

早稲田大学審査学位論文

博士（スポーツ科学）

Effects of antioxidant vitamins and polyphenols on  
skeletal muscle adaptation and endurance capacity

抗酸化ビタミンおよびポリフェノールが骨格筋の  
適応および持久力に及ぼす影響

2018年1月

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# Chapter I

## Background

Endurance exercise increases production of reactive oxygen species (ROS) in the working skeletal muscles (Abadi et al., 2013; Davies et al., 1982). Oxidative stress is a condition in which the cellular production of ROS exceeds endogenous antioxidant defense system (Powers and Jackson, 2008). ROS react with cellular components including DNA, lipid and protein and these damaged components can lead to cell dysfunction (Powers and Jackson, 2008). Additionally, acute vigorous exercise-induced oxidative stress may decrease the performance of athletes because oxidative stress may be associated with the decrease of muscle contractile function (Reid, 2001), inflammation and muscle damage (Aoi et al., 2004). Therefore, antioxidants intake is recommended for athletes. Indeed, animal studies show that antioxidants can protect skeletal muscles against oxidative stress and also prevent fatigue during prolonged strenuous exercise (Powers and Jackson, 2008; Reid, 2008).

On the other hand, several studies have shown that ROS act as signal molecules. The following explanation about ROS and skeletal muscle adaptation is a proof of our previous papers. Endurance training induces several adaptations in skeletal muscle such as mitochondrial biogenesis, glucose transporter 4 (GLUT4) expression, and angiogenesis (Lira et al., 2010). The coactivator peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) is involved in these adaptations (Baar et al., 2002;

Lira et al., 2010; Norrbom et al., 2004; Pilegaard et al., 2003). The expression and/or activation of PGC-1 $\alpha$  are regulated by multiple intracellular signaling, including AMP-activated kinase (AMPK), p38 MAPK, and Ca<sup>2+</sup> (Akimoto et al., 2005; Handschin et al., 2003; Jager et al., 2007). ROS also regulate the PGC-1 $\alpha$  expression *in vitro* (Irrcher et al., 2009; Silveira et al., 2006; St-Pierre et al., 2006). The effects of ROS on endurance training-induced adaptation of skeletal muscle have been evaluated *in vivo* by antioxidant supplementation. Gomez-Cabrera et al. (2008) reported that vitamin C supplementation prevented the increase in endurance training-induced protein and gene expression of PGC-1 $\alpha$ , nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor A (mTFA) and protein expression of cytochrome c (Gomez-Cabrera et al., 2008). Additionally, Ristow et al. (2011) has reported that vitamin C and E supplementation may inhibit exercise training-induced improvement of insulin sensitivity and muscle adaptation in humans (Ristow et al., 2009). Since then, evidence supporting that antioxidant supplementation attenuates exercise training-induced adaptations in human and rodent skeletal muscle has been published (Meier et al., 2013; Paulsen et al., 2014). However, we and other research groups have shown that antioxidant supplementation does not alter exercise training-induced adaptations (Higashida et al., 2011; Strobel et al., 2011; Yada and Matoba, 2014; Yfanti et al., 2010;

Yfanti et al., 2011). Thus, there is little agreement on the effects of antioxidant supplementation on exercise-induced skeletal muscle adaptation.

Several chemical compounds such as polyphenols, carotenoids and vitamins have antioxidant capacity. Polyphenols have several effects as well as antioxidant capacity. For instance, polyphenols have anti-obesity, anti-inflammatory, and anti-cancer effects.

Therefore, the purpose of this study was to clarify 1) the effects of vitamin C on low-intensity exercise training-induced skeletal muscle adaptation, 2) the effects of antioxidant vitamin supplementation on acute exercise-induced change of skeletal muscle adaptation-related gene expression and these signaling pathways, and 3) the effects of taheebo polyphenol supplementation on endurance capacity.

# Chapter II

## Study 1

## INTRODUCTION

Acute vigorous exercise-induced oxidative stress may decrease the performance of athletes because oxidative stress may be associated with the decrease of muscle contractile function (Reid, 2001), inflammation and muscle damage (Aoi et al., 2004). Therefore, antioxidants intake is recommended for athletes. It has been reported that antioxidant supplementation hampers exercise training-induced mitochondrial biogenesis and improvement of insulin sensitivity (Gomez-Cabrera et al., 2008; Ristow et al., 2009). These results suggest that ROS acts as exercise adaptation signaling and excess removal of ROS by antioxidants may attenuate exercise training adaptation. On the other hand, it was also reported that antioxidant supplementation did not prevent the endurance training-induced adaptation in skeletal muscle (Higashida et al., 2011; Strobel et al., 2011; Yfanti et al., 2010; Yfanti et al., 2011). Furthermore, we also reported that vitamin C supplementation did not change the high-intensity swimming exercise-induced mitochondrial biogenesis (Yada and Matoba, 2014). Therefore, the effects of antioxidant supplementation on endurance training-induced adaptation in skeletal muscle have not been elucidated because of differences in the study design; mode, intensity or duration of the training program, and the amount or type of the antioxidants used and the length of supplementation. Furthermore, Strobel et al.



indicates that these data have been somewhat misinterpreted because of the absence of an untrained antioxidant supplemented condition in the study design (Strobel et al., 2011). Also, it is possible that many of the effects previously observed with antioxidants preventing training-increased mitochondrial biogenesis were not actually due to attenuation of the exercise training response but to an overall down-regulation of basal mitochondrial biogenesis (Strobel et al., 2011). Indeed, Strobel et al. demonstrated that supplementation of vitamin E and  $\alpha$ -lipoic acid does not prevent the endurance training-induced mitochondrial biogenesis but decreases the baseline levels of mitochondrial biogenesis markers (Strobel et al., 2011).

On the other hand, it has been reported that  $\alpha$ -lipoic acid and coenzyme Q10 have positive effects for skeletal muscle adaptations (Abadi et al., 2013; Wagner et al., 2012). Furthermore, quercetin which is a kind of flavonoid supplementation also increases mitochondrial biogenesis in skeletal muscle and brain (Davis et al., 2009). These results may suggest that effects other than antioxidants of supplements are affecting the skeletal muscle adaptations. Hence, it is important to consider the influence of antioxidant supplementation on exercise training adaptation in order to accommodate the applicability of antioxidants. However, previous studies did not determine whether

vitamin C supplementation changes the skeletal muscle adaptation regardless of exercise training. In the present study, we hypothesized that vitamin C supplementation prevents exercise training-induced muscle adaptation. The aim of this study was to determine the effects of vitamin C supplementation on low-intensity prolonged endurance training-induced change of antioxidant and energy metabolic enzyme activities.

## MATERIALS AND METHODS

### *Animals*

Three-week-old male Sprague-Dawley-strain rats (n=28) were purchased from CLEA Japan, Inc. (Osaka, Japan). The rats were housed in a room with an 8:00-20:00 light and 20:00-8:00 dark cycle and fed a chow (Oriental Yeast Co., Ltd; MF) that contains vitamin C (4 mg/100 g) and water *ad libitum*. The rats took about 0.76 mg vitamin C per day from the diet. Room temperature was maintained at 22±1 °C. The animal use protocol was approved by the Animal Studies Committee of the University of Tokushima. The rats were divided into two groups (non-supplemented (N) and vitamin C supplemented (VC), n=14/ groups). After 15 days vitamin C supplementation, the rats in the N and VC groups were allocated to either an exercise training (T; n=7) or a

sedentary (S; n=7) group.

### ***Vitamin C supplementation***

The rats in the VC group were given 500 mg ascorbic acid/kg body weight/day. The dose of 500 mg ascorbic acid/kg/day has been reported to prevent the training-induced mitochondrial biogenesis (Gomez-Cabrera et al., 2008). The rats in the VC group were given the ascorbic acid for a total of 25 days. During a pre-training period for 15 days, they were given ascorbic acid dissolved in their drinking water. In the training period for 10 days, they were administered ascorbic acid solution with a feeding needle 1 h before each training session. They were given the ascorbic acid until 24 h before the dissection. They were also allowed to have free access to drinking water containing no ascorbic acid. The rats of the N group were given water *ad libitum* for the experimental period of 25 days. Furthermore, in the training period, they were administered water with a feeding needle 1 h before each training session.

### ***Training protocol***

Low-intensity prolonged swimming training was employed for this study. The study used the training protocol described by Terada et al. (Terada et al., 2002; Terada and

Tabata, 2004). The rats of exercise groups swam without a load for 6 h in two 3-h sessions separated by a 45-min period of rest. Training period was consecutive 10 days. Four or six rats swam in a barrel filled with water to a depth of 50 cm and with an average surface area of 200 cm<sup>2</sup>/rat. Water temperature in the barrel was maintained at 36 °C.

### ***Tissue preparation***

All rats were anesthetized with the sodium pentobarbital (40 mg/kg) 48 h after the last training session, to avoid the effect of the acute exercise. The triceps brachii muscle was excised, quickly frozen in liquid nitrogen and stored at -50 °C until analysis. Blood samples were collected from tail vein and centrifuged at 980 xg for 3 min. Then, plasma samples were stored at -34 °C for later analysis.

First, the frozen triceps brachii muscles were homogenized in ice-cold 50mM phosphate buffer (pH 7.4) containing 1mM EDTA. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. This supernatant was used for the measurement of activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) and trolox equivalent antioxidant capacity (TEAC). Second, the frozen triceps brachii muscles

were homogenized in ice-cold 50mM phosphate buffer (pH 6.8) containing 1 mM EDTA. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. This supernatant was used for the measurement of protein carbonyl and catalase activity. Then, this supernatant was added 10% metaphosphoric acid and centrifuged at 10,000 rpm for 5 min at 4°C. This supernatant was used for the measurement of total glutathione. Third, the frozen triceps brachii muscles were homogenized in ice-cold 0.17 M phosphate buffer (pH 7.4) containing 0.05% BSA. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. This supernatant was used for the measurement of citrate synthase (CS) and hexokinase (HK) activities.

### ***Measurement of oxidative stress markers***

ROS production in skeletal muscle was measured using 2', 7'-dichlorofluorescein diacetate (H2DCFDA : Invitrogen-Molecular Probes, D399, Invitrogen, Carlsbad, CA) and according to the method of Koltai et al. (Koltai et al., 2012). Protein carbonyl, a marker of protein oxidation, in triceps brachii muscle was measured with Protein Carbonyl Assay Kit (Cayman Chemical Co., Ann Arbor, MI). Plasma d-ROMs, blood marker of oxidative stress were measured according to the method of Verde et al. (Verde et al., 2002).

### ***Measurement of antioxidant enzyme activities***

Activities of SOD, GPx and CAT were measured with commercially available assay kit (Cayman Chemical Co., Ann Arbor, MI).

### ***Measurement of marker of non-enzymatic antioxidant capacities***

Non enzymatic antioxidant capacity TEAC in triceps brachii muscle was measured according to the methods of Re et al., and total glutathione (TGSH) was measured with Glutathione Assay Kit (Cayman Chemical Co., Ann Arbor, MI). Ferric reducing antioxidant power (FRAP) was measured according to the methods of Benzie and Strain (1999), for evaluation of blood non-enzymatic antioxidant capacity (Benzie and Strain, 1999).

### ***Measurement of blood vitamin C concentration***

Plasma vitamin C concentration was measured using the method described by Benzie and Strain (Benzie and Strain, 1999).

### ***Measurement of skeletal muscle enzyme activities***

CS and HK activities of the triceps brachii muscle were measured according to the methods of Srere and Simoneau et al., respectively (Simoneau et al., 1985; Srere, 1969).

### ***Statistical analysis***

Data are presented as means  $\pm$  standard errors (SE). A two-way analysis of variance (ANOVA) was performed (SPSS V17.0, IBM Japan, Ltd, Tokyo, Japan) to assess the main effects of exercise and/or VC supplementation. If this analysis revealed a significant interaction, Bonferroni's post-hoc test was utilized to determine the significance among the means. Statistical significance was set at  $P < 0.05$ .

## **RESULTS**

### ***Effects of low-intensity swimming training and vitamin C supplementation on markers of oxidative stress***

The muscle protein carbonyl concentration in the VC+T group was significantly lower in comparison to that of the N+T group ( $P < 0.05$ , Fig. 1 A). Plasma d-ROMs concentration was significantly increased by low-intensity swimming training ( $P < 0.05$ , Fig. 1 B). However, vitamin C supplementation did not alter plasma d-ROMs concentration (Fig. 1 B). In skeletal muscle, low-intensity swimming training

significantly increased ROS production ( $P < 0.05$ , Fig. 1 C). However, vitamin C supplementation did not affect ROS production in skeletal muscle (Fig. 1 C).

