

HIBISCUS PROTOCATECHUIC ACID SUPPLEMENTATION REDUCES OXIDATIVE STRESS INDUCED BY EXHAUSTIVE EXERCISE IN RAT MUSCLE

City Chin-Cheng Hsieh¹, Meng-Yin Lee¹, Chang-Che Chen², Jen-Jeng Hsu³,
Hsueh-Kuan Lu³, Chau-Jong Wang²

¹*Department of Physical Education, National Hsinchu University of Education, Hsin Chu, TAIWAN*

²*Institute of Biochemistry, College of Medicine, Chung Shan Medical University, Taichung, TAIWAN*

³*Department of Physical Education, National Taiwan College of Physical Education, Taichung, TAIWAN*

The purpose of this study was to investigate the exhaustive exercise (CE) induced oxidative damage and the protective effect of Hibiscus protocatechuic acid (PCA) supplementation on malonyldialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GRd) in rat skeletal muscle. Thirty-two male Sprague–Dawley rats were randomly divided into the following four groups: (i) control (C, $n = 8$), (ii) exhaustive exercise (CE, $n = 8$), (iii) PCA (P, $n = 8$), and (iv) PCA-exhaustive exercise (PE, $n = 8$). The amount of PCA supplementation was 1 mg per kg mass body per day for 7 days. The exhaustive exercise started at 10% grade, 15 m min⁻¹ for 15 min followed by a gradual increase of treadmill speed and times as 25 m min⁻¹ for 15 min, 30 m min⁻¹ for 30 min, 35 m min⁻¹ for 60 min, 40 m min⁻¹ for 30 min, 45 m min⁻¹ for 30 min until exhaustion. Exercised duration in CE and PE groups were 91.8 ± 23.6 and 107.6 ± 22.3 min ($p > 0.05$), respectively. Two-way analysis of variance was performed in this study. The results of this study showed that MDA activity was 192% higher in CE versus C rats and 42% lower in PE versus CE rats ($p < 0.05$). SOD activity was 49% lower in CE versus C rats and 69% higher in PE versus CE rats ($p < 0.05$). Moreover, GRd activity was 50% higher in PE versus CE rats ($p < 0.05$). It was concluded that exhaustive exercise could result in oxidative stress. The PCA supplementation was beneficial in enhancing antioxidant status and inhibiting oxidative stress induced by exhaustive exercise.

Keywords: glutathione peroxidase, glutathione reductase, malonyldialdehyde, superoxide dismutase

Introduction

Free radicals (molecules or atoms with an unpaired electron) and their metabolites have been known for their effect on mechanisms regulating cellular function.

Several metabolic events can account for free radical formation, such as lipid and protein metabolism. In 1982, Demopoulos proposed that the free radical theory of disease will be an important breakthrough in the field of medicine than that of the germ theory (Demopoulos 1982). Recently, many kinds of diseases result not because of the germ, but from the free radicals in the human body, such as cancer, atherosclerosis, diabetes, cataract, aging, Parkinson's disease (Fehrenbach & Northoff 2001). The oxygen free radical is an oxygen molecule with unpaired electrons in its outer

Corresponding Author

Chau-Jong Wang, Institute of Biochemistry, College of Medicine, Chung Shan Medical University, Taichung, TAIWAN.

Tel: (886) 4 24730022 (ext. 11883)

Fax: (886) 4 23248167

E-mail: wcj@csmu.edu.tw

orbits. A small fraction (2–5%) of the oxygen consumed by the cells can be converted to oxygen free radicals (Ernster 1986). It is well known that the oxygen consumption is a direct function of workload (Kanter et al. 1993; Alessio & Goldfarb 1988). One consistent finding in the tissue is that exhaustive exercise may increase the production of oxygen free radicals which in turn increases lipid peroxidation (Gunduz et al. 2003).

