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# Anti-Fatigue Activity of a Mixture of *Stauntonia hexaphylla* (Thunb.) Decaisne and *Vaccinium bracteatum* Thunb. Fruit Extract

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**ABSTRACT:** *Stauntonia hexaphylla* (Thunb.) Decaisne and *Vaccinium bracteatum* Thunb. are commonly used in traditional herbal medicine and food and both exhibit antioxidant and anti-inflammatory effects. Herein, hot-water extracts of *Stauntonia hexaphylla* (Thunb.) Decaisne and *Vaccinium bracteatum* Thunb. fruits (1:1 mixture) were used to produce a complex extract NET-1601. The anti-fatigue activity of NET-1601 was evaluated in an *in vitro* oxidative stress model induced by treating C2C12 myotubes with $H_2O_2$. An exhaustive swimming test (EST) *in vivo* model was established using ICR mice. NET-1601-treated C2C12 myotubes (50, 100, and 200 μg/mL) with $H_2O_2$-induced oxidative stress displayed significantly increased cell viability and ATP content, but significantly decreased levels of reactive oxygen species. All NET-1601-treated EST models demonstrated significantly higher maximum swimming rates than control mice. Furthermore, serum lactate, lactate dehydrogenase activity, non-esterified fatty acid, and intramuscular glycogen levels were higher in NET-1601-treated mice than in control mice. In addition, mRNA levels of regulatory factors involved in muscle mitochondrial fatty acid β-oxidation increased upon NET-1601 treatment. Moreover, catalase, superoxide dismutase, glutathione-*S*-transferase, and liver glutathione content, and antioxidant activity were higher in NET-1601-treated mice than in control mice. Reduced malondialdehyde levels indicated that NET-1601 treatment inhibited exercise-induced lipid peroxidation. Together, these results suggest that NET-1601 retains antioxidant enzyme activity during oxidative stress, simultaneously enhancing both muscle function via glycogen and fatty acid oxidation, thereby exerting a positive effect on recovery from fatigue.

**Keywords:** anti-fatigue activity, exhaustive swimming test, oxidative stress, *Stauntonia hexaphylla* (Thunb.) Decaisne, *Vaccinium bracteatum* Thunb.

## INTRODUCTION

Fatigue refers to the physical or mental state of feeling tired or helpless, caused by various factors including lack of sleep or rest, anemia, thyroid dysfunction, aging, depression, stress, and strenuous exercise. Fatigue exerts a negative impact on quality of life, task efficiency, social relationships, and physical activity (Kang et al., 2017). Fatigue can also refer to a state of reduced exercise performance due to weakened muscular strength caused by the physical exhaustion upon strenuous exercise. Rapid consumption of adenosine triphosphate (ATP) during periods of fatigue increases levels of inorganic acids, lactate, lactate dehydrogenase (LDH), and ammonia in serum (Finsterer, 2012). In muscle, the glycogen levels decrease when it is used as an energy source (Ørtenblad et al., 2013). The natural substances red ginseng (Kim et al., 2016), *Rhodiola rosea* (Kang et al., 2015), and garlic (Morihara et al., 2007) are known to exhibit a positive effects on fatigue.

Reactive oxygen species (ROS) are generated in the body during normal metabolic processes and by external factors such as excessive stress, alcohol, drug, or ultraviolet light (Rani et al., 2016). Overproduction of ROS induces oxidative stress that inflicts oxidative damages to intracellular lipids, proteins, and nucleic acids (DNA and RNA), which may lead to development of diseases such as cancer, diabetes, arteriosclerosis, Alzheimer's or Parkinson's diseases, and accelerate aging (Essick and Sam, 2010; Huang et al., 2016; Papaconstantinou, 2019; Trist et al., 2019). During strenuous exercise, ROS accumulates as a metabolite of mitochondrial oxygen reduction to produce ATP in skeletal muscle fibers (Filler et al., 2014; Jackson et al., 2016). Over-accumulation ROS in-

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hibits release of $Ca^{2+}$ from the sarcoplasmic reticulum, which lowers the $Ca^{2+}$ sensitivity of myofilaments and reduces the affinity between myosin and actin filaments. The resulting suppression of the muscle contraction function (muscular strength) consequently results in fatigue (Debold, 2015). Therefore, ROS scavengers are likely to be efficacious in reducing fatigue due to physical exercise.

The fruits of *Stauntonia hexaphylla* (Thunb.) Decaisne and *Vaccinium bracteatum* Thunb. have long been used in traditional medicine and food ingredients, and have been shown to exhibit a variety of bioactivities, including anti-inflammatory and antioxidant activities. *Stauntonia hexaphylla* (Thunb.) Decaisne is an evergreen climber of the Lardizabalaceae family that has palm-shaped leaves, small flowers, and reddish black edible fruits. The plant is native to Korea, China, and Japan, and contain chemical compounds including triterpenoids, glucosides, flavonoids, and phenylpropanoids (Hwang et al., 2017). The fruit of *Stauntonia hexaphylla* (Thunb.) Decaisne has long been used as a diuretic agent and in the treatment of rheumatic diseases. In addition, effects on enhancing blood circulation and vision have been reported since the fruit exhibits both antioxidant and anti-inflammatory activity (Park et al., 2009; Kim et al., 2018; Ba Vinh et al., 2019).

*Vaccinium bracteatum* Thunb. is a shrub or small tree naturally growing in East Asian countries that has long been used to treat inflammation, diarrhea, and skin diseases (Fan et al., 2019). The fruit of *Vaccinium bracteatum* Thunb. contains flavonoids such as anthocyanin (Tsuda et al., 2013) and is reported to exhibit anti-inflammatory, anticancer, anti-depressant, and antioxidant activities (Landa et al., 2014; Oh et al., 2018).

In this study, we mixed the hot water extracts of the fruits of *Stauntonia hexaphylla* (Thunb.) Decaisne and *Vaccinium bracteatum* Thunb. (1:1 ratio) to obtain NET-1601, which exhibits the efficacy of the two ingredients. We evaluated the antioxidant and anti-fatigue activities of NET-1601 using an *in vitro* models of oxidative stress and fatigue.

## MATERIALS AND METHODS

### Plant material and preparation of NET-1601

The fruit of *Stauntonia hexaphylla* (Thunb.) Decaisne used in this study was purchased from the Jeongnamjin Stauntonia Farming Association Corporation (Jangheung, Korea) and the fruit of *Vaccinium bracteatum* Thunb. was purchased from the Georhim Botanical Garden (Goheung, Korea). The fruit extracts from *Stauntonia hexaphylla* (Thunb.) Decaisne and *Vaccinium bracteatum* Thunb. were each obtained following 8 h of reflux extraction at 100± 10°C through addition of water (1:10～15, w/v). Following extraction with hot water, the mixture was filtered using a 10-μm paper filter and the extracts were decompressed to 25°brix using a rotary evaporator (R-215, BÜCHI Labortechnik, Flawil, Switzerland). NET-1601 was formulated as a 1:1 (w/w) mixture and was homogenized and dried to a powdered form for use in subsequent experiments.

### Cell culture

The C2C12 myoblasts used in this study were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (Tissue Culture Biologicals, Inc., Seal Beach, CA, USA) and 1% penicillin/streptomycin, and incubated at 37°C with 5% $CO_2$. To induce differentiation into myotubes, culture medium was replaced with DMEM containing 2% horse serum (Gibco). Medium was replaced every two days.

### Cell viability assays

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) assays were used to examine the effects of NET-1601 on cell viability and the protective effects against oxidative damage induced by treatment with $H_2O_2$. C2C12 cells were aliquoted into 48-well plates ($5\times10^4$ cells/well) for cell culture. After stabilization for 24 h, the medium was replaced with differentiation medium for three days to induce differentiation. On day 3, cells were treated with three different concentrations of NET-1601 (50, 100, and 200 μg/mL) and incubated for 24 h. Next, cells were treated with MTT (Sigma-Aldrich Co., St. Louis, MO, USA) diluted to 0.5 mg/mL and allowed to react for 3 h at 37°C. MTT reagent was then removed and the formazan in each well was dissolved with dimethyl sulfoxide. The absorbance of the mixture was measured at 450 nm using the microplate reader (SYNERGY H1, BioTek Instruments Inc., Winooski, VT, USA). To determine the protective effect of NET-1601 against oxidative stress-induced cell damage, cells on day 3 of differentiation were pre-treated with NET-1601 for 1 h, followed by 1.2 mM of $H_2O_2$ (Sigma-Aldrich Co.). After 24 h culture, MTT assays was carried out to evaluate the cytoprotective effect.

### Measurement of intracellular ATP content

ATP assays were performed to examine if NET-1601 reduces the levels of ATP produced by $H_2O_2$-induced oxidative stress. C2C12 cells were aliquoted to 48-well plates ($5\times10^4$ cells/well) and cultured for 24 h. The medium was then replaced with differentiation medium for 3 days to induce differentiation. The cells were then treated with three different concentrations of NET-1601 (50, 100, and 200 μg/mL) for 1 h, followed by 1.2 mM of $H_2O_2$ (Sigma-Aldrich Co.). After 24 h culture, cells were harvested. To

measure the ATP content, cell lysates were treated with 4 M perchloric acid and 2 M KOH for deproteinization, and the supernatant was evaluated using ATP assay kits (Abcam, Cambridge, MA, USA).

**Measurement of ROS generation**

To examine changes in the generation of intracellular ROS, C2C12 cells were aliquoted to a 96-well white plates ($2.5\times10^4$ cells/well). Following 24 h culture, the medium was replaced with differentiation medium for 3 days to induce differentiation. Cells were pre-treated with NET-1601, 30 μM of a non-fluorescent probe, and 2',7'-dichlorofluorescin diacetate (Sigma-Aldrich Co.) for 1 h, and then treated with 600 μM of $H_2O_2$ (Sigma-Aldrich Co.) dissolved in Hanks' balanced salt solution (Gibco). After 2 h, the fluorescence (excitation wavelength, 485 nm; emission wavelength, 530 nm) was measured using a microplate reader (SYNERGY H1, BioTek Instruments Inc.).

**Animal experiments (exhaustive swimming test)**

The loaded forced swimming test described by Moriura et al. (1996) was followed, with modification (Matsumoto et al., 1996). An acrylic-based transparent plastic pool ($90\times45\times45$ $cm^3$) was filled up to 38 cm in height with distilled water. A pump was used to create a one-way flow of 7.5 L/min and the temperature was maintained at $34\pm1^{\circ}C$. ICR mice (5 weeks old male, 20.6±1.6 g) were acclimatized in the animal room for a week, then acclimatized to the swimming test once a day (from 1 to 3 p.m.) from three days prior to the experiment until completion of swimming tests (the time point when the noses of the mice were immersed below the surface of water for 5 s). One hour before the experiment, mice received an AIN-93M isocaloric diet with either saline (control) or NET-1601 (50 mg/kg/d or 200 mg/kg/d). The experiment was carried out until completion of swimming, repeated every day for 14 days. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) in the Kyung Hee University (approval no. KHGASP-19-104). All animal experiments were conducted in accordance with the IACUC guidelines.

**Determination of biochemical indices**

Following exhaustive swimming, the ICR mice were anesthetized using isoflurane and their blood was sampled via heart clipping. Organs including the liver and muscle were dissected from the euthanized mice. Serum was isolated from blood to measure levels of LDH, lactate, and non-esterified fatty acids (NEFA) using enzyme-linked immunosorbent assay (ELISA) assays (BioVision Inc., Milpitas, CA, USA). The glycogen levels in muscle were measured in using chemical ELISA assays (BioVision Inc.). Expression of peroxisome proliferator-activated receptor-delta (PPAR-δ), carnitine palmitoyltransferase-1 (CPT1), β-hydroxyacyl coenzymes A dehydrogenase (HADH), and uncoupling protein-3 (UCP3) in muscle were measured after RNA extraction using real-time-polymerase chain reaction (RT-PCR).

**RT-PCR analysis**

Total RNA was isolated from muscle using the RNeasy extraction kit (QIAGEN Sciences Inc., Germantown, MD, USA) following manufacturer's instructions. cDNA was synthesized using the iScript Select cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following manufacturer's instructions using 5 μg of total RNA. The Step One Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) was used for real-time PCR, following the iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.) protocol. The mRNA sequences are shown in Table 1.

**Analysis of antioxidant enzyme activity in mouse liver**

Antioxidant enzyme activity was measured as follows: liver tissue was homogenized and total protein in the supernatant was quantified in accordance with the Bradford method. The value obtained was set as the standard. Catalase (CAT), superoxide dismutase (SOD), and glutathione-*S*-transferase (GST) enzyme activities were measured using ELISA assays (BioVision Inc.), using 100 μL of each enzyme. Liver samples were harvested to quantify levels of glutathione (GSH; an antioxidant substance) and malondialdehyde (MDA; an intermediate in tissue lipid oxidation). Levels were quantified by measuring chemical conversion using colorimetry.

**Table 1.** Primer set sequences used for real-time polymerase chain reaction

| Sequence name | Forward (5′→3′) | Reverse (5′→3′) | NCBI reference |
|---|---|---|---|
| GAPDH | TGG CCT CCA AGG AGT AAG AAA C | CAG CAA CTG AGG GCC TCT CT | BC087743.1 |
| PPAR-δ | CGC AAG CCC TTC AGT GAC AT | CGC ATT GAA CTT GAC AGC AAA | NM_011145 |
| CPT1 | GTG ACT GGT GGG AGG AAT AC | GAG CAT CTC CAT GGC GTA G | MN_013495 |
| HADH | GAC AGG GTC ATG CTA TGA TTG TG | TCG GTC GCC TCC TTC TAG AG | MN_145558 |
| UCP3 | CCA GAG CAT GGT GCC TTC GCT | CTC GTG TCA GCA GCA GTG | MN_009464 |

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PPAR-δ, peroxisome proliferator-activated receptor-delta; CPT1, carnitine palmitoyltransferase-1; HADH, β-hydroxyacyl coenzymes A dehydrogenase; UCP3, uncoupling protein-3.

### Statistical analysis

All experiments were carried out in triplicate. Statistically significant differences were analyzed by ANOVA using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Post-hoc analysis was performed using Bonferroni tests, with differences considered statistically significant at 5% ($P<0.05$).

## RESULTS AND DISCUSSION

### Evaluation of cytotoxicity of NET-1601

The MTT assay is based on reduction of the yellow tetrazolium bromide reagent to purple formazan through activity of mitochondrial dehydrogenase in cells. The assay allows cell viability to be evaluated by measuring absorbance (Kang et al., 2017). In this study, C2C12 myotubes were treated with three different concentrations of NET-1601 (50, 100, and 200 μg/mL). The results indicated a lack of cytotoxicity after 24-h treatment with NET-1601 of concentrations of up to 200 μg/mL (Fig. 1A).

### Evaluation of the cytoprotective effect of NET-1601 against oxidative stress in myotubes

Upon strenuous exercise, ROS damages cells and proteins to cause physical fatigue (Jackson et al., 2016). The differentiation-induced C2C12 myotubes were treated with $H_2O_2$ to induce oxidative stress, allowing us to investigate the cytoprotective effect of NET-1601. The cells were pre-treated with NET-1601 for 1 h and anti-fatigue activity was evaluated. The results indicated that cell viability was approximately 40% lower in the group treated with $H_2O_2$ compared with the control group. Treatment of $H_2O_2$-induced cells with NET-1601 increased cell viability by 16.0% ($P<0.05$), 17.0% ($P<0.01$), and 20.1% ($P<0.01$) at concentrations of 50, 100, and 200 μg/mL, respectively (Fig. 1B). Therefore, NET-1601 demonstrated a protective effect against oxidative stress in C2C12 myotubes.

### Evaluation of ATP content in myotubes following NET-1601 treatment

During exercise, ATP hydrolysis in muscles occurs when the affinity between myosin and actin is enhanced through repeated muscle contractions. Long periods of strenuous exercise lead to sudden ATP consumption and accumulation of ROS in muscle (Debold, 2015). High levels of ROS can lead to dysfunction of the mitochondrial respiratory chain, resulting in low levels of ATP (Couillard and Prefaut, 2005). Because mitochondria are the main producers of ATP, it is important to remove ROS from muscle cells (Ábrigo et al., 2018). C2C12 myotubes were treated with $H_2O_2$ to induce oxidative stress and ATP assays were used to measure changes in intracellular ATP content. The results indicated an approximately 50% reduction in ATP following treatment with $H_2O_2$. However, the ATP content was significantly increased following treatment with 50 μg/mL ($P<0.05$) and 200 μg/mL ($P<0.05$) NET-1601 (Fig. 1C). Therefore, NET-1601 helps restore ATP levels in C2C12 myotubes following induction of oxidative stress.

### Evaluation of NET-1601 activity against ROS generation in myotubes

To investigate whether the cytoprotective effect of NET-1601 against $H_2O_2$-induced oxidative stress in C2C12

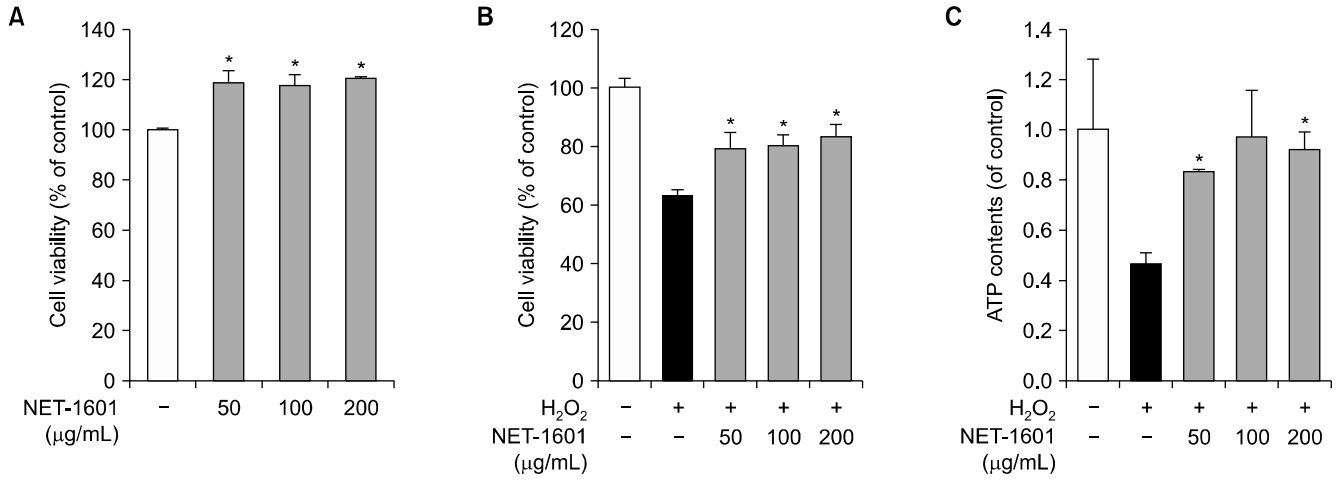


**Fig. 1.** Anti-fatigue effect of NET-1601 in C2C12 myotubes. (A) Cytotoxicity of NET-1601 on C2C12 myotubes. The cells were treated with three different concentrations of NET-1601 and incubated for 24 h. Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) assay. Data are presented as the mean±SD (n=3). *$P<0.05$ compared with the control. (B) Protective effect of NET-1601 in $H_2O_2$-treated C2C12 myotubes. (C) Effect of NET-1601 on adenosine triphosphate (ATP) content in $H_2O_2$-treated C2C12 myotubes. The cells were pre-treated with three different concentrations of NET-1601 for 1 h followed by a treatment with 1.2 mM $H_2O_2$ for 24 h at 37°C. Cell viability was determined by MTT assay and ATP content was determined by ATP assay. Data are presented as the mean±SD (n=3). *$P<0.05$ compared with the $H_2O_2$-treated cells.

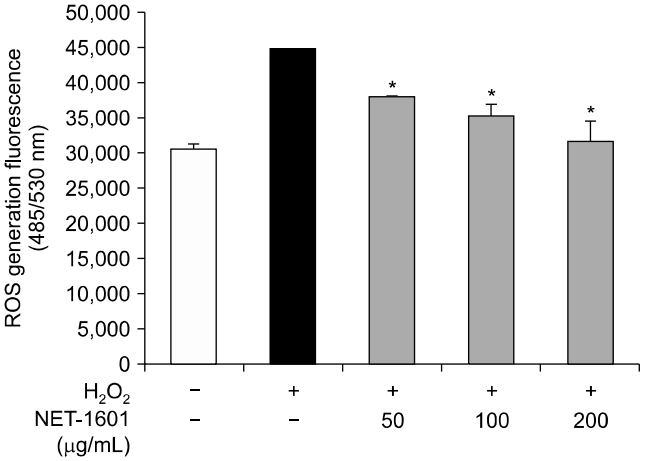


**Fig. 2.** Effect of NET-1601 on $H_2O_2$-induced reactive oxygen species (ROS) generation in C2C12 myotubes. The cells were pretreated with three different concentrations of NET-1601 (50, 100, and 200 μg/mL) and 30 μM 2′,7′-dichlorofluorescin diacetate for 1 h. The cells were then treated with 600 μM $H_2O_2$ for 2 h at 37°C. ROS generation was determined by fluorometry. Data are presented as the mean±SD (n=3). *$P$<0.05 compared with the $H_2O_2$-treated cells.

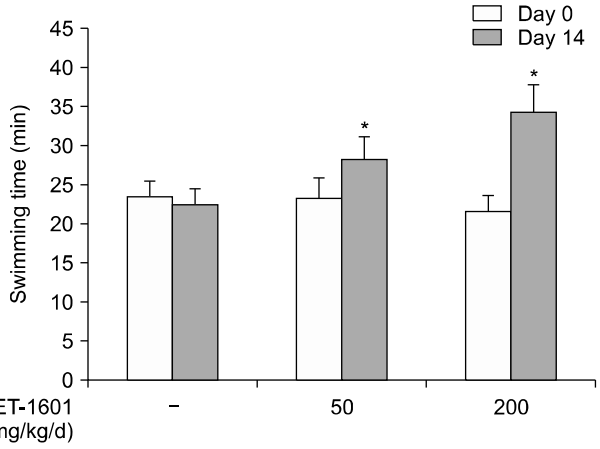


**Fig. 3.** Effect of NET-1601 on exhaustive swimming capacity in mice. Data are presented as the mean±SD for 10 mice in each group. *$P$<0.05 compared with the control by Bonferroni test following one-way ANOVA.

myotubes acts by inhibiting ROS generation, we evaluated ROS generation in relation to NET-1601 activity. The results indicated an increase in ROS in cells treated with $H_2O_2$ only compared with the control, whereas NET-1601 significantly decreased ROS in a concentration-dependent manner ($P$<0.05) (Fig. 2). Thus, we demonstrated NET-1601 has a powerful ROS scavenging activity against $H_2O_2$-induced oxidative stress in C2C12 myotubes. These results indicate the cytoprotective effect of NET-1601 against $H_2O_2$-induced oxidative stress is correlated to inhibition of ROS generation.

**Evaluation of NET-1601 activity on myokinetics during EST**

To evaluate the effect on NET-1601 on myokinetics, we carried out exhaustive swimming tests on mice. On day 0, the day before treatment, we minimized bias caused by mice by setting the baseline values for reference in further experiments. These results did not reveal any significant difference between control mice and mice treated with NET-1601. On day 14, the maximum swimming time was 22.4 min for the control group, whereas swimming time was increased for all NET-1601 treatment groups [50 mg/kg NET-1601: 28.2 min ($P$<0.05); 200 mg/kg NET-1601: 34.2 min ($P$<0.05)] (Fig. 3). We administrated NET-1601 in doses of 50 and 200 mg/kg/d to clearly distinguish the efficacy of low and high doses, and to estimate the dose for use in clinical trials. Based on these results, NET-1601 treatment was shown to extend the maximum swimming time, thereby demonstrating NET-1601 enhances myokinetics.

**Evaluation of NET-1601 activity on intramuscular LDH activity and serum lactate levels in mice during EST**

LDH, an enzyme that catalyzes formation of lactate from pyruvate in an anaerobic state, is a key factor in muscle fatigue. LDH enzymatic activity increases upon high-intensity exercise. During strenuous exercise, excess amounts of pyruvate are produced to facilitate formation of lactate and there is an increase in LDH activity, which catalyzes conversion of pyruvate into lactate (Gibson and Edwards, 1985). Our results show that after exhaustive swimming LDH activity was 3,420±275 U/L for control mice, which is higher than the mean activity in normal mice (1,488±202 U/L). Compared to the control, both NET-1601 treatment groups exhibited significantly lower LDH activity [NET-1601 50 mg/kg: 2,348±172 U/L ($P$< 0.05); NET-160 1,200 mg/kg: 1,863±199 U/L ($P$<0.05)], with dose-dependent differences observed between groups (Fig. 4A).

During strenuous exercise, when oxygen supply does not meet the amount of oxygen consumed in muscle, the level of lactate in increases. Accumulation of lactate leads to acidification, which causes fatigue (Allen et al., 2008). The results from this study show that levels of lactate in serum significantly decreased following treatment with NET-1601 at 50 mg/kg (5.27±0.54 mM/L, $P$<0.05) and 200 mg/kg (4.25±0.58 mM/L, $P$<0.05) compared with the control (6.42±0.78 mM/L). The dose-dependent response observed between groups was statistically significant (Fig. 4B). These results are consistent with the exhaustive swimming times, further supporting that myokinetics impact lactate levels and that NET-1601 decreases LDH activity and reduces accumulation of lactate and fatigue, thereby improving the limit of exercise.

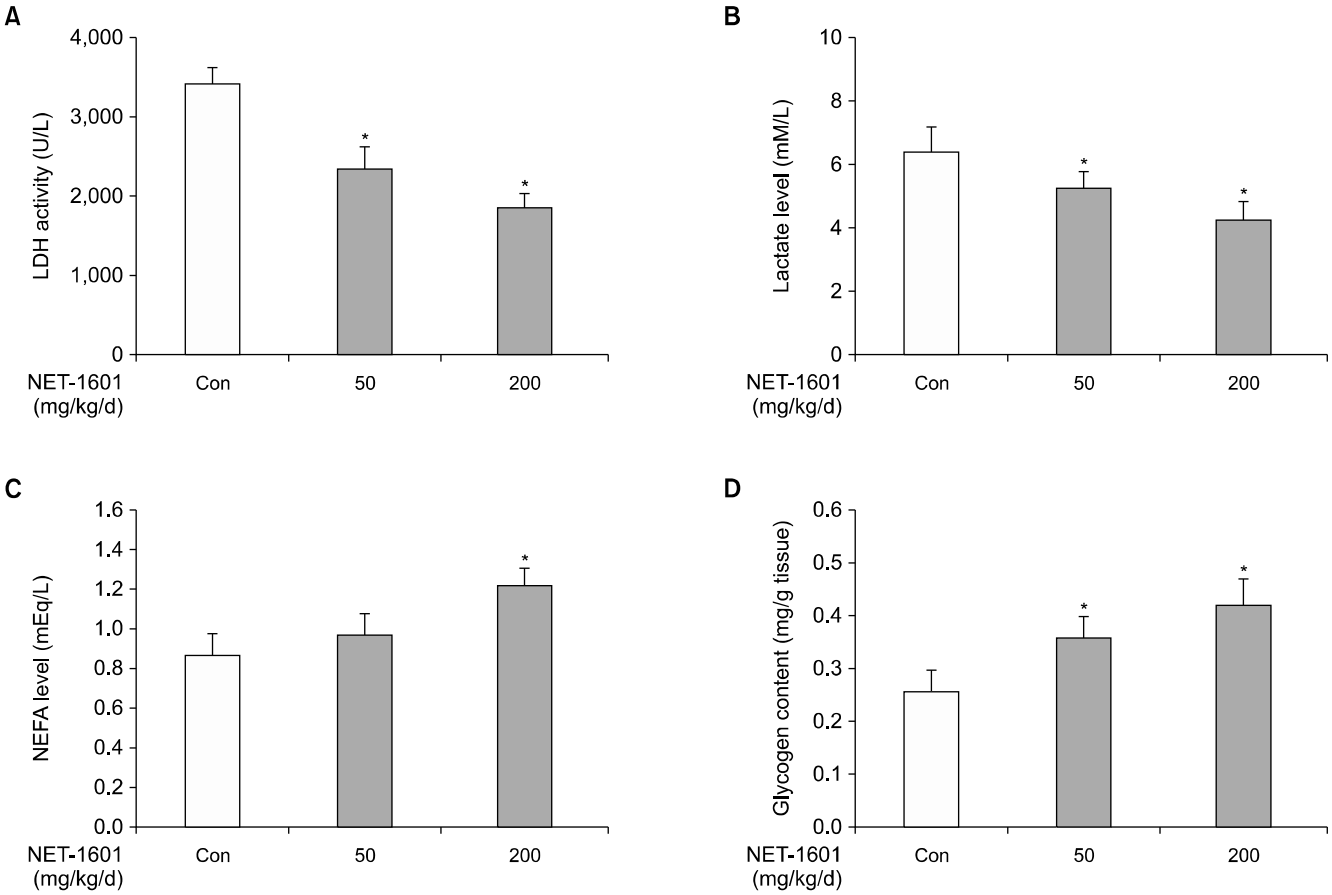


**Fig. 4.** Effect of NET-1601 on (A) serum lactate dehydrogenase (LDH) activity, (B) lactate, and (C) non-esterified fatty acids (NEFA) levels in exhaustive swimming test mice. (D) Intramuscular glycogen content in mice. Data are presented as the mean±SD for 10 mice in each group. $^{*}P<0.05$ compared with the control by Bonferroni test following one-way ANOVA.

**Evaluation of NET-1601 activity on serum NEFA levels and intramuscular glycogen contents in mice during EST**

NEFA account for 5% of total fatty acids; however, fast conversion allows NEFA to serve as a critical energy source in peripheral tissues. In muscle tissue, inadequate levels of energy and reduced intramuscular glycogen are aided by energy from NEFA to maintain muscular activity (van Hall, 2015). Our results showed that treatment with 200 mg/kg NET-1601 significantly increased NEFA levels compared with control mince (1.22±0.09 mEq/L compared with 0.87±0.11 mEq/L, $P<0.05$) (Fig. 4C).

Glycogen in muscle is degraded upon strenuous exercise to generate energy, which leads to a trend of decreased intramuscular glycogen. Therefore, enhancing exercise performance plays an important role in improving fatigue by increasing the glycogen content in muscle (Ørtenblad et al., 2013). The results of the current study indicate similarities between intramuscular glycogen and NEFA contents, with a significant increase in the glycogen content in all NET-1601 treatment groups compared with the control [50 mg/kg NET-1601: 0.36±0.04 mg/g ($P<0.05$); 200 mg/kg NET-1601: 0.42±0.05 mg/g ($P<0.05$); control: 0.26±0.04 mg/g] (Fig. 4D). Therefore, NET-1601 treatment was shown to both increase intramuscular glycogen content and help promote muscular activities through supplying serum NEFA in response to inadequate levels of energy resulting from glycogen depletion.

**Evaluation of NET-1601 activity on expression of regulatory factors involved in recovery from fatigue in muscle tissue during EST**

CPT1 and HADH help regulate fatty acid β-oxidation during mitochondrial transport. The transcription factor PPAR-δ regulates enzymes involved in fatty acid oxidation and transport in muscle tissue, and the transcription factor UCP3 is involved in energy metabolism in muscle tissue while inhibiting accumulation of ROS (Samec et al., 1999; Tanaka et al., 2003; Xia et al., 2019). In this study, both NET-1601 treatment groups (50 mg/kg and 200 mg/kg) showed significantly higher mRNA expression of PPAR-δ, HADH, and UCP3 compared with the control ($P<0.05$). Significant changes were also observed for different doses of NET-1601. For example, while expression of CPT1 did not significantly differ upon administration of NET-1601 50 mg/kg compared with the control, we observed a concentration-dependent trend, with significantly higher expression observed for the 200 mg/

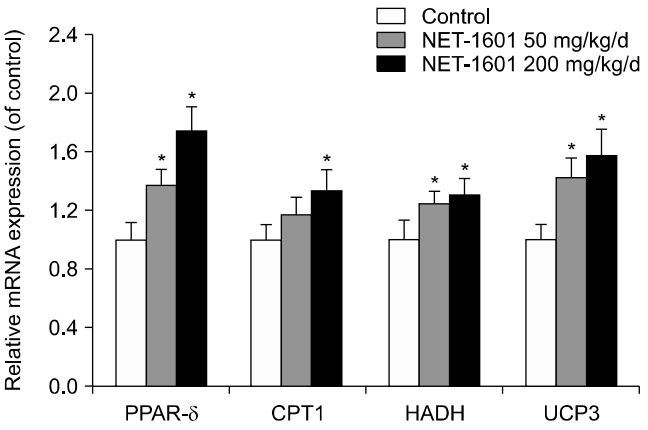


**Fig. 5.** Genetic expression of peroxisome proliferator-activated receptor-delta (PPAR-δ), carnitine palmitoyltransferase-1 (CPT1), β-hydroxyacyl coenzymes A dehydrogenase (HADH), and uncoupling protein-3 (UCP3) with NET-1601 supplementation in exhaustive swimming test mice muscle. Data are presented as the mean±SD for 10 mice in each group. *$P$<0.05 compared with the control by Bonferroni test following one-way ANOVA.

kg treatment group ($P$<0.05) (Fig. 5). Therefore, NET-1601 treatment increased expression of the regulatory factors CPT1, HADH, PPAR-δ, and UCP3, which are involved in recovery from fatigue by increasing fatty acid β-oxidation in mitochondria, thereby reducing fatigue and impacting exercise performance.

### Evaluation of NET-1601 on antioxidant enzyme activity and MDA in liver tissue during EST

The liver is key organ affected by exercise. During strenuous exercise, hepatic blood flow decreases, whereas metabolism and ROS generation increase help to restore hepatic blood flow (Bejma et al., 2000; Heinonen et al., 2014). An increase in ROS can cause oxidative stress, resulting in physical fatigue. Therefore, antioxidant enzymes in the liver play an important role in strenuous exercise-induced fatigue (Sun et al., 2010). SOD is an antioxidant enzyme that catalyzes the conversion of superoxide into oxygen and hydrogen peroxide, and plays a role in removal of free radicals from the blood. Whereas CAT is an enzyme that catalyzes degradation of hydrogen peroxide into oxygen and water (Gibson and Edwards, 1985), and GST is an enzyme that protects cells against oxidative damage (Raza, 2011). Furthermore, GSH is a crucial intracellular substance with non-enzymatic antioxidant activity, which functions to detoxify radicals at the final stage of antioxidant activity (Finsterer, 2012).

Significant increases in both CAT and SOD activity were observed following treatment with NET-1601 compared with the control ($P$<0.05). In addition, there was a significant dose-dependent response of SOD between treatment groups. For GST, absolute activity was much greater than that of CAT or SOD, and was significantly increased in the NET-1601 treatment groups compared with the control ($P$<0.05), with a significant dose-dependent response observed between treatment groups. Furthermore, GSH content in the liver tissue was significantly changed from the control in the 200 mg/kg NET-1601 group only ($P$<0.05) (Table 2).

MDA is an oxide used as an indicator of tissue damage. An increase in MDA occurs due to oxidative stress upon strenuous exercise damages tissues (Jackson et al., 2016). The results from this study show that, compared with the control (6.91±0.35 mole/g), a treatment with NET-1601 significantly decreased MDA content [50 mg/kg NET-1601: 5.67±0.21 mole/g ($P$<0.05); 200 mg/kg NET-1601: 4.57±0.09 mole/g ($P$<0.05)] (Table 2). Based on these results, treatment with NET-1601 both promoted antioxidant enzyme activity and alleviated tissue damage. These findings suggest that when fatigue is induced by increased ROS during strenuous exercise, administration of NET-1601 is likely to have a positive effect on recovery from fatigue by enhancing antioxidant enzyme activity.

In this study, we used cell-based assays to explore the cytoprotective effect of NET-1601 against $H_2O_2$-induced oxidative stress by measuring the recovery of ATP. These findings may be attributed to the inhibitory effect of NET-1601 on ROS generation, which suppresses oxidative stress. In this study, the EST model identifies transcription factors, lactate and NEFA in the blood, all of which are involved in recovery from fatigue and inhibition of energy and glycogen consumption in muscle tissue. Following exhaustive swimming, the maximum swimming times for mice in the NET-1601 treatment groups were increased compared with the control. Furthermore, fatigue-related biomarkers demonstrated positive dose-dependent effects of NET-1601. It is conceiva-

**Table 2.** Effect of NET-1601 on CAT, SOD, GST, and GSH activities and MDA levels in mice liver

| Group (mg/kg/d) | CAT (U/mg protein) | SOD (U/mg protein) | GST (U/mg protein) | GSH (μmoles/mg protein) | MDA (moles/mg protein) |
|---|---|---|---|---|---|
| Control | 15.28±1.21 | 14.12±0.94 | 38.41±3.03 | 12.79±1.22 | 6.91±0.35 |
| NET-1601 50 | 18.95±1.37* | 18.62±1.25* | 44.81±2.79* | 13.30±1.57 | 5.67±0.21* |
| NET-1601 200 | 27.23±1.14* | 24.85±2.01* | 51.33±3.77* | 16.21±2.14* | 4.57±0.09* |

Data are presented as mean±SD for 10 mice in each group.
*$P$<0.05 compared with the control by Bonferroni test following one-way ANOVA.
CAT, catalase; SOD, superoxide dismutase; GST, glutathione-*S*-transferase; GSH, glutathione; MDA, malondialdehyde.

ble that the levels of lactate (a fatigue-inducing factor) and LDH activity (a related factor) were reduced by rapid supplementation of the energy required for muscular activity via oxidation of glycogen and fatty acids. Furthermore, the NET-1601 treatment groups exhibited powerful antioxidant activities in the liver, thereby indicating NET-1601 induces an environment that promotes muscle tissue function.

Oxidative stress is associated with the various symptoms of menopause in women, including heart disease, osteoporosis, and vasomotor disturbances. Some antioxidants such as vitamins C and E are known to help women in the perimenopausal and postmenopausal phases. Thus, supplementation with antioxidants may improve symptoms of menopause (Doshi and Agarwal, 2013). In addition, we are currently developing NET-1601 as a functional healthy food ingredient that functions to alleviate symptoms of menopausal; clinical trials (IRB No. KUMC 2019-07-033-001, 2019AN0423) are ongoing. The findings from this study demonstrate that the potential benefits of NET-1601 are based on its antioxidant activity against $H_2O_2$-induced oxidative stress (*in vitro*) and exercise-induced physical fatigue (*in vivo*).

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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