

## Exhaustive Exercise Test and Oxidative Stress Response in Athletic and Sedentary Subjects

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### ABSTRACT

*Exhaustive exercise induces oxidative stress in human with imbalance between free radical generation and antioxidant. The response to exhaustive exercise in human who has irregular or regular exercise (athletics) is very interesting. The aim of this study was to determine the changes of oxidative stress, malondialdehyde (MDA), protein hydroperoxide, glutathione (GSH) and total antioxidant capacity (TAC) in the blood of athletes before and after exhaustive exercise by modified Bruce protocol compared with sedentary group. MDA, protein hydroperoxide and TAC in plasma were detected by TBARs, FOX and ABTS decolorization methods respectively. The GSH in erythrocyte was determined by DTNB. A Mann-Whitney U-test was used for statistical analysis. Before exercise, athletic group showed higher TAC, lower protein hydroperoxide and MDA. After exercise, sedentary and athletic groups showed slight increase of GSH, significant reduction of TAC, MDA and protein hydroperoxide. The changes of all parameters between sedentary and athletic groups were not statistically different except the decrease in TAC after exercise in sedentary group compared with athletic group. This study represented similar response to oxidative stress from exhaustive exercise between sedentary and athletic groups.*

**Key words:** Exhaustive exercise, Glutathione, Malondialdehyde, Protein hydroperoxide, Total antioxidant capacity

### INTRODUCTION

Free radicals are capable of independent existence and are produced in all living cells. Reactive oxygen species (ROS) or reactive nitrogen species (RNS), e.g., superoxide (O<sub>2</sub><sup>•</sup>), hydroxyl (OH<sup>•</sup>), alkoxy (RO<sup>•</sup>), peroxy (ROO<sup>•</sup>), and hydroperoxide (ROOH) can oxidize other biological molecules, including carbohydrates, amino acids, fatty acids and nucleotides. Previous data shows the high level of lipid peroxidation from detection the malondialdehyde (MDA) represented the oxidative stress in the body (Halliwell and Gutteridge, 1999). Scavenging of all free radicals produced *in vivo* by both enzymatic- and non-enzymatic antioxidants usually occurs. Antioxidant enzymes include superoxide dismutase, glutathione peroxidase and catalase. The main non-enzymic antioxidants include glutathione (GSH), vitamin E and vitamin C (Cooper et al., 2002) proposes to total antioxidant capacity (TAC) in the biological system. The potential sources of free radical generation in exercising muscle are mainly from mitochondria, xanthine oxidase, prostanoid metabolism, catecholamines, NAD (P) H oxidase and secondary sources such are phagocytosis or calcium accumulation

(Jackson, 2000). Physical exercise requires adenosine triphosphate (ATP) as an energy source which is primarily produced by oxidative phosphorylation. Re-oxygenation or reperfusion with oxygenated blood results in local and systemic effects which may cause more tissue damage (Tisi and Shearman, 2000). Free radical induces many protein oxidation and MDA products in the blood circulation (Blommer et al., 2005). Protein hydroperoxide (prOOH) is stable and a propagation of radical reactions on other proteins, lipids and DNA (Hawkins and Davies, 2001). In this study, we determined the response of oxidative stress by measurement of GSH, protein hydroperoxide, MDA and TAC between sedentary and athletic groups after exhaustive treadmill exercise.

## MATERIALS AND METHODS

### Materials and chemicals

Glassware were cleaned with warm concentrated nitric acid and thoroughly rinsed with double-distilled water before experiments. Xylenol orange [*o*-cresolsulfonaphthalein-3,3'-bis(sodium methylimino diacetate)], Perchloric acid (AR grade; 70–72%), Guanidine hydrochloride, tert-butyl hydroperoxide, 5,5'-Dithiobis 2-nitrobenzoic acid (DTNB), Thiobarbituric acid (TBA) were from Sigma (St. Louis, MO, USA). Tetramethoxypropane (TMP) and ammonium ferrous sulfate were purchased from Aldrich (USA). All reagents used were at least of reagent grade.

### Subjects

This study was approved by Ethic Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand. Blood before and after exercise tests was collected by using heparin as anticoagulant.

### Blood samples

A total of 40 subjects, 20 control and 20 athletic groups were studied in an exhaustive exercise by modified Bruce protocol with a Series 200 treadmill (Marquette medical systems, Inc. Milwaukee, USA). The testing protocol composed of 10 steps and 3 minutes in each step. The heart rate at 85% of maximum heart rate or rate perception exertion (RPE) at 9 was a final target to stop testing. Blood at pre- and immediately post-exercise test was collected by vein puncture in heparinized tube. Blood was centrifuged at 3000 rpm for 5 min and erythrocyte was separated from plasma. Glutathione in erythrocyte, protein hydroperoxide, malondialdehyde and total antioxidant capacity in plasma were determined by DTNB, FOX, TBARS, and ABTS decolorization methods, respectively.

### The Exercise protocol

The exhaustive exercise was followed from the Modified Bruce protocol and target heart rate (Target MHR) was performed by the recommendation of American College Sport Medicine (ACSM)'s Guidelines (2000).

Step I: 5 minutes for warm up with hip flexion/extension, stretching the quadriceps, hamstrings and gastrocnemius under 35% of maximum heart rate and score of rate perceived exertion (RPE) less than 10.

Step II: Protocol testing as following steps. Each step was performed for 3 min.

- |    |                                |
|----|--------------------------------|
| I  | velocity 1.7 mph with 0% slope |
| II | velocity 1.7 mph with 5% slope |



III	velocity 1.7 mph with 10 % slope
IV	velocity 2.5 mph with 12% slope
V	velocity 3.4 mph with 14% slope
VI	velocity 4.2 mph with 16% slope
VII	velocity 5.0 mph with 18% slope
VIII	velocity 5.5 mph with 20% slope

Step III: 5 minutes for cool down with slow walking on treadmill. The target heart rate was at 85% of maximum heart rate and RPE at 14–15. Any symptom as leg pain, muscle thigh unwilling of subjects were criteria to stop exercise.

### **Estimation of glutathione in erythrocyte**

The glutathione in erythrocyte was determined by using the Dithiobisnitrobenzoic acid (DTNB) reagent (Beutler et al., 1963). 400 µl of erythrocyte was precipitated with 3 ml of precipitating solution (0.2 g EDTA, 1.67g meta-phosphoric acid and 30 g sodium chloride in 100 ml distilled water) and 1.6 ml of distilled water. After filtration with Millipore membrane (0.22 µM) (Millex®-GV, Ireland), 200 µl of clear filtrate was mixed with 400 µl of 0.3 M phosphate buffer (pH. 8) and 400 µl of DTNB solution (40 mg DTNB in 100 ml distilled water containing 1% sodium citrate). Within 5 min absorbance, color development was read at 412 nm. The glutathione concentration was calculated by comparing with standard reduced glutathione (Sigma).

### **Estimation of plasma malondialdehyde**

Malondialdehyde (MDA), the lipid peroxidation product, was determined by modified thiobarbituric acid (TBAR)s. The 100 µl of plasma was precipitated with trichloroacetic acid (TCA) (100%) and mixed with 450 µl of normal saline solution (0.9%), 200 µl of thiobarbituric acid (TBA) solution. The whole mixture was incubated in 90°C water bath for 30 min, then cooled in water. After centrifugation at 6000 rpm for 5 min at 4°C, the absorbance was read at 532 nm. The concentration of malondialdehyde was calculated from the 0–20 µM of standard malondialdehyde (Sigma).

### **Estimation of plasma protein hydroperoxide**

Protein hydroperoxide concentration was estimated by modified Ferrous oxidation-Xylenol orange (FOX) method from Gay (2003). To perform modified FOX assay, 100 µl of sample plasma was taken and protein were precipitated with 500 µl of 0.2 M perchloric acid (PCA) and centrifuged at 6500 rpm for 10 min at 4°C. The protein pellet was redissolved in 1000 µl of 6 M guanidine hydrochloride. The lipid was removed twice by 100 µl of chloroform, containing 2% of Butylated hydroxytoluene (BHT). The protein hydroperoxide in upper layer was mixed with 40 µl of 0.5 M PCA, 25 µl of 5 mM xylenol orange and 5 mM ammonium ferrous sulfate. After keeping at room temperature in the dark for 30 min, the mixture was centrifuged at 10,000 rpm for 3 min at 4°C. The absorbance of the solution was measured at 560 nm. Plasma protein hydroperoxide was calculated by comparing to standard *tert*-butyl hydroperoxide (Sigma) (1–10 µM).

### **Total antioxidant capacity assay**

Total antioxidant capacity (TAC) was assayed by modified 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) decolorization method (Re et al., 1999). ABTS was dissolved in water to a 7 mM final concentration. ABTs radical cation (ABTS<sup>•+</sup>) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) overnight in the dark at room temperature. The starting absor-

bance of ABTS radical cation was approximately  $0.7 \pm 0.02$  at 734 nm by spectrum scanning. The percentage of reduction of the absorbance at 734 nm was calculated against a standard curve of Trolox (0–10 mmol/L).

### Statistical analysis

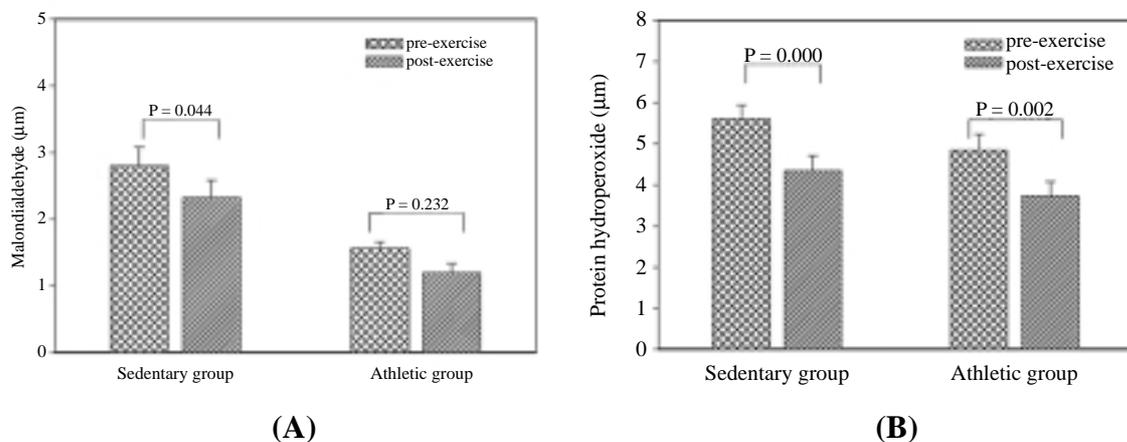
All parameter changes of erythrocyte glutathione, plasma protein hydroperoxide, malondialdehyde and total antioxidant capacity at pre- and post-exercise testing between sedentary and athletic group were compared by a Mann-Whitney U-Test.

## RESULTS

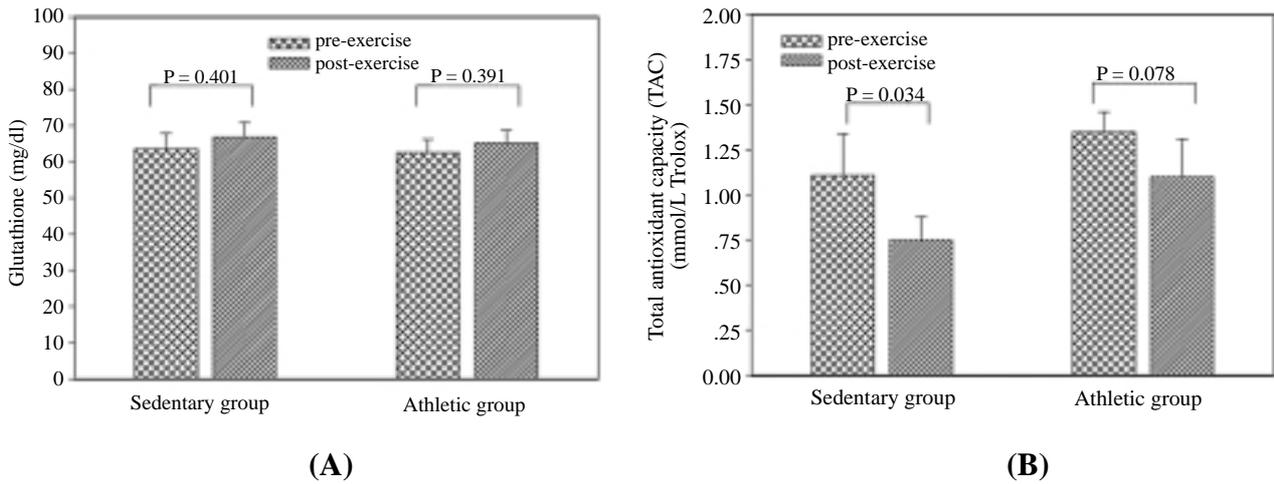
In the present study, 40 subjects; 20 sedentary subjects with mean  $\pm$ SD of age =  $19.75 \pm 1.7$ , range = 18–24 years, Body Mass Index (BMI) =  $20.53 \pm 1.84$  kg/m<sup>2</sup> and 20 athletics with mean  $\pm$ SD of age =  $21.75 \pm 0.34$ , range = 16–25 years, BMI =  $20.64 \pm 2.9$  kg/m<sup>2</sup> were studied. In athletic group, all subjects performed regular exercise of 5–7 days per week for more than three months prior to the study.

Figure 1.A shows the plasma malondialdehyde levels before exhaustive exercise of athletic group ( $1.56 \pm 0.10$   $\mu$ M) and sedentary group ( $2.8 \pm 0.28$   $\mu$ M). After exercise, the level slightly decreased in both athletic group ( $1.2 \pm 0.14$   $\mu$ M) and sedentary group ( $2.32 \pm 0.27$   $\mu$ M) significantly. The protein hydroperoxide before exhaustive exercise of the athletic group ( $4.35 \pm 0.36$   $\mu$ M) and sedentary group ( $5.6 \pm 0.34$   $\mu$ M) are shown in Figure 1.B and the level significantly decreased after exercise in both the athletic ( $3.73 \pm 0.45$   $\mu$ M) and sedentary group ( $4.84 \pm 0.62$   $\mu$ M).

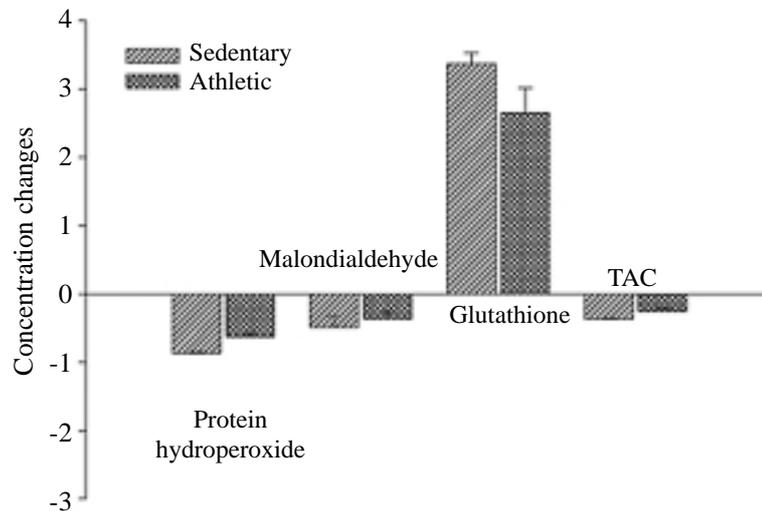
Average red blood cell glutathione levels before exercise in the athletic group was  $62.56 \pm 3.63$  mg/dl) and sedentary group was  $63.51 \pm 4.33$  mg/dl and after exercise, they slightly increased both in athletic group ( $65.17 \pm 3.66$  mg/dl) and sedentary group ( $66.88 \pm 3.93$  mg/dl) as shown in Fig 2.A. Figure 2.B represents the total antioxidant capacity (TAC) in sedentary ( $1.11 \pm 0.23$  mmol equivalent Trolox/L) and athletic group ( $1.35 \pm 0.11$  mmol equivalent Trolox/L) before the exercise and after exhaustive exercise, the TAC in sedentary ( $0.71 \pm 0.13$  mmol equivalent Trolox/L) decreases more than in athletic group ( $0.10 \pm 0.21$  mmol equivalent Trolox/L).



**Figure 1.** The level of malondialdehyde (A) and protein hydroperoxide (B) at pre- and post-exercise of 20 sedentary and 20 athletic subjects.



**Figure 2.** The level of glutathione (A) and total antioxidant capacity (TAC) (B) at pre- and post-exercise of 20 sedentary and 20 athletic groups.



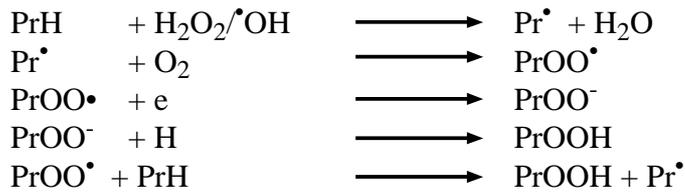
**Figure 3.** Changes in concentrations of malondialdehyde ( $\mu\text{M}$ ), protein hydroperoxide ( $\mu\text{M}$ ), glutathione (mg/dl erythrocyte) and total antioxidant capacity (mmol equivalent Trolox/L) in sedentary and athletic groups.

Figure 3 summarize the changes of all parameters after exercise in both groups. Protein hydroperoxide in sedentary group changed less than in athletic group ( $-0.62 \mu\text{M}$  vs  $-0.86 \mu\text{M}$ ) as well as malondialdehyde ( $-0.48 \mu\text{M}$  vs  $-0.36 \mu\text{M}$ ). The increase of glutathione in athletic group was less than in sedentary group ( $2.65 \text{ mg/dl}$  vs  $3.32$ ). The net changes of total antioxidant capacity in athletic group were smaller than in sedentary group ( $-0.25 \text{ mmol equivalent Trolox/L}$  vs  $-0.36 \text{ mmol equivalent Trolox/L}$ ).

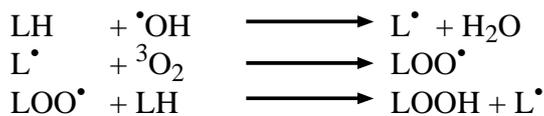
### DISCUSSION

Exercise-induced changes in levels of antioxidants had been studied but their significance to oxidative stress was hardly determined. While oxidative stress could cause a primary decrease in antioxidants, mobilization from secondary source could occur. The initial reaction of protein hydroperoxide is activating protein (PrH) with various oxidants  $\text{H}_2\text{O}_2$  or  $\cdot\text{OH}$  in biological system. The carbon-centered free radical ( $\text{Pr}\cdot$ ) produced will be

oxidized to form protein peroxy radical (PrOO<sup>•</sup>) and finally to protein hydroperoxide (PrOOH) (Gebicki, 1997).

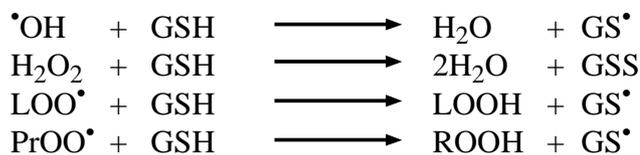


Malondialdehyde (MDA) that is the end product of lipid oxidation is also formed after oxidation of lipid due to peroxy radicals, hydrogen peroxide or non-radical species (Girotti, 1998).



Although, the results of this study showed slightly reduced of MDA in both groups but is not difference between before or after exercise testing that is similar to the previous study in 30 minutes of aerobic and anaerobic exercise in trained men (Bloommer et al., 2005).

Oxidative stress in human erythrocyte is controlled by glutathione- independent and -dependent systems. (Ogus et al., 1998). Total thiol protein in erythrocyte, 80–85% of the reactive thiols belong to hemoglobin, 10–15% glutathione and the remaining 5% in the form of membrane proteins and other thiol compounds (Morell et al., 1964). The GSH is the major thiol redox system in the erythrocyte which controls free radicals or non-radicals in the circulation such as hydroxyl radical (<sup>•</sup>OH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), lipid hydroperoxide (LOOH) or protein hydroperoxide (PrOOH) (Jones., 2002).



This study found the higher statistically significant difference of total antioxidant capacity (TAC) in athletic group compared to sedentary group. Previous data showed an advantage of endurance training in athletes by increasing the antioxidant enzymes, as catalase (CAT), glutathione peroxidase (GRX), superoxide dismutase (SOD), including the total glutathione, vitamin E, and C (Miyazaki et al., 2001). This study, however, also represented opposite results to the report (Aslan et al., 1997) that after exercise, the level of glutathione increased while malondialdehyde, protein hydroperoxide and total antioxidant capacity decreased. The possible explanation of this response was that exhaustive exercise in this study induced the antioxidant system by enhancing the glutathione in order to scavenge the lipid and protein hydroperoxide in the body and this result supported the previous data of Cooper and co-workers (2002). That report indicated that the GSH level in erythrocyte increased for 24 hr after exercise whereas the total antioxidant capacity that reflects other antioxidants as vitamin E, C, uric acid, including glutathione, decreased after exercise. However, this study showed various differences between athletic group and sedentary group in changing of biochemical parameters i.e, MDA, PrOOH and TAC which were less in athletic group than in sedentary group while GSH did not change.

This study concluded that in athletes who have regular exercise showed the benefit of exercise by gaining a higher total antioxidant capacity, lower oxidation on protein and lipid and moreover, against the free radicals from exhaustive exercise better than in non-exercised people.

### ACKNOWLEDGEMENTS

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