Hibiscus protocatechuic acid (PCA) is a simple phenolic compound isolated from the dried flowers of *Hibiscus sabdariffa* L., a Chinese herbal medicine; reported to be antiseptic, aphrodisiac, astringent, cholagogue, demulcent, digestive, diuretic, emollient, purgative, refrigerant, resolvent, sedative, stomachic, and tonic. *Hibiscus sabdariffa* L. is a folk remedy for abscesses, bilious conditions, cancer, cough, debility, dyspepsia, dysuria, fever, hangover, heart ailments, hypertension, neurosis, scurvy, and strangury (Chewonarin et al. 1999; Haji & Haji 1999; Dafallah & al-Mustafa 1996). PCA has been reported to reduce the formation of the lipid peroxidation and to be an effective antioxidant (Lee et al. 2002). The effect of PCA supplementation on oxidative stress induced by exhaustive exercise is still poorly understood. Therefore, the purpose of this study was to investigate the effects of the exhaustive exercise-induced oxidative stress on malonyldialdehyde (MDA), and the protective effects of supplementation of PCA on superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GRd) in rat muscle.

Materials

Animals

Thirty-two Sprague–Dawley male rats (250–280 g, 8 g weeks old) were purchased from the National Laboratory Animal Breeding and Research Center Taipei, Taiwan. Rats were randomly divided into the following four groups: (i) control (C, $n = 8$), (ii) exhaustive exercise (CE, $n = 8$), (iii) PCA (P, $n = 8$), and (iv) PCA-exhaustive exercise (PE, $n = 8$). The rats were housed individually in hanging wire-mesh cages in a room regulated for temperature ($23 \pm 1^\circ\text{C}$), humidity (45–50%), and light (lights kept on from 06:00 to 18:00 h). They were fed with laboratory rat chow and water *ad libitum* throughout the

study. The animals of the PCA and PCA-exhaustive exercise groups were supplemented with PCA (Sigma Chemical Co., St Louis, MO, USA). The amount of PCA supplementation was 1 mg per kg mass body per day for 7 days by using oral feeding needle. The study was approved by the Institutional Animal Care and Usage Committee of National Hsinchu University of Education and followed the guidelines established by the National Laboratory Animal Breeding and Research Center in Taiwan.

Experimental protocol

Exhaustive exercise was performed on a rodent treadmill with the following protocols. In the adaptive period, rats were accustomed to treadmill running for a week. Then, the rats of exhaustive exercise group were subjected to graded treadmill running starting at 10% grade, 15 m min^{-1} for 15 min followed by a gradual increase in the treadmill speed and time to 25 m min^{-1} for 15 min, 30 m min^{-1} for 30 min, 35 m min^{-1} for 60 min, 40 m min^{-1} for 30 min, 45 m min^{-1} for 30 min until exhaustive (Figure 1).

Electrical shocks were used sparingly in exhaustive exercise groups to motivate the animals to run. Exhaustion was defined as the inability of the rats to run on the treadmills, despite electrical shock.

Tissue preparation

At the end of exhaustive exercise, the rats were anesthetized with ether (an anesthesia chamber was utilized as the induction method of delivering volatile anesthetic agent to the rats); ether was volatilized by placing it on cotton balls at the bottom of the jar. The rats after anesthetization were killed by decapitation. The rat gastrocnemius muscle was isolated to prepare the muscle homogenate. Control rats were also killed at the same time as the exhaustive exercise

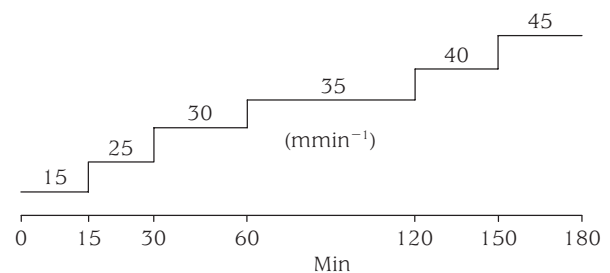


Fig. 1 Exhaustive exercise protocol.

group. All muscle samples were stored at -70°C until various assays were performed.

