EFFECTS OF EXERCISE TRAINING AND ANTIOXIDANT SUPPLEMENTATION ON STREPTOZOTOCIN INDUCED DIABETES IN RATS

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PIYANAT BUNNITIGON : EFFECTS OF EXERCISE TRAINING AND ANTIOXIDANT SUPPLEMENTATION ON STREPTOZOTOCIN INDUCED DIABETES IN RATS. THESIS ADVISORS: BUARONG LEWCHALERM-WONGSE, M.Sc., KK SHIDA PANG, Ph.D., CHAIVAT TOSKULKAO, D.V.M., Ph.D., VIJITTRA LEARDKAMOLKARN Ph.D. 177 p. ISBN 974-664-935-3.

This study investigated the effect of long-term exercise training and/or vitamin E supplementation on the onset and severity of diabetes induced by streptozotocin (STZ), and on skeletal muscle antioxidant enzymes (superoxide dismutase-SOD, glutathione peroxidase-GPX, catalase-CAT). 64 male Sprague-Dawley rats were divided into 8 groups: sedentary, exercise trained (12 weeks running on motor driven treadmill, 1 hr/day, 5 days/week), vitamin E supplemented (12 weeks daily i.g. feeding of 70 IU/kg BW/day of alpha-tocopherol acetate), and exercise trained plus vitamin E supplemented groups, with and without diabetic induction by injection of 35 mg/kg BW of STZ. Onset and severity of diabetes were determined by measuring plasma glucose level for 6 consecutive days after STZ injection. Scavenging enzymes and lipid peroxidation, using malondialdehyde (MDA) as an indicator, were measured in the gastrocnemius muscle, 7 days after STZ.

Vitamin E supplemented diabetic rats had levels of plasma glucose that were significantly lower than sedentary diabetic rats, throughout 7 days of measurement. Although the training program employed in this study is sufficient to cause physiological adaptation to exercise training, indicated by lowered resting heart rate and increased in citrate synthase activity, the trained diabetic rats showed no improvement in diabetic condition in both onset and severity when compared to sedentary diabetic rats. Plasma glucose level in exercise trained plus vitamin E supplemented diabetic rats was higher than in vitamin E supplemented-diabetic rats but was still lower than that in sedentary-diabetic rats.

Our results suggest a beneficial effect of vitamin E supplementation. However, exercise training itself did not appear to improve the diabetic condition although there was a beneficial effect in increasing selected muscle scavenging enzymes in exercise trained diabetic rats and non-diabetic rats.

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ปิยะนาถ บุญนิธิกร : ผลของการฝึกออกกำลังกายและการให้สารแอนติออกซิแดนท์ต่อภาวะเบาหวานในหนูที่เหนี่ยวนำโดยสารสเตรปโทโซโทซิน (EFFECTS OF EXERCISE TRAINING AND ANTIOXIDANT SUPPLEMENTATION ON STREPTOZOTOCIN INDUCED DIABETES IN RATS) คณะกรรมการควบคุมวิทยานิพนธ์: บัวรอง ลิ่วเฉลิมวงศ์, วท.ม., Kathleen K.S. Pang, Ph.D., วิจิตรา เลิศกมลกาญจน์, Ph.D., ชัยวัฒน์ ต่อสกุลแก้ว, สพ.บ., ปร.ด., 177 หน้า ISBN 974-664-935-3

การวิจัยนี้ มีจุดประสงค์เพื่อศึกษาผลของการออกกำลังกายระยะยาวและการให้สารต่อต้านอนุมูลอิสระ (แอนติออกซิแดนท์: วิตามิน อี) ต่อการเกิดและความรุนแรงของภาวะเบาหวาน ซึ่งเหนี่ยวนำโดยสเตรปโทโซโทซิน (STZ) ในหนูขาว นอกจากนี้ยังศึกษาผลต่อเอนไซม์ที่ช่วยทำลายอนุมูลอิสระ (สคาเวนจิงเอ็นไซม์: SOD, CAT, GPX) ในภาวะพัก หนูขาวเพศผู้จำนวน 64 ตัว ถูกแบ่งออกเป็น 8 กลุ่มคือ 1) กลุ่มปกติที่ไม่ได้รับการออกกำลังกายหรือวิตามินอี, 2) กลุ่มที่ได้รับการออกกำลังกาย, 3) กลุ่มที่ได้รับวิตามินอี, 4) กลุ่มที่ได้รับการออกกำลังกายร่วมกับวิตามินอี, 5) กลุ่มที่ไม่ได้รับการออกกำลังกายและเป็นเบาหวาน, 6) กลุ่มที่ได้รับการออกกำลังกายและเป็นเบาหวาน, 7) กลุ่มที่ได้รับวิตามินอีและเป็นเบาหวาน, 8) กลุ่มที่ได้รับการออกกำลังกายร่วมกับวิตามินอีและเป็นเบาหวาน หลังจากฝึกออกกำลังกายโดยการวิ่งบนลู่วิ่งสำหรับหนู 1 ชั่วโมงต่อวัน, 5 วันต่อสัปดาห์หรือได้รับวิตามินอี 70 ไอยูต่อน้ำหนักตัว 1 กิโลกรัมเป็นเวลา 12 สัปดาห์ หนูกลุ่มที่ 5, 6, 7 และ 8 จะได้รับการฉีด STZ ในปริมาณ 35 มิลลิกรัมต่อน้ำหนักตัว 1 กิโลกรัมทางเส้นเลือดดำที่หาง ตรวจวัดระดับของน้ำตาลในเลือดก่อนการฉีด STZ และหลังการฉีดทุกๆ 24 ชั่วโมง จนครบ 7 ครั้ง เพื่อบ่งชี้การเกิดและความรุนแรงของภาวะเบาหวาน เอนไซม์ SOD, CAT และ GPX รวมทั้งระดับของลิปิดเปอร์ออกซิเดชันซึ่งบ่งชี้โดยมาลอนไดอัลดีไฮด์ (MDA) ได้ถูกวัดในกล้ามเนื้อแกสทรอคนีเมียส ในวันที่ 7 หลังการฉีดสาร STZ

พบว่าหนูที่ได้รับวิตามินอีมาก่อนการฉีด STZ (กลุ่มที่ 7) มีระดับน้ำตาลในเลือดต่ำกว่าหนูกลุ่มที่ไม่ได้ออกกำลังกายและไม่ได้รับวิตามินอีมาก่อนฉีด STZ (กลุ่มที่ 5) อย่างมีนัยสำคัญทางสถิติ ตลอด 6 วันหลังการฉีด STZ อย่างไรก็ดีถึงแม้ว่าระดับและระยะเวลาของการออกกำลังกายที่ใช้ในการทดลองนี้จะทำให้มีการปรับตัวของการทำงานของร่างกายซึ่งบ่งชี้โดยมีการเพิ่มอย่างมีนัยสำคัญทางสถิติของเอ็นไซม์ซิเตรทซินเทส และการลดลงอย่างมีนัยสำคัญทางสถิติของอัตราการเต้นของหัวใจในขณะพัก แต่พบว่าการออกกำลังกายระยะยาวเพียงอย่างเดียวไม่ช่วยชะลอการเกิดและลดความรุนแรงของภาวะเบาหวานในหนูกลุ่มที่ได้รับการออกกำลังกายและได้รับ STZ (กลุ่มที่ 6) เมื่อเทียบกับกลุ่มที่ไม่ได้ออกกำลังกาย ไม่ได้รับวิตามินอีและได้รับ STZ (กลุ่มที่ 5) ความรุนแรงของภาวะเบาหวานในหนูกลุ่มที่ออกกำลังกายร่วมกับได้รับวิตามินอีและได้รับ STZ (กลุ่มที่ 8) น้อยกว่าในหนูกลุ่มที่ไม่ได้ออกกำลังกายไม่ได้รับวิตามินอี และได้รับ STZ (กลุ่มที่ 5) แต่ยังคงมากกว่าหนูกลุ่มที่ได้รับวิตามินอี และ STZ (กลุ่มที่ 7) นอกจากนี้พบว่า สคาเวนจิงเอนไซม์ SOD เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ และ GPX มีแนวโน้มเพิ่มขึ้นหลังจากออกกำลังกายเป็นเวลา 12 สัปดาห์ (กลุ่มที่ 2) และการให้วิตามินอี 12 สัปดาห์จะทำให้สคาเวนจิงเอนไซม์ SOD เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ (กลุ่มที่ 3) เมื่อเทียบกับกลุ่มปกติที่ไม่ได้ออกกำลังกายและไม่ได้รับวิตามินอี (กลุ่มที่ 1)

ผลการทดลองนี้ชี้ให้เห็นว่าการให้วิตามินอีสามารถลดความรุนแรงของภาวะเบาหวาน แต่การออกกำลังกายไม่มีผลเปลี่ยนแปลงระยะเวลาการเกิดและความรุนแรงของภาวะเบาหวานซึ่งเหนี่ยวนำโดยการฉีด STZ ในหนูชนิดนี้ อย่างไรก็ตามการออกกำลังกายระยะยาวมีผลดีในการเพิ่มขีดความสามารถของระบบเอ็นไซม์ที่ใช้ทำลายอนุมูลอิสระ (scavenging enzyme) ของกล้ามเนื้อลายในหนูที่เป็นเบาหวาน

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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
CS	citrate synthase
Cu	copper
°C	degree celcius
DM	diabetes
DNA	deoxyribonucleic acid
et al.,	and colleagues
Ex	exercise
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
g	gram
GDM	gestational diabetes mellitus
g/kg BW	gram per kilogram body weight
GPX	glutathione peroxidase
GSH	glutathione

LIST OF ABBREVIATIONS (Cont.)

GSSG	glutathione disulfide
h	hour
H_2O	water
H_2O_2	hydrogen peroxide
IDDM	insulin dependent diabetes mellitus
i.g.	intragastric
IU	international unit
i.v.	intravenous
kg	kilogram
l	litre
$L^{\bullet}$	lipid radical
LH	lipid
$LO_2^{\bullet}$	peroxyl radical
LOOH	lipid hydroperoxide
M	molar or male per litre
MDA	malondialdehyde
mg	milligram
mg%	milligram percent
min	minute
ml	millitre
mM	millimolar

LIST OF ABBREVIATIONS (Cont.)

Mn	manganese
MRDM	malnutrition-related diabetes mellitus
µmole	micromole
N	normal or molar
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide 3′-phosphate
NADPH	nicotinamide adenine dinucleotide 3′-phosphate, reduced form
NIDDM	non-insulin-dependent diabetes mellitus
mmole	nanomole
O_2	oxygen molecule
$O_2^{\bullet-}$	superoxide anion radical
O.D.	optical density
OH^-	hydrogen ion
$OH^\bullet$	hydroxy radical
pH	log concentration of $(H^+)^{-1}$
RNA	ribonucleotide acid
ROS	reactive oxygen species
rpm	round per minute
Se	sedentary

LIST OF ABBREVIATIONS (Cont.)

SEM	standard error of means
SOD	superoxide dismutase
STZ	streptozotocin
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substance
TMP	1,1,3,3 tetramethoxypropane
TRX	thioredoxin
vol	volume
w/v	weight by volume
$\bar{X}$	mean
Zn	zinc

CHAPTER I

INTRODUCTION AND EXPERIMENTAL OBJECTIVE

Insulin-dependent diabetes mellitus (IDDM) is a chronic disorder that usually results from the selective destruction of pancreatic beta cells, which subsequently leads to a loss of endogenous insulin secretion. The low level of plasma insulin decreases the rate of glucose uptake into cells, resulting in high plasma glucose level (hyperglycemia), a metabolic shift from using glucose to fatty acids for energy, and subsequently, ketoacidosis. Insulin treatment is therefore necessary, not only to control hyperglycemia, but also to prevent spontaneous ketosis and possibly death.

It is accepted that an autoimmune mechanism plays a central role in the development of this disease in humans. A major feature of the pathogenesis of IDDM are macrophage and lymphocyte infiltration of the pancreatic islets. Both types of cells exhibit cytotoxic effects on beta cells by raising the level of oxygen free radicals. These reactive oxygen free radicals are capable of damaging biological macromolecules such as DNA, protein and carbohydrate, inducing lipid peroxidation and subsequently damaging the membrane of the islet cells.

The primary biological defenses against free radicals are antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). The especially low antioxidant capacity of beta cells may play a significant role in the pathogenesis of IDDM.

As an adjunct to hormone (insulin) therapy, exercise training is considered beneficial for DM patients in several aspects. The first benefit is to increase the rate of glucose uptake into contracting skeletal muscle and improve long-term glycemic control.

The increase in glucose uptake has been shown to be due to an increase in GLUT-4 expression. GLUT-4 is the major insulin-regulatable glucose transporter, a transmembrane protein expressed predominantly in tissues which exhibit rapid changes in response to insulin. Rodnick and co-workers in 1990 reported that rats running on an exercise wheel for 6 weeks showed a 60% increase in the number of insulin-regulatable glucose transporters in plantaris muscle (1). Ploug and co-workers in 1990 showed that swim training can increase the maximum insulin-stimulated glucose transport in both fat and muscle cells of rats (2). A similar experiment in humans showed a 3-fold increase in glucose transporter protein, GLUT-4 after only 7 days of exercise training (3).

Besides increasing glucose transport, exercise training can result in a reduction in lipid peroxidation level by increasing the activity of major free radical scavenging enzymes. In 1998, Alessio and Goldfarb reported that running for 18 weeks resulted in a reduction in lipid peroxidation level and an increase in catalase enzyme activity in rats (4). In 1996, Venditti and Di Meo, also experimenting with rats, showed that after 10 weeks of swimming, glutathione peroxidase, glutathione reductase activities, and the overall antioxidant capacity of the tissue was elevated (5).

Vitamin E, an exogenous antioxidant found naturally in food also showed beneficial effects on IDDM. Vitamin E supplementation was found to reduce the

incidence of diabetes in NOD mice (6). In 1997, Pozzilli and co-workers studied the effect of vitamin E and another scavenging molecule, nicotinamide (NA), on residual beta cell function and found that vitamin E and NA produced similar effects in protecting residual beta cell functions in IDDM patients (7). It has also been found that combined deficiency of vitamin E and selenium in adult male rats enhances their sensitivity to the ill-effects of streptozotocin (8).

In conclusion, both exercise training and vitamin E have antioxidant effects. Exercise training has an additional effect in increasing a specific glucose transporter, thus increasing the rate of glucose uptake. However, no one has shown a protective role of exercise training plus vitamin E supplementation on the development or severity of IDDM. The present study, therefore I will examine the separate and combined effects of vitamin E supplementation and exercise training on the onset and severity of diabetes mellitus in rats treated with streptozotocin (STZ).

The objectives of this study are as follows;

1. To evaluate the effects of long-term exercise training on the onset and severity of diabetes.

2. To evaluate the effects of long-term antioxidant supplementation (vitamin E) on the onset and severity of diabetes.

3. To evaluate the combined effects of exercise training and antioxidant supplementation on the onset and severity of diabetes.

4. To propose a possible protective role of exercise training and antioxidant supplementation against diabetes.

CHAPTER II

LITERATURE REVIEW

I. Diabetes

As a disease, diabetes mellitus (DM) is a syndrome possessing metabolic, vascular and neuropathic components. The metabolic component is characterized by the alteration in carbohydrate, fat and protein metabolism due to the absence or diminuation of insulin secretion or its effective action. This abnormality leads to abnormally high plasma glucose (hyperglycaemia) both in the fasting state and in response to a glucose load. The persistence of high plasma glucose (chronic hyperglycemia) is the major indicative symptom of DM and is the parameter normally used for diagnosis of the disease.

The vascular component of DM involves both large and small vessels and normally leads to other severe complications such as myocardial infarction, peripheral vascular disease, cerebral vascular accidents, and widespread damage in almost every tissue of the body.

