

Nigella sativa seed extract attenuates the fatigue induced by exhaustive swimming in rats

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Abstract. In previous studies, *Nigella sativa* (NS) has been studied due to its various physiological and pharmacological activities. However, evidence on the effects of NS on physical fatigue following exhaustive swimming remains limited. In the present study, the authors evaluated the potential beneficial effects of NS against the fatigue activity following exhaustive swimming. Rats were orally administered with NS extract (2 g/kg/day) for 21 days, and the anti-fatigue effect was assessed by exhaustive swimming exercise. The presented results indicated that pre-treatment of NS extract significantly increased the time to exhaustion. In hemodynamic parameters, NS extract increased blood pO_2 and O_{2sat} , but decreased pCO_2 . For underlying mechanisms, NS extract protected depletion of energy, indicated by increased levels of blood pH, glucose and tissue glycogen contents, and decreased levels of blood lactate, tissue lactic dehydrogenase and creatine kinase, when the NS extract was pre-treated. In addition, the NS extract inhibited oxidative stress following exhaustive swimming, as reflected by the results of increased levels of superoxide dismutase and redox ratio, and decreased the level of malondialdehyde when the NS extract was pre-treated. Collectively, the present study demonstrated that NS extract has an anti-fatigue activity against exhaustive swimming by energy restoration and oxidative-stress defense.

Introduction

Nigella sativa (NS) seed, commonly known as a black cumin, belongs to the Ranunculaceae family (1). It is an herbaceous plant, which is composed of several constituents, including moisture, oil, proteins, carbohydrates, vitamins and minerals (2,3). Among these, fixed oils and unsaturated fatty acids comprise 30 and 85% of the ingredients in NS seed (2,4). In addition, NS seed contains various chemical compounds with pharmacological properties, including thymoquinone, nigellone, melanthin, damascenone, p-cymene and pinene (5-7). NS seed has been traditionally used as a folk medicine in North Africa, Southeast Asia and Mediterranean countries for many centuries. Indeed, it is used for the treatment of asthma, bronchitis, cough, fever and headaches (8). Previous studies have demonstrated that NS seed has numerous therapeutic activities, such as anti-inflammatory (9), antioxidant (5,10), immune-modulatory (11), cardioprotective (12,13) and hepatoprotective effects (14). However, the effects of NS seed on the fatigue following exercise have not yet been fully elucidated.

Fatigue is defined as a feeling of extreme physical or mental tiredness, resulting from severe stress and hard physical or mental work (15,16). It may be associated with many activities, such as exercise, aging, tumor growth, multiple sclerosis and Parkinson's disease (17-19). In particular, physical fatigue induced by strenuous exercise is thought to lead to a deterioration in performance, causing a decrease in muscular power and endurance, as well as in mental functions (20,21). The underlying mechanism suggests that strenuous exercise accumulates metabolic products (e.g., reactive oxygen species (ROS), lipid peroxides and lactic acid), and leads to oxidative stress, which can potentially contribute to fatigue (22,23).

Accordingly, many studies have focused on the development of drugs or therapies with respect to fatigue (24,25). In addition, considering the limitations of available therapies for fatigue in modern medicine, potential alternatives from traditional medicine are worth investing because of their safety, availability, and ease of administration (26). In the current study, the authors sought to investigate the anti-fatigue activities of NS seed extract in rats subjected to the exhaustive swimming test as a fatigue model.

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Materials and methods

Preparation of NS extract. NS seeds were obtained from a local market in Mymensingh, Bangladesh. The extraction of NS seeds was performed as previously described (27). Briefly, the black seeds were washed with running tap water, air-dried and ground to powder form (0.2–0.3 mm particle size, to ensure homogeneity). A total of 800 g powdered seeds were extracted in a Soxhlet apparatus with petroleum ether. The extract obtained was evaporated to a viscous liquid at 40°C under reduced pressure (yield of 300 ml, 310.7 g and 39.2%). Gas chromatographic analysis of the final extract failed to identify significant amounts of *n*-hexane and *n*-heptane (0.1%), which are the major indicators for this distillate.

