

Hot Water Extract of *Vaccinium corymbosum* Increases Endurance Exercise Capacity by Improving Fatty Acid β -Oxidation and Antioxidant Defense System in Mice

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Received January 12, 2019; Revised February 19, 2019; Accepted March 12, 2019

Abstract The aim of this study was to evaluate the effects of water extract from *Vaccinium corymbosum* (VCW) on endurance exercise capacity. Exhaustive swimming time in the exercise and orally administered VCW group (Ex-VCW) was significantly increased compared to that of the exercise only group (Ex-CON). Compared to the Ex-CON group, the Ex-VCW group displayed lower lactate and higher non-esterified fatty acid levels after 10 min post-exercise and exhibited increased muscular glycogen level. VCW enhanced antioxidant activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione (GSH) in the experimental group compared to those of the Ex-CON group. Muscular malondialdehyde (MDA) level in Ex-VCW group appeared to be slightly decreased compared to that of the Ex-CON group; however, this difference was not statistically significant. VCW enhanced mRNA expression levels of mitochondrial biogenesis (PGC-1 α , NRF, and Tfam) and fatty acid β -oxidation (CPT-1, β -HAD, and PPAR- δ) related genes. These results suggest that VCW increases endurance exercise capacity *via* enhanced antioxidant activities, energy metabolism, and mitochondrial biogenesis-related gene expression.

Keywords: *Vaccinium corymbosum*, endurance exercise, antioxidant, fatty acid β -oxidation, mitochondrial biogenesis

Cite This Article: Shintae Kim, Jeongjin Park, Yeon Hyang Kim, Jeongmin Lee, Kyungmi Kim, and Woojin Jun, "Hot Water Extract of *Vaccinium corymbosum* Increases Endurance Exercise Capacity by Improving Fatty Acid β -Oxidation and Antioxidant Defense System in Mice." *Journal of Food and Nutrition Research*, vol. 7, no. 3 (2019): 205-212. doi: 10.12691/jfnr-7-3-5.

1. Introduction

Endurance exercise prevents cardiovascular diseases, metabolic disorders and increases muscular endurance [1,2,3]. Exercise causes depletion of energy sources and accumulation of reactive oxygen species (ROS) and in the case of endurance exercise, fatty acids are used instead of glucose as the primary energy source [4]. The use of fatty acids reduces glucose utilization and lactate production, and this in turn, increases endurance exercise capacity. Several previous studies have demonstrated that the ingestion of functional materials promotes fatty acid oxidation, resulting in improved endurance exercise capacity [4,5,6]. Also, energy production within the body occurs in the mitochondria. High density of mitochondria within the muscle results in the increased energy production to facilitate increased exercise duration [7,8]. Factors that

regulate mitochondria density are peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α , key regulator of mitochondrial biogenesis), nuclear respiratory factor (NRF), and mitochondrial transcription factor A (Tfam, factors of necessary for mitochondrial transcription) [9].

Generally, ROS produced during normal metabolism are eliminated by the antioxidant defense system in the body, however, exercise disrupts the balance of this defensive system [10]. The accumulation of ROS causes lipid peroxidation, enzyme inactivation and DNA damage, all of which can be suppressed by the administration of antioxidants [11,12].

Vaccinium corymbosum, known as blueberry, is a rich source of phytochemicals such as anthocyanin, flavones, and elagic acid [13]. These phytochemicals are multifunctional and can act as anti-oxidant, anti-viral, and anti-bacterial [14,15]. Some studies have indicated that *V. corymbosum* possesses anti-aging, anti-cancer, anti-obesity, and anti-diabetic properties [16,17,18].

The purpose of this study was to investigate the endurance exercise capacity of *V. corymbosum* water extract (VCW). We determined the endurance exercise capacity and the blood lactate, non-esterified fatty acid (NEFA), muscular glycogen, lipid peroxidation, antioxidant enzyme activities in mice treated with VCW, and we evaluated the effect of VCW on exercise by measuring the amount of mRNA expression of genes within the lipid β -oxidation and mitochondria biogenesis pathways.