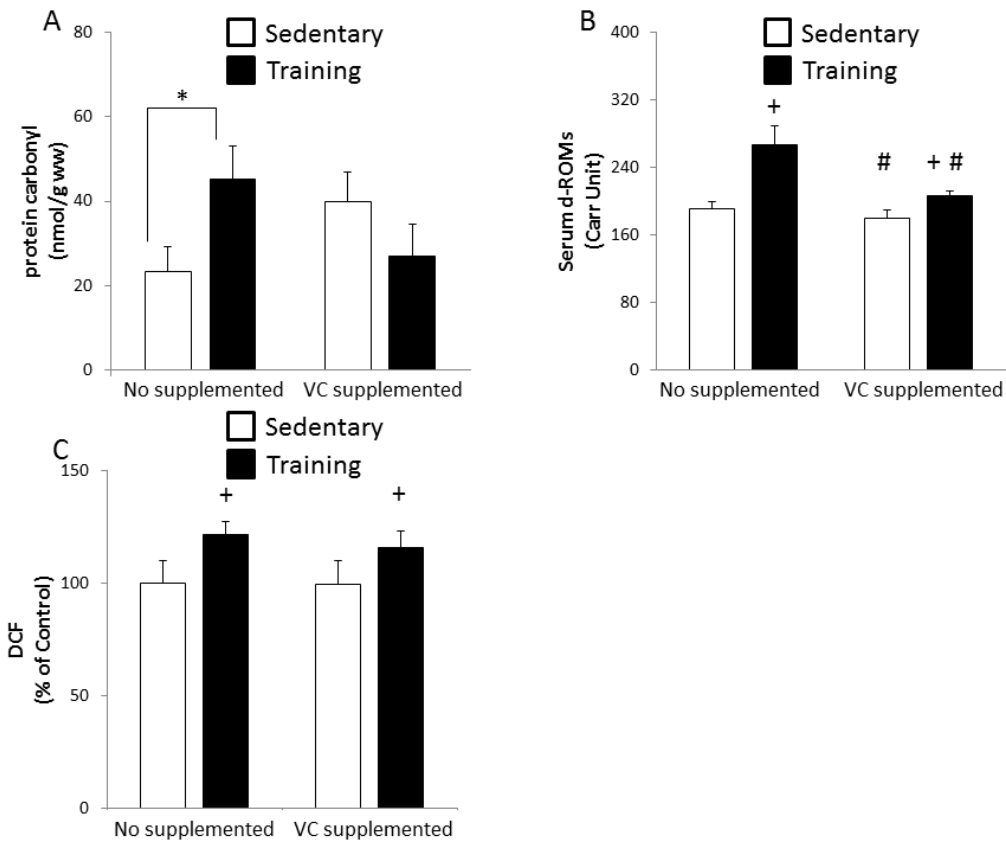


Figure 1. Markers of oxidative stress in skeletal muscle and plasma after training and vitamin C (VC) supplementation. means $\pm$ SE. \*,  $P < 0.05$ , vs no supplemented exercise group. +,  $P < 0.05$ , main effect for training. #  $P < 0.05$  main effect for VC supplementation.

***Effects of low-intensity swimming training and vitamin C supplementation on muscle antioxidant activities***



After consecutive 10 days low-intensity swimming training, plasma vitamin C concentration was significantly decreased ( $P<0.05$ , Fig. 2 A). However, vitamin C supplementation did not alter plasma vitamin C concentration (Fig. 2 A). TEAC in skeletal muscle and plasma TEAC were not changed by exercise training and vitamin C supplementation (Fig. 2 B, C). Total glutathione in skeletal muscle was increased by vitamin C supplementation but was not altered by exercise training ( $P<0.05$ , Fig. 2 D).

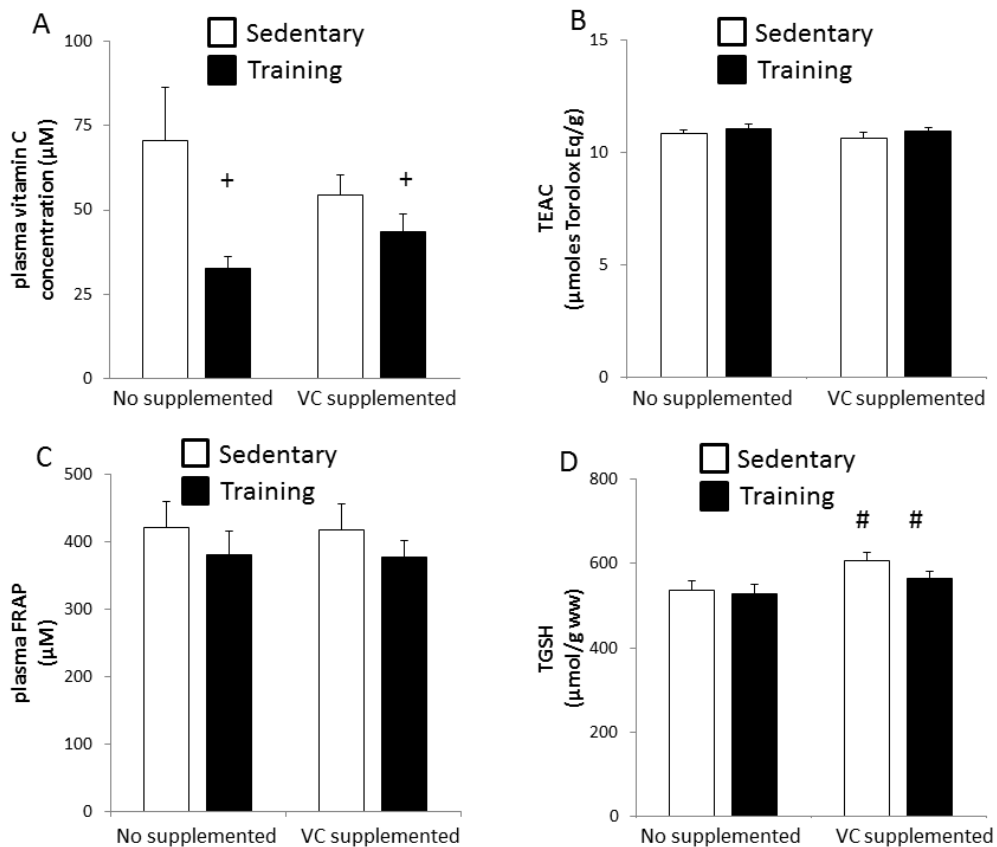


Figure 2. Non-enzymatic antioxidant in skeletal muscle and plasma after training and vitamin C (VC) supplementation. means $\pm$ SE. +,  $P<0.05$ , main effect for training. #,  $P<0.05$ , main effect for VC supplementation.

P<0.05, main effect for VC supplement.

*Effects of low-intensity swimming training and vitamin C supplementation on enzymatic antioxidant capacity*

In skeletal muscle, SOD activity was increased by exercise training (P<0.05, Fig. 3 A). But, vitamin C did not alter the muscle SOD activity (Fig. 3 A). On the other hand, CAT activity in skeletal muscle was decreased by low-intensity swimming training and increased by vitamin C supplementation (P<0.05, Fig. 3 B). GPx activity in skeletal muscle was not changed by training and vitamin C supplementation (Fig. 3 C).

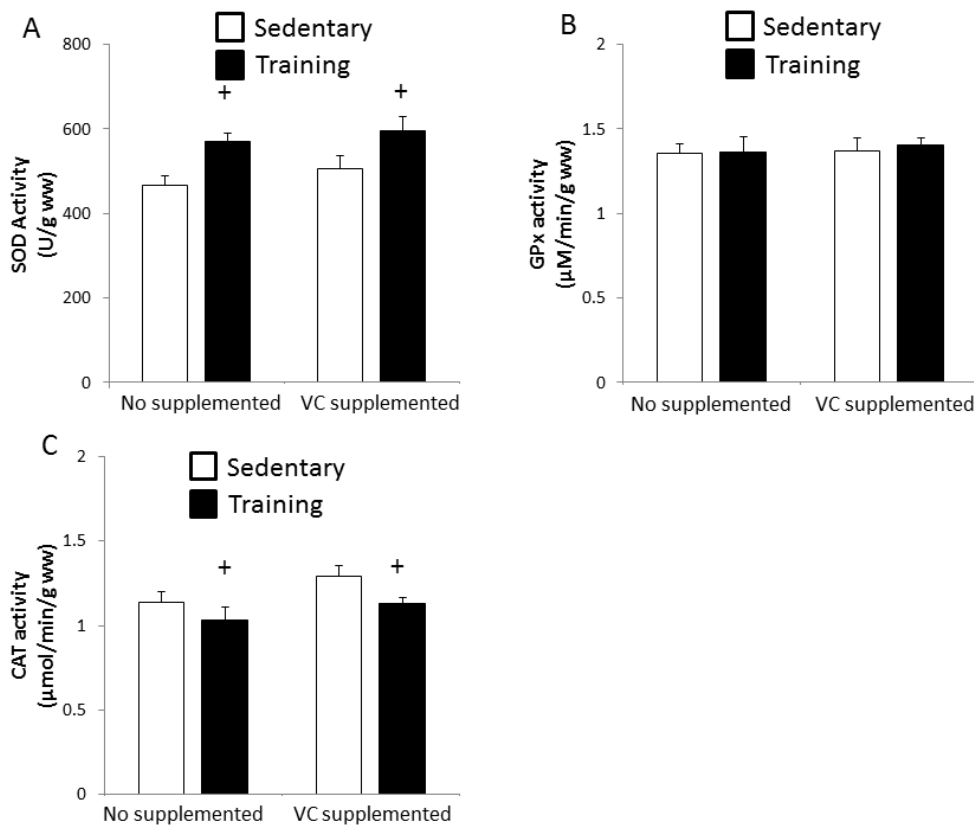


Figure 3. Antioxidant enzyme activities in skeletal muscle after training and vitamin C (VC) supplementation. means±SE. +, P<0.05, main effect for training. #, P<0.05, main effect for VC supplementation.

*Effects of low-intensity swimming training and vitamin C supplementation on skeletal muscle enzyme activities*

CS and HK activities in skeletal muscle were increased by exercise training (P<0.05, Fig 4 A, B). However, vitamin C did not alter the skeletal muscle CS and HK activities (Fig 4 A, B).

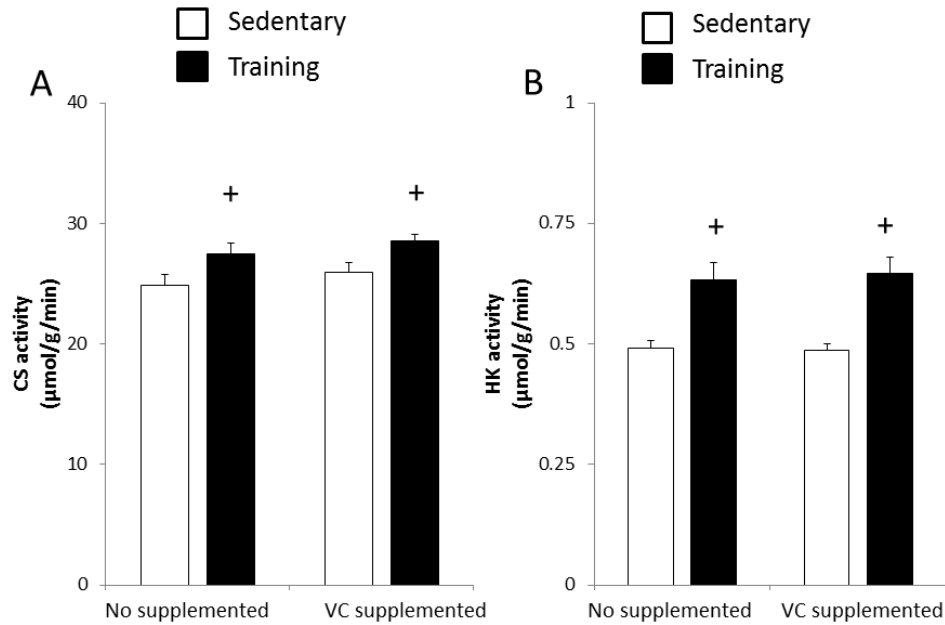


Figure 4. Metabolic enzyme activities in skeletal muscle after training and vitamin C (VC) supplementation. means $\pm$ SE. +,  $P < 0.05$ , main effect for training.

## DISCUSSION

In present study, we investigated the effects of VC supplementation on the change of skeletal muscle enzyme activities by long-term swimming exercise training for 10 days.

We found that CAT activity in skeletal muscle was increased by vitamin C supplementation but decreased by exercise training. On the other hand, SOD, CS and HK activities were increased by exercise training but not changed by vitamin C

supplementation.

The rats of the training groups in the current study worked on low-intensity prolonged swimming training. It has been reported that this training model induces increase in PGC-1 $\alpha$  protein content, CS, HK and  $\beta$ -HAD activities, and SOD2, GPx protein content in rat skeletal muscle (Higashida et al., 2011; Terada and Tabata, 2004). In agreement with previous studies, the skeletal muscle CS, HK and SOD activities of the training group rats in the present study were increased by consecutive training for 10 days.

Endurance exercise increases production of ROS in skeletal muscle. Also, produced ROS induces oxidative stress. Furthermore, Higashida et al. (Higashida et al., 2011) showed that acute low-intensity swimming exercise increased plasma malondialdehyde (MDA) concentration. This oxidative stress induced by low intensity exercise was inhibited by antioxidant vitamin supplementation. Recently, Higashida et al. evaluated the effects of antioxidant supplementation on low-intensity prolonged swimming training-induced adaptation in rat skeletal muscle (Higashida et al., 2011). However, low-intensity swimming exercise differs from high-intensity swimming exercise in the

ROS production or the increase of oxidative stress during the exercise. Low-intensity prolonged swimming exercise did not alter the blood lactate concentration (data not shown). These findings suggested that ROS production during low-intensity swimming exercise was smaller than the production during high-intensity swimming exercise.

