

## Effects of unaccustomed downhill running on muscle damage, oxidative stress, and leukocyte apoptosis

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Kyung-Shin Park and Man-Gyoon Lee. Effects of unaccustomed downhill running on muscle damage, oxidative stress, and leukocyte apoptosis. *JENB.*, Vol. 19, No. 2, pp.55-63, 2015 **[Purpose]** The purpose of this study was to investigate the effect of unaccustomed downhill running on muscle damage, oxidative stress, and leukocyte apoptosis. **[Methods]** Thirteen moderately trained male subjects performed three 40 min treadmill runs at ~70%  $VO_{2max}$  on separate days: a level run (L) followed by two downhill runs (DH1 and DH2). Blood samples were taken at rest (PRE) and immediately (POST), 2 h, 24 h, and 48 h after each run. Data were analyzed using 2-way repeated measures ANOVA with post hoc Tukey tests. **[Results]** Creatine kinase (CK) activity and oxidative stress level were significantly elevated at 24 h and 48 h following DH1 ( $P < 0.05$ ). The level of oxidative stress at the POST measurement following DH1 and DH2 was greater than PRE. The rate of leukocyte apoptosis was significantly increased at the POST measurement following all three runs, and remained elevated for up to 48 h following DH1 ( $P < 0.01$ ). **[Conclusion]** CK activity and oxidative stress were elevated following an acute bout of moderate intensity downhill running, resulting in a greater apoptotic response at 24 h and 48 h post-exercise in comparison with level grade running or a second downhill run. These elevations were blunted following DH2. Although the link between exercise-induced muscle damage and leukocyte apoptosis is currently unknown, the differential response to DH1 vs. L and DH2 indicates that it may be mediated by the elevation of oxidative stress. **[Key words]** Muscle damage, oxidative stress, apoptosis, eccentric exercise.

### INTRODUCTION

Exercise is a form of physiological stress that has a marked effect on the immune system. An increase in the number of immune cells is observed with the performance of high intensity exercise [1-2]. When exercise ends, leukocyte subsets show nonsynchronous behavior. Lymphocytes decrease rapidly to a level well below the pre-exercise value, whereas granulocytes can remain increased for several hours after exercise [3-4]. This decline in lymphocyte count may last from several hours up to days and may, at least in part, account for post-exercise immune suppression [5-6]. The majority of studies investigated the effects of exercise on lymphocyte apoptosis and proposed exercise intensity and duration as two determinants of lymphocyte apoptosis [7-10]. It was reported that high intensity or exhaustive exercise can elicit increases in muscle damage [11-12] and oxidative stress [13-14], which

consequently induce leukocyte apoptosis in blood [15-16].

Repetitive eccentric muscle contractions overstretch and disrupt sarcomeres, which can spread to adjacent areas including the sarcoplasmic reticulum. This cascade activates proteolytic pathways related to muscle fiber degradation, with a consequent increase in muscle damage [17]. The symptoms of exercise-induced muscle damage includes delayed onset muscle soreness, muscle malfunctioning, and increases in muscle proteins such as creatine kinase and troponin in the circulation [18]. Unaccustomed downhill running is known to induce more muscle damage compared to uphill or level runs [6,19,20]. A causative role for eccentric exercise-induced muscle damage is ascribed to the mechanical stress that occurs when muscles lengthen and generate break force upon foot strike to maintain balance against gravity [6,11]. Even though exercise-induced muscle damage is known to be associated with an increase in leukocyte apoptosis in peripheral blood,

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the mechanism that connects exercise-induced muscle damage with changes in circulating immune cells is not clear.

It has been suggested that acute eccentric exercise may produce free radicals not only by an enhanced rate of oxygen utilization but also by anaerobic metabolism and secondary inflammation due to the phagocytic activity of immune cells [14,21,22]. Activated neutrophils and macrophages infiltrate damaged muscle, which may be beneficial to muscle repair [23,24]; however, it may also trigger further inflammatory processes and additional muscle damage, in part through an enhanced formation of uncontrolled free radical reactivity in the vicinity of phagocytic activity [25-26]. Additionally, lysosomes can store the free radicals produced in muscle cells and later release them into the blood [27]. These reactive species increase oxidative stress and subsequently disrupt the mitochondrial transmembrane potential and DNA structure [2,28]. It is well known that exercise-induced apoptosis in leukocytes mainly contributes to an increase in reactive oxygen or nitrogen species following vigorous exercise [14, 18,28]. However, no studies have investigated whether an elevation of oxidative stress from free radical production can mediate exercise-induced muscle damage and an increased rate of leukocyte apoptosis in circulating blood.