Animal models and NS seed extract administration. All experimental protocols employed herein were approved by the Committee on the Care of Laboratory Animal Resources of Chonbuk National University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Bethesda, MA, USA; NIH Publication no. 85-23, revised 1996). A total of forty male Sprague-Dawley rats (220–250 g, Samtako Bio Korea Co., Ltd., Daejeon, Korea) were used for all experiments. Animals were housed in cages maintained at 23±2°C with 50±5% humidity and subjected to a 12-h light/dark cycle. Food and water was available *ad libitum* prior to exercise. The NS extract was freshly dissolved in either distilled water and orally administered using a gavage with a dose of 2 g/kg/day. In addition, NS extract was administered on the 21st day, 1 h prior to the initiation of swimming exercise. Distilled water was administered for control group.

Exhaustive swimming. A pool for exhaustive swimming was designed especially for rats. The system consisted of a glass chamber (70 cm in height, 60 cm in length and 90 cm in width) filled with water to a height of 55 cm. The pool was equipped with a heating system and an air pumping system. To prevent floating during swimming, water bubbles were produced by tubes connected to the air pumping system. The temperature of the water within the glass chamber was maintained at 36±1°C via a thermostatically controlled heater located at the base of the chamber. The rats were individually applied to forced-swimming until exhaustion, which was defined as failure to rise to the surface of the water to breathe for 7 sec (28).

Analysis of blood and serum biochemical parameters. Blood was collected from the tail vein just before swimming and from the caudal vena cava following swimming. A Nova Stat Profile® pHox Ultra Analyzer system (Nova Biomedical, Waltham, MA, USA) was used to measure pH and to quantify levels of partial pressure of carbon dioxide ($p\text{CO}_2$), the partial pressure of oxygen ($p\text{O}_2$) and oxygen saturation ($\text{O}_{2\text{sat}}$). In addition, the concentrations of glucose, lactate and hemoglobin (Hb) were measured, along with hematocrit (Hct). Serum was immediately separated by centrifugation at 1,500 x *g* for 10 min following swimming and stored at -80°C until biochemical analysis. A Hitachi 7020 system (Hitachi Corporation, Tokyo, Japan) was used for analyses of total protein (TP), albumin, lactic dehydrogenase (LDH) and creatine kinase (CK) levels.

Analysis of glycogen contents in liver and gastrocnemius muscle (g. muscle) tissues. The liver and g. muscle were cut, weighed and homogenized in cold perchloric acid. The homogenate was centrifuged for 15 min at 15,000 x *g* at 4°C. The supernatant was carefully decanted. A standard glycogen (Sigma; St.Louis, MO, USA) and tissue extract were mixed with iodine-potassium iodide reagent for binding iodine to glycogen. The mixtures were measured by the SpectraMax M3 ELISA reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 460 nm wavelength.

Analysis of antioxidant defense. Levels of malondialdehyde (MDA) in serum, liver and g. muscle were measured with an OXI-TEK TBARS assay kit (Enzo Life Sciences Inc., Farmingdale, NY, USA). Reaction products were quantitated by measuring the absorbance at 532 nm according to the manufacturer's protocol. The levels of superoxide dismutase (SOD) in the serum, liver and g. muscle were quantitated using a SOD activity kit (cat. no. ADI-900-157, Enzo Life Sciences Inc.) by measuring the absorbance of the reaction products at 450 nm. The total glutathione (tGSH) and oxidized glutathione (GSSG) levels were measured using a glutathione (total) detection kit (cat. no. ADI-900-160) from Enzo Life Sciences Inc. by measuring the absorbance of the reaction products at 405 nm. The concentration of reduced glutathione (GSH) was calculated using the formula $\text{GSH} = \text{tGSH} - \text{GSSG}$, which was provided in the kit protocol. The redox ratio was calculated using the formula $\text{GSH}:\text{GSSG} = (\text{tGSH} - 2\text{GSSG})/\text{GSSG}$, as described previously (29).

Statistical analysis. All data are reported as mean ± standard error of the mean. Statistical significance was analyzed using the Student's *t* test or, where applicable, two-way analysis of variance with Bonferroni post-hoc analysis for multiple group comparisons using GraphPad Prism software (version, 5.03; GraphPad Software Inc., La Jolla, CA, USA), and $P < 0.05$ was considered statistically significant.