On the other hand, it is suggested that ROS is related to skeletal muscle adaptation (St-Pierre et al., 2006). The expression and/or activation of PGC-1 $\alpha$  (master regulator of skeletal muscle adaptation) are regulated by multiple intracellular signaling, including AMP-activated kinase (AMPK), p38 MAPK, and Ca<sup>2+</sup> (Akimoto et al., 2005; Handschin et al., 2003; Jager et al., 2007). ROS also regulate the PGC-1 $\alpha$  expression via activation of AMPK or p38 MAPK *in vivo* and *in vitro* (Irrcher et al., 2009; Kang et al., 2009). Furthermore, the effects of ROS on endurance training-induced adaptation of skeletal muscle have been evaluated *in vivo* by antioxidant supplementation. Gomez-Cabrera et al. reported that vitamin C supplementation prevented the increase in endurance training-induced protein and gene expression of PGC-1 $\alpha$ , nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor A (mTFA) and protein expression of cytochrome c (Gomez-Cabrera et al., 2008).

In this study, the rats in the VC group were given 500 mg ascorbic acid/kg body weight/day. The dosage of 500 mg ascorbic acid/kg/day has been reported to prevent the training-induced mitochondrial biogenesis (Gomez-Cabrera et al., 2008). However, in the present study, vitamin C supplementation did not influence the exercise training-induced change of HK, CS and antioxidant enzyme activities in skeletal muscle. CS, HK and SOD gene expression levels are directly or indirectly regulated by PGC-1 $\alpha$ . Wende et al. has shown that CS gene expression and activity and HK protein content were increased in PGC-1 overexpression mice (Wende et al., 2007). Also, the H<sub>2</sub>O<sub>2</sub>-induced increases of SOD and CAT gene expression were inhibited in PGC-1 knockout mice (St-Pierre et al., 2006). Thus, it is considered that these enzyme activities did not change by vitamin C supplementation in the present study because the gene expression or activity of PGC-1  $\alpha$  was not changed by VC administration.

After the training period, plasma vitamin C concentration was decreased by exercise training. In addition, vitamin C supplementation did not change the plasma vitamin C concentration. Previous studies have shown that plasma vitamin C level was decreased for three days after the strenuous exercise (Gleeson et al., 1987). This decrease of vitamin C level is believed to be due to the increase of oxidative stress. Also, after 24

hours of the vitamin C supplementation, plasma vitamin C level was returned to baseline (Makanae et al., 2013). In this study, blood samples were collected 48 h after the last training session. Therefore, vitamin C level might be changed by vitamin C supplementation.

In this study, CAT activity and TGSH were increased by vitamin C supplementation. Previous studies have shown that CAT activity was increased by vitamin C in skeletal muscle, liver, brain and heart (Shireen et al., 2008; Suresh et al., 1999). Vitamin C also increases GSH in red blood cells (Johnston et al., 1993). Also, tissue vitamin C concentration is decreased by the deficiency of glutathione (Martensson and Meister, 1991). Hence, vitamin C concentration is related with glutathione concentration. These results suggest that vitamin C not only has radical scavenging capacity but also enhances the other antioxidant systems. Vitamin C supplementation decreased the skeletal muscle protein carbonyl and plasma d-ROM concentration in this study. This inhibition of oxidative stress due to vitamin is considered that there was a contribution of the vitamin C-induced increase of TGSH and CAT activity in skeletal muscle.

In conclusion, vitamin C supplementation increases antioxidant enzyme CAT activity



and antioxidant GSH concentration in skeletal muscle but did not change the exercise training-induced increase of CS and HK enzyme activities in skeletal muscle. Furthermore, vitamin C reduced skeletal muscle and blood oxidative stress. These results suggest that vitamin C supplementation reduces oxidative stress by increasing antioxidant capacity, but does not change the exercise training-induced increase of enzyme activities of energy metabolism in skeletal muscle.

# Chapter III

## Study 2

## INTRODUCTION

Endurance exercise training induces adaptations in skeletal muscle such as mitochondrial biogenesis (Holloszy, 1967), angiogenesis (Chinsomboon et al., 2009), increase of antioxidant enzymes activity (Higuchi et al., 1985) and increase of glucose transporter 4 gene and protein expression (Daugaard et al., 2000). These adaptations are regulated by acute exercise-induced several intracellular signaling pathways, including AMP activated kinase (AMPK) (Jager et al., 2007), p38 mitogen-activated protein kinase (p38 MAPK) (Akimoto et al., 2005), calcium/calmodulin-dependent protein kinase (CaMK) (Handschin et al., 2003) and sirtuin 1 (SIRT1) (Lagouge et al., 2006). Such signaling pathways activate peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) (Akimoto et al., 2005; Handschin et al., 2003; Jager et al., 2007; Lagouge et al., 2006). PGC-1 $\alpha$  has been known as a master regulator of mitochondrial biogenesis in skeletal muscle and this cofactor binds to the transcription factors related with skeletal muscle adaptations and enhances these transcriptional activities (Ugucioni et al., 2010). Recently, it has been reported that transcription coregulators nuclear corepressor 1 (NCoR1) also regulates oxidative metabolism in skeletal muscle (Yamamoto et al., 2011). NCoR1 negatively modulates the skeletal muscle adaptations by suppressing transcription activity of transcription factors related to oxidative

adaptation. Thus, exercise-induced skeletal muscle adaptation is regulated by several intracellular signaling pathways (Yamamoto et al., 2011).

Acute endurance exercise induces the generation of reactive oxygen species (ROS) in muscle (Davies et al., 1982). To further understand the role of exercise-induced ROS, it is necessary to investigate the effects of antioxidants on intracellular signaling and markers of skeletal muscle adaptation during acute exercise, because exercise training adaptation reflects an accumulation of acute exercise stimulus. The effects of acute exercise-induced ROS on skeletal muscle signals and adaptations have been evaluated using allopurinol inhibitor of xanthine oxidase (XO) or nonspecific antioxidant vitamin C. It was shown that ROS generated by XO during acute exercise regulates some intracellular signals (Kang et al., 2009; Wadley et al., 2013). However, these results only reflect the effects of ROS derived from XO on exercise-induced mitochondrial biogenesis but do not reflect the effects of other sources of ROS such as mitochondria (Barja, 1999) and leukocytes (Suzuki et al., 2003). Wadley and McConell (Wadley and McConell, 2010) reported that vitamin C does not alter the acute exercise-induced increases in markers of mitochondrial biogenesis. Also, they reported that vitamin C supplementation did not prevent the exercise-induced increase of oxidative stress. Thus, it is likely that their methodology was not suitable to determine the effects of exercise-induced ROS on

mitochondrial biogenesis. Vitamin C is a water-soluble antioxidant and scavenges free radicals (Rose and Bode, 1993). However, it is possible that vitamin C acts as a pro-oxidant (Carr and Frei, 1999). On the other hand, vitamin C can react with vitamin E radicals to regenerate vitamin E (Packer et al., 1979) and a combination of vitamin C and E has high antioxidant capacity and decreases exercise-induced oxidative stress (Higashida et al., 2011; Ryan et al., 2010). In addition, to our knowledge, there are no studies investigating the effects of antioxidant supplementation and/or non-exhaustive endurance exercise on skeletal muscle gene expression of NCoR1 that is a coregulator of oxidative adaptation.

The aim of this study was to investigate the effects of vitamin C and E supplementation on acute exercise-induced changes of makers of skeletal muscle adaptation and its signaling pathways in mice. We hypothesized that vitamin C and E supplementation would inhibit the activation of AMPK and p38 MAPK during acute exercise and prevent increases in the markers of muscle adaptation after acute exercise.

## MATERIALS AND METHODS

### *Experimental animals and protocol*

Male C57BL/6 mice (8 weeks old) were purchased from Takasugi experimental animals supply (Kasukabe, Japan). Five animals were housed together in 1 cage (27×17×13cm) in a controlled environment under a light-dark cycle (lights on at 0900 and off at 2100). The experimental procedures followed the Guiding Principles for the Care and Use of Animals in the Waseda University Institutional Animal Care and Use Committee. The mice were derived into two groups (non-supplemented (NS) and vitamin C and E supplemented (VS) groups (n=40), and then allocated to either an exercise (n=20) or a sedentary (n=20) group.

Mice in the VS group were given vitamin C (750 mg/kg weight/day) and vitamin E (150 mg/kg weight/day) with a feeding needle. Mice in the NS group were given vehicle (200 mg of triolein and 20 mg of Tween in 1 ml of saline). Mice were received the antioxidants or vehicle for two weeks. One hour after the last supplementation exercise group mice ran on a treadmill at 25 m/min, 8% grade for 120 min. Immediately or three hours after the treadmill run exercising and sedentary mice were sacrificed under light anesthesia with the inhalant isoflurane (Abbott, Tokyo, Japan). The gastrocnemius muscle was excised, quickly frozen in liquid nitrogen and stored at -80 °C until analysis. A portion of gastrocnemius was quickly immersed in RNAlater (Applied Biosystems,

Foster City, CA) and stored at -80 °C.

Gastrocnemius muscle was homogenized in tissue protein extraction reagent (T-PER; Pierce, Rockford, IL) containing protease inhibitor (Complete mini protease inhibitor cocktail tablets; Roche, Mannheim, Germany) and phosphatase inhibitor (Roche) at 4°C. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and the protein content of the supernatant was determined by a bicinchoninic acid (BCA) Protein Assay Kit (Thermo, Rockford, IL). This supernatant was used for the measurement of hydroperoxide (H<sub>2</sub>O<sub>2</sub>), thiobarbituric acid reactive substances (TBARS) and trolox equivalent antioxidant capacity (TEAC) and immunoblot analysis.

#### ***Measurement of oxidative stress markers***

H<sub>2</sub>O<sub>2</sub> level in gastrocnemius muscle was measured with SensoLyte ADHP Hydrogen Peroxide Assay Kit (Ana Spec, San Jose, CA). TBARS, a marker of lipid peroxidation, in gastrocnemius muscle was measured with TBARS Assay Kit (Cayman Chemical Co, Ann Arbor, MI). Non-enzymatic antioxidant capacity TEAC in gastrocnemius muscle was measured according to the methods of Re et al. (Re et al., 1999).

### ***Real-time quantitative PCR***

Measurement of gene expression levels were performed according to previous study of our laboratory (Kawanishi et al., 2016). Total RNA was extracted from the gastrocnemius muscle homogenate using Trizol reagent (Invitrogen, Carlsbad, CA) and the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of total RNA was assessed using the NanoDrop system (NanoDrop Technologies, Wilmington, DE). Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed with the Fast 7500 real-time PCR system (Applied Biosystems) using the Fast SYBR® Green PCR Master Mix (Applied Biosystems). The thermal profiles consisted of 10 min at 95 °C for denaturation followed by 40 cycles of 95 °C for 3 s and annealing at 60 °C for 15 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the housekeeping gene, and the  $\Delta\Delta CT$  method was used as previously described (Livak and Schmittgen, 2001) to quantify target gene expression. All data are represented relative to its expression as fold change based on the values of the sedentary group. Specific PCR primer pairs for each studied gene are shown in Table 1.



Table 1. Primer sequences for real-time RT-PCR analysis.

Gene	Forward	Reverse
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
CS	GGAGCCAAGAACTCATCCTG	TCTGGCCTGCTCCTTAGGTA
VEGF	CCCCAGAATGAAGGTTACACA	TGTCAAAGGGAGAAGGGTTTT
PGC-1 $\alpha$	GACTGGAGGAAGACTAAACGGCCA	GCCAGTCACAGGAGGCATCTTT
NCoR1	GACCCGAGGGAAGACTACCATT	ATCCTTGTCCGAGGCAATTTG

GAPDH; glyceraldehyde-3-phosphate dehydrogenase, CS; citrate synthase, VEGF; vascular endothelial growth factor, PGC-1 $\alpha$ ; Peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$ , NCoR1; nuclear receptor corepressor 1

### ***Immunoblot analysis***

Homogenized samples were diluted with homogenizing buffer to 5.5 mg/mL protein. Samples were then mixed with 4  $\times$  Laemmli sample buffer (Bio-Rad, Richmond, CA) in 10 % 2-mercaptoethanol to 4 mg protein/mL and heated at 60 °C for 10 min. Aliquots of samples were separated by electrophoresis on 8 % SDS–polyacrylamide gels and then transferred to polyvinyl difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature in Tris-buffered saline/0.1 % Tween 20 (TBST) with 5 % skim milk. Then, membranes were incubated overnight in the TBST in 5 % BSA containing anti-phospho-AMPK $\alpha$  Thr172 (#2535; Cell Signaling Technology, Beverly, MA), anti-AMPK $\alpha$  (#2532; Cell Signaling), anti-phospho-p38MAPK Thr180/Tyr182

(#9211; Cell Signaling) and anti-p38 MAPK (#9212; Cell Signaling). The membranes were washed with TBST and incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (1:30000; Bio-Rad), visualized by enhanced chemiluminescence (ECL; GE Healthcare, Arlington Heights, IL) and quantified by densitometry (Las-3000, Fuji photo, Tokyo, Japan).