Classification of DM

In 1985, the World Health Organization (WHO) classified diabetes mellitus according to its clinical features into 5 classes as follows:

1. Insulin-dependent diabetes mellitus (IDDM)
2. Non-insulin-dependent diabetes mellitus (NIDDM)
3. Malnutrition-related diabetes mellitus (MRDM)

4. Gestational diabetes mellitus (GDM)

5. Other types of diabetes mellitus associated with specific conditions and syndromes.

Insulin Dependent Diabetes Mellitus (IDDM)

The classical symptoms of insulin-dependent diabetes mellitus (IDDM) or type I DM are: high level of blood glucose or hyperglycaemia, thirst, passage of large amounts of urine (polyuria), fatigue, overwhelming tiredness and weight loss. Minor symptoms include muscular cramps, skin infections, constipation and blurred vision due to osmotic changes in the lens. High levels of blood and urine ketone bodies due to ketoacidosis can be detected in some IDDM patients. Features of ketoacidosis are nausea, vomiting, drowsiness and abdominal pain. The duration of symptoms is short, usually only 2-3 weeks or less. Some patients may present with ketoacidosis and coma.

Insulin treatment in this case in necessary, not only to control hyperglycaemia, but to prevent spontaneous ketosis and death.

Non-Insulin-Dependent Diabetes Mellitus (NIDDM)

Non-insulin-dependent diabetes mellitus (NIDDM) or type II DM may also present with classical hyperlgycaemic symptoms (thirst, polyuria, and fatigue) but ketone bodies are present in only low concentrations in the blood and urine. Coma can sometimes occur due to hyperglycaemia and hyperosmolarity but is relatively uncommon in NIDDM.

The recommended treatment is dietary management with or without oral hypoglycaemic agents.

Malnutrituion-Related Diabetes Mellitus (MRDM)

Malnutrition-related diabetes mellitus (MRDM), is the third major class, and usually occurs in malnourished adolescents or young adults. Possible etiological factors include malnutrition, dietary toxins and genetic susceptibility. There are two subclasses, fibro calculous pancreatic diabetes and protein-deficient pancreatic diabetes.

These patients have high levels of blood glucose but ketosis is usually absent. Most of these patients require insulin, at conventional dosages.

Gestational Diabetes Mellitus (GDM)

Gestational diabetes mellitus is defined as the diabetes that first presents in a pregnant woman who has never been diabetic before.

Many women will recover from GDM and return to normal after parturition. However, some may remain diabetic after pregnancy.

Other Type of Diabetes

Other types of diabetes include those associated with various specific conditions and syndromes, such as;

1. Diabetes due to pancreatic disease.
    - Chronic or recurrent pancreatitis
    - Haemochromatosis
2. Diabetes due to other endocrine disease.
    - Cushing's Syndrome
    - Hyperaldosteronism
    - Acromegaly

- Thyrotoxicosis
- Phaeochromocytoma
- Glucagonoma

3. Diabetes due to drugs and toxins
   - Glucocorticoids and ACTH
   - Diazoxide
   - Diuretics
   - Phenytoin
   - Pentamidine
   - Vacor (rodenticide)
4. Diabetes due to abnormalities of insulin or its receptor
   - Insulinopathies
   - Receptor defects
   - Circulating antireceptor antibodies
5. Diabetes associated with genetic syndromes such as:
   - DIDMOAD syndrome
   - Myotonic dystrophy and other muscular disorders
   - Lipoatrophy
   - Type I glycogen storage disease
   - Cystic fibrosis

Pathogenesis of Insulin Dependent Diabetes Mellitus (IDDM)

The most obvious histological finding in the pancreas of a patient who has had IDDM for many years is an almost total lack of insulin-secreting beta cells (9).

The finding that insulin-containing islets were over 20 times more likely to be affected by insulitis than insulin-deficient islets provided the first real evidence that beta cell destruction was caused by the insulitis process (10). IDDM fulfills the criteria of an organ specific, autoimmune disease where the beta cell is the target (11).

Autoantibodies in IDDM

Autoantibodies reactive with antigens contained in pancreatic islet cells are common in IDDM. There are several antibodies reactive with a variety of islet cell components, include antibodies to insulin, proinsulin, glutamic acid decarboxylase (GAD), carboxy-peptidase H, a sialoglycolipid, and several other incompletely defined antigens of different molecular weights: 37, 38, 52, 64, 69 and 150 kd.

Although antibodies could damage beta cells by anitbody-dependent complement cytotoxicity or by targeting natural killer cells to beta cell antigens, the large number of autoantibodies against many different islet antigens found in the sera of newly daignosed IDDM patients raises question regarding the role of these antibodies in the IDDM disease process, and which antigen is most important.

Even though the understanding of the relationship between antibodies and IDDM is not complete, antibody measurements can now be used to identify nondiabetic subjects at high risk of developing IDDM and can identify IDDM among patients clinically diagnosed as having NIDDM (12).

Autoimmune Mechanism of IDDM

Inflammation of pancreatic cells can be found in almost all Type I diabetes. One interpretation of histopathologic specimens is that the Islets of

Langerhans at the time of clinical onset are end-stage islets with signs of chronic inflammation.

Immunocytochemical investigations in rare specimens of pancreatic tissue from patients dying shortly after the clinical onset of IDDM indicate that all cell types that are considered part of the immune response, macrophages, as well as granulocytes and NK cells have all been reported (4).

How the autoimmune-attack against beta cells in the islet is initiated and carried out remains, at present, unresolved.

As generally known, the physiological immune response is initiated by HLA class II-positive cells, such as macrophages, which present antigens to T-helper lymphocytes which then trigger the chain of immune reactions. It has been postulated that as yet unrecognized agents, which could be environmental factors, are responsible for triggering the "inappropriate" or "aberrant" expression of class II molecules on the autoimmune target cells, including the beta cells of islets affected by insulitis.

This expression of class II MHC molecules would cause pancreatic beta cells to become antigen-presenting cells and thereby trigger an autoimmune response. If this phenomenon occurs in genetically susceptible individuals, for example, those who develop IDDM, the configuration of certain class II products (HLA-DR3 or DR4 or both) may be ideal for presentation of autoantigens which are normally expressed on the surface of beta cells. This initial event could lead to activation of T-helper cells which, in turn, stimulate effectors such as B and cytotoxic T lymphocytes (13).

Both macrophages and lymphocytes which infiltrate the pancreatic islets exhibit cytotoxic effects on beta cells (14-17). Activated macrophages and lymphocytes secrete cytokines including IL-1, IFN-γ and TNF-α which have been found to cause functional impairment and lysis of beta-cell via reactive oxygen intermediates such as nitric oxide (NO), hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and superoxide radical (O^{2-}) (15, 18, 19).

Moreover, the characteristically low level of key scavenging enzymes in islet cells cause insulin-producing cells to suffer even more from oxidative stress (20-23).

Alpha and delta cells do not express class II products in these islets, which is consistent with the sparing of these cells in the killing process. However, the exact role of the aberrant expression of class II molecules in the pathogenesis of autoimmune disease is not yet fully understood.

Besides what was previously described, it has been reported that there are other factors which may participate in the pathogenesis of IDDM, including viral infection, dietary factors and also chemical modification. Therefore, the diabetic condition can be induced in some animals to be models for further study.

Ketogenesis

In the normal fed state, the ketone bodies acetoacetate and 3-hydroxybutyric acid, play a small role in body metabolism. The primary circulating nutritional substrates are diet-derived glucose and mobilized free fatty acids. On the other hand, if food is unavailable for example, under diabetic conditions, ketones rapidly become of major importance as an alternative energy source, particularly for the brain. In

contrast to most other tissues in the body, the central nervous system cannot use plasma free fatty acids as metabolic fuel. Therefore, the mechanism to convert fat into a form utilizable for energy by the brain, is critical. The process involved is ketogenesis, the conversion of long-chain fatty acids into acetoacetate and 3-hydroxy-butyrate by liver.

Although the increased production of acetoacetate and 3-hydroxybutyrate that characterizes starvation is a beneficial adaptive response, larger accumulations of ketones are dangerous. This is because they are powerful organic acids capable of producing a profound metabolic acidosis. This point is illustrated by the fact that untreated diabetic and alcoholic ketoacidoses, the major pathologic ketotic states, are potentially fatal illnesses.

The physiological ketosis of fasting or prolonged starvation never progresses to life-threatening acidosis. The pathologic transition is prevented by the presence of intact pancreatic beta cells. The ketogenic sequence is initiated by a fall in plasma glucose and a rise in plasma glucagon. Free fatty acids are mobilized from adipose tissue stores and transported to the liver, which has been activated for ketone body production. When plasma ketone concentrations reach 4 to 6 mmole, insulin release from the pancreas in stimulated. This insulin decreases (but does not normalize) lipolytic activity in adipose cells such that plasma free fatty acid levels are fixed at about 0.7 to 1.0 mM, which are sufficient to allow moderate production of acetoacetate and 3-hydroxybutyrate by the liver, but insufficient to allow the maximum rates of production required to develop ketoacidosis; thus, a metabolic acidosis does not occur. In Type I diabetic subjects, the protective ketone-insulin

feedback loop cannot operate because of beta-cell destruction in the Islets of Langerhans. As a consequence, plasma free fatty acids reach much higher concentrations, driving ketone production to maximal rates, thereby leading to a lethal ketoacidotic state (24).

Islets of Langerhans: Morphology

The Islets of Langerhans are clusters of endocrine tissue scattered throughout the exocrine pancreas. The islets vary in size and are most numerous in the tail of the pancreas. It's mass is dynamic, adjusting to meet the changing need of the individual, whose size and level of activity may vary at different stages of life. It contains a variety of cell types, of which about 82% are insulin-producing beta cells, 13% glucagon-producing alpha cells, 4% somatostatin-producing delta cells and 1% pancreatic polypeptide-producing PP-cell. The latter three will be referred to collectively as the non-beta-cells. The beta-cells are polyhedral, being truncated pyramids and are usually well granulated. The alpha cells are usually smaller and more columnar than the beta cells, the delta cells are usually smaller than either alpha and beta cells and are often dendritic in shape while PP cells are the most variable among species.

The distribution of the endocrine cells is nonrandom, with a core of beta cells surrounded by a discontinuous mantle of non-beta cells.

The islet is highly vascularized and has a direct arteriolar blood supply. Islet capillaries are fenestrated, whereas fenestration is decreased or absent in capillaries in the surrounding exocrine tissues (25).

Islets of Langerhans in Diabetes Mellitus

In IDDM there is selective destruction of beta cells within the islets. The destruction of beta cells proceeds very slowly and it may occur for years preceeding any signs of clinical onset.

Even in the islets from individuals with a very short history of IDDM, the number of beta cells which represent the major tissue of the islets, is greatly reduced. These islets, like islets in long-standing IDDM, consist mostly of alpha, delta, and PP cells, and thus are devoid of insulin. Infiltration of the islets with mononuclear inflamatory cells (insulitis or isletitis) is a common finding, especially in individuals younger than 10 years of age.

In long-standing IDDM the islets are small, with a concurrent major reduction of islet volume, which depends largely on the almost complete loss of islet beta cells. This beta cell atrophy agrees well with very low extractable insulin in such pancreas. In cases of IDDM of longer duration, insulitis may be absent. When present, the inflammatory infiltrate consists mainly of T-lymphocytes of certain subsets, B lymphocytes and macrophages (26).

II. Animal Models of Insulin-Dependent Diabetes Mellitus

There are several ways to induce the diabetic condition in animals. Other than pancreatectomy, (the method of choice a hundred years ago) strains of spontaneously diabetic animals and the use of chemical toxins have subsequently been adopted to provide access to experimental insulin-dependent diabetes in different animal species.

Animal models with spontaneous IDDM

It is now generally accepted that the two main contributory factors involved in the pathogenesis of IDDM are the immune system and genetically determined predisposition to the disease. Therefore, two spontaneously diabetic rodents, the bio-breeding (BB) rat and the non-obese diabetic (NOD) mouse, which develop diabetic syndromes closely resembling that in human IDDM, have been used.

The BB rat

The diabetic syndrome of the BB rat shares many characteristics with human IDDM. There is a genetic predisposition to develop the disease. The onset of symptoms is at around 1 month of age and is usually accompanied by a pancreatic mononuclear cell infiltration (insulitis) (27). The insulitis is similar to that described in human IDDM (28), with T cells (including both helper and cytotoxic/suppressor subsets), macrophages, natural killer cells and B lymphocytes being present at the onset of diabetes (29).

There are many studies indicating direct involvement of both cellular and humoral immunity in the pathogenesis of diabetes in the BB rat.

The NOD (non-obese diabetic) mouse

The NOD mouse has been derived from a subline of outbred Jc1-IRC mice which develop spontaneous cataracts (30). The diabetic syndrome of the NOD mouse occurs at approximately 14 weeks of age (30). The insulitis, with both helper and cytotoxic T cells and natural killer cells in the infiltrate is present as in the BB rat (31). There is a marked sex difference in NOD mice since diabetes develops in 80% of females and only 20% of male mice (30).

Animal model of IDDM induced by diabetogenic and toxic chemicals

Several diabetogenic chemicals, including zinc chelators, alloxan and streptozotocin, have been used to induce permanent diabetes in animals.

Zinc chelator-induced DM

The zinc chelating compounds, dithizone and 8-hydroxy guinoline, were found to induce permanent diabetes in mice and rabbits (32). After dithizone was injected into the animal, the rapid deposition of zinc dithizonate granules within the islets occurs, followed by progressive hyperglycaemia.

Alloxan-induced DM

Several studies have shown that alloxan alters the properties of the beta cell plasma membrane. Rodent islets treated *in vitro* with alloxan displayed abnormal membrane morphology (33) and altered ion flux (34), which can be prevented by high glucose concentrations. The precise location of action of alloxan on the beta cell membrane is unclear, but some studies suggest that its action involves the glucose transport mechanism in the membrane. Following its uptake by the beta cell, alloxan has been shown to interact with sulphydryl-containing cellular components (35). Sulphydryl enzymes are known to be essential for beta cell function (36). Glucokinase, which has a signal-recognition function in coupling glucose concentration to insulin release (37) is particularly sensitive to inhibition by alloxan (38). Other enzymes, including hexokinase (39) and protein kinase (40) are also inhibited by alloxan.

Other proposed mechanisms for alloxan cytotoxicity include the induction of DNA fragmentation both *in vivo* (41) and *in vitro* (42), direct induction

of mitochondrial abnormalities (43) and extreme sensitivity of beta cells to the cytotoxic effects of free radicals generated during the reduction/re-oxidation cycle of alloxan (44-46).

Streptozotocin-induced DM

Streptozotocin is one of several diabetogenic agents which produces much less toxic side effects than other chemicals and becomes a chemical of choice for the induction of DM in experimental animal models. It has been reported to selectively destroy pancreatic beta cells, resulting in permanent diabetes when given either as a single large dose (as with alloxan) or as multiple subdiabetogenic doses. With the latter, hyperglycaemia and symptoms develop gradually (47).

III. Streptozotocin

Streptozotocin [STZ; 2-deoxy-2(3-methyl-3-nitrosoureido)-D-glucopyranose] is a broad-spectrum antibiotic which consists of a D-glucopyranose and a methyl-nitrosourea group (48-50). STZ is derived from a fermentation broth of streptomyces achromogenes variety. The molecular weight is approximately 265 with the empirical formula $C_8H_{15}N_3O_7$ (Figure 1).

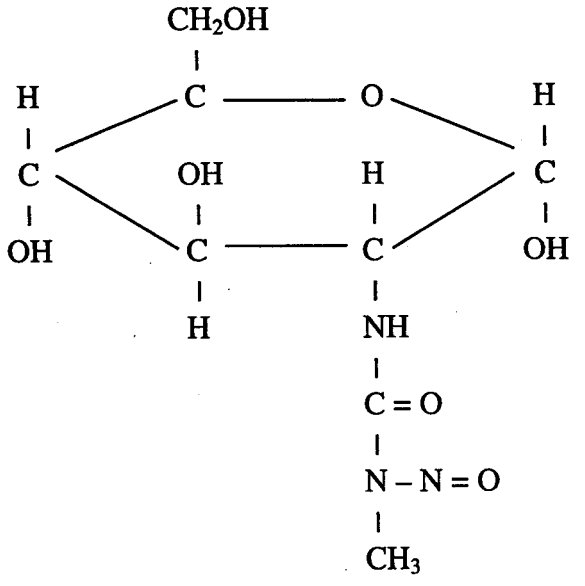


Figure 1. The chemical structure of streptozotocin

STZ was initially developed as an antibiotic drug although subsequent studies showed that the drug also exerted some other biological effects and was too toxic to be used as an antibiotic agent. At present, it has also been used as an important antitumor agent against malignant carcinoid tumor and is most actively used for the treatment of islet cell carcinomas (51). STZ was also reported to be a beta cytotoxic or diabetogenic agent. Severity of the diabetogenic state as a result of STZ was found to be closely related to the dosage used (52, 53). The acute response to STZ has been described by a triphasic blood glucose curve. This triphasic blood curve includes 1) an initial hyperglycemia which may be a result of direct effect of STZ itself on the beta cell (54) 2) a hypoglycemic phase which has been attributed to the increase of insulin in plasma from lysing pancreatic beta-cells, and 3) permanent hyperglycemia due to insulin deficiency resulting from beta-cell destruction (55, 56).