Measurement of lipid peroxidation

Thiobarbituric acid (TBA) reacts with MDA to form a diaduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm using the method of Wallin et al. (1993). To each tube containing 0.55 mL of incubated low-density lipoprotein, 0.5 mL of 25% (w/v) trichloroacetic acid (TCA) was added to denature the protein. The samples were centrifuged (10,000 rpm) at 10°C for 30 min to remove the pellet. TBA (1%, 0.5 mL) in 0.3% NaOH was added to the supernatant, and the mixed reagents reacted at $90\text{--}95^{\circ}\text{C}$ for 40 min in the dark. After completing the reactions, samples were detected with excitation at 532 nm and emission at 600 nm in a Hitachi F-2000 spectrofluorimeter. One unit of lipid peroxidation activity was defined as the amount of TBA that converts to thiobarbituric acid reacting substance (TBARS). The concentration was expressed in $\mu\text{m}/\text{mg}$ protein in tissue samples.

Measurement of SOD

SOD activities of the tissue samples were measured with a commercial kit (SD 125; Randox Laboratories, Antrim, UK). Fifty microliters of diluted sample was added to 1.7 mL of mixed substrate ($50\ \mu\text{mol L}^{-1}$ of xanthine and $25\ \mu\text{mol L}^{-1}$ of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride). Two hundred and fifty microliters of xanthine oxidase was added to the mixture and SOD activity was measured at 37°C on a Hitachi U-2000 Spectrophotometer at 505 nm for 3 min. The activity was expressed in U mg^{-1} protein in tissue samples.

Measurement of GPx

Glutathione peroxidase activities of skeletal muscle tissue samples were determined with a commercial kit (RS 504; Randox Laboratories, Antrim, UK). Twenty microliters of diluted sample was added to 1 mL of mixed substrate ($4\ \text{mmol L}^{-1}$ glutathione, $0.5\ \text{U L}^{-1}$ GRd and $0.34\ \text{mmol L}^{-1}$ NADPH dissolved in $50\ \text{mmol L}^{-1}$ phosphate buffer, pH 7.2, $4.3\ \text{mmol L}^{-1}$ EDTA). Forty microliters of cumene hydroperoxide (diluted in deionized water) was added to the mixture and GPx activity was measured at 37°C on a Hitachi U-2000

Spectrophotometer at 340 nm for 3 min. The activity was expressed in U mg^{-1} protein in tissue samples.

Measurement of GRd

Glutathione reductase activities of skeletal muscle tissue samples were measured with a commercial kit (Calbiochem 359962; Calbiochem-Novabiochem). Two hundred microliters of the diluted sample was added to $400\ \mu\text{L}$ of $2.4\ \text{mmol L}^{-1}$ GSSG buffer (dissolved in $125\ \text{mmol L}^{-1}$ potassium phosphate buffer, pH 7.5, $2.5\ \text{mmol L}^{-1}$ EDTA). Four hundred microliters of $0.55\ \text{mmol L}^{-1}$ NADPH (dissolved in deionized water) was added to the mixture and GRd activity was measured at 340 nm for 5 min on a Hitachi U-2000 Spectrophotometer. The activity was expressed in U g^{-1} protein in tissue samples.

Determination of protein

In order to express the antioxidant enzyme activities per gram protein, total protein concentration of tissue samples was estimated spectrophotometrically according to the method of Lowry et al. (1951) using a Bio-Rad DC protein assay kit (Cat. No. 500-0116; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The experiments were carried out in triplicate. Data are expressed as mean \pm SEM. To determine whether there were significant differences in MDA, SOD, GPx, and GRd activities between control, exhaustive exercise, PCA, and PCA-exhaustive exercise groups, two-way ANOVA was run in the presence of a significant *F* value. *Post hoc* comparisons of means were provided by Tukey's range test. Statistically significant value was $p < 0.05$.