### ***Statistical analysis***

Data are presented as means±SE. A two-way analysis of variance (ANOVA) was performed (SPSS V17.0, IBM Japan, Ltd, Tokyo, Japan) to determine the main effects of exercise and/or antioxidant supplementation. If this analysis revealed a significant interaction, Bonferroni's post-hoc test was used to determine the significance among the means. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### ***Effects of acute exercise and antioxidant supplementation on oxidative stress marker and antioxidant capacity***

Single bout of endurance exercise increased  $H_2O_2$  and TBARS in skeletal muscle ( $P < 0.01$ , Fig. 1 A and B). The acute exercise-induced increase in  $H_2O_2$  and TBARS were

prevented in mice given antioxidants ( $P < 0.01$ , Fig. 1 A and B). In addition, 2 weeks of vitamin C and E supplementation resulted in significantly higher TEAC in skeletal muscle ( $P < 0.05$ , Fig. 1 C).

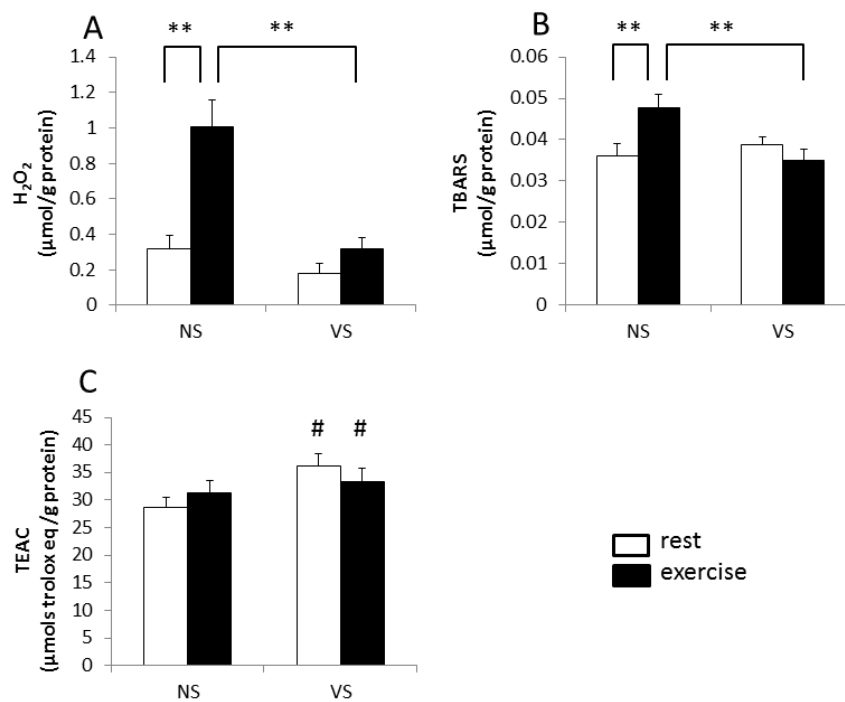


Fig. 1. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; A), thiobarbituric acid reactive substances (TBARS; B) and trolox equivalent antioxidant capacity (TEAC; C) in the gastrocnemius muscle of mice after 2 weeks of vehicle (NS) or vitamin C and E supplementation (VS) under sedentary condition (sedentary) or immediately after 120 min of treadmill running (exercise). Values are means±SE. #,  $P < 0.05$  main effect for vitamin C and E supplemented. \*\*,  $P < 0.01$ , vs NS exercise group.

*Effects of acute exercise and antioxidant supplementation on markers of skeletal muscle adaptation*

Gene expression levels of citrate synthase (CS), a marker of mitochondrial biogenesis, and vascular endothelial growth factor (VEGF), a marker of angiogenesis, were significantly increased 3 h following 120 min treadmill running ( $P < 0.01$ , Fig. 2 A and B). However, antioxidant supplementation did not alter expression levels of CS and VEGF (Fig. 2 A and B).

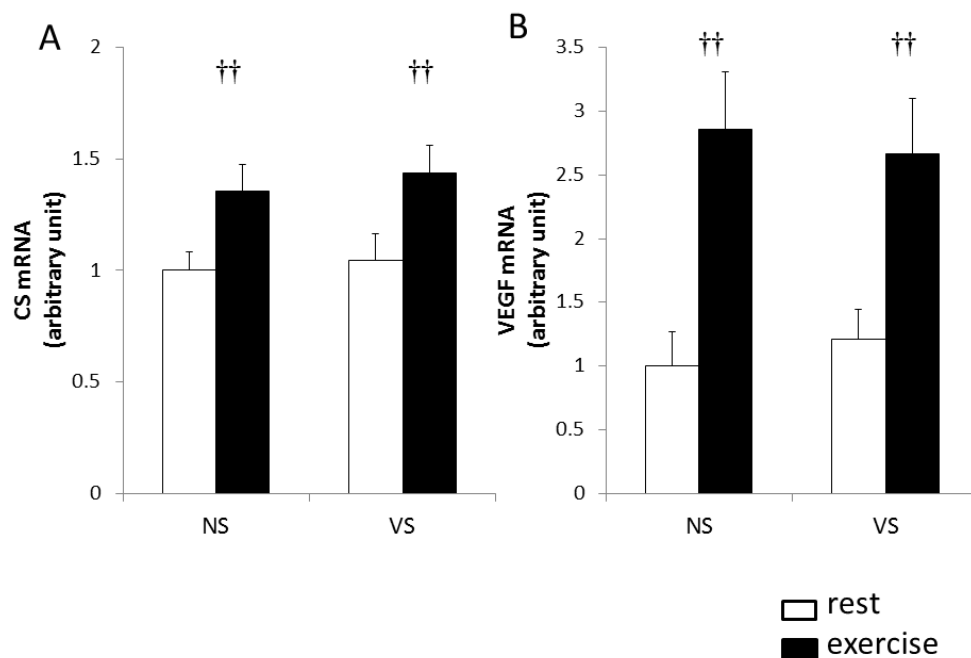


Fig. 2. Citrate synthase (CS; A) and vascular endothelial growth factor (VEGF; B)

mRNA in the gastrocnemius muscle of mice after 2 weeks of vehicle (NS) or vitamin C and E supplementation (VS) under sedentary condition (sedentary) or 3 h after 120 min of treadmill running (exercise). Values are means $\pm$ SE. ††, P<0.01, main effect for exercise.

***Effects of acute exercise and antioxidant supplementation on phosphorylation of protein kinase***

Phosphorylation of AMPK and p38 MAPK were significantly increased immediately following endurance exercise (P<0.01, Fig. 3 A and B). Antioxidant supplementation prevented the increase in phosphorylation of AMPK and p38 MAPK (P<0.05, Fig. 3 A and B).

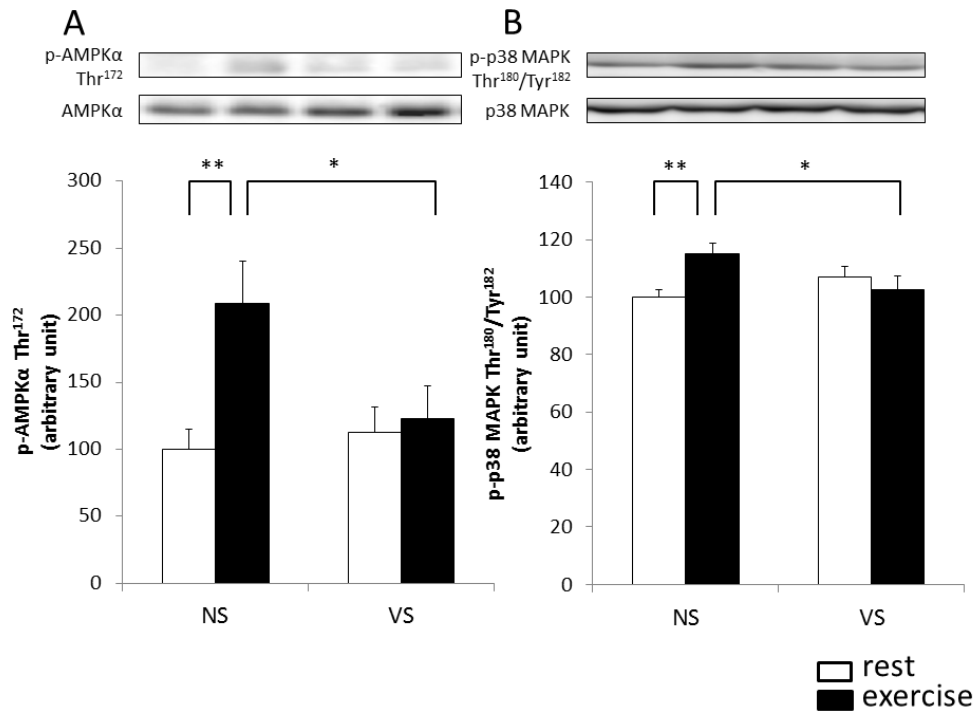


Fig. 3. Phosphorylation of AMP activated kinase (AMPK)- $\alpha$  (A) and p38 MAPK (B), in the gastrocnemius muscle of rats after 2 weeks of vehicle (NS) or vitamin C and E supplementation (VS) under sedentary condition (sedentary) or immediately after 120 min of treadmill running (exercise). Values are means $\pm$ SE. \*, P<0.05, vs NS exercise group. \*\*, P<0.01, vs NS exercise group.

*Effects of acute exercise and antioxidant supplementation on skeletal muscle adaptation signaling pathway gene expression*

Three hours after the endurance exercise, PGC-1 $\alpha$  and NCoR1 mRNA was significantly decreased compared to sedentary mice (P<0.01, Fig. 4 A and B). However,

antioxidant supplementation did not alter gene expression of PGC-1 $\alpha$  and NCoR1 (P<0.01, Fig. 4 A and B).

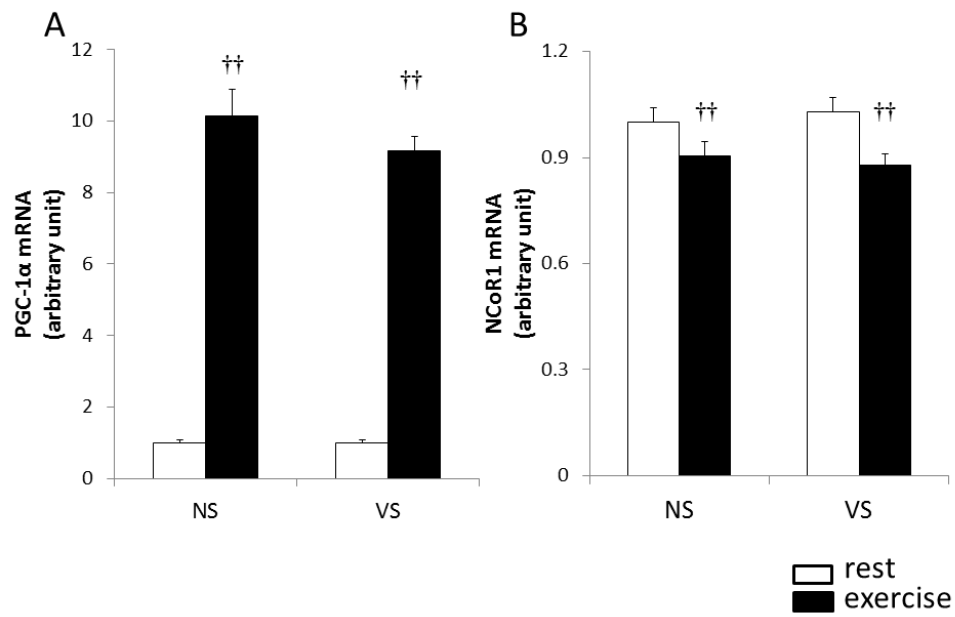


Fig. 4. Peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ; A) and nuclear corepressor 1 (NCoR1; B) mRNA in the gastrocnemius muscle of mice after 2 weeks of vehicle (NS) or vitamin C and E supplementation (VS) under sedentary condition (sedentary) or 3 h after 120 min of treadmill running (exercise). Values are means $\pm$ SE. ††, P<0.01, main effect for exercise.

## DISCUSSION

The purpose of the present study was to investigate whether antioxidant supplementation affects acute exercise-induced change in makers of skeletal muscle adaptation and its signaling pathways. In contrast to our hypothesis, the present study showed that combined supplementation with vitamins C and E did not alter the acute exercise-induced increase in markers of mitochondrial biogenesis and angiogenesis. However, interestingly, vitamin C and E supplementation prevented the phosphorylation of AMPK and p38 MAPK following the treadmill running. These results are in accordance with the rodent study of Wadley et al. (Wadley et al., 2013).