2. Materials and Methods

2.1. Sample and Chemicals

Powdered *V. corymbosum* fruit (50 g) was refluxed with 1 L of water at 250°C for 3 h. Each extract was filtered through Whatman paper No. 6 and concentrated in a rotary evaporator under reduced pressure. The concentrate was then lyophilized. The extract was named VCW and was stored at -20°C until further analysis. Anthrone, hydrogen peroxide, bovine serum albumin (BSA), bovine liver glycogen, Bradford reagent, potassium chloride (KCl), potassium hydroxide (KOH), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), malondialdehyde (MDA), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals

Four-week-old male ICR mice (Orient Bio, Seongnam, Korea) were adapted for 1 week prior to the initiation of experiments. All mice were housed in a temperature controlled (23°C \pm 2°C) and humidity controlled (50% \pm 5%) room with a 12-hour light and a 12-hour dark cycle. All mice received a 5L79 diet (Orient Bio, Seongnam, Korea) and water *ad libitum*.

2.3. Exhaustive Swimming Capacity

The swimming capacity of mice was determined in an adjustable-current swimming pool (90 \times 45 \times 45 cm) [19]. Swimming time was measured at a flow rate of 8 L/min. The mice were measured for exhaustive swimming time. The mice were divided into three groups with similar mean exhaustive swimming times and body weights as control group (CON), the exercise only group (Ex-CON), and the exercise and orally administered VCW group (Ex-VCW). The CON group, however, could not perform exhaustive swimming during the swimming periods. Each group was administered distilled water or VCW for 28 days. VCW treatment occurred 3 h prior to exhaustive swimming, and the exhaustive swimming capacity was measured once every 7 days. The mice were removed from the swimming pool when they failed to rise to the surface within a 5-7 s period.

2.4. Analyses of Energy Metabolic Parameters

Blood lactate and NEFA levels were measured using commercial test strips and kits. On day 24, blood lactate

and NEFA were both collected from the tail (pre-exercise) and then collected again 10 min after swimming (post-exercise). Blood lactate level was measured using commercial test strips (ARKRAY, Kyoto, Japan) by an enzymatic method. NEFA level was detected in plasma collected from blood that was centrifuged at 1000 rpm at 4°C for 10 min. NEFA level was assayed using a commercial kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) by an enzymatic method. The mice were sacrificed 4 h following the last exhaustive swimming exercise. The gastrocnemius muscle glycogen concentration was measured as described in previous reports [20,21]. Briefly, muscle pieces were washed and homogenized. Homogenized muscle was centrifuged at 10,000 rpm for 30 min, and following centrifugation the pellet was assayed for glycogen. The pellet was hydrolyzed in 30% KOH at 100°C for 30 min. Then, 0.1 mL of hydrolyzed tissues was transferred to a test tube and 0.4 mL of distilled water was added. The 0.2% anthrone solution was also added to each test tube. The mixture was maintained at room temperature for 15 min, and absorbance was measured at 620 nm. Glycogen standard curve was generated to determine the absorbance of bovine liver glycogen at a range of 0-200 μ g/m. The values are expressed as mg/g tissue.

2.5. Antioxidant Activities

Muscular antioxidant enzyme activities were determined from homogenized gastrocnemius muscle. Homogenized muscle was centrifuged at 10,000 rpm for 30 min, and the supernatant was used to determine antioxidant activities. The catalase (CAT) activity was determined as described by Aebi [22]. The superoxide dismutase (SOD) activity was examined using the method described by McCord and Fridovich [23]. The glutathione peroxidase (GPx) activity was estimated by the spectrophotometric method reported by Thomson *et al* [24]. The glutathione reductase (GR) activity was analyzed according to the spectrophotometric method with some modification [25]. Glutathione (GSH) level was determined by the enzymatic method described by Akerboom *et al* [26]. MDA concentration was assayed by monitoring thiobarbituric acid reactive substance (TBARS) formation. The MDA concentration method was described by Draper and Hadley [27].

2.6. Real-time Polymerase Chain Reaction

Total RNA was isolated by easy-BLUE™ (INtRON biotechnology, Seongnam, Korea) according to the manufacturer instructions. Real-time polymerase chain reaction (RT-PCR) incorporated SYBR Green PCR Master Mix (Qiagen, Valencia, CA) according to the manufacturer instructions. Primer sequences used in this study are reported in Table 1.

2.7. Statistical Analysis

All data are expressed as mean \pm standard error (S.E). The results were statistically evaluated using one-way analysis of variance (ANOVA) with Duncan multiple comparison test and one-way ANOVA with Student's t-test. $p < 0.05$ was considered as statistically significant.

Table 1. Real-time PCR primer sequences.

Target	Primer sequence (5'→3')	
PGC-1 α	Forward	CACGTTCAAGGTCACCCTACAG
	Reverse	CTACCGACTTCAACCTGCTTC
NRF-1	Forward	GGCCAGCACCTTTGG
	Reverse	GGTCTTCCAGGATCATGCTCTT
Tfam	Forward	GGATGATTCCGGCTCAGGGAA
	Reverse	CTACCGACTTCAACCTGCTTC
CPT-1	Forward	GTGACTGGTGGGAGGAATAC
	Reverse	GAGCATCTCCATGGCGTAG
β -HAD	Forward	GACAGGGTCATGCTATGATTGTG
	Reverse	TCGGTCGCCTCCTTCTAGAG
PPAR- δ	Forward	CGCAAGCCCTTCAGTGACAT
	Reverse	CGCATTGAACCTTGACAGCAA

3. Results

3.1. Effect of VCW on Endurance Exercise Capacity

In our study, endurance exercise capacity was measured by swimming time. As shown in Figure 1, the Ex-VCW

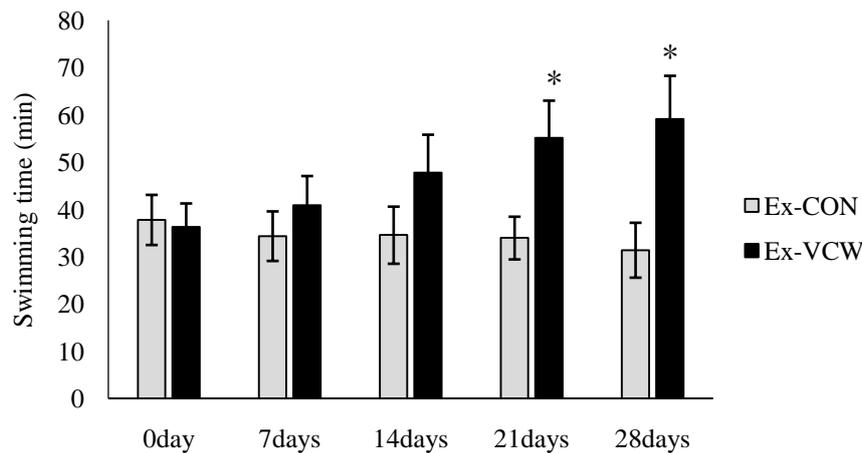


Figure 1. Effect of *Vaccinium corymbosum* hot water extract (VCW) on endurance swimming capacity. Mice were given distilled water and VCW (1 g/kg body weight/day). The swimming time was measured at 8 L/min. Each value represents the mean \pm S.E. in each group. The asterisk above the bar presents a statistically different value compared to the Ex-CON group as assessed by Student's t-test ($p < 0.05$)

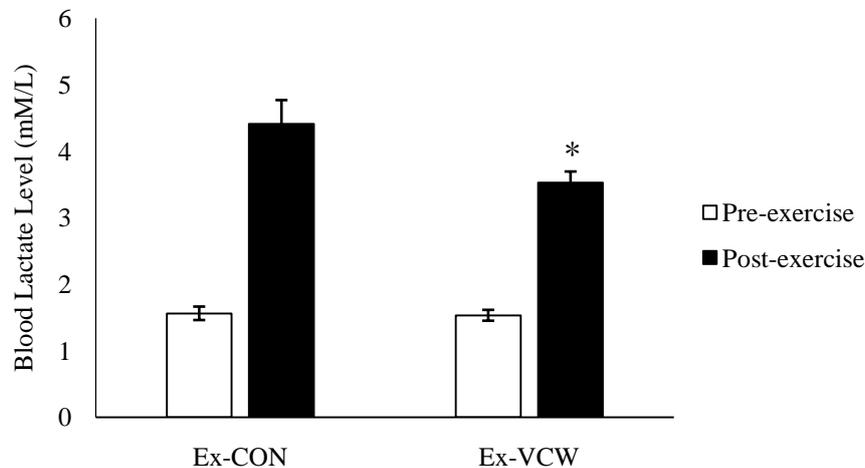


Figure 2. Effect of *Vaccinium corymbosum* hot water extract (VCW) on blood lactate levels. Mice were administered distilled water and VCW (1 g/kg body weight/day). Blood lactate levels were evaluated before and 10 min after swimming exercise. Each value represents the mean \pm S.E. in each group. The asterisk above the bar indicated a statistically different value compared to that of the Ex-CON group as assessed by Student's t-test ($p < 0.05$)

group exhibited increased swimming times on the 21st and 28th days compared to those of the Ex-CON group. Swimming time was increased by approximately 65% compared to the Ex-CON group at 21 days by VCW administration.

3.2. Effect of VCW on Energy Metabolism

As exercise time increases, the use of fatty acid increases compared to glucose, and both glycogen and NEFA levels are important factors in determining exercise capacity. Lactate and NEFA levels were measured before swimming (pre-exercise) and after 10 min of swimming (post-exercise) on day 24. The blood lactate and NEFA levels at pre-exercise were similar for each group, but the blood lactate level of the Ex-VCW group at post-exercise was approximately 20% lower than that of the Ex-CON group (Figure 2). The NEFA level in post-exercise was increased by approximately 7% in the Ex-VCW group compared to that of the Ex-CON group (Figure 3).

Muscular glycogen levels can be used a fatigue indicator. When comparing the Ex-VCW and Ex-CON groups, it was confirmed that these levels were significantly higher in the Ex-VCW group (Figure 4).

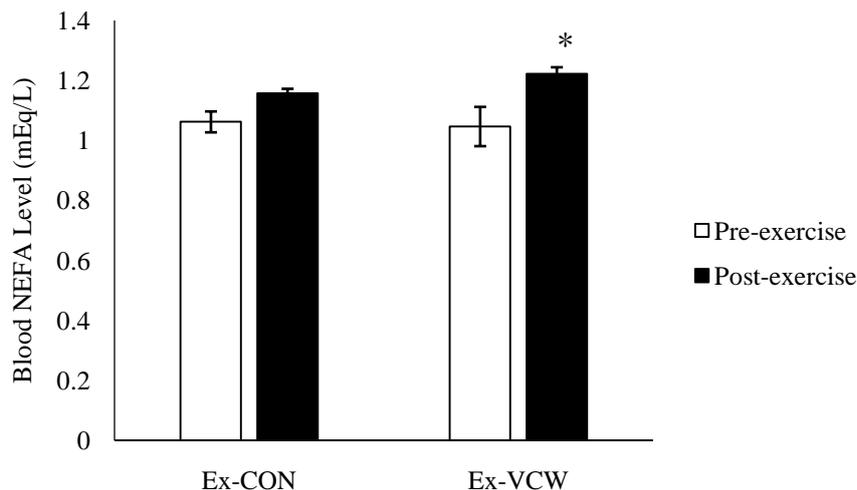


Figure 3. Effect of *Vaccinium corymbosum* hot water extract (VCW) on blood NEFA levels. Mice were administered distilled water and VCW (1 g/kg body weight/day). Blood NEFA levels were evaluated before and 10 min after swimming exercise. Each value represents the mean \pm S.E. in each group. The asterisk above the bar indicates a statistically different value compared to that of the Ex-CON group as assessed by Student's t-test ($p < 0.05$)

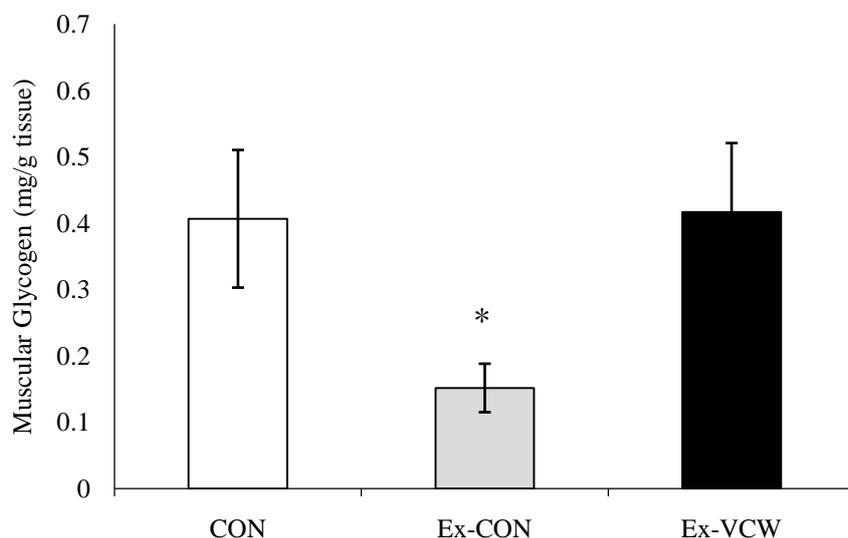


Figure 4. Effect of *Vaccinium corymbosum* hot water extract (VCW) on muscular glycogen levels. Mice were administered distilled water and VCW (1 g/kg body weight/day). After the final exhaustive exercise test, the mice were sacrificed. Each value represents the mean \pm S.E. in each group. The asterisk above the bar represents statistically different values compared to those of the CON group as assessed by Duncan's multiple range test ($p < 0.05$)

Table 2. Effect of *Vaccinium corymbosum* hot water extract (VCW) on muscular antioxidant enzyme activities and MDA level

	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	GR (U/mg protein)	GSH (nmoles/g tissue)	MDA (μ g/mg protein)
CON	1.59 \pm 0.36	16.35 \pm 0.89	0.19 \pm 0.01	0.06 \pm 0.00	1.72 \pm 0.12	0.25 \pm 0.04
Ex-CON	0.97 \pm 0.10*	11.93 \pm 1.15*	0.16 \pm 0.01*	0.04 \pm 0.00*	1.14 \pm 0.08*	0.79 \pm 0.06*
Ex-VCW	1.39 \pm 0.12	15.32 \pm 0.77	0.18 \pm 0.00	0.06 \pm 0.00	1.50 \pm 0.07	0.42 \pm 0.05

Each value represents the mean \pm S.E. in each group. Mice were administered distilled water and VCW (1 g/kg body weight/day). After the final exhaustive exercise test, the mice were sacrificed. The various superscripts asterisks represent statistically different values compared to those of the CON group as assessed by Duncan's multiple range test ($p < 0.05$).

3.3. Effect of VCW on Muscular Antioxidants

The effect of VCW on exercise-induced ROS was evaluated by measuring the antioxidant enzyme and non-enzyme activities in muscle. The muscular antioxidant enzyme and non-enzyme activities in the Ex-VCW group were significantly increased compared to those in the Ex-CON

group (Table 2).

The lipid peroxidation caused by ROS was also evaluated by measuring MDA. The muscular MDA level in the Ex-VCW group was decreased compared to that of the Ex-CON group, and no significant difference was observed between the Ex-VCW and Ex-CON groups (Table 2).

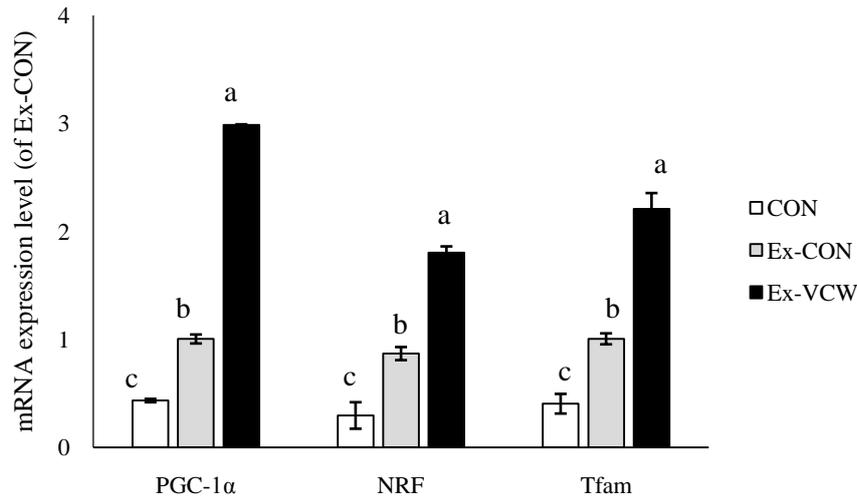


Figure 5. Effect of *Vaccinium corymbosum* hot water extract (VCW) on mRNA expression levels in muscle for mitochondrial biogenesis factors determined by RT-PCR. Mice were administered distilled water and VCW (1 g/kg body weight/day). Each value represents the mean \pm S.E. in each group. The various letters represent statistically different values compared to those of the CON group as assessed by Duncan's multiple range test ($p < 0.05$).

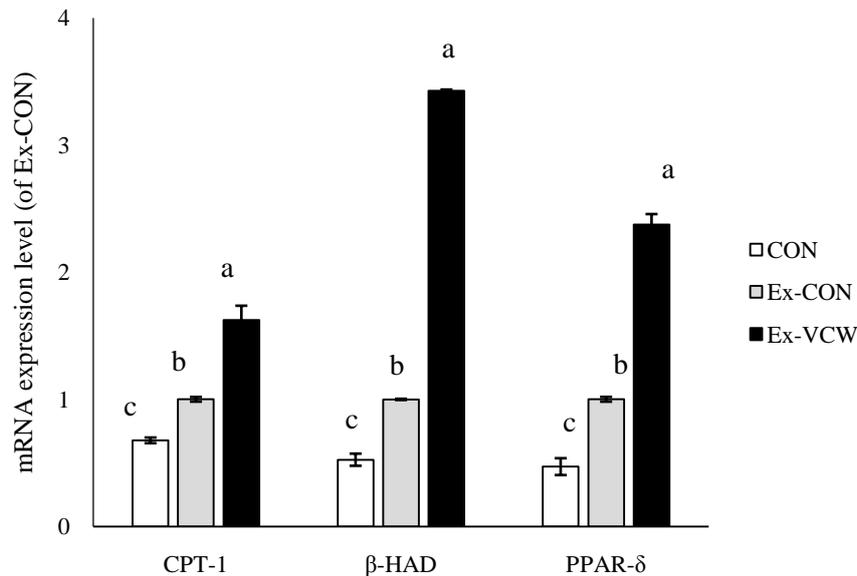


Figure 6. Effect of *Vaccinium corymbosum* hot water extract (VCW) on mRNA expression levels in muscle for fatty acid β oxidation as determined by RT-PCR. Mice were administered distilled water and VCW (1 g/kg body weight/day). Each value represents the mean \pm S.E. in each group. The various letters represent statistically different values compared to those of the CON group as assessed by Duncan's multiple range test ($p < 0.05$).

3.4. Effects of VCW on mRNA Expression Level of Mitochondrial Biogenesis and Fatty Acid β Oxidation Factor in Muscle

Muscular mitochondrial biosynthesis was assessed by measuring the mRNA expression levels of PGC-1 α , NRF, and Tfam. Additionally, mRNA expression of fatty acid β -oxidation factors was evaluated by measuring carnitine palmitoyltransferase-1 (CPT-1), β -hydroxyacyl coenzyme A dehydrogenase (β -HAD), and peroxisome proliferator-activated receptor δ (PPAR- δ) mRNA level. As a result, mRNA expression levels of mitochondrial biosynthesis factors in the Ex-CON and Ex-VCW groups were significantly increased compared to those in the CON group (Figure 5). The levels in the Ex-VCW group were

higher than those in the Ex-CON group. The results of the mRNA expression levels of fatty acid β -oxidation assays were similar to those of mitochondrial biosynthesis assays (Figure 6).

4. Discussion

In this study, we demonstrated that supplementation with VCW improved endurance exercise capacity. This is consistent with the previous finding that supplementation with blueberries increases antioxidant levels and the β -oxidation of fat. Supplementation with VCW improved endurance exercise by promoting changes in energy metabolism, antioxidant activity, and by increasing mitochondrial biogenesis.

Lactate, NEFA, and glycogen levels were measured to assess energy metabolite levels. Blood lactate is produced within the muscle and can be utilized as an indicator of fatigue [7]. During intense exercise, glucose metabolism produces excessive amounts of lactate when muscles require a large amount of energy in a short period of time. This lactate then accumulates in muscles and blood, causing acidification and fatigue. In this study, VCW supplementation reduced lactate concentration after exercise compared to levels observed in the Ex-CON group, suggesting that endurance exercise capacity was improved by VCW supplementation. NEFA is a fatty acid produced by hydrolysis of adipocytes and degradation of triglyceride by lipoprotein lipase [6]. NEFA can generate a large amount of energy and reduce the use of glucose to inhibit the production of lactate [4]. Therefore, increased NEFA levels during exercise are important indicators of endurance exercise. Glycogen is a form of glucose storage in the body, and depletion of glycogen is an important indicator due to its role in the homeostasis of blood glucose levels [28]. Several studies have also reported that endurance exercise increases when consumption of fatty acids increases while glycogen is preserved. In this study, supplementation with VCW inhibited the accumulation of blood lactate levels and promoted the decomposition of fatty acids, thus contributing to endurance exercise. As the consumption of glucose was reduced, the amount of glycogen observed in the Ex-VCW group was higher than that of the Ex-CON group.

The increase in NEFA level was believed to increase β -oxidation of adipocytes, and the levels of β -oxidation related mRNA products were measured. Fatty acid β -oxidation related mRNAs include CPT-1, β -HAD, and PPAR- δ [29,30,31]. CPT-1 is a transferase that exists in the mitochondria outer membrane and helps the mitochondrial entry of fatty acids when they are converted to Acyl-CoA [32]. β -HAD is a dehydrogenase that aids in the oxidation of L-3-hydroxyacyl CoA to 3-ketoacyl CoA by NAD⁺ [33]. PPAR- δ is increased by exercise and is one of the factors that helps to break down fat [34]. mRNA expression level of CPT-1, β -HAD, and PPAR- δ were increased in the Ex-CON and Ex-VCW groups compared to those of the CON group. The expression in the Ex-VCW group, however, was higher than that of the Ex-CON group. As a result, supplementation with VCW increased the level of fatty acid β -oxidation, and thereby improved endurance exercise capacity.

ROS within the body are produced in the mitochondria and are removed by the antioxidant system. The generation and elimination of ROS are both balanced in the body. During endurance exercise, fat is a more important energy source than is glucose, however, more ROS are produced by fatty acid β -oxidation. Eventually, endurance exercise causes an imbalance in ROS levels. Also, fatty β -oxidation in mitochondria produces more ROS than energy production through glucose [35]. Eventually, endurance exercise causes accumulation of ROS [36]. The antioxidant system acts on various enzymes. SOD in the antioxidant system produces hydrogen peroxide from the superoxide radical (O₂⁻), and this metabolite is highly reactive. Other enzymes are CAT and GPx, which remove hydrogen peroxide by converting it to water and oxygen.

Given this, it is clear that SOD, CAT, and GPx play important roles in removing ROS [37]. GSH transfers electrons to the ROS to remove active oxygen from the body. By transferring electrons in this way, GPx can facilitate the conversion of ROS into water. When GSH transfers electrons, it becomes inactivated, and inactivates GSH binds to other GSH molecules to form GSSG. GR functions to convert this generated GSSG back into GSH. In this study, we confirmed that the antioxidant system, reduced through endurance exercise, is maintained due to supplementation with VCW. Lipid peroxides are produced by the production of ROS that subsequently attack cellular lipids [38]. One of the byproducts produced by lipid peroxidation is MDA [27]. The high level of MDA indicates that high levels of ROS result in lipid peroxidation. Given this, we identified ROS by measuring MDA levels. As a result, MDA level was increased due to endurance exercise, and we confirmed that supplementation with VCW lowered MDA level. Noticeably, VCW inhibited the formation of ROS during endurance exercise.

Mitochondria are an important factor in generating energy, and the density of mitochondria in the context of endurance exercise is important [9]. In many studies, endurance exercise increased mitochondrial density by increasing mRNA expression levels of factors associated with mitochondrial biogenesis, including PGC-1 α , NRF, and Tfam [39-40]. Therefore, we measured the expression of mRNA associated with mitochondrial biogenesis. PGC-1 α was found to be the most important factor in mitochondrial biogenesis and was activated by exercise and other factors [41]. Activated PGC-1 α activates NRF, and NRF acts as a regulator of the Tfam promoter. Tfam is an important factor in transcription in the context of mitochondrial biogenesis [40]. The expression of mRNA associated with mitochondrial biogenesis was measured, and expression was increased in response to exercise, and these levels were further increased by VCW. Ultimately, mitochondrial biogenesis in the muscle was increased by supplementation with VCW.

In conclusion, supplementation with VCW inhibited lactate production in the blood, reduced consumption of glycogen, promoted fat decomposition, and improved endurance exercise capacity. Additionally, VCW inhibited the accumulation of ROS, increased the antioxidant system, and increased expression of mitochondrial biogenesis related factors, resulting in enhanced endurance exercise capacity.

Acknowledgements

This research was supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT) through the Encouragement Program for the Industries of Economic Cooperation Region (R0004933).

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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