The diabetogenic action of STZ is mediated by selective destruction of pancreatic beta-cells. Its nitrosourea moiety is responsible for beta-cell toxicity, while the deoxyglucose moiety facilitates its transport across the cell membrane (57). It has been considered that STZ exerts its cytotoxic effects via the reduction of islet NAD content. The NAD depletion is linked to stimulation of the activity of the nuclear enzyme poly (ADP-ribose) synthase, which is involved in the excision and repair of broken DNA strands, and requires NAD as substrate. STZ produces the acute NAD loss, which becomes irreplaceable. The low level of NAD inhibits proinsulin synthesis and results in virtual cessation of NAD dependent energy, protein metabolism, and leads to cell necrosis. Nuclear enzyme poly (ADP-ribose) synthase can be inhibited by nicotinamide and picolinamide, which prevent the STZ-induced DNA damage *in vivo* and helps to maintain the NAD content and insulin biosynthesis *in vitro* (57, 58). There is considerable evidence that free radicals play an important role in the diabetogenic action of STZ. Free radicals, such as nitric oxide (NO), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet}$), either administered exogenously or induced endogenously in beta cells by cytokines, have been shown to cause the destruction of beta-cells, *in vitro*, and apoptosis has recently been reported (59). The presumption was expressed that STZ causes "nitric oxide" (NO) scattering, and with the help of NO or through its reaction with superoxide radical, peroxinitrite ($ONOO^-$) occurs. Then the decomposition of peroxinitrite can produce $OH^{\bullet}$ radicals.

$$ONOO^- + H^+ \rightarrow NO_2 + OH^{\bullet} \qquad (60)$$

The accumulation of superoxide and hydroxyl radicals and/or DNA alkylation are considered to lead to fragmentation of beta cell DNA. Breaks in DNA strands are responsible for the deterioration in insulin synthesis and secretion. A DNA break also immediately initiates the processes of excision and repair, involving the activation of poly (ADP-ribose) synthase and further associated NAD depletion (Figure 2). This concept is supported by the protective effects of nicotinamide and OH scavengers, as mentioned above, which inhibit the activity of poly (ADP-ribose) synthase (57). Thioredoxin (TRX), an anti-oxidative and anthiapoptosis protein, has also recently been shown to protect beta-cells from oxidative stress and apoptosis induced by STZ, and in autoimmune diabetes (59).

In fact, such mechanisms may also apply to beta-cell destruction by processes initiated by autoimmune insulitis following lymphocyte infiltration. Therefore, the pathological and biochemical features of this model may be compatible to those of type I diabetes in humans (61).

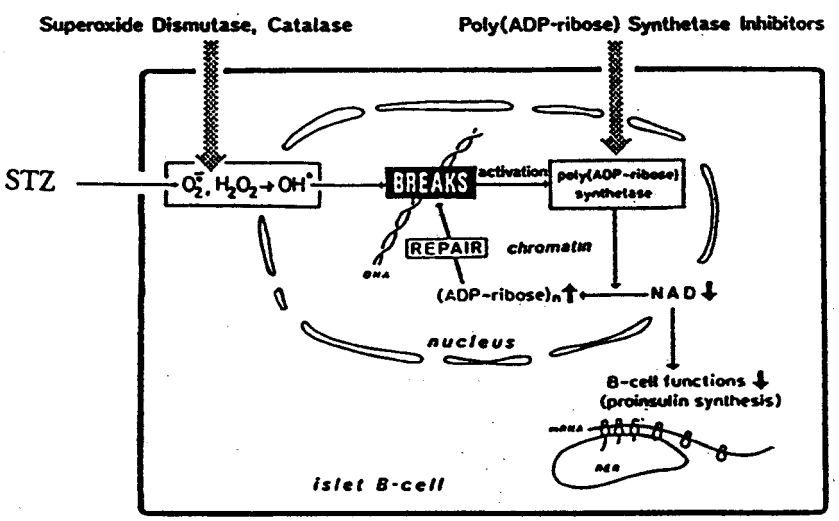
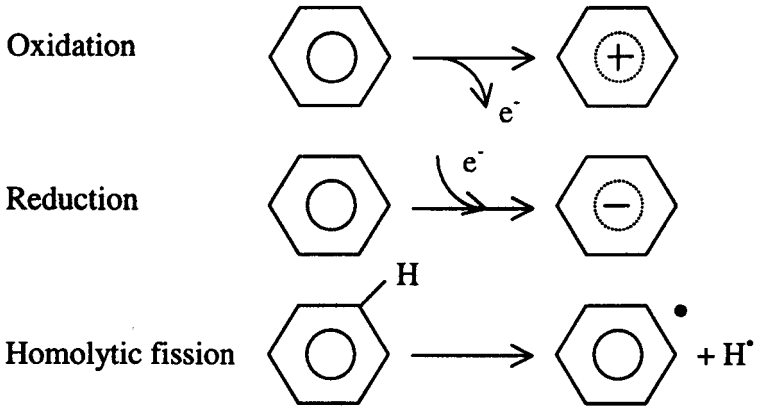


Figure 2. Diagrammatic representation of how streptozotocin excert its biological action on beta cells.

IV. Free Radicals and Cell Damage

Free radicals are defined as molecules that contain a single, unpaired electron in their outer shell. Free radicals are highly reactive chemical species (organic or inorganic), unstable, and can be positively or negatively charged, or electrically neutral. For example, benzene can be converted into three chemically distinct free radicals:

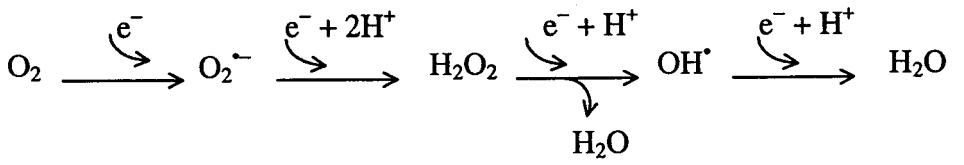


The removal of a π-electron from benzene (one-electron oxidation) generates the benzene cation radical. The addition of an electron (one-electron reduction) results in the formation of the benzene anion radical. Finally, homolytic cleavage of one of the C-H bonds (e.g., by ultraviolet radiation) yields two radicals, the phenyl radical and a hydrogen atom (62).

The presence of the unpaired electron in the free radical is conventionally represented by a superscript bold dot at the right side: $R^{\bullet}$, the symbol R will be used throughout to represent an unspecified radical.

In general, human cells are aerobic and consume molecular oxygen in energy-producing processes. Current evidence shows that oxygen free radical intermediates

partly yielded from the electron transfer process in the respiratory chain are harmful to aerobic cells. Under normal conditions, most molecular oxygen in biological systems undergoes tetravalent reduction (four electron reduction) to H_2O. The three most important oxygen-derived free radicals are superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (57, 61-64).



By means of the univalent pathway for the reduction of molecular oxygen, involving a series of single-electron transfers, molecular oxygen is first reduced to superoxide free radical ($O_2^{\bullet}$). Then from superoxide, with the addition of two protons and another electrons these are reduction to hydrogen peroxide (H_2O_2). Hydrogen peroxide is itself then univalently reduced, and with the addition of another proton, yields water and the hydroxyl radical ($OH^{\bullet}$). A final univalent reduction and the addition of another proton converts the hydroxyl radical to water (63).

Sources of free radicals within cells can be divided into exogenous and endogenous sources.

Exogenous Sources of Free Radicals:

Oxygen radicals produced by activated phagocytes are microbiocidal and can inadvertantly cause collateral tissue damage. Many anthracyclic antineoplastic agents such as adriamycin, duanorubicin, doxorubicin and other antibiotics that depend on quinoid groups or bound metals for activity are able to generate oxygen radicals. Many of the chemotherapeutic effects and cytotoxic side effects of these drugs can be

attributed to their ability to reduce oxygen to $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$. Irradiation of organisms with electromagnetic radiation and particulate radiation (electrons, protons, neutorns, deuterons and α and β particles) generate primary radicals by transferring their energy to cellular components such as water. These primary radicals can then undergo secondary reactions with dissolved O_2 or with cellular solutes. In addition, a wide variety of environmental agents including photochemical air pollutants, hyperoxia, pesticides, tobacco smoke, solvents, anaesthetics and the general class of aromatic hydrocarbons also cause free radical damage to cells (64).

Endogenous sources of free radicals

- **Autoxidation of small molecules**

Superoxide can be generated by nonenzymatic reaction involving the autoxidation of cellular components by molecular oxygen. A wide variety of soluble cell components, capable of undergoing oxidation-reduction contribute to intracellular free radical production. These include thiols, hydroquinones, catecholamines, flavins and tetrahydropterins. In all cases, $O_2^{\bullet-}$ is the primary radical formed by the reduction of dioxygen by these molecules. Chelated Fe (III) can be reduced to Fe (II) by thiols, ascorbate and a host of other reductants. Fe (II) can then autoxidize, producing $O_2^{\bullet-}$. Hydrogen peroxide is a secondary product of one-electron autoxidations, via spontaneous or enzymatically catalyzed dismutation of $O_2^{\bullet-}$ (64).

- **Soluble enzymes and protein:**

Several enzymes generate free radicals during their catalytic cycling. Xanthine oxidase are flavoproteins containing FAD, iron-sulfur centers and molybdenum, that can undergo redox cycling. Molecular oxygen can serve as an

efficient oxidizing substrate for these enzymes leading to production of both $O_2^{\bullet-}$ and H_2O_2. In a typical reaction, xanthine oxidase generates $O_2^{\bullet-}$ and H_2O_2 during the conversion of hypoxanthine to xanthine and then to uric acid as shown below:

$$\text{Hypoxanthine} + H_2O + O_2 \rightarrow \text{xanthine} \rightarrow H_2O_2 \text{ (or } O_2^{\bullet-})$$

$$\text{Xanthine} + H_2O + O_2 \rightarrow \text{uric acid} + H_2O_2 \text{ (or } O_2^{\bullet-})$$

The relative proportion of $O_2^{\bullet-}$ and H_2O_2 released from the active site of the enzyme depends on pH, oxygen concentration and substrate concentration (65, 66). Interestingly, xanthine oxidase can be converted from a dehydrogenase (non-superoxide producing) to the oxidase (superoxide producing) form during tissue hypoxia (67-69).

Aldehyde oxidase, which is structurally similar to xanthine oxidase, is present in liver cytosol and shares many of the same substrates and also generates superoxide (67).

Dihydroorotate dehydrogenase (70), flavoprotein dehydrogenase (71) and tryptophan dioxygenase (72) also utilize superoxide during their catalytic cycle.

Mitochondrial electron transport

The ubiquinone cytochrome b region is the major site of $O_2^{\bullet-}$ production (73-77). The transport of electrons from substrate through the mitochondrial respiratory chain involves the sequential reduction of flavo protein, ubiquinone and mitochondrial cytochromes. The reduction of ubiquinone (and possibly flavin) occurs by a single electron transfer (78). The ubisemiquinone generated is, in large part, reoxidized by the cytochrome b complex, but may also react with molecular oxygen to generate $O_2^{\bullet-}$ (79).

Superoxide and H_2O_2 production normally accounts for one to two percent of mitochondrial oxygen consumption under reduced conditions (64).

Endoplasmic reticulum and nuclear membrane electron transport

The endoplasmic reticulum and nuclear membrane share many of the same elements (80). Both of these intracellular membranes contain the cytochromes P_{450} and b_5 that can oxidize unsaturated fatty acids (81), xenobiotics (82) and reduce dioxygen (83), among other substrates. These mixed function oxidases convert nonpolar compounds into hydroxylated derivatives by electron transfer reactions. The more polar products are better excreted by the kidney. NADH or NADPH are required cofactors for these reactions. Flavoprotein-containing cytochrome reductase, which provides the electrons for the cytochrome P_{450} and b_5 mediated reactions are also capable of autoxidizing to produce $O_2^{\bullet-}$ and H_2O_2 (84).

Peroxisomes

Peroxisomes are potent sources of cellular H_2O_2 because of high concentrations of oxidases. Some peroxisomal H_2O_2-generating enzymes include D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase and fatty acyl-coA oxidase (64).

Plasma Membranes

Free radical production by microsomal and plasma membrane-associated enzymes such as lipoxygenase and cyclooxygenase is of current interest because it is involved in arachidonic acid metabolism, the substance which is converted into biologically potent products. These products include prostaglandins,

thromboxanes, leukotrienes and slow-reacting substances of anaphylaxis. The enzymatic oxidation of arachidonic acid by membrane-bound enzymes, cyclooxygenase and lipoxygenase, are reportedly involved in some free radical intermediates include hemoprotein-, oxygen-, and carbon-centered free radicals (64).

Potential for Cell Damage

The reactivity of free radicals has a major influence on diffusion distances. $OH^{\bullet}$ has a high reactivity, so this free radical is not likely to diffuse away from cellular sites of production. Less reactive free radicals may be capable of reacting distally from the site of generation. Superoxide, much less reactive with cell components than $OH^{\bullet}$, could potentially diffuse further away from where it is generated. The charged $O_2^{\bullet-}$ molecule can cross membranes and enter cells via transmembrane anion channels. Hydrogen peroxide can cross membranes almost as readily as water (85).

Extensive studies with model systems and with biological materials *in vitro*, have clearly shown that reactive free radicals are able to produce chemical modification, and damage to proteins, carbohydrates, nucleotides and cell membranes (86, 87).

Cellular Components at Risk from Free Radical Damage

- Proteins

Proteins are present inside and outside cells in relatively high concentrations. The presence, inside the protein molecules, of complexed iron or copper ions that catalyze the decomposition of H_2O_2 may facilitate the initiation of a radical reaction chain. That process has been named the metal ion-catalyzed oxidation of proteins (MCO). Hydroxyl radical is the main radical that initiates the radical

reaction chain in proteins. Such changes in the primary structure of polypeptide chains generally lead to changes in the net charge and conformation of proteins (thus promoting changes in secondary and tertiary structures) including protein unfolding. Possible consequences are the following:

- Changes (usually increases) in hydrophobicity and thus modifications in the intrinsic capacity of a protein to interact with membranous lipid bilayers, to bind specifically to a receptor, or to bind specifically to a ligand.

- Changes in biological activity, generally inactivation of an enzyme or indirect activation via the inhibition of an inhibitor.

- Changes in immunogenicity

- Induction of the synthesis of "stress proteins" due to stable interaction of denatured proteins with the so-called 70-kD heat shock protein (HSP 70) and, consequently, the release of a transcription factor (HSF 70) that turns on specific genes.

- Increased susceptibility to proteolysis (probably due to changes in hydrophobicity) so that it is now considered that such oxidation of protein is one way cells "mark" their proteins for degradation.

- Nucleic Acids

Polyribonucleotides as well as polydesoxyribonucleotides are sensitive targets for radicals and reactive oxygen species. The primary molecular sites for an attack are the heterocyclic bases, at the (desoxy) ribosyl moiety, but also single- and/or double strand breaks. Modifications of the bases include addition of chemical groups and opening of the ring(s) and molecular rearrangements, leading to single

base changes [mainly guanine/cytosine (GC) to adenine/thymine (AT)] or base deletions in deoxyribonucleic acid (DNA). Some of these are known to be mutagenic. Reaction at the (desoxy) riboxyl moiety leads to oxidative degradation of the carbohydrate structure, followed by rupture of the phosphoribose backbone. DNA strand breaks are likely to cause aberrations in chromosomes and DNA strand breaks may indirectly cause cell death. Finally, it is worth mentioning that as a consequence of an intracellular oxidative stress, increases in intracellular free Ca^{2+} concentration may lead to the activation of nucleases and the subsequent hydrolysis of nucleic acids in apoptosis (88).