Results

Effects of NS extract on the exhaustive swimming on rats. To evaluate the effect of NS seed extract on exercise durability, the exhaustive swimming test was performed using rats, which were pre-treated with either distilled water as a control or NS extracts for 21 days. As demonstrated in Fig. 1, the swimming time to exhaustion was significantly increased in the NS-treated group compared with control group ($P < 0.001$; 24.6% increase vs. control). These data indicated that NS seed extract may prolong the swimming time in rats.

Effects of NS seed extract on hemodynamic parameters following exhaustive swimming. Exhaustive swimming resulted in significant decreases of pH ($P < 0.001$), $p\text{O}_2$ ($P < 0.001$) and $\text{O}_{2\text{sat}}$ levels ($P < 0.001$), but an increase of $p\text{CO}_2$ level ($P < 0.001$), when compared with the pre-swimming control group. However, these changes were significantly attenuated by pre-treatment with NS seed extract compared with the post-swimming control group ($P < 0.01$). Hb and Hct levels were slightly increased ($P > 0.05$) following swimming in the control group. Notably, in pre-swimming and post-swimming

Table I. Effects of NS extract on blood biochemical parameters after forced-swimming.

	Control		NS	
	Pre-swimming	Post-swimming	Pre-swimming	Post-swimming
pH	7.30±0.03	7.09±0.03 ^c	7.30±0.02	7.13±0.03 ^e
Hb (g/dl)	12.5±0.5	13.0±0.4 ^a	15.5±0.4 ^d	15.9±0.4
Hct (%)	42±1	43±1 ^a	45±1 ^d	46±1
pCO ₂ (mmHg)	44.8±2.5	78.7±4.9 ^c	42.0±2.4	68.2±3.0 ^e
pO ₂ (mmHg)	58.6±3.0	24.7±1.7 ^c	67.9±2.9 ^a	37.9±2.78 ^e
O _{2sat} (%)	81.6±2.2	33.0±4.9 ^c	85.3±3.5	46.4±3.9 ^e
TP (g/dl)	5.0±0.2	6.4±0.3 ^b	4.7±0.2	5.6±0.3 ^d
Albumin (g/dl)	2.9±0.2	3.1±0.2	2.6±0.1	3.0±0.1

The data are reported as the mean ± standard error of the mean (n=10). Significance was measured via two-way analysis of variance. ^aP<0.05, ^bP<0.01 and ^cP<0.001 vs. pre-swimming control group; ^dP<0.05, ^eP<0.01 and ^fP<0.001 vs. pre-swimming NS-treated group. Control, control group; NS, NS-treated group; Hb, hemoglobin; Hct, hematocrit; pCO₂, partial pressure of carbon dioxide; pO₂, partial pressure of oxygen; O_{2sat}, oxygen saturation; TP, total protein.

of NS-pre-treated groups, these values had a tendency to increase compared with pre-swimming-control group, even though values in post-swimming group with pre-treatment of NS extract were not significantly changed compared with post-swimming control group (P>0.05; Table I). Finally, TP was markedly elevated in both control and NS-pre-treated groups following exhaustive swimming, when compared with pre-swimming control group. Moreover, there was no obvious difference in albumin levels between control and NS-treated groups following exhaustive swimming (Table I). These data indicated that NS seed extract could maintain the blood homeostasis following exhaustive swimming.

Effects of NS seed extract on fatigue-related serum biomarkers after exhaustive swimming. Fatigue can be evaluated by several important biochemical indicators, including glucose, lactate, LDH and CK. Exhaustive swimming caused depleted glucose, as an energy source, and the accumulation of lactate, LDH and CK (30). Thus, the authors examined the levels of fatigue-related blood biomarkers to test the anti-fatigue effect of NS seed extract. The exhaustive swimming led to a significant decrease in serum glucose level (from 127 to 103.2 mg/dl following exercise; P<0.001) but significant increases in lactate (3.5 to 5.5 mmol/l; P<0.001), LDH (202.4 to 811.6 IU/l; P<0.001) and CK (405.6 to 1288.4 IU/l; P<0.001) levels (Fig. 2) compared with the pre-swimming-control group. However, pre-treatment with NS extract significantly protected these exercise-induced alterations after exhaustive swimming (Fig. 2). The serum glucose level was significantly increased in the NS-treated group compared with control group following exhaustive swimming (P<0.01). Conversely, increased levels of serum lactate, LDH and CK, due to exhaustive swimming, were significantly attenuated by pre-treatment of NS extract (lactate, P<0.05; LDH and CK, P<0.001; Fig. 2). Taken together, the authors also demonstrated the anti-fatigue effects of NS extract on exhaustive swimming from the analysis of typical fatigue-related indicators (e.g., glucose, lactate, LDH and CK).