It has been reported that a combination of vitamin C and vitamin E reduces oxidative stress in blood and organs (Higashida et al., 2011; Ryan et al., 2010). In agreement with these studies, we have shown that supplementation with vitamin C (750mg/kg body weight) and E (150mg/kg body weight) inhibited exercise-induced oxidative stress ( $H_2O_2$  and TBARS) and increased the basal level of antioxidant capacity (TEAC). Thus, in this study, it seems that combination of vitamin C and E was appropriate for the evaluation of the effects of inhibiting exercise-induced ROS production on the adaptations in skeletal muscle.



Acute exercise increases markers of skeletal muscle adaptations such as mitochondrial biogenesis (Safdar et al., 2011) and angiogenesis (Biro et al., 2003). In this study, we measured CS and VEGF gene expression as markers of skeletal muscle adaptation. Indeed, it has been known that CS protein content and activity reflect the mitochondrial density (Reichmann et al., 1985) and VEGF is involved in the regulation of angiogenesis (Wilting et al., 1993). The results of this study indicate that exercise increased CS and VEGF gene expression but vitamin C and E did not affect the exercise-induced increase in CS and VEGF gene expression. These results are consistent with the studies suggesting that antioxidant supplementation did not alter exercise training-induced adaptations (Higashida et al., 2011; Strobel et al., 2011; Yada and Matoba, 2014; Yfanti et al., 2010; Yfanti et al., 2011).

In addition, we evaluated the effects of antioxidant supplementation on exercise-induced changes in intracellular signaling i.e., AMPK, p38 MAPK, PGC-1 $\alpha$  and NCoR1. We observed that vitamin C and E supplementation attenuated the exercise-induced phosphorylation of AMPK and p38 MAPK. These proteins regulate activation and/or expression of PGC-1 $\alpha$  (Akimoto et al., 2005; Jager et al., 2007).

However, despite these effects of supplementation, in the present study, gene expression of PGC-1 $\alpha$  was not affected by antioxidant supplementation. PGC-1 $\alpha$  is the key factor of skeletal muscle adaptations and modulates CS (Wu et al., 1999) and VEGF (Arany et al., 2008) expression. Thus, it is likely that significantly increased CS and VEGF gene expression levels observed in both exercise groups (with or without vitamin C and E) arose from the augmentation of the PGC-1 $\alpha$  by exercise, irrespective of antioxidant supplementation. These observations agree with findings of the study of Wadley et al. (Wadley et al., 2013), who reported that XO inhibition by allopurinol prevented the exercise-induced phosphorylation of p38 MAPK and ERK but did not alter the increases in acute exercise-induced signal gene expression levels such as PGC-1 $\alpha$  and the training adaptations in rat skeletal muscle. In the present study, it is unclear why PGC-1 $\alpha$  and makers of skeletal muscle adaptation were not affected by antioxidant supplementation, even though phosphorylation of AMPK and p38 MAPK were prevented by the antioxidant supplement. It has been reported that PGC-1 $\alpha$  is regulated by several intracellular signals such as AMPK (Jager et al., 2007), p38 MAPK (Akimoto et al., 2005), CaMK (Handschin et al., 2003) and SIRT1 (Lagouge et al., 2006). The limitation of the present study is that we were unable to examine the effect of vitamin supplementation on intracellular signals except for AMPK and p38 MAPK. Hence, it

remains unclear what kind of signal activated PGC-1 $\alpha$  in present study. However, it is possible that the increase in PGC-1 $\alpha$  gene expression was induced by intracellular signals except for AMPK and p38 MAPK.

NCoR1 is a transcription coregulator that negatively regulate skeletal muscle oxidative metabolism (Yamamoto et al., 2011). However, it is unclear whether expression level of this gene is changed by exercise and/or antioxidants. Especially, to our knowledge, there are no studies investigating the effects of antioxidant supplementation and/or non-exhaustive acute exercise on skeletal muscle NCoR1. Our results show that NCoR1 mRNA was decreased 3 h after the non-exhaustive treadmill exercise but was not altered by antioxidant supplementation. Thus, it is likely that exercise-induced gene expression of CS in this study does not only result from the increase in PGC-1 $\alpha$  but also the decrease in NCoR1 with exercise.

In conclusion, the combined supplementation with vitamin C and E prevented the acute exercise-induced phosphorylation of AMPK and p38 MAPK. However, despite these effects on signaling, supplementation with vitamin C and E did not alter the exercise-induced change in gene expression of coregulator PGC-1 $\alpha$  and NCoR1, and

makers of adaptation, CS and VEGF. These results suggest that acute exercise-induced ROS does not alter mitochondrial biogenesis and angiogenesis in skeletal muscle.

# Chapter IV

## Study 3

## INTRODUCTON

Endurance capacity is important for not only the performance of the endurance athletes but also maintenance of quality of life. Indeed, it has been reported that high-endurance capacity is associated with lower blood pressure, visceral adipose fat, plasma triglyceride, insulin and glucose levels compared with low-endurance capacity (Wisløff et al., 2005) and longer life span in rats (Koch et al., 2011). The main sources of energy during endurance exercises are carbohydrate (CHO) and lipids. During low-intensity prolonged exercise, fat is the main source for ATP synthesis. However, demand of CHO utilization increases as exercise intensity increases. It is known that exercise-induced exhaustion can be occurred when muscle glycogen is almost depleted, especially when exercise is done with moderate intensity and long duration (Ahlborg et al., 1967; Karlsson and Saltin, 1971). The level of glycogen stores and the ability to increase glycogen stores are crucial limiting factors for aerobic endurance. On the other hand, when exercise is done with high intensity, the reason of fatigue is due to the accumulation of different metabolites, such as lactic acid, Pi, ammonia or  $\text{Ca}^{2+}$  (Allen et al., 2002; Cairns, 2006; Fitts, 1994; Wilkerson et al., 1975).

It is well known that exercise training and intake of nutritional supplement are both important strategies for the improvement of endurance capacity. Various studies have

shown that several nutritional supplements such as green tea extracts, polyphenols, L-carnitine, taurine and capsaicin could improve endurance capacity (Jisu et al., 2016). These supplements enhance the endurance capacity via several mechanisms. For example, green tea extracts increase running time via enhancement of fatty acid oxidation (Murase et al., 2005; Murase et al., 2006). Quercetin increases endurance performance via activation and/or up-regulation of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 $\alpha$ ) and mitochondrial biogenesis (Davis et al., 2009). Other polyphenols have also been reported to inhibit oxidative stress in several conditions such as obesity, strenuous exercise and ultraviolet radiation (Alessio et al., 2002; Bogdanski et al., 2012; Feillet-Coudray et al., 2009; Katiyar et al., 2001; Morillas-Ruiz et al., 2006). Since oxidative stress is one of the main fatigue mediators during exercise (Powers and Jackson, 2008), polyphenols may improve the endurance capacity via not only the enhancement of substrate oxidation but also inhibition of oxidative stress.

*Tabebuia avellanedae* is a tree native to the tropical rain forest from Brazil to North Argentina in South America. The extract obtained from inner bark of *Tabebuia avellanedae* is called taheebo. Taheebo has been traditionally used for the treatment of several diseases in South America. Recently, taheebo has been reported to have

beneficial effects for treatment of cancer, inflammation, oxidative stress and obesity (Byeon et al., 2008; Iwamoto et al., 2016; Mukherjee et al., 2009; Ohno et al., 2015). However, it is unclear whether the signaling pathways that are modulated by taheebo extracts can contribute to enhancement of endurance capacity. Therefore, the aim of the present study was to investigate the effects of taheebo supplementation on endurance capacity using the polyphenol fraction (taheebo polyphenols; TP).

## MATERIALS AND METHODS

### *Animals*

Seventy-eight male C57BL/6J mice (8 weeks old) were purchased from Takasugi experimental animals supply (Kasukabe, Japan). Four or five animals were housed together in 1 cage (27×17×13cm) in a controlled environment under a light-dark cycle (lights on at 0900 and off at 2100). The experimental procedures followed the Guiding Principles for the Care and Use of Animals in the Waseda University Institutional Animal Care and Use Committee and were approved by the Institutional Animal Care and Use Committee in the university.

### *Extraction of polyphenol fraction of *Tabebuia avellanedae**



The dried inner bark of *Tabebuia avellanedae* (2.0 kg), which was generously provided by Taheebo Japan Co., Ltd (Osaka, Japan) was extracted using with boiling water (12.8 L) two times for 1 h. The water solution was subjected to polyamide (400 g) column chromatography and eluted by water (6.0 L), 30% MeOH aq. (1.5 L), 40% MeOH aq. (1.5L), 50% MeOH aq. (2.5 L) and 100% MeOH. The 50% MeOH aq. elution was concentrated in vacuo. The residue (12.6 g) was followed by silicagel column chromatography and eluted by CHCl<sub>3</sub> - MeOH - H<sub>2</sub>O (14 : 6 : 1). Then, we used the concentrated fraction (7.7 g) which exhibited coloration in a dark green color by a mist of the ferric chloride reagent on thin-layer chromatography (TLC; Silica gel G) from the eluent and is demonstrated to be polyphenols especially acteoside as the main constituent (Suo et al., 2012).

### ***Experimental protocol***

One week before the exhaustive exercise, all mice were accustomed to treadmill running at 15 m/min for 10 min. On the day of the experiment, all mice were randomly assigned to one of the four groups: control group (Con; water administrated + rest, n = 14), TP administrated group (TP; TP administrated + rest, n = 14), exercise group (Ex; water administrated + exercise, n = 25) or TP administrated plus exercise group (TP +

Ex; TP supplemented + exercise, n = 25). One hour before the exhaustive exercise on a motorized treadmill (Natsume, Kyoto, Japan), mice in TP and TP + Ex group were given TP (200 mg/kg weight) with a feeding needle. Mice in the Con and Ex group were given water. Then, mice in the Ex and TP + Ex group were subjected treadmill running at 10 m/min for 15 min, followed by 15 m/min and 20 m/min for 15 min each, and then 24 m/min and 7 % grade until exhaustion. The exhaustion was defined as the inability to continue regular treadmill running despite the stimulation of repeated tapping on the back of the mouse. The running time of exercised mice were recorded.

Immediately and 4 h after the exhaustion, mice (Con and TP group mice, n = 7, Ex and TP + Ex group mice, n = 8, respectively) were sacrificed under light anesthesia with the inhalant isoflurane (Abbott, Tokyo, Japan). Blood sample was taken using heparin from the abdominal aorta under inhalant isoflurane-induced mild anesthesia and the gastrocnemius muscle and liver were immediately excised and frozen in liquid nitrogen. Plasma was obtained from blood samples by centrifugation at 1600 g for 10 min at 4°C. These samples were stored at -80°C until analyses. For the measurement of glycogen, gastrocnemius muscle and liver were homogenized in distilled water. For the measurement of tissue glycerol, skeletal muscle and liver were homogenized in Glycerol Assay Buffer contained in the assay kit. Then, the homogenate was centrifuged at

10000xg for 5 min at 4°C and the supernatant was used. For the measurements of antioxidant enzymes activities, protein carbonyl, and immunoblot analysis, skeletal muscle was homogenized in tissue protein extraction reagent (T-PER; Pierce, Rockford, IL) containing protease inhibitor (Complete mini protease inhibitor cocktail tablet; Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor (Roche). Then, the homogenate was centrifuged at 10000xg for 15 min at 4°C and the supernatant was used. For the measurements of glutathione and trolox equivalent antioxidant capacity (TEAC), skeletal muscle was homogenized in phosphate buffered saline (PBS; pH 7.4).

***Measurement of plasma biochemical parameters, tissue glycogen and markers of oxidative stress***

Plasma concentrations of glucose, lactic acid (LA), non-essential fatty acids (NEFA), triglyceride (TG), blood urea nitrogen (BUN), total ketone body (T-KB), uric acid (UA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK) and lactate dehydrogenase (LDH) were measured by Oriental Yeast Co. (Tokyo, Japan). Plasma glycerol concentration was measured with Glycerol Colorimetric Assay Kit (Cayman Chemical Co., Ann Arbor, MI). Skeletal muscle and liver glycerol levels were measured with Free Glycerol Assay Kit II (BioVison, Inc., Milpitas, CA). Tissue

glycogen concentration was measured with Glycogen Assay kit (BioVison, Inc.). Protein carbonyl, a marker of oxidative stress, in muscle was measured with Protein Carbonyl Assay Kit (Cayman Chemical Co.). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were measured with SOD Assay Kit (Cayman Chemical Co.), Catalase Assay Kit (Cayman Chemical Co.) and GPx Assay Kit (Cayman Chemical Co.), respectively. Reduced glutathione (GSH) was determined using a commercially available assay kit (Biooxytech GSH/GSSG-412; Oxis Health Products, Portland, OR). Non enzymatic antioxidant capacity TEAC in muscle and plasma were measured according to the assay described by Re, et al. (Re et al., 1999).