- Unsaturated Fatty Acyl Moieties in Lipid

Biomembranes and subcellular organelles are major sites of lipid peroxidation damage (89). Membranes are complex mixtures of lipids and proteins. The plasma membrane is basically a biomolecular leaflet of phospholipids and other amphipathic substances like cholesterol. Proteins interact with membrane lipid by ionic and hydrophobic forces. Segments of protein composed of aromatic amino acids will form hydrophobic associations with lipids in the mid zone of membranes, whereas ionogenic groups on proteins will react with the polar head groups at the membrane's surface.

The membrane of organelles are lipid-protein complexes, with a relatively greater amount of protein and phospholipid than the plasma membrane. The phospholipid of organelles contain more polyunsaturated fatty acids than do the phospholipids of the plasma membrane (90). Mitochondrial and microsomal

membranes contain relatively large amounts of polyunsaturated fatty acids in their phospholipids (89).

In biomembranes, unsaturated fatty acyl moieties have a major role contribution to membrane fluidity, which increases with increasing numbers of double bonds of the lipid bilayer.

Natural unsaturated fatty acids have one or more cis c=c double bonds starting from the ninth C upward, each double bond being separated from the other by an allylic methylene (CH_2) group (88). Unsaturated and, in particular, polyunsaturated fatty acids are sensitive targets for the initiation of radical chain reactions (91) or so-called lipid peroxidation. Lipid peroxidation is a process by which any free radical in the presence of molecular oxygen, with sufficient energy, abstracts a hydrogen atom from a methylene group ($-CH_2$) of an unsaturated fatty acid (92-95). The process of lipid peroxidation can be divided into three distinct steps as follows:

1) Initiation. The first step is the initiation process by which the Bis-allylic H is abstracted from a methylene ($-CH_2$) group located between two double bonds (a position that weakens the C-H bond) in a polyunsaturated fatty acyl group, thus generating a lipid radical ($L^{\bullet}$), as in the reaction below:

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH$$

2) Propagation. The second steps are series of propagation reactions. Lipid radical ($L^{\bullet}$) usually reacts with molecular oxygen, which bonds to the radical to form a peroxyl radical ($LOO^{\bullet}$). The peroxy radical can abstract a H atom

from another polyunsaturated fatty acid producing a lipid hydroperoxide (LOOH) as well as another $L^{\bullet}$ so as to propagate the radical chain reaction; as shown below:

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

3) Termination. Propagation of the radical chain reaction will continue until the substrate is depleted or the process is interrupted by an antioxidant (AH) (mainly vitamin E). The antioxidant will react with the free radicals and terminate the chain reaction process. The termination reactions are shown below (91, 96-98):

$$LOO^{\bullet} \text{ (or } L^{\bullet}) + AH \rightarrow LOOH \text{ (or LH)} + A$$

$$LOO^{\bullet} \text{ (or } L^{\bullet}) + L^{\bullet} \rightarrow LOOL \text{ or } L_2$$

Extensive lipid peroxidation can result in membrane disorganization by peroxidizing mainly the highly unsaturated polyunsaturated fatty acids, thereby changing the composition of the (polyunsaturated) fatty acid and phospholipid fractions, leading to changes in the ratio of polyunsaturated to other fatty acids (99).

Oxidation of polyunsaturated fatty acids can produce aldehydic compounds, in particular the volatile low molecular weight aldehyde, malondialdehyde (MDA) (100, 101) (Figure 3). MDA can induce polymerization of membrane components and a variety of crosslinking reactions such as lipid-lipid cross-linking and lipid-protein cross-linking (102). These effects will result in deteriorative changes in the cellular membrane such as decrease in membrane fluidity (99). Another deleterious change is the inability to maintain ionic gradients leading to

cellular swelling, a loss of cell integrity and cell inflammation (101, 103), perhaps sufficient to cause cell death (99).

Lipid peroxidation in important organelles such as mitochondrial membranes can lead to swelling, lysis and disintegration of the mitochondria. Free radical damage to microsomal membranes results in disaggregation of polyribosomes and inhibition of protein synthesis. Since lysosomes contain hydrolytic enzymes, damage to lysosomal membranes will result in enzymatic digestion of other cell components (104).

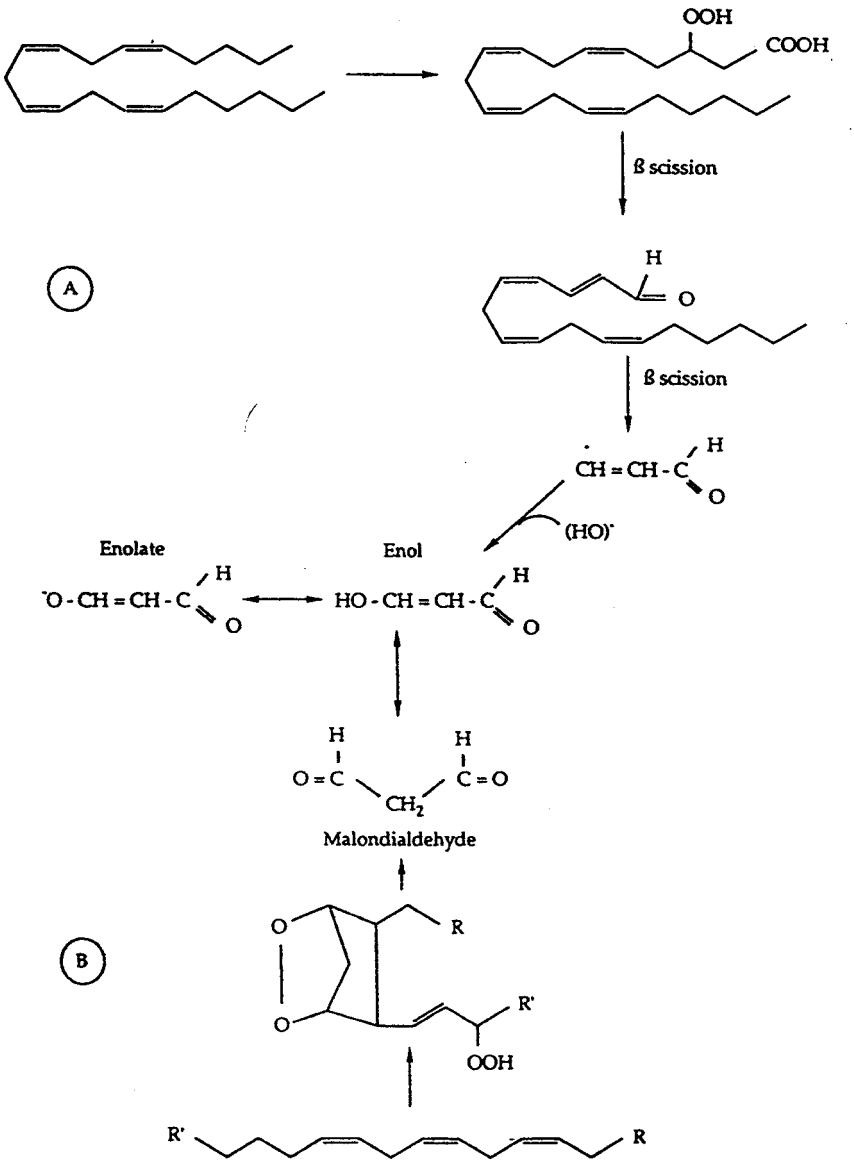


Figure 3. Malondialdehyde, its tautomeric forms (enol, enolate), and the proposed molecular formation, as a result of peroxidation of polyunsaturated lipids containing more than two double bonds. A: adapted from Esterbauer et al. [29]; B: adapted from Pryor and Stanley [105].

V. Defense Systems

From a biological standpoint, O_2 is a poisonous molecule. Experimental studies reveal that cells and organisms require complex defenses against oxidative stress, without which survival under aerobic conditions would be impossible. The defense systems include enzymatic and nonenzymatic antioxidants (106, 107).

Enzymatic Antioxidants

Antioxidant enzymes or scavenging enzymes that are directly involved in the detoxification of reactive oxygen species are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (108-112).

Superoxide dismutase (SOD)

It is now generally accepted that the biological role of SOD is to scavenge $O_2^{\bullet-}$, and is known to be generated *in vivo* in amounts increasing with O_2 exposure (113). Thus, the principle function of SOD is to catalyze the conversion of $O_2^{\bullet-}$ to H_2O_2.

SOD can be divided into four types namely, Copper-Zinc-containing superoxide dismutase (CuZn-SOD), manganese superoxide dismutase (Mn-SOD), iron-containing superoxide dismutase (Fe-SOD) and cambialistic superoxide dismutase. Mammalian tissues contain the first two types, CnZn-SOD and Mn-SOD. The other two types, Fe-SOD and cambialistic SOD have been found in animal tissue; cambialistic SOD is a hybrid enzyme containing subunits of the manganese enzyme and of the iron enzyme in the same dimeric molecule and found in several bacteria.

CuZn-SOD is present in virtually all eukaryotic cells. In animal cells, most CuZn-SOD is located in the cytosol. Cyanide and diethyldithiocarbamate

are powerful inhibitors of CuZn-SOD. The copper ions in CuZn-SOD appear to function in the dismutation reaction by undergoing alternate oxidation and reduction:

$$\text{Enzyme-Cu}^{2+} + O_2^{\bullet-} \rightarrow \text{Enzyme-Cu}^{2+} + O_2$$

$$\underline{\text{Enzyme-Cu}^{2+} + O_2^{\bullet-} + 2H^+ \rightarrow \text{Enzyme-Cu}^{2+} + H_2O_2}$$

Net reaction: $O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$

Mn-SOD is not inhibited by cyanide or diethyldithiocarbamate, and is largely located in the mitochondria. Mn-SOD catalyses essentially the same reaction as CuZn-SOD. The relative activities of Mn-SOD and CuZn-SOD depend on the tissue and on the species.

Catalase (CAT)

CAT is an enzyme present in most aerobic cells (114). In animals CAT is present in all major body organs, being especially concentrated in the liver. The CAT activity of animal and plant tissues is largely or completely located in subcellular organelles bounded by a single membrane and known as peroxisomes. CAT directly catalyses decomposition of H_2O_2 to ground-state O_2 which reaction may be written as follows:

$$\text{Catalase} - \text{Fe (III)} + H_2O_2 \rightarrow \text{compound I} + H_2O$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{catalase} - \text{Fe (III)} + H_2O + O_2$$

One H_2O_2 is reduced to H_2O and the other is oxidized to O_2.

Glutathione peroxidase (GPX)

GPX has been found in animal liver and lung and human erythrocytes. It consists of four protein subunits, each of which contains one atom of the element selenium (Se) at its active site. Traces of Se are essential in the diet. An

important role of dietary Se is to provide the selenium-containing cofactor for the GPX enzyme family. GPX uses GSH as a hydrogen donor and removes H_2O_2 by coupling its reduction to H_2O.

$$H_2O_2 + 2GSH \xrightarrow{GPX} GSSG + H_2O$$

However, GPX can also act on peroxides other than hydrogen peroxide (H_2O_2); in all cases the peroxide group is reduced to an alcohol (114).

$$LOOH + 2GSH \xrightarrow{GPX} GSSG + H_2O + LOH \quad (115)$$

Nonenzymatic Antioxidants

An important source for non-enzymatic antioxidants is the diet, which contains numerous compounds exhibiting antioxidant activity (116-118), such as ascorbate (vitamin C), α-tocopherol (vitamin E) and β-carotene (vitamin A).

Vitamin C:

Vitamin C is water-soluble and can be found in high concentration in many tissues. The major sources for vitamin C in the diet are fruits.

Upon interaction with reactive oxygen species, vitamin C is oxidized to dehydroascorbate via the intermediate ascorbyl free radical. Dehydroascorbate is recycled back to ascorbic acid by dehydroascorbate reductase (119).

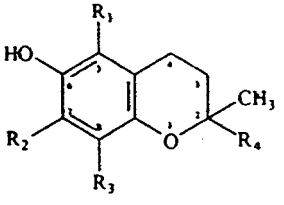
Carotenoids:

Carotenoids are natural colorants with pronounced antioxidant activity (118). Reactive oxygen species that are efficiently scavenged by carotonoids are superoxide radical and peroxyl radicals. Two different pathways that operate with respect to the deactivation of O_2 are physical and chemical quenching. Physical

quenching implies the deactivation of O_2 by energy transfer from the excited oxygen species to the carotenoid. Chemical quenching contributes less than 0.05% to total O_2 quenching by carotenoids. Carotenoids are also reported to scavenge peroxyl radical by chemical interaction.

Vitamin E:

Vitamin E, an essential fat-soluble vitamin, occurs in two classes, designated as tocopherols and tocotrienols, with different biological activities. The basic structure consists of hydroxylated ring system (chromanol ring) and an isoprenoid (phytyl) side chain.



Tocopherols	Tocotrienols	CH_3-position
α	α	5, 7, 8
β	β	5, 8
γ	γ	7, 8
δ	δ	8

Tocopherols : $R_4 = CH_2-(CH_2-CH_2-\underset{\displaystyle CH_3}{\underset{|}{CH}}-CH_2)_3-H$

Tocotrienols : $R_4 = CH_2-(CH_2-\underset{\displaystyle CH_3}{\underset{|}{CH}}=CH-CH_2)_3-H$

Figure 4. The chemical structure of tocopherol vitamers.

The isoprenoid side chain (R_4 position) is saturated in the tocopherols. While it is unsaturated in the tocotrienols

d–α-tocopheral has the highest biological activity and is the most widely-available form of vitamin E in food.

Vegetable and seed oils are the main sources of the tocopherols whereas animal products are generally poor sources of this vitamin. Relative to other fat-soluble vitamins, vitamin E is safe.

Vitamin E is lipophilic, operative in membrane or lipoprotein particles. The most widely accepted biological function of vitamin E is its antioxidant property (120). Its most important antioxidant function appears to be the inhibition of lipid peroxidation, scavenging lipid peroxyl radicals to yield lipid hydroperoxides and the tocopheroxyl radical (121).

Vitamin E appears to be located within membranes with the phytyl tail closely aligned with acyl chains of lipids in the membrane. The chromanol head of vitamin E appear to be close to the surface of the membrane. When a peroxyl radical is formed in the tail region, it is forced or projected out of the nonpolar region toward the polar region where the chromanol head is located. This enables the chromanol region to be oxidized, yielding the tocopheroxyl radical (122).

There is evidence shows that vitamin C is capable of interacting with and regenerating the chromanol region of the tocopheroxyl radical to form tocopherol again (119, 122).

VI. Exercise Training

Exercise training or chronic exercise is characterized by repeated bouts of exercise over several days or months (123). In general, physical training imposes stress to the body tissues, in particular, the muscle. Chronic muscular activity, which

occurs during training, can be considered a positive form of stress because it results in adaptations, changes that are gradual and occur over several weeks, within and around the muscle, making them more tolerant of specific tasks (124).

Principles of Training

The three principles of training are overload, specificity and reversibility.

Overload

The term overload refers to the observation that the system or tissue must be exercised at a level beyond which it is presently accustomed in order for a training effect to occur. The system or tissue gradually adapts to this increase demand. This pattern of overload, followed by adaptation continues until the system or tissue can no longer adapt. The typical variables that constitute overload include the intensity, duration and frequency of exercise.

Specificity

The training effect is specific to the muscle fibers involved in the activity. However, this also means that if an individual participates in a long, slow distance running program that utilizes slow-twitch muscle fibers, there will has little or no training effect in the fast-twitch fibers of the same muscle. Therefore, training effects are clearly specific to the type of muscle.

Specificity also refers to the type of adaptations occurring in the muscle as a result of training. If the muscle is engaged in endurance type of exercise, the primary adaptations is the increase in capillary and mitochondria numbers, which will increase the capacity of the muscle to produce energy aerobically. If the muscle is engaged in heavy resistive training, the primary adaptation is an increase in the

quantity of contractile proteins, and the decrease in mitochondrial and capillary densities. Therefore, training programs need to deal with specificity by addressing not only the muscle groups to be trained, but also the systems that will be providing the energy.