Results

Exercise duration

The time of running to exhaustion was not significantly different for E (91.8 ± 8.34 min) and PE (107.6 ± 7.88 min) rats ($p > 0.05$).

Lipid peroxidation

The main effect of exercise resulted in a significant increase in muscle lipid peroxidation. Exercise-induced MDA activity was 347% higher than in nonexercised

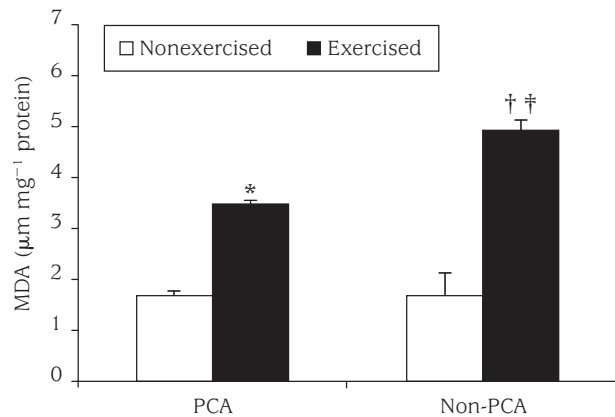


Fig. 2 MDA activity in rat skeletal muscle. Each bar presents the mean \pm SEM ($n = 8$).

* $p < 0.05$, PE vs. CE.

† $p < 0.05$, CE vs. C.

‡ $p < 0.05$, nonexercised vs. exercised.

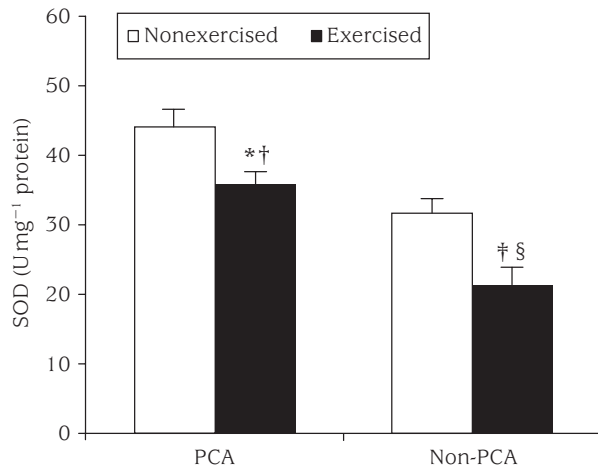


Fig. 3 SOD activity in rat skeletal muscle. Each bar presents the mean \pm SEM ($n = 8$).

* $p < 0.05$, PE vs. CE.

† $p < 0.05$, PCA vs. non-PCA.

‡ $p < 0.05$, CE vs. C.

§ $p < 0.05$, nonexercised vs. exercised.

rats. Meanwhile, MDA activity was 192% higher in CE versus C rats and 42% lower in PE versus CE rats ($p < 0.05$) (Figure 2).

Antioxidant enzyme activities

The main effects of exercise and PCA supplementation on SOD activities were 33% higher in nonexercised versus exercised rats and 59% higher in PCA versus non-PCA rats ($p < 0.05$). Further, SOD activity was 49% lower in CE versus C rats and 69% higher in PE versus CE rats ($p < 0.05$); (Figure 3). There was no significant

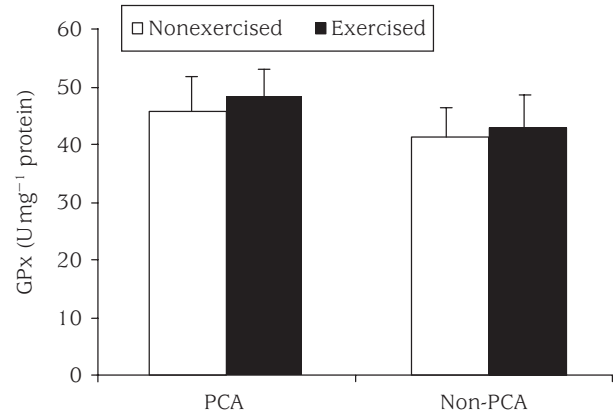


Fig. 4 GPx activity in rat skeletal muscle. Each bar presents the mean \pm SEM ($n = 8$). There was no significant difference among groups ($p > 0.05$).