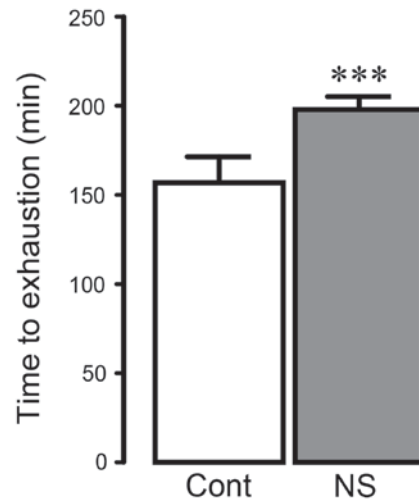


Figure 1. Effect of NS extract on the exhaustive swimming on rats. Time to exhaustion was measured in rats pre-treated with the distilled water as a control or NS extract. Data are expressed as the means ± standard error of the mean (n=10 per group). Significance was measured via paired Student's t-test. ***P<0.001. NS, *Nigella sativa*; Cont, control group; NS, NS-treated group.

Effect of NS extract on glycogen contents in liver and muscle. Glycogen content, which is main storage form of glucose, is an integral determining factor in fatigue following exhaustive swimming. Many studies have reported that glycogen is significantly depleted in both liver and muscles during exhaustive swimming (31). In the present study, following exhaustive swimming, glycogen levels in liver and g. muscle were significantly diminished compared with pre-swimming-control group (21.5 and 11.5% decreases in liver and g. muscle vs. control; P<0.001, respectively; Fig. 3). However, in NS extract-treated group, glycogen content was restored in both liver and g. muscle (39.4 and 10.2% increases in liver and g. muscle vs. swimming-control; P<0.001, respectively). In particular, the liver glycogen in NS-pre-treated-swimming group was significantly increased compared with control group (9.5% increase vs. control; P<0.05; Fig. 3A). These results demonstrated that

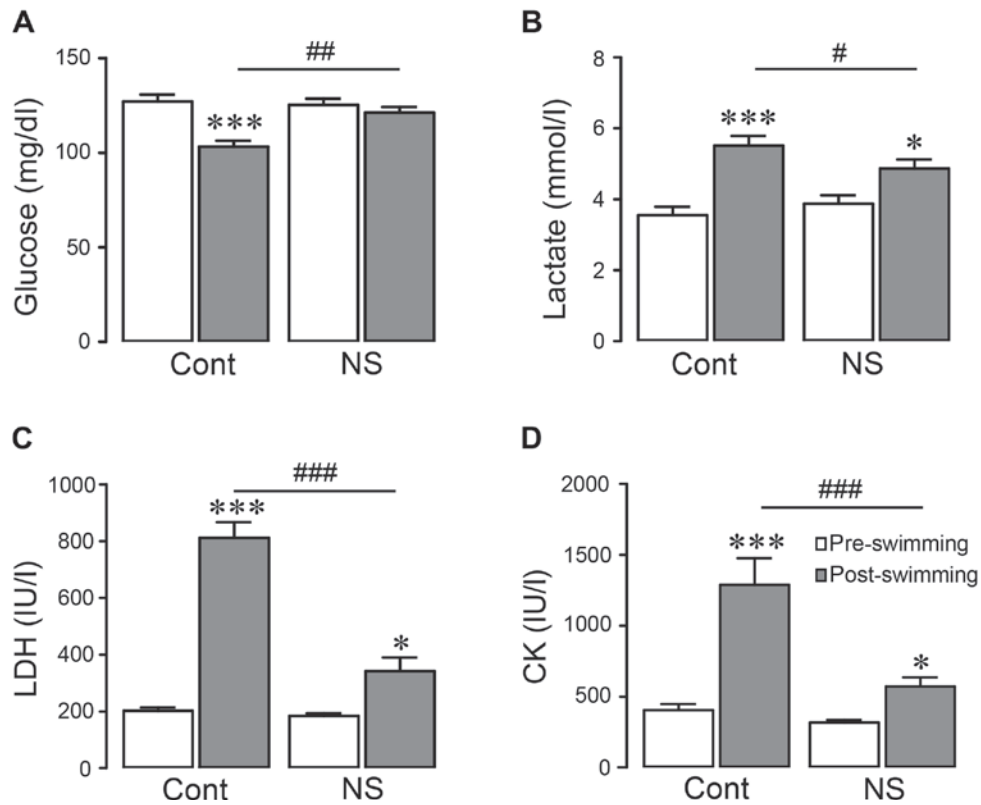


Figure 2. Effects of NS extract on serum biomarkers related to fatigue. Serum levels of (A) glucose, (B) lactate, (C) LDH and (D) CK were measured following the collection of blood just prior to and following swimming. The Cont group were treated with distilled water, while the experimental group was treated with NS extract. Data are expressed as the means \pm standard error of the mean (n=10 per group). *P<0.05, **P<0.01 and ***P<0.001, measured via Bonferroni post hoc test following two-way analysis of variance vs. the pre-swimming Cont; #P<0.05, ##P<0.01 and ###P<0.001, measured via Student's t-test between post-swimming groups. NS, *Nigella sativa*; LDH, lactate dehydrogenase; CK, creatine kinase; Cont, control group; NS, NS-treated group.

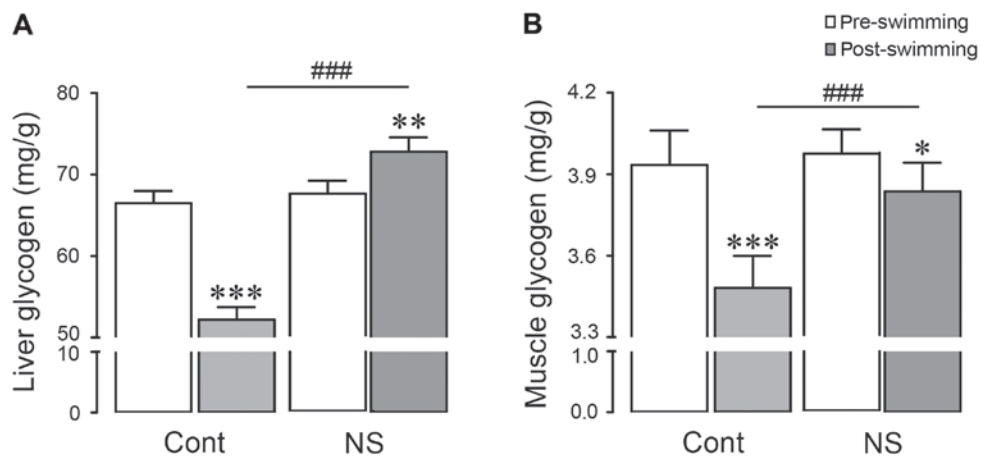


Figure 3. Effect of NS extract on glycogen contents in liver and muscle. Glycogen levels in (A) liver and (B) g. muscle were measured by enzyme-linked immunosorbent assay following the collection of liver and g. muscle tissues just prior to swimming or after swimming. Tissues were treated with distilled water (control) or NS extract. Data are expressed as the means \pm standard error of the mean (n=10 per group). *P<0.05, **P<0.01 and ***P<0.001, measured via Bonferroni post hoc test following two-way analysis of variance vs. the pre-swimming Cont; #P<0.05, ##P<0.01 and ###P<0.001, measured via Student's t-test between post-swimming groups. NS, *Nigella sativa*; g. muscle, gastrocnemius muscle; Cont., control group; NS, NS-treated group.

NS extract administration may reserve glycogen in liver and g. muscle.