Reversibility

When an athlete stops training, the training effect is quickly lost, or reversed (125).

Anaerobic and Aerobic Training

There are three energy production systems available during exercise. These are the adenosine triphosphate-phosphocreatine (ATP-PC) system, the glycolytic or lactic acid system and the aerobic or oxygen system. These three systems will operate at different times during exercise (126). Exercise is broadly classified, in terms of duration and the perdominating energy pathways, into either of two categories, anaerobic or aerobic. In reality the energy to perform most types of exercise comes from a combination of anaerobic and aerobic sources (127-130).

Contribution of Aerobic and Anaerobic Production of ATP During Maximal Exercise as a Function of the Duration of the Event

	Duration of Maximal Exercise								
	Seconds			Minutes					
	10	30	60	2	4	10	30	60	120
Percent aerobic	10	20	30	40	65	85	95	98	99
Percent anaerobic	90	80	70	60	35	15	5	2	1

(126)

The aerobic, or oxygen, system releases energy for ATP production from the breakdown mainly of carbohydrates and fats, and sometimes, protein. In order to accomplish an aerobic training effect, two important factors are required. First, training must provide a sufficient cardiovascular overload to stimulate an increase in stroke volume and cardiac output. Second, this central circulatory overload should be accomplished by exercising the sport-specific muscle groups to enhance their local circulation and metabolic machinery. For example, runners should run, cyclists should bicycle, rowers should row and swimmers should swim. In conclusion, the two major goals of aerobic training are to develop the capacity of the central circulation to deliver oxygen and to enhance the capacity of the local musculature to supply and process oxygen (131). The improvement in endurance that occur with daily aerobic training appear to be the result of changes in both central and peripheral circulation and muscle metabolism. Figure 5 shows a schematic relationship between cardiovascular factors that contribute to an augmentation in cardiac output. This is the overall effect of increasing circulation to exercising muscle.

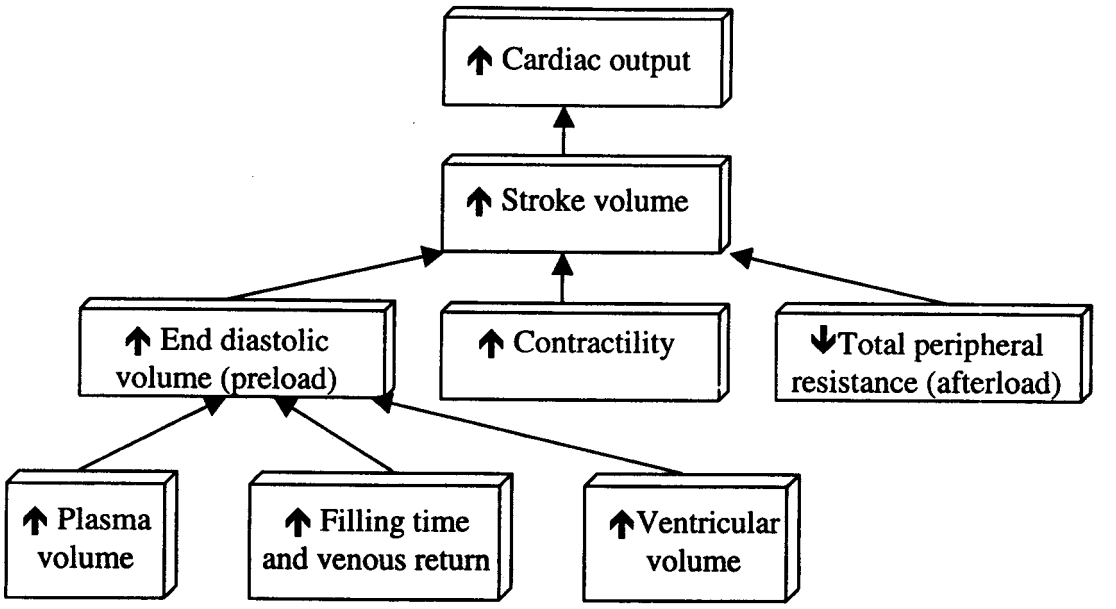


Figure 5. Contribution of various factors to an increase in cardiac output after exercise training.

Stroke volume (SV), is equal to the difference between end diastolic volume (EDV) and end systolic volume (ESV). The three factors that influence SV are EDV, contractility and total peripheral resistance (TPR).

There is evidence that left ventricle size increases as a result of endurance training, with little change in ventricular wall thickness. This effect is believed to be due to "volume loading" experienced by the heart during exercise. However, Rowell (132) raises the question that the increase in stroke volume that occurs with endurance training may be due to the chronic stretch of the myocardium at rest because of the increased filling time associated with a lower resting heart rate (bradycardia). Besides that, an increase in plasma volume due to endurance training also influences EDV and leads to an increase in stroke volume. Cardiac contractility refers specifically to the strength of the cardiac muscle contraction when the fiber length (EDV), afterload

(peripheral resistance), and heart rate are constant. While an acute exercise bout increases cardiac contractility due to the action of the sympathetic nervous system on the ventricle, it is difficult to conclude whether or not the inherent contractility of the heart changes with endurance training. Afterload refers to the peripheral resistance against ventricular contraction as it tries to pump blood into the aorta. If the heart contracts with the same force while the peripheral resistance decreases, a greater stroke volume will result. During maximal work with trained muscles following an endurance training program, there is a decrease in the resistance of the vascular bed to match the increase in maximum cardiac output, maintaining blood pressure (125).

Several adaptations can occur follow the aerobic training program.

Oxygen Delivery

One of the most important changes that occurs during training is an increase in the number of capillaries surrounding each muscle fiber. This change allows greater exchange of gases, heat, fuel, and by-products between the blood and the working muscle fibers. Aerobic training also increases the myoglobin content of the muscle. Myoglobin's main function is to deliver oxygen from the cell membrane to the mitochondria and also acts as a storage compartment for oxygen.

Energy Production

Aerobic energy production is the exclusive responsibility of the mitochondria. Endurance training induces changes in the mitochondria, improving the capacity of the muscle fibers to produce ATP. The ability to use oxygen and produce ATP oxidatively depends on the number, size and efficiency of the mitochondria. It has been demonstrated that endurance training produce a weekly

increase of approximately 5% in the number of mitochondria over a 27-week period. At the same time, the average size of the mitochondria increases by about 35%. As a result of aerobic training, there is also increase in the amount of muscle's oxidative enzymes. The concentrations of muscle enzymes such as succinate dehydrogenase (SDH) and citrate synthase (CS) are dramatically influenced by endurance training.

Fuel Storage

Endurance training places repeated demands on the muscle's energy supplies of glycogen and fat. Muscle glycogen, in particular, may be severely reduced after each training session. As a result, the mechanisms responsible for the resynthesis of glycogen stimulate greater storage when the athlete has adequate rest and sufficient dietary carbohydrate. In addition to its greater glycogen stores, endurance trained muscle has substantially more fat (triglyceride) than can be found in untrained fibers. Many of the muscle's enzymes responsible for the oxidation of fat are also increased by endurance training (124).

Antioxidant Capacity

Exercise is associated with a large increase in oxygen consumption and therefore, in free radical production (133), resulting in elevated levels of lipid peroxidation as indicated by expired breath samples of ethane and pentane (134).

An increase in lipid peroxidation level, leading to muscle damage during acute exercise may indicate that the body's defense system is unable to regulate lipid peroxidation during periods of increased O_2 consumption (135).

Because of this finding, one might expect that repeated bouts of exercise would result in cumulative damage and accelerated aging of muscle in

individuals who exercise regularly. However, evidence suggests that the metabolic potential (136) and functional capacity (137) of muscle are not impaired in individuals exposed to chronic, regular exercise over a period of many years. This raises the possibility that regularly performed exercise might induce an adaptive enhancement of the defense mechanisms that protect against free radical damage in skeletal muscle (138).

Alessio et al (135), studying running trained rats, found that endurance training resulted in reduced lipid peroxidation after moderate acute exercise. This was attributed to the activation of the scavenging enzyme, catalase. Venditti et al (57) also studied the effect of endurance training on lipid peroxidation during rest and after acute exhaustive exercise in swim trained rats. They found that malondialdehyde (MDA) and hydroperoxide content in trained rats was increased less than sedentary animals, and overall scavenging enzymes were higher in rats after 10 weeks of training. Experiments in humans also show the effect of training on lipid peroxidation. Toskulkao et al (58) measured the level of lipid peroxidation, as MDA, and scavenging enzymes (SOD, GPX, CAT) in plasma after endurance exercise in trained and untrained subjects. It was found that plasma MDA, CPK and LDH levels were significantly higher in sedentary subjects than in trained subjects, whereas scavenging enzyme activities were higher in trained subjects.

It seems to be accepted that exercise training results in decreased free radical damage after exercise due to the high protective capacity of scavenging enzymes. However, several different patterns of scavenging enzyme response to training have been reported. Higuchi et al (138) reported that only mitochondrial

SOD activity was increased in 3 month treadmill-running trained rats. Hammeren et al (139) found an increase in GPX activity after 10 weeks of running training in rats. Laughlin et al (140) reported that exercise training induced an increase in GPX, a decrease in CAT and no change in SOD activity in running trained rats. Most recently, Toskulkao et al (141) reported that short and long distance running training for about 3 years in humans induced increases in most plasma scavenging enzymes, SOD, GPX and CAT.

Up to now, regularly performed exercise or exercise training is believed to have several positive effects including an increase in antioxidant enzyme activity to further enhance the muscle tissue's resistance to oxidative damage, both in animals and humans, although the mechanisms responsible for enhancing the body's antioxidant enzymes after training are still unclear.

Glucose Uptake

Under normal physiological conditions, glucose transport into skeletal muscle is the rate limiting step in glucose utilization (142). The transport of glucose molecules across the plasma membrane of muscle occurs primarily by a process of facilitated diffusion, which is an energy-independent process and uses a carrier protein. Glucose transport in muscle is stereospecific for D-glucose (143) and the major isoform of transmembrane glycoproteins carrier in skeletal muscle is the GLUT-4 protein (144). The GLUT-1 isoform also appears to be expressed in skeletal muscle but at lower abundance.

The major mediators of glucose transport activity in muscle are insulin (145, 146) and fiber contraction (145, 147), although there are numerous other

factors including hypoxia, catecholamines, growth factors corticosteroids, thyroid and growth hormones and glucose itself, which can alter glucose transport.

Insulin increases glucose transport in a dose-dependent manner, whereas exercise increases transport proportionally to the frequency of muscle contraction. The effect of exercise on glucose transport can persist for several hours following the cessation of exercise (148). In contrast, following the removal of an acute insulin stimulation, glucose transport rapidly returns to basal rates (149). Rates of basal, insulin-stimulated and contraction-stimulated glucose transport generally are greater in muscle composed of predominantly red, oxidative fibers, as compared to white, more glycolytic fibers (150, 151).

Insulin exerts its biological action on tissues by first binding to a specific cell-surface membrane receptor. This binding presumably initiates signaling events involving phosphorylation of the receptor itself and other endogenous proteins. These phosphorylation events may then result in several metabolic and growth processes including the stimulation of glucose transport. With muscle contraction, glucose transport processes can occur independent of insulin (152), although the initial signaling event that eventually leads to a stimulation of glucose transport has not been conclusively determined.

It has been demonstrated that insulin stimulates glucose transport by the translocation of glucose transporters from an intracellular microsomal membrane pool to the plasma membrane (153, 154). Similar to insulin, the exercise-induced increase in plasma membrane glucose transporter number has been shown to be associated with a significant decrease in glucose transporter numbers in an

intracellular microsomal pool. Evidence showed that besides an increase in the number of glucose transporters in the plasma membrane, this insulin-independent contractile activity also showed a rise in the "average functional activity" of glucose transporters in plasma membrane. Exercise does not alter insulin receptor function (155).

Both insulin and exercise can increase glucose transporter synthesis after chronic stimulation. It was recently reported that insulin infusion for 3 hours increases the GLUT-4 mRNA in human skeletal muscle. Furthermore, exercise training has been found to increase the mRNA for both GLUT-4 and GLUT-1 in rat skeletal muscle. In 1990, Rodnick and co-workers, in an experiment in rats, reported that after 6 weeks of running exercise, the concentration of glucose transporters per unit of muscle protein or DNA was increased by 60% in plantaris muscle from exercise trained rats (156). In the same year, Ploug and his co-workers showed that there were approximately two fold increases in concentration of mRNA for GLUT-1 and GLUT-4 in 10 weeks swim trained rats (157). In 1995, A. Houmard and his co-workers in an experiment with human. Subjects determined the level of GLUT-4 protein content in skeletal muscle after 7 days of training. Using needle biopsy samples from the vastus lateralis muscle, they found a 2.8 ± 0.5 fold increased in muscle GLUT-4 after 7 days of training (158). Therefore GLUT-4 content in skeletal muscle can be increased by both long and short-term exercise training.

CHAPTER III

MATERIAL AND METHODS

A. Animals

Sixty-four male Sprague-Dawley rats, 4 weeks old, were purchased from the National Animal Center at Salaya, Mahidol University, Thailand. They were allowed to rest for a few days after arrival at the Animal Center, Faculty of Science, Mahidol University before being used in the experiment. The animals were kept in a room where temperature was 25±2°C and relative humidity at approximately 65%. Two rats were housed in stainless steel cage and fed *ad libitum* with regular dry rat chow [Animal Nutrition Research and Development Center (ANRDC), Kasetsart University, Thailand] and water. Heart rates of all animals were measured both at the beginning and at the end of the experiment. Prior to sacrifice, rats were fasted overnight (about 16-20 hours) but allowed continuous access to water.

B. Chemicals

All chemicals and reagents used throughout the investigation were analytical grade. Reagents for determination of CS, SOD, GPX, CAT, MDA, GSH and protein were purchased from Sigma Chemical Company (St. Louis, USA). Glucose oxidase and peroxidase kits were purchased from Bio-Medical Laboratory (Bangkok, Thailand). Diethyl ether, hydrogen peroxidc (30% w/v) and acetic acid (100%) were purchased from Merck (Darmstadt, Germany).

C. General Procedures

1. Exercise training program

The rats were subjected to a running program, 5 days/week on a motor-driven rat treadmill. The intensity and duration of exercise was gradually increased, on a weekly basis beginning from the first week of training, until the 9th week. By the time, daily exercise consisted of running for 1 hour at a speed of 18 meters/minute up a 20% grade. This intensity was maintained until the 12th week.

Week	Speed (m/min)	Duration (min)	% Grade
1	10	20	0
2	15	25	5
3	18	30	10
4	18	35	15
5	18	40	15
6	18	45	15
7	18	50	20
8	18	55	20
9	18	60	20
10	18	60	20
11	18	60	20
12	18	60	20

2. Vitamin E

Natural Vitamin E (Vit E), supplementation was purchased from Sigma Chemical Company (St. Louis, USA), 1360 IU/g. The dose of 70 IU/kg body weight

was used and was prepared by dissolving in corn oil. The Vitamin E was orally administered by means of a small oral-gastric intubation needle, once a day, every day. The intubation needle (3 inches, 16 gauge) with a rounded tip as attached to a syringe and passed down the esophagus to the stomach. The controls and other, none supplemented, groups were orally fed with corn oil in a similar manner to the Vitamin E supplementation group.

3. Diabetic Induction

To induce diabetes mellitus, streptozotocin (STZ) (Sigma Chemical Co.) was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma Chemical Co.) and immediately injected into the tail vein of non-fasted rats, at a dose of 35 mg/kg body weight.

4. Plasma glucose determination

To evaluate the state of diabetes, plasma glucose level was determined at 24 hours after STZ injection and every 24 hours thereafter for 1 week, using a glucometer and strips (Advantage, blood glucose monitor, Boehringer Mannheim Co.). Before the glucometer and strips were used in the experiment, they were calibrated against 100, 200 and 300 mg% standard glucose. The plasma glucose level on each measured was three times and averaged. Rats which had plasma glucose levels higher than 200 mg% were considered to be diabetic.

