2, 3.4 ± 0.3 and 2.9 ± 0.3 cm/s respectively, significant decrease was observed in velocity($p <0.05$, Fig. 1F). In OTC-exposed group at concentration of 200 mg/L, zone transition was significant decreased by 5.6 ± 1.4 times compared to control group ($p <0.05$, Fig. 1G).

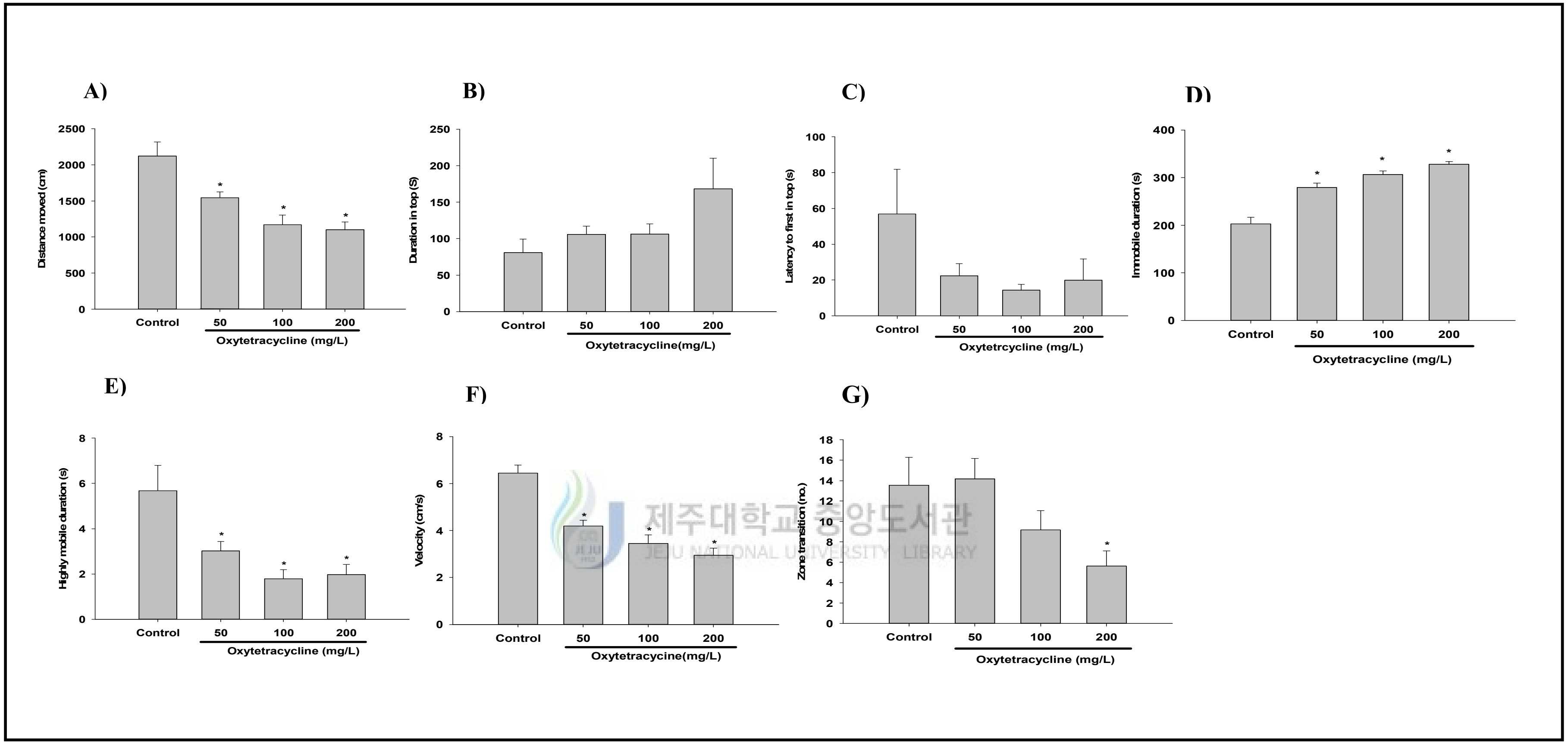

Figure 1. Effects of Oxytetracycline (50, 100 or 200 mg/L) on (A) distance moved, (B) duration in top, (C) latency to top, (D) immobile duration, (E) highly mobile duration, (F) velocity and (G) zone transition in the novel tank test in zebrafish. Each bar represents mean ± S.E.M. of 10-12 animals. P values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test (*$p < 0.05$ vs control).

3.2 Effect of OTC on open field test

OFT is an experimental method measures the swimming pattern and locomotor activity of the zebrafish. This method is similar to that of reodent. In the control group, the distance moved was 3050.2 ± 466.2 cm. In OTC-exposed groups, we observed that 1510 ± 251.6 cm at 100 mg/L, 1362.4 ± 137.6 cm at 200 mg/L. This result was statistically significant ($p < 0.05$, Fig. 2A.). By the exposure to OTC, the immobile duration in OFT were significantly increased by 270.1 ± 15.4 seconds (200 mg/L), versus the control group . ($p<0.05$, Fig. 2B). By the exposure to OTC, the mobile duration in OFT were significantly reduced by mobile duration 112.4 ± 22.3 seconds (100 mg/L), 87.5 ± 15.1 seconds (200 mg/L), respectively, versus the 167.9 ± 36.5 seconds control group. ($p<0.05$, Fig. 2C). In the control group velocity, control value was 8.5 ± 1.2 cm/s. In OTC-exposed group, it was observed that 5.6 ± 0.7 cm/s at 50 mg/L, 4.1 ± 0.6 cm/s at 100 mg/L and 3.7 ± 0.3 cm/s at 200 mg/L. This result was statistically significant ($p<0.05$, Fig. 2D). However, duration in center zone and duration in outter zone were not statistically significant (Fig. 2E and 2F). In meandering and turn angle, significant difference were not found (Fig. 2G and 2H).

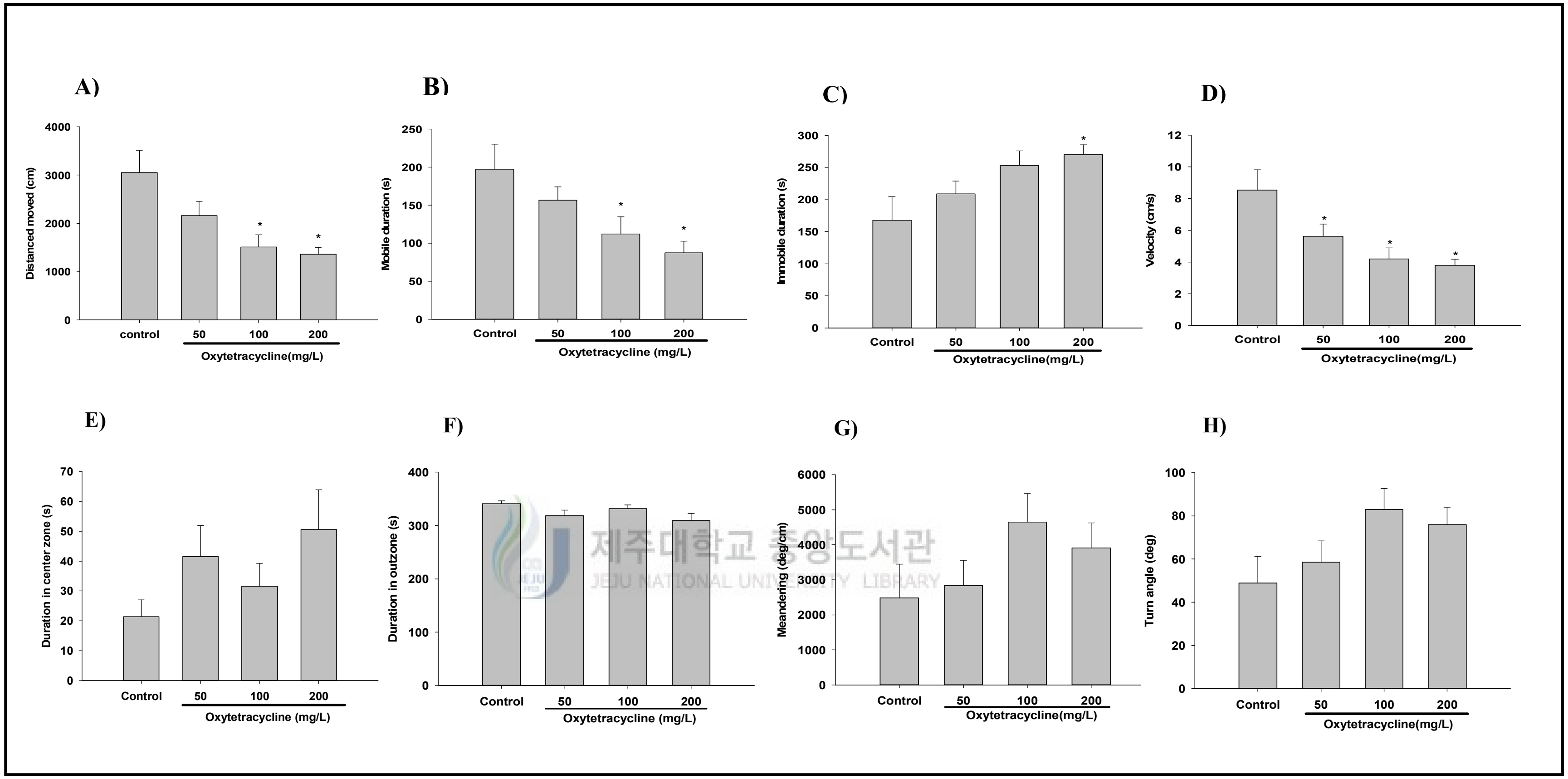

Figure 2. Effects of Oxytetracycline (50, 100 or 200 mg/L on (A) distance moved, (B) mobile duration, (C) immobile duration, (D) velocity, (E) duration in center zone, (F) duration in outzone, (G) meandering, (H) turn angle on the open field test in zebrafish. Each bar represents mean ± S.E.M. of 10-12 animals. P values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test (*$p < 0.05$ vs control).

3.3 Effect of OTC on whole-body cortisol level

To investigate whether changes in biochemical parameters after the exposure to OTC, we measured whole-body cortisol level in zebrafish. In the control group, the mean whole-body cortisol level was 152.6 ± 70.8 ng/g. Interestingly, the exposure to OTC at concentration of 200 mg/L significantly increased the whole-body cortisol level compared with control group (526.4 ± 105.2 ng/g, $p < 0.05$, Fig. 3).

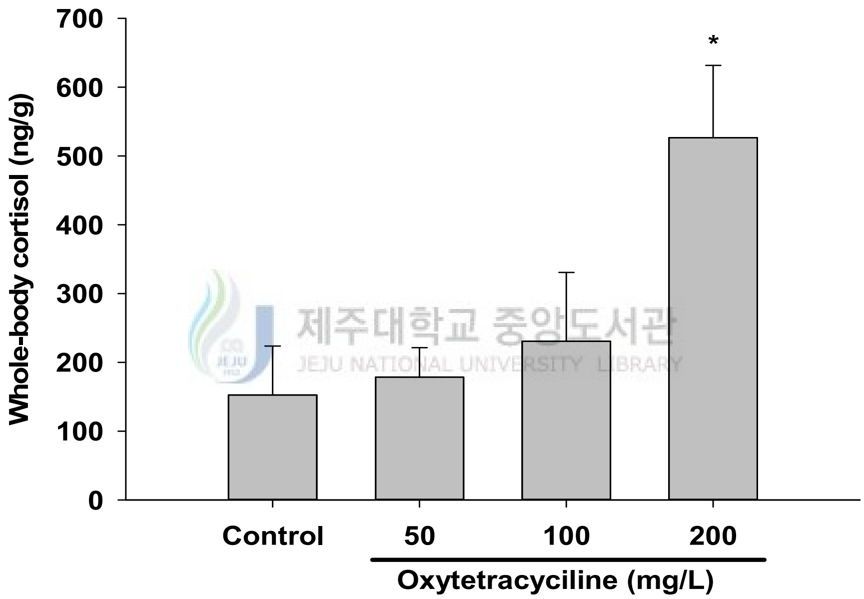

Figure 3. Whole-body cortisol level after exposure to oxytetracycline (50, 100 or 200 mg/L) for 6 min in zebrafish. Each bar represents mean ± S.E.M. of 4 animals. P values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test (*$p < 0.05$ vs control).

4. Discussion

Stress is a key factor closely related to the changes in behavior and in the endocrine system. One of the changes is to the immune system. Acute stress activates the innate immune system and leads to an increase level of white blood cells and its mobility and Th1 cell reactivity [13, 25]. Moderate levels of stress to the fish are necessary for them to survive, but the long-term exposure to stress is known to cause adverse changes in their body When exposed to chronic stress, however, it is known to cause an overall inhibition reaction within the body by rather suppressing innate immunity, oppressing the differentiation and distribution of white blood cells, and increasing Th2 reactivity [11, 26].

In this study, the stress responses of zebrafish to the OTC-exposure were observed throughout NTT, OFT and whole-body cortisol level. NTT and OFT are behavioral experiments that are commonly used to measure states of stress and anxiety [14, 17, 20]. As a result of NTT after an exposure to OTC in zebrafish, we observed decreases in the distance moved, highly mobile duration, average speed and zone transition, and increase in immobile duration. Furthermore, from OFT, we observed decreases of the distance moved and average velocity, and increase of immobile duration (Fig. 1 and 2).

Exposure to toxic compound such as microcystins acts as chemical stressor to fish. Chemical stressors induce the behavioral changes including decrease of locomotor activity or endocrine changes like increase of cortisol level in fish [15]. Microcystins are a class of toxins produced by certain freshwater *cyanobacteria*; primarily *Microcystis aeruginosa* but also other *Microcystis*, as well as members of the *Planktothrix*, *Anabaena*, *Oscillatoria* and *Nostoc* genii [7]. Kist et al. [15] demonstrated that microsystins act as a chemical stressor to zebrafish and make effets on behavior of them. In zerafish exposed to microcystins at the concentration of 100 μg/L, the immobile duration increased in NTT [15]. Immobility is one of the anxiety like (or stressed) behaviors of zebrafish and immobile duration was increased when the fish was exposed to OTC. The phenomenon by exposure to OTC is similar to that of microcystins. Therefore, we assumed that OTC also acted as a chemical stressor which induces anxiety-like behaviors.

In OFT, we observed that the distance moved, moving duration and velocity were reduced by the exposure to the OTC and increase immobile duration (Fig. 2A, 2E, 2F and 2D). Stress produces robust increases in cortisol levels and long-lasting behavioral changes, such as, decreased exploratory activity [1]. Mamczarz et al. [19] demonstrated that restraint stress makes axiety-like effects including increased immobile duration and reduced distance moved on OFT in guinea pig. Similarly to previous study, the exposure to OTC influenced exploratory activity of zebrafish in OFT. Therefore, It was believed that OTC acted as stressor like restraint stress.

Acute stress initially trigger the innate immune defenses and induces rapid elevation of white blood cell counts and Th1 cell reactivity [8, 25]. However, chronic stress suppresses innate immunity and distribution and differentiation of white blood cells therefore it causes a long-term immunosuppression by inducing an increase of Th2 reactivity [11, 26]. As a result of measuring whole-body cortisol level, which is known as a stress indicator, increase of whole-body cortisol level was observed in zebrfaish exposed to OTC. This result showed a similar to exposure to lead. Rogers et al [23]. demonstrated that acute toxicity of lead may have triggered the release of cortisol in rainbow trout. In this study, it is estimated that OTC acted as chemical stressors to zerafish like lead or microcystins.

OTC is a medicine which is now widely used in the environment of an aquaculture and fisheries, and is utilized to treat various diseases including vibriosis in flatfish, streptococcus, Pseudotuberculosis, furunculosis, vibrio infections or gill disease [3]. The use of antibiotics is an important method in the aquaculture environment which can protect the fish from the virulent diseases. It increases the commercial value of fish in market and reduce the economic loss. However, this study revealed that the excessive use of antibiotics might cause stress to fish. This study may contribute to the future research on welfare issues in fish farming and stress responses due to the use of antibiotics in aquaculture and fishery industry.

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Abstract in Korean

Zebrafish는 약리학 연구에 널리 사용되는 실험 동물 중 하나이다. Oxytetracycline은 넓은 항균 범위를 가지는 항생제로써 단백질 합성을 억제하여 정균 작용을 가진 항생제이다. 본 연구의 목적은 OTC에 노출이 되었을 때 zebrafish에게서 나타나는 행동변화와 내분비계의 변화를 관찰하였다. OTC(50, 100 or 200 mg/L)에 노출된 zebrafish의 행동변화를 확인하기 위해 novel tank test와 open field test를 진행하였다. 또한 내분비계의 변화를 확인하기 위하여 whole-body cortisol의 수치를 cortisol kit를 이용하여 측정하였다. Novel tank test 결과 상층부에서 머문 시간과 부동 시간에서 OTC를 약욕하였을 때 통계적으로 유의성이 있는 증가현상이 확인되었다. 반면에, 총 이동거리, 과행동 시간, 유영 속도 그리고 상하 변위에서 OTC를 약욕하였을 때 통계적으로 유의성 있는 감소 현상이 확인되었다. Open field test 결과 부동시간에서 OTC를 약욕 하였을 때 통계적으로 유의성 있는 증가 현상이 확인 되었다. 그러나 OTC를 약욕 하였을 때 총 이동거리, 움직인 시간, 유영 속도에서 통계적으로 유의성이 있는 감소 현상이 확인 되었다. Whole-body cortisol에서는 zebrafish에게 약욕을 하였을 때 증가하는 경향이 관찰되었다. 이러한 결과는 OTC가 zebrafish에게 화학적 스트레스로 작용한 것으로 사료된다.

Acknlowedgement

학사를 졸업하고 대학원에 입학한지가 엊그제 같은데 벌써 졸업을 앞두고 있는점이 매우 시간이 빠르게 지나간거 같습니다. 다들 논문을 주시면서 마지막 감사의 글에 '나두 졸업을 할 때 이렇게 글을 써야 겠다'라는 생각을 많이 했는데 벌써 시간이 지나서 이렇게 글을 쓰고 있는 저를 보면서 감회가 새롭습니다. 또한 저에게 도움을 주신분들이 많아서 무슨 말을 해야할지 모르겠습니다.

학사 시절 저에게 관심을 주시고, 연구의 길을 걸을 수 있도록 이끌어 주신 실험실에서 늘 많은 조언을 주신 이승헌 교수님 진심으로 감사합니다. 또한 재미있는 어류 생리학 수업과 졸업 발표 당시 저에게 많은 조언을 주신 여인규 교수님, 또한 학사 때 저에게 자신감을 갖으라고 조언해주시고 또한 대학원 전체 세미나 에서도 저에게 많은 조언을 해주신 정준범 교수님 감사합니다. 학부시절에 분자세포를 알려주신 송춘복 교수님 정말로 감사드립니다.

그리고 실험실에서 저와 같이 석사 과정을 같이 한 친구 원보 같이 학회다니면서 재미 있었던거 같아서 정말로 재미있었고, 같이 졸업 할 수 있어서 고맙다. 그리고 학부 시절 연구실 짐부터 같이 옮겼던 준영이 정말 같이 있다보니 정두 많이 든거 같고 학회 다니면서 놀면서 재미있게 놀았던거 같아 2년 동안 정말로 고생했고 고맙다. 그리고 막내 성은이 실험실에 늦게 들어왔지만 같이 실험하면서 재미 있었고 잘 알려 주지 못해서 미안했는데 많이 따라와 줘서 정말로 고맙다.

제가 대학원 들어갈 때 많은 조언을 해주신 정태형, 그리고 윤정이누나 많이 감사드립니다. 먼곳에 있어서 메일과 보이스톡으로 연락을 했는데 많은 도움이

되었습니다. 또한 앞으로 제가 나아갈 방향에 대해서 진지하게 같이 고민해주셔서 감사합니다. 그리고 사랑하는 나의 여자친구 진정아 이렇게 감사의 글에 너의 이름을 쓸수 있다는 영광을 줘서 고마워 대학원 동안 만나서 많이 놀러가지도 못했고 그런데 항상 오빠 옆에 있어줘서 고맙고, 사랑한다.

그리고 내가 이 논문을 쓰는데 까지 많은 도움을 준 덕훈이, 중건이, 성욱이, 우영이, 성범이, 경근이, 홍성이, 용준이 정말로 너희들이 없었다면 '내가 이 논문을 마무리 할 수 있었을까?' 라는 생각이 들었다 정말로 고맙고 앞으로 우리 우정 변치 말고 잘 지냈으면 좋겠다.

이외에도 저와 같이 수산질병 관리사 시험을 준비하면서 사회생활에 조언을 주신 현성일 선생님, zebrafish 해부 및 pcr하는데 도움을 주신 송헌이형, 늘 친동생처럼 챙겨주시고 많은 고민을 해결해주신 승민이형, 그리고 동생 같은 다원이, 현경이, 이외에도 모든 분들께 진심으로 감사드립니다.

마지막으로 제가 사랑하는 아버지, 저를 바르게 키워주신 어머니, 어릴적에 투닥투닥했지만 지금은 많은 조언을 주는 은아, 그리고 하늘에서 저를 많이 응원해주신 할머니 넘치는 사랑에 다시한번 감사드립니다. 그리고 저에게 힘내라고 늘 말씀해주시던 큰 고모, 작은 고모 진심으로 감사합니다.