Effects of NS seed extract on antioxidant parameters in serum, liver and g. muscle. Oxidative stress occurs following exhaustive swimming, and subsequently may lead to pathology and clinical symptoms of fatigue (32). Therefore, the authors

investigated the antioxidant activity of NS extract following exhaustive swimming by examining the SOD, MDA and serum redox ratio (GSH/GSSG) values as typical oxidative stress related parameters. Serum SOD level was significantly decreased in the swimming control-group, when compared with the control group (4.9% decrease vs. control; P<0.05). As expected, in the NS extract-pre-treated group, serum SOD

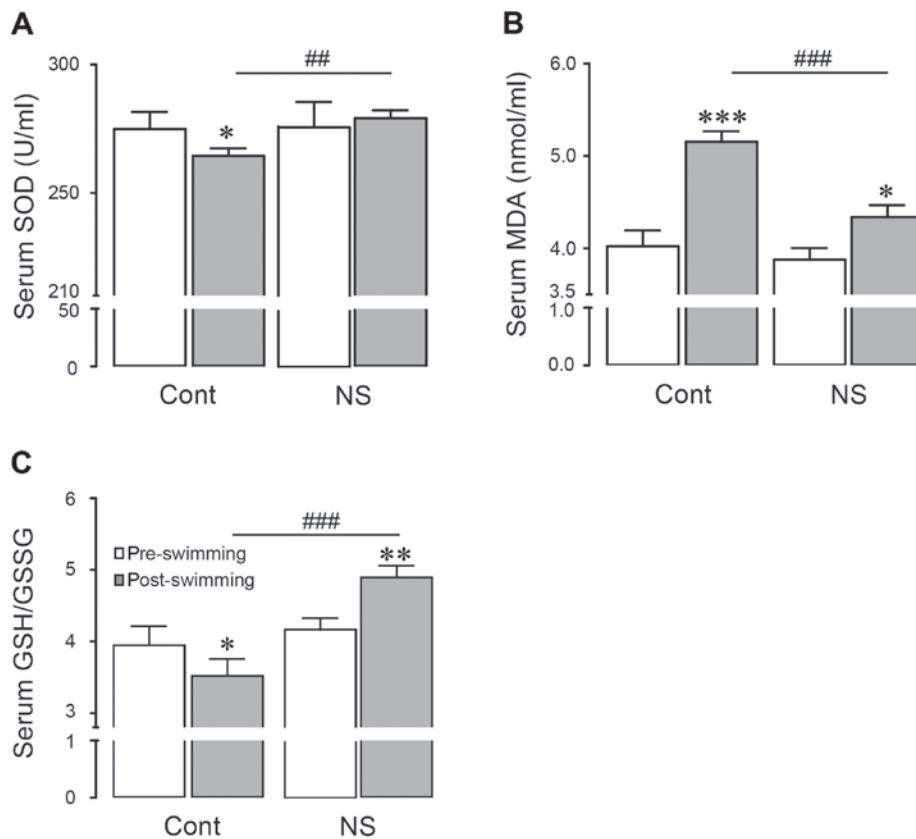


Figure 4. Effects of NS extract on serum antioxidant parameters. Serum levels of (A) SOD and (B) MDA were measured by enzyme-linked immunosorbent assay after collecting blood just prior to swimming or following swimming. Tissues were treated with distilled water (control) or NS extract. (C) The redox ratio (GSH/GSSG) was calculated following measuring serum GSH and GSSG. Data are expressed as the means \pm standard error of the mean (n=10 per group). *P<0.05, **P<0.01 and ***P<0.001, measured via Bonferroni post hoc test following two-way analysis of variance vs. the pre-swimming Cont.; #P<0.05, ##P<0.01 and ###P<0.001, measured via Student's t-test between post-swimming groups. NS, *Nigella sativa*; SOD, superoxide dismutase; MDA, malondialdehyde; GSH, reduced glutathione; GSSG, oxidized glutathione; Cont, control group; NS, NS-treated group.

level was significantly increased compared with the swimming control-group following exhaustive swimming (5.8% increase vs. swimming-control; P<0.01; Fig. 4A). Serum MDA level was dramatically increased following exhaustive swimming (28.1% increase vs. control; P<0.001). However, pre-treatment with NS extract inhibited the serum MDA level following exhaustive swimming (15.8% decrease vs. swimming-control; P<0.001; Fig. 4B). Similarly, the serum redox ratio (GSH:GSSG) was significantly decreased in the swimming-control group, when compared with the control group (10.8% decrease vs. control; P<0.05), but was increased in the exercise-NS extract-treated group (39.1% increase vs. swimming-control; P<0.001; Fig. 4C).

