นิพนธ์ต้นฉบับ

การตอบสนองภาวะออกซิเดทีฟสเตรสจากการออกกำลังกายอย่างหนักในปัสสาวะและเลือดของคนปกติ: การศึกษานำร่อง

นัฏฐกาล ลือสารุ่งระยับ*, จิตตรัตน์ ตันเสนีย์*, ประภาส โพธิ์ทองสุนันท์*, นันทยา ชนะรัตน์**

บทคัดย่อ

การศึกษาครั้งนี้สนใจถึงผลกระทบจากการออกกำลังกายอย่างหนักในคนปกติต่อการกระตุ้นการออกซิเดชันของเหล็กและกระบวนการออกซิเดชันของไขมันที่เกิดขึ้นในเลือดและในปัสสาวะ โดยให้อาสาสมัครที่มีสุขภาพดี จำนวน 15 คน ออกกำลังกายอย่างหนักตามโปรแกรมการออกกำลังกายที่ได้ประยุกต์จากโปรแกรมของ Bruce ทำการเจาะเลือดก่อนและหลังการออกกำลังกายทันที ปัสสาวะได้ถูกเก็บก่อนและหลังหยุดออกกำลังกายทันทีและนาทีที่ 20 นำเลือดและปัสสาวะมาทำการตรวจวัดปริมาณของมาลอนไดออลดีไฮด์ และการเร่งปฏิกิริยาออกซิเดชันของเหล็กด้วยวิธี TBARs และ FOX. นำผลที่ได้มาวิเคราะห์ทางสถิติโดยใช้ Wilcoxon Signed Rank Test และ Related Paired t-test ผลการศึกษาพบว่าการออกกำลังกายอย่างหนัก สามารถเพิ่มปฏิกิริยาการเกิดออกซิเดชันของเหล็กทั้งในเลือดและในปัสสาวะที่นาทีที่ 20 อย่างมีนัยสำคัญ ส่วนปริมาณของมาลอนไดออลดีไฮด์ในเลือดเพิ่มขึ้นเพียงเล็กน้อยและไม่แตกต่างกันทางสถิติ แต่ในปัสสาวะพบว่ามีค่าเพิ่มขึ้นอย่างมีนัยสำคัญในนาทีที่ 20 เมื่อเปรียบเทียบกับก่อนออกกำลังกาย การศึกษาครั้งนี้แสดงให้เห็นว่าการตรวจวัดภาวะออกซิเดทีฟสเตรสจากการออกกำลังกายอย่างหนักสามารถตรวจวัดได้ทั้งในเลือดและในปัสสาวะ สามารถนำมาใช้ในงานวิจัยด้านวิทยาศาสตร์การกีฬาในอนาคตได้ต่อไป **วารสารเทคนิคการแพทย์เชียงใหม่ 2549; 39: 2-9.**

คำสำคัญ: การออกกำลังกายอย่างหนัก ภาวะออกซิเดทีฟสเตรส มาลอนไดออลดีไฮด์ ปฏิกิริยาออกซิเดชันของเหล็ก

---

* กลุ่มวิจัย Clinical Biochemistry and Movement Sciences Research Group. ภาควิชากายภาพบำบัด,

** ภาควิชาเคมีคลินิก คณะเทคนิคการแพทย์ มหาวิทยาลัยเชียงใหม่

Abstract : Oxidative Stress Response to Exhaustive Exercise in Urine and Blood of Healthy Subjects : Preliminary Study

Nuttakaan leelarugnrayub*, Jittarat Tansenee*, Prapas Pothongsunun*, Nantaya Chanarat**

This study aimed to determine the effects of exhaustive exercise in sedentary subjects on the activation of the ferrous oxidation and lipid peroxidation in the blood and urine. Exhaustive exercise program was followed by a modified Bruce protoco in 15 healthy subjects. Blood and urine were collected at pre and post exercise immediately. Only urine was additionally collected at 20 minutes post exercise. Malondialdehyde and Ferrous oxidation were determined by Ferrous oxidation xylenol-orange (FOX) and TBARs assays. Data wre statistically analysed by using a Wilcoxon Signed Rank test and related paired t-test. The results showed that exhaustive exercise increased significantly the ferrous oxidation in blood and urine at 20 minutes after stop exercise immediately. Whereas lipid peroxidation slightly increased. In urine, the malondialdehyde increased significantly at 20 minutes after stop exercise. Therefore, this study showed the evaluation of the oxidative stress from exhaustive exercise would be analyzed from the blood and urine which could be applied to sport sciences researches in the future. **Bull Chiang Mai Assoc Med Sci 2006; 39: 2-9.**

Key words: Exhaustive exercise, Oxidative Stress, Malondialdehye, Ferrous Oxidation.

* Clinical Biochemistry and Movement Sciences Research Group. Departments of Physical Therapy and
** Clinical Chemistry, Faculty of Associated Medical Sciences, Chiang Mai University.

Introduction

Nowadays, many reports show the exhaustive exercise-induced oxidative stress[1]. 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 as phagocytosis or calcium accumulation in the cell[2]. Physical exercise requires energy source as adenosine triphosphate (ATP) which is primary produced by mitochondial oxidative phosphorylation. Re-oxygenation or reperfusion with oxygenated blood results in local and systemic effects which may cause more tissue damage[3]. Free radicals are capable of independent existence and produced in all living cells. Reactive oxygen species (ROS) or reactive nitrogen species (RNS), e.g., superoxide (O_2), hydroxyl ($OH^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), hydroperoxide (-OOH), and peroxyl nitrite ($NOO^{\bullet}$) are capable of readily oxidizing another biological molecules, including carbohydrates, amino acids, fatty acids, and nucleotides. Scavenging mechanism of all free

radical produced *in vivo* by both enzyme- and non-enzyme antioxidants are presented. Antioxidant enzymes include superoxide dismutase, glutathione peroxidase and catalase, The main non-enzymic antioxidants include glutathione (GSH), vitamin E and vitamin C[1]. Free radical induces many protein and lipid oxidation products in the blood circulation. Some reports showed the physical exercise lead to an increase in lipid peroxidation.[4] Protein hydroperoxide (PrOOH) is a stable and propagation of radical reactions on other protein, lipid, and DNA[5]. Thus, this study aimed to determine the response of oxidative stress in sedentary subjects by evaluating the lipid peroxidation and ferrous oxidation status in blood and urine from exhaustive exercise.

Methods and Materials

Materials, glass wares and chemicals

Glassware were cleaned with warm concentrated nitric acid and thoroughly rinsed with double distilled water before experiments. All other reagents were at least of reagent grade. Xylenol orange [o-cresolsulfonphthalein-3, 3-bis (sodium methylimino diacetate)], Guanidine hydrochloride, Perchloric acid (AR grade; 70-72%), Thiobarbituric acid (TBA), Malonaldehyde (bis) and ammonium ferrous sulfate were purchased from Aldrich (USA).

Subjects

This study was approved by Ethic Committee of Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand. A total of 15 subjects was 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 steps. The heart rate at 85% of maximum heart rate or rate perceived exertion (RPE) at 9 was a final target for stop testing. Blood at pre- and immediately post-exercise test from vehous puncture (# 21) were collected in non-heparinized tube. Serum was separated by gentle

centrifuge at 1000 rpm shortly and kept in cool (4°C) for 2 hours. Urines at pre-, and post-exercise (immediately and 20 minutes) were collected and kept in cool (4°C).

The Exercise Protocol

The exhaustive exercise was followed from the Modified Bruce protocol and target heart rate (Target MHR) was performed from recommendation of American College Sport Medicine (ACSM)'s guidelines[6].

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 by following steps and each step performed for 3 minutes.

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 maximal heart rate and RPE at 14-15. Any symptoms as leg pain, muscle thigh, unwilling of subjects were criteria to stop exercise.

Serum and urine malondialdyde analysis

Malondialdehyde (MDA), the lipid peroxidation product was determined with modified Thiobarituric acid reactive substance (TBARs)[7]. The 100 µl of sample (urine or serum) was precipitated with 100% trichloroacetic acid (TCA) in 6 M HCl and mixed with 450 µl of normal saline solution (0.9% NSS), 200 µl of (4%) thiobarbituric acid (TBA) reagent. The whole mixture was incubated in 90°C water bath for 30 minutes then cooled in water. After centrifugation at 6,000 rpm for 5 minutes at 4°C, the absorbance was read at 532 nm. The concentration of malondialdhyde was calculated from the standard malondialdehyde (Sigma).

Ferrous oxidation analysis

The activity of blood and urine on activation the ferrous oxidation were evaluated by FOX method from Gay's protocol[8]. To perform modified FOX assay, 100 µl of serum was taken and precipitated the protein with 500 µl of 0.2 M perchloric acid (PCA) and centrifuged at 6500 rpm for 10 minutes (4°C). The pellet protein was dissolved in 1,000 µl of 6 M guanidine hydrochloride. The lipid was removed twice by 100 µl of absolute chloroform containing 2%BHT. The 700 µl of upper layer was mixed with 40 µl of 0.5 M PCA, 25 µl of 5 mM xylenol orange and 5 mM ferrous solution. For urine method, 700 µl of sample was incubated directly with 0.5 M PCA, 5 mM xylenol orange and 5 mM ferrous solution. After keeping at room temperature in the dark for 30 min, the mixture was centrifuged at 10,000 rpm for 3 min (4°C). The oxidative activity in blood and urine was calculated by compared with the absorbance at 560 nm of the standard t-butyl hydroperoxide (1-10 µM) (Sigma).

Statistical analysis

All data were presented as mean±standard error of mean (SEM). The levels of malondialdehyde and ferrous oxidation in blood before and after exercise were compared with paired t –test, whereas MDA and Ferrous oxidation in urine at various periods were statistically analyzed by a Wilcoxon

Signed Rank Test or repeated multiple variables test.

Results

This experiment was studied in 15 subjects with mean±SD of age = 20.75±1.89, range = 19-24 years, and BMI = 19.75±1.71 kg/m^2. The mean±SD of RPE and a percentage of heart rate compared with maximal HR were 14.13±2.45 and 77.67±4.92.

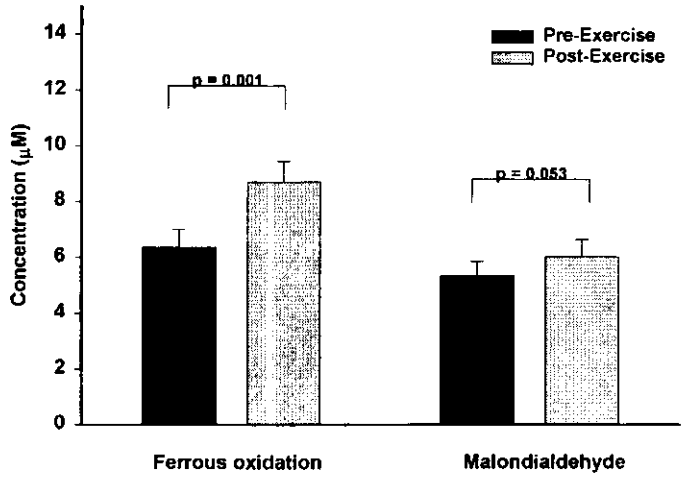

Figure 1. showed mean±SEM of serum malondiadehyde levels and ferrous oxidation relative to tert-butyl hydroperoxide levels before and post-immediately exercise). Note; p value was calculated by related paired t-test.

The levels of serum MDA were increase without significant difference: 5.32±0.54 vs 6.01±0.61 μM respectively before and after exercise. Whereas ferrous oxidation increased significantly (6.36±0.63 & 8.71±0.74 μM, $p < 0.05$).

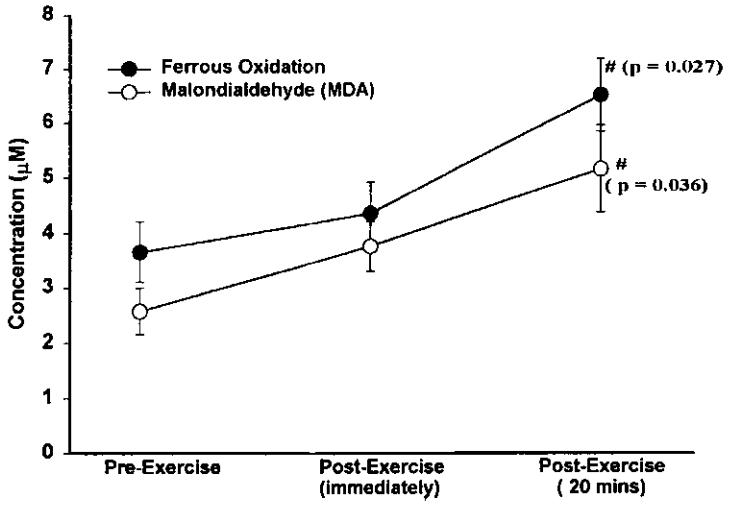

Figure 2. The levels of urine malondialdehyde and ferrous oxidation relative to tert-butyl hydroperoxide at pre-exercise, and post-exercise (immediately and at 20 min). Note; # represented different statistics compared with pre-exercise period and p was calculated with repeated variable measurement.

Urinary malondialdehyde and ferrous oxidation levels before and, immediately and at 20 minutes after stop exercise were shown in figure 2. The results showed increased tendency with time response in both MDA and ferrous oxidation; malondialdehyde was 2.58±0.42, 3.77±0.72, and 5.17±0.56 μM respectively, and ferrous oxidation was 3.66±0.85, 4.36±0.7, and 6.52±0.8 μM respectively. Both levels significantly by increased at 20 minutes after exercise termination compared with before exercise, whereas immediately post exercise termination showed no any difference.

Discussion

Oxidative stress from exhaustive exercise from this study showed that two biochemical parameters MDA and ferrous oxidation product were increased. The malondialdehye is the end product of lipid oxidation is formed after oxidation of lipid due to peroxyl radicals, and hydrogen peroxide, or non-radical species [9,10].This results was similar to the previous reports that represented higher lipid peroxidation on low density lipoprotein (LDL) and very low density lipoprotein (VLDL) fraction in plasma of marathon runner 42 kilometers (male=21, female= 25) [11].

The activity of blood and urine on oxidation the ferrous as determined by FOX method which is ax evaluation a Fe^{3+}-XO complex and detectable at 560 nm.

$$Fe^{2+} + \text{Oxidant} \longrightarrow Fe^{3+}$$

$$Fe^{3+} + XO \longrightarrow Fe^{3+}\text{-XO complex (560 nm)}$$

Source of Ferrous Oxidation

$$Fe^{2+} + LOOH \longrightarrow LO^{\bullet} + Fe^{3+}$$

$$Fe^{2+} + PrOOH \longrightarrow PrO^{\bullet} + Fe^{3+}$$

$$Fe^{2+} + H_2O_2 + H^{+} \longrightarrow Fe^{3+} + H2O + {}^{\bullet}OH$$

Various oxidant molecules that can motivate this system in the body, for example; protein hydroperoxide (PrOOH), lipid hydroperoixde (LOOH), H_2O_2, or excessive ferrous ion (Fe^{2+}) in the fluid[12].

In this study, the method presumed the ferrous oxidation from protein hydroperoxide (PrOOH) only by extraction the lipid out and precipitate the protein. Generally the activating protein (PrH) with various oxidants H_2O_2 or $^{\bullet}OH$ occurs in biological system. The carbon-centered free radical ($Pr^{\bullet}$) produced will be oxidized to form protein peroxyl radical ($PrOO^{\bullet}$) and finally to protein hydroperoxide (PrOOH) [12].

$$PrH + H_2O_2/{}^{\bullet}OH \longrightarrow Pr^{\bullet} + H_2O$$

$$Pr^{\bullet} + O_2 \longrightarrow PrOO^{\bullet}$$

$$PrOO^{\bullet} + e \longrightarrow PrOO$$

$$PrOO^{-} + H \longrightarrow PrOOH$$

$$PrOO^{\bullet} + PrH \longrightarrow PrOOH + Pr^{\bullet}$$

Interesting in the results represented the oxidative stress in the urine. Normally, urine contains with various substances, mainly urea and many metabolites that possible give a false positive result. However, the determination of these aldehydes from measurements of thiobarbituric acid reactive substance (TBARS) adducts clearly lacks specificity. It may contaminate with proteins, oxidized lipids, amino acids or sialic acid. As same as the ferrous oxidation, this method is widely used to detect the hydroperoixde. But this stituation used this protocol to detect the oxidative stress situation whether, hydroperoixde, hydrogen peroxide, ferrous or ferric ions, although there may be any molecules that gives the positive results in this test. Nevertheless, we found the responsive results after exhaustive exercise with time dependent that reflects to the body response to exercise. It means that detection the lipid peroxidation and ferrous oxidation products in urine can be applied to investigate the oxidative stress in sport researches in the future, especially to evaluate the body injury from some special training program, nutrition supplement, or medical treatment.

Acknowledgement

This study was granted by Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand.

References

1. Cooper CE, Vollaard NBJ, Choueiri T, Wilson MT. Exercise, free radicals and oxidative stress. Biochem. Soc. Transac. 2002: 30: 280-384.
2. Jackson MJ. Exercise and oxygen radical production by muscle. In Sen, C.K., Packer, L., Hanninen, O (eds). Handbook of oxidants and antioxidant in exercise. Marcel Dekker, Inc. New York. 2000: 57-66.
3. Tisi PV, Shearman CP. Claudication exercise and antioxidant. In. Sen LCK, Hanninen PO (eds). Handbook of Oxidants and Antioxidant in Exercise. Marcel Dekker, Inc. New York.; 2000: p1973.
4. Dillard CJ, Litov RE, Savin WM, Dumelin EE, Tappel AL. Appl. Physiol 1978; 45: 927-932.
5. Hawkins CL, Davies MJ. Generation and propagation of radical reactions on protiens. Biochem. Biophys. Acta 2001; 1504: 196-219.
6. ACSM's guidelines for exercise testing and prescription.6th ed. Lippincott Willians&Wilkins, Philadelphia, 2000.
7. Hensagvilai K, Leelarungrayub N, Chanarat N. The relationship between gross motor function measurement score (GMFMS) and glutathione (GSH), malondialdehyde (MDA) levels in children with cerebral palsy. 14th International WPCT congress, Barcelona, Spain 2003.
8. Gay CA, Gebicki JM. Meaurement of protein and lipid hydroproxides in biological systems by the feric-xylenol orange method. Anal Biochem 2003: 315: 29-35.
9. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J. Lipid Res. 1998: 39: 1529-1542.
10. Aslan R, Sekeroglu MR, Tarakcioglu M, Bayiroglu F, Meral I. Effect of acute and regular exercise on antioxidative enzymes, tissue damage markers and membrane lipid peroxidation of erythrocytes in sedentary students. Trop. J. Med. Sci. 1998: 28: 411-414.
11. Kaikkonen J, Porkkala-Sarataho E, Tuomainen TP, Nyyssonen K, Kosonen L, Ristonmaa U, Lakka HM, Salonen R, Korpela H, Salonen JT. Exhaustive exercise increases plasma/serum total oxidation resistance in moderately trained

men and women, whereas their VLDL + LDL lipoprotein fraction is more susceptible to oxidation. Scand J. Clin. Lab. Invest. 2002; 62: 599-607.

12. Gebicki JM. Protein hydroperoxide as new reactive oxygen species. Redox Rep. 1997: 3: 99-110.