EFFECTS OF DIALLYL SULFIDE ON ACUTE EXHAUSTIVE EXERCISE INDUCED OXIDATIVE STRESS IN PLASMA, DIAPHRAGM, FAST AND SLOW TWITCH MUSCLES IN RATS

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ABSTRACT

This study investigated the effects of acute exhaustive exercise on the time course of changes in the contents of malondialdehyde (MDA), index of lipid peroxidation in plasma, diaphragm, rectus femoris (fast twitch), and soleus (slow twitch) muscles of male Sprague Dawley rats that had received garlic extract; diallyl sulfide (DAS) orally at 2 different doses (50 mg/kg BW, 200 mg/kg BW) for 28 days, and untreated control rats. Animals were sacrificed at 0, 6, 12, 24, 36, 48, and 72-hrs after exercise. The second part of this study involved 108 rats, which were divided into 2 groups; DAS supplemented and control. The optimum dose of DAS (50 mg/kg BW) and the high responsiveness organ (diaphragm) obtained from the previous study were used to identify the time course effect of DAS supplementation. Rats from each treated group were subjected to an acute bout of exhaustive exercise and sacrificed at the pre-determined time post exercise. The third part studied the effects of DAS on antioxidative enzyme activities (superoxide dismutase, SOD; glutathione peroxidase, GPX;catalase,CAT)and antioxidative content (glutathione).

Exhaustive exercise led to increase in MDA levels in all tissues examined, which showed peak productions at 6-hrs post exercise. Rats treated with 50 mg/kg BW had significantly lower levels of MDA than untreated control rats at 6-hrs postexercise, while 200 mg/kg BW induced less protection. DAS supplementation plus exercise induced significant increase in intracellular SOD and GPX enzyme activities in the diaphragm of DAS administered rats when compared with the corresponding non-supplemented group, but did not influence the intracellular CAT activity and glutathione content.

Our results showed that exhaustive exercise induced oxidative damaged occurred not during but following exhaustive exercise. DAS supplementation can help deplete the amount of oxidative stress, suggesting a beneficial effect of DAS as food additive. DAS possesses mild antioxidative capacity indicated by an increase in intracellular SOD and GPX activities.

KEY WORDS: DIALLYL SULFIDE / EXHAUSTIVE EXERCISE / LIPID PEROXIDATION / ANTIOXIDANT

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ผลของ Diallyl sulfide จากกระเทียมกับการออกกำลังกายจนล้าและการเปลี่ยนแปลงอนุมูลอิสระในพลาสมา กระบังลม กล้ามเนื้อหดตัวเร็วและช้าในหนูออกกำลังกายจนล้า (EFFECTS OF DIALLYL SULFIDE ON ACUTE EXHAUSTIVE EXERCISE INDUCED OXIDATIVE STRESS IN PLASMA, DIAPHRAGM, FAST AND SLOW TWITCH MUSCLES IN RATS)

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บทคัดย่อ

การวิจัยนี้มีจุดประสงค์เพื่อศึกษาผลของการออกกำลังกายจนล้าต่อการเปลี่ยนแปลงระดับของลิปิดเปอร์ออกซิเดชันซึ่งบ่งชี้โดยวัดระดับของมาลอนไดอัลดีไฮด์ (MDA) ในพลาสมา กระบังลม กล้ามเนื้อหดตัวเร็วและช้า ซึ่งวัด ณ เวลา 0, 6, 12, 24, 36, 48, และ 72 ชั่วโมงหลังสิ้นสุดการออกกำลังกาย นอกจากนี้ยังศึกษาผลของสารสกัดจากกระเทียม (diallyl sulfide, DAS) ต่อเอนไซม์ต้านอนุมูลอิสระ (superoxide dismutase, SOD; glutathione peroxidase, GPX; catalase, CAT) และปริมาณกลูตาไทโอน (GSH) หนูขาวเพศผู้ แบ่งออกเป็น 3 กลุ่มคือ 1) กลุ่มที่ได้รับ DAS (50 มก./ก.ก.) 2) กลุ่มที่ได้รับ DAS (200 มก./ก.ก.) 3) กลุ่มควบคุม หลังจากได้รับ DAS ติดต่อกันเป็นเวลา 28 วัน นำหนูไปวิ่งบนลู่วิ่งจนล้า ปริมาณของ DAS ที่ใช้และกล้ามเนื้อที่ตอบสนองถูกเลือกมาเพื่อศึกษาต่อในส่วนที่ 2 ซึ่งเป็นการศึกษาถึงระยะเวลาเกิดประสิทธิผลของการให้ DAS หนูขาวแบ่งเป็น 2 กลุ่มคือกลุ่มที่ได้รับ DAS และกลุ่มควบคุม ทุก 1 สัปดาห์ หนูจากแต่ละกลุ่มจะถูกนำไปวิ่งจนล้าเพื่อวัดปริมาณ MDA ในเนื้อเยื่อต่างๆ จนกระทั่งครบ 8 สัปดาห์ ส่วนที่ 3 ศึกษาผลของ DAS ต่อระดับแอนติออกซิเดนท์เอนไซม์ (SOD, GPX, CAT) และปริมาณกลูตาไทโอน (GSH)ในกล้ามเนื้อที่ตอบสนองมากที่สุด

พบว่าการออกกำลังกายจนล้าทำให้ระดับ MDA ใน พลาสมา กล้ามเนื้อกระบังลม กล้ามเนื้อ soleus และ กล้ามเนื้อ rectus femoris เพิ่มขึ้นสูงที่สุดที่ 6 ชั่วโมงหลังออกกำลังกาย หนูที่ได้รับ DAS ปริมาณ 50 มก/ก.ก. เป็นเวลา 28 วันมีปริมาณ MDA น้อยกว่าหนูที่ไม่ได้รับ ($p< 0.05$) ส่วนหนูที่ได้รับปริมาณ 200 มก/ก.ก. มีประสิทธิภาพน้อยกว่าในการลดการเกิดลิปิดเปอร์ออกซิเดชัน นอกจากนี้การได้รับ DAS ร่วมกับการออกกำลังกายจนล้า ทำให้ปริมาณเอนไซม์ SOD และ GPX ในกล้ามเนื้อกระบังลมเพิ่มขึ้นแต่ไม่ทำให้ปริมาณกลูตาไทโอนและเอนไซม์ CAT เปลี่ยนแปลง

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LIST OF ABBREVIATIONS

ADP	adenosine 5′- diphosphate
ATP	adenosine 5′- triphosphate
ATP-PC	adenosine triphosphate-phosphocreatine
BW	body weight
Ca^{2+}	calcium ion
CAT	catalase
Cu	copper
^{o}C	degree celcius
DAS	diallyl sulfide
DNA	deoxyribonucleic acid
et al.,	and colleagues
Exs	exercise
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
G	gram
mg/kg BW	milligram per kilogram body weight
GPX	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione-*S*-transferase
hr	hour
H_2O	water
H_2O_2	hydrogen peroxide
i.g.	intragastric
IU	international unit
i.v.	intravenous

LIST OF ABBREVIATIONS (Cont.)

kg	kilogram
l	litre
$L^{\bullet}$	lipid radical
LDH	lactate dehydrogenase
LH	lipid
$LO_2^{\bullet}$	peroxyl radical
LOOH	lipid hydroperoxide
M	molar or mole per litre
MDA	malondialdehyde
mg	milligram
mg%	milligram percent
min	minute
ml	millitre
mM	millimolar
Mn	manganese
μmol	micromole
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide 3′-phosphate
NADPH	nicotinamide adenine dinucleotide 3′-phosphate, reduced form
nmol	nanomole
O_2	oxygen molecule
$O_2^{\bullet-}$	superoxide anion radical
O_3	ozone
O.D.	optical density
OH^-	hydrogen ion
$OH^{\bullet}$	hydroxyl radical
pH	log concentration of $(H^+)^{-1}$

LIST OF ABBREVIATIONS (Cont.)

RNA	ribonucleotide acid
ROS	reactive oxygen species
rpm	round per minute
SEM	standard error of means
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substance
TMP	1,1,3,3 tetramethoxypropane
vol	volume
w/v	weight by volume
$\overline{x}$	mean
Zn	zinc

CHAPTER 1
INTRODUCTION

It is believed that garlic (Allium sativum) contains important dietary and medicinal roles. For example, various pharmacological effects of garlic are presented as hypolipidemic, hypoglycemic (Sheela *et al*. 1995; Chang & Johnson 1980), antiartherosclerosis (Steiner & Lin 1998; Efendy *et al.,* 1997), antihypertensive (Ogawa *et al.,* 1993), antimicrobial (Cellini *et al*. 1996; Farbman *et al*., 1993), and antitumor (Hatano *et al*., 1996; Amagase & Milner 1993), as an antidote for heavy metal poisoning, hepatoprotective and as an immunomodulator (Lawson 1994; Lau *et al.,* 1991; Abudullah *et al.,* 1989). Moreover, investigators have recently reported that garlic also enclose high antioxidant property (Amagase 1997; Kourounakis and Rekka 1991), which inhibited lipid peroxidation (Ohnishi and Kojinra 1997; Prasad *et al.,* 1996; Torok *et al.,* 1994). This activity has been shown to be related to sulfur-containing components in garlic, which include diallyl sulfides (DAS), diallyl disulfides (DADS), triallyl sulfides (TAS), allicin, alliin, allixin. (Prasad *et al*., 1995; Horie *et al*., 1992; Kourounakis and Rekka, 1991). Among these sulfur-containing components in garlic, DAS and allicin have been proven as the most potent antioxidant compounds (Imai *et al*., 1994). Allicin, transient lipid–soluble compound, is unstable (Freeman and Kodera, 1995) and not preferably used by most investigations. Diallyl sulfide (DAS) is, mostly, presented within studies in which it is used to define antioxidant properties in animal model. Underlying mechanisms of DAS protective roles have been proposed by it ability to suppress superoxide anion (O_2^-) to scavenge peroxyl or alkoxyl radicals (Rekka & Kourounakis, 1994). These reports, however, have been investigated in resting condition.

Physical exercise training is known to exhibit various beneficial roles including higher rate of oxygen uptake, lower blood pressure (Gallagher *et al.,* 1996), and enhance body's temperature regulation (Glen *et al*., 1996). These alterations, either with structural or physiological changes, depend upon type and intensity of

physical training (Gambelunghe *et al*., 2001). While acute bout of exercise at low to moderate intensity demonstrates positive physiologic benefits to the body (Power *et al*., 1999). Single bout of exhaustive exercise, on the other hands, obviously causes damages to various organs (Jiankang *et al*., 2000). Indeed, both the voluntary and involuntary muscles are subjected to exercise-induced oxidative stress (Anzueto *et al*., 1993). Itoh and coworkers (1998) reported increased in hydroxyl radical levels in the plasma and soleus muscle of rats after exhaustive exercise. Strenuous exercise increases the releasing of cytosolic enzymes, the hallmark of muscle damage, from skeletal muscle (Salminen and Vihko 1983, Krotkiewski and Brzeinska, 1996, Venditti and Meo, 1996). It has been postulated that the generation of free radicals is enhanced during exercise as a result of increases in mitochondrial oxygen consumption where higher rate of electron transport flux is accompanied by higher tendency of lipid peroxidation (Clutton *et al*., 1997; Jackson *et al*., 1993; Singh *et al*., 1982). Studies of isolated muscle tissue indicate that loss of muscle cell viability is associated with a loss of calcium-dependent degradation pathways (Smith *et al*., 1989; Jones *et al*., 1984). Calcium-dependent proteases are known to cause conversion of the enzyme xanthine dehydrogenase to xanthine oxidase, an oxygen reactive species form, which causes damage to the skeletal muscle (Fridovich 1983; Halliwell and Gutteridge 1984). For example, previous reports have shown that slow twitch oxidative fibers (Type I, soleus) contain higher antioxidant enzymes which compose of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) at rest and after exercise than fast-twitch fibers (type IIa or IIb, recurs femoris muscle) (Jenkins *et al*., 1984; Ji *et al*., 1988; Lawler *et al*., 1993; Oh-ishi *et al*., 1995). Thus, oxidative stress induced by acute exercise elicits difference responses, which likely depend on tissue type of organ and its endogenous antioxidant level.

It is believed that DAS may possibly suppress lipid peroxidation in muscles subjected to exhaustive exercise-induced oxidative stress. Accordingly, none of the previous study investigated the effect of muscle lipid peroxidation and antioxidant enzyme activities followed by pretreated diallyl sulfide and exhaustive exercise. Therefore, the protective roles of diallyl sulfide on acute exhaustive exercise induced-lipid peroxidation in diaphragm, fast, and slow twitch muscles in rats will be determined in the study. It is also hypothesized that respiratory muscle and skeletal

muscle may not response to oxidative stress in the similar pattern. Dissimilarity in the magnitudes of muscle lipid peroxidation of diaphragm, fast and slow twitch muscles induced by acute exhaustive exercise is also expected.

Objectives

1. To determine the effects of exhaustive exercise on lipid peroxidation profiles in plasma, diaphragm, fast, and slow twitch muscles in rats.

2. To investigate the effects of diallyl sulfide supplementation on lipid peroxidation from acute exercise induced-oxidative stress in plasma, diaphragm, fast, and slow twitch muscles in rats.

3. To define the protective roles of diallyl sulfide supplementation on antioxidant contents and enzymes activity in plasma, diaphragm, fast, and slow twitch muscles in rats.

Benefits of the Study:

It is expected that:

- Daily consumption of DAS may significantly enhance antioxidative effect in some skeletal muscles.
- These studies will provide information on the effective dose and duration of DAS supplementation.
- Adverse effects of exhaustive exercise-induced deterioration of muscles will be diminished by continuously administration of DAS.

CHAPTER 2
LITERATURE REVIEW

1. Reactive Oxygen Species

Reactive oxygen species is a general term for molecular oxygen-derived molecules that are reactive species or that are converted easily to reactive species; some reactive oxygen species are free radicals (Radak, 2000).

1.1 Free radicals

1.1.1 Superoxide radical

The superoxide radical is produced from one electron reduction of oxygen, as shown previously (Bielski 1978) and can pass through biomembranes only via an anion channel (Lynch and Fridovich 1978). It also undergoes a dismutation reaction, which can be written overall as:

$$2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

The superoxide radical can be produced chemically and enzymatically. The superoxide radical is formed in almost all aerobic cells (Fridovich 1983). A major source is leakage from the oxygen reduction pathways in the electron transport chains of mitochondria and endoplasmic reticulum. The oxygen uptake is due to the activation of a nicotinamide adenine dinucleotide phosphate (reduced form, NADPH) oxidase complex bonded to the plasma membrane (Babior 1978). The xanthine oxidase system damages biomolecules, including deoxyribonucleic acid (DNA), hyaluronic acid, collagen, in addition to, organells, cells, and organs. Reactive oxygen species relevant to biological systems are listed in Table 1.

Table 1. Reactive oxygen species relevant to biological system

Reactive Oxygen Species	Molecular formula	Formation
Free radical species		
Superoxide radical	O_2^-	One-electron reduction of ground state molecular oxygen
Nitrogen monoxide	NO	One-electron reduction of nitrite
Hydroperoxyl radical	HOO•	Protonation of the superoxide radical.
Hydroxyl radical	HO•	One-electron reductionof hydrogen peroxide and three-electron reduction of the ground state molecular oxygen.
Peroxyl radical	ROO•	One-electron oxidation of hydroperoxide.
Nonradical species		
Hydrogen peroxide	H_2O_2	Two-electron reduction of ground state molecular oxygen, followed by protonation, and protonation of the peroxide ion.
Peroxynitrite	$ONOO^-$	Reaction of nitrogen monoxide with the superoxide radical
Hydroperoxide	ROOH	Autoxidation and singlet oxygen oxygenation of unsaturated compounds
Ozone	O_3	Oxidation of ground state molecular oxygen with atomic oxygen formed by photolysis of ground state molecular oxygen
Hypochlorous acid	HClO	Hydrolysis of molecular chlorine

(source: Radak, 2000)

1.1.2 Hydrogen peroxide

Hydrogen peroxide is a relatively stable compound. Hydrogen peroxide can pass through biological membranes (Fridovich 1983; Halliwell and Gutteridge 1984). Microsomes from animal tissues have shown to produce the superoxide radical and hydrogen peroxide at high rate in the presence of NADPH. These species largely arise from the NADPH-cytochrome P-450 reductase-cytochrome- P-450 system (Terelius and Ingelman-Sundberg, 1988).

1.1.3 Hydroxyl radical

The hydroxyl radical is produced by radiolysis of water under high-energy ionizing radiation and is produced mainly in biology systems by reductive cleavage of hydrogen peroxide in the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$$

Once formed *in vivo*, the hydroxyl radical may react rapidly with almost any biomolecule that is near its formation site. However, the hydroxyl radical has extremely high reactivity and hence must be short-lived *in vivo* (Mello Filho *et al*., 1984).

2. Lipid Peroxidation

Lipid peroxidation may be a potent attribute of the injury caused by free radical attack of cells. Lipid peroxidation is initiated when free radicals rapidly abstract hydrogen atom from esterified polyunsaturated fatty acid in cell membranes and lipoproteins as well as free fatty acids (Hochstein and Emster, 1963).

2.1 Radical chain reactions

2.1.1 Initiation

When a free radicals ($\mathbf{R}^{\bullet}$) removes a bis-allytic hydrogen atom from a polyunsaturated lipid (**LH**), a carbon-centered free radical ($\mathbf{L}^{\bullet}$). Thus generate and the process of lipid peroxidation is initiated. The carbon-centered, $\mathbf{L}^{\bullet}$ reacts at a diffusion-controlled rate with oxygen to form a peroxyl radical. This reaction is accompanied by rearrangement of the double bonds to produce a conjugated diene (Aust *et al.,* 1985). Initial process is present in Figure 1.

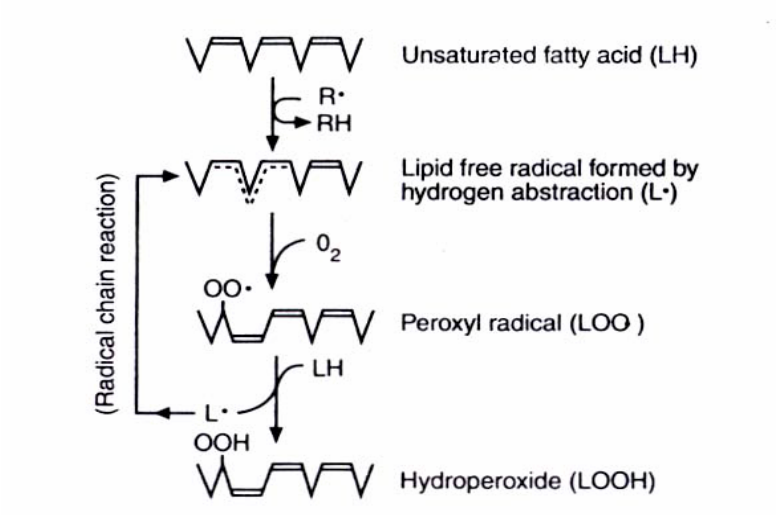


Figure 1. Initial process of lipid peroxidation in unsaturated fatty acids. (Radak, 2000)

2.1.2 Propagation

The peroxyl radical, **LOO•**, formed in the initial reaction may then remove a hydrogen atom from another polyunsaturated fatty acids, consequentially producing a lipid hydroperoxide (**LOOH**) and another **L•**. The stable products of this process are hydroperoxides (Barber and Bemheim, 1967; Dahle *et al.*, 1962; Pryor, 1973).

2.1.3 Termination

When two free radicals meet and interact, nonradical species can be formed terminating the lipid peroxidation process (equations 1-3):

1. $L^{\bullet} + L^{\bullet} \rightarrow LL$
2. $LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2$
3. $LOO^{\bullet} + L^{\bullet} \rightarrow LOOL$

3. Exercise Induced-Biomarkers of Oxidative Stress

3.1 Indicator of muscle damage

Elevations of muscle enzymes such as lactate dehydrogenase (LDH) in plasma are characteristic responses to strenuous exercise and often used as indicators of muscle damage.

3.1.1 Lactate dehydrogenase

Lactate dehydrogenase (LDH) is serum enzyme used as indirect indicator of tissue membrane damage (Yagi 1992). LDH catalyze inter-conversion of lactate and pyruvate in the presence of NADH or $NADH_2$. LDH consists of five separable isoenzymes, each made of tetramer of two subunits, H and M. LDH-1 is present in high concentration in heart muscle, whereas LDH-2 is present in kidney cortex. LDH-3, -4, -5 are present in skeletal muscle and liver (Yagi 1992).

3.2 Muscle damage and lipid peroxidation

Lipid peroxidation appears to be an important mechanism underlying exercise-induced muscle damage. Maughan and co-workers (1989) had subjects performed a 45-min bout of downhill running and found that plasma TBARS were significantly elevated at 6 h and CK (Creatine Kinase) peaked at 24 h post-exercise. It is worth noting that those subjects with the greatest increased in CK also had the most TBARS.

In animals, a number of studies have demonstrated the direct measurement of lipid peroxidation in tissue with exhaustive exercise during the last decades. Frankiewicz-Jozko and co-workers (1996) reported that exhaustive running increased TBARS in soleus muscle, and heart on untrained rats.

3.3 Exercise-induced production of free reactive oxygen species

It is now widely accepted that many of the disorders at the cell, tissue, or organ levels observed either immediately after heavy exercise or during post-exercise recovery may be attributed to ROS generation. Several biochemical pathways, which may be activated under different physiological conditions and in different organs,

tissues, and cellular locations, have been either identified (Jenkins, 1988; Jenkins, 1993; Meydani and Evans, 1993; Ji, 1995).

3.3.1 Mitochondrial electron-transport chain

It is hypothesized that ROS is produced within mitochondria (Figure 2). The majority of oxygen consumed is reduced in the mitochondria through the electron-transport chain (ETC) where both NADPH-ubiquinone reductase and ubiquinone-cytochrome *c* reductase generate O_2^- and H_2O_2 (Chance *et al.,* 1979). During maximal exercise, whole-body oxygen consumption (VO_2max) can increase up to 20-folds, while VO_2 at the muscle fiber level is elevated by much as 100-folds above the resting level (Meydani and Evans 1993). From these findings, two ideas of direct evidence supporting the mitochondrial hypothesis of ROS production are: (a) the ROS detected in the various studies are indeed generated in the mitochondria from exercised animals; and (b) mitochondial production of ROS is quantitatively related to O_2 consumption and workload (Meydani and Evans 1993; Ji *et al.* 1992).

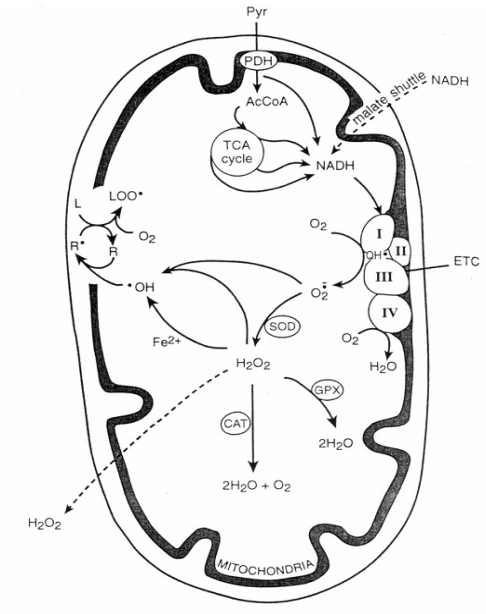


Figure 2. Generation of reactive oxygen species in the mitochondria. CAT, catalase; ETC, electron-transport chain; GPx, glutathione peroxidase; LOO^-, lipid peroxy radical; PDH, pyruvate dehydrogenase complex; QH^-, semiquinone; R^-, alkyl radical; SOD, superoxide dismutase. (Ji, 1995).

3.3.2 Xanthine/Xanthine oxidase pathway

Xanthine oxidase (XO) catalyzed reactions have been well established as one of the major sources of free radical generation in the ischemic-reperfused (I-R) heart (Downey 1990). During ischemia, adenosine triphosphate (ATP) is degraded to adenosine diphosphate (ADP) and adenosine monophosphate (AMP). Without sufficient oxygen to replenish ATP by oxidative phosphorylation, AMP is continuously degraded leading to accumulation of hypoxanthine, which is converted to xanthine and uric acid by xanthine oxidase (XO), couple with the one-electron reduction of O_2 and generation of O_2^- and H_2O_2 (Kuppasamy and Zweier 1989; Hearse *et al*., 1986). The model of I-R tissue apparently provides all these requirements (Figure 3).

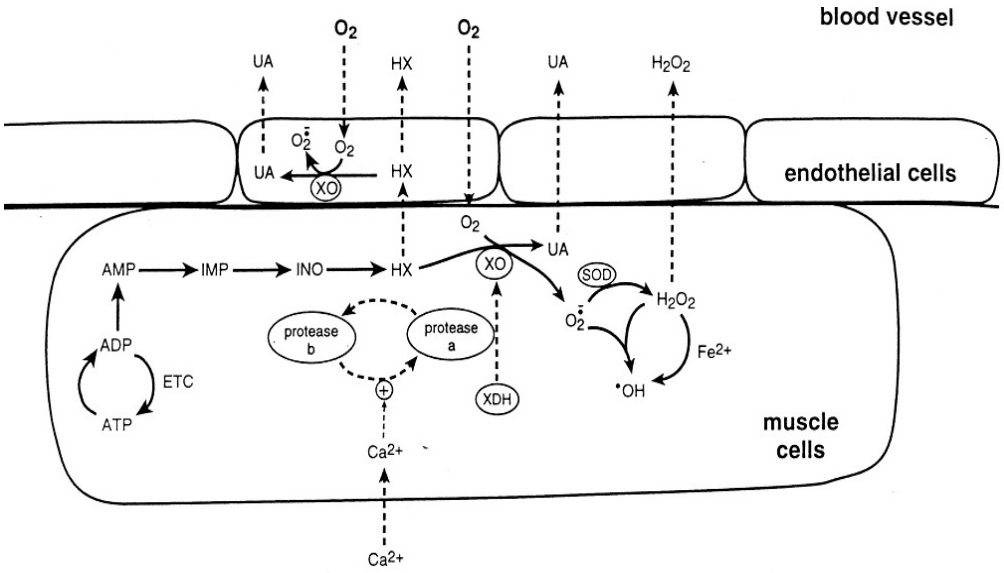


Figure 3. This figure showed the role of xanthine oxidase (XO) in free radical generation in the muscle and endothelial cells. ECT, electrons transport chain; HX, hypoxanthine; UA, uric acid; XDH, xanthine dehydrogenase (Ji, 1995).