5. Heart rate determination

All rats, heart rates were measured at the beginning and the end of experiments. Three copper wiring transcutaneous pins were attached to the rat. Electrodes were attached in a bipolar limb lead pattern, anode at left anterior axillary

line at heart level, cathose at right anterior axillary line at heart level and ground line at mid-line of lower back. The rats were allowed to rest in a dark, quite restrainer for about 5 minutes before measuring. Heart rate was calculated from the standard ECG recording on paper, averaging the beats over a period of several seconds duration.

6. Body weight determination

All rats were weighed once a week on the first day of each week throughout the experiment, before STZ injection and before sacrifice.

7. Euthanasia

At the time of sacrifice, the animals were anesthetized with intra-peritoned sodium pentobarbital, 55 mg/kg body weight. After waiting for about 15 minutes and confirming the absence of motor reflexes, both of gastrocnemius muscles were excised, the abdominal wall was opened, and the diaphragm and pancreas were immediately excised. Gastrocnemius muscle was used to determine CS, SOD, GPX, CAT, GSH, MDA and protein. Diaphragm was used to determine glucose uptake and pnacreas was subjected to study histology.

8. Collection of muscle and organs

8.1 Gastrocnemius muscle: After the rat was anesthetized, the skin on both hindlimbs was reflected. Then the gastrocnemius muscle was excised by sectioning the tendoarchialis tendon and immersed in ice-cold 0.9% NaCl. The tendon and fibrous tissues of these muscles were removed. The muscles was blotted on filter paper and weighed

8.2 Diaphragm muscle: The abdominal wall was opened, then the whole diaphragm was excised and immersed in ice-cold 0.9% NaCl. The fibrous

tissues and central tendon were removed. The muscle was blotted on filter paper and weighed.

8.3 Pancreas: The pancreas was excised, immersed in an ice-cold 0.9% NaCl then fat tissue was removed. The pancreas was blotted on filter paper and immersed in 10% formalin.

9. Glucose uptake determination

Whole diaphragm without the central tendon was incubated for 30 minutes in a 25 ml Erlenmeyer flask, containing 180 mg% glucose, 1 mg/ml albumin and 60 mU insulin (Regular type insulin 100 units/ml, Eli Lilly, Mexico) in Krebs Henseleit-HCO_3^- solution [NaCl, 116.1; $NaHCO_3$, 24.5; KCl, 4.65; $CaCl_2$, 2.49; KH_2PO_4, 1.17 and $MgSO_4$: $7H_2O$, 1.17 mM (pH 7.4)]. Krebs Henseleit-HCO_3^- solution was freshly prepared and oxygenated with 95% O_2 - 5% CO_2 at least 30 minute before use. During incubation temperature was maintained at 37°C in a shaking water-bath incubator, 120 rpm and oxygenized all the time. The glucose concentrations in Kreb Henseleit-HCO_3^- solution were determined by using a glucose oxidase and peroxidase kit (Bic-Medical Laboratory Co.)

10. Preparation of whole muscle homogenate and subcellular fraction

The gastroenemius (right side) was homogenized in 5 volumes (w/v) of 1.15% KCl in 0.1 M phosphate buffer, pH 7.4 by using a glass Potter-Elvehjem homogenizer. The homogenization was performed for 10 strokes in 2 minutes at 4°C then separated into 3 parts.

Part 1) 1 ml of whole homogenate was used for the citrate synthase assay. 9 ml of 1 mM EDTA was added, then homogenizede 5 stokes in 30 seconds at

4°C to rupture the mitochondrial membrane. After the homogenate was prepared, it was centrifuged at 1,000 rpm for 10 minutes at 4°C to remove fibrous tissue.

Part 2) 1 ml of whole homogenate was used for determining glutathione content by mixing with 1 ml of 4% sulfosalicylic acid. After mixing, the mixuter was centrifuged at 4,500 rpm for 30 minutes at 4°C, and the supernatant was subjected to assay.

Part 3) The remainder was used to assay SOD, GPX and CAT activity by adding phosphate buffer, pH 7.4 to yield 1:9 (w/v). This was then homogenized 5 stokes in 30 seconds at 4°C and centrifuged at 2,500 rpm for 10 minutes (to remove unbroken cells and nuclei, and cell debris). The supernatant fraction was subjected to centrifugation at 10,000 rpm for 30 minutes at 4°C by using Refrigerator Kontron Centrifuge (A24-24) to remove mitochondria and lysosomes. The resultant supernatant fraction was then centrifuged at 40,000 rpm for 60 minutes at 4°C by using Beckman Ultracentrifuge (Rotor 60 Ti). The final pellet, as microsomal fraction and supernatant, as cytosolic fraction were obtained.

D. Chemical Procedures

1. Citrate synthase (CS) activity was determined by a modification of the method described by Srere, 1969 (Appendix VI).

2. Lipid peroxide (MDA) content in whole muscle homogenates was determined by the thiobarbituric acid reaction as described by Ohgawa et al., 1979 (Appendix I).

3. Cytosolic superoxide dismutase (SOD) activity was determined by the modified method of Winterbourn, 1975 (Appendix II).

4. Cytosolic glutathione peroxidase (GPX) activity was determined by a modification of the method of Paglia and Valentine described by Tepple, 1978 (Appendix IV).

5. Cytosolic catalase (CAT) activity was determined by spectophotometric method as described by Luck, 1965 (Appendix III).

6. Glutathione (GSH) content was determined by a colorimetric method reported by Boyland and Chasseaud, 1970 (Appendix V).

7. Protein was determined by a modification of the Lowry method (Appendix VII).

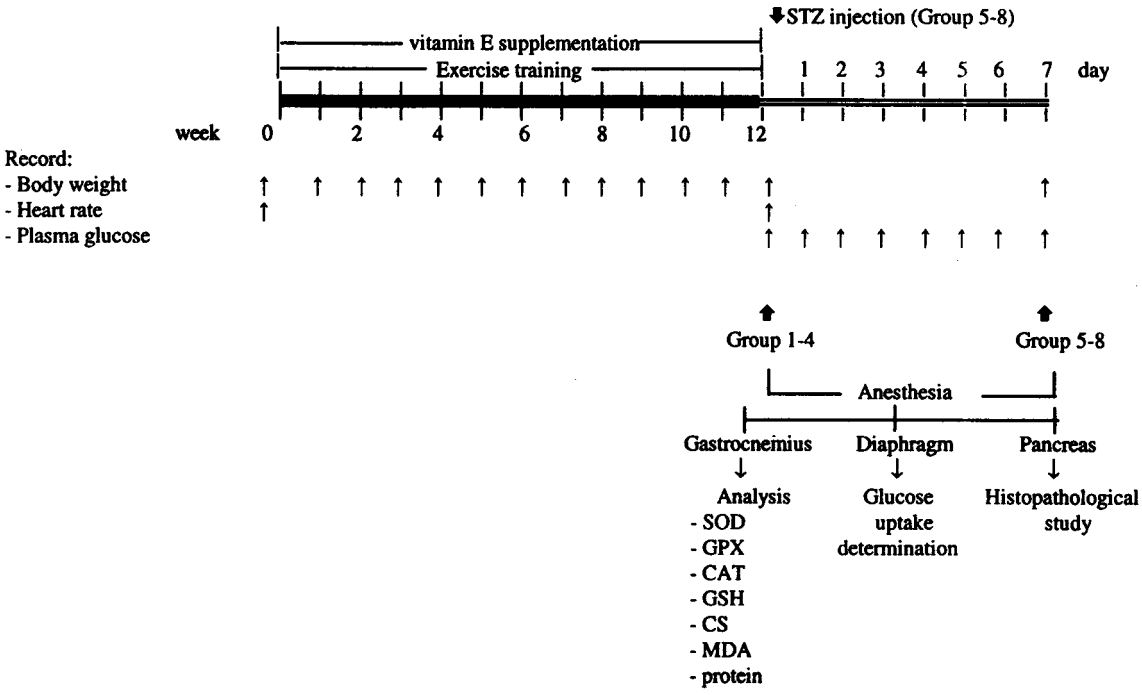
E. Histopathological Study

In the study, the histopathological change of pancreas was examined under the light microscope. After abdominal incision, the pancreas was immediately excised carefully stripped of adhering fat and immersed in 10% neutral buffered formalin (pH 7.4). The fixative was changed on the next day. Then the tissue was processed under the standard paraffin technique and stained with hematoxylin and eosin (Appendix VIII).

F. Statistical Analysis

All data were presented as means and standard error of the mean (SEM). Significance at differences between groups was determined by using one way analysis of variance (one way ANOVA) and the differences in pair of means among groups were evaluated by a least significant difference (LSD). Differences were considered to be statistically significant if the p-value was less than 0.05 ($p<0.05$).

G. Experimental Protocols



All rats were randomly assigned into one of 8 experimental groups. Each group consisted of 6-8 rats, as follows:

Group 1. Sedentary group

2. Exercise trained group

3. Vitamin E supplemented group

4. Exercise trained plus vitamin E supplemented group

5. Diabetic group

6. Exercise trained, Diabetic group

7. Vitamin E Supplemented, Diabetic group

8. Exercise trained plus vitamin E supplemented, Diabetic group

Body weight of all rats was recorded once a week, heart rate was recorded two times, at the 1st week and 12th week. Rats were administered corn oil in groups 1, 2, 5

Body weight of all rats was recorded once a week, heart rate was recorded two times, at the 1st week and 12th week. Rats were administered corn oil in groups 1, 2, 5 and 6 while groups 3, 4, 7 and 8 were orally administered a mixture of corn oil, plus vitamin E at a dose of 70 IU/kg BW/day, every day, for 12 weeks. In groups 2, 4, 6 and 8, rats were subjected to the exercise training program for 12 weeks. The rats in groups 5, 6, 7 and 8 were induced with diabetes by using STZ at the dose of 35 mg/kg BW, by intravenous injection into the lateral tail vein. To follow up, the severity of diabetes was monitored daily by plasma glucose level measurement for one week than sacrifice.

After sacrifice, bitateral gastrocnemius muscle, diaphragm and pancreas were collected. The pancreas were subjected to histopathology study, the diaphragm was used to determine glucose uptake and gastrocnemius muscle tissue was subjected to assay of SOD, GPX, CAT, GSH, CS and MDA.

CHAPTER IV

RESULTS

1. Dose Response of Streptozotocin (STZ)

The level of plasma glucose in rats, before and after injection with various doses of STZ, are shown in Table 1 and Figure 6. There were no statistical significant difference in plasma glucose when comparing pre-STZ control values among the three groups of rats. The plasma glucose concentration in rats given a dose of 25 mg/kg BW of STZ before and at 1, 2, 3, 4, 5 and 6 days after STZ injection were 101.5±15.8, 86.5±6.6, 103.0±8.5, 104.0±4.8, 91.0±13.3, 106.5±14.9 and 89.0±75 mg%, respectively. None of these values was significantly different from the plasma glucose value before the 25 mg/kg BW STZ injection. Plasma glucose levels before and at 1-6 days after 35 mg/kg BW STZ were 99.2±10.6, 330.3±43, 300.0±7.7, 314.7±14, 279.5±15, 314.2±25, 288.2±34, and for 45 mg/kg BW STZ were 84.7±11.5, 393.02±8, 353.0±26, 358.0±32, 364.0±24, 369.6±30, 409.3±46 mg%, respectively.

Plasma glucose levels of rats following the doses of 35 and 45 mg/kg BW were found to be significantly higher than after the dose of 25 mg/kg BW at all six days after each dose of STZ injections ($p<0.001$). There was no significant difference in plasma glucose when comparing between the dose at 35 and 45 mg/kg BW at all points of measurement.

Table 1 Level of plasma glucose (mg%) in rats treated with 25, 35 and 45 mg/kg BW of streptozotocin (STZ) before, and 1st to 6th days after STZ induced diabetes.

Group	Plasma glucose level (mg%)						
	Before	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
25mg/kg (n=5)	101.5±15.5	86.5±6.6	103.0±8.5	104.0±4.8	91.0±13.3	106.5±14.9	89.0±7.5
35mg/kg (n=5)	99.2±10.6	330.0±43***	300.0±7.7***	314.7±14***	279.5±15***	314.2±25***	288.2±34**
45mg/kg (n=5)	84.7±11.5	393.2±8***	353.0±26***	358.0±32***	364.0±24***	369.6±30***	409.3±46***

Values are means ± SEM.

**p<0.01, ***p<0.001; significant differences from the corresponding day of rats given a dose of 25 mg/kg BW.

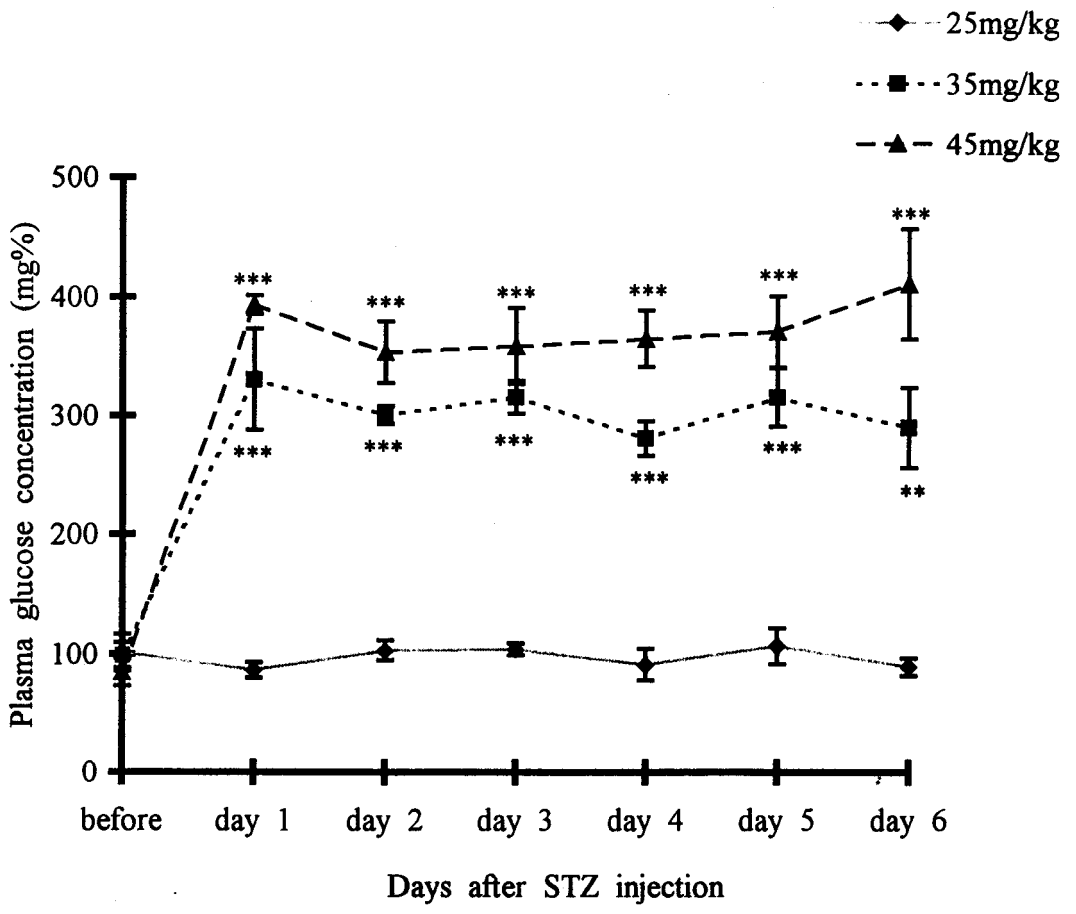


Figure 6 Level of plasma glucose (mg%) in rats treated with 25, 35 and 45 mg/kg BW of streptozotocin (STZ) before and at 1st to 6th days after STZ injection. Values are means ± SEM.

**p<0.01, ***p<0.001; significant differences from the dose of 25 mg/kg BW.

2. Body Weight Gain

Values for the body weight gain of sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups are shown in Table 2 and Figure 7.

The weight gain of rats in the sedentary group from the 1st to the 12th weeks of experiment were 61.4±3, 99.6±4, 154.7±4, 189.8±4, 215.4±4, 239.3±5, 256.8±5, 273.5±5, 289.2±6, 288.4±4, 303.5±5 and 298.5±6 grams, respectively. Rats in the vitamin E supplemented group displayed a similar pattern of weight gain when compared to the sedentary group and there indeed was no significant difference between these two groups of rats throughout the experiment. The mean body weight gain of rats in the exercise trained and exercise trained plus vitamin E supplemented group at the 1st, the 2nd, and the 3rd experimented week were 54.8±2, 860±3, 128.3±5 and 56.5±2, 86.6±3 and 138.6±4 grams, respectively which were not significantly different from the sedentary group. From the 4th to the 12th weeks of experiment, however, both exercise trained and exercise trained plus vitamin E supplemented group have a significantly lower mean body weight gain as compared to the sedentary group ($p<0.05$ at the 4th week and $p<0.001$ from the 5th to the 12th week). There was no significant differences in body weight gain when comparing between exercise trained and exercise trained plus vitamin E supplemented rats for all 12 weeks.

Table 2 Body weight gain (gram) of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E), and exercise trained plus vitamin E supplemented (Ex + Vit E) groups at 1st to 12th weeks of experiment.

Group	Body weight gain (grams)												
	Initial wt.	Week1	Week2	Week3	Week4	Week5	Week6	Week7	Week8	Week9	Week10	Week11	Week12
Se (n=16)	110.1±4	61.4±3	99.6±4	154.7±4	189.8±4	215.4±4	239.3±5	256.8±5	273.5±5	289.2±6	288.4±4	303.5±5	298.5±6
Ex (n=16)	115.6±5	54.8±2	86.0±3	128.3±5	161.6±4*	185.7±3***	209.6±4***	227.5±5***	235.3±4***	251.9±5***	253.2±5***	262.3±5***	263.8±6***
Vit E (n=16)	105.4±6	58.6±2	97.7±4	150.1±6	194.8±4	222.2±4	242.5±4	260.1±4	268.1±4	275.3±5	287.9±5	295.8±5	294.6±5
Ex + Vit E (n=16)	115.3±4	56.5±2	86.6±3	138.6±4	173.3±5*	187.1±5***	210.0±4***	222.6±5***	226.7±4***	238.2±5***	246.4±5***	248.0±6***	247.4±7***

Values are means ± SEM.

*$p<0.05$, ***$p<0.001$; significant differences from sedentary group.

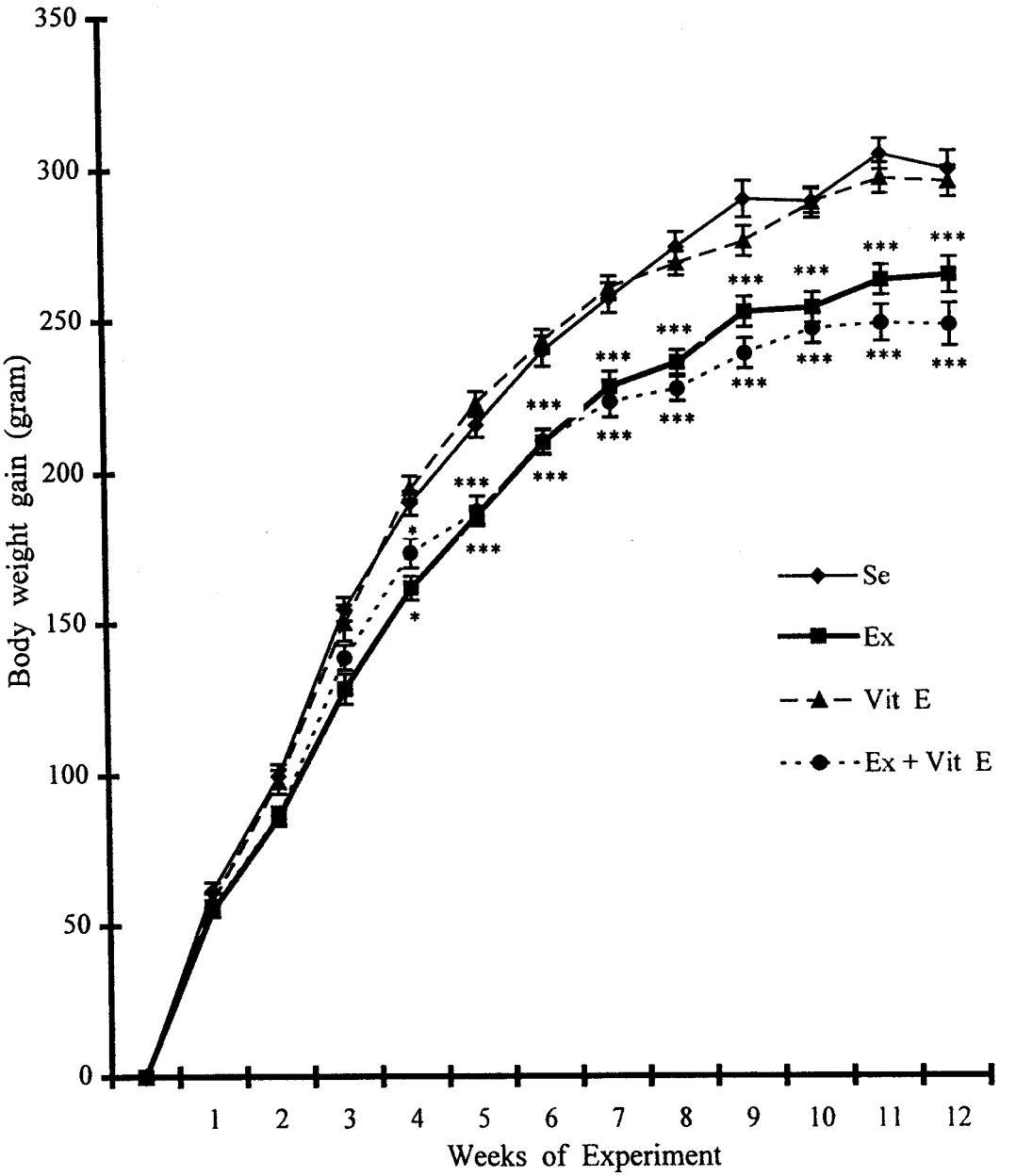


Figure 7 Body weight gain (gram) of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups at 1st to 12th weeks of experiment.

Values are means ± SEM.

*p<0.05, ***p<0.001; significant differences from sedentary group.

3. Heart Rate

Table 3 and Figure 8 show heart rate of sedentary (Se) and exercise trained (Ex) rats at the 12^{th} week of experiment.

The heart rate of rats in exercise trained group was 431.6±8.9 beats/min which significantly lower than in sedentary group (465.8±6.6 beats/min, $p<0.05$).

Table 3 Heart rate (beats / min) of rats in sedentary (Se) and exercise trained (Ex) groups at 12th week of experiment.

	Se (n=16)	Ex (n=15)
Heart rate (beats/min)	465.8±6.6	431.6±8.9*

Values are means ± SEM.
*p<0.05; significant differences from sedentary group.

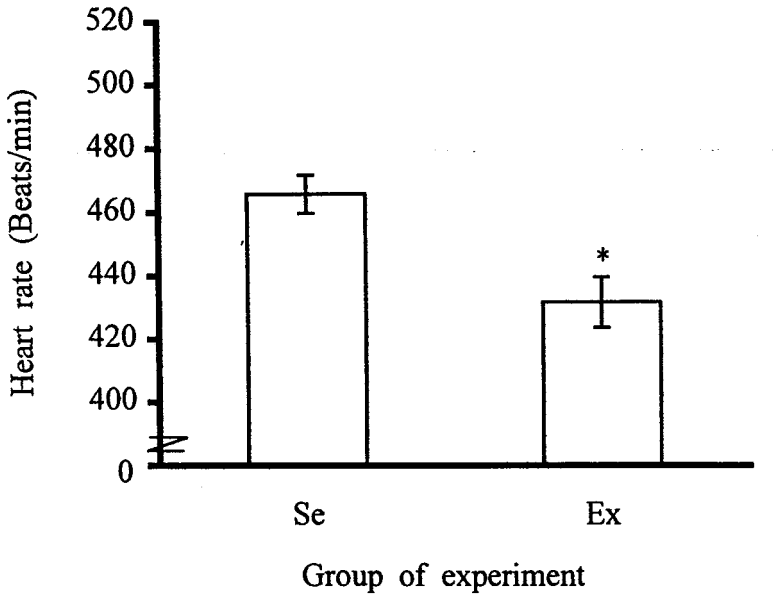


Figure 8 Heart rate of rats in sedentary (Se) and exercise trained (Ex) groups at 12th week of experiment.
Values are means ± SEM.
*p<0.05; significant differences from sedentary group.

4. Citrate Synthase Activity

Table 4 and Figure 9 show citrate synthase activity in rats in the sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without diabetic induction. Values for citrate synthase enzyme activity of rats in the sedentary, exercise trained, vitamin E supplemented and exercise trained plus vitamin E supplemented groups were 42.7±2, 61.1±2, 46.4±1 and 58.4±2 nmol/min/mg protein, respectively. Citrate synthase activity in rats in the exercise trained and exercise trained plus vitmin E supplemented groups was significantly higher than that of the sedentary group ($p<0.001$ and $p<0.05$, respectively) whereas citrate synthase activity in rats in the vitamin E supplemented group was not significantly different from sedentary group.

The citrate synthase enzyme activity of rats in the sedentary diabetic, exercise trained diabetic, vitamin E supplemented diabetic and exercise trained plus vitamin E supplemented diabetic groups were 41.9±1, 52.6±1, 41.6±1 and 51.7±2 nmol/min/mg protein, respectively. Citrate synthase activity in rats in both exercise trained diabetic and exercise trained plus vitamin E supplemented diabetic groups were significantly higher than that in the sedentary diabetic group ($p<0.05$) while citrate synthase activity in rats in the vitamin E supplemented diabetic group was not statistically different from the sedentary diabetic group.

Table 4 Citrate synthase activity (nmol/min/mg protein) of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups, with and without STZ - induced diabetes.

	Citrate synthase activity (nmol/min/mg protein)	
Experimental Treatment	Non DM	DM
Sedentary	42.7±2 (n=8)	41.9±1 (n=8)
Exercise training	61.1±2*** (n=7)	52.6±1+ (n=8)
Vitamin E supplementation	46.4±1 (n=8)	41.6±1 (n=8)
Exercise training plus vitamin E supplementation	58.4±2 * (n=6)	51.7±2+ (n=8)

Values are means ± SEM.

*p<0.05, ***p<0.001; significant differences from sedentary group.

+p<0.05; significant differences from sedentary diabetic group.

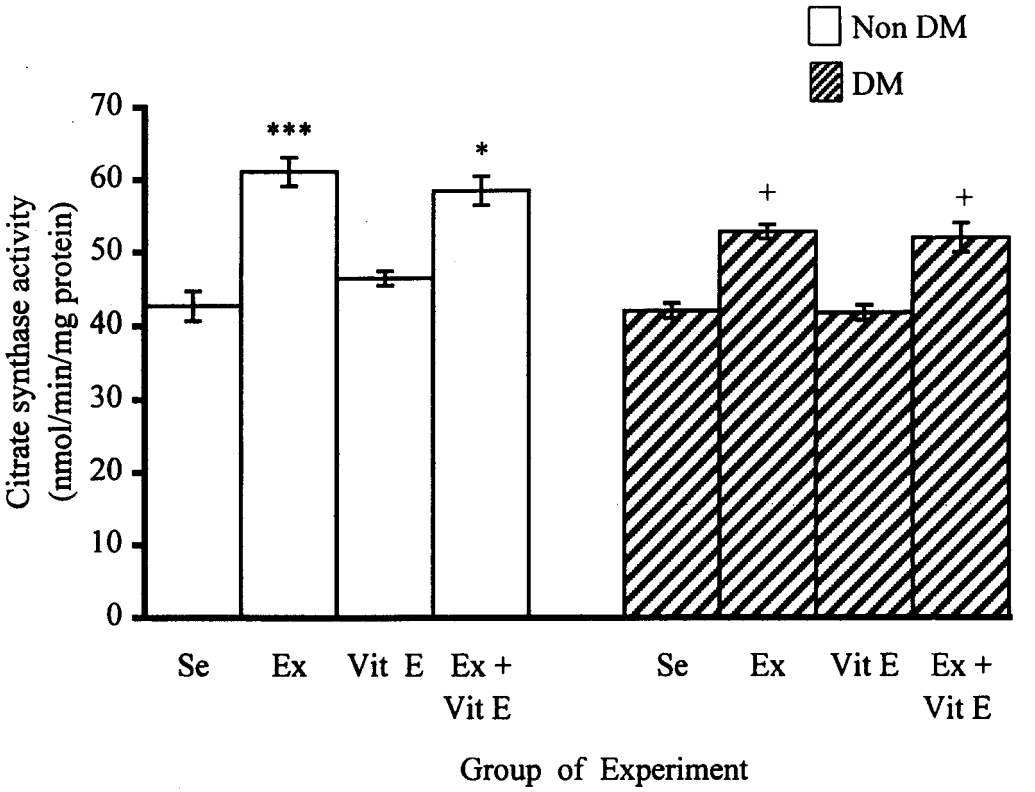


Figure 9 Citrate synthase activity of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Exs + Vit E) groups, with and without STZ - induced diabetes.

Values are means ± SEM.

*$p<0.05$, ***$p<0.001$; significant differences from sedentary group.

+$p<0.05$; significant differences from sedentary diabetic group.

5. Glucose Uptake

Table 5 and Figure 10 show level of glucose uptake in eight groups of rats, sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E), exercise trained plus vitamin E supplemented (Ex + Vit E), sedentary diabetic (Se + Dm), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm), exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm) groups. There were no statistically significant differences among the eight groups, although there was a tendency towards an increase in exercise trained and exercise trained plus vitamin E supplement groups without diabetes.

Table 5 Glucose uptake (mg/g tissue) in muscle of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without STZ - induced diebetes.

	Glucose uptake (mg/g tissue)	
Experimental Treatment	Non DM	DM
Sedentary	3.0 ±0.2 (n=8)	3.1 ±0.3 (n=8)
Exercise training	3.4 ±0.2 (n=7)	3.0 ±0.3 (n=8)
Vitamin E supplementation	2.8 ±0.1 (n=8)	3.2 ±0.2 (n=8)
Exercise training plus vitamin E supplementation	3.3 ±0.3 (n=6)	3.4 ±0.3 (n=8)

Values are means ± SEM.

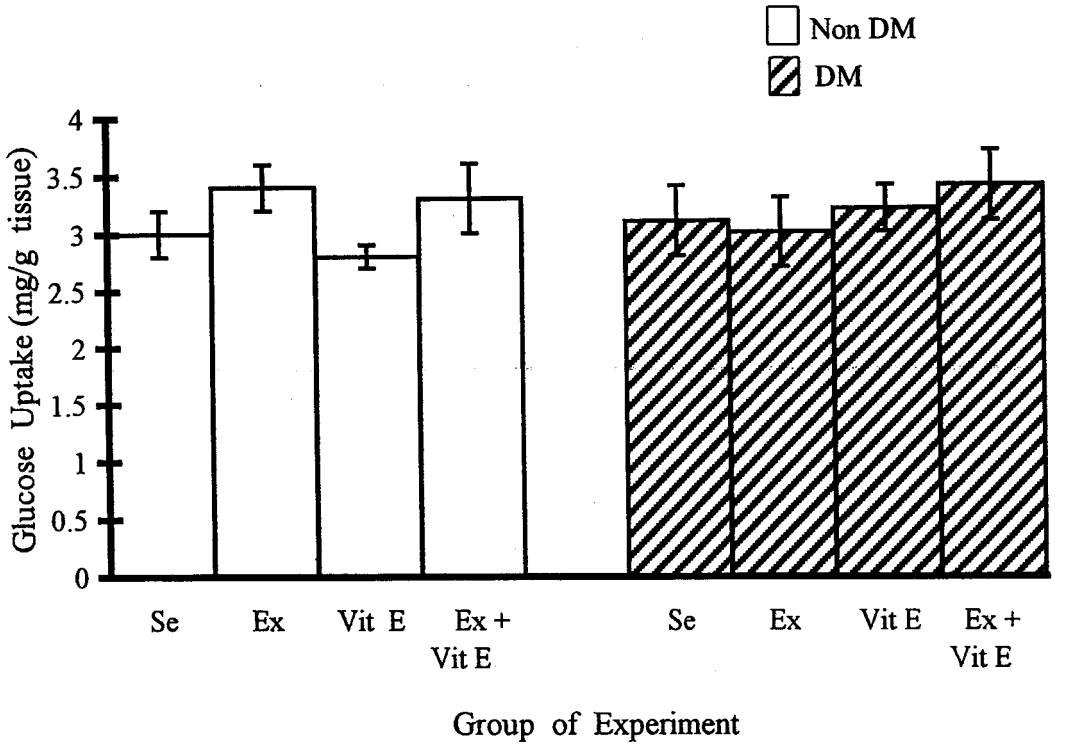


Figure 10 Glucose uptake (mg/g tissue) in muscle of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without STZ - induced diabetes. Values are means $\pm$ SEM.

6. Plasma Glucose

Level of plasma glucose in rats in the sedentary diabetic (Se + Dm), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm) and exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm) group before and at 1st, 2nd, 3rd, 4th, 5th and 6th days after STZ injection are shown in Table 6 and Figure 11.

Levels of plasma glucose in rats in the sedentary diabetic group before and on days 1 to 6 were 87.61±4, 340.34±12, 339.11±8, 371.81±17, 407.70±30, 360.75±18 and 359.63±20 mg%, respectively. Plasma glucose level the day before STZ injection was similar in all groups of rats and considered to be not diabetes. Also there were no significant differences observed in the level of plasma glucose among sedentary diabetic, exercise trained diabetic and exercise trained plus vitamin E supplemented diabetic group throughout the six days of measurement.

In the vitamin E supplemented group, the change in plasma glucose from the control (78.2±3) was greatest on day 1. After the first day it appeared to increase at a slower, but continuous rate until the last day measured, day 6. Plasma glucose on days 1-6 after STZ injection was 199.2±19, 240.5±15, 239.9±21, 275.1±27, 274.5±29 and 279.6±30 mg%, respectively.

The sedentary diabetic, exercise trained diabetic and exercise trained plus vitamin E supplemented diabetic groups all showed a similar plasma glucose response after injection with STZ. The first day after injection correlated with the largest increase in plasma glucose, followed by smaller changes over the subsequent days. However, the levels of plasma glucose in these three groups were consistently higher

than the vitamin E supplemented group. This was statistically significant for all groups on all days.

On day 1 and 2 after STZ injection, the plasma glucose of rats in the vitamin E supplemented group was significantly lower than the other three groups, sedentary diabetic, exercise trained diabetic and exercise trained plus vitamin E supplemented diabetic ($p<0.001$). As found in Table 11 the plasma glucose on day 1 was 199.2±19, 340.3±12, 344.6±17, 343.21±14 and on day 2 was 240.5±15, 339.1±8, 352.5±12, 321.2±14 mg%, respectively. On the 3rd day after STZ injection, plasma glucose level of rats in the vitamin E supplemented diabetic group was also significantly less than the sedentary diabetic, exercise trained diabetic and exercise trained plus vitamin E supplemented diabetic rats, with glucose levels of 239.8±21, 371.81±17, 366.3±12, 325.6±14 mg% ($p<0.001$, $p<0.01$, $p<0.05$), respectively. At the 4th day after STZ injection plasma glucose level of rats in vitamin E supplemented diabetic groups was still significantly less then the other three groups ($p<0.001$, $p<0.01$ and $p<0.05$, respectively). The levels were 275.1±27, 407.7±30, .89.6±14, 344.6±15, respectively. A 5th day, the plasma glucose level of rats in vitamin E supplemented diabetic group was 274.5±29 mg% which significantly less than sedentary diabetic group, $p<0.01$ (361.7±18 mg%) exercise trained diabetic group, $p<0.05$ (355.7±20 mg%) and exercise trained plus vitamin E supplemented group, $p<0.05$ (352.1±17 mg%). The 6th day after STZ injection, the plasma glucose level of rats in vitamin E supplemented group was 279.6±30 mg%, significantly less than sedentary diabetic group, exercise trained diabetic group, exercise trained plus vitamin E supplemented diabetic group, $p<0.05$ (359.6±20, 404.4±24 and 351.5±22, respectively)

Table 6 Plasma glucose (mg%) in rats in sedentary diabetic (Se + Dm), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm) and exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm) groups before and at 1st to 6th days after injection of 35 mg/kg BW streptozotocin (STZ).

Group	Plasma glucose level (mg%)							
	Before							
	1st	2nd	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Se + Dm (n=8)	80.6±2	87.61±4	340.34±12	339.11±8	371.81±17	407.70±30	360.75±18	359.63±20
Ex + Dm (n=8)	74.7±4	85.08±5	344.56±17 +++ fff ***	352.54±12 +++ fff ***	366.36±12 + fff ***	389.64±14 + ff ***	355.70±20 + f **	404.38±24 + f *
Vit E + Dm (n=8)	80.3+2	78.16±3	199.25±19	240.50±15	239.88±21	275.13+27	274.5±29	279.63±30
Ex + Vit E + Dm (n=8)	79.6±5	79.01±5	343.25±14	321.25±14	325.63±14	344.63±15	352.13±17	351.50±22

Values are means ± SEM.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; significant differences from sedentary diabetic group on corresponding days.

$^{f}p<0.05$, $^{ff}p<0.01$, $^{fff}p<0.001$; significant differences from exercise trained diabetic group on coresding day.

$^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$; significant differences from exercise trained plus vitamin E supplemented diabetic group on corresponding day.

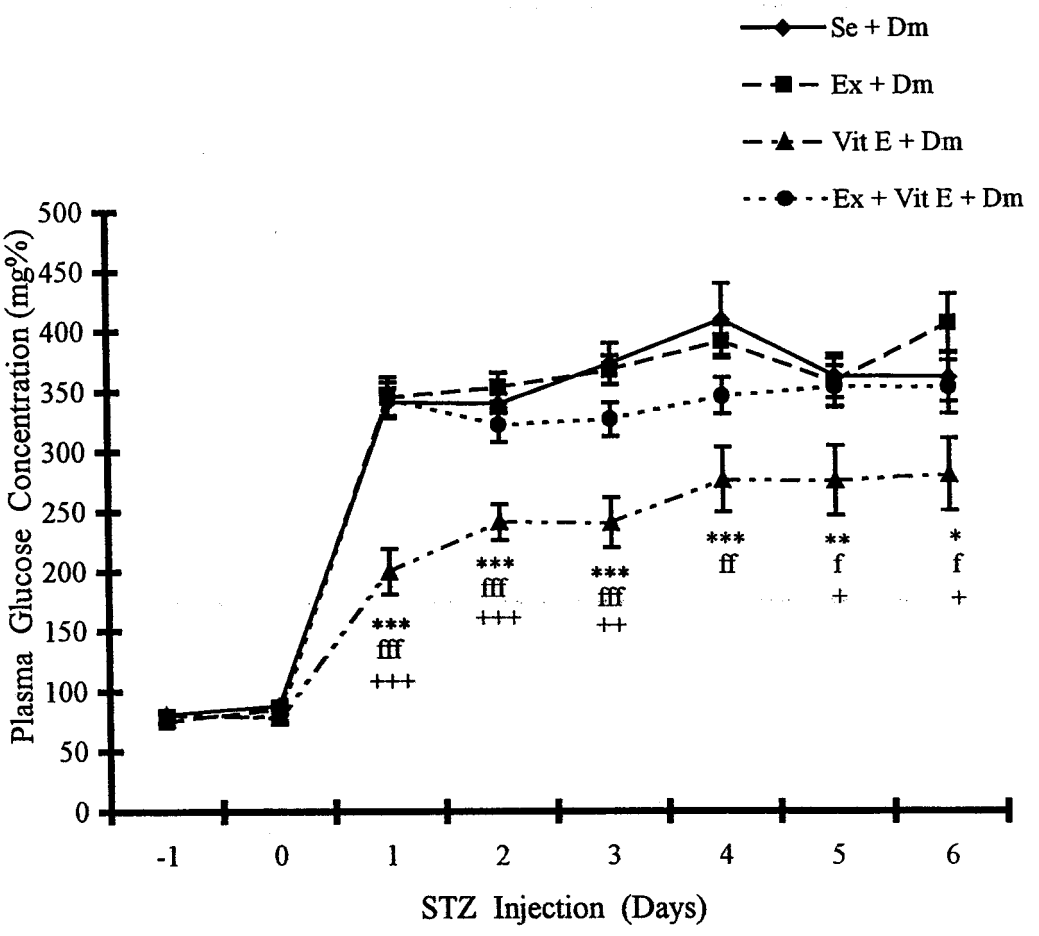


Figure 11 Level of plasma glucose of rats in sedentary diabetic (Se + Dm), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm) and exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm) group before and at 1st to 6th days after injection of 35 mg/kg BW streptozotocin (STZ). Values are means + SEM.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; significant differences from sedentary diabetic group.

$^{f}p<0.05$, $^{ff}p<0.01$, $^{fff}p<0.001$; significant differences from exercise trained diabetic group.

$^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$; significant differences from exercise trained plus vitamin E supplemented diabetic group.

7. Superoxide Dismutase Activity

Table 7 and Figures 12 show superoxide dismutase activity of rats in eight groups, sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E), exercise trained plus vitamin E supplemented (Ex + Vit E), sedentary diabetic (Se + Dm), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm), exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm) groups.

The superoxide dismutase activity of rats in sedentary, exercise trained, vitamin E supplemented and exercise trained plus vitamin E supplemented were 23.1±1, 30.5±1, 33.6±4, 38.1±6 unit/mg protein, respectively. Superoxide dismutase activity of rats in sedentary diabetic, exercise trained diabetic, vitamin E supplemented diabetic and exercise trained plus vitamin E supplemented diabetic group were 26.4±1, 28.9±2, 23.5±2 and 25.0±1 unit/mg protein, respectively (Table 7).

Seen in Figure 12 (upper), the rats in exercise trained, vitamin E supplemented and exercise trained plus vitamin E supplemented groups had superoxide dismutase activity levels that were significantly higher than in the sedentary group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). Superoxide dismutase activity of rats in the exercise trained plus vitamin E supplemented group was also significantly higher than the group that was only exercise trained ($p < 0.05$). There were no significant differences in superoxide dismutase activity in the four groups of diabetic induced rats.

Figure 12 (lower) shows that although there was a small decrease in superoxide dismutase activity in the sedentary diabetic group, compared to the non-diabetic sedentary group, this was not significant. In contrast, in the exercise trained groups, superoxide dismutase activity in the diabetic group was significantly lower ($p < 0.05$). Superoxide dismutase activity of rats in the vitamin E supplemented diabetic group was significantly lower than the vitamin E supplemented group ($p < 0.01$). Also SOD activity in the exercise trained plus vitamin E supplemented diabetic groups was also significantly lower than exercise trained plus vitamin E supplemented group ($p < 0.001$).

Table 7 Superoxide dismutase activity (units/mg protein) of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without STZ - induced diabetes.

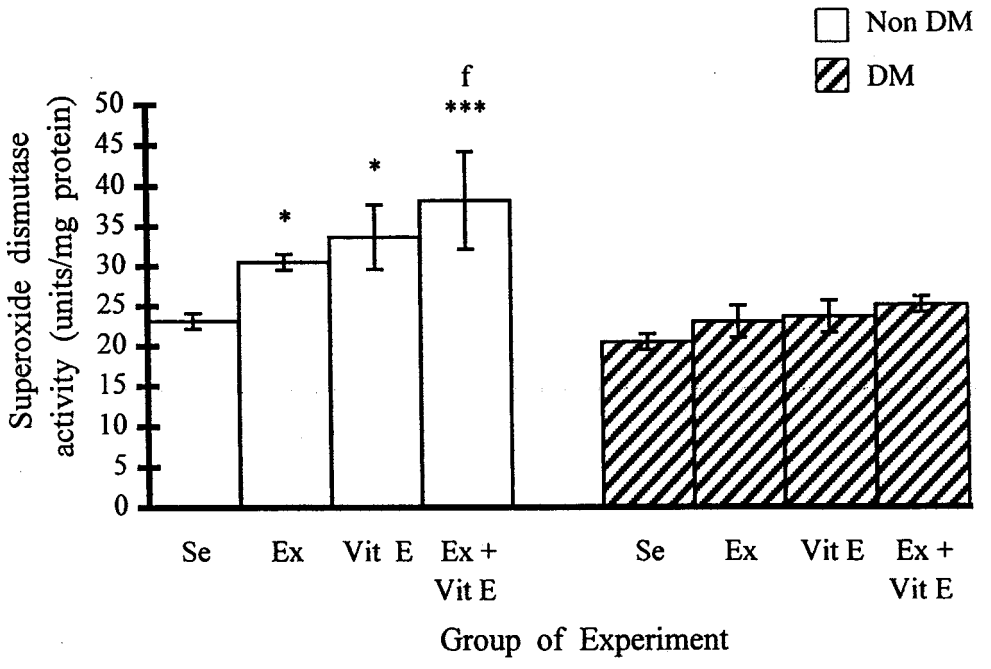
	Superoxide dismutase activity (units/mg protein)	
Experimental Treatment	Non DM	DM
Sedentary	23.1±1 (n=8)	20.4±1 (n=8)
Exercise training	$30.5\pm1^{*}$ (n=7)	$22.9\pm2^{+}$ (n=8)
Vitamin E supplementation	$33.6\pm4^{**}$ (n=8)	$23.5\pm2^{++}$ (n=8)
Exercise training plus vitamin E supplementation	$38.1\pm6^{f***}$ (n=6)	$25.0\pm1^{+++}$ (n=8)

Values are means ± SEM.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; significant differences from sedentary group.

$^{f}p<0.05$; significant differences from exercise trained group.

$^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$; significant differences from corresponding non-diabetic group.



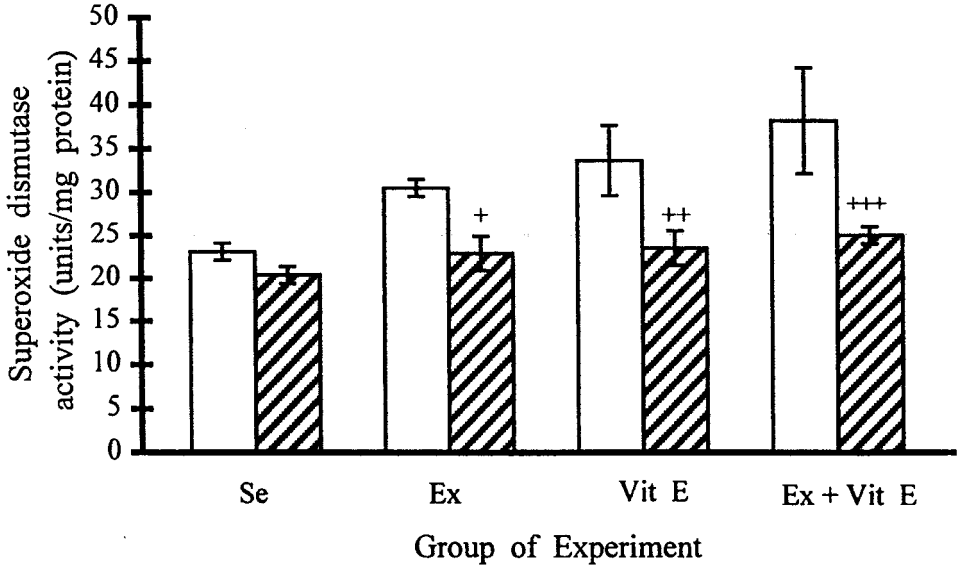


Figure 12 Superoxide dismutase activity of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) group with and without diabetic induction.

Values are means ± SEM.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; significant differences from sedentary group.

$^{f}p<0.05$; significant differences from exercise trained group.

$^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$; significant differences from corresponding, non-diabetic group.

8. Glutathione Peroxidase Activity

Table 8, Figure 13 show levels of glutathione peroxidase activity in eight groups of rats, sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E), exercise trained plus vitamin E supplemented (Ex + Vit E), sedentary diabetic (Se + DM), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm) and exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm) groups.

The levels of glutathione peroxidase enzyme activity of rats in sedentary, exercise trained, vitamin E supplemented and exercