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Changes in hematoserological profiles and leukocyte redistribution in rainbow trout (*Oncorhynchus mykiss*) under progressive hypoxia

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In recent years, global warming is causing dramatic environmental changes and deterioration, such as hypoxia, leading to reduced survival rate and growth performance of farmed aquatic animals. Hence, understanding systemic immuno-physiological changes in fish under environmental stress might be important to maximize aquaculture production. In this study, we investigated physiological changes in rainbow trout exposed to hypoxic stress by monitoring changes in blood chemistry, leukocyte population, and expression levels of related cytokine genes. Hematological and serological factors were evaluated in blood obtained from rainbow trout sampled at a dissolved level of 4.6 mg O_2 L^{-1} and 2.1 mg O_2 L^{-1}. Blood and head kidney tissue obtained at each sampling time point were used to determine erythrocyte size, leukocyte population, and cytokine gene expression. The level of LDH and GPT in fish under progressive hypoxia were significantly increased in plasma. Likewise, the (Granulocyte + Macrophage)/lymphocyte ratio (%) of fish exposed to hypoxia was significantly lower than that in fish in the control group. Such changes might be due to the rapid movement of lymphocytes in fish exposed to acute hypoxia. In this study, significant up-regulation in expression levels of IL-1β and IL-6 gene appeared to be involved in the redistribution of leukocytes in rainbow trout. This is the first study to demonstrate the involvement of cytokines in leukocyte trafficking in fish exposed to hypoxia. It will help us understand systemic physiological changes and mechanisms involved in teleost under hypoxic stress.

Key words: Progressive hypoxia; Hematology; Leukocyte redistribution; Rainbow trout

Introduction

Climate change affects marine and inland ecosystems with physical-chemical consequences such as hypoxia, stratification and salinity changes (Barange et al. 2009). Hypoxia has a wide range of effects on behavior, survival rate, and growth performance of fish (e.g., Goldberg, 1995; Diaz and Rosenberg, 2008; Levin et al., 2009; Somero, 2012). It has been shown that exposure to hypoxia can increase pathogen-related mortality and impair immunity of fish and invertebrates (Welker et al., 2007; Breitburg et al., 2015). Hence, low dissolved oxygen (DO) level and

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human-induced hypoxia could threaten hypoxia-sensitive organisms such as rainbow trout (*Oncorhynchus mykiss*), grayling (*Thymallus thymallus*), asp (*Aspius aspius*), pike (*Esox Lucius*), flounder (*Platichthys flesus*), and Russian sturgeon (*Acipencer gueldenstaedtii*) (Lai et al., 2006; Rytkönen et al., 2007). Moreover, most farmed fish are potentially in danger because they have to face progressive hypoxia (continuously decreasing DO level with times) due to oxygen pump failure, electricity dysfunction and influx of oxygen deficient water. Unfortunately, understanding immunological influence and hemato/serological changes under progressive hypoxia is very limited (Poulsen et al., 2011; Nishizawa et al., 2017). Poulsen et al. (2011) and Nishizawa et al. (2017) have investigated fish behavior and physiological characteristics of marbled sole and rainbow trout under progressive hypoxia for 260 and 240 min, respectively. They found that progressive hypoxia affected behavioral activity of fish, suggesting that physiological and immunological imbalances might have been triggered by hypoxia.

Several studies (e.g., Van Raaij et al., 1996; Petersen and Gamperl, 2010; Ni et al., 2014) have shown numerous physiological adjustments in fish after acute exposure to hypoxia, including increased levels of cortisol, glucose, erythrocytes, and/or hemoglobin. Saint-Paul (1984) found that hypoxic conditions induced changes of hematological index (e.g., hemoglobin and RBC count) in characoid fish. Taken together, hypoxia stress can be a critical factor that leads to an imbalance of cortisol and other hematological/serological factors (e.g., glucose, lactate) (O'Connor et al., 2011). Such changes can affect various physiological and immunological characteristics. Maule and Schreck (1990) have found that the number of leukocytes is significantly increased in the kidney but significantly decreased in blood and spleen of cortisol-fed coho salmon (*Oncorhynchus kisutch*), indicating that serological changes under hypoxia can broadly change fish immune system and physiological characteristics rather than local tissue devastation. Therefore, understanding immunological changes including leukocyte movement, cytokines, and hemato/serological factors under progressive hypoxic stress is crucial.

In rainbow trout, previous studies have demonstrated that hypoxia affects their physiological responses, including reduction of digestive and growth performance, modification of erythropoietin level, and delay of hematopoiesis and embryo development (See Table S1) (Van Raaij et al., 1996; Lai et al., 2006; Bianchini and Wright, 2013; Eliason and Farrel, 2014; Liu et al., 2017). However, those studies did not investigate changes in leukocyte distribution or cytokines expression, although influences of stress on leukocyte trafficking in mammals are well-documented (Davis et al., 2008). A few studies (Saint-Paul, 1984; Maule and Schreck, 1990) have tried to understand changes in leukocytes distribution of rainbow trout after exposure to hypoxia, but there is still lack of data. The objectives of this study were: 1) to understand systemic changes occurring in fish under hypoxia stress using varying serological parameters, and 2) to monitor morphological changes of erythrocytes and movement of leukocyte known to interact with cytokine expression in the head kidney.

Materials and Methods

Hypoxia stress

Rainbow trout (*Onchorhynchus mykiss*) (body weight = 76.1 ± 12.6 g) were purchased from a commercial farm in Korea and maintained in aerated, dechlorinated fresh water at 15°C. Health status of fish was examined immediately upon arrival in the aquaria and at one week thereafter. During one week of acclimation, water pumped from a water cooler (Fish Cooler, Daeil Cooler Co. CTD) created a clockwise current in the tank. Half of the rearing water was exchanged daily Fish were fed with commercial dry-pellet at a rate of 1% of their body weight during the acclimation period. Feeding was stopped one day before starting

Table S1. Comparison of experimental conditions and results of studies using rainbow trout

Dissolved oxygen (mg O_2 L^{-1})		Exposure duration	Temperature (℃)	Results	References
Hypoxic	Normoxic				
4.4, 6.7, and 8.9	11	~1.5 h	10-11	Hypoxic stress reduced digestive performance of rainbow trout.	Eliason and Farrel, (2014)
2.5+	9.1+	3 h	15	Fish exposed to hypoxia showed dramatic increased behavior index, and were killed.	Van Raaij et al., 1996
5.0 and 2.5	11	24 h	8	Hypoxic stimulus during early development increased glucose metabolism in juvenile fish.	Liu et al., 2017
5.9 and 3.2+	10.8+	4, 8, 12, 24, 48, 72, 144, 216 h	12	Erythropoietin levels was increased in the head-kidney but decreased in the spleen of fish under hypoxia.	Lai et al., (2006)
3.4+	11.3+	60-65 d	10	Hypoxia delays hematopoiesis and embryo development of fish.	Bianchini and Wright (2013)
Sampled when DO level reached to 4.6 and 2.1	8.6	1, 2.5 h (Time to reach 4.6, 2.1 mg O_2 L^{-1})	15	Hypoxia induced significant decrease of granulocyte/lymphocyte ratio, which might be related to significant increase of proinflammatory cytokines (IL-1β and IL-6), and production of different isoforms of HSP70.	This study

+DO level was inferred from % saturation based on DO calculator (MIT License, 2015).

the experiment. Thirty-six rainbow trout were separated into two ovoid tanks (125L), and they were acclimated for a week. Hypoxia stress was induced by stopping aeration to fish tank. Dissolved oxygen (DO) was continuously monitored using a DO-meter (Multi 90*i*, *iSTEK*, Korea) at 10, 20, 30, 60, 90, 120, and 150 min after stopping aeration. Total ten fish from randomly selected five fish in two tanks for the hypoxia group were sampled when DO levels reached 4.6 and 2.1 mg O_2 L^{-1} at 60 and 150 min after stopping aeration, respectively. Ten trout with DO level at 8.6 mg O_2 L^{-1} (normoxia) were achieved before starting the experiment as control. Sampled fish were euthanized with an excess of anesthetic (Ethyl 3-aminobenzoate methanesulfonate; MS-222; Sigma). Blood collected from the caudal vein was anticoagulated with either heparin or 3.8% sodium citrate depending on the purpose of further experiment. Head kidney was taken and divided into three parts. One part was treated with RNAlater™ (Invitrogen, Lithuania) and stored at -80°C. One part was fixed with 10% neutral buffered formalin while the other part was kept on ice for further analysis.

Hemato-serological analysis

Blood (approximately 2 ml each) was collected from a total of 30 fish for hematological and biochemical tests in this study. After mixing 75 μl of blood obtained from each fish with 25 μl of 3.8% sodium citrate, the anticoagulated blood was transferred into a plane capillary tube which was then vertically placed for an hour to measure erythrocyte sediment rate (ESR). Except for ESR measurement, heparin was used as an anticoagulant for all other serological tests. Venous blood was transferred into a heparinized capillary tube and hematocrit (Hct) was de-

termined after centrifuging at 12,000 rpm for 10 min (Digital Centrifuge, Digisystem Laboratory Instruments Inc., Taiwan). For RBC count and total hemoglobin concentration, blood was diluted 200 to 1,000 fold, respectively with phosphate buffered saline (PBS, pH 7.2-7.4) and analyzed using a hemocytometer and hemoglobin assay kit (Sigma, USA). For biochemical analysis, plasma was used to determine levels of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), lactate dehydrogenase (LDH), glucose (GLU), total protein (TP), total cholesterol (TCHO), and calcium (Ca) using an automated dry chemistry analyzer (FUJI DRI-CHEM 3000).

Mean corpuscular volume (MCV, μm^2) was obtained using the following formula: MCV = 10 × Hct (%) divided by RBC count (as million unit). Mean corpuscular hemoglobin (MCH, pg) was calculated by dividing total mass of Hb ($g \cdot dl^{-1}$) by RBC count (as million unit): MCH = $(Hgb \times 10) \cdot RBC^{-1}$. Mean corpuscular hemoglobin concentration (MCHC) was used to measure the concentration of hemoglobin. It was calculated with the following equation: MCHC = [Hb ($g \cdot dl^{-1}$) / Hct (%)] × 100.

Flow cytometry

Leukocytes were isolated from the head kidney using a modified method as described previously (Kim and Austin, 2006) to determine the ratio of macrophage to granulocyte and lymphocyte population. Briefly, the head kidney of each trout was grinded onto a 70-μm nylon mesh in RPMI-1640 (Sigma, USA) supplemented with 2% fetal bovine serum (FBS, Gibco), 1% Anti-anti (Antibiotic-Antimycotic; Gibco), and 10 IU · ml^{-1} heparin. Filtered suspensions were then placed onto 34/51% percoll gradient diluted by Hank's balanced salt solution and centrifuged at 400 g for 25 min at 4°C. Leukocyte band layered between 34% and 51% percoll was obtained and washed twice with RPMI-1640 medium as followed by previous study (Kim and Austin, 2006). Head kidney leukocytes and whole blood diluted 200 to 1,000 folds with Leivovitz's L-15 media were then analyzed with a flow cytometer (Accuri C6™ Flow Cytometer, BD Biosciences) by randomly selecting 10,000 cells within 1 hr. Head kidney leukocytes attached onto slide-glass were stained with May-Giemsa. Different types of leukocytes were observed under a light microscope.

Histopathology

After the head kidney was fixed in 10% neutral buffered formalin (BBC biochemical, USA) for histopathological observation and quantification of different kinds of leukocytes. After fixed organ was dehydrated and washed with graded alcohol and xylene, it was embedded in paraffin wax. Sections (approximately 4 μm in thickness) from each embedded tissue block were stained with hematoxyline-eosin (BBC biochemical, USA). Possible changes of types of leukocytes population in head kidney were analyzed by counting macrophages and lymphocytes in magnified (x400) microphotographs. In histopathological examination, lymphocytes and granulocytes/macrophages in × 400 magnified head kidney sections originated from 7, 6, and 6 fish sampled from normoxia group, sub-hypoxia, and lethal hypoxia groups, respectively, were counted because not enough head kidneys remained.

RNA extraction and cDNA synthesis

Total RNA was isolated using Trizol (Life Technologies™) according to the manufacturer's protocol. One microgram of total RNA after treating with DNase (Sigma, USA) was used to synthesize cDNA using a MMLV-reverse transcriptase kit (Bioneer, Korea). Briefly, 100 pmol of oligo dT was supplemented to 1μg of RNA suspension, and 10μl of DEPC water was added. RNA was supplemented with 5X M-MLV reverse transcriptase reaction buffer. Then 100 mM DTT, dNTP, and reverse transcriptase were

added. The reaction mixture was brought up to a final volume of 20 μl. After heating at 65°C for 10 min, cDNA was synthesized at 40°C for 1 h and 95°C for 5 min.

Real-time PCR (Quantitative PCR)

Real-time PCR was performed to determine expression levels of IL-1β (Interleukin-1β), IL-2 (Interleukin-2), IL-6 (Interleukin-6), IL-10 (Interleukin-10) and EF-1α (Elongation factor-1α) genes using 2 μl of synthesized cDNA as template. PCR mixtures were prepared as follows: 12.5 μl of SYBR Green 2X master premix (Mbiotech, Korea), 8.5 μl of distilled water, 2 μl of cDNA, and 1 μl of forward/reverse primer (10 μM). Primer sequence are listed in Table 1. For all genes except IL-2, PCR was performed as follows: 1 cycle of pre-denaturation at 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 60 s at 60°C according to previously described method (Castro et al. 2014). Real-time PCR for quantification of IL-2 transcripts was performed by incubating at 94°C for 3 min followed by 42 cycles of 20 s at 94°C, 20s at 60°C, and 30 s at 72°C. Expressed levels of genes were normalized against the level of EF-1α (Castro et al. 2014). Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method and melting curve of each reactions was observed to verify non-specific amplification.

Statistical analysis

Hematological and serological results, qPCR results and leukocyte population were subjected to one-way analysis of variance (ANOVA) in SPSS (20.0) based on Duncan's multiple range test or Student's-t test. Significant differences among groups and/or control were represented by different letters or asterisk when *P*-value was less than 0.05. Results are expressed as mean ± standard deviation (SD).

Results

Hematology

Since two dead rainbow trout were observed when decreasing DO level up to 2.1 mg L^{-1}, 2.1 mg L^{-1} of DO group was considered as a lethal group in this study. All hematological and serum biochemical parameters (RBC count, Ht, Hb, MCV, MCHC, ESR, GOT, GPT, ALP, BUN, GLU, TCHO, TP, LDH, and Ca) in rainbow trout exposed to hypoxic stress (sub-hypoxia and lethal hypoxia groups) or control (normoxia) are shown in Table 2. RBC counts were increased after exposure to hypoxia stress compared to those observed in control animals (pre-exposure). Hb concentration also tended to increase when DO level in rearing water was decreased. LDH and GPT levels in the lethal hypoxia group were significantly higher than those in the control group, although they were not significantly different between the control group

Table 1. Primers used in this study

Target gene	Primer	References
EF-1α	F: 5'- GATCCAGAAGGAGGTCACCA -3' R: 5'- TTACGTTCGACCTTCCATCC -3'	Castro et al., 2014
IL-1β	F: 5'- GACATGGTGCGTTTCCTTTT -3' R: 5'- ACCGGTTTGGTGTAGTCCTG -3'	
IL-2	F: 5'- CATGTCCAGATTCAGTCTTCTATACACC -3' R: 5'- GAAGTGTCCGTTGTGCTGTTCTC -3'	Díaz-Rosales et al., 2009
IL-6	F: 5'- CCTTGCGGAACCAACAGTTTG -3' R: 5'- CCTCAGCAACCTTCATCTGGTC -3'	Castro et al., 2014
IL-10	F: 5'- CTGCTGGACGAAGGGATTCTAC -3' R: 5'- GGCCTTTATCCTGCATCTTCTC -3'	

Table 2. Serological and hematological changes in rainbow trout exposed to hypoxic stress

	Groups (dissolved oxygen level (mg O_2 L^{-1}))		
	Normoxia (8.6)	Sub-hypoxia (4.6)	Lethal hypoxia (2.1)
RBC (10^8 cells mL^{-1})	7.90 ± 0.94^a	10.5 ± 2.36^b	9.75 ± 2.12^b
Hematocrit (%)	39.2 ± 4.4	42.4 ± 4.2	39.6 ± 6.7
Hemoglobin (g dL^{-1})	5.28 ± 1.32	6.07 ± 2.25	6.55 ± 2.31
MCV (μm^2)	504 ± 83	429 ± 118	418 ± 73
MHC (pg)	68 ± 20	58 ± 14	71 ± 30
MCHC (%)	14 ± 4	15 ± 6	17 ± 7
ESR (%)	96 ± 2	97 ± 3	96 ± 1
GOT (U L-1)	619 ± 102	698 ± 224	662 ± 167
GPT (U L -1)	29 ± 6^a	36 ± 10^{ab}	38 ± 10^b
ALP (U L -1)	243 ± 81	269 ± 124	230 ± 60
BUN (mg dL^{-1})	2.34 ± 0.45^a	2.91 ± 0.61^b	2.41 ± 0.32^a
GLU (mg dL^{-1})	81 ± 13	97 ± 15	94 ± 43
TCHO (mg dL^{-1})	265 ± 39	311 ± 69	260 ± 46
TP (g dL^{-1})	4.52 ± 0.41	4.85 ± 0.44	5.11 ± 1.04
LDH (U L-1)	2230 ± 883^a	2942 ± 1435^a	5358 ± 2865^b
Ca (mg dL^{-1})	11.92 ± 1.07	12.28 ± 0.56	12.88 ± 1.92

[+]Superscripted different letters indicated statistically significant difference determined by Duncan's multiple range test ($P<0.05$).

and the sub-hypoxia group.

Leukocyte population in the head kidney

Distribution of leukocytes in the head kidney is shown in scatter plots (Fig. 1). Populations in P7 and P8 regions shown in Fig. 1 represent macrophages/granulocytes and lymphocytes, respectively, as confirmed by May-Giemsa staining. Granulocyte+Macrophage/lymphocytes ratio ((G+M)/L ratio) using cell number in each gating (P7 or P8) was significantly decreased from 157% to 84% when DO level was 2.1 mg L^{-1}. Although there was no significant difference in the number of granulocyte/macrophages between experimental and control groups, lymphocyte counts in fish exposed to hypoxia were significantly higher than those in the control group (Fig. 2).

Gene expression level

Expression levels of cytokine genes (IL-1β, IL-2, IL-6 and IL-10) in normoxia, sub-hypoxia, and lethal hypoxia groups are shown in Fig. 3. Expression levels of IL-1β and IL-6 in head kidney were significantly increased when dissolved oxygen level in rearing water was decreased.

Discussion

Defining hypoxic environment and normoxia condition is very complicated because they can be affected by DO level, exposure time under hypoxia, fish species, water salinity, temperature, and other various environmental factors (Richards et al., 2009). For these reasons, standards for normoxia remain controversial (Remen et al., 2013; Eliason et al., 2014). As summarized in Table S1, various hypoxic conditions have been applied to rainbow trout studies. Remen et al. (2013) have argued that 80% of air saturation is considered normoxia for Atlantic salmon (*Salmo salar*). Richards et al. (2009) have defined DO concentration of less than 2-3 mg $O_2 \cdot L^{-1}$ and 5-6 mg $O_2 \cdot L^{-1}$ as a hypoxic environment for freshwater and marine fish, respectively. Although many studies have used DO levels less than 2 mg $O_2 \cdot L^{-1}$ as hypoxic condition, Vaquer-Sunyer and Duarte (2008)

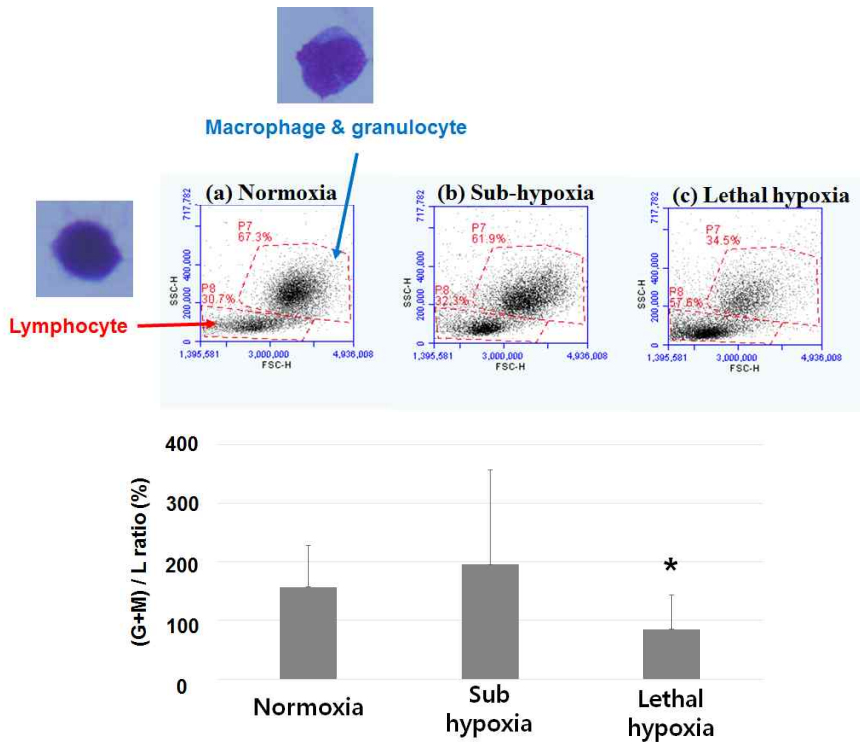

Fig. 1. Leukocyte population in head kidney of normoxia (a), sub-hypoxia (b), and lethal hypoxia (c) groups. Bottom part of graph indicates (Granulocyte + Macrophage) / Lymphocyte ratio (%) in normoxia, sub-hypoxia (4.6mg O_2 L^{-1}), and lethal hypoxia (2.1mg O_2 L^{-1}) groups. *, Significant difference, $P < 0.05$.

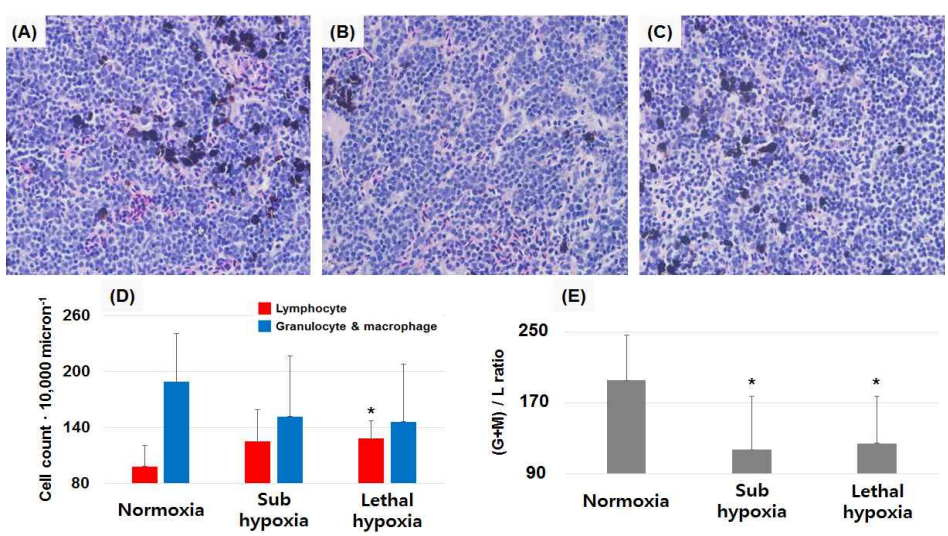

Fig. 2. Histopathological sections of the head kidney from normoxia (A), sub-hypoxia (4.6 mg O_2 L^{-1}) (B), and lethal hypoxia (2.1 mg O_2 L^{-1}) (C) groups (×400). Lymphocyte counts and granulocyte & macrophage counts in 10,000 microns of head-kidney tissue (D). (Granulocyte + Macrophage) / Lymphocyte ratio (%) was calculated by dividing granulocyte and macrophage counts to lymphocyte counts in the section (E). *, Significant difference, $P<0.05$.

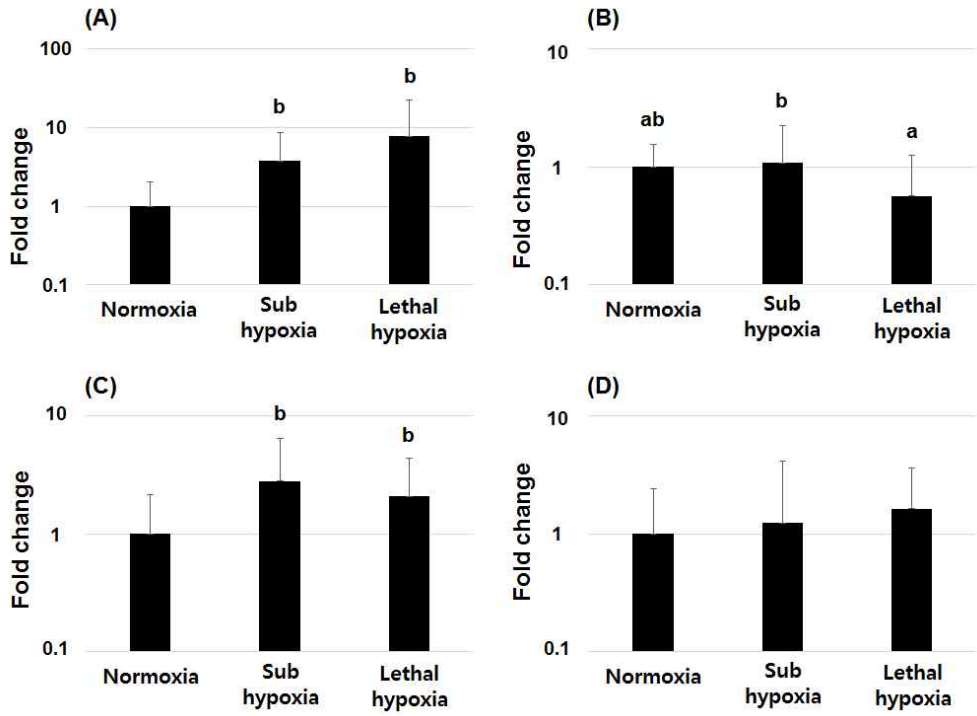

Fig. 3. Expression levels of IL-1β (A), IL-2 (B), IL-6 (C) and IL-10 (D) in the head kidney. Different letters indicate statistically significant difference determined by Duncan's multiple range test ($P < 0.05$).

have suggested that 4-5 mg $O_2 \cdot L^{-1}$ should be included as a threshold of hypoxic condition based on results of 872 published papers with 206 species. In the present study, it took 150 min for DO level to reach hypoxia (2.1 mg O_2 L^{-1}) from normoxia and two fish died at the end of hypoxic condition. This gradual hypoxia has been barely used for fish including rainbow trout. Also, Thorarensen et al. (2010) investigated blood partial pressure of oxygen (PO_2) were decreased from 8.58 to 6.00 with reducing the oxygen saturation from 100 % to 57 % in *Atlantic halibut*, which indicated that lower dissolved oxygen significantly affected oxygen saturation in trout blood. Based on studies described above and our study, 4.6 mg O_2 L^{-1} and 2.1 mg O_2 L^{-1} in this study were defined as sub-hypoxia and lethal hypoxia conditions, respectively.

Various stress related factors and catecholamines released in rainbow trout exposed to hypoxia can cause RBC swelling in order to increase Hb-O_2 affinity (Bianchini and Wright, 2013) along with increased number of circulating erythrocytes in rainbow trout and neotropical characoid fish (Saint-Paul, 1984; Lai et al., 2006). Although previous studies have shown changes in erythrocyte and leukocyte population in fish under various stressful conditions (e.g., fish exposed to the air, and drug and toxic chemical, and fed with feed containing cortisol) (Maule and Schreck, 1990; Yang and Chen, 2003; Girasole et al., 2012), none has shown changes under hypoxic stress.

In biochemical analysis of the plasma in this study, levels of LDH and GPT in fish of the lethal hypoxia group were significantly higher than those in fish of the control group. Increased LDH value in rainbow trout exposed to hypoxia has been reported in previous studies (Milligan et al., 1993; Vijayan et al., 1997). LDH can catalyze the interconversion of pyruvate to lactate, and regulate levels of these metabo-

lites in accordance with oxygen availability. Limited oxygen availability is also known to drive a switch in metabolism from mitochondrial aerobic oxidative phosphorylation to an anaerobic glycolytic pathway, resulting in induction of enzyme LDH and increased lactate (Omlin and Weber, 2010; Liu et al., 2017). Significantly increased GPT and LDH levels in the plasma of fish under hypoxia suggest that hypoxic stress can affect lactate metabolism and induce cell damage as described previously (Kim et al., 2008; Talas and Gulhan, 2009). Since liver cell damages have been much more highly associated with increasing GPT than GOT level, significantly high level of GPT not GOT has been interpreted that remarkable liver injury would have occurred during hypoxic stress (Lott and Wolf, 1986; Wolf, 1999).

Previous studies (Angelidis et al. 1987; Maule and Schreck, 1990) have already found that lymphopenia or lymphocyte redistribution in teleost can occur after bacterial infection or when cortisol mixed feed is provided. In general, the movement of leukocyte is closely related to cytokines that are known as key factors that recruit different kinds of leukocytes (Takase et al. 2006; Marques-Rocha et al. 2015). Despite the importance of cytokine function, there is little information about relationship between leukocyte populations and cytokine production in teleost. This study successfully demonstrated that interaction of leukocyte movement and cytokine expression as well as systemic changes of various hematological and serological factors under hypoxic environment. We successfully observed that the (G+M)/L ratio in fish exposed to acute hypoxia stress was decreased compared to that in fish exposed to normoxia. Reduced (G+M)/L ratio (%) (Fig. 2E) in stress-induced fish indicates that hypoxic condition can cause significant increase in the number of lymphocytes. In general, (G+M)/L ratio in vertebrate plasma can change according to cortisol and glucocorticoid levels (Angelidis et al., 1987; Maule and Schreck, 1990; Davis et al., 2008). Increased glucocorticoid in stress-induced animal can stimulate activation of β-adrenergic receptors in lymphocytes.

Subsequent interaction between these receptors and catecholamines can promote redistribution of lymphocytes (DeBlasi et al., 1986; Maule and Schreck, 1990). Such adrenergic response in lymphocytes can stimulate their traffic and cell-mediated immunity since immune surveillance and defense are activated to overcome stressed circumstance (Dhabhar, 2002; Krüger et al., 2008). Expression levels of IL-1β and IL-6 in fish exposed to hypoxia were increased significantly in this study, suggesting that these cytokines might be involved in redistribution of leukocytes. Indeed, it has been revealed that the production of stress hormone and IL-6 (also known as B lymphocyte stimulatory factor) is highly related to leukocyte redistribution in humans (Hirano et al., 1986; Lütticken et al., 1991; Kishimoto et al., 1995; Li and Gleeson, 2004; McLoughlin et al., 2005). McLoughlin et al. (2005) have demonstrated that IL-6 deficient mice show impaired recruitment of lymphocytes. IL-1β is a chemotactic factor for T-lymphocyte and a lymphocytic activation molecule (Hunninghake et al., 1987; Kruse et al., 2001). Although (G+M)/L ratio in the head kidney was significantly dropped at 150 min post hypoxic stress (lethal hypoxia group), increased expression levels of cytokines IL-1β and IL-6 were first observed at 60 min post hypoxia, indicating their possible vital roles in lymphocyte movement in fish. However, no significant expression of IL-10 known to major anti-inflammatory cytokine has been inferred that hypoxia stress did not stimulate anti-inflammatory responses in rainbow trout (Grayfer et al., 2011).

Although previous studies (Angelidis et al., 1987; Maule and Schreck, 1990; Davis et al., 2008) have shown that glucocorticoids are key factors for lymphocyte redistribution in stress-induced fish, the present study indicates that cytokines might also play important roles in trafficking of lymphocytes. Notably, (G+M)/L ratio was much more sensitive biomarkers in fish exposed to progressive hypoxia than sero-

logical parameters (GPT and LDH).

In conclusion, this study successfully demonstrated that acute hypoxia could induce increase of erythrocyte number and levels of GPT and LDH. This is the first study to show that hypoxic condition can induce significantly increased IL-1β and IL-6 expression in the head kidney that can lead to leukocyte redistribution in fish. Our results could help us understand systemic physiological response of fish against low oxygen environment.

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