Radak and co-workers (1995, 1996) showed that XO activity was increased 10-folds in the plasma and skeletal muscle of the rats after repeated high-intensity running to exhaustion, and plasma XO activity correlated to lactate concentration. Thus, XO hypothetically merit at least under the conditions that

skeletal muscle encounters an adenine nucleotide deficit and /or hypoxia followed by reoxygenation (Ji, 1995).

3.3.3 Involvement of neutrophils

Number of researches on exercise and immune function has been reported it became clear only recently that ROS might be involved in tissue inflammatory response to injury and that polymorphoneutrophils (PMNs) play a key role in this process (Meydani and Evans, 1993; Cannon and Blimberg, 1994). Figure 4 shows the process of neutrophil activation and ROS production during an acute-phase response in the cell.

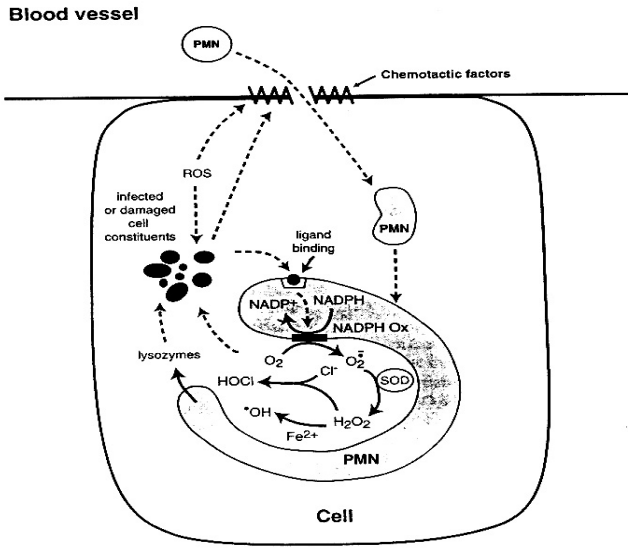


Figure 4. This figure showed process of polymorphoneutrophil (PMNs) infiltration and activation in the cell. NADPH OX, NADPH oxidase; ROS, reactive oxygen species; SOD, superoxide dismutase. (Ji, 1995).

Strenuous exercise has long been recognized to cause of muscle injury accompanied by post-exercise soreness and inflammatory response, which is characterized by increased protease and lysozymal enzyme activities in working muscle (Salminen and Vihko, 1983).

4. Antioxidant Defense Mechanisms

Cellular antioxidant defenses are conventionally classified into two categories: enzymatic and non-enzymatic types. Primary antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) (Chance *et al*., 1979). A number of enzymes involve in the supply of substrates and reducing power (NADPH) for primary antioxidante enzyme, such as glutathione reductase (GR). Glutathione sulfur-transferase (GST) conjugates glutathione (GSH). Non-enzymeatic antioxidants such as vitamin E, vitamin C, and β-carotene directly scavenge O_2^- and •OH (Yu, 1994; Meister and Anderson, 1983).

4.1 Classification of Antioxidant enzymes

4.1.1 Superoxide dismutase (SOD)

Superoxide dismutase was discovered by McCord and Fridovich in 1969 (McCord and Fridovich, 1969). It represents a family of metalloenzymes that catalyzes a common one-electron dismutation of O_2^- to H_2O_2.

$$\mathbf{2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2}$$

There are three types of SOD, depending on the metal ion bound to its active site. Copper- and zinc-containing SOD (CuZn-SOD) is a highly stable enzyme found primarily in the cytosolic compartment of the eukaryotic cells. CuZn-SOD is a dimmer (Fridovich, 1995). Manganese-containing SOD (Mn-SOD) is a tetramer with a much larger. Mn-SOD is present in the mitochondrial matrix of eukaryotes. Its primary function is to remove O_2^- generated outside cell membrane (Fridovich, 1995; Ohno *et al*., 1994). Among different muscle fibers, CuZn-SOD mRNA levels are the highest in type 1 muscle (soleus), followed by mixed muscle fiber type (diaphragm) and then type2 muscle (vastus lateralis and gastrocnemius) (Ohno *et al*., 1994).

4.1.2 Glutathione peroxidase (GPX)

Glutathione peroxidase (GPX) catalyzed the reduction of H_2O_2 and organic hydroperoxide to H_2O and alcohol, respectively, by using GSH as the electron donor. (Flohe, 1982)

$$2GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$$

In mammalian cells, GPX catalyzed the reduction of both hydrogen and organic peroxides such as lipid peroxide by GSH, forming water and alcohol, respectively. GSH is oxidized to glutathione disulfide (GSSG) (Reed, 1986). GPX is highly specific for its hydrogen donor GSH but has low specificity for hydroperoxide (Flohe, 1982). GPX is located in both the cytosol and mitochondrial matrix of the cell. The activity of GPX is high in the liver and erythrocytes; moderate in the brain, kidney, and low in skeletal muscle (Chance *et al*., 1979).

4.1.3 Catalase (CAT)

The primary reaction that catalase (CAT) catalyzes is the decomposition of H_2O_2 to H_2O. CAT has other biological functions (Chance *et al*., 1979). It shares this function with GPX, but the substrate specificity and affinity as well as the cellular location of the two antioxidants enzyme.

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Catalase is a tetramer. Heme (Fe^{3+}) is a ligand required to bind to the enzyme's active site for its catalytic function (Halliwell and Gutteridge, 1989). CAT is located primarily in the organelle called peroxisome (Aebi, 1984). Among the various muscle types, type 1 muscle (soleus) displays the highest of CAT activity, followed by type IIa muscle, deep vastus lateralis (DVL). Type IIb muscle, superficial vastus lateralis (SVL) has the lowest of CAT activity (Halliwell and Gutteridge, 1989). The primary function of CAT is removed H_2O_2 which produced in the peroxisomes due to enzymes such as flavoprotein dehydrogenase in the β-oxidation of fatty acid (Chance *et al.*, 1979).

4.2 Antioxidant protection during acute exercise

Adequate antioxidant protection is crucial for the cell to avoid oxidative damage caused by ROS. Strenuous aerobic exercise is known to be associated with increased ROS production in skeletal muscle (Davies *et al*. 1982; Jackson *et al.* 1985). SOD, CAT, and GPX provide the primary defense against ROS generated during exercise, where activities of these enzymes increase in response to exercise in both animal and human studies (Jenkins 1983; Ji 1995; Sen *et al*. 1994). It is reported that acute bout of exercise increases SOD activity in the skeletal muscle (Ji et al. 1990; Lawler et al. 1993; Quintanilha and Packer 1983). The most studies also indicate that acute exercise increases CuZn-SOD rather than Mn-SOD activity (Quintanilha and Packer 1983).

GPX activity has demonstrated variable responses to an acute bout of exercise in various types of skeletal muscle. Several studies have shown no change in this enzyme in skeletal muscle after acute exercise (Brady *et al*., 1979; Ji *et al*., 1990; Leeuwenburgh and Ji 1995; Vihko *et al*., 1978), whereas others have reported significant elevation of GPX activity (Ji and Fu, 1992; Ji *et al*., 1992; Leeuwenburgh and Ji, 1996; Oh-ishi *et al*., 1996). Muscle fiber-specific responses of GPX have also been noticed. For example, Ji and co-workers (1992) showed that GPX activity increased as a function of treadmill speed in deep vastus lateralis and superficial vastus lateralis but not in the soleus. The mechanism responsible for the increased of GPX activity with acute exercise is stilled unknown.

The CAT activity, most studies reported no significant alteration in CAT activity with acute exercise (Ji 1995; Meydani nad Evans, 1993). CAT activity was found to increases significantly alter an acute bout of exercise to exhaustion or at high intensity in rats. Only in DVL muscle showed this activation, whereas SVL, soleus did not demonstrate any appreciable change with exercise (Ji and Fu 1992; Ji *et al.*, 1992).

4.3 Vitamins and low-molecular weigh antioxidants

Antioxidant vitamins and other low-molecular-weight antioxidants play an important role in breaking free radical chain-reaction and keeping cellular homeostasis during acute exercise.

4.3.1 Vitamin E

The important of vitamin E during exercise is best illustrated in studies. Davies and co-workers (1982) found that vitamin E-deficiency exacerbated muscle free radical production and enhanced lipidperoxidation and mitochondrial dysfunction in exhaustively exercised rats.

4.3.2 Vitamin C

The important of vitamin C in protecting against exercise-induced oxidative stress is not well established. Vitamin C also performs numerous functions that are not related to those of an antioxidant (Bendich and Langseth 1995). This is ascorbate react with transition metal ions to form ROS, including •OH (Halliwell and Gutteridge 1989; Yu 1994).

4.3.3 Ubiquinone (Q_{10})

As an electron carrier, ubiquinone is abundant in the mitochondrial inner membrane. Gohil and coworkers (1987) showed that exercise training could significantly increase ubiquinone content in skeletal muscle and adipose tissue. The reduced ubiquinone acts as an antioxidant in vitro, and exerts its role as an antioxidant in vivo has been proposed (Beyer 1994).

4.3.4 α-Lipoic Acid

α-Lipoic acid is a well-known cofactor for the oxidative decarboxylation catalyzed by ketoacid dehydrogenase. The α-Lipoic acid has exhibited specific scavenging capacity for a variety of free radicals, such as O_2^-, •OH, $^1O_2$, peroxyl radical, and hydrochlorous radical (Packer *et al.*, 1995).

4.4 Glutathione homeostasis

Glutathione (GSH) is a thiol-containing tripeptide found in high concentrations in virtually all animal and plant cells. The most important antioxidant function of GSH is to serve as a substrate for GPX to remove hydrogen and organic

peroxides such as lipid peroxidation. This reaction takes place in conjunction with GPX, thus providing a redox cycle for the regeneration of GSH (Flohe, 1982; Asuncion *et al*., 1996; Vina *et al*., 1995).

In skeletal muscle GSH concentration varies, depending on muscle fiber type and animal species (Ji, 1995; Ji *et al*., 1992). In the case of rat skeletal muscle, type I fiber (soleus muscle) contain 6-folds higher GSH content than type IIb fiber (white vastus lateralis muscle) (Deneke and Fanburg, 1989). Skeletal muscle is an important for GSH pool. Instead, the large muscle mass of the body (approximately 40% of the body weight) (Kretzschwmar *et al*., 1992). Figure 5 illustrates the inter-organ GSH transport and regulation in mammals.

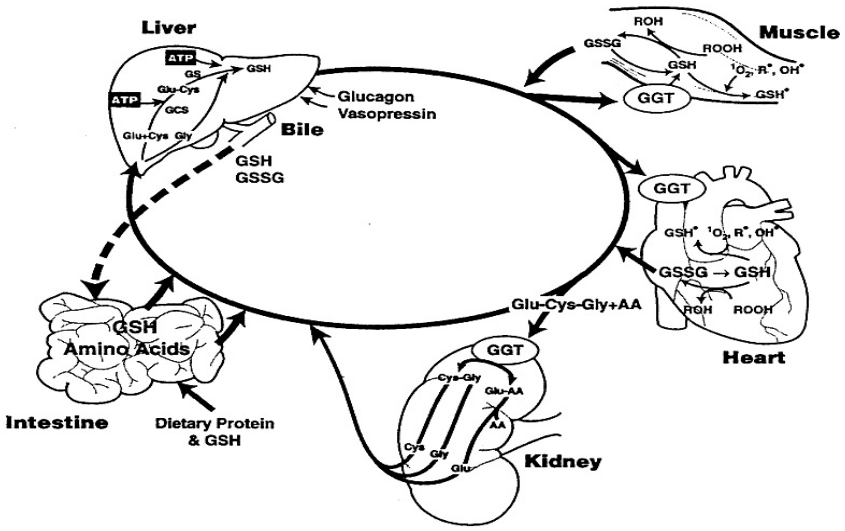


Figure 5. Postulated interorgan transport of glutathione. GSH, glutathione; GSSG, glutathione disulfide; GS, glutathione thiyl radical; ROOH, hydrogen peroxide; ROH, alcohol; GGT, γ-glutamyl transpeptidase (Ji, 1995).

4.5 Glutathione homeostasis during exercise

During heavy aerobic exercise, ROS production is increased in skeletal muscle, which requires more NADPH to regenerate GSH. All of these factors contribute to decreased GSH and an accumulation of GSSG in the cell. Indeed, an acute bout of exhaustive exercise has been shown to increases of GSSG content significantly in rat skeletal muscle. Accumulation of GSSG in exercise muscle is

associated with an activation of GPX and GR (Ji *et al.,* 1992; Ji and Fu 1992; Lew *et al*., 1985). On the other hand, prolong exercise at moderate intensity fails to change the level of GSSG in skeletal muscle in mice (Leeuwenburgh and Ji, 1995), and rat (Leeuwenburgh and Ji 1996), which indicating a relatively stable GSH homeostasis. In rat studies, showed that no changed in the GSH to GSSG ratio after acute bout of exhaustive exercise (Ji *et al.*, 1992, Leeuwenburgh and Ji 1995, Ji and Fu 1992, Lew *et al.*, 1985). It is now clear that some body tissues are capable of adapting to chronic exercise by increasing their GSH content.

5. Garlic

5.1 General description

Garlic (*Allium sativum*) has been used as a spice or medicinal herb. It was used as remedy for heart disease, tumors, and headaches are documented in the Egyptian Codex Ebers, dating from 1550 BC (Block, 1985; Leung, 1980).

5.2 Chemical composition

Garlic contains 0.1-0.36% of a volatile oil composed of sulfur-containing compounds: allicin, diallyl disulfide, diallyl trisulfide, and others. Other constituents of garlic include: alliin (S-allyl-L-cysteine sulfoxide), S-methyl-L-cysteine sulfoxide, protein (16.8%, dry weight basis), high concentrations of trace minerals (particularly selenium), vitamins, glucosinolates, and enzymes (alliinase, peroxidase, and myrosinase) (Leung, 1980; Raj and Parmar, 1977) Dimitrov and co-workers (1997) and Gwilt and colleagues (1994) also quantified various organosulfur compounds in aged garlic extract shows in Table 2.

Table 2. Organosulfur Compounds in Aged Garlic Extract.

Organosulfur Compounds in Aged Garlic Extract	
γ -glutamyl S-allyl cysteine	Ethyl -propenesulfinate
Alliin	Methyl disulfide
Allyl methyl disuflide	Methyl trisulfide
Allyl methyl sulfide	S-propenylcysteine
Allyl methyl trisuflide	S-allyl cysteine
Diallyl disulfide	S-methyl cysteine
Diallyl sulfide	S-allyl mercaptocysteine
Diallyl trisulfide	Cycloalliin

5.3 Pharmacological properties of aged garlic extract

Recent studies have validated many of the medicinal properties attributed to garlic. Epidemiological studies show an inverse correlation between garlic consumption and reduced risk of gastric and colon cancer (Steinmetz *et al*., 1994). Garlic has been shown to have antithrombotic activity (Block, 1985), lower blood lipids and have a cardio-protective effect (Neil and Sigali, 1994). The mechanisms of garlic have been ascribed to its potent antioxidant action (Wei and Lau, 1998; Yang *et al.,* 1993).

5.3.1 Antioxidant effect of aged garlic extract

Aged garlic extract and its various constituents have demonstrated an array of antioxidant and radio-protective effects in many studies (Horie *et al.*, 1989; Ide *et al.*, 1996; Amagase *et al*., 2000). Dially sulfide (DAS), one of a number of organosulfur compounds accounting for the flavor and odor, is a lipophilic thioether, which is derived from oxidize allicin. They have been shown to protect liver cells from lipid peroxidation and vascular endothelial cells from oxidant injury. Further, they have been shown to enhance antioxidative enzyme systems in cells. They have

been shown to scavenge hydrogen peroxide, to inhibit the formation of TBARS. Horie and colleagues (1989) found a reduction in TBARS suggesting an antioxidant effect of the aged garlic extract. Effectiveness of DAS could be due to its ability to scavenge oxygen free radicals (Fanelli *et al*., 1998; Horie *et al*., 1992; Imai *et al*., 1994). The antioxidant protection of DAS is exerted via modulating antioxidant-related enzymes such as 3-hydroxy-3-methyglutaryl-COA reductase, glutathione-s-transferase, and catalase (Dwieli *et al*., 1996; Borek, 2001; Yeh *et al*., 2001).

The antioxidant enzymes superoxide disumutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) play an important role in scavenging oxidants and preventing cell injury.

5.3.2 Antibacterial activity of aged garlic extract

Previous studies have demonstrated that both garlic juice and allicin inhibited the growth of *Staphylococcus, Streptococcus, Bacillus, Brucella*, and *Vi-brio* species at low concentrations (Huddleson *et al*, 1944; Cavallito and Bailey, 1944). In more recent studies were found that effective antibiotic agents against many bacteria, as listed in Table 3 (Vahora *et al.*, 1973).

Table 3. Microbes Inhibited by Garlic.

Bacteria
Staphylococcus aureus
Alpha- and beta-hemolytic Streptococcus
Escherichia coli
Proteus vulgaris
Salmonella enteritidis
Citrobacter sp.
Klebsiella pneumoniae
Mycobacteria
Fungi
Candida albicans
Cryptococcus neoformans
Helminths
Ascaris lumbricoides
Hookworms
Viruses
Human rhinovirus type 2

(Sources: Adetumbi and Lau, 1983; Koch, 1993; Hughes BG, Lawson, 1991; Elnima *et al*., 1983)

CHAPTER 3
MATERIALS AND METHODS

A. Animals

Male Sprage Dawley rats (n = 210), 50-65 grams body weight, 3 weeks olds were supplied by the National Laboratory Animal Center, Mahidol University, Salaya Nakonpathom. The animals were allowed to familiar with the new environment for one week after arrival at the Animal Center, Faculty of Science, Mahidol University. The animals were kept in a control condition room where temperature was approximately 25 ±2 C° and relative humidity at approximately 65%. All animals were separately housed in stainless steel cages and fed *ad libitum* with standard regular rat chow diet [Animal Nutrition Research and Development Center (ANRDC), Kasetsart University, Thailand] and with free access to water. Every afternoon rats were brought together to the treadmill to make them recognizable with the running track. Mahidol Ethics Committee on Animal Experiment approved the experimental protocol for this study. Rats were weighed at the beginning of experiment and every week throughout the experiment. Diet compositions revealed that there was no garlic-related component (Table 4).

B. Chemical Reagents

All analytical grade chemical reagents were used throughout the investigation. Reagents for determination of Glutathione peroxidase (GPX), Catalase (CAT), Superoxide dismutase (SOD), Malondialdehyde (MDA), Glutathione (GSH), and protein content were obtained from Sigma Chemical Co. (St. Louis, MO, USA); Lactate dehydrogenase (LDH) was obtained from Randox Laboratory (Antrim, United Kingdom); Hydrogen peroxide (30% w/v) and acetic acid (100%) were obtained from Merck (Darmstdt, Germany).

Table 4. Compositions of rat chow diet

Composition	Amounts	Composition	Amounts
Biotin	0.4 mg/kg	Pantothenic acid	60 mg/gk
Calcium	1.0 %	Phosphorus	0.9 %
Choline Chloride	1,500 mg/kg	Potassium	1.17 %
Cobalt	1.82 ppm.	Potassium iodide	1 ppm.
Copper	22 ppm.	Selenium	0.1 ppm.
Crude Protein	24 %	Sodium	0.20 %
Fat (minimum)	4.5 %	Vitamin A	20,000 i.u./kg
Fiber (Maximum)	5 %	Vitamin B_1	20 mg/kg
Folic acid	6 mg/kg	Vitamin B_2	20 mg/kg
Iron	180 ppm.	Vitamin B_6	20 mg/kg
Magnesium	0.23 %	Vitamin B_{12}	0.036 mg/kg
Manganese	171 ppm.	Vitamin D	4,000 i.u./kg
Metabolizable energy	3,040 kcal/kg	Vitamin E	100 mg/kg
Moisture (maximum)	12 %	Vitamin K	5 mg/kg
Niacin	100 mg/kg	Zinc	100 ppm.

C. Acute Exercise Protocol

To ensure that animals would complete the exercise protocol, each animal in exercise group was familiarized with running trial on an animal motor-driven treadmill for 5 min/day at a speed of 20 m/min., 0% grade, for one week prior to the experiment (Ramires and Ji, 2001). To keep animal running, stimulation was achieved using hair- dryer whenever it was needed. On the final day, rats in exercise groups were subjected to running exercise on the same motor driven-treadmill at speed 20 m/min, 0% grade until exhaustion. Result from the pilot study revealed that time to

exhaustion was approximately 60 ±15 minutes. Thus animals in exercise group were exposed to exhaustive run up to 75 minutes. Exhaustion was identified when the animals repeatedly failed to sustain their running pace of over than five time within two minutes period despite the stimulation by the hair-dryer and the righting reflex was loss when animal was being turned on their back (Ji *et al*., 1999).

D. Diallyl Sulfide (DAS)

Oil soluble component of garlic extract, diallyl sulfide (DAS) with 97% purity was obtained from Fluka Chemical, USA. The dose of 50 mg/kg body weight (Hu *et al.,* 1996) or 200mg/ kg body weight (Chen *et al*., 1999) was separately used and freshly prepared by dissolving in 4 ml corn oil/kg body weight. Corn oil was a product of the Mazola, CPC International, Inc. (Englewood Cliffs, NJ, USA), which was normally used as mixture in similar experiments (Numagami *et al*., 1996; Guyonnet *et al*., 1999; Sheen *et al*., 1999). Daily feeding of DAS was orally performed using a small oral-gastric intubation needle for the entire 4 weeks period. The intubation needle (3 inches, 16 gauge) with a round tip attached to a syringe was passed down via esophagus to the stomach. The control rats received the same amount of pure corn oil in a similar manner to DAS supplementation group.

E. Supplement Protocol

Rats are orally administered DAS on the daily basis at 50 and 200 mg/kg body weight in 4 ml corn oil for the periods specified in each study. Control rats were received 4 ml corn oil/kg body weight only.

F. Body Weight Determination

Body weight was determined, using Triple Beam Balance (Ohaus Scale Cooperation, NI, USA), every week in all treatment groups throughout the

experimental period, before the supplementation with DAS and/or corn oil and before exercise.

G. Euthanasia

The time of the day when rats were sacrificed was consistent to eliminate the possible diurnal effects on experimental result. At the time of sacrifice, the animal was anesthetized with intravenous (tail vein) sodium pentobarbital (S.S.N.A-La Ballastiere, France) injection, at the dose of 20-35 mg/kg body weight. After anesthetized and loss of motor reflex was confirmed, a small incision at the lateral side of neck was exposed, and the jugular vein was cannulated and perfused with 0.9% NaCl for 5 minutes at constant pressure of 15 cmH_2O (Wang *et al*., 2000). The posterior vena cava was cut as an outlet for fluid drainage at opening abdominal wound. This procedure removes most of the red blood cells, which resulted in a light tan in color of organs.

H. Tissue Collection

Diaphragm, soleus, and rectus femoris muscles were excised immediately. Adipose tissues and connective tissues are subsequently trimmed and excluded. The selected tissue was rinsed in ice-cold saline, weighed and rapidly submerged in dry ice. Muscles preparation was performed within 2 minutes. Samples were stored at – 80 °C for subsequent biochemical analysis.

I. Preparation of Whole Tissue Homogenate and Subcellular Fraction

Diaphragm, soleus, and rectus femoris muscles were minced with scissors. Pieces of the tissue sample were then put as fast as possible in homogenizing buffer at the ratio 1 gram tissue per 9 ml. Homogenization was performed in a glass Potter-Elvehjem tissue grinder. The homogenization was performed for 15 strokes with the

pestle rotating at 450 rpm in 2 minutes at 4 °C. Whole homogenates were kept on ice and used for lipid peroxidation assay (Malondialdehyde content assay). To remove unbroken cells, nuclei, and cell debris, the cytosol fraction that was used in the assay of SOD, GPX, and CAT activities were centrifuged at 3,000 rpm for 10 minutes at 4 °C using Sorvall RT 6000D. The supernatant fraction was further centrifuged at 15,000 rpm for 30 minutes at 4 °C by using Refrigerator Sorvall Super T21 Centrifuge to removed mitochondria and lysosomes. The resultant supernatant fraction was then centrifuged at 45,000 rpm for 60 minutes at 4 °C using Optima ™ LE-80K Ultracentrifuge (Beckman Coulter). The final cytosolic supernatant was stored at –80 °C for later analysis.

J. Blood Collection

Before rat was perfused, 4-5 ml of blood was withdrawn from the aorta at abdominal part into heparinized syringe. Plasma was obtained by centrifugation of the collecting blood at 3,000 rpm for 10 minutes at 4 °C. The plasma supernatant was collected and kept at –80 °C for later analysis.

K. Experimental Procedure

Male Sprague-Dawley rats were randomly separated into group 1 (control group: Four ml corn oil/kg body weight), group2: acute exhaustive exercise (4 ml corn oil/kg body weight), group3: DAS supplementation (with 50 and 200 mg in 4 ml corn oil/kg body weight), and group 4: DAS + exhaustive exercise (same amount of DAS at 50 and 200 mg in 4 ml corn oil/kg body weight). The supplementation periods were routinely and continuously performed for 28 days in study A, and 8 weeks for study B and C. Numbers of rats in each group in study A were serially sacrificed at the end of 28 days pre-treated period, 0, 6, 12, 24, 36, 48, and 72 hours after exhaustive exercise. Rats in each group of studies B and C were sacrificed at the end of every week throughout the 8 weeks period, and the lowest MDA level, group 2, specified from study A.

Study A

- To find out muscle MDA responses following exhaustive exercise.
- To determine MDA profile in muscles and plasma in rats treated with DAS and exercise.

In this study, rats were divided into 4 groups:

Group 1: Control group: corn oil 4 ml /kg body weight

Group 2: Acute exhaustive exercise + corn oil 4 ml /kg body weight

Group 3: DAS supplementation for 4 weeks

3.1: 50 mg in corn oil 4 ml /kg body weight

3.2: 200 mg in corn oil 4 ml /kg body weight

Group4: DAS supplementation 4 weeks + acute exhaustive exercise

4.1: 50 mg in corn oil 4 ml /kg body weight

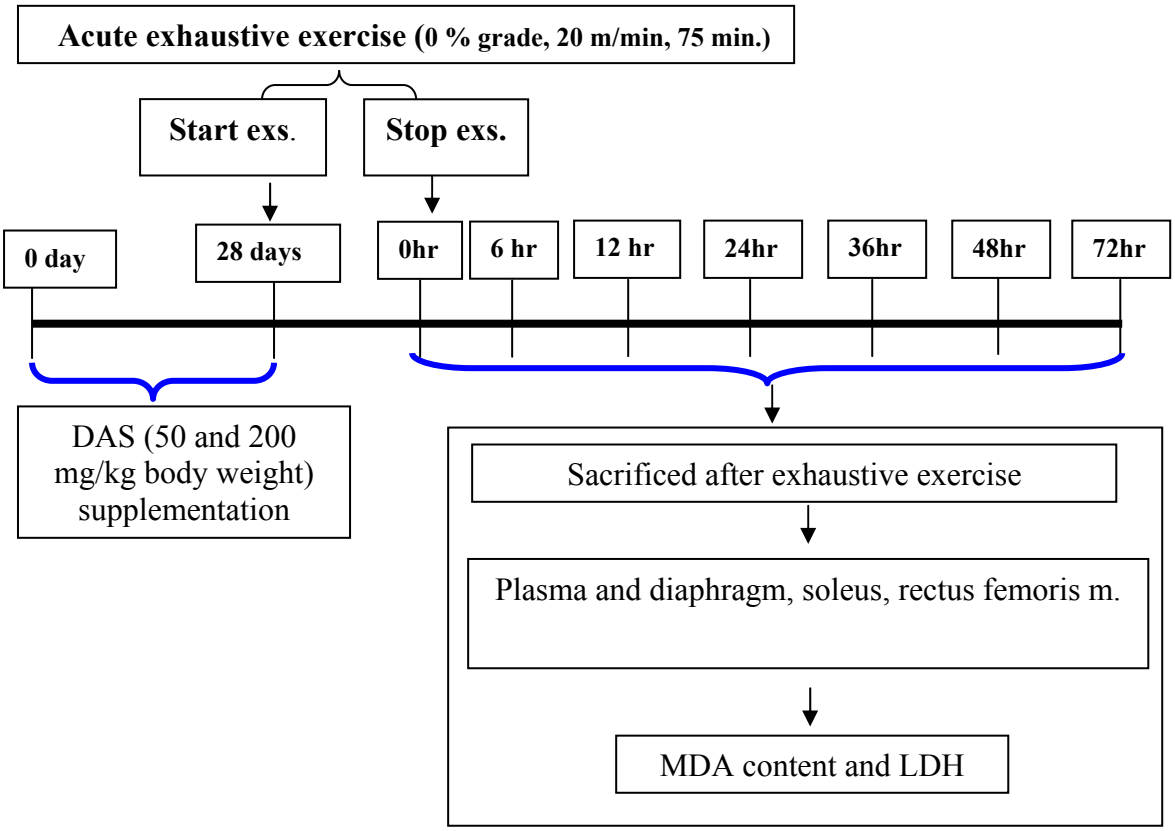
4.2: 200 mg in corn oil 4 ml /kg body weight

Body weight is determined every week throughout treatment in all groups. Rats are randomly sacrificed for muscle and blood collection at 0, 6, 12, 24, 36, 48, and 72 hours post-exercise.

Biochemical analysis

Plasma, diaphragm, soleus, and rectus femoris muscles were analyzed for MDA content and lactate dehydrogenase (LDH) at 0, 6, 12, 24, 36, 48, and 72 hours post-exercise.

Experimental Diagram for Study A:



Study B:

- To investigate time-course response of the most effective dose of DAS supplementation in selected muscle, specified from study A.

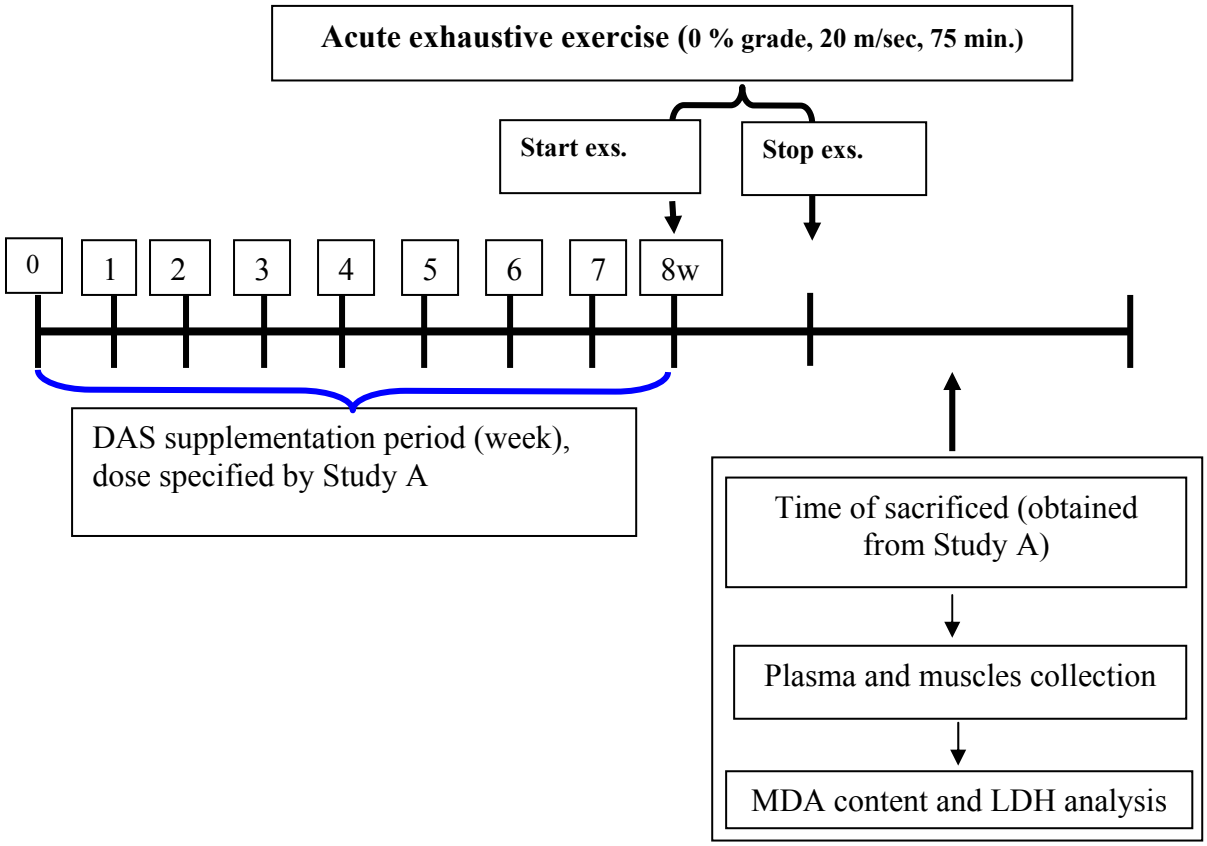
In this study, rats were divided into two groups:

Group B1 (control): corn oil 4ml/ kg body weight (8 wks) + acute exhaustive exercise

Group B2 (DAS: dose obtained from study A): corn oil 4 ml/kg body weight (8wks) + acute exhaustive exercise.

On a weekly basis, plasma, diaphragm, soleus, and rectus femoris muscles of each group were analyzed for MDA content and lactate dehydrogenase (LDH).

Experimental Diagram for Study B:



Study C:

- To determine the effects of DAS on antioxidant enzyme activity and protein contents.

In this study, rats were divided into 4 groups:

Group C1: Control group: corn oil 4 ml /kg body weight

Group C2: Exhaustive exercise group: corn oil 4 ml /kg body weight

Group C3: DAS supplementation group (dose specified from Study A) in corn oil 4 ml /kg body weight:

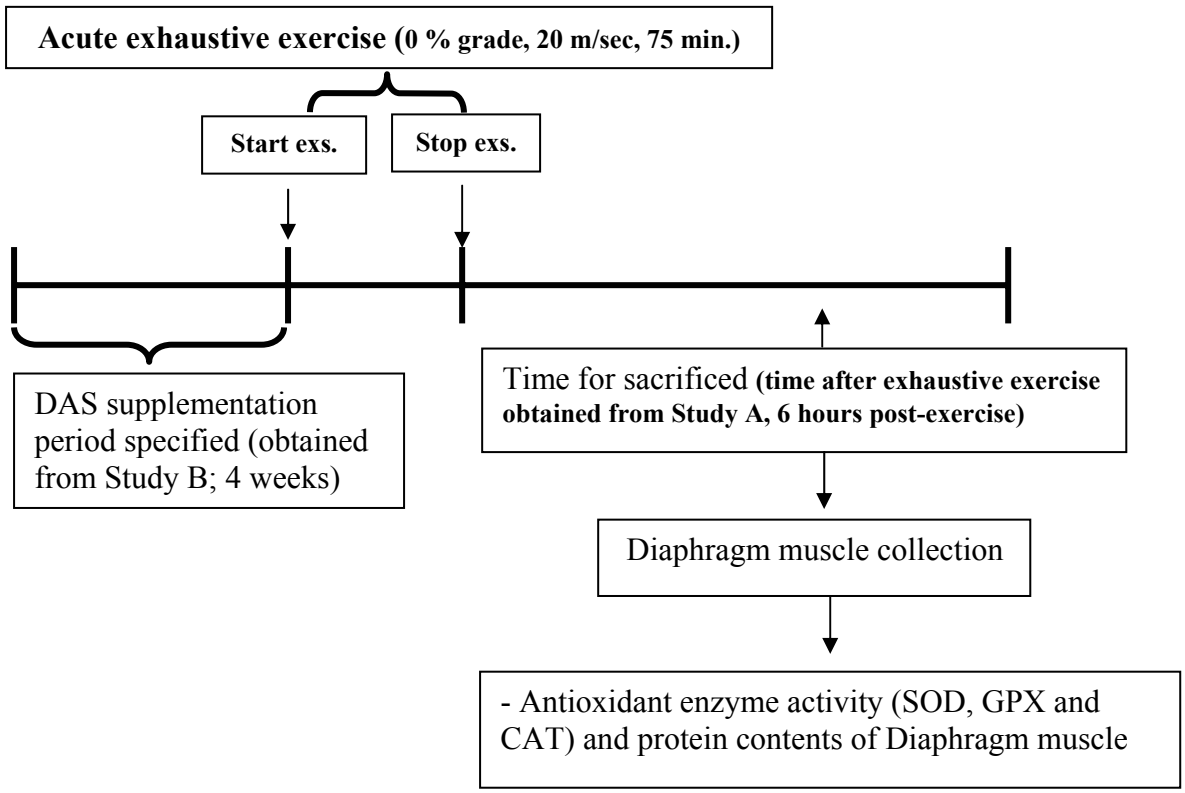
Group C4: DAS supplementation group (dose specified from Study A) in corn oil 4 ml /kg body weight + exhaustive exercise.

Body weight is determined every week throughout treatment in all groups. Rats were supplemented with DAS and were randomly sacrificed for muscle and blood collection specified by group 2 of Study A.

Biochemical analysis

Plasma is analyzed for MDA content and lactate dehydrogenase (LDH). The muscle, which specified by study1, is analyzed for MDA content, lactate dehydrogenase (LDH), and antioxidant enzymes (SOD, GPX, and CAT) activity and protein contents.

Experimental Diagram for Study C:



L. Chemical Analyses

Standard analyses of sample were used for the experiments as follow:

1. Lipid peroxidation (MDA) content in whole homogenates was determined by the thiobarbituric acid reaction as described by Ogawa and co-worker in 1979 (Appendix A).

2. Lactate Dehydrogeanse in plasma was determine by modified method of Amador *et al*. (Appendix B).

3. Cytosolic superoxide dismutase (SOD) activity was determined using the modified method of Winterbourn (1975) (Appendix C).

4. Cytosolic catalase (CAT) activity was determined using spectrophotometric method as described by Luck (1965) and Lew (1991) (Appendix D).

5. Cytosolic glutathione peroxidase (GPX) activity was determined by a modified method of Paglia and Valentine described by Tapple (1978) (Appendix E).

6. Glutathione (GSH) content was determined by a colorimetric method reported by Bouland and Chasseaud (1970) and Ellman (1959) (Appendix F).

7. Protein content was determined by a modification of the Lowry's method (Appendix G).

M. Statistical Analysis

Results of all biochemical determination were presented as means and standard error of the mean (SEM) using standard procedure. The statistical significance of differences between groups were investigated by General Linear Model (GLM) using *Post hoc* Multiple Comparison test. The statistical significance difference within the same group was detected by the repeated unpaired *t*-test. GLM contrast was applied to detect significant differences between rest and post-exercise states. The 0.05 probability level was used to determine the significance of all statistical tests.

CHAPTER 4
RESULTS

1. Body Weight Gain

Effects of two doses of DAS on the growth characteristics are shown in Table 5. The initial weight of rats in the control groups, 50 mg/kg BW DAS supplemented (DAS_{50}) and 200 mg/kg BW DAS supplemented (DAS_{200}) were 75 ±1.49, 76 ±1.79, and 73.7 ±1.67 grams, respectively, which showed no significant difference among the groups. Body weight of the three groups increased at the similar rate throughout the entire 4 weeks period with showed no significant difference among all groups. Rats, which received DAS supplementation showed a similar pattern of weight gain when compared to the control group and there were no significant differences between these three groups throughout the entire period of 4 weeks supplementation. The final mean body weight of rats in control, DAS_{50} and DAS_{200} groups were 244 ±3.59, 244.1 ±3.92 and 237.4 ±3.71 grams respectively.

Thus, daily DAS supplementation during the experimental 4 weeks period did not affect on body weight gain of rat in all treatment groups. At the end of the experiment, the body weight gain of the rats was not significantly different among three treatment groups.

Table 5. Body weight gain (grams) of rats in control, 50mg/kg body weight (DAS_{50}) and 200 mg/kg body weight (DAS_{200}) DAS supplemented groups at initial to 4^{th} week of experiment.

Group	Body weights (grams)				
	Initial	Week 1	Week 2	Week 3	Week 4
Control (n=42)	75.9 ±1.49	121.7 ±1.13	162.1 ±1.95	205.3 ±3.28	244.3 ±3.59
DAS_{50} (n=42)	76.1 ±1.79	123.1 ±1.28	170.6 ±2.19	209.5 ±3.15	244.1 ±3.92
DAS_{200} (n=42)	73.7 ±1.67	120.6 ±1.18	165.4 ±1.83	204.5 ±3.12	237.4 ±3.71

Values are mean ±SEM. [a] Significant difference between control and DAS_{50} at $p< 0.05$; [b] significant difference between DAS_{50} and DAS_{200} at $p< 0.05$; and [c] significant difference between control and DAS_{200} at $p< 0.05$.

2. Malondialdehyde (MDA) Responses

Changes in the MDA content, an index of lipid peroxidation in diaphragm, soleus, rectus femoris muscles and plasma were studied in control, DAS_{50} and DAS_{200} treated rats at immediate, 6, 12, 24, 36, 48 and 72 hrs post-exercise.

2.1 Diaphragm and MDA

The resting values of MDA level in sedentary control, DAS_{50}, and DAS_{200} supplemented groups were similar. The values were 104 ±7.59, 102 ±6.08, and 98.53 ±2.72 nmol/100 mg protein, respectively which showed no significant among the groups. The level of MDA of the diaphragm muscle in six subgroups of the rats, sedentary control, exercise control, DAS_{50}, DAS_{50}-Exs, DAS_{200}, and DAS_{200}-Exs groups were presented in Figure 6.

As illustrated in Figure 6, exhaustive running caused of a significant increased in lipid peroxide contents of the diaphragm muscle in all groups of rats when compared to their corresponding sedentary control subgroups. In exercise control subgroup, the significantly increasing of post-exercise MDA content was initially presented at 6-hr, 12-hr, and 24-hr compared to the sedentary control

subgroup (Figure 6A) ($p< 0.05$). The MDA content were at 303.21 ± 9.22, 238.08 ± 9.62, and 193.34 ± 18.68 nmol/100 mg protein, respectively.

The MDA levels in DAS_{50}-Exs subgroup was significantly elevated at immediate, 6-hr, 12-hr, 24-hr, and 36-hr (Figure 6B) ($p< 0.05$). The MDA concentration were 133.48 ± 5.87, 186.92 ± 9.38, 121 .90 ± 9.35, 129.28 ± 6.88, and 115.81 ± 5.15 nmol/100 mg protein. Rats in DAS_{200}-Exs group showed slightly similar characteristic, in which it was significantly increased at 6-hr and 12-hr (Figure 6C) ($p< 0.05$) post-exhaustive exercise. The values were 222.80 ± 6.31 and 160.65 ± 3.99 nmol/100 mg protein.

The peak MDA content in exercise subgroups of treatment group was significantly observed at 6-hr ($p< 0.05$) after exhaustive exercise and the contents returned to a level near to the sedentary control group at 36-hr, 48-hr, and 24-hr in exercise control, DAS_{50}-Exs, and DAS_{200}-Exs groups.

In the DAS supplemented groups, lower levels of MDA were detected in diaphragm muscle where significantly lower MDA contents were found in DAS_{50}-Exs and DAS_{200}-Exs groups when compared to exercise control at 6-hr after exercise ($p< 0.05$). Moreover, MDA concentration in DAS_{50}-Exs was significantly lower than the MDA content DAS_{200}-Exs subgroup (186.92 ± 9.38 and 222.80 ± 6.31 nmol/100 mg protein, ($p< 0.05$)).

2.2 Soleus muscle and MDA

The level of MDA of the soleus muscle in six subgroups of rats, sedentary control, exercise control, DAS_{50}, DAS_{50}-Exs, DAS_{200}, DAS_{200}-Exs groups were presented in Figure 7.

The resting value of MDA content in sedentary, DAS_{50} and DAS_{200} supplemented groups were similar. The values were 83.14 ± 10.24, 93.92 ± 10.26, and 95.82 ± 12.47 nmol/100 mg protein, respectively. The resting values were slightly higher, but insignificantly.

As illustrated in Figure 7, exhaustive running caused a significant increased in MDA content of the soleus muscle in all groups when compared to control subgroups. In exercise control subgroup, the significantly increasing of post-exercise

MDA content was presented at immediate, 6-hr, and 12-hr when compared to the sedentary control subgroup (Figure 7A) ($p< 0.05$). The MDA contents were at 134.72 ± 11.04, 279.96 ± 4.26, and 168.74 ± 19.74 nmol/100 mg protein, respectively.

Table 6. Time course of 72 hours changes on MDA levels of diaphragm muscle after a acute exhaustive exercise in rats. Values are Mean ± SEM, (n=3).

Time after Exercise (hrs)	MDA levels (nmol/100mg protein)					
	Control		DAS 50 mg/ kg BW		DAS 200 mg/ kg BW	
	Sedentary	Exercise	Sedentary	Exercise	Sedentary	Exercise
0	104.98 ± 7.59	108.75 ± 13.45	102.76 ± 6.08	133.48 ± 5.87 [c]	98.53 ± 2.72	108.20 ± 10.70
6	105.33 ± 8.35	303.21 ± 9.22 [c, *]	106.61 ± 4.50	186.92 ± 9.38 [a,c]	121.01 ± 6.47	222.80 ± 6.31 [a,b,c,*]
12	57.95 ± 6.26	238.08 ± 9.62 [c, *]	90.59 ± 4.71	121.90 ± 9.35 [a,c]	95.64 ± 7.29	160.65 ± 3.99 [a,b,c]
24	67.90 ± 9.92	193.34 ± 18.68 [c, *]	75.03 ± 9.03	129.28 ± 6.88 [a,c]	79.71 ± 2.00	96.30 ± 8.07 [a,b]
36	80.36 ± 9.14	99.81 ± 27.01	72.68 ± 6.85	115.81 ± 5.15 [c]	71.37 ± 11.19	44.93 ± 4.75 [b,*]
48	74.74 ± 9.18	60.34 ± 17.75	52.54 ± 2.88	57.30 ± 3.14	75.44 ± 4.19	65.44 ± 6.89 *
72	47.63 ± 6.54	43.85 ± 11.93	43.84 ± 5.99	46.38 ± 4.81 *	53.57 ± 3.32	29.16 ± 1.22 [b,c, *]

[a] Significant difference from the control at $p< 0.05$

[b] Significant difference from the DAS_{50} at $p< 0.05$

[c] Significant difference between sedentary and exercise within the control, DAS_{50} and DAS_{50} at $p< 0.05$

* Significant difference from initial within the correspondent subgroup at $p< 0.05$

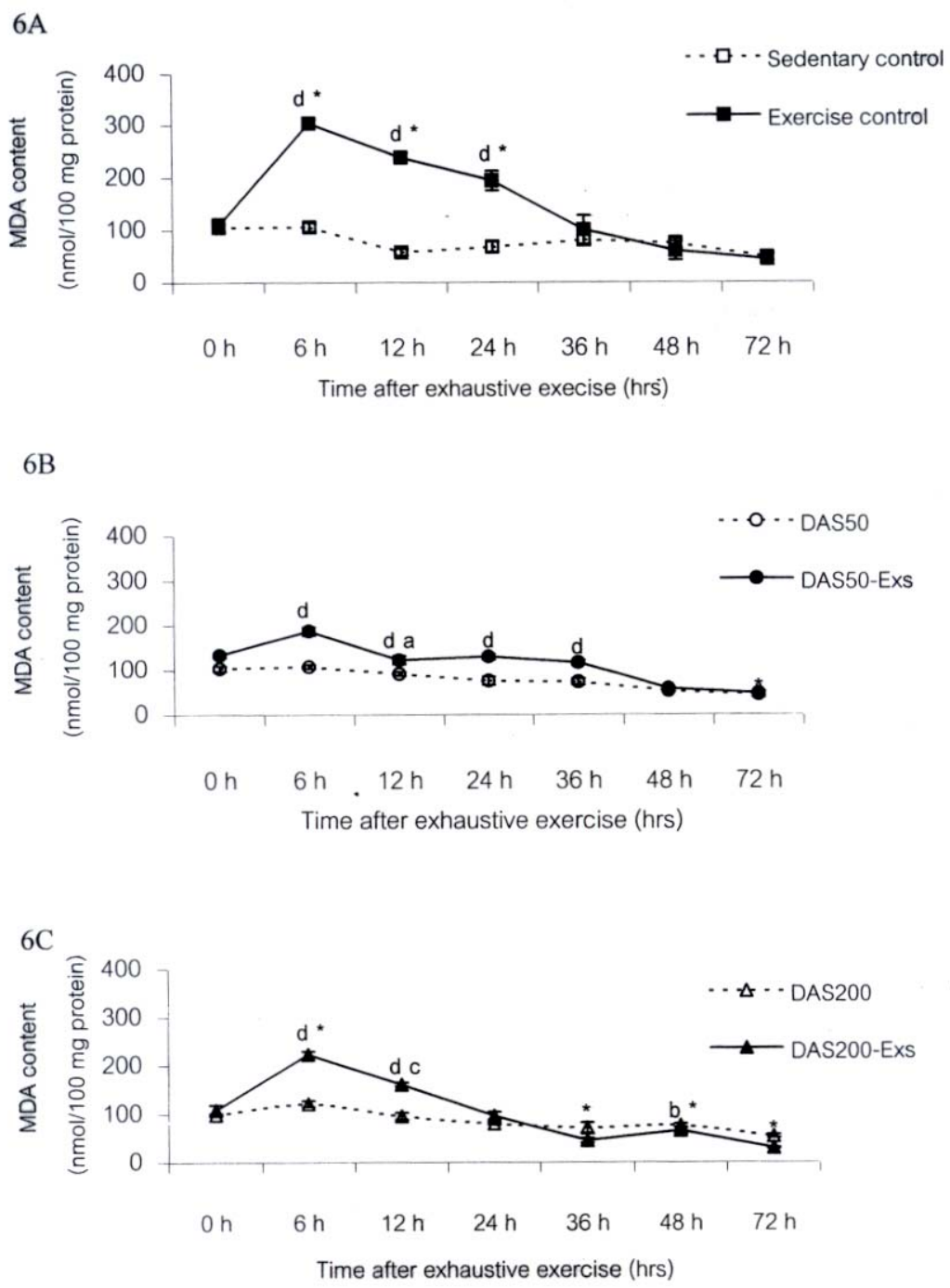


Figure 6. Time course change on MDA contents of the diaphragm tissues in control (A), DAS_{50} (B), and DAS_{200} (C) groups, (n=3). Open and closed symbols represent control and exercise subgroups respectively; [a] represents significant difference between control and DAS_{50} rats at $p< 0.05$; [b] represents significant difference between DAS_{50} and DAS_{200} rats at $p< 0.05$; [c] represent significant difference between control and DAS_{200} rats at $p< 0.05$; [d] represents significant difference between sedentary and exercise subgroups at $p< 0.05$; and [*] significant difference from the corresponding initial (time 0) values at $p< 0.05$.

The MDA levels in DAS_{50}-Exs subgroup was significantly elevated at 6-hr, 12-hr, 24-hr, and 36-hr (Figure 7B) ($p< 0.05$). The MDA contents were 227.49 ± 16.98, 134.34 ± 5.6, 111.87 ± 5.56, and 101.51 ± 4.9 nmol/100 mg protein, respectively. Rats in DAS_{200}-Exs subgroup presented similar pattern of MDA content, in which it was significantly increased at 6-hr post exercise (Figure 7C) ($p< 0.05$).

The peak MDA content in exercise subgroups of the three treated groups was significantly presented at 6-hr ($p< 0.05$) after post exhaustive running and the content of MDA returned to a level close to the level of sedentary subgroup at 36-hr in exercise control and DAS_{200}-Exs subgroups, whereas the significantly MDA elevation persisted to 48-hr in DAS_{50}-Exs ($p< 0.05$).

In the DAS supplemented groups, the MDA contents were detected in soleus muscle slightly significant lower in DAS_{50}-Exs and DAS_{200}-Exs subgroups when compared to exercise control at 6-hr after exhaustive exercise ($p< 0.05$). Moreover, the MDA content in DAS_{50}-Exs was insignificantly difference when compared with the MDA content of DAS_{200}-Exs subgroup.

2.3 Rectus femoris muscle and MDA

The level of MDA of the rectus femoris muscle in six subgroups of rats, sedentary control, exercise control, DAS_{50}, DAS_{50}-Exs, DAS_{200}, DAS_{200}-Exs groups were presented in Figure 8.

The resting values of MDA level in sedentary control, DAS_{50} groups were similar, whereas the MDA level in DAS_{200} group showed slightly higher than the other one. The resting MDA values were 46.16 ± 5.90, 59.43 ± 10.16, and 84.79 ± 5.92 nmol/100 mg protein. The resting value MDA content of DAS_{50} group was insignificantly difference when compared with sedentary control, whereas it was significantly difference in DAS_{200} group.

Table 7. Time course of 72 hours changes on MDA levels of soleus muscle after a acute exhaustive exercise in rats, (n=3).

Time after Exercise (hours)	MDA levels (nmol/100mg protein)					
	Control		DAS 50 mg/ kg BW		DAS 200 mg/ kg BW	
	Sedentary group	Exercise group	Sedentary group	Exercise group	Sedentary group	Exercise group
0	83.15 ± 10.42	134.72 ± 11.04 [c]	93.92 ± 10.26	74.26 ± 6.69 [a]	95.82 ± 12.47	125.12 ± 10.26 [b]
6	92.02 ± 4.18	279.96 ± 4.26 [c, *]	113.46 ± 1.28	227.49± 16.98 [a,c,*]	145.55 ± 8.71	215.67± 12.41 [a,c,*]
12	62.13 ± 6.55	168.74 ± 19.47 [c]	95.18 ± 5.49	134.34 ± 5.60 [c]	124.15 ± 9.66	194.01 ± 3.95
24	106.62 ± 15.49	143.17 ± 11.31	74.78 ± 7.59	111.87 ± 5.56 [c]	110.53 ± 9.99	102.57 ± 5.44 [a]
36	59.51 ± 8.13	70.76 ± 10.09	68.55 ± 5.51	101.51 ± 4.90 [c]	72.30 ± 9.23	54.92 ± 5.33 *, [b]
48	71.57 ± 15.77	56.24 ± 4.36 *	63.78 ± 7.25	72.62 ± 11.70	48.79 ± 10.33	62.14 ± 11.81
72	36.95 ± 14.93	45.72 ± 11.21*	34.69 ± 1.78	48.81 ± 9.37	54.71 ± 5.06	44.63 ± 10.87 *

Values are Mean ± SEM

[a] Significant difference from the control at $p< 0.05$

[b] Significant difference from the DAS_{50} at $p< 0.05$

[c] Significant difference between sedentary and exercise within the control, DAS_{50} and DAS_{50} at $p< 0.05$

* Significant difference from initial within the correspondent subgroup at $p< 0.05$.

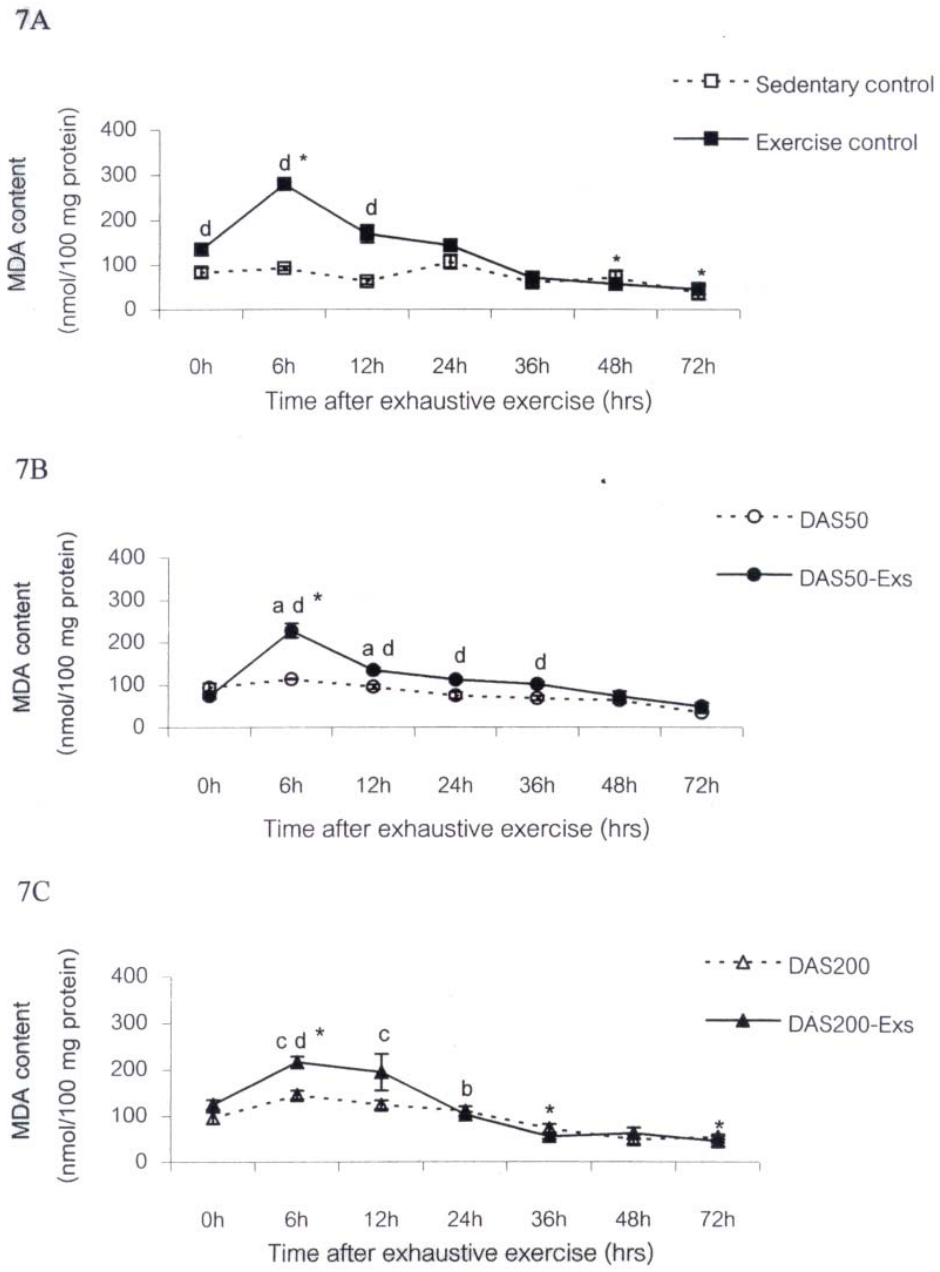


Figure 7. Time course change on MDA contents of the soleus tissue in control (A), DAS_{50} (B), and DAS_{200} (C), (n=3). Open and closed symbols represent control and exercise subgroups; [a] represents significant difference between control and DAS_{50} rats at $p< 0.05$; [b] represents significant difference between DAS_{50} and DAS_{200} rats at $p< 0.05$; [c] represent significant difference between control and DAS_{200} rats at $p< 0.05$; [d] represents significant difference between sedentary and exercise subgroups at $p< 0.05$; and [*] significant difference from initial (time 0) values at $p< 0.05$.

As illustrated in Figure 8, the exhaustive exercise motive significant increasing in MDA contents of rectus femoris muscle in all groups when compared to control groups. In exercise control group, the significantly increasing of post-exhaustive exercise MDA content were showed at immediate, 6-hr, and 24-hr when compared to the sedentary control group (Figure 8A) ($p< 0.05$). The MDA contents were at 128.33 ± 9.28, 246.46 ± 16.68, and 146.88 ± 15.99 nmol/100 mg protein.

The MDA levels in DAS_{50}-Exs subgroup was significantly elevated at 6-hr, 12-hr, and 24-hr (Figure 8B) ($p< 0.05$). The MDA contents were 210.15 ± 11.41, 162.96 ± 15.96, and 146.34 ± 12.71 nmol/100 mg protein. Rats in DAS_{200}-Exs group showed slightly similar pattern of MDA content, which was significantly increased at 6-hr, 12-hr, 24-hr, and 36- hr (Figure 8C) ($p< 0.05$). The values were 184.34 ± 7.35, 136.83 ± 1.27, 115.57 ± 5.29, and 76.83 ± 3.38 nmol/100 mg protein.

The peak MDA content in exercise groups of all groups was significantly presented at 6-hr ($p< 0.05$) after post exhaustive exercise. The MDA contents returned to level near to the sedentary control group at 36-hr in exercise control, whereas significantly MDA elevation persevered to 48-hr in DAS_{50}-Exs and DAS_{200}-Exs subgroups ($p< 0.05$).

In the DAS supplemented groups, the MDA levels were detected in rectus femoris muscle significant lower in DAS_{50}-Exs and DAS_{200}-Exs groups when compared to exercise control group at 6-hr ($p< 0.05$). Moreover, MDA content in DAS_{50}-Exs was insignificantly difference when compared with DAS200-Exs group.

Table 8. Time course of 72 hours changes on MDA levels of rectus femoris muscle after an acute exhaustive exercise in rats, (n=3).

Time after Exercise (hours)	MDA levels (nmol/100mg protein)					
	Control		DAS 50 mg/ kg BW		DAS 200 mg/ kg BW	
	Sedentary group	Exercise group	Sedentary group	Exercise group	Sedentary group	Exercise group
0	46.16 ± 5.91	128.33 ± 9.28 [c]	59.43 ± 10.16	95.13 ± 20.64	84.79 ± 5.92	105.97 ± 8.22
6	80.86 ± 13.11	246.46 ± 16.68 [c, *]	75.09 ± 14.80	210.15± 11.41 [a,c,*]	91.82 ± 13.47	184.34 ± 7.35 [c, *]
12	109.32 ± 25.50	136.14 ± 17.34	40.13 ± 7.00	162.96 ± 15.24 [c,*]	77.23 ± 5.75	136.83 ± 1.27 [c]
24	67.04 ± 13.79	146.88 ± 15.99 [c]	69.05 ± 19.71	146.34 ± 12.70 [c]	77.61 ± 9.49	115.57 ± 5.29 [c]
36	80.86 ± 13.81	77.26 ± 17.00	64.12 ± 24.64	104.33 ± 21.93	57.92 ± 5.28	76.83 ± 3.38 [c]
48	79.07 ± 4.05	58.61 ± 9.11*	41.98 ± 14.67	50.71 ± 14.33	58.49 ± 10.27	54.48 ± 7.16
72	37.60 ± 4.46	35.38 ± 14.93 *	61.25 ± 12.74	89.51 ± 12.74	24.64 ± 0.59	41.40 ± 1.94 [b,c,*]

Values are Mean ± SEM

[a] Significant difference from the control at $p< 0.05$

[b] Significant difference from the DAS_{50} at $p< 0.05$

[c] Significant difference between sedentary and exercise within the control, DAS_{50} and DAS_{50} at $p< 0.05$

* Significant difference from initial within the corresponding subgroup at $p< 0.05$

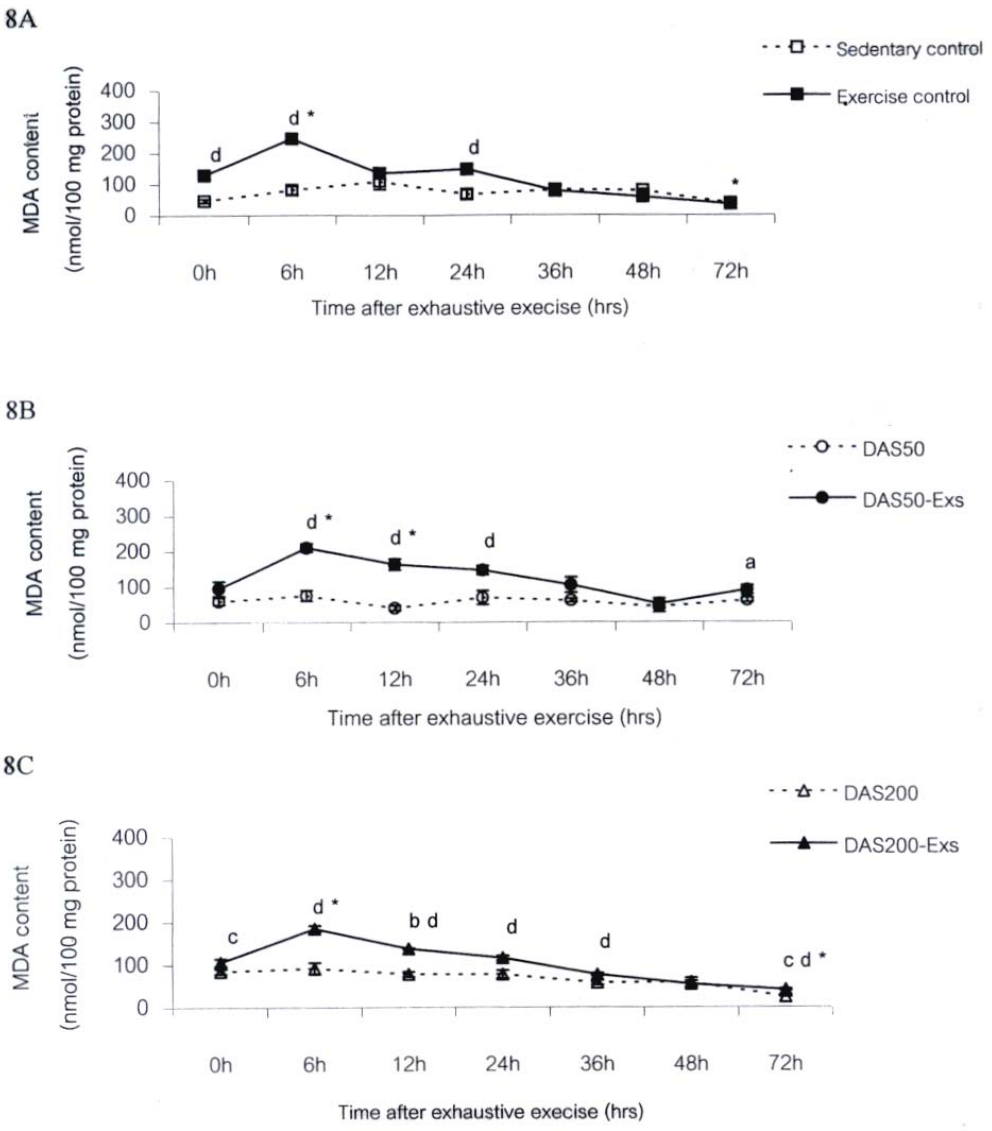


Figure 8. Time course change on MDA contents of the rectus femris tissue in control (A), DAS_{50} (B), and DAS_{200} (C), (n=3). Open and closed symbols represent control and exercise subgroups; [a] represents significant difference between control and DAS_{50} rats at $p< 0.05$; [b] represents significant difference between DAS50 and DAS_{200} rats at $p< 0.05$; [c] represent significant difference between control and DAS_{200} rats at $p< 0.05$; [d] represents significant difference between sedentary and exercise subgroups at $p< 0.05$; and [*] significant difference from initial (time 0) values at $p< 0.05$.

2.4 Plasma and MDA

The MDA levels of plasma in groups of rats, sedentary control, exercise control, DAS_{50}, DAS_{50}-Exs, DAS_{200}, DAS_{200}-Exs groups were presented in Figure 9.

The resting MDA levels in sedentary control, DAS_{50} and DAS_{200} groups were similar. The values were 67.24 ± 3.91, 80.18 ± 9.75, and 87.78 ± 9.88 nmol/100 mg protein, respectively. The resting MDA values were slightly insignificantly higher in DAS treated rats.

As illustrated in Figure 9, exhaustive exercise caused a significant rise in MDA contents of plasma in all groups of rats when compared to their control groups. In exercise control group, significantly increasing of MDA content was presented at immediate, 6-hr, 12-hr, and 24-hr when compared to sedentary control group (Figure 9A) ($p< 0.05$). The MDA content were at 97.50 ± 3.50, 161.98 ± 2.17, 109.68 ± 7.73, and 107.12 ± 3.99 nmol/100 mg protein.

The MDA level in DAS_{50}-Exs group was significantly elevated at 6-hr, 24-hr, and 48-hr (Figure 9B) ($p< 0.05$). The MDA concentration were 100.58 ± 3.85, 94.62 ± 0.65, and 82.15 ± 5.41 nmol/100 mg protein. Rats in DAS200-Exs group was showed similar characteristic, in which was significantly increased at immediate, 6-hr, 24-hr, and 48-hr (Figure 9C) ($p< 0.05$). The values were 100.47 ± 2.24, 90.65 ± 9.39, 106.36 ± 6.49, and 108.98 ± 9.39 nmol/100 mg protein, respectively.

The peak MDA contents in exercise groups of all groups was significantly observed at 6-hr ($p< 0.05$) after exhaustive exercise and the content returned to level near to sedentary group at 36-hr in exercise control and DAS_{50}-Exs, whereas it was returned to closed the MDA of sedentary control subgroup at 72-hr in DAS_{200}-Exs subgroup.

In the DAS supplemented subgroups, lower levels of MDA were detected in plasma significantly lower in DAS_{50}-Exs and DAS_{200}-Exs groups when compared to exercise control subgroup at 6- hr after exercise ($p< 0.05$). Moreover, the MDA concentration in DAS_{50}-Exs was significantly lower than MDA content of DAS_{200}-Exs subgroup.

Table 9. Time course of 72 hours changes on plasma MDA levels acute exhaustive exercise in rats, (n=3).

Time after Exercise (hours)	MDA levels (nmol/100mg protein)					
	Control		DAS 50 mg/ kg BW		DAS 200 mg/ kg BW	
	Sedentary	Exercise	Sedentary	Exercise	Sedentary	Exercise
0	67.24 ± 3.91	97.50 ± 3.50 [c]	80.18 ± 9.75	86.93 ± 1.24 [a]	87.78 ± 9.88	100.47 ± 2.24 [b]
6	74.97 ± 11.23	161.98 ± 2.17 [c,*]	74.01 ± 7.29	100.58 ± 3.85 [a,c]	68.88 ± 8.09	124.10± 3.69 [a,b,c,*]
12	42.03 ± 3.94	109.68 ± 7.73	40.13 ± 9.14	82.14 ± 5.45 [a,c]	48.42 ± 16.40	90.65 ± 9.39 [c]
24	52.92 ± 7.86	107.12± 3.99 [c]	50.23 ± 5.11	94.62 ± 0.65 [a,c]	62.36 ± 2.41	106.36 ± 6.49 [c]
36	74.99 ± 1.06	83.18 ± 3.93	72.22 ± 12.34	74.10 ± 7.85	91.33 ± 2.57	83.45 ± 8.57 [c]
48	52.69 ± 5.70	77.53 ± 7.76	62.72 ± 5.29	84.65 ± 5.45	70.25 ± 5.27	108.98 ± 9.39
72	60.68 ± 5.35	67.97 ± 4.97	45.45 ± 8.79	75.55 ± 15.82	67.52 ± 13.41	64.74 ± 6.56 [c]

Values are Mean ± SEM

[a] Significant difference from the control at $p< 0.05$

[b] Significant difference from the DAS_{50} at $p< 0.05$

[c] Significant difference between sedentary and exercise within the control, DAS_{50} and DAS_{50} at $p< 0.05$

* Significant difference from initial within the correspondent subgroup at $p< 0.05$

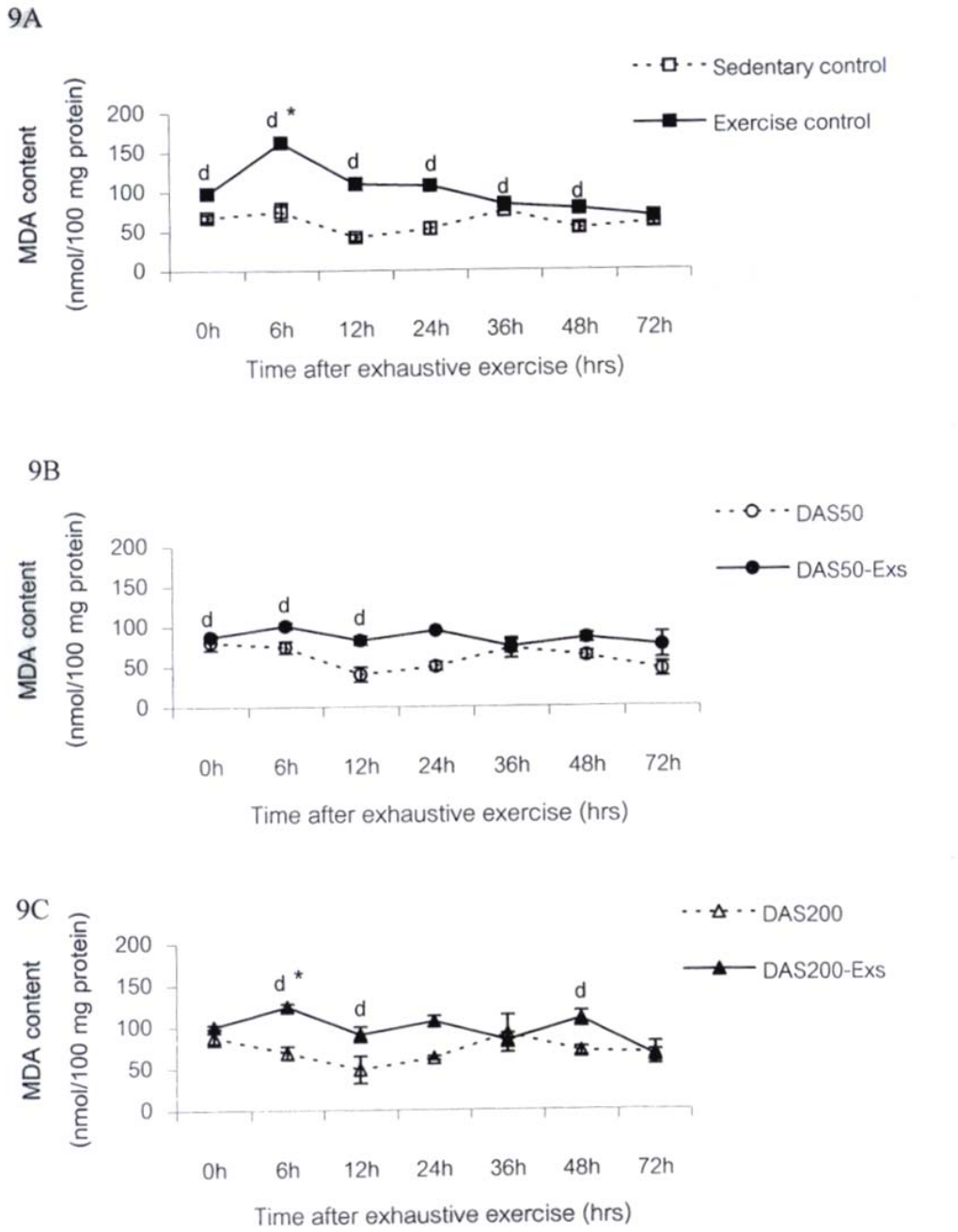


Figure 9. Time course change on MDA contents of the plasma in control (A), DAS_{50} (B), and DAS_{200} (C), (n=3). Open and closed symbols represent control and exercise subgroups; [a] represents significant difference between control and DAS_{50} rats at $p< 0.05$; [b] represents significant difference between DAS50 and DAS_{200} rats at $p< 0.05$; [c] represent significant difference between control and DAS_{200} rats at $p< 0.05$; [d] represents significant difference between sedentary and exercise subgroups at $p< 0.05$; and [*] significant difference from initial (time 0) values at $p< 0.05$.

3. Dose Responses of DAS

As MDA contents in sedentary subgroups of rats were accordingly unchanged (Figures 6, 7, 8, and 9), in this study is aimed to described only in the effects of DAS in exhaustive exercise rats.

3.1 Diaphragm muscle responses to DAS

The MDA content in diaphragm muscle of rats subjected to exhaustive treadmill running in control, 50mg/ kg body weight DAS supplemented plus exercise (DAS_{50}-Exs), 200mg/ kg body weight DAS supplemented plus exercise (DAS_{200}-Exs) subgroups were presented in Table 10 (absolute values) and Figure 10 (% from initial).

As showed in Table 10, DAS supplementation emerged to reduce the MDA content in diaphragm muscle induced by exhaustive exercise. Rats received DAS_{50}-Exs and DAS_{200}-Exs effectively showed less MDA content at 6-hr post-exhaustive exercise when compared to exercise control group ($p< 0.05$). MDA levels were 133.48 ± 5.87 and 222.80 ± 6.31 compared to 303.21 ± 9.22 nmol/100 mg protein, respectively. Moreover, rats in DAS_{50}-Exs group showed significantly higher MDA content than DAS_{200}-Exs group ($p< 0.05$).

In Figure 10, MDA contents were presented as percent change from initial values. The MDA content in exercise control subgroup was increased to 287.31 ± 36.20 % of value from its initial at 6-hr and then returned to initial level at 36-hr. From 48-72 hrs, the contents were decreased to half of initial value (54.41 ± 14.69, 24-hr) and (43.67 ± 14.27, 72-hr). The MDA contents in DAS_{50}-Exs and DAS_{200}-Exs groups increased to 140.43 ± 8.42 and 210.08 ± 22.35 % of value from its initial at 6-hr, respectively, and then these contents decreased to half of initial values.

Table 10. Dose responses of DAS on MDA levels of diaphragm muscle after exhaustive exercise in exercise control, 50 mg/kg DAS plus exercise (DAS_{50}-Exs) and 200 mg/kg DAS plus exercise (DAS_{200}-Exs) groups, (n=3).

Time (hours)	MDA contents (nmol/100 mg protein)		
	Exs	DAS_{50}-Exs	DAS_{200}-Exs
0	108.75 ± 13.45 (100 %)	133.48 ± 5.87 (100 %)	108.20 ± 10.70 (100 %)
6	303.21 ± 9.22 * (↑187.31 %)	186.92 ± 9.38 ^{e,*} (↑40.00 %)	222.80 ± 6.31 ^{f,g,*} (↑110.08 %)
12	238.08 ± 9.62 * (↑126 %)	121.90 ± 9.35 ^{e} (↓8.48 %)	160.65 ± 3.99 ^{f,g,*} (↑ 51.39 %)
24	193.34 ± 18.68 * (↑86 %)	129.28 ± 6.88 ^{e} (↓2.35 %)	96.30 ± 8.07 ^{f,g} (↓9.91 %)
36	99.81 ± 2.70 (↓12.07 %)	115.81 ± 5.15 (↓12.57 %)	44.93 ± 4.75 ^{g,*} (↓58.38 %)
48	60.34 ± 17.75 (↓45.59 %)	57.30 ± 3.14 (↓57.11 %)	65.44 ± 6.89 (↓37.43 %)
72	43.85 ± 11.93 * (↓56.33 %)	46.38 ± 4.81 * (↓63.12 %)	29.16 ± 1.22 ^{g,*} (↓71.75 %)

Values are mean ± SEM. Symbol ^{e} represents significant difference between exercise control and DAS_{50}-Exs rats at $p< 0.05$; ^{f} represents significant difference between exercise control and DAS_{200}-Exs rats at $p< 0.05$; ^{g} represents significant difference between DAS_{50}-Exs and DAS_{200}-Exs rats $p< 0.05$; and * represents significant difference from initial (time 0) value at $p< 0.05$.

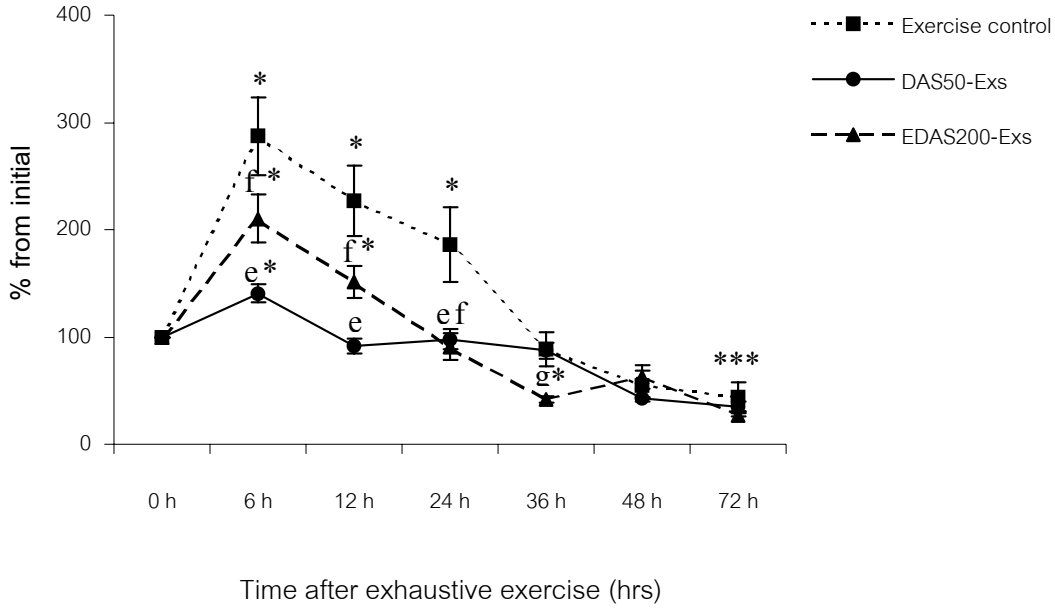


Figure 10. MDA contents in diaphragm muscle of rats in exercise control, DAS_{50}-Exs and DAS_{200}-Exs subgroups. Concentrations are represented as percentage from initial values. Values are mean ± SEM, (n=3). Symbol ^{e} represents significant difference between exercise control and DAS_{50}-Exs rats at $p<0.05$; ^{f} represent significant difference between exercise control and DAS_{200}-Exs rats at $p<0.05$; ^{g} represents significant difference between DAS_{50}-Exs and DAS_{200}-Exs rats at $p<0.05$ and * represents significant difference from initial (time 0) values, $p<0.05$.

3.2 Soleus muscle responses to DAS

The MDA content in soleus muscle of rats subjected to exhaustive exercise in control, DAS_{50}-Exs, DAS_{200}-Exs groups were presented in Table 11 (absolute values) and Figure 11 (% from initial).

As shown in Table 11, at daily DAS supplementation for four weeks revealed to suppress on the MDA content induced by acute exhaustive exercise. Rats in DAS_{50}-Exs showed lower MDA content at 6-hr post exercise compared to exercise control subgroup ($p< 0.05$). The MDA levels were 227.49 ± 16.98 and 279.96 ± 4.26 nmol/100 mg protein. Rats in DAS_{200}-Exs presented lower MDA contents compared

to exercise control group at 6-hr post exercise ($p< 0.05$). MDA levels were 215.67 ± 12.41 and 279.96 ± 4.26 nmol/100 mg protein.

In Figure 11, MDA contents were presented in percentage of changes from initial values. The significant difference was shown at 6-hr after exhaustive exercise, MDA contents in exercise control group was 2-folds increasing then reduced to initial level at 24-hr. From 36-72 hr, the contents were decreased to half of initial value 54.31 ± 12.19 (36-hr), 41.79 ± 1.03 (48-hr), 33.52 ± 7.45 (72-hr) % from its initial values. On the other hand, MDA content in DAS_{50}-Exs was 3-flods increased (316.03 ± 54.96 % from initial), whereas rats in DAS_{200}-Exs group was showed increasing to 173.39 ± 8.72 % from its initial value at 6-hr after attainment exercise, the contents decreased to half of initial values thereafter.

Table 11. Dose responses of DAS changes on MDA levels of soleus muscle after exhaustive exercise in exercise control, DAS_{50}-Exs and DAS_{200}-Exs groups, (n=3).

Time (hours)	MDA contents (nmol/100 mg protein)		
	Exs	DAS_{50}-Exs	DAS_{200}-Exs
0	134.72 ± 11.04 (100 %)	74.26 ± 6.69 [e] (100 %)	125.12 ± 10.26 [g] (100 %)
6	279.96 ± 4.26 [*] (↑110.85 %)	227.49 ± 16.98 [e,*] (↑216.03 %)	215.67 ± 12.41 [f,*] (↑73.39 %)
12	168.74 ± 19.47 (↑25.21 %)	134.34 ± 5.60 [*] (↑84.13 %)	194.01 ± 39.59 [*] (↑55.55 %)
24	143.17 ± 11.30 (↑6.31%)	111.87 ± 5.56 (↑54.34 %)	102.57 ± 5.44 [f] (↓17.63 %)
36	70.76 ± 10.09 [*] (↓44.69%)	101.51 ± 4.90 (↑39.30 %)	54.92 ± 5.33 [g,*] (↓56.14 %)
48	56.24 ± 4.36 [*] (↓58.21%)	72.62 ± 11.70 (↓0.81 %)	62.14 ± 11.81 (↓49.67 %)
72	45.72 ± 11.20 [*] (↓66.48%)	48.81 ± 9.37 (↓ 32.92 %)	44.63 ± 10.87 [*] (↓63.18 %)

Values are mean ± SEM. Symbol [e] represents significant difference between exercise control and DAS_{50}-Exs rats at $p< 0.05$; [f] represents significant difference between exercise control and DAS_{200}-Exs rats at $p< 0.05$; [g] represents significant difference between DAS_{50}-Exs and DAS_{200}-Exs rats $p< 0.05$; and [*] represents significant difference from initial (time 0) value at $p< 0.05$.

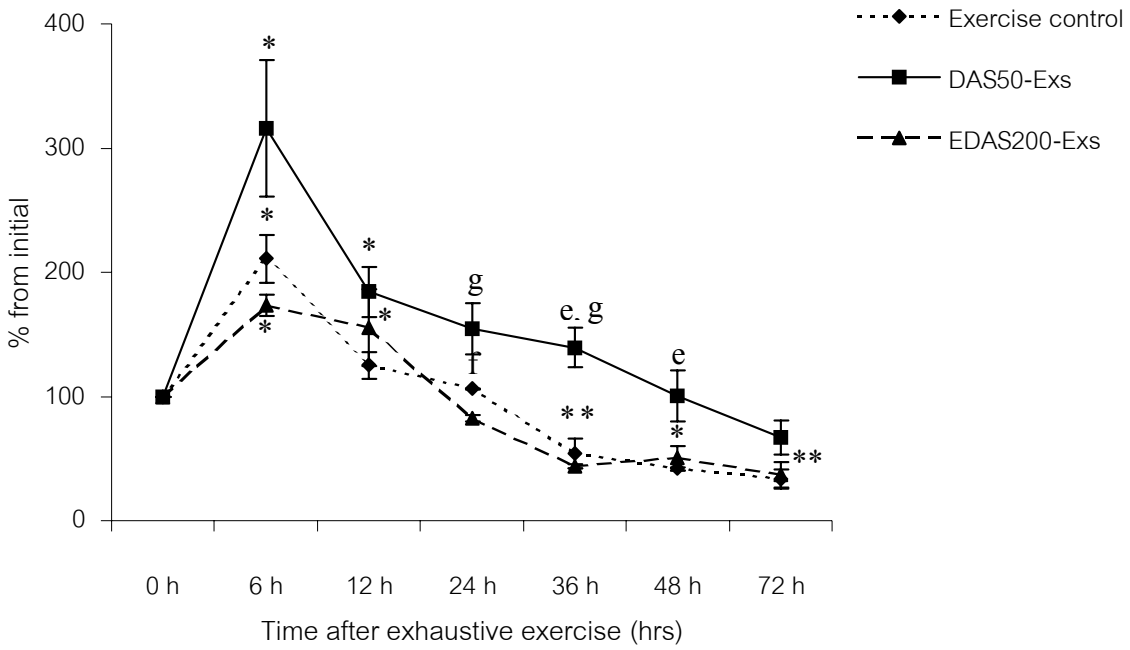


Figure 11. MDA contents in soleus muscle of rats in exercise control, DAS_{50}-Exs and DAS_{200}-Exs groups. Concentrations are represented as percentage from initial values. Values are mean ± SEM, (n=3). Symbol ^{e} represents significant difference between exercise control and DAS_{50}-Exs rats at $p<0.05$; ^{f} represent significant difference between exercise control and DAS_{200}-Exs rats at $p<0.05$; ^{g} represents significnt difference between DAS_{50}-Exs and DAS_{200}-Exs rats at $p<0.05$; and * represents significant difference from initial (time 0) values, $p<0.05$.

3.3 Rectus femoris muscle responses to DAS

The MDA content in rectus femoris muscle of rats subjected to exhaustive exercise in control, DAS_{50}-Exs, DAS_{200}-Exs groups were presented in Table 12 (absolute values) and Figure 12 (% from initial).

As shown in Table 12, daily DAS supplementation of 200 mg/kg body weight DAS supplementation for four weeks revealed the suppressive effect on MDA content induced by exhaustive exercise. The MDA content reduction showed at 6-hr after exercise. The MDA level was 184.34 ± 7.35 compared to 246.46 ± 16.68 nmol/100 mg protein in exercise control group ($p< 0.05$). Rats in DAS50-Exs showed significant suppression of MDA content at 6-hr after exhaustive exercise ($p< 0.05$). The MDA level was 210.15 ± 11.41 compared to 246.46 ± 16.68 nmol/100 mg protein. On the other hand, MDA contents was showed higher at 12-hr in DAS_{50}-Exs subgroup.

In Figure 12, MDA contents were presented in percent change from initial values. The significantly difference was observed at 6-hr after exercise, MDA contents in exercise control subgroup was increased 192.69 ± 10.79 % from its initial value, then declined to initial value at 24-hr post exercise. From 36-72 hrs, the MDA contents decrease to half of initial values were 59.12 ± 9.09 (36-hr), 45.20 ± 4.01 (48-hr), and 26.25 ± 9.08 (72-hr) % from its initial values. MDA contents in DAS_{50}-Exs and DAS_{200}-Exs subgroups were increased to 235.14 ± 33.29 and 175.07 ± 7.59 % from its initial values at 6-hr after attainment exercise, then the contents decreased to half of initial values thereafter.

Table 12. Dose response of DAS changes on MDA levels of rectus femoris muscle after exhaustive exercise in exercise control, DAS_{50}-Exs and DAS_{200}-Exs groups, (n=3).

Time (hours)	MDA contents (nmol/100 mg protein)		
	Exs	DAS_{50}-Exs	DAS_{200}-Exs
0	128.33 ± 9.28 (100%)	95.13 ± 20.64 (100%)	105.97 ± 8.22 (100%)
6	246.46 ± 16.68 * (↑92.31 %)	210.15 ± 11.41* (↑135.14 %)	184.34 ± 7.35 f,* (↑75.07 %)
12	136.14 ± 17.34 (↑6.61 %)	162.96 ± 15.27 * (↑80.37 %)	136.83 ± 1.27 * (↑50.64 %)
24	146.88 ± 15.99 (↑14.55 %)	146.34 ± 12.71* (↑64.59 %)	115.57 ± 5.29 (↑9.62 %)
36	77.26 ± 17.00 (↓40.88 %)	104.33 ± 21.93 (↑10.57 %)	76.83 ± 3.38 (↓26.09 %)
48	58.61 ± 9.11 * (↓54.80 %)	50.71 ± 14.33 (↓47.72 %)	54.48 ± 7.16 (↓47.23 %)
72	35.38 ± 14.93 * (↓73.75 %)	89.51 ± 12.74 (↓6.65 %)	41.40 ± 1.94 g,* (↓60.74 %)

Values are mean ± SEM. Symbol ^{e} represents significant difference between exercise control and DAS_{50}-Exs rats at $p< 0.05$; ^{f} represents significant difference between exercise control and DAS_{200}-Exs rats at $p< 0.05$; ^{g} represents significant difference between DAS_{50}-Exs and DAS_{200}-Exs rats $p< 0.05$; and * represents significant difference from initial (time 0) value at $p< 0.05$.

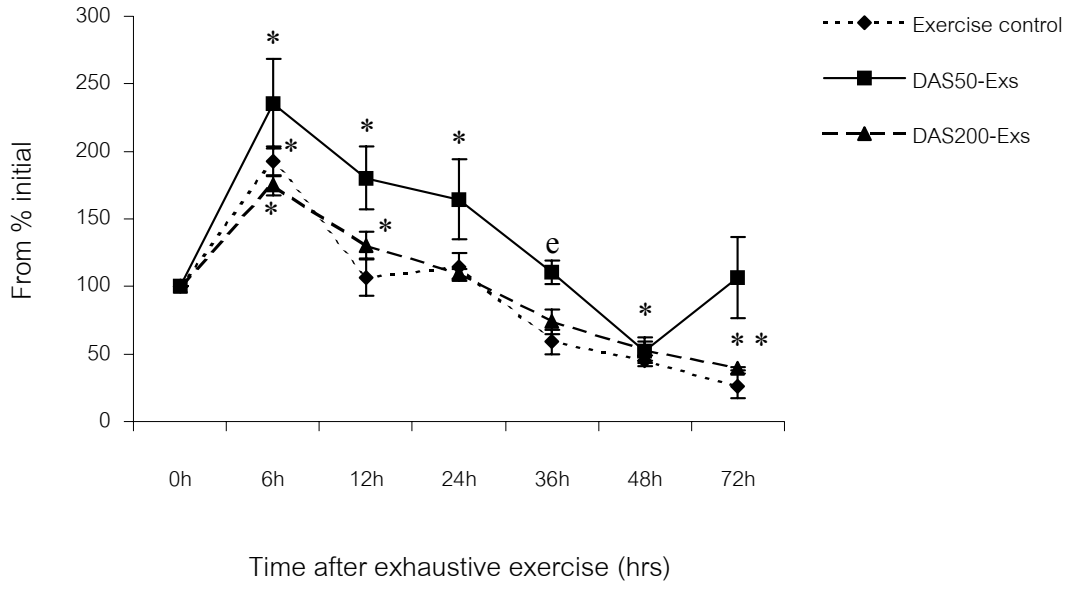


Figure 12. MDA contents in retus femoris muscle of rats in exercise control, DAS_{50}-Exs and DAS_{200}-Exs subgroups. Concentrations are represented as percentage from initial values. Values are mean ± SEM, (n=3). Symbol ^{e} represents significant difference between exercise control and DAS_{50}-Exs rats at $p<0.05$; ^{f} represent significant difference between exercise control and DAS_{200}-Exs rats at $p<0.05$; ^{g} represents significant difference between DAS_{50}-Exs and DAS_{200}-Exs rats at $p<0.05$ and * represents significant difference from initial (time 0) values, $p<0.05$.

3.4 Plasma responses to DAS

The MDA content in plasma of rats subjected to exhaustive exercise in control, DAS_{50}-Exs, DAS_{200}-Exs groups were presented in Table 13 (absolute values) and Figure 13 (% from initial).

As showed in Table 13, at daily DAS supplementation for four weeks presented reduction of the MDA production induced by exhaustive exercise. Rats in DAS_{50}-Exs showed suppressive of MDA formation at 6-hr post exercise compared to exercise control group. The MDA level was 100.58 ± 3.85 compared to 161.98 ± 2.17 nmol/100 mg protein, ($p< 0.05$). Rats in DAS_{200}-Exs showed reduction of MDA content compared to exercise control subgroup at 6-hr post exercise ($p< 0.05$). The MDA content was 124.10 ± 3.69 compared to 161.98 ± 2.17 nmol/100 mg protein.

In Figure 13, MDA content were presented in percent change from initial values. The significantly difference was shown at 6-hr after exhaustive exercise, MDA contents in exercise control group was 1.5-folds increased (166.43 ± 4.42 % from initial value) then reduced to initial value at 48-hr after exercise. At 72-hr, the content decreased to half of initial value 69.59 ± 3.44 % from initial value. The MDA contents in DAS_{50}-Exs and DAS_{200}-Exs groups were increased to 115.69 ± 3.99 and 123.79 ± 6.16 % of value from its initial at 6-hr, and then these contents decreased to initial values.

Table 13. Dose responses of DAS changes on MDA levels of plasma after acute exhaustive exercise in exercise control, DAS_{50}-Exs and DAS_{200}-Exs groups, (n=3).

Time (hours)	MDA contents (nmol/100 mg protein)		
	Exs	DAS_{50}-Exs	DAS_{200}-Exs
0	97.50 ± 3.50 (100%)	86.93 ± 1.24 (100%)	100.47 ± 2.24 ^{g} (100%)
6	161.98 ± 2.17 * (↑66.43 %)	100.58 ± 3.85 ^{e} (↑15.69 %)	124.10 ± 3.69 ^{f,g} (↑23.79 %)
12	109.68 ± 7.73 (↑12.27 %)	82.14 ± 5.45 ^{e} (↓5.54 %)	90.65 ± 9.39 (↓ 9.53)
24	107.12 ± 3.99 (↑10.00)	94.62 ± 0.65 ^{e} (↑8.91 %)	106.36 ± 6.49 (↑6.15 %)
36	83.18 ± 3.93 (↓14.76 %)	74.10 ± 7.85 (↓14.74 %)	83.45 ± 8.57 (↓16.70 %)
48	77.53 ± 7.76 (↓12.27 %)	84.65 ± 5.45 ^{e} (↓5.54 %)	108.98 ± 9.39 (↑9.53 %)
72	67.97 ± 4.97 (↓30.41 %)	75.55 ± 15.82 (↓13.05 %)	64.74 ± 6.65 (↓35.27 %)

Values are mean ± SEM. Symbol ^{e} represents significant difference between exercise control and DAS_{50}-Exs rats at $p< 0.05$; ^{f} represents significant difference between exercise control and DAS_{200}-Exs rats at $p< 0.05$; ^{g} represents significant difference between DAS_{50}-Exs and DAS_{200}-Exs rats $p< 0.05$; and * represents significant difference from initial (time 0) value at $p< 0.05$.

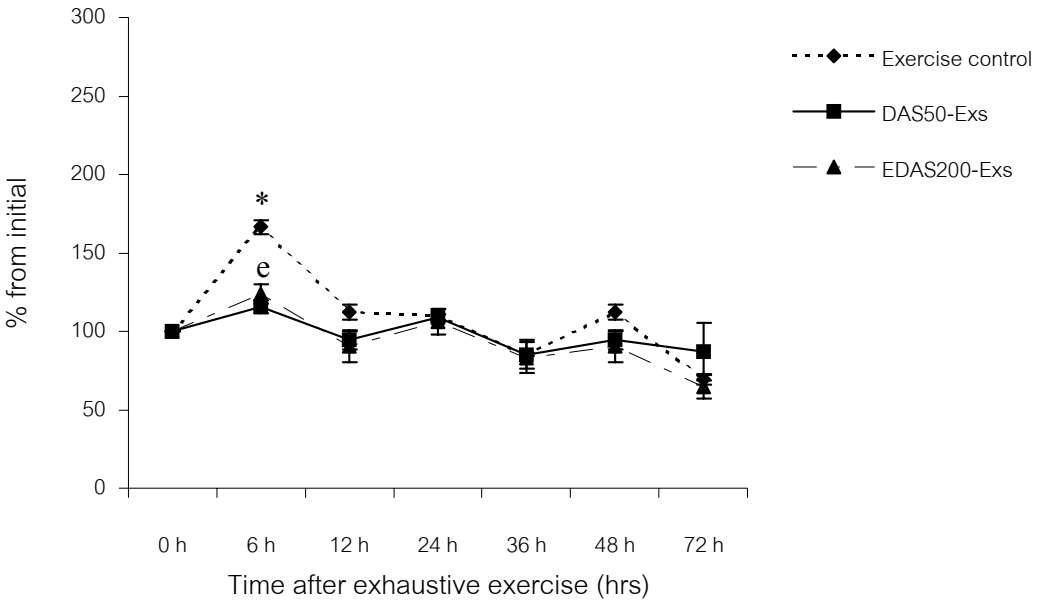


Figure 13. MDA contents in plasma of rats in exercise control, DAS_{50}-Exs and DAS_{200}-Exs subgroups. Concentrations are represented as percentage from initial values. Values are mean ± SEM, (n=3). Symbol ^{e} represents significant difference between exercise control and DAS_{50}-Exs rats at $p<0.05$; ^{f} represent significant difference between exercise control and DAS_{200}-Exs rats at $p<0.05$; ^{g} represents significant difference between DAS_{50}-Exs and DAS_{200}-Exs rats at $p<0.05$; and * represents significant difference from initial (time 0) values, $p<0.05$.

4. Time Course Effects of DAS Supplementation

As 50 mg/ kg body weight of DAS supplementation showed the remarkable suppressive effect on the MDA levels during post exercise period. This dose was then selectively used to investigate the time course of such diminishing of MDA levels in diaphragm muscle and plasma. Time period in this study was performed up to 8 weeks period.

4.1 Time course effects of DAS supplementation on diaphragm

Table 14 showed MDA contents in diaphragm muscle of rats in exercise control and DAS_{50}-Exs from the initial (no treatment rats) to the 8^{th} week of supplementation period.

After treated for the selected period, rats were subjected to the same experimented protocal. Following the exhaustive exercise, rats were kept for 6-hr after attainment of exercise and then sacrificed for MDA contents measurements. Results demonstrated that MDA contents obtained from rat's diaphragm in exercise control group showed the increasing pattern (Table14). The initial value (no treatment) was of 57.32 ± 3.55 nmol/100 mg protein. The MDA contents of exercise control group then increased about 2-flods during 1^{st} to 7^{th} weeks. MDA levels were 140.02 ± 6.19 (wk1), 130.38 ± 3.57 (wk2), 131.12 ± 4.91 (wk3), 143.39 ± 0.65 (wk4), 154.98 ± 11.38 (wk5), 130.82 ± 11.22 (wk6), and 154.44 ± 5.00 (wk7) nmol/100 mg protein. MDA level increased approximately 3-folds on the 8^{th} week, 173.78 ± 9.81 nmol/100 mg protein. In the DAS_{50}-Exs group, the initial MDA content was similar to the exercise control group ($p< 0.05$). MDA also showed an increasing pattern, but less in magnitude. MDA contents were up to 2 folds until week 3 (50.12 ± 3.39 (initial), 112.79 ± 12.95 (wk 1), 125.19 ± 11.41 (wk2), and 110.31 ± 6.12 (wk3) nmol/100 mg protein). Thereafter MDA contents reduced to initial level until week 8 (50.21 ± 2.92 (wk4), 56.10 ± 2.16 (wk5), 74.92 ± 4.66 (wk 6), 69.19 ± 8.12 (wk7), and 68.31 ± 2.56 (wk8) nmol/100 mg protein).

Results from within the group comparison presented that exercise control group had significantly higher MDA level from initial values, from the 1^{st} week

throughout the 8th week of the study. In the other group, DAS_{50}-Exs showed reducing of the MDA content at 4th week throughout the 8th week of the study.

Table 14. MDA contents in diaphragm muscle of rats subjected to exercise and exercise with pre-treated with 50mg/kg body weight, from the initial to 8th week of supplementation period. Data were collected from the beginning of the study (untreated, initial) and at a week interval at 6-hr post exhaustive exercise, (n=3).

Duration	MDA contents (nmol/100 mg protein)	
	Control exercise	DAS_{50}-Exs
Initial	57.32 ± 3.55	50.12 ± 3.39
Week 1	159.93 ± 5.80 *	112.79 ± 12.95 e
Week 2	173.61 ± 8.81 *	125.19 ± 11.41 e,*
Week 3	154.78 ± 6.85 *	110.31 ± 6.12 e,*
Week 4	155.16 ± 10.45 *	50.21 ± 2.92 e
Week 5	150.98 ± 3.97 *	56.10 ± 2.16 e
Week 6	186.02 ± 12.07 *	74.92 ± 4.66 e
Week 7	180.11 ± 11.37 *	69.19 ± 8.12 e
Week 8	192.95 ± 4.76 *	68.31 ± 2.56 e

Values are mean ± SEM. Symbols e represents significant difference between exercise control and DAS50-Exs rats at $p< 0.05$ and * represents significance difference from initial (time 0) value, $p< 0.05$.

4.2 Time course effects of DAS supplementation on plasma

The Table 15 showed MDA contents in plasma of rats in exercise control and DAS_{50}-Exs from the initial to the 8^{th} week of supplementation period.

After treated for the determined period, rats were subjected to the same experimental procedure. Results showed that MDA contents obtained from rats plasma in exercise control group increased in the stepwise pattern (Table 15). First of all, the initial value of 65.32 ± 3.85 nmol/100 mg protein, the MDA contents in exercise control group increased about 2-folds within 7^{th} week (140.02 ± 6.19 (wk1), 130.38 ± 3.57 (wk2), 131.12 ± 4.91 (wk3), 143.39 ± 0.65 (wk4), 154.98 ± 11.38 (wk5), 130.82 ± 11.22 (wk6), and 151.44 ± 5.00 (wk7) nmol/100 mg protein, respectively) and 3-folds at 8^{th} week of 173.78 ± 9.81 nmol/100 mg protein.

Even though, the initial MDA content in DAS_{50}-Exs group was similar to the exercise control group, increasing in this DAS treated group was also observed throughout 8 weeks period, but the magnitude was less. In DAS_{50}-Exs group, MDA contents were sustained up to 2 folds, until 3^{rd} week (54.53 ± 3.39 (initial), 101.85 ± 6.42 (wk1), 121.09 ± 3.92 (wk2), and 98.80 ± 3.55 (wk3) nmol/100 mg protein). Thereafter MDA contents stepwise decreased at 4^{th} week until 8^{th} week (76.92 ± 7.03 (wk4), 85.70 ± 5.57 (wk5), 76.67 ± 1.18 (wk6), 88.05 ± 5.89 (wk7), and 100.64 ± 12.51 (wk8) nmol/100 mg protein).

Results obtained from within the group comparison showed that either exercise control or DAS_{50}-Exs groups had significantly higher MDA levels from their initial values, from the 1^{st} week throughout the 8^{th} week of the study. The MDA contents were presented that significantly decreasing when compared between DAS_{50}-Exs with exercise control groups.

Table 15. MDA contents in plasma of rats subjected to exercise and supplemented with 50mg/kg body weight, from the initial to 8th week of supplementation period. Data were collected from the beginning of the study (untreated, initial) and at one week interval at 6-hr post exhaustive exercise, (n=3).

Duration	MDA contents (nmol/100 mg protein)	
	Control exercise	DAS_{50}-Exs
Initial	63.32 ± 3.85	54.53 ± 3.39
Week 1	140.02 ± 6.19 *	101.85 ± 6.42 e,*
Week 2	130.38 ± 3.57 *	121.09 ± 3.92 *
Week 3	131.12 ± 4.91 *	98.80 ± 3.55 e
Week 4	143.39 ± 0.65 *	76.92 ± 7.03 e
Week 5	154.98 ± 11.38 *	85.70 ± 5.57 e
Week 6	130.82 ± 11.22 *	76.67 ± 1.18 e
Week 7	151.44 ± 5.00 *	88.05 ± 5.89 e
Week 8	173.78 ± 9.81 *	100.64 ± 12.51 e,*

Values are mean ± SEM. Symbol e represents significant difference between exercise control and DAS50-Exs rats at $p< 0.05$; and * represents significance difference from initial (time 0) value, $p< 0.05$.

5. Effect of DAS on Skeletal Muscle Damage Induced by Exhaustive Exercise.

5.1 Plasma lactate dehydrogenase and dose response

Tissue damage induced by acute exhaustive treadmill running exercise in this study was determined by using activity of plasma enzyme or lactate dehydrogenase (LDH) as an indicator. The LDH activity in rat treated orally with corn oil, DAS 50 and 200mg/kg body weight/day for 4 weeks following by 75 minutes exhaustive treadmill running was summarized in Table 16. The plasma LDH was determined at 0, 6, 12, 24, 36, 48, and 72 hours after exercise.

The level of LDH activity of the plasma in sedentary control, exercise control, DAS_{50}, DAS_{50}-Exs, DAS_{200}, DAS_{200}-Exs groups were presented in Figure 14.

The resting state value of LDH level in sedentary control, DAS_{50}, and DAS_{200} supplemented groups were similar. The values were 56.34 ± 3.39, 59.51 ± 7.17, and 73.81 ± 3.63 U/I. The resting values were slightly, although insignificantly, higher in the DAS treated rats.

As illustrated in Figure 14, exhaustive caused of a significant increased in LDH activity of the plasma in all groups of rats when compared to their sedentary control subgroups. In exercise control group, the significantly increasing of post-exercise LDH activities was presented at immediately, 6-hr, 12-hr, 24-hr, 36-hr, and 48-hr compared to the sedentary control group (Figure 14A) ($p< 0.05$). The LDH activities were at 113.87 ± 2.77, 211.48 ± 3.52, 166.64 ± 4.18, 143.63 ± 4.41, 149.98 ± 5.18, and 114.66 ± 4.87 U/I.

The LDH activity level in DAS_{50}-Exs group was significantly elevated at an immediate, 6-hr, and 12-hr (Figure 14B) ($p< 0.05$). The LDH activities were 99.19 ± 6.78, 127.36 ± 8.44, 113.87 ± 6.77 U/I. The rats in DAS_{200}-Exs group was showed slightly similar characteristics of LDH activities level, in which it was significantly increased at 6-hr, 12-hr and 24-hr (Figure 14C) ($p< 0.05$) post-exhaustive exercise. The values were 158.31 ± 6.55, 157.52 ± 3.09, and 152.75 ± 4.36 U/I.

The peak of LDH activity in exercise groups of the treatment group was significantly observed at 6-hr ($p< 0.05$) after exhaustive exercise and the activity

returned to a level to the sedentary control group at 72-hr, 24-hr, and 36-hr in exercise control, DAS_{50}-Exs, and DAS_{200}-Exs subgroups.

In the DAS supplemented groups, lower levels of LDH activities were detected in plasma significantly lower in DAS_{50}-Exs and DAS_{200}-Exs supplemented groups when compared to exercise control at 6-hr after exercise ($p< 0.05$). Moreover, LDH activity level in DAS_{50}-Exs was significantly lower than the LDH activity level of DAS_{200}-Exs subgroup. The LDH activity levels were 127.36 ± 8.44 and 158.31 ± 6.55 U/I ($p< 0.05$).

Table 16. Time course of 72 hours changes on LDH activities of plasma after an acute exhaustive exercise in rats, (n=3).

Time after Exercise (hours)	LDH levels (U/I)					
	Control		DAS 50 mg/ kg BW		DAS 200 mg/ kg BW	
	Sedentary	Exercise	Sedentary	Exercise	Sedentary	Exercise
0	56.34 ± 3.39	113.87 ± 2.77 [c]	59.51 ± 7.17	99.19 ± 6.78 [a,c]	73.81 ± 3.63	103.55 ± 11.25
6	89.27 ± 7.65	211.48 ± 3.52 [c,d] *	90.86 ± 2.60	127.36 ± 8.44 [a,c]	102.76 ± 3.09	158.31± 6.55 [a,b,c,d]
12	103.55 ± 2.47	166.64 ± 4.18 [c,d]	92.05 ± 1.43	113.87 ± 6.78 [a,c]	121.81 ± 6.88	157.52 ± 3.09 [b,d]
24	108.71 ± 6.38	143.63 ± 4.41 [c]	126.17 ± 7.27	112.68 ± 4.41[a]	130.93 ± 9.91	152.75 ± 4.36 [b,c,d]
36	116.65 ± 4.18	149.66 ± 5.18 [c]	94.82 ± 3.78	100.78 ± 3.45 [a]	121.81± 7.57	133.31 ± 5.36 [b]
48	97.21 ± 3.39	114.66 ± 4.87 [c]	103.55 ± 3.82	93.63 ± 4.14 [a]	99.59 ± 3.45	121.41 ± 4.81[b,c]
72	95.22 ± 2.47	98.00 ± 3.39	98.41 ± 4.82	91.25 ± 4.02	117.84 ± 4.81	106.73 ± 5.76

Values are Mean ± SEM

[a] Significant difference from the control at $p< 0.05$

[b] Significant difference from the DAS_{50} at $p< 0.05$

[c] Significant difference between sedentary and exercise within the control, DAS_{50} and DAS_{50} at $p< 0.05$

* Significant difference from initial within the correspondent subgroup at $p< 0.05$

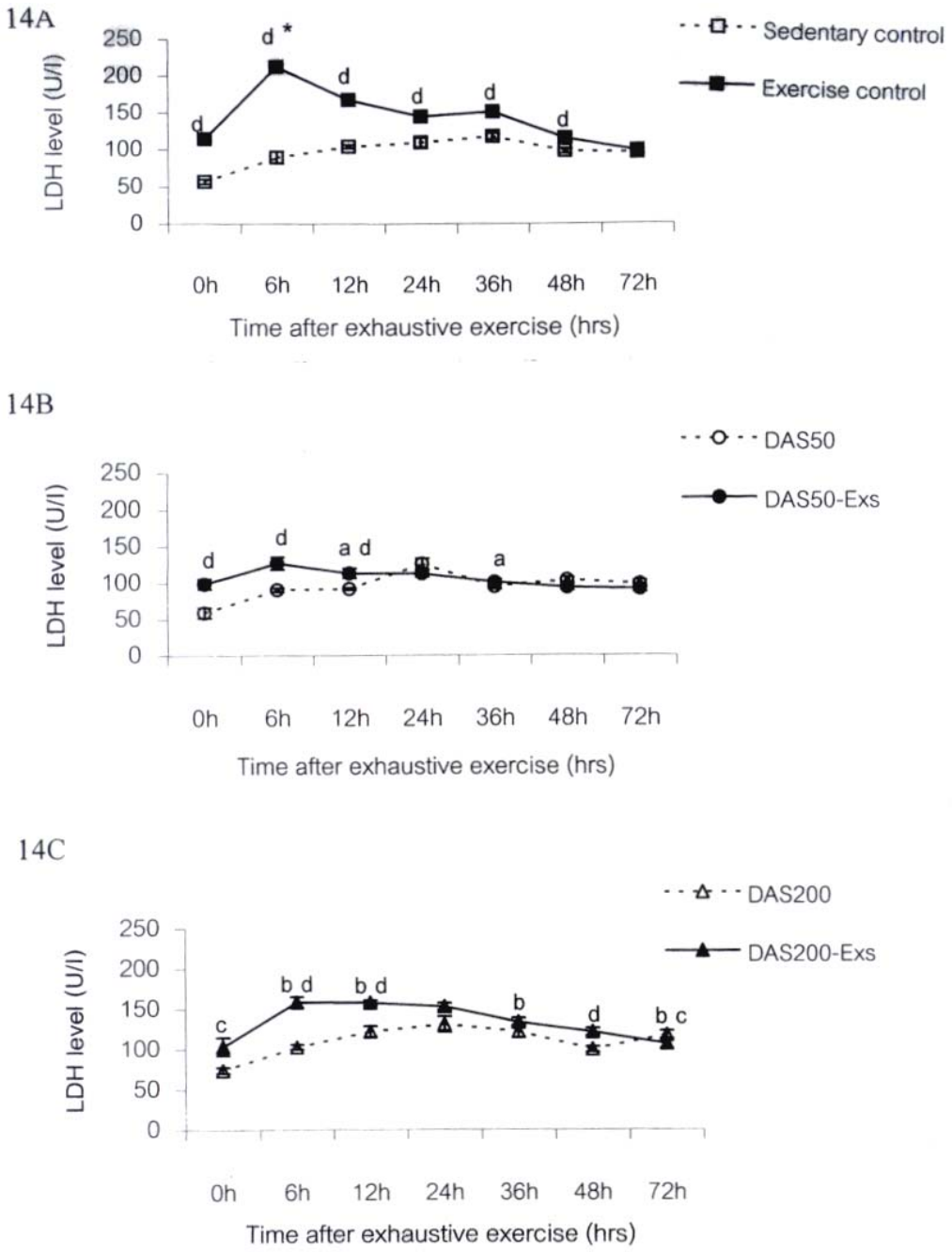


Figure 14. Time course changes on LDH activity of the plasma in control (A), DAS_{50} (B), and DAS_{200} (C). The values are mean ± SEM, (n=3). [a] represents significant difference between control and DAS_{50} rats at $p< 0.05$; [b] represents significant difference between DAS_{50} and DAS_{200} rats at $p< 0.05$; [c] represent significant difference between control and DAS_{200} rats at $p< 0.05$; [d] represents significant difference between sedentary and exercise subgroups at $p< 0.05$; and [*] significant difference from initial (time 0) values at $p< 0.05$.

5.2 Time course effects of DAS supplementation and plasma lactate dehydrogenase.

As 50mg/ kg body weight of DAS supplementation showed the remarkable effect on the diminished of LDH activity levels during post exercise period. This dose was then selectively used to investigate the time course of such diminishing of MDA levels in diaphragm muscle and plasma. In this study was performed up to 8 weeks period.

Table 17 showed LDH activities levels in plasma of rats in exercise control and DAS_{50}-Exs from the initial to the 8^{th} week of supplementation period.

After treated for the selected period, rats were subjected to the same experimented protocol. Following the exhaustive exercise, rats were kept for 6-hr after attainment of exercise and then LDH enzyme activity analysis. Results showed that LDH activity obtained from rat's plasma in exercise control group increasing pattern (Table17). In initial value (no treatment rats) was 65.47 ± 4.56 U/I. The LDH activity of exercise control group increased 3-flods during 1^{st} to 8^{th} weeks. LDH activity levels were 134.50 ± 7.19 (wk1), 150.37 ± 4.41 (wk2), 167.44 ± 5.59 (wk3), 174.58 ± 7.74 (wk4), 150.37 ± 1.22 (wk5), 158.71 ± 9.58 (wk6), 157.52 ± 2.93 (wk7), and 171.01 ± 6.91 (wk8) U/I, respectively. In the DAS_{50}-Exs group, the initial LDH activity was similar to the exercise control group ($p< 0.05$). DAS50-Exs's LDH activity also showed increasing, but less in magnitude. In DAS_{50}-Exs group, LDH activity levels were contained up to 2-folds until week 3 (59.33 ± 7.21 (initial), 117.05 ± 1.48 (wk 1), 133.31 ± 1.45 (wk2), and 115.06 ± 7.91 (wk3) U/I, respectively). Thereafter LDH activities reduced to closed initial level until week 6 (97.21 ± 4.51 (wk4), 96.41 ± 3.66 (wk5), 91.25 ± 2.67 (wk 6) U/I, respectively), but LDH activity levels were increased at 7^{th} to 8^{th} week (123.01 ± 4.61 (wk7), 112.28 ± 1.01 (wk 8) U/I). Results from within the group comparison presented that exercise control group had significantly higher LDH activity level from corresponding initial values, from the 1^{st} week throughout the 8^{th} week of the study. In the other group, DAS_{50}-Exs showed reducing of the LDH activities at 4^{th} week throughout the 8^{th} week of the study.

Table 17. LDH levels in diaphragm muscle of rats subjected to exercise and supplemented with 50mg/kg body weight, from the initial to 8th week of supplementation period. Data were collected from the beginning of the study (untreated, initial) and at one week interval at 6-hr post exhaustive exercise, (n=3).

Duration	LDH activity (U/I)	
	Control exercise	DAS_{50}-Exs
Initial	65.47 ± 4.56	59.33 ± 7.21
Week 1	134.50 ± 7.19 *	117.05 ± 1.48 *
Week 2	150.37 ± 4.41 *	133.31 ± 1.45 *
Week 3	167.44 ± 5.59 *	115.06 ± 7.90 e
Week 4	174.58 ± 7.74 *	97.21 ± 4.51 e
Week 5	150.37 ± 1.22 *	96.41 ± 3.66 e
Week 6	158.71 ± 9.58 *	91.25 ± 2.67 e
Week 7	157.52 ± 2.92 *	123.00 ± 4.61 e,*
Week 8	171.01 ± 6.91 *	112.28 ± 1.01 e,*

Values are mean ± SEM. Symbol e represents significant difference between exercise control and DAS50-Exs rats at $p< 0.05$; and * represents significance difference from initial (time 0) value, $p< 0.05$.

6. Effect of DAS on Antioxidative Enzymes and Non-Enzyme Content.

The antioxidative-related variables were investigated in similar to the time course effect of DAS supplementation. These variables were obtained from exercise and control conditions after 4 weeks of DAS supplementation.

6.1 DAS and superoxide dismutase enzyme activity

Figure 15 showed level of superoxide dismutase activity in four groups of rats. These composed of sedentary control, control exercise, 50 mg/kg body weight DAS supplemented (DAS_{50}), and 50 mg/kg body weight DAS supplemented with exercise (DAS_{50}-Exs) groups.

The superoxide dismutase activities of rat's diaphragm tissues in sedentary control and DAS_{50} groups were 27.30 ± 0.70 and 35.41 ± 1.17 unit/mg protein, respectively. In exercise groups, the superoxide dismutase activities were 36.12 ± 0.80 and 54.39 ± 1.79 unit/mg protein of exercise control and DAS_{50}-Exs groups, respectively.

The superoxide dismutase activity in DAS supplementation and DAS supplementation plus exercise groups showed higher significant ($p< 0.05$) than sedentary control and exercise control groups, respectively. The superoxide dismutase activity in both of exercise groups represented significantly increasing when compared with control groups ($p< 0.05$).

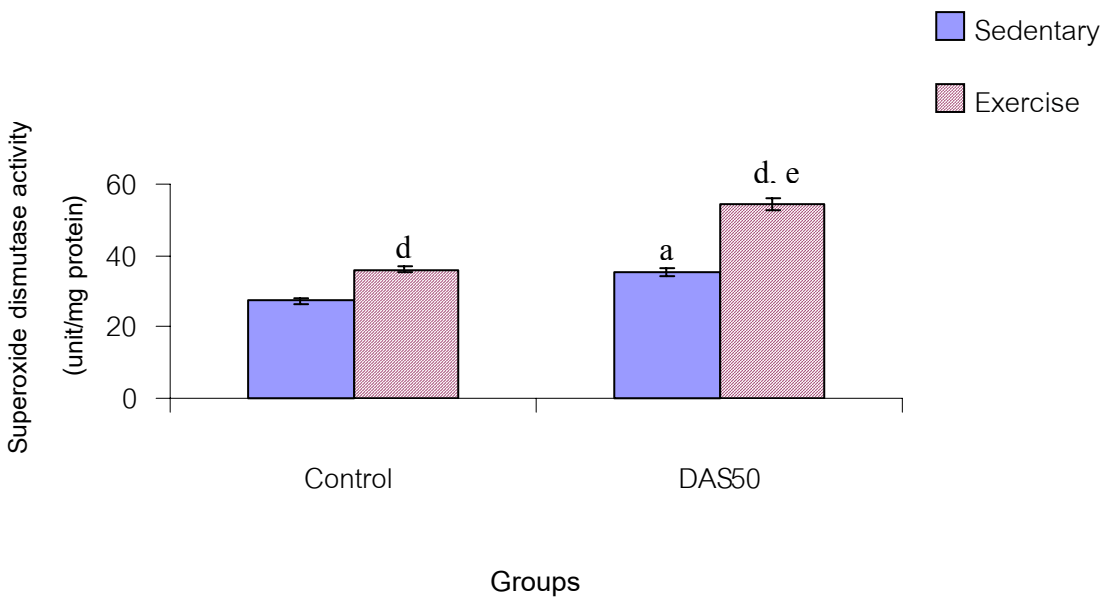


Figure 15. Superoxide dismutase activity (unit/mg protein) of diaphragm muscle of rats in sedentary control, exercise control, DAS_{50} and DAS_{50}-Exs groups for 4 week. Values are mean ± SEM, (n=3). [a] showed significant difference between sedentary control and DAS_{50} at $p< 0.05$. [d] showed significant difference between sedentary and exercise groups at $p< 0.05$. [e] represents significant difference between exercise control and DAS_{50}-Exs at $p< 0.05$.

6.2 DAS and glutathione peroxidase enzyme activity

Figure 16 showed level of glutathione peroxidase activity in four groups of rats. These composed of sedentary control, control exercise, DAS_{50}, and DAS_{50}-Exs groups.

The glutathione peroxidase activities of rat's diaphragm tissue in sedentary control and DAS_{50} groups were 220.53 ± 10.73 and 259.58 ± 8.94 μmol/mg protein/min, respectively. In exercise groups, the glutathione peroxidase activities were 349.67 ± 5.21 and 439.67 ± 5.62 μmol/mg protein/min of exercise control and DAS_{50}-Exs groups, respectively.

The glutathione peroxidase activity in DAS supplementation plus exercise groups showed higher significant ($p< 0.05$) than exercise control groups, respectively. The glutathione peroxidase activity in both of exercise groups represented significantly increasing when compared with control groups ($p< 0.05$).

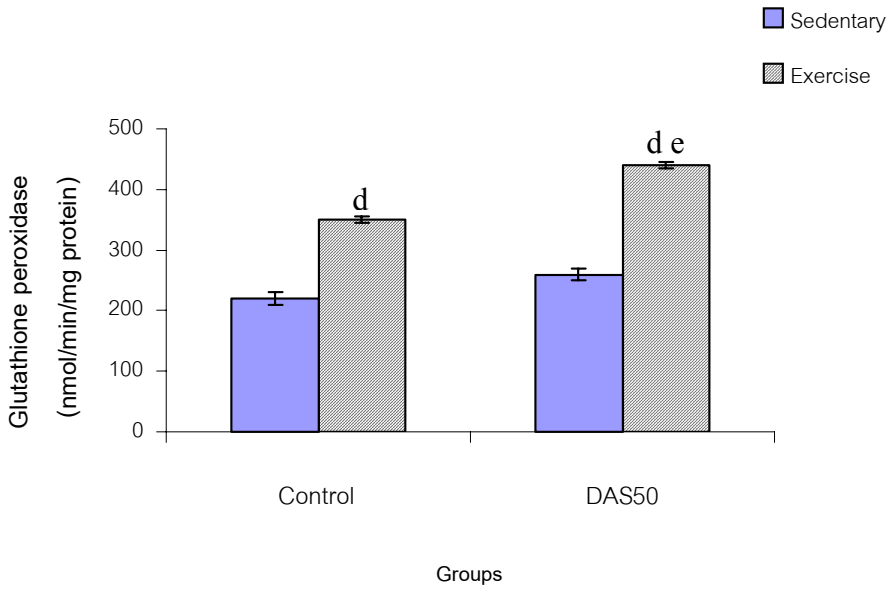


Figure 16. Glutathione peroxidase activity in diaphragm muscle of rats in sedentary control, exercise control, DAS50 and DAS50-Exs groups for 4 weeks. Values are mean ± SEM, (n=3). [a] showed significant difference between sedentary control and DAS_{50} at $p< 0.05$. [d] showed significant difference between sedentary and exercise groups at $p< 0.05$. [e] represents significant difference between exercise control and DAS_{50}-Exs at $p< 0.05$.

6.3 DAS and catalase enzyme activity

Figure 17 showed level of catalase activity in four groups of rats. These composed of sedentary control, control exercise, DAS_{50}, and DAS_{50}-Exs groups.

The catalase activities of rat's diaphragm tissue in sedentary control and DAS_{50} groups were 0.32 ± 0.07 and 1.03 ± 0.29 μmol/mg protein/min, respectively. In exercise groups, the catalase activities were 0.54 ± 0.29 and 1.28 ± 0.16 μmol/mg protein/min of exercise control and DAS_{50}-Exs groups, respectively.

The catalase activity of DAS supplementation plus exercise groups represented significantly increasing when compared with DAS supplementation group ($p< 0.05$).

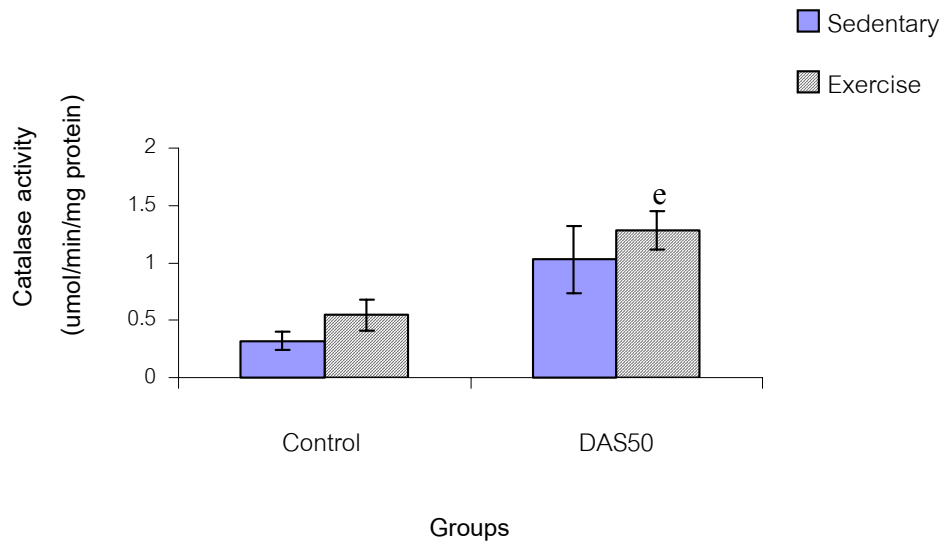


Figure 17. Catalase activity in diaphragm muscle of rats in sedentary control, exercise control, DAS50 and DAS50-Exs groups for 4 weeks. Values are mean ± SEM, (n=3). [a] showed significant difference between sedentary control and DAS_{50} at $p< 0.05$; [d] showed significant difference between sedentary and exercise groups at $p< 0.05$; [e] represents significant difference between exercise control and DAS_{50}-Exs at $p< 0.05$.

6.4 DAS and glutathione contents

Figure 18 showed level of glutatione content in four groups of rats. These composed of sedentary control, control exercise, DAS_{50}, and DAS_{50}-Exs groups.

The glutathione content of rat's diaphragm tissue in sedentary control and DAS_{50} groups were 0.506 ± 0.001 and 0.548 ± 0.02 μmol/g wet weight, respectively. In exercise groups, the glutathione content were 0.492 ± 0.01 and 0.587 ± 0.015 μmol/g wet weight of exercise control and DAS_{50}-Exs groups, respectively.

The glutathione content in DAS supplementation plus exercise group showed higher significant ($p< 0.05$) than exercise control groups.

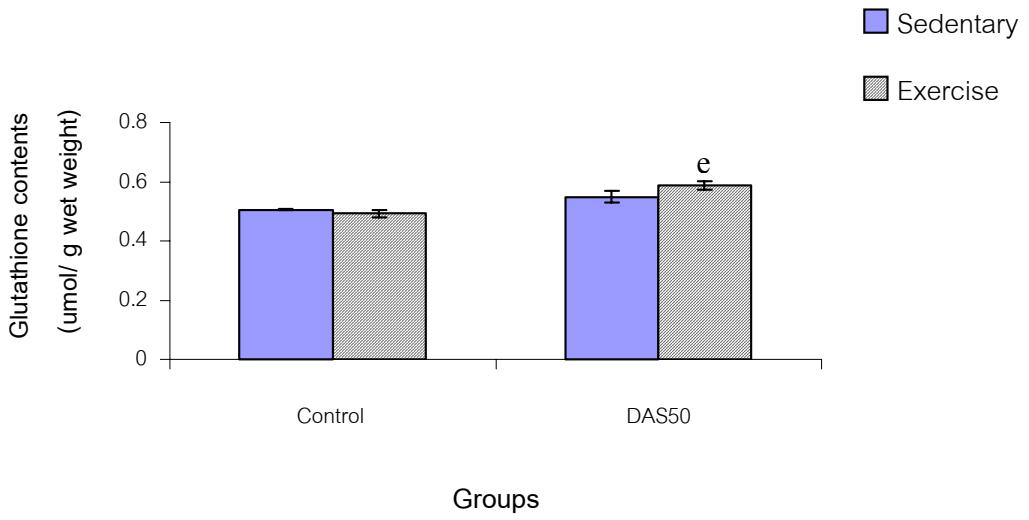


Figure 18. Glutathione contents in diaphragm muscle of rats in sedentary control, exercise control, DAS50 and DAS50-Exs groups for 4 weeks. Values are mean ± SEM, (n=3). [a] showed significant difference between sedentary control and DAS_{50} at $p< 0.05$; [d] showed significant difference between sedentary and exercise groups at $p< 0.05$; [e] represents significant difference between exercise control and DAS_{50}-Exs at $p< 0.05$.

CHAPTER 5
DISCUSSION

Initial body weights of male Sprague Dawley rats in this study (aged 3 wks), as well as final body weights (7-8 wks), were in the similar ranges of normal growth curve reported by previous studies (Chen *et al.*, 1994; Wu *et al.*, 2001).

1. Effect of DAS Supplementation on Body Weight Gain

In the current study, it appeared that either corn oil or DAS supplementation caused no significant difference in body weight gain of rats throughout the supplementation period. Basically, rats in all groups had the same type of diet and water. There was no change of their behaviors in all groups. No sign of toxicity or side effect were observed. Corn oil, DAS or combined corn oil-DAS exerted no positive or negative effect on body weight gained than their normal growth period.

High dose of DAS is known to harm to animals. The maximum safety dose had ever been determined was 200 mg/kg body weight (Chen *et al*., 1999). This dose was selectively used in the present study, which showed no net loss of animal during the feeding period. This study confirms results from previous studies in that different concentrations of DAS supplemented did not affect body weight gained. It was indicated that body weight gained was free from duration of DAS supplementation (Chen *et al*., 1994; Sheen *et al*., 1999; Wu *et al*., 2001). With the similar ranges of body weight gained in this study, it is concluded that changes in variables were the results from exercise, DAS and their combined effects.

2. Effect of Acute Exhaustive Exercise on Lipid Peroxide Levels

In this study, the effect of acute exhaustive exercise was induced by treadmill running, which was specifically designed for rodent. The investigator concentrates only post exhaustive exercise changes of MDA concentrations in diaphragm (as aerobic involuntary muscle), soleus and rectus femoris (limb muscles), and plasma in control and DAS supplemented rats throughout a period of 0-72 hrs following treadmill exhaustive running.

Free radicals are known as toxic agents created, as by-products, of metabolic activity. Under aerobic conditions, the participation of oxygen in redox reactions is remarkably enhanced in which reactive oxygen species such as hydroxyl radical, hydrogen peroxide, lipid peroxides, nitric oxide, and superoxide are possibly produced (Sjodin *et al*., 1990). The predominant processes resulting from oxidative stress include oxidative lipid peroxidation, disturbance of calcium homeostasis, and alteration of metabolic pathways (Carlson and Sawada, 1995).

Free radical stress has been implicated in the functional inhibition or down regulation of important metabolic enzymes with acute exhaustive exercise (Lawler *et al*., 1993). One indicator of free radical stress, which was malondialdehyde (MDA), has been postulated as the higher generation of free radicals during exercise (Jackson and O' Farrell, 1993; Clutton, 1997).

Acute bouts of exhaustive exercise have been repoted to result in damage to locomotor skeletal muscle cellular constituents such as membrane lipid (Alessio and Goldfarb 1988), protein (Bostrom *et al*., 1974), and sacroplasmic reticulum (Byrd, 1992). One possible mechanism to cellular damage in exercising skeletal muscle is free radical stress (Byrd, 1992; Jackson *et al*., 1985; Ji *et al.,* 1988). Calcium-dependent proteases are also known to cause conversion of the enzyme xanthine dehydrogenase to an oxygen free radical-generating form, xanthine oxidase, which could cause damage to the muscle (Rice, 1994). It has been found that extensive lipid peroxidation in cell can results in impaired functions of membrane bound receptors, ion channels, and reduced enzyme activities (Halliwell and Gutteridge, 1990; Kukreja and Hess, 1992).

Prolonged strenuous exercise was proposed to be an oxidative stress to the body due to the generation of oxygen-center free radicals (Alessio *et al.*, 1988) which

would be enhanced by a several-fold increase in oxygen consumption (Ashton *et al.*, 1999), high metabolism of catecholamines (Singh *et al.*, 1969), high hydrogen ion concentration thus promoting conversion of a weakly toxic radical O_2^- into a highly toxic free radical ^{-}OOH (Singh, 1998), local ischemia with subsequent reoxygenation, and infiltration of neutrophil (Sen *et al.*, 1989). Higher post-exercise MDA was found in both working muscles and plasma (Ashton *et al.*, 1999) within 10 minutes in animals (Brady *et al.*, 1987) and 30 minutes in human (Sen *et al*., 2001). Even in the same organ, some authors have found controversial levels of lipid peroxidation markers during an intense physical effort (Caillaud *et al.*, 1999; Maughan *et al.*, 1989). It is suggested that antioxidant function is adequately adjusted in some organs, lung and diaphragm, but not the others. It would suggest that the process of lipid peroxide formation takes time to achieve and might be related to reperfusion during the recovery period. The present study shows that all either diaphragm or skeletal muscles are susceptible to injury by oxidative stress during the recovery phase after exercise.

Generation of free radicals is possibly described by the following mechanisms (Figure19). First mechanism relies on the increases in epinephrine and other catecholamines, which are metabolically inactivated (Singh *et al.*, 1969). It has been estimated that 4-5% of the oxygen uptake during mitochondrial respiration leaked from electron transport chain (Clarkson and Thompson, 2000; Sen *et al.*, 2001). They proposed that the greater oxygen consumed during exercise, the higher oxygen may possibly leak from mitochondria. As a result, oxygen free radical is formed and named as oxidative stress. Second mechanism is the inflammatory responses secondary to muscle damage incurred with overexertion (Clarkson and Thompson, 2000) with subsequent infiltration of neutrophils (Sen *et al.*, 1989). Third, previous studies have demonstrated that change in the distribution of blood flow during intense exercise may cause a transient ischemic-hypoxic state in the liver and kidney which in turn reduce to half of normal and leading to exercise induced ischemia or hypoxia (Fleig and Wahlen 1971; Ohnishi *et al.*, 1985; Adams and Best, 2002). There is reperfusion during the recovery period after exercise leading to conversion of xanthine dehydrogenase (XDH) to the oxygen free radical generating oxidase form (xanthine oxidase, XO). This has been finally elicited the formation of

superoxides, which cause a rapid production of the most reactive metabolites, hydroxyl radicals. There are many reports of free radical generation causing oxidative tissue injuries in cells following ischemia-reperfusion insults (McCord, 1985). Hellsten-Westing and colleagues (1994) suggested that free radicals stemming from XO might impair the structure and function of the cells. However, there were some evidences that the conversion of XDH into XO did not occur to any significant extent in vivo ischemia has strongly supported the hypothesis that the liver and kidney were potently resistant to hypoxia. Exhaustive physical exercise associated with the increased production of reactive oxygen species (ROS) and oxidative stress is known to cause cellular dysfunction either alterations of protein and lipid membrane integrity or modification of DNA (Davies *et al*., 1982). As a consequence, lipid and protein peroxidation are induced (Criswell *et al.*, 1993). Li and co-workers (1999), using the fluorescense polarization method, found decreasing of mitochondrial fluidity immediately and 24 hr after exhaustive exercise, which later indicated to be related to higher level of TBARS.

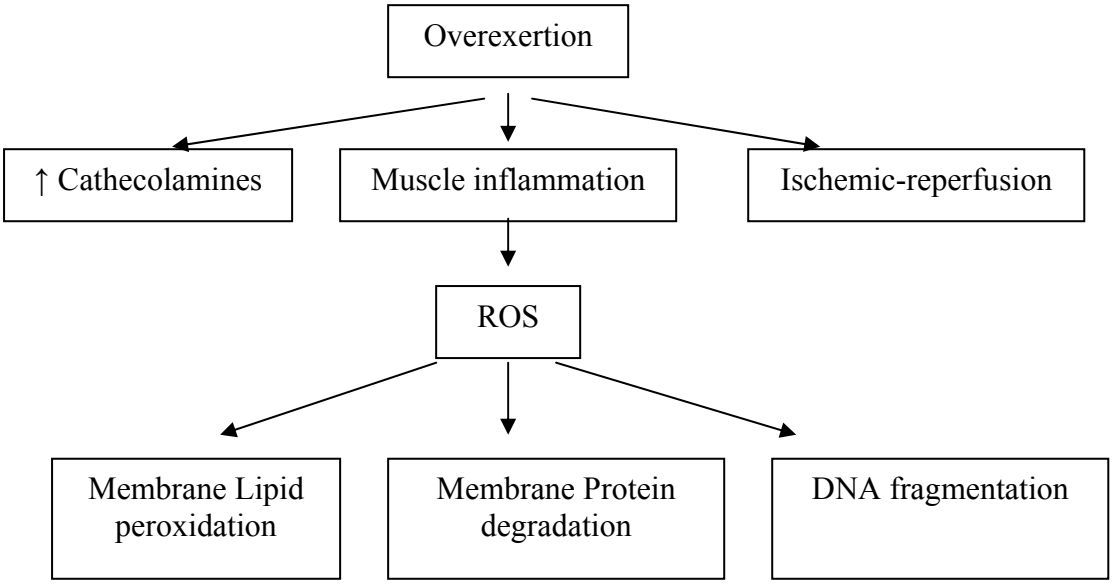


Figure 19. Proposed possibly exertion-related mechanisms and ROS induction, which affect cell and nuclear membrane integrities.

The present study reveals that acute bout of exhaustive exercise causes induction of MDA contents of the diaphragm, soleus, rectus femoris muscles (Figure 6, 7, 8 respectively), which may result in higher MDA in plasma (Figure 9). It has been repoted that free radicals and other reactive oxygen species (ROS) are produced during muscle contraction in the diaphragm (Davies *et al*., 1982; Reid *et al*., 1992; Diaz *et al.,* 1993). MDA concentration was increased with acute exercise in young exercise group's costal diaphragmatic muscle (Lawler *et al*., 1994). Other investigation reported that some free radical stress occurred in the diaphargm with acute exercise (Anzueto *et al*., 1992). Furthermore, production of ROS during repeated diaphragm contraction in vitro or resistive breating in vivo has been shown to contribute to diaphragm oxidative injury, such as lipid and protein peroxidation (Anzueto *et al*., 1992 and 1993; Vincent *et al*., 1999). Supinski and colleagues (1991) reported that MDA concentrations increased in the diaphragm with *vitro* electrical stimulation.

Previous studies showed that the increasing of level of a marker of lipid peroxidation (MDA) in the locomotor skeletal muscles such as soleus and quadriceps muscles (Alessio and Goldfarb, 1988; Ji and Fu, 1992).

3. Time Course of Changes of Lipid Peroxide Levels

The present findings uniquely define time course of changes of lipid peroxide levels of involuntary and voluntary muscle and plasma. Results obtained from previous studies controversially found that plasma TBARS were significantly elevated at 3-hrs (Frankiewiez-Jozko *et al.*, 1996), 6-hr after exercise (Maughan *et al.*, 1989). MDA in deep vastus lateralis muscle significantly increased at immediately after exhaustive exercise (Ji and Fu, 1992). Rats in this study were subjected to exercise-induced lipid peroxidation of diaphragm, soleus, rectus femoris muscles, and plasma where peaks MDA levels of all specified tissues were showed at 6-hrs after exercise. It is indicated that exhaustive running induced lipid peroxidation during post-exercise period (0-36 hrs) in exercise control group. It is indicated from the present study that there is time-lag between the stimulation and appearance of MDA. It would suggest that the process of lipid peroxidation formation, of both voluntary

and involuntary muscles, takes time to achieve and might be related to the recovery period.

When rats was treated with DAS, MDA contents showed similar pattern but less in magnitudes and returned to normal level during 12-36 hrs post-exercise. Thus, DAS significantly attenuated lipid peroxidation when compared with control groups. This compound derived from garlic, involves extensively in oxidation processes due to three possible chemical characteristics: sulfur atom, allylic carbon and terminal double bonds. Sulfur atom of DAS is induced by Cytochrome P_{450}2E1 (CYP2E1) enzyme-mediated oxidation, which produces diallyl sulfoxide (DASO) and diallyl sulfone ($DASO_2$) (Brady *et al.*, 1991). It was proposed that the chemoprotective effects of DAS and its metabolites might be attributed to the induction of phase II detoxification enzymes (Sparnin *et al.*, 1988; Ji *et al.*, 1996). It is believed that DAS with greatly lipid solubility could interact deeply with biomembranes and might be effective in scavenging radicals in the lipid phase (Kwak *et al.*, 1994).

Diaphragm

Rat diaphragm, the principal muscle of inspiration, that contains combination of slow oxidative, fast glycolytic, and fast oxidative glycolytic fibers (Brooke and Kaiser, 1970; Keens *et al*., 1978). These different muscle fiber types appear to be sequentially recruited at different time during the inspiratory phase, serving to generate the requirements for rapid force development necessary to overcome the progressive increase in elastic resistance and to permit smooth airflow to cope with fluctuated dynamic resistance during respiratory cycle (Iscoe *et al*., 1976). This muscle, therefore, contains special property of unlimited contraction despite the changes in respiratory rate. The high frequency of ventilation in the adult rat is about 100 beat/minute but also highly fatigue resistance (Crossfill and Widdicombe, 1961).

Diaphragm is the unique involuntary skeletal muscle that considered as the main part of metabolic capacity (Powers *et al*., 1990). As a major pressure generator during ventilation, this muscle itself requires appropriate energy supply and might possibly prone to free radicals and other reactive oxygen species (ROS) producing during repeated rhythmic contractions (Davies *et al*., 1982; Reid *et al*., 1992; Diaz *et al.,* 1993). Moreover, this muscle can temporarily adapt among its fiber type (Smith *et*

al., 1988). In contrast to this adaptive capability, the present study defines that diaphragm is susceptible to cell damaged in the similar extents as those found in soleus and rectus femoris muscles (Figure 6A, 7A, and 8A). Such cell damaged is subsided as animals were treated with DAS at either 50 or 200 mg/kg BW doses (Figure 6B, C; 7B, C; and 8B,C).

Some investigators have been explained possible mechanisms of oxidative formation in muscle, which is based on cellular respiration and amount of mitochondria. There are several potential sources of oxygen-derived radicals during energy metabolism within the muscle, either intracellular or extracellular level. Superoxide radical is formed from the reactions of ubiquinone and NADH dehydrogenase during electron transport in mitochondria (Girotti, 1985; Gristman and Granger, 1989). Hydrogen peroxide (H_2O_2) can then be formed from the dismutation of superoxide radical. Superoxide radical can also react with H_2O_2 to produced hydroxyl radical (OH^-) in the presence of low concentration of iron (Fridovich, 1982). Another potential cytosolic source of oxygen-derived free radicals during strenous contraction is an intracellular accumulation of xanthine by-products. These by-products are formed during exertion as intracellular ATP is consumed and catabolized (Lew *et al*., 1985). Superoxide anions are formed from the reaction of oxygen and xanthine by-products in the present of xanthine oxidase (Parks and Granger, 1986).

The possibility that free radicals formed in working diaphragm muscle contributed to muscle damage is supported by evidence from studies by other investigators of in vivo and in vitro diaphragm and peripheral skeletal muscle (Gristman and Granger, 1989; Diaz *et al.,* 1993). The present study additionally confirms that a marker of diaphragm cell damaged is remarkably appeared after extremely high physical stress. Diaphragmatic damaged is not reduced with DAS treatment, either 50 or 200 mg/kg BW doses.

Soleus

Soleus muscle is basically a postural muscle, persistently active and composes primarily of slow contracting and fatigue-resistant fiber with high oxidative potential (Kugelberg, 1976). Previous studies reported that acute exercise might cause large-scale deletion of mitochondrial DNA and ultrastructural mitochondrial changes

in the soleus muscle (Sakai *et al*., 1999). Frankiewitch-Jozko and colleagues (1996) showed that exhausting exercise led to a considerable increase in TBARS concentrations in the soleus muscle of the untrained rat. Bejma and Ji (1999) reported the high lipid peroxidations of soleus and vastus lateralis muscles after an acute bout of exercise in rats. The present finding determines that postural voluntary muscle, soleus, is liable to cell damaged despite the fact that it contains highly aerobic property. Soleus damaged is subsided with DAS treatment, either 50 or 200 mg/kg BW doses.

Rectus femoris

Rectus femoris is highly fatiquable and is composed almost exclusively of fast-contracting fibers, high in the activities of enzymes involved in glycolysis and low in mitochondrial enzymes (Eddinger *et al*., 1985; Vrbova *et al*., 1985). Previous reports have unquestionably shown that the fats-twich oxidaitive fiber (Type II, rectus femoris muscle) showed exhaustive exercise-induced lipid peroxidation (Salminen and Vihko, 1983; Goldfarb *et al*., 1994; Leeuwenburgh and Ji, 1995, 1996; Hara *et al*., 1996). The present finding reveals that exercise-induced lipid peroxidation takes place in the similar pattern in fatigue labile as well as in fatigue resistant muscle. When DAS is applied, rectus femoris damaged is not reduced with DAS treatment, either 50 or 200 mg/kg BW doses.

Plasma

In this study, plasma MDA levels are consistent in all exercise control subgroups despite the fact that rats were treated with DAS. Plasma MDA is found to be related to exercise mode and intensity in animals (Alessio *et al.*, 1988). In human, it was significantly correlated with an increase in serum creatine kinase after an 80-km running race (Kanter *et al.*, 1988) or at 24-hr after 90 min of strenous exercise (Krotkiewski and Brzezinska, 1996). Itoh and colleagues (1998) reported increased hydroxyl radical level in the plasma in a group, which ran faster and for a shorter distance. Alessio and colleages (2000) who noted increased lipid peroxide in serum after isometric contractions and increase in protein carbonyls and oxygen radial absorbance capacity after aerobic exercise. Lovin and co-workers (1987) measured

serum thiobarbituric acid-reactive substance (TBARS) after graded cycle ergometry to exhaustion and found no difference in serum during progressive intensities (40% to 70% maximum oxygen consumption, VO_{2max}) but found significantly elevated at the end of the exercise (100 % VO_{2max}).

4. Effects of DAS Supplementation on Exercise Induced-Lipid Peroxidation

Diallyl sulfide (DAS) lipophic thioether, is one of these compounds, found exclusively in garlic. As previously mentioned about anti-oxidative properties of DAS at three positions: sulfur atom, allylic carbon, and terminal double bonds, it is reported that DAS contains greatly biomembranes scavenging radicals in the lipid phase. DAS and diallyl disulfide (DADS), all sulfur rich constituents of garlic, are known to induced activities of phase II enzymes such as glutathione S- tranferase and quinoe reductase, which in turn reduce the genotoxicity of several carcinogens (Guyonnet *et al*., 2001).

In animal studies, results showed that DAS did not only effective in modulating phase I and phase II metabolizing enzyme (Pan *et al*., 1990; Reicks and Crankshaw, 1996; Jeong and Lee, 1998) but also the antioxidant system capacity (Dwivedi *et al*., 1998; Chen *et al*, 1999, 2001). Study of Qu and co-workers (2003) showed that DAS exhibited antioxidant activities against copper- and amphotericin B-induced low density lipoprotein (LDL) oxidation and the author suggested that DAS are potent agent for protecting LDL against oxidation and glycation. Gaed and colleagues (2003) reposted that DAS inhibits lipid peroxidation induced by exposure to diethylstilbestrol (DES), a synthetic estrogen that causes of breast cancer in breast tissue of rat model. Horie and co-workers (1992) reported that the antioxidant capacity of DAS against ascorbic acid induced lipid peroxidation in rat liver microsomes. The anioxidative properties of DAS was studied in rat liver microsome, DAS inhibited the formation of thiobarbituric reactive substance (TBARS) initiated by t-butylhydroperoxide (Imai *et al*., 1993). The protective effect was observed when DAS was given before, during, or soon after chemical treatment (Yang *et al*., 2001).

This study also shows the positive effects of DAS treated, which DAS can reduce of concentration of malondialdehyde (MDA) induced by exhaustive exercise in muscles and plasma in rats. In DAS_{50} group showed the tendency to reduce formation of MDA in skeletal muscles and plasma. The uniqueness of the present study is that DAS effectively suppresses MDA formation during the peak period of the formation in examined skeletal muscles and plasma particularly at the highest peak of MDA formation (6 hour, after exercise). Lipid peroxide concentrations were not different in DAS treated and untreated rats in sedentary control groups, thus DAS may benefit only during exposed of oxidative stress. However, in DAS_{200} (200 mg/kg body weight) supplemented group shows no consensus result in which high dose of supplementation might be the cause of tissues damaged and higher of MDA production in rectus femoris muscle. Thereafter, the suppression of DAS on MDA concentration might depends on the appropriate of dose and treated time periods. The exhaustive exercise resulted in increased of MDA formation and DAS supplementation can suppresses on this process. This study proved this effect of two doses of DAS, thus the precise appropriated dose of DAS supplemented requires further investigation.

5. Muscle Damage and Lactate Dehydrogenase Induced by Exhaustive Exercise

Plasma LDH in the present study is used to approximate muscle cell damaged. The increase in plasma enzyme activities after exhaustive exercise has been well established (Komulainen *et al*., 1994; Clarkson *et al*, 1992; Armstrong *et al*., 1983; Kuipers, 1994; Maughan *et al*., 1989). Several hypotheses have been proposed to account for the presence in plasma of high levels of enzyme activities with a generally intracellular origin (Cotran *et al*., 1989; Kuipers, 1994; Anderson *et al.,* 1993). The present results show that increases in plasma lactate dehydrogenase (LDH) activity is a consequence of exhaustive exercise, which significantly highest at 6 hrs. The number of reports demonstrated that plasma enzyme activities increased after exercise (Komulainen *et al*., 1994; Armstrong *et al*., 1983; Van Der Meulen *et al*.,

1991). The possible cause for the risen in plasma enzyme activities at 6 hrs after exercise is associated with an acute and transient increased sacrolemmal permeability due to a decline in the pool of energy-rich phosphates (Armstrong *et al*., 1983; Kuipers, 1994; Van Der Meulen *et al*., 1991). Mena and colleagues (1996) showed a significant increased in plasma LDH, AST (Aspatate amino transferase) and ALT (Amino Alanine transferase) at immediately after exercise and suggest that the presence of these enzymes in the blood is probably due to mechanical damaged of muscle cells leaking their content into the interstitial fluid.

The other possibility that might explain the first, almost immediately, peak of plasma enzyme activities post-exercise is the lymph hypothesis. Exercise causes a continuous lymphatic output through pulsations by muscle contractions. It is known that exercise increases lymph flow in human (Olszewski *et al*., 1977) and that the variation in lymph protein concentration is considerable, such as 90% in the case of lactate dehydrogenase (Szabo *et al*., 1972). Moreover, there is a relationship between increased free radical activity and muscle damage following exercise (Maughan *et al*., 1989; Kanter *et al*., 1988). Kanter and colleagues (1988) demonstrated the significant correlation between post-race MDA concentration and serum CK-MB levels.

In the present study, the peak of LDH activity reached at 6 hour after exhaustive exercise as well as plasma and skeletal muscles lipid peroxidation concentration found at 6 hour after the exercise. This might indicate that exhaustive exercise induced tissues damage by mechanical stress resulting in the highest significantly increased either of LDH activity and lipid peroxidation concentration in plasma.

6. Dose Responses of DAS Supplementation

The present study demonstrates that an appropriate dose of 50 mg/kg BW DAS supplementation significantly attenuated lipid peroxidation. Supplementation of DAS above this level has adverse effect only in soleus muscle. The probable explanation for the pronounce increase in MDA content in diaphragm and muscles during exercise is their prolonged rhythmic contractions and increases cellular respiration to meet the high energy demand. There are several sources of oxygen-

derived radicals during exercise include formation of superoxide radical from the reactions of ubiquinone and NADH dehydrogenase during electron transport in mitochondria; hydrogen peroxide (H_2O_2) from the dismutation of superoxide radical (Girotti, 1985; Gristman and Granger, 1989).

Previous studies reported that at appropriate concentration, DAS protects against the toxicity of different xenobioticc (Morri *et al*., 2000). Adversely, at higher DAS concentration this substance might induce mild genotoxicity (Musk *et al*., 1997). Lohani and co-workers (2003) investigated the protective effects of DAS (5 or 10 μM) against abestose-induced genotoxicity in human mesothelial cells, which showed the significant reduction after treated with 5 μM but not with 10 μM of DAS. The authors proposed that at appropriates concentrations of DAS can attenuate asbestos induced genotoxicity which was mediated by reactive oxygen species.

Supplementation of DAS causes the reduction formation of MDA at the peak period in skeletal muscles and plasma ($p<0.05$). Thus results revealed that after daily dose of 50 mg/kg body weight for four weeks, DAS inhibited MDA but not in the dose-dependent pattern. However our results contradict with some previous reports, in which the protective effects of DAS was both dose and time dependent. Garlic extract exhibits a concentration and dose-dependent inhibition of free radical generation triggered by hydrogen peroxide in vascular endothelial cells (Wei and Lau, 1998). Horie and co-workers (1992) reported that the antioxidant capacity of DAS against ascorbic acid induced-lipid peroxidation in rats liver microsomes. The antioxidant protection from this organosulfur compound increased significantly with increasing the concentration from 5 μM to 10 μM (Yin *et al*., 2002).

7. Time Course Effects of DAS Supplementation

Repeated daily intra-gastric administrations of either corn oil or DAS over a period of 4 weeks tend to reduce exercise-induced peroxidation in both diaphragm and plasma. The reduction was observed from the first week of administered but did not reach that statistical significant level. The significant protection was observed at 4^{th} week of supplementation in diaphragm and plasma ($p<0.05$) of rats subjected to exhaustive exercise. However, the MDA levels at week 1^{st} were increased higher than initial value (no treatment) about 2 folds, the possible mechanism has been explained in that stress produced by gavage administration (feeding by insertion of a long tube) in rats for four weeks which might relate to stress hormone responses, Cortisol, which induced oxidative stress in week 1^{st} both of diaphragm and plasma in rats (Brown *et al.*, 2000).

The present results showed that pre-treatment with DAS (50 to 200 mg/kg body weight) significantly protected rats from hepatotoxicity as indicated by lactate dehydrogenase leakage. This is in good agreement with the finding of Hu and colleagues (1996), which showed LSH as hepatic protection from acetaminophen (an analgesic; APAP)-induced liver toxicity in a time and dose dependent fashion.

This study uniquely demonstrates the results of pretreatment with DAS in skeletal muscle. Effect of DAS on exercise induced-lipid peroxidation is quite complicated in that repeated doses may result in responses that differ from that of a single dose. Chen and co-workers (1994) indicated that effects of chronic ingestion of DAS (50 and 200 mg/kg body weight) given daily for 29 days had no apparent cumulative effects in plasma due to the multiple treatments of DAS. Investigation conducted by Sai-Kato and colleagues (1995) demonstrated that repeated dosing of DAS (100 mg/kg body weight) for 5 days can significantly protect against pentachlorophenol (PCP) induced hepatic oxidative DNA damaged.

8. Effects of DAS on Glutathione Content

The observed antioxidant protection of DAS discloses that this organosulfur compound is a potent agent for enhancing membrane lipid stability via Glutathione (GSH) enhancement. In 2003, Scharf and Co-workers investigated the responses of hepatoma cell line incubated with diallyl sulfide (175-700 mM) and the result showed that exposure to DAS led to dose dependent increase in GSH concentration. DAS (50 mg/kg) supplementation tended to significantly increased the level of diaphragm GSH in the DAS-Exs groups when compared with control exercise group (p<0.05). Results of the present study are consensus with previous finding where DAS increases the level of GSH (Srivastava *et al*., 1997). However, different doses of DAS were consistently reported to enhance GSH levels. DAS supplementation (80 mg/kg body weight) increased GSH content in red blood cells and liver of rats (Wu *et al*., 2001). Oral DAS (100 mg/kg body weight) treated three times per week for seven weeks results in significantly increased of GSH levels in rat hepatic cell (Sheen *et al*., 1999). Horie and colleagues (1991) revealed that DAS inhibited the formation of TBARS in isolated liver microsome membrane.

The mechanism of DAS to enhance intracellular content of the endogenous GSH is still unknown.

9. Effects of DAS on Antioxidant Enzymes Activities

In rat diaphragm, DAS supplementation did influence the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX). DAS_{50} and DAS_{50}-Exs showed significantly increase SOD and GPX when compared with sedentary control and exercise control group respectively. However, catalase (CAT) activity and glutathione (GSH) content were significantly increased in DAS_{50}-Ex compared with exercise control groups. The higher GPX activity could enhance the ability of the cell to remove hydroperoxides (Maurya and Singh, 1991).

Conflicting results still remain concerning GPX, SOD, and CAT activities. Previous studies have found neither change nor significant increase in GPX, SOD and CAT activities. Sheen and colleagues (1996) showed significant decrease in

glutathione-S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPX) activities in cultured rat hepatocytes. Chen and co-workers (1999) found no change in GPX or SOD activities in DAS treated (50 or 200 mg/kg for 29 days) rat liver, kidney, lung, and brain. The antioxidative effects of DAS could firstly be attributed to its ability to modulate phase I and phase II metabolizing enzymes (Pan *et al*., 1990; Singh *et al*., 1998; Chen *et al*., 1999) which increases the intracellular levels of GSH and modulating GSH-dependent detoxification enzymes; such as GPX and GR in any tissues. DAS also poses nonenzymatic antioxidant property.

We noted increases in SOD and GPX activities in the diaphragm with exercise. This is the indication, to pervious investigations, that some oxidative stress does occur in the diaphragm with *in vivo* exercise. Increases in the level of markers of lipid peroxidation (MDA) have been previously reported in locomotor skeletal muscle with exercise (Alessio and Goldfarb, 1988; Ji and Fu, 1992). In diaphragm, Supinski and colleagues (1991) reported that MDA levels increase in diaphragm with *in vitro* electrical stimulation. Some reports demonstrated that increases in antioxidant enzyme activities in rat hind limb muscle with an acute bout of exercise (Ji and Fu, 1992; Lawler *et al*., 1993). Increases in antioxidant enzyme activity in locomotor muscle and in the diaphragm as a result of acute exercise are likely too rapid to be a result simply of new protein synthesis (Ji and Fu, 1992). However, acute exercise induced a clear metabolic stress on the diaphragm as a ventilation and work of breathing increase at an elevated rate (Coast and Krause, 1993).

In present study, we found that significantly higher in activity levels of the superoxide dismutase and glutathione peroxidase both of exercise control and DAS_{50}-Ex groups. Previous investigations showed similar results, Power and colleagues (1990) showed that greater activity levels of the antioxidant enzyme glutathione peroxidase in both costal and crural diaphragm when compared to plantaris muscle. However, in 1997, Oh-ishi and co-workers reported that acute exercise increased the activities of GPX and CAT in diaphragm of untrained rats.

CHAPTER 6
CONCLUSION

In conclusion, the present study identifies that exhaustive exercise induces free radicals in diaphragm (involuntary muscle), rectus femoris (fast-twitch), soleus (slow-twitch) muscle and plasma. It is demonstrated that different tissues showed different responsiveness to the same oxidative stimuli. Rectus femoris shows higher lipid peroxidation (malondialdehyde, MDA) than soleus, diaphragm, and plasma. The dynamic profiles of lipid peroxidation in that the peaks of all tissues MDA are at 6 hrs with recovery periods are about 24 to 36 hrs post-acute exhaustive exercise. Results emphasize that exercise-induced oxidative stress takes place not during but following exhaustive exercise.

Long term supplementation of diallyl sulfide (DAS), at appropriate concentration of 50 mg/kg body weight, shows its protective roles against exhaustive exercise-induced lipid peroxidation in diaphragm, soleus, rectus femoris and plasma. The antioxidative effects of DAS could be attributed to its ability to modulate antioxidant enzyme by increasing the intracellular superoxide dismutase and glutathione peroxidase.

Results support hypothesis that DAS, the kitchen additive herb benefits as antioxidant protection against oxidative stress. In addition, the present study specifies the effective dose of DAS at 50mg/kg body weight.

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APPENDIX

APPENDIX A
LIPID PEROXIDATION

Reagents

1. 8.1% Sodium dodecyl sulfate (SDS)

Dissolve 8.1 g of SDS in distilled water to a final volume of 100 ml. Store in refrigerator. Stir and heat before use.

2. 20% Acetic acid solution

Mix 20 ml of pure acetic acid with distilled water, adjust to pH 3.5 with 20 N and 1 N NaOH. Add distilled water to a final volume of 100 ml.

3. 0.8% Thiobarbituric acid (TBA)

Dissolve 0.8 g of TBA in distilled water, to a final volume of 100 ml. Stir and heat before use.

4. Mixture of n-butanol and pyridine (15:1 v/v)

Mix 750 ml of n-nutanol with 50 ml of pyridine, store in refrigerator.

5. 1,1,3,3-Tetramethoxypropane (TMP) or malondialdehyde bis (dimethyl acetal) solution is used as an external standard.

Store this solution in refrigertor. The level of lipid peroxide is expressed as nmol of MDA (malondialdehyde). Prepare 10^3 nmol/ml of stock TMP solution by pipette 16.4 μl pure TMP (MW = 164.2) and add distilled water to a final volume of 100 ml. Then pipette 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, ml of this stock, resulting in the following concentrations of standard TMP: 10, 20, 30, 40, 50, 60, 70, and 80 nmol/ml.

6. 1.15% KCl in 0.1 M Phosphate buffer (pH 7.4)

Mix 0.1 M K_2HPO_4 in M KH_2PO_4 to make phosphate bufferpH 7.4 add 1.15 g of KCl to 100ml of this 0.1 M phosphate buffer (pH 7.4), mix thoroughly.

Procedure

1. After washing the isolated muscle in ice-cold 0.9% NaCl, the muscle homogenate is prepared by homogenizing each gram of wet tissue in 4 ml of 1.15% KCl in 0.1 M phosphate buffer, pH 7.4.

2. Pipette the following solutions into a series of glass tubes with screw caps:

Solutions	Blank (ml)	Standard (ml)	Unknown (ml)
Sample	-	-	0.5
8.1% SDS	0.2	0.2	0.2
20% Acetic acid (pH3.5)	1.5	1.5	1.5
0.8% TBA	1.5	1.5	1.5
TMP stock solution	-	0.5	-
Distilled water	0.8	0.3	0.3

3. Heat tubes in a water-heat at 100 °C for 30 min.

4. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine are added. Tubes are shaken for 1 minute.

5. After centrifugation at 3,500 rpm for 15 minutes, the organic layer is taken and its absorbance at 532 nm is measured.

6. The content of lipid peroxide is expressed in term of nmol MDA/ml.

Calibration Curve

1. Prepare a series of tube containing TMP stock standard in water in the following concentrations:: 5.0 nmol/0.5 ml, 15.0 nmol/0.5 ml, 20.0 nmol/ 0.5 ml, 25nmol/0.5 ml, 30.0nmol/0.5 ml, and 40.0 nmol/0.5 ml.

2. Perform step 2 in procedure.

3. Determine the absorbance at 532 nm. Then plot the optical density as a function of nmol of MDA/ml.

Reference

Ohgawa H, Ohishi N, Yaki K. Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-8.

APPENDIX B
LACTATE DEHYDROGENASE

(Method of Amador et al., modified)

Reagents

1. Lactic acid buffer solution
2. NAD (Nicotnamide adenine dinucleotide) poder

Reagents Preparation

Lactic acid-NAD Reagent. For every 100 ml of working lactic acid solution to be used, add 400 mg NAD. Prepare each day a sufficient volume for the number of specimens to be assayed. Keep solution in refrigerator or on ice at all times.

Procedure

The procedure is described for a Gilford 2400 recording spectrophotometer equipped with a temperature controlled cuvette compartment held at 32 °C by a circulating constant temperature bath.

1. Place 2.9 ml lactic acid-NAD reagent into a test tube and incubate in a water bath 32 °C ± 0.5 °C for 4-5 min. During this period the cuvette are kept in the instrument's cuvette compartment so they reach temperature equilibrium (spectrophotometer and circulating water bath turned an at least 2 hours prior to use.)

2. Add 0.1 ml plasma by TC pipette, mix, and transfer as quickly as possible to the prewarmed cuvette, which is rapidly reinserted into the cuvette compartment. The cuvette compartment lid is left open for as sort a time as possible. Immediately start automatic recording of the change in absorbance at 340 nm.

3. If the resulting recorded curve is linear for at least 6 min, proceed with calculations as described below. If the rate is too rapid to give a linear curve, rerun using the sample diluted with 0.85 % saline (dilution required if result is over 300 units.)

Calculation:

$$\text{Unit}(\mu\text{mol NADH/min/liter}) = \frac{\sigma \text{ A for } T \text{ min}}{} \times \frac{1}{T} \times \frac{483}{\text{ml sample used in test}}$$

The term 483 is used to convert A_{340}/min/ml to μmol NADH/min/liter and is based on 6.22×10^3 as the molar absorptivity of NADH, the use of a 3 ml assay volume, and correction to 1 liter of plasma

$$\frac{\sigma \text{ A per min}}{6.22 \times 10^3} \times 3 \times 10^3 \times 10^3 = 483$$

Reference

Henry RJ, Cannon DC, Winkelman JW, wditors. Clinical Chemistry: principles and technics. 2 nd ed. New York : Haper & Row, 1974.

APPENDIX C
SUPEROXIDE DISMUTASE

(Modify from the method of Winterbourn 1975)

Reagents

1. 0.1m EDTA (ethylene diaminetetraacetic acid)
2. 1.5 mg KCN in 100 ml distilled water
3. 1.5 mM NBT (Nitroblue tetrazolium)
4. 0.12 mM Riboflavin
5. 0.067 M Phosphate buffer pH 7.8

Mix 0.067 M of K_2HPO_4 in 0.067 M KH_2PO_4 to make phosphate buffer ph 7.8

Procedure

1. For each sample to be assayed, the tubes were set up containing 0, 10, 20, 40, 60, 80, 200, and 500 µl of SOD extract. The reagents were added into these tubes as following:

0.2 ml of 0.1 M EDTA

0.2 ml of 1.5 mg of KCN in 100 ml distilled water

0.1 ml of 1.5 ml NBT

0.05 ml of 0.12 mM riboflavin (lastly added)

0.067 phosphate buffer pH 7.8 was added to give a total volume of 3 ml.

2. The tube which contained no extract were a control for each run. These tubes were then illuminated with a light box for 12 min at room temperature (25°C).

3. Optical density was measured at 560 nm.

Calculation

Results were expressed as units of superoxide dismutase per mg protein of tissue and 1 unit was defined for a particular system as the amount of enzyme (1 ml) causing half the maximum inhibition of NBT reduction. The percent inhibition of NBT reduction versus the amount of SOD extract was plotted on linear graph paper.

The volume of extract (μl) required to inhibited the reduction of NBT by 50 % was used in the following equation.

$$E = \frac{1000}{\mu l \text{ of SOD extract} \times \text{mg protein}}$$

where E = the enzyme activity expressed as 50 % inhibition in units/ mg protein.

As percentage inhibition could be calculated from this formula:

$$\% \text{ inhibition} = \frac{\text{O.D. of control} - \text{O.D. of experiment}}{\text{O.D. of control}} \times 100$$

Reference

Winterbourn CC, Hawkins RE, Brain M, Carrell RW. The estimation of res cell superoxide dismutase activity. J Lab Clin Med 1975; 85: 337-41.

APPENDIX D

CATALASE

(Modify from the method of Luck, 1965)

Reagents

1. 67 mM phosphate buffer pH 7.0

Dissolve 3.522 g of KH_2PO_4 and 7.268 g of $Na_2 HPO_4 \bullet 2H_2O$ in distilled water and make up to 1000 ml.

2. H_2O_2 –Phosphate buffer (67 mM phosphate : 1.25×10^{-2} M H_2O_2, ph 7.0)

Dilute 0.16 ml hydrogen peroxide (30% W/V) to 100 ml with phosphate buffer (solution 1) freshly. The optical density of this solution should be about 0.500+0.015 at 240 nm and with a 1 cm light path.

Procedure

1. Into the cuvette, pipette each solution as follows:

1.1 Add phosphate buffer (solution1) 3 ml into a blank cuvette and add H_2O_2 –Phosphate buffer (solution 2) 3 ml into the experimental cuvette.

1.2 The appropriate enzyme fraction (10-40 μl) which give a linear function of O.D. is added into both a blank cuvette and experimental cuvette.

2. The mix this solution with a glass rod.

3. Read the optical density in spectrophotometer at 240 nm against a blank cuvette.

4. Measure the rate of a decrease in the optical density.

Calculation

The activity of the catalase muscles are calculated from the optical density change in 1 min and the molar extrinction coefficient for H_2O_2 at 240 nm of 0.017 mM^{-1} cm^{-1}. The activity was expressed as μmole/mg protein/min.

$$A = \frac{\sigma O.D. \times TV \times \text{Dilution factor}}{0.017 \times EV \times \text{protein (mg)}}$$

A = Specific enzyme activity (μmole/mg protein/min)

TV = Total reaction volume (ml)

EV = Enzyme volume (ml)

References

Luck H. Catalase. Method for Enzymatic Analysis, Vol. 3. Edited by Han-Ulrich Bergmeyer. New York and London : Academic Press, 1965: 885-88.

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APPENDIX E
GLUTATHIONE PEROXIDASE

Reagents

1. 50 mM Tris buffer, pH 7.6 will 0.1 mM EDTA

Dissolve Tris (Hydroxymethyl) methylamine 6.057 g in distilled water and make up to 1,000 ml. Adjust pH of buffer with HCL until pH 7.6.

2. Stock solution.

Add 9.603 mg (0.25 mM) glutathione (reduced form), 12.5 mg (0.12 mM), NADPH and gllutathione reductase (1 unit of R/ml) in 125 Tris buffer pH 7.6 (solution1). This stock solution should be freshly prepared in ice-buckets.

3. Cumene Hydroperoxide 1.0 mg/ml of distilled water.

Pipette cumene hydroperoxide 25 μl into 20 ml distilled water and mix, this solution should be freshly prepared before use.

Procedure

1. After washing in ice-cold 0.9% NaCl, muscle tissue is homogenized in 0.1 M Phosphate buffer at a ratio of 1 g of wet tissue in 9 ml of buffer.

2. The supernatant fract on that obtained after centrifugation of the homogenate at 10,000 rpm and 4°C for 30 minutes (Refrigerator Kontron Centrifuge A24-42) is subjected to centrifuge at 40,000 rpm and 4°C for 60 minutes (Beckman Ultracentrifuge-Roter 60 Ti). The resultant supernatant fraction is collected for enzyme assay.

3. Add the solution into each tube which immersed in ice, as follows

	Blank tube(ml)	Experimental tube(ml)
Stock solution	1.55	1.55
Diluted enzyme	0.10	0.10

4. Mix and incubate in the water bath for 5 min at 37 °C.

5. Then this mixture solution was set zero absorbance at 340 nm by spectrophotometer.

6. Add 0.05 ml of cumene hydropeoxide into the cuvette in sample cell and rapidly mix.

7. The decrease in optical density of the reaction mixture at 340 nm as the conversion of NADPH to NADP is following by spectrophotometer with continuous recorder.

8. The sample of enzyme should be appropriated diluted to get a linear function curve of the optical density.

Calculation

The activity of glutathione peroxide was calculated from the change in optical density in 1 min and the molar extinction coefficient for NADPH at 340 nm of 6.22 mM^{-1} cm^{-1}. The activity was expressed as nmol/mg protein/min.

$$A = \frac{\sigma O.D. \times TV \times \text{Dilution factor} \times 1000}{6.22 \times EV \times \text{protein (mg)}}$$

A = Specific enzyme activity (μmole/mg protein/min)

TV = Total reaction volume (ml)

EV = Enzyme volume (ml)

1000=Conversion of units μmole to nmol.

References

Tappel AL. Glutatione Peroxidase and hydroperoxides In: Method in enzymology, Vol. II.Edited by Sidney F, Lester P.New York: Academic Press, 1978:506.

Splittgerber AG, Tappel AL. Inhibition of glutathione peroxidase by cadmium and other metal ions. Arch Biochem Biophys 1979; 197:534-42.

Paglia DE, Valentine WN. Studies on the quantitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1976; 70:158.

APPENDIX F

GLUTATHIONE

(Ellman, 1959; Boyland and Chasseaud, 1970)

Reagents

1. 0.1 M Phosphate buffer

Mix 0.1 M Na_2HPO_4 solution and 0.1 KH_2PO_4 solution until pH 7.0, 7.4 and 8.0.

2. Stock standard glutathione (GSH, reduced form) "Freshly prepared"

Dissolve GSH 10 mg/ 10 ml 0.1 M phosphate buffer, pH 8.0.

3. Color reagent

Dissolve 39.6 mg of 5-5 –dithiobis (2-nitrobenzoic acid) (DTNB) in 10 ml 0.1 M phosphate buffer pH 7.0 "Freshly prepared".

4. 4% sulfosalicylic acid

Dissolve 4 g sulfosalicylic acid in distilled water to make 100 ml.

Determination of Glutathione

"All operations were carried out below 10 °C"

1. Sample tissue are immediately removed and homogenized in 5 volume of 0.1 M phosphate buffer, pH 7.4, and equal volume of 4 % sulfosalicylic acid is added.
2. The mixture is centrifuged at approximately 4500 rpm for 30 min.
3. The supernatant is assayed for GSH by DTNB method.
4. Add solution into each tube which is immersed in ice, as follows:

	Blank (ml)	Standard (ml)	Unknown (ml)
0.1M Posphate buffer,pH8.0	1.5	1.48	1.4
Supernatant	-	-	0.1
Standard GSH	-	0.02	-
Distilled water	1.5	1.5	1.5

5. Mix and add color reagent 25 µl into mixture, mix well and allow the color to develop for 20 min at room temperature.

6. Read the optical density by a spectrophotometer at 410 nm.

7. The amount of glutathione level is expressed as µmole/ g wet weight.

Calibration Curve

1. Prepare series of tubes containing GSH in the following concentrations: 10 µg/3.0ml, 15µg/3.0 ml, 20µg/3.0 ml, 25µg/3.0 ml, 30µg/3.0 ml.

2. Perform the procedure as in step 5.

3. Determine the optical density at 410 nm.

4. Plot a clibration curve of optical density versus µg of GSH.

References

Boyland E,Chasseaud LF. The effect of some carbonyl compounds on rat liver glutathione level. Biochem Pharmacol 1970; 19: 1526-8.

Ellman GL. Tissue sulfhydryl group. Arch Biochem Biophys 1959; 82: 70-7.

APPENDIX G
PROTEIN CONTENTS

Reagents

1. Solution A: 2% Na_2CO_3 in 0.1 N NaOH

Dissolved 4.0 g NaOH in distilled water. While stirring, add 20g Na_2CO_3, and adjusted distilled water to a volume of 1,000 ml.

2. 4% Na-K^+ Tartate

Dissolve 4 g of Na-K^+ Tartate in 100 ml distilled water.

3. 2% Copper sulfate

Dissolve 2 g of $CuSO_4 \cdot 5H_2O$ in distilled water make up to 100 ml.

4. Folin Reagent

Dilute 2.0 N Folin & Ciocaten's phenol reagent 1:1 with distilled water before use.

5. Standard protein solution

Dissolve 50 mg of bovine serum albumin in distilled water to make 100 ml of solution.

6. Lowry E solution

Freshly prepared, mix 25 ml of solution A with 125 μl of 4% tartate solution and 125 μl of 2% copper sulfate solution.

Procedure

1. Add the following solutions into a series of tubes.

Solutions	Standard tubes (μl)						Experimental tubes (μl)
	0	10	20	30	40	50	
BSA solution	0	20	40	60	80	100	-
Solution A	200	180	160	140	120	100	150
Unknown	-	-	-	-	-	-	50
Lowry E	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Folin	100	100	100	100	100	100	100

2. Mix and allow standing for 30 minutes at room temperature.
3. The optical density was measured at 770 nm.

Calculation

The concentration of standard BSA is plotted versus absorbance at 770 nm and concentration of the unknown were determined from the standard curve.

Reference

Lowry OH, Rosebrough NJ, Farr Randall J. Protein measurement with folin phenol reagent. *J Bio Chem* 1951; 193: 265-75.

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