The primary purpose of this study was to determine the effect of an acute bout of moderately high eccentric exercise on the induction of muscle damage and leukocyte apoptosis in peripheral blood. Secondly, we measured changes in oxygen radical absorbance capacity in the blood to investigate whether an elevation of oxidative stress in the blood serves as a mediator between exercise-induced muscle damage and leukocyte apoptosis. We hypothesized that the elevation of leukocyte apoptosis following an initial downhill run would be sustained for a longer period of time compared to elevation following a second downhill or running at level grade due to different degrees of muscle damage following each run. Moreover, we hypothesized that apoptosis would occur via the mitochondrial pathway due to increased oxidative stress following the unaccustomed downhill run.

## METHODS

### *Subjects*

Subjects were apparently healthy, moderately trained males between the ages of 21-32 yrs. Participants who were non-smokers, not taking medication that would alter metabolic, cardiovascular or immune functions, and had no musculoskeletal limitations conducted a  $VO_{2max}$  screening test. The

subjects whose  $VO_{2max}$  categorized them as moderately trained (49-55 ml·kg<sup>-1</sup>·min<sup>-1</sup>, 70-90 percentile in ACSM's guideline) were selected for this study [29]. All documents and procedures were approved by the institutional IRB (protocol# 050400 1901) and participants provided written informed consent.

### *Pre-testing*

Subjects completed a physical activity readiness questionnaire, and performed a  $VO_{2max}$  test to determine eligibility for this study.  $VO_{2max}$  was measured during a continuous, progressive, treadmill running protocol. Initial speed was 3.5 mph at 0% grade with increases of 0.5 mph every two min up to 7.5 mph after which the grade was increased by 2% every two min without any change in speed. Maximal  $VO_2$  was assumed if subjects attained at least two of the following criteria: volitional fatigue, an increase in workload with little or no increase in heart rate and  $VO_2$ , and RER greater than 1.15.

### *Study design*

On separate occasions and at least one week following  $VO_{2max}$  testing, eligible subjects performed three 40 min running bouts on a motorized treadmill at a moderately high intensity (~70%  $VO_{2max}$ ): once on a level grade (L) and twice at -10% downhill grade (DH1 and DH2). Level running was performed first, DH1 occurred two weeks after L, and DH2 occurred three weeks following DH1 to minimize any effects from previous running trials.

Participants were asked to refrain from any strenuous exercise for two days prior to the exercise tests. For each subject, all three running trials were scheduled at the same time of day and day of the week. Before running, each subject rested in a chair for 20 min after which a blood sample was obtained (PRE) from an antecubital vein. Blood samples were obtained four additional times following each run, i.e., immediately postexercise (POST), and at 2 h, 24 h, and 48 h of recovery. Metabolic variables were measured during exercise by indirect calorimetry using an automated gas analysis system (Parvo Medics TrueMax 2400; Salt Lake City, UT) that was calibrated prior to each testing session.

### *Sample preparation*

#### *Blood sampling and preparation*

Ten ml of blood was collected at each sampling period to measure serum creatine kinase (CK) activity, level of oxygen radical absorbance capacity (ORAC), and rate of leukocyte apoptosis (apoptotic index : AI). Five ml of whole blood in

plain tubes was centrifuged at  $1000 \times g$  for 15 min (Allegra X-15R Refrigerated Centrifuge, Beckman Coulter, Irving, TX, USA) and serum was stored at  $-80^{\circ}\text{C}$  until further analysis for CK and ORAC.

#### *Leukocyte isolation*

Leukocytes were isolated using an ammonium chloride lysing technique. Two ml of whole blood was mixed with 48 ml of 1X ammonium chloride lysing reagent in a 50 ml centrifuge tube. Each tube was set on a rocker for 10 min at room temperature and then centrifuged for 10 min at  $400 \times g$ . After decanting, the supernatant samples were washed twice for 5 min at  $200 \times g$ . After cell counting, each sample was mixed with two ml Tri Reagent (Sigma, St. Louis, MO, USA).

Cell fixation for TUNEL assay: Leukocytes in two ml Tri reagent was mixed with 10 ml of 1 % paraformaldehyde in PBS (approximately  $1-2 \times 10^6$  cells/ml) and placed on ice for 45 min. The cells were centrifuged for 5 min at  $300 \times g$ , and the supernatant was discarded. The cells were washed in 5 ml PBS for 5 min at  $300 \times g$  and resuspended in the residual PBS after discarding the supernatant. Washing and centrifugation were repeated twice. The cell concentration was adjusted to  $1-2 \times 10^6$  cells/ml in 70% (v/v) ice cold ethanol (approximately 5 ml), the samples were put on ice for another 45 min, then stored at  $-20^{\circ}\text{C}$  until used.

#### *Measurements*

##### *Serum creatine kinase activity*

Serum CK activity was determined using a colorimetric enzymatic assay with a creatine kinase reagent set (Pointe Scientific Inc. Lincoln Park, MI) according to the manufacturer's instructions. One ml CK reagent was incubated for 5 min at  $37^{\circ}\text{C}$  in a spectrophotometer equipped with a temperature controlled cuvette (Perkin-Elmer Lambda, Wellesley, MA). The sample (25  $\mu\text{l}$ ) was added and mixed by inversion, then incubated for 2 min at a constant temperature ( $37^{\circ}\text{C}$ ). Absorbance was recorded at 340 nm at 1 min intervals for a period of 2 min. Change in absorbance per minute ( $\Delta A$ ) was used to determine CK activity.

##### *Oxygen radical absorbance capacity (ORAC)*

Total antioxidant activity indicated by ORAC in the serum was measured as previously described [30]. The assay utilizes the free radical sensitive fluorescent indicator, phycoerythrin, to monitor the effectiveness of various serum antioxidants protecting the beta phycoerythrin from becoming damaged by free radicals. To make 0.075 M potassium phosphate buffer,

10.3 g  $\text{NaH}_2\text{PO}_4$  and 13.06 g  $\text{K}_2\text{HPO}_4$  were added to 1 L of distilled water. One mg of  $\beta$ -phycoerythrin protein (Sigma, St. Louis, MO, USA) was diluted into 25.25 ml distilled water. Then, 0.0026 g of Trolox was mixed with 100 ml distilled water (0.1mM), heated and stirred until the powder was completely dissolved. Afterward, 0.162 g of Azobis was mixed with 10 ml of distilled water. To test ORAC in the serum samples, 200  $\mu\text{l}$  of potassium phosphate buffer was first added to all wells followed by the addition of 25  $\mu\text{l}$  of fresh  $\beta$ -phycoerythrin. Ten  $\mu\text{l}$  of diluted serum sample (1:100 dilution) was added to each well and 25  $\mu\text{l}$  of Azobis was added to all wells right before plate reading. For the blank and control, the following mix was added to each well: blank (buffer only), Trolox control 1 (10  $\mu\text{l}$  of Trolox), and Trolox control 2 (5  $\mu\text{l}$  of Trolox). The optical density of each well was determined immediately using a microplate reader (Synergy<sup>TM</sup> HT multi-detection microplate reader, Biotek, Vermont) and KC version 5.0 software. The wave length for excitation and emission were set to 540nm and 565nm. Absorbance was measured every 5 min for 1 h.

##### *Apoptosis: TUNEL assay technique*

Terminal deoxynucleotidyltransferase was used to incorporate fluorescein-12-dUTP with nuclei to assess the amount of DNA fragmentation as an index of leukocyte apoptosis, following the manufacturer's protocol (APO-BRDUTM Kit, PharMingen, San Diego, CA). The rate of apoptosis (apoptotic index: AI) was analyzed using flow cytometry and calculated as the number of leukocytes in the high fluorescence intensity population divided by the total number of leukocytes counted [9]. Briefly, about 1ml of fixed leukocytes in 70 % ethanol (approximately  $1 \times 10^6$  cells) was placed in  $12 \times 75$  mm flow cytometry centrifuge tubes and centrifuged for five minutes at  $300 \times g$ . After removing ethanol by aspiration, the leukocytes were resuspended in 1ml of wash buffer and centrifuged for 5 min at  $300 \times g$  followed by supernatant aspiration. Wash buffer treatment was repeated twice. Leukocytes were resuspended in 50  $\mu\text{l}$  of DNA Labeling Solution and incubated in a temperature controlled bath ( $37^{\circ}\text{C}$ ) for 60 min. Each tube was shaken for resuspension every 15 min. After incubation, leukocytes were rinsed twice with one ml of rinse buffer (provided with the kit) with centrifugation for 5 min at  $300 \times g$ . The cell pellet was resuspended in 100  $\mu\text{l}$  of the antibody solution and incubated in the dark for 30 min at room temperature. Then, 500  $\mu\text{l}$  of PBS was added after incubation. Leukocyte apoptosis was analyzed within three hours of staining using flow cytometry (Beckman, Fullerton, CA).

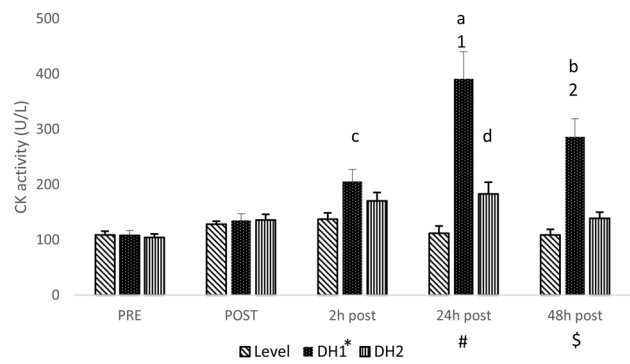
### Statistical analysis

A power analysis indicated that eleven subjects were needed to detect a 8.5% change in lymphocyte apoptosis with 90% power at  $\alpha = .05$ . The operating characteristic curve [31] was used to calculate power from the data reported by Mooren *et al.* [10]. Data are presented as the mean  $\pm$  SE. All statistical analyses were conducted using SPSS 22 (SPSS inc., Chicago, USA). Two-way analysis of variance (ANOVA) with repeated measures was used to analyze changes in CK activity, ORAC, and leukocyte apoptosis. Post hoc tests using Tukey *post hoc* test were performed when appropriate. Statistical significance was accepted for all tests at  $P < 0.05$ .

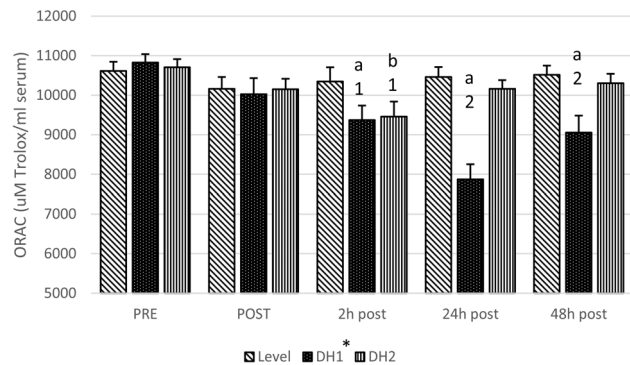
## RESULTS

A total of fifteen subjects participated in this study and thirteen of them completed all three running trials. Two participants dropped out after the first downhill run due to complaints of muscle soreness (data not included). Their physical characteristics are shown in Table 1.

CK activity was expressed as international units (U/L and, indicates the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute. Results are shown in Fig. 1. CK activity was significantly greater in DH1 than Level and DH2 ( $p = 0.0032$ ). There were also significant time effects: CK activity at 24 h post was significantly greater than PRE, POST, 2 h, and 48 h post ( $p < 0.01$ ), while activity at 48 h post was significantly greater than PRE ( $p < 0.01$ ) and POST ( $p < 0.05$ ). There was a significant interaction effect (trial  $\times$  time). Within DH1, CK activity was significantly higher at 24 h post ( $391 \pm 49$ ,  $p < 0.01$ ) than at PRE ( $108 \pm 9$ ), POST ( $135 \pm 12$ ), 2 h ( $206 \pm 22$ ) and 48 h post ( $286 \pm 33$ ), and was significantly elevated at 48 h post ( $p < 0.05$ ) compared to PRE and POST. CK activity at 2h post was also greater than PRE ( $P = .042$ ). In DH2, CK activity measured



**Fig. 1.** Creatine kinase (CK) activity measured at PRE, POST, 2 h, 24 h and 48 h post in level (L) and downhill running trials at 70%  $VO_{2max}$  (first downhill trial = DH1, second trial = DH2). Values are mean  $\pm$  SE. \* DH1: significantly greater than L and DH2; #, significantly greater than PRE, POST, 2 h, and 48 h post; \$, significantly greater than PRE and POST; Between groups: 1, significantly greater than L and DH2 at 24 h post; 2, significantly greater than L and DH2 at 48 h post; Within group: a, significantly greater than PRE, POST, 2 h and 48 h post in DH1; b, significantly greater than PRE and POST in DH1; c, significantly greater than PRE in DH1; d, significantly greater than PRE in DH2.



**Fig. 2.** Oxygen radical absorbance capacity (ORAC) measured at PRE, POST, 2 h, 24 h and 48 h post in Level (L), and downhill running trials at 70%  $VO_{2max}$  (first downhill trial = DH1, second trial = DH2). Values are mean  $\pm$  SE. \*DH1: significantly lower than Level and DH2; Between groups: 1, significantly lower than L at POST; 2, significantly lower than L and DH2 at 24 h and 48 h post; Within group: a, significantly lower than PRE and POST in DH1; b, significantly lower than PRE in DH2.

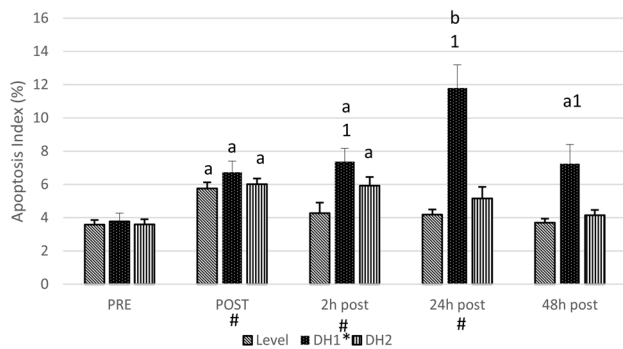
**Table 1.** Physical characteristics of subjects

Variables	All participants (n = 15)	Subjects that completed three runs (n = 13)
Age (year)	24.9 $\pm$ 1.1	24.6 $\pm$ 1.1
Height (cm)	179.1 $\pm$ 1.3	179.3 $\pm$ 1.3
Weight (kg)	71.9 $\pm$ 1.8	71.6 $\pm$ 1.8
% body fat (%)	9.0 $\pm$ 1.2	8.9 $\pm$ 1.2
$VO_{2max}$ (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	53.3 $\pm$ 0.7	53.1 $\pm$ 0.7
Running speed at level grade (km·h <sup>-1</sup> )	10.8 $\pm$ 0.5	10.7 $\pm$ 0.5
Running speed at -10% downhill (km·h <sup>-1</sup> )	15.0 $\pm$ 0.7	14.9 $\pm$ 0.6

Values are mean  $\pm$  SE.

at 24 h post ( $183 \pm 21$ ) was significantly greater compared to PRE ( $104 \pm 7$ ,  $p < 0.027$ ). CK activity following DH1 was significantly greater than following Level and DH2 at 24 h post ( $p < 0.01$ ) and 48 h post ( $p < 0.05$ ).

ORAC was measured to indirectly estimate changes in oxidative stress. One ORAC unit is equal to the net protection afforded by 1  $\mu$ M Trolox per ml serum ( $\mu$ M Trolox/ml serum). Results are displayed in Fig. 2. There were significant trial, time and interaction (trial  $\times$  time) effects. ORAC for the DH1 trial was significantly lower than Level and DH2 ( $p < 0.01$ ). ORAC was significantly reduced at 2h post ( $p = 0.038$ ) and remained low up to 48 h following DH1 (24 h post:  $p =$



**Fig. 3.** Apoptotic index (%) of total leukocytes measured at PRE, POST and 2 h, 24 h and 48 h post in level (L) and downhill running trials at 70%  $VO_{2max}$  (first downhill trial = DH1, second trial = DH2) using TUNEL assay. Values are mean  $\pm$  SE, \* DH1: significantly greater than L and DH2; # significantly greater than PRE; Between groups: 1, significantly greater than L and DH2 at 2 h, 24 h, and 48 h post; Within group: a, significantly greater than PRE in each trial; b, significantly greater than PRE, POST, 2 h, and 48 h post in DH1.

0.0014; 48 h post:  $p = 0.017$ ). ORAC was significantly lower only at 2 h post as compared to PRE following DH2 ( $p = 0.033$ ; time effect). ORAC at 2h post following DH1 ( $9371 \pm 368$ ,  $p = 0.041$ ) and DH2 ( $9458 \pm 380$ ,  $p = 0.046$ ) was significantly lower in comparison with Level ( $10349 \pm 358$ ). At 24 h post, ORAC in DH1 ( $7869 \pm 384$ ) was significantly lower than Level ( $10463 \pm 249$ ,  $p = 0.006$ ) and DH2 ( $10162 \pm 219$ ,  $p = 0.005$ ). At 48 h post, ORAC in DH1 ( $9052 \pm 431$ ) was significantly lower than Level ( $10516 \pm 232$ ,  $p = 0.027$ ) and DH2 ( $10302 \pm 240$ ,  $p = 0.0340$ ).

The AI of total leukocytes was measured by the TUNEL assay using flow cytometry. Results of the analysis are shown in Fig. 3. Significant main effects were found for both trial and time. AI for DH1 was significantly greater than Level and DH2 ( $p < 0.01$ ). POST and 2 h and 24 h post were significantly greater than PRE and 48 h post ( $p < 0.01$ ; time effect). There was also a significant interaction. In the Level trial, AI at POST ( $5.76 \pm 0.4\%$ ) was significantly greater than PRE ( $3.58 \pm 0.3$ ,  $P = 0.006$ ). AI following DH1 was significantly increased above PRE ( $3.78 \pm 0.5$ ) at POST ( $6.74 \pm 0.7$ ,  $p = 0.015$ ), 2 h post ( $7.40 \pm 0.8$ ,  $p = 0.004$ ), 24 h post ( $11.8 \pm 1.4$ ,  $p = 0.001$ ), and 48 h post ( $7.25 \pm 1.16$ ,  $p = 0.031$ ). The peak was recorded at 24 h post and was significantly greater than POST, 2 h and 24 h post. Significant differences were found in DH2: POST ( $6.02 \pm 0.3$   $p = 0.009$ ) and 2 h post ( $5.92 \pm 0.5$ ,  $p = 0.018$ ) were significantly greater than PRE ( $3.60 \pm 0.3$ ). AI obtained at 2 h post ( $p < 0.05$ ), 24h ( $p < 0.01$ ) and 48h post ( $p < 0.05$ ) were significantly higher in DH1 than Level and DH2.

## DISCUSSION

The current study demonstrated that exercise-induced muscle damage, at least in part, accounts for the elevation of leukocyte apoptosis following exercise via an increase in oxidative stress. The majority of apoptosis studies in humans thus far have been focused on elucidating the effects of exercise intensity and duration on lymphocyte apoptosis. A number of researchers have reported a significant increase in lymphocyte apoptosis in peripheral blood following maximal intensity exercise [7,32,33]. Few studies on humans have assessed lymphocyte apoptosis at exercise intensities other than maximal [8,34]. Due to lack of information, it is not possible to identify the percentage of  $VO_{2max}$  needed to elicit a significant increase in cell death with physical activity. The lowest intensity reported to significantly increase the rate of apoptosis in a single bout of exercise was 75%  $VO_{2max}$  for a 2.5 h treadmill run [34]. In addition, other studies that employed a single bout of exercise at 60%  $VO_{2max}$  did not observe an increase in apoptosis [9-10]. The present study was designed to investigate the effects of exercise-induced muscle damage on leukocyte apoptosis in peripheral blood, therefore, 70%  $VO_{2max}$  was selected as the exercise intensity for all three runs to eliminate the influence of exercise intensity on leukocyte apoptosis. Our subjects conducted two downhill runs to investigate whether leukocyte apoptosis is affected by exercise-induced muscle damage or exercise mode (downhill run) itself. From previous studies and the present study, it was concluded that an unaccustomed bout of downhill running at moderate intensity induces significant muscle damage and this muscle damage is attenuated following a second bout of downhill running and does not occur following a run at level grade due to muscle adaptation [20,35].

Exercise-induced muscle damage is frequently experienced following unaccustomed exercise, particularly if the exercise is exhaustive or includes eccentric muscle action [12]. The initial injury is followed by secondary degradation in the 2-5 days following muscle damaging exercise with a consequent increase in the number of damaged fibers [19,36]. The present study demonstrated that an unaccustomed eccentrically-biased exercise (DH1) at a moderately high intensity results in an elevation in CK activity as an index of muscle damage up to 48 h, with a peak at 24 h post, whereas CK activity was unaltered following a level run and was attenuated following a repeated bout of downhill running (DH2). Results of the present study correspond with previous studies. A downhill study conducted with ten well trained male subjects found that CK activity peaked at 24 h post, which was 420% greater than pre-exercise [35]. A similar result was reported in

untrained subjects, in which CK activity was significantly increased up to 48 hour following downhill running at -8 degrees and peaked at 24 h following the run [20]. Although the mechanism that induces muscle damage during downhill running has not yet been elucidated, it was suggested that the mechanical stress that occurs when the muscle lengthens and generates braking forces upon foot strike to maintain balance against gravity might initiate the muscle damage [11].

Several researchers investigated the effect of physical exercise on free radical production and found elevated oxidative stress [2,14,18]. It is known that two to four percent of the total oxygen consumed by the mitochondria undergoes one electron reduction with the production of a superoxide radical, consequently producing hydrogen peroxide [37]. Secondary muscle degradation following a single bout of intense and acute exercise induces oxidative stress in muscle [14]; however, the effect of exercise-induced muscle damage on oxidative stress in circulating blood has not been studied extensively. In the present study, the results of the ORAC assay were a mirror image of CK activity, i.e., ORAC was significantly lower following DH1 as compared to Level and DH2 at 24 h post. Sacheck *et al.* [38] also found that a decrease in ORAC correlated with an increase in CK activity in subjects who ran downhill for 45 min at 75%  $VO_{2max}$ , suggesting that free radical production increases during prolonged or intense exercise. This may induce muscle damage and consequently decrease the number of antioxidant compounds. Close *et al.* [39] reported greater increases in CK activity up to 24 hours and increases in oxidative stress up to 72 hours following downhill running at 65%  $VO_{2max}$  as compared to level running and suggested that exercise-induced muscle damage may increase oxidative stress due to the production of reactive oxygen species, which later initiate damaging mechanism. Studies with eccentric resistive exercise confirmed that an increase in oxidative stress following repeated eccentric contraction is associated with simultaneous increase in indices of muscle damage [21,22,40].

It has been suggested that cellular damage accompanies circulatory oxidative stress in a dose dependent manner [2, 7,14,21]. However, no studies have demonstrated the interaction between cellular damage and oxidative stress in circulation. Results of the present study suggest that exercise-induced muscle damage may increase oxidative stress in the blood through as yet unknown mechanisms. Since infiltrated leukocytes in muscle are unable to return to the blood circulation [23,24], exercise-induced muscle damage and muscle oxidative stress was not believed to directly contribute to an increase in oxidative stress in blood. Knowledge regarding the interaction between circulating and infiltrated skeletal

muscle immune cells may be helpful in understanding increased oxidative stress in blood. It is possible that fragments of damaged cells leak through muscle membrane lesions and rapidly initiate complement activation through an antibody independent manner in circulation [27,41]. Free radicals produced by phagocytosis in muscle may be released into the extracellular space and then into the circulation [27]. Another oxidative stress inducer in blood may be cytokines from muscle. It is known that pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 are released from muscle in response to exercise stress [42,43] or muscle damage [44,45] and increase oxidative stress while inducing cell death in circulating immune cells [46,47].

In the present study, the AI of total leukocytes at 24 h and 48 h following DH1 were significantly greater than L and DH2, which corresponded with a significant increase in CK activity and a decrease in ORAC at 24 h and 48 h following DH1. Although the underlying mechanism of this elevation in leukocyte apoptosis following an unaccustomed downhill run is not known yet, the results of this study indicate that exercise-induced muscle damage via elevating oxidative stress may contribute to an increase in AI via a mitochondrial pathway.

It was reported that a single bout of downhill running elevated the level of CK activity and the rate of apoptosis in both lymphocyte and neutrophils in the blood, with up to 48 h of recovery [6]. The effect was attenuated following a second bout of downhill running and was not observed following level running, indicating that exercise-induced muscle damage at least contributes in part to the elevation of apoptosis in the circulating blood. On the contrary, Simpson *et al.* [48] observed lymphocytopenia following intensive exercises on both flat and -10 % downhill grade surfaces at 80%  $VO_{2max}$  with no evidence of lymphocyte apoptosis and suggested that exercise-induced muscle damage is not associated with the elevation of lymphocyte apoptosis following intensive exercise. However, the rate of apoptosis in this study using Annexin-V was surprisingly low and even the peak value was lower than the resting values in other studies [5,10,28,34], indicating potential problems in detection or blood treatment techniques. Phagocytosis in damaged muscle cells may aggravate the initial mechanical stress injury. Additionally, free radicals produced by phagocytic activity may induce secondary muscle degradation in the days following exercise [2]. Even though there is no direct evidence that oxidative stress induces apoptosis, it was found in *in vitro* studies that a high level of oxidative stress induces apoptosis, and additional antioxidants delay cell death [13,49].

Early elevation in AI appeared immediately following all

three running trials while CK activity and oxidative stress remained unaltered, indicating that there might be other factors responsible for the AI elevation. The results of previous studies suggest that the effect might be induced by the elevation of stress hormones such as catecholamine and glucocorticoids as a function of exercise intensity, with an apparent threshold of about 60%  $\text{VO}_{2\text{max}}$  for the release of epinephrine. The mechanism through which an increase in stress hormones may produce leukocyte apoptosis in circulating blood is not known yet; however, several cell culture studies induced cell apoptosis by adding catecholamines [50, 51] or glucocorticoids [51,52]. Combined with the hormonal response to exercise and the results from the aforementioned *in vitro* studies, it can be postulated that either elevation of epinephrine or glucocorticoids following exercise may contribute to the early elevation of leukocyte apoptosis immediately after exercise regardless of exercise mode.

## CONCLUSIONS

An acute bout of moderate intensity downhill running altered CK activity and the level of oxidative stress in the peripheral blood, resulting in a greater apoptotic response at 24 h and 48 h of recovery compared to level grade running or a second downhill run, indicating that oxidative stress via an increase in free radical production may interact with muscle and circulating leukocytes. The present study did not elucidate the mechanisms through which the early elevation in leukocyte apoptosis immediately after all three runs occur; however, it is suggested that elevated concentrations of stress hormones such as epinephrine and glucocorticoids may be involved in this elevation. Although the mechanism that links exercise-induced muscle damage with increased apoptosis following unaccustomed eccentric exercise is currently unknown, it may be related to oxidative stress. Since very few studies have investigated the effects of exercise-induced muscle damage on immune cell apoptosis in peripheral blood, further study is warranted to investigate the mechanisms that link exercise-induced muscle damage and apoptosis in the circulating blood.

## ACKNOWLEDGMENTS

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