### ***Real-time quantitative polymerase chain reaction (PCR)***

Measurement of gene expression levels were performed according to previous study of our laboratory (Kawanishi et al., 2016). Total RNA was extracted from the gastrocnemius muscle and liver using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of total RNA was assessed using the NanoDrop system (NanoDrop Technologies, Wilmington, DE). Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

PCR was performed with the Fast 7500 real-time PCR system (Applied Biosystems) using the Fast SYBR® Green PCR Master Mix (Applied Biosystems). The thermal profiles consisted of 10 min at 95°C for denaturation followed by 40 cycles of 95°C for 3 s and annealing at 60°C for 15 s. 18s mRNA was used as the housekeeping gene, and the  $\Delta\Delta CT$  method was used as previously described [30] to quantify target gene expression. All data are represented relative to its expression as fold change based on the values of the Con group. Specific PCR primer pairs for each studied gene are shown in Table 1.

Table 1. Primer sequence for real-time PCR analysis

Genes	Forward	Reverse
GS	ACTGCTTGGGCGTTATCTCTGTG	ATGCCCGCTCCATGCGTA
GP	TGGCAGAAAGTGGTGAACAATGAC	CCGTGGAGATCTGCTCCGATA
HK2	CTGTCTACAAGAAACATCCCCATTT	CACCGCCGTCACCATAGC
G6Pase	GTGGCAGTGGTCGGAGACT	ACGGGCGTTGTCCAAAC
PEPCK	CACCATCACCTCCTGGAAGA	GGGTGCAGAATCTCGAGTTG
HADH	ACTACATCAAAATGGGCTCTCAG	AGCAGAAATGGAATGCGGACC
MCAD	GCTCGTGAGCACATTGAAAA	CATTGTCCAAAAGCCAAACC
ACO	TGTTAAGAAGAGTGCCACCA	ATCCATCTCTTCATAACCAAATTT
CPT1 $\alpha$	CCAGGCTACAGTGGGACATT	GAACCTGCCCATGTCCCTTGT
CPT1 $\beta$	CCCATGTGCTCCTACCAGAT	CCTTGAAGAAGCGACCTTTG
CPT2	GAAGAAGCTGAGCCCTGATG	GCCATGGTATTTGGAGCACT
ACC1	ATTGGGCACCCCAGAGCTA	CCCGCTCCTTCAACTTGCT
ACC2	GGGCTCCCTGGATGACAAC	TTCCGGGAGGAGTTCTGGA
MCD	ACTCCATCAGCCTGACCCAG	ACCCCTTGAGGCTCTCGTGA
PGC-1 $\alpha$	GACTGGAGGAAGACTAAACGGCCA	GCCAGTCACAGGAGGCATCTTT
SIRT1	GCAACAGCATCTTGCCTGAT	GTGCTACTGGTCTCACTT
Cyto c	CACGCTTTACCCTTCGTTCT	CTCATTTCCCTGCCATTCTC
18S	CGGCTACCACATCCAAGGA	AGCTGGAATTACCGCGGC

GS; glycogen synthase, GP; glycogen phosphorylase, HK2; hexokinase 2, G6Pase; glucose-6-phosphase, PEPCK; phosphoenolpyruvate carboxykinase, HADH; hydroxyacyl-CoA dehydrogenase, MCAD; medium chain acyl-CoA dehydrogenase, ACO; acyl-CoA oxidase, CPT; carnitine palmitoyltransferase, ACC; acetyl-CoA carboxylase , MCD; malonyl-CoA decarboxylase, PGC-1 $\alpha$ ; peroxisome proliferator-activated receptor gamma coactivator-1 alpha, SIRT1; sirtuin 1, Cyto c; cytochrome c, 18S; 18s ribosomal RNA

### ***Statistical analysis***

Data are presented as means  $\pm$  SE. For comparison of means between two groups, Students' unpaired t-test was performed. A two-way analysis of variance (ANOVA) was performed to determine the main effects of TP administration and/or exercise. Statistical analysis was done using SPSS V22.0 (IBM Japan, Ltd, Tokyo, Japan). When this analysis revealed significant interaction, Bonferroni's post-hoc test was performed to determine the significance among the means. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### *Effect of TP administration on endurance capacity*

The running time of the mice of the TP + Ex group was significantly higher than that of the mice of the Ex group ( $263 \pm 9.4$  min vs  $232 \pm 9.9$  min,  $P=0.023$ , Fig. 1). Thus, single dose administration of TP improved the endurance capacity of mice on treadmill running.

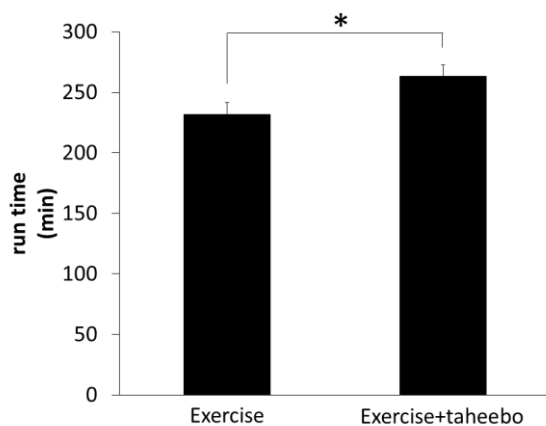


Fig. 1. The run time to exhaustion in the Ex group (n = 25) and the TP + Ex group (n = 25). Values are means  $\pm$ SE. \*  $P<0.05$ , compared with the Ex group.

### *Effects of exhaustive exercise and TP administration on plasma glucose level, skeletal muscle and liver glycogen concentrations*

Plasma glucose and skeletal muscle and liver glycogen concentrations were decreased

by exhaustive exercise ( $P < 0.001$ , Fig. 2A, B and C). TP administration increased plasma glucose and glycogen concentrations in skeletal muscle ( $P < 0.05$ , Fig. 2A and B).

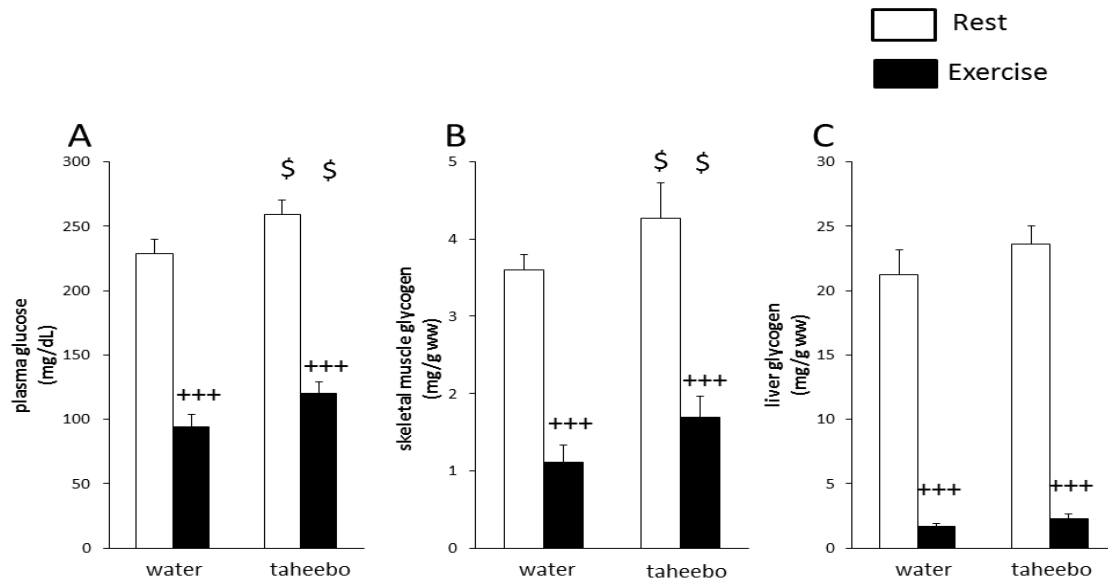


Fig. 2. Plasma glucose (A), skeletal muscle (B) and liver (C) glycogen concentrations of the Con (n = 7), TP (n = 7), Ex (n = 8) and TP + Ex (n = 8) groups immediately after the exercise session. Values are means  $\pm$ SE. +++  $P < 0.001$  main effect for exercise, \$  $P < 0.05$  main effect for taheebo polyphenol (TP) administration.

***Effects of exhaustive exercise and TP administration on plasma, skeletal muscle and liver glycerol concentrations***

Exhaustive exercise increased plasma glycerol concentration (Fig. 3A). On the other



hand, skeletal muscle and liver glycerol concentrations were decreased by TP administration ( $P < 0.05$ , Fig. 3B and C).

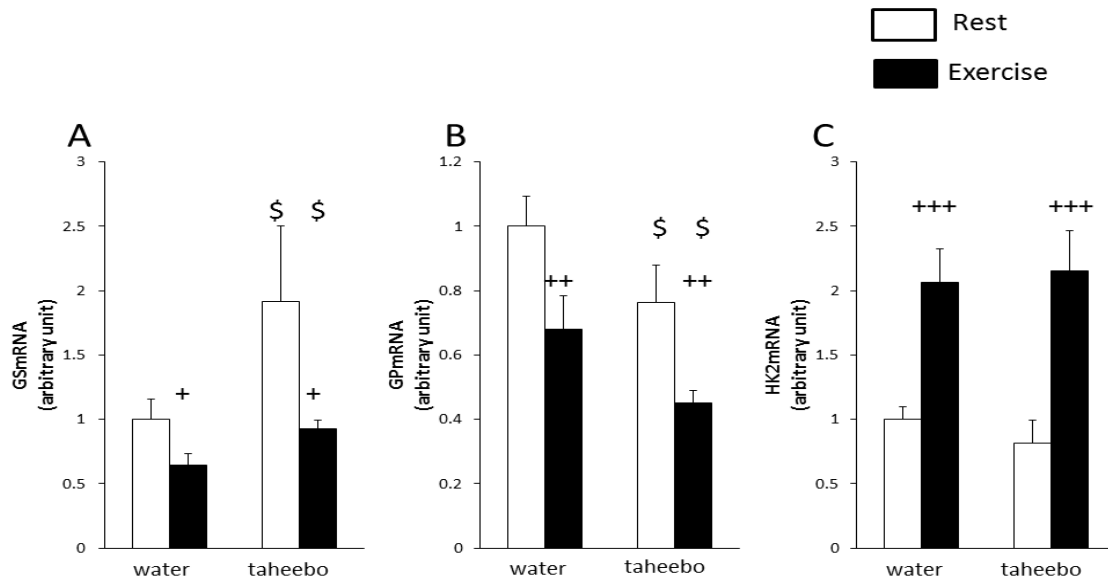


Fig. 3. Glycogen synthase (GS) (A), glycogen phosphorylase (GP) (B) and hexokinase 2 (HK2) (C) mRNA expression levels in skeletal muscle of the Con ( $n = 7$ ), TP ( $n = 7$ ), Ex ( $n = 8$ ) and TP + Ex ( $n = 8$ ) groups immediately after the exercise session. Values are means  $\pm$ SE. +, ++, +++  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$  main effect for exercise, \$  $P < 0.05$  main effect for taheebo polyphenol (TP) administration.

***Effects of exhaustive exercise and TP administration on blood biomarkers***

Exhaustive exercise increased NEFA, BUN and T-KB levels and reduced LA

concentration in plasma (P<0.001, Table 2). Moreover, BUN, a marker of protein degradation, was decreased by TP administration (P<0.05, Table 2). We measured AST and ALT as markers of liver injury, CK and LDH as markers of muscle injury. These injury markers increased immediately after the exhaustive exercise (P<0.001, Table 2). However, TP administration did not alter these injury markers (Table 2).

Table 2. Plasma biochemistry data

	Con	Ex	TP	TP + Ex
LA (mg/dL)	49.5 ± 6.2	25.3 ± 3.8 <sup>+++</sup>	44.2 ± 7.3	26.0 ± 5.3 <sup>+++</sup>
NEFA (µEq/L)	641 ± 118	1477 ± 130 <sup>+++</sup>	695 ± 135	1492 ± 74 <sup>+++</sup>
TG (mg/dL)	56.3 ± 8.2	67.8 ± 6.3	67.7 ± 11.5	65.4 ± 3.4
BUN (mg/dL)	26.5 ± 1.8	67.8 ± 4.9 <sup>+++</sup>	23.3 ± 1.6 <sup>\$</sup>	56.2 ± 3.0 <sup>\$+++</sup>
T-KB (µmol/L)	141 ± 23	3049 ± 92 <sup>+++</sup>	197 ± 29	3048 ± 218 <sup>+++</sup>
UA (mg/dL)	0.60 ± 0.09	0.89 ± 0.07	0.69 ± 0.11	0.70 ± 0.07
AST (IU/L)	59 ± 2	191 ± 18 <sup>+++</sup>	58 ± 6	164 ± 15 <sup>+++</sup>
ALT (IU/L)	28.7 ± 8.7	99.3 ± 19.1 <sup>+++</sup>	27.0 ± 2.0	71.8 ± 10.0 <sup>+++</sup>
CK (IU/L)	143 ± 26	1844 ± 383 <sup>+++</sup>	80 ± 13	1363 ± 161 <sup>+++</sup>
LDH (IU/L)	158 ± 10	855 ± 126 <sup>+++</sup>	173 ± 23	798 ± 72 <sup>+++</sup>

Values are means ± SE. +++ P<0.001 main effect for exercise, \$ P<0.05 main effect for taheebo polyphenol (TP) supplementation.

LA; lactic acid, NEFA non-essential fatty acid, TG; triglyceride, BUN; blood urea nitrogen, T-KB; total ketone body, UA; uric acid, AST; aspartate aminotransferase, ALT; alanine aminotransferase, CK; creatine kinase, LDH; lactate dehydrogenase.

*Effects of exhaustive exercise and TP administration on glycogen metabolism-related*

*gene expression in skeletal muscle*

Exhaustive exercise increased hexokinase 2 (HK2) gene expression level and decreased glycogen synthase (GS) and glycogen phosphorylase (GP) gene expression level in skeletal muscle ( $P < 0.001$ ,  $P < 0.05$  and  $P < 0.01$ , respectively, Fig. 4A, B and C). TP administration increased GS gene expression level and decreased GP gene expression level ( $P < 0.05$ , Fig. 4A and B).

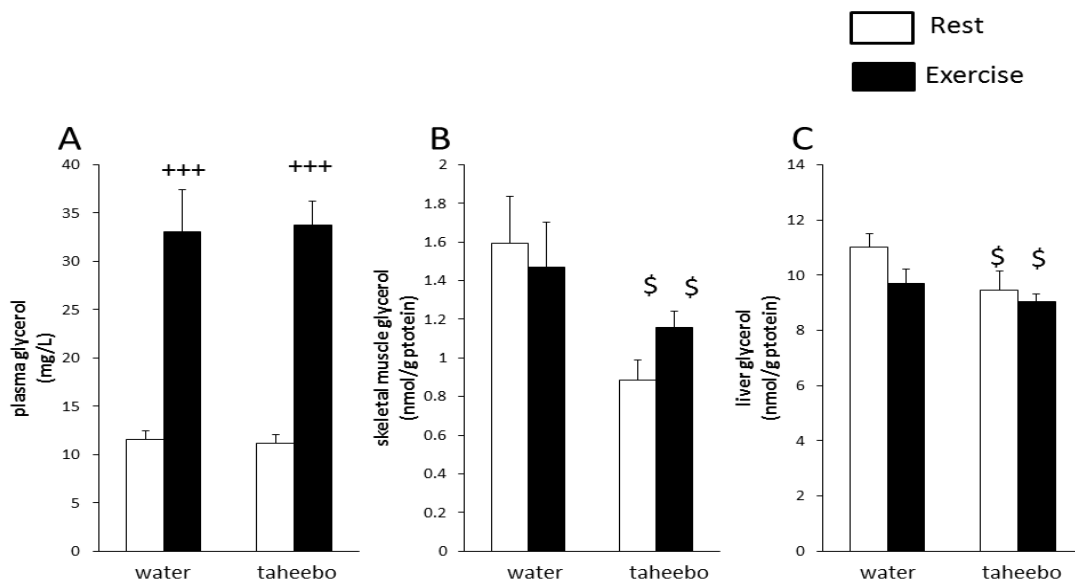


Fig. 4. Glycerol concentrations in plasma (A), skeletal muscle (B) and liver (C) of Con (n = 7), TP (n = 7), Ex (n = 8) and TP + Ex (n = 8) groups immediately after the exercise session. Values are means  $\pm$ SE. +++  $P < 0.001$  main effect for exercise, \$  $P < 0.05$  main effect for taheebo polyphenol (TP) administration.

*Effects of exhaustive exercise and TP administration on the gene expression level of gluconeogenesis-related genes in liver*

Glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) gene expression levels in liver were increased by exhaustive exercise ( $P < 0.001$ , Fig. 5A and C). Moreover, in the rest condition, liver G6Pase gene expression level of TP administrated group mice was higher compared with that of control group mice ( $P < 0.05$ , Fig. 5B).

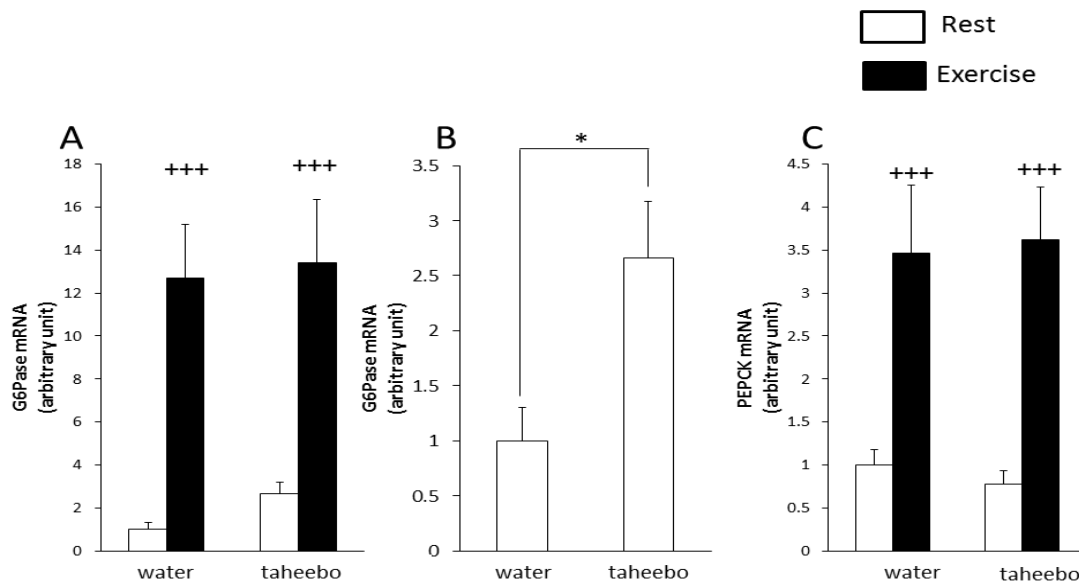


Fig. 5. Glucose-6-phosphatase (G6Pase) mRNA expression levels in skeletal muscle of the Con (n = 7), TP (n = 7), Ex (n = 8) and Ex + TP (n = 8) groups (A) and G6Pase mRNA

expression level in rest condition mice (Con vs TP) (B) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression levels in skeletal muscle of the Con (n = 7), TP (n = 7), Ex (n = 8) and TP + Ex (n = 8) groups (C) immediately after the exercise session. Values are means  $\pm$ SE. \* P<0.05, +++ P<0.001 main effect for exercise.

***Effects of exhaustive exercise and TP administration on the gene expression of fatty acid metabolism-related genes in skeletal muscle***

We measured the gene expression of lipid oxidation and fatty acid metabolism-related genes to determine the effect of TP on fatty acid metabolism. Gene expression of hydroxyacyl-CoA dehydrogenase (HADH) and medium chain acyl-CoA dehydrogenase (MCAD), which are  $\beta$ -oxidation-related genes, decreased by exhaustive exercise (P<0.01, Fig. 6A and B). However, acyl-CoA oxidase (ACO), which is another  $\beta$ -oxidation-related gene, was not altered by exercise (Fig. 6C). However, TP administration did not alter these gene expression levels (Fig. 6A, B and C). Gene expression levels of carnitine palmitoyltransferase (CPT) 1 $\alpha$ ,  $\beta$  and CPT2, which relates with the import of fatty acyl-CoA into mitochondria, were not altered by exercise and TP administration (Fig. 6D, E and F). Acyl-CoA carboxylase (ACC) is an enzyme that produces malonyl-CoA from acyl-CoA. Also, malonyl-CoA is known as an inhibitor of CPT1. The gene

expression levels of ACC 1 and 2 were not altered by exercise (Fig. 6G and H). ACC 1 was decreased by TP administration ( $p < 0.05$ , Fig. 6G). Malonyl-CoA decarboxylase (MCD) is an enzyme that carboxylates malonyl-CoA and produces acetyl-CoA. Gene expression of MCD was not changed by exercise and TP administration (Fig. 6I).

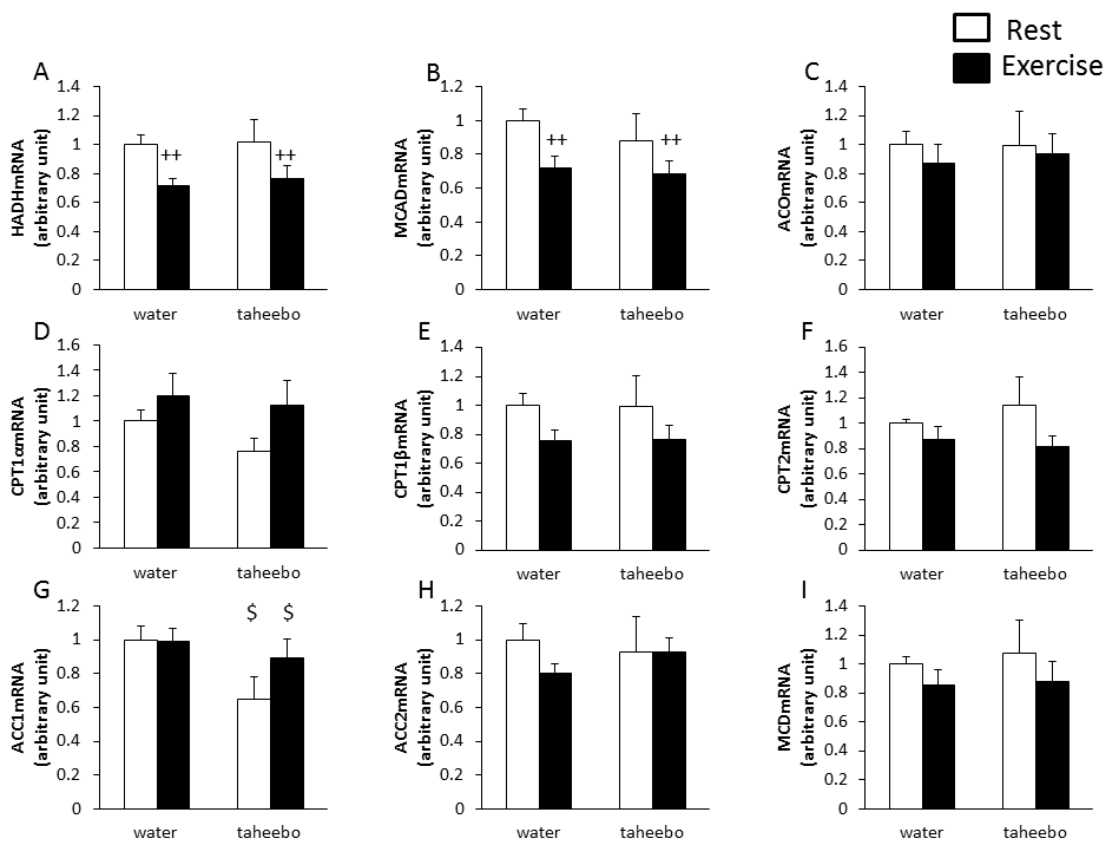


Fig. 6. Expression of fatty acid metabolism-related genes in skeletal muscle of the Con (n = 7), TP (n = 7), Ex (n = 8) and TP + Ex (n = 8) groups immediately after the exercise session. Values are means  $\pm$ SE. ++  $P < 0.01$  main effect for exercise, \$  $P < 0.05$  main effect for taheebo polyphenol (TP) administration.

*Effects of exhaustive exercise and TP administration on oxidative stress in skeletal muscle*

Protein carbonyl level in skeletal muscle was increased by exhaustive exercise ( $P < 0.01$ , Fig. 7). However, TP administration reduced protein carbonyl level in skeletal muscle ( $P < 0.05$ , Fig. 7).

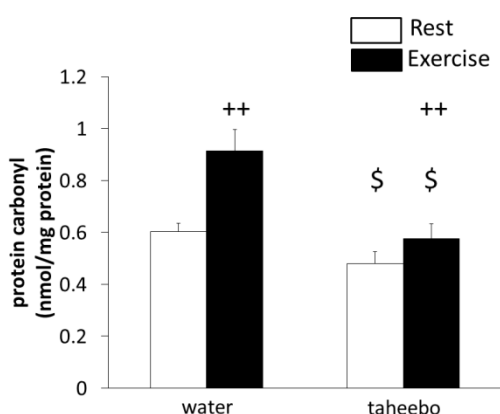


Fig. 7. Protein carbonyl levels in skeletal muscle of the Con ( $n = 7$ ), TP ( $n = 7$ ), Ex ( $n = 8$ ) and TP + Ex ( $n = 8$ ) groups immediately after the exercise session. Values are means  $\pm$ SE. ++  $P < 0.01$  main effect for exercise, \$  $P < 0.05$  main effect for taheebo polyphenol (TP) administration.

*Effects of exhaustive exercise and TP administration on markers of antioxidant capacity*

Exhaustive exercise increased GPx activity and TEAC in skeletal muscle and decreased plasma level of TEAC (P<0.001, P<0.05 and P<0.001 respectively, Table 3). On the other hand, TP administration did not affect these antioxidant markers (Table 3).

Table 3. Markers of antioxidant in skeletal muscle and plasma

	Con	Ex	TP	TP + Ex
SOD activity (U/mg protein)	0.49 ± 0.02	0.41 ± 0.03	0.42 ± 0.02	0.39 ± 0.02
GPx activity (µmol/mg protein)	51.8 ± 1.1	55.2 ± 0.7 <sup>+++</sup>	52.3 ± 0.3	55.2 ± 0.4 <sup>+++</sup>
CAT activity (µmol/g protein)	22.5 ± 2.6	29.3 ± 3.0	26.2 ± 3.3	24.8 ± 1.3
GSH (µmol/g protein)	3.05 ± 0.14	3.02 ± 0.12	3.07 ± 0.22	2.94 ± 0.12
TEAC (µmol Trolox Eq/g protein)	129.9 ± 3.6	136.1 ± 2.1 <sup>+</sup>	131.6 ± 2.5	140.7 ± 3.1 <sup>+</sup>
plasma TEAC (µmol Trolox Eq/mL)	0.84 ± 0.01	0.76 ± 0.02 <sup>+++</sup>	0.85 ± 0.01	0.77 ± 0.01 <sup>+++</sup>

Values are means ± SE. +, +++ P<0.05, P<0.001 main effect for exercise.

SOD; superoxide dismutase, GPx; glutathione peroxidase, CAT; catalase, GSH; glutathione, TEAC; trolox equivalent antioxidant capacity.

***Effects of exhaustive exercise and TP administration on gene expression levels of skeletal muscle adaptation-related genes.***

We measured gene expression of some key proteins that play an important role in adaptive response to endurance exercise in skeletal muscle samples which were obtained 4 h after the end of the exhaustive exercise. The gene expression of sirtuin



(SIRT) 1 in skeletal muscle of the Ex group and TPS group were significantly higher compared with the Con group ( $P < 0.001$  and  $P < 0.05$ , respectively, Fig. 8A). However, there was no significant difference between the expression of SIRT1 in skeletal muscle of the Ex + TPS group and Ex group or TPS group (Fig. 8A). The gene expression levels of PGC-1 $\alpha$  and cytochrome c (Cyto c) were increased by exhaustive exercise ( $P < 0.001$  and  $P < 0.05$ , respectively, Fig. 8B and C). However, these gene expression levels were not affected by TP supplementation (Fig. 8B and C).

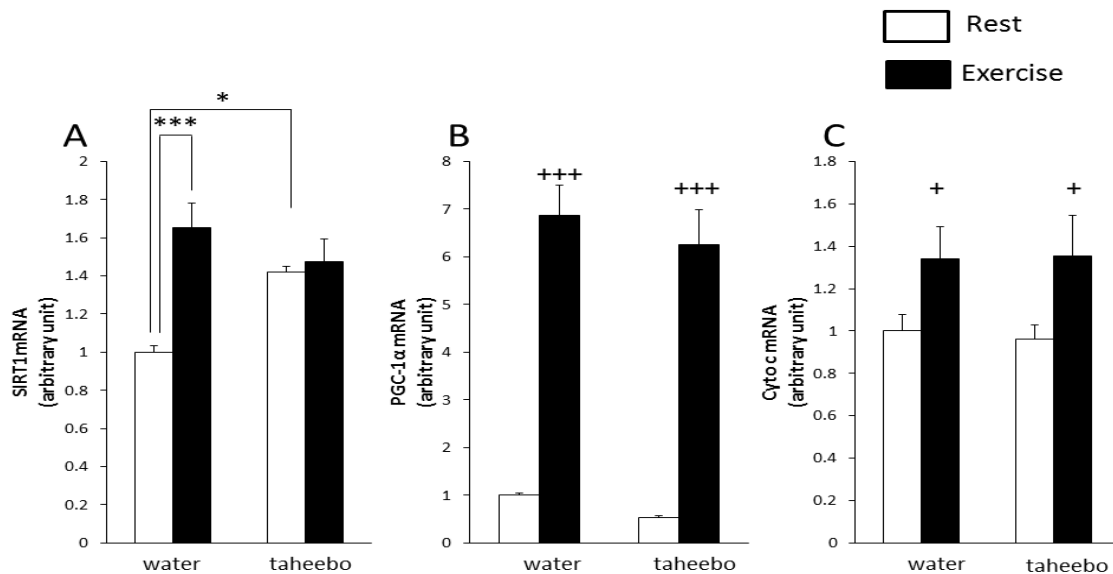


Fig. 8. Gene expression of sirtuin 1 (SIRT1) (A), peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 $\alpha$ ) (B) and cytochrome c (Cyto c) (C) of the Con (n = 7), TP (n = 7), Ex (n = 8) and TP + Ex (n = 8) groups four hours after the

exercise session. Values are means  $\pm$  SE. \*, \*\*\* P<0.05, P<0.001. +, +++ P<0.05, P<0.001 main effect for exercise.

## DISCUSSION

In the present study, we investigated the effects of TP on endurance capacity and the related molecular factors. The main finding of the present study is that TP administration enhanced endurance capacity via up-regulation of muscle glycogen levels. Several studies which investigated the effects of taheebo extract have reported that taheebo has anti-cancer, anti-obesity, anti-inflammation and antioxidant effects (Byeon et al., 2008; Iwamoto et al., 2016; Mukherjee et al., 2009; Ohno et al., 2015). The present study is the first report that lights up the mechanisms by which TP benefits endurance capacity.

In this study, a single dose administration of TP improved endurance capacity, up-regulated blood glucose and muscle glycogen levels and decreased BUN concentration. Aerobic exercise-induced exhaustion is caused by the depletion of muscle glycogen store (Ahlborg et al., 1967; Karlsson and Saltin, 1971) which induces protein degradation and increases BUN level. BUN is known as the marker of kidney injury as well as the indicator of exercise tolerance (Zhang et al., 2006). TP supplementation also

decreased skeletal muscle and liver concentrations of glycerol, which is one of the sources of gluconeogenesis. Gluconeogenesis is an important pathway to produce glucose from lactate, amino acid and glycerol in liver or kidney. During fasting or exercise, blood glucose level is maintained through the increase of gluconeogenesis or fat utilization. Therefore, it is suggested that endurance capacity was increased by TP via up-regulation of muscle glycogen and blood glucose levels through acceleration of gluconeogenesis particularly from glycerol. Glycogen synthesis and degradation are regulated by GS and GP. In this study, gene expression of GS was increased whereas gene expression of GP was decreased by TP. It has been reported that green tea polyphenol increased GS expression in murine liver cells (Kim et al., 2013). Furthermore, Kamiyama et al. (2010) reported that catechin gallates from green tea decreased GP expression in *in vitro* study (Kamiyama et al., 2010). Thus, polyphenols could regulate glycogen synthesis. Therefore, it is considered that TP supplementation-induced accumulation of muscle glycogen could be mediated by up-regulation of GS and down-regulation of GP. Moreover, we measured gene expression of gluconeogenesis-related enzymes because glycerol, a substrate for gluconeogenesis, was decreased with TP administration in this study. TP increased G6Pase gene expression in rest condition. G6Pase, which removes phosphate from

glucose-6-phosphate and generates glucose, is one of the key enzymes in gluconeogenesis. Therefore, it is suggested that TP decreased the tissues glycerol and increased plasma glucose level through activation of gluconeogenesis. Additionally, TP did not alter plasma NEFA and TG concentrations. Moreover, we measured fatty acid oxidation-related genes. However, TP scarcely affected these genes expression. Consequently, these results suggest that TP directly affected glycogen accumulation and gluconeogenesis without affecting fatty acid metabolism. However, tissue injury markers, ALT, AST, CK and LDH were increased by exhaustive exercise regardless of administration of TP. These results suggested that exercise-induced tissue damage was not related with running time until exhaustion or the leakage of these small molecular size proteins could be due to increased permeability of cell membrane.

It is known that oxidative stress is also associated with exercise-induced fatigue (Powers and Jackson, 2008). This involvement of oxidative stress on exercise-induced fatigue has been appraised by examining the influence of antioxidant supplementation on exercise performance. It has been reported that increased reactive oxygen species (ROS) reduced muscle tension (Reid, 2001; Reid et al., 1992). However, there is a bell-shape curve between ROS levels and force generation of skeletal muscle, and large level of ROS leads to decreased muscle function, which is probably the case during

exhaustive exercise. In fact, it was reported that some antioxidants could improve endurance performance (Clarkson, 1995; Radak et al., 2017).

Several *in vitro* studies reported that Taheebo extract has antioxidant capacity (Lee et al., 2012; Ohno et al., 2015; Park et al., 2003). Consistent with those reports, TP supplementation inhibited muscle oxidative stress in this study. However, TP did not affect the activities of antioxidant enzymes or non-enzymatic antioxidant capacity. These results suggested that TP directly reduced the generation of ROS but did not modify the antioxidant enzyme activities. In this study, mice of TP supplemented group were administered TP 1 h before exhaustive exercise. In addition, the time to exhaustion of Ex + TPS group mice were  $263 \pm 9.4$  min. Therefore, tissue sampling and blood collection immediately after the exhaustive exercise were approximately 320 min after the TP supplementation. It is possible that antioxidant ability of TP has not been reflected in the antioxidant ability because it had already been utilized during exercise. These results suggest that TP directly curbs the activities of some enzymes that generate ROS but does not change the antioxidant capacity. The activity of NADPH oxidase has been shown to be attenuated by the administration of polyphenols, hence this enzyme could be a potential target of TP (Laurent et al., 2012; Pignatelli et al., 2006; Sarr et al., 2006). Thus, it is considered that TP supplementation improves

endurance performance by both accumulation of muscle glycogen and attenuation of oxidative stress.

Some studies have shown that long term administration of polyphenol or other plant extract increases endurance performance via mitochondrial biogenesis (Davis et al., 2009; Eguchi et al., 2013; Minegishi et al., 2011). In this study, we measured gene expression of PGC-1 $\alpha$ , a master regulator of skeletal muscle adaptation, and Cyto c, a marker of mitochondrial biogenesis. However, these gene expression levels did not change by TP supplementation. On the other hand, gene expression of SIRT 1 was increased by TP administration. SIRT 1 is known as activators of PGC-1 $\alpha$ . Single dose administration of TP increased the AMPK phosphorylation and gene expression level of SIRT1, but may be insufficient to change the gene expression level of PGC-1 $\alpha$  or Cyto c.

In conclusion, polyphenol fraction of taheebo improved endurance capacity of mice by acceleration of gluconeogenesis, increase of blood glucose level and up-regulation of glycogen content in skeletal muscle. Moreover, TP decreased exercise-induced oxidative stress. Hence, TP can improve the endurance capacity via accumulation of muscle glycogen as well as attenuation of oxidative stress. Single dose administration of TP also increased phosphorylation of AMPK and gene expression level of SIRT1 but did not alter the maker of mitochondrial biogenesis. Therefore, long term administration of TP

may improve endurance capacity via the increase of mitochondrial biogenesis.

# Chapter V

## Conclusions



## CONCLUSIONS

In the first study, we investigated the effects of VC supplementation on the change of skeletal muscle enzyme activities by long-term swimming exercise training for 10 days. The finding of the present study was that vitamin C supplementation increases antioxidant enzyme catalase activity and antioxidant glutathione concentration in skeletal muscle but did not change the exercise training-induced increase of CS and HK enzyme activities in skeletal muscle. Furthermore, vitamin C reduced skeletal muscle and blood oxidative stress. These results suggest that vitamin C supplementation reduces oxidative stress by the increase of antioxidant capacity, but does not change the exercise training-induced increase of enzymatic activities of energy metabolism in skeletal muscle.

In the second study, we investigated whether antioxidant supplementation affects acute exercise-induced change in makers of skeletal muscle adaptation and its signaling pathways. The combined supplementation with vitamin C and E prevented the acute exercise-induced phosphorylation of AMPK and p38 MAPK. However, despite these effects on signaling, supplementation with vitamin C and E did not alter the exercise-induced change in gene expression of coregulator PGC-1 $\alpha$  and NCoR1, and

makers of adaptation, CS and VEGF. These results suggest that acute exercise-induced ROS does not alter mitochondrial biogenesis and angiogenesis in skeletal muscle.

In the third study, we investigated the effect of TP on endurance capacity and the related factors. Polyphenol fraction of taheebo improved endurance capacity of mice by acceleration of gluconeogenesis, increase of blood glucose level and up-regulation of glycogen content in skeletal muscle. Moreover, TP decreased exercise-induced oxidative stress. Hence, TP can improve the endurance capacity via accumulation of muscle glycogen as well as attenuation of oxidative stress. Single dose administration of TP also increased gene expression level of SIRT1 but did not alter the maker of mitochondrial biogenesis. Long-term administration of TP may improve endurance capacity via the increase of mitochondrial biogenesis.

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## ACKNOWLEDGEMENTS

I would like to grateful all members of the preventive medicine laboratory. Dr. Kawanishi, Dr. Okuda, Dr. Miyazaki gave me advice on my experiments. I discussed about may research with Mr. Hung, Mr. Mizokami, Mr. Niihara, Dr. Sako and Ms, Oginome, and they supported my studies. Thanks to their supports, I was able to finish writing this doctoral dissertation. In particular, I would like to thank Professor Katsuhiko Suzuki whose enthusiastic guidance including various advices, many supports, valuable discussion and considerable encouragement made my research more high quality. Finally, I would like to thank Professor Mitsuru Higuchi and Professor Takao Akama for taking on sub-chief examiner of my doctoral dissertation out of busy schedule.