In addition, SOD levels in liver and g. muscle were significantly decreased compared with the control group following exhaustive swimming (21.9 and 6.0% decreases in liver and g. muscle vs. control; P<0.001, respectively; Fig. 5A and C). Notably, SOD levels in both tissues were significantly increased by pre-treatment of NS extract compared with the swimming-control-group (50.5 and 8.0% increases in liver and g. muscle vs. swimming-control; P<0.001, respectively; Fig. 5A and C). MDA levels in liver and g. muscle were significantly increased by pre-treatment of NS extract compared with the control group following exhaustive swimming (58.7 and 36.9% increases, in liver and g. muscle vs. control; P<0.001, respectively). However, when the NS extract was

pre-treated, MDA levels in both tissues were significantly inhibited compared with exercise-control group (30.4 and 14.2% decreases in liver and g. muscle vs. swimming-control; P<0.001, respectively; Fig. 5B and D). These data suggested that NS extract may have a beneficial role against oxidative stress to alleviate physical fatigue following exercise.

Discussion

NS seeds possess many pharmacological activities, and are especially well known for their potent antioxidant effects. Many previous studies have reported that NS seeds may reduce toxicity in a number of diseases including diabetes, neural disease, renal disease, cardiovascular disease and cancer, due to their antioxidant activities from both *in vitro* and *in vivo* approaches (6,33-36). Therefore, in the present study, the authors focused on determining the protective effects of NS seeds against exhaustive swimming-induced fatigue.

To evaluate the anti-fatigue effects of NS extract in rats, the authors performed the exhaustive swimming, which has been commonly used as a fatigue model (37). The results demonstrated that pre-treatment of NS extract significantly increased the swimming time to exhaustion (Fig. 1). Further, in hemodynamic parameters, NS extract may attenuate decreased values of pO_2 and O_{2sat} and decrease values of pCO_2 , resulting in impaired oxygen supply during fatigue (Table I). Thus, the

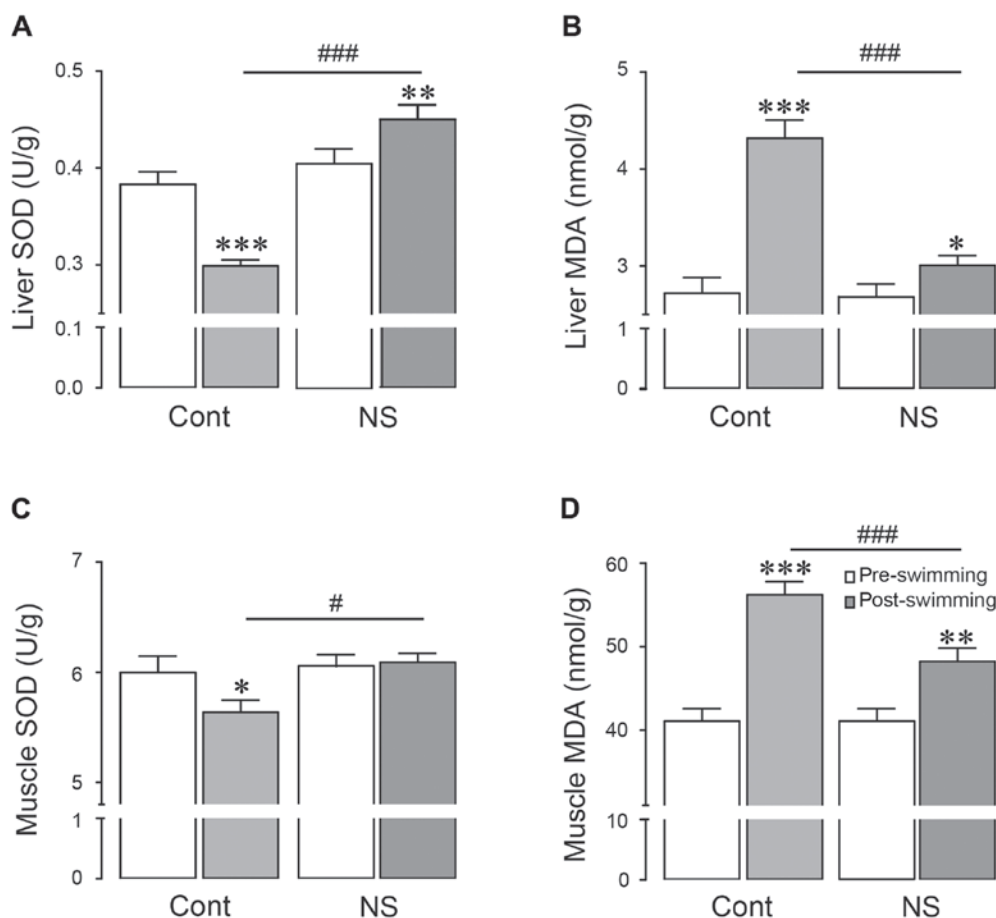


Figure 5. Effects of NS extract on antioxidant parameters in the liver and g. muscle tissues. (A and C) SOD and (B and D) MDA levels were measured by enzyme-linked immunosorbent assay following the collection of liver and g. muscle tissues just before swimming or after swimming. Tissues were treated with distilled water (control) or NS extract. Data are expressed as the means \pm standard error of the mean (n=10 per group). * P <0.05, ** P <0.01 and *** P <0.001, measured via Bonferroni post hoc test following two-way analysis of variance vs. the pre-swimming cont.; # P <0.05, ## P <0.01 and ### P <0.001, measured via Student's t-test between post-swimming groups. NS, *Nigella sativa*; SOD, superoxide dismutase; MDA, malondialdehyde; Cont, control group; NS, NS-treated group.

presented results indicated that NS extract may improve the swimming capacity against the fatigue.

Main causes of fatigue after exhaustive swimming are the depletion of energy sources and dysregulation of anti-oxidant defenses system (30). Regarding the depletion of energy sources, many studies have been demonstrated that energy sources, such as glucose and glycogen are depleted during exhaustive swimming, which in turn cause to physical fatigue (38). Here, it was observed that glycogen contents in both the liver and g. muscle were restored upon administration of NS extract (Figs. 2A and 5). In addition, under normal conditions, ATP, as an energy source, is produced by glycolysis (conversion of glycogen into glucose), which is, in turn, broken down into pyruvate (39). However, the muscles obtain energy from anaerobic glycolysis (conversion of pyruvate to lactate) during intense exercise. This intense exercise leads to accumulation of lactate, and lactate then drops the blood and muscle pH, and consequently, fatigue is occurred (40). The results demonstrated that levels of serum glucose, lactate and LDH (key enzyme for lactate production) were increased following exhaustive swimming. Notably, these increases were dramatically attenuated when NS extract pre-treated (Fig. 2B-D). In addition, it was demonstrated that glycogen contents in liver and g muscle tissues were dramatically

preserved when pre-treated NS extract following exhaustive swimming (Fig. 3). Therefore, the authors suggested that NS extracts may contribute to fatigue retardation through preservation of glycogen content and reduction of lactate accumulation.

Secondly, the other important factor in fatigue is dysregulation of the antioxidant defense system (30). Exhaustive swimming may release reactive oxygen species (ROS) due to increased oxygen consumption, resulting in the development of fatigue. Therefore, MDA (an oxidative degradation product of cell membrane lipids) and SOD (major component of anti-oxidant defense system) are considered to be physiological markers relevant for fatigue. In the present study, the results revealed that elevated MDA levels and decreased levels of SOD, as well as the serum redox ratio (GSH:GSSG) in liver and g. muscle tissues were completely reversed by pre-treatment of NS extract (Fig. 4). These findings suggested that anti-fatigue effect of NS extract is involved in the modulation of oxidative stress following exhaustive swimming.

In conclusion, the current study demonstrated that NS may be able to alleviate the physical fatigue by inhibiting energy depletion and oxidative stress. The authors further propose that NS may be a potential strategy for prevention and treatment of physical fatigue.

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