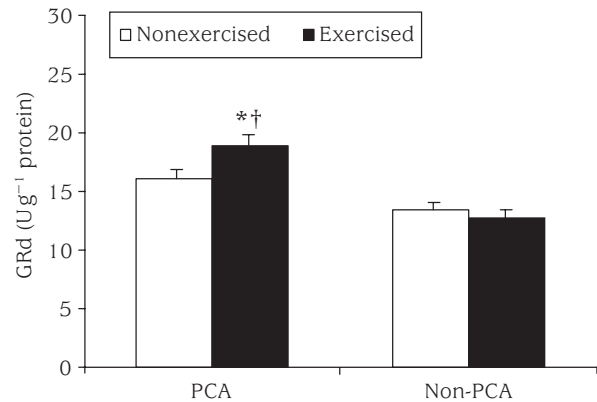


Fig. 5 GRd activity in rat skeletal muscle. Each bar presents the mean \pm SEM ($n = 8$).

* $p < 0.05$, PE vs. CE.

† $p < 0.05$, PCA vs. non-PCA.

change in GPx activity among various groups (Figure 4). The main effect of PCA supplementation on GRd activity was 34% higher in PCA versus non-PCA rats ($p < 0.05$). Moreover, GRd activity was 50% higher in PE versus CE rats ($p < 0.05$), but no significant difference in CE versus C rats (Figure 5).

Discussion

The rats in the CE and PE groups ran in the rodent treadmill until exhaustion. Durations in the CE and PE groups were 91.8 ± 8.34 and 107.6 ± 7.88 min ($p > 0.05$), respectively, while the average exercise intensity in both groups were 35 m min^{-1} in this study. The

duration and intensity were longer and higher than those, respectively, in a previous study by Ji et al. (1992). It has been reported that exercise intensity could be 92.3% $\dot{V}O_{2max}$, when Sprague–Dawley ran at 26.8 m min⁻¹ and 10% grade (Bedford et al. 1979). Obviously, the intensity of exhaustive exercise in this study was very strenuous.

During exercise there is an increase in the requirement of oxygen. The process of delivering the oxygen to the working muscles may actually result in damage to polyunsaturated fatty acids in membrane structures. This has been documented by numerous investigations demonstrating increases in the byproducts of lipid peroxidation following exercise (Bejma & Ji 1999; Starnes et al. 1989). When a hydroxyl radical reacts with an unsaturated fatty acid, a lipid peroxy radical is formed. In the presence of oxygen this new free radical incites a chain of events referred to as lipid peroxidation. Lipid peroxidation of cell membranes results in decreased membrane fluidity, inability to maintain ionic gradients, cellular swelling, and tissue inflammation (Alessio 1993). Demonstration of free radical activity in biological tissues is a difficult process because the superoxide radical is very unstable, existing for just milliseconds at neutral pH (Freeman 1984). The commonly used MDA levels were analyzed as an indicator of the lipid peroxidation activities (Ozbay & Dulger 2002). This study showed that MDA activity was 192% higher in CE versus C rats and 42% lower in PE versus CE rats ($p < 0.05$) (Figure 2). The current data indicated that exhaustive exercise-induced lipid peroxidation due to excess oxygen radical reacting polyunsaturated acid in the muscle. The present study is in agreement with some of the previous studies (Ji & Fu 1992; Alessio & Goldfarb 1988), which demonstrated that intense or exhaustive exercise resulted in a significant increase in MDA. It seems reasonable to interpret this finding as an indication that the muscle is vulnerable to oxidative damage because of their continuous exposure to oxygen.

PCA is a simple phenolic compound isolated from the dried flowers of *H sabdariffa* L., a Chinese herbal medicine. PCA has been reported to reduce the formation of lipid peroxidation and to be an effective antioxidant (Lee et al. 2002). In the present study, PCA supplementation was 1 mg per kg mass body per day for

7 days. SOD activity was 49% lower in CE versus C rats and 69% higher in PE versus CE rats ($p < 0.05$) (Figure 3). Among the various antioxidant defense mechanisms, SOD is the front line of defense against oxidative damage (Hassan & Schellhorn 1988). As mentioned earlier, exhaustive exercise is associated with a higher rate of lipid peroxidation, since increased oxygen consumption may rise several-fold with high levels of exercise. It has been proposed that physically active individuals have higher requirements for the antioxidants. The findings in this study showed that SOD activity was 69% higher in PE versus CE rats, and MDA activity 42% lower in PE versus CE rats. These results indicated that PCA play the role of antioxidant to prevent oxidative damage in rat skeletal muscle.

The physiological role of GSH is as an essential intracellular reducing agent for the maintenance of thiol groups on intracellular protein and antioxidant molecules. It was well established that GSH, the most important biomolecule protecting against chemically induced cytotoxicity, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction. The ratio of GSH to GSSG would be expected to be a more sensitive marker of oxidative stress, because a small increase in GSSG and decrease in GSH can appear to be more amplified by examining the ratio than by measuring either of them separately (Kadiiska et al. 2000). In addition, the GSH antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. This system consists of GSH and an array of functionally related enzymes, of which GRd is responsible for the regeneration of GSH, whereas GPx worked together with GSH in the decomposition of hydrogen peroxide or other organic hydroperoxides (Mark et al. 1996). Thus, the levels of GSH and activities of the GPx and GRd were used to monitor the peroxidative balance (Velmurugan et al. 2001). This study showed that GRd activity was 50% higher in PE versus CE rats ($p < 0.05$), with no significant difference in CE versus C rats (Figure 5). In view of the present findings, it seems likely that such metabolites of PCA might have contributed to the skeletal muscle antioxidant defenses. In PCA-supplemented rats, exercising was associated with a tendency to increase tissue GRd activity. This would result in an improved antioxidant defense in that tissue as well.

To conclude, the results in this study demonstrated that exhaustive exercise could result in oxidative stress and PCA supplementation was beneficial in enhancing the antioxidant status and inhibiting oxidative stress induced by exhaustive exercise.

Acknowledgments

This study was supported by grants from the National Science Council of Taiwan (NSC 88-2413-H-134-010).

References

- Alessio HM, Goldfarb AH (1988). Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. *J Appl Physiol: Resp Environ Exerc Physiol* 64:1333–6.
- Alessio HM (1993). Exercise-induced oxidative stress. *Med Sci Sports Exerc* 25:218–24.
- Bedford TG, Tipton CM, Wilson NC (1979). Maximum oxygen consumption of rats and its changes with various experimental procedures. *J Appl Physiol: Resp Environ Exerc Physiol* 47:1278–83.
- Bejma J, Ji LL (1999). Aging and acute exercise enhance free radical generation in rat skeletal muscle. *J Appl Physiol* 87:465–70.
- Chewonarin T, Kinouchi T, Kataoka K, Arimochi H, Kuwahara T, Vinitketkumnuen U, Ohnishi Y (1999). Effects of roselle (*Hibiscus sabdariffa* L.), a Thai medicinal plant, on the mutagenicity of various known mutagens in *Salmonella typhimurium* and on formation of aberrant crypt foci induced by the colon carcinogens azoxymethane and 2-amino-1-methyl-6-phenylimidazo(4, 5-b) pyridine in F344 rats. *Food Chem Toxicol* 37:591–601.
- Dafallah AA, al-Mustafa Z (1996). Investigation of the anti-inflammatory activity of *Acacia nilotica* and *Hibiscus sabdariffa*. *Am J Chin Med* 24:263–9.
- Demopoulos H Quoted in Pearson D, Shaw S (1982). *Life Extension a Practical Scientific Approach*. Warner Books, New York, p 100.
- Ernster L (1986). Oxygen as an environmental poison. *Chem Scr* 26:525–34.
- Fehrenbach E, Northoff H (2001). Free radical, exercise, apoptosis, and heat shock protein. *Exerc Immunol Rev* 7:66–89.
- Freeman BA (1984). Biological sites and mechanisms of free radical production. In: *Free Radicals in Molecular Biology, Aging, and Disease*. In: Armstrong D, Sohal RS, Cutler RG, Slater TF (eds). Raven Press, New York, pp 43–52.
- Gunduz UK, Senturk O, Kuru O, Aktekin B, Aktekin MR (2003). The effect of one year's swimming exercise on oxidant stress and antioxidant capacity in aged rats. *Physiol Res* 53:171–6.
- Haji FM, Haji TA (1999). The effect of sour tea (*Hibiscus sabdariffa*) on essential hypertension. *J Ethnopharmacol* 65:231–6.
- Hassan HM, Schellhorn HE (1988). Superoxide dismutase an antioxidant defense enzyme. In: Cerruti PA, Fridovich I, McCord JM (eds). *Oxyradicals in Molecular Biology and Pathology*. Alan R. Liss Inc, New York, pp 183–93.
- Ji LL, Fu RG (1992). Responses of glutathione system and antioxidant enzymes to exhaustive exercise and hydroperoxide. *J Appl Physiol* 72:549–54.
- Ji LL, Fu R, Mitchell EW (1992). Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *J Appl Physiol: Resp Environ Exerc Physiol* 73:1854–9.
- Kadiiska MB, Gladen BC, Baird DD, Dikalova AE, Sohal RS, Hatch GE, Jones DP, Mason RP, Barrett JC (2000). Biomarker of oxidative stress study: are plasma antioxidant markers of CCl4 poisoning? *Free Rad Biol Med* 28:838–45.
- Kanter MM, Nolte LA, Holloszy JO (1993). Effects of an antioxidant vitamin mixture on lipid peroxidation at rest and postexercise. *J Appl Physiol: Resp Environ Exerc Physiol* 74:965–9.
- Lee MJ, Chou FP, Tseng TH, Hsieh MH, Lin MC, Wang CJ (2002). Hibiscus protocatechuic acid or esculetin can inhibit oxidative LDL induced by either copper ion or nitric oxide donor. *J Agric Food Chem* 50:2130–6.
- Lowry OH, Rosebrough NJ, Farr AI, Randall JL (1951). Protein measurement with Folin-phenol reagent. *J Biol Chem* 193:265–75.
- Mark DH, Ip SP, Li PC, Poon MK, Ko KM (1996). Alterations in tissue glutathione antioxidant system in Streptozotocin-induced diabetic rats. *Mol Cel Biochem* 162:153–8.
- Ozbay B, Dulger H (2002). Lipid peroxidation and antioxidant enzymes in Turkish population: relation to age, gender exercise, and smoking. *T J Exp Med* 197:119–24.
- Starnes JW, Cantu G, Farrar RP, Kehrer JP (1989). Skeletal muscle lipid peroxidation in exercise and food-restricted rats during aging. *J Appl Physiol* 67:69–75.
- Velmurugan B, Bhuvanewari V, Balasenthil S, Nagini S (2001). Lycopene, an antioxidant carotenoid modulates glutathione dependent hepatic biotransformation enzymes during experimental gastric carcinogenesis. *Nutri Res* 21:1117–24.
- Wallin B, Rosengren B, Shertzer HG, Camejo G (1993). Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtiter plate: its use for evaluation of antioxidants. *Anal Biochem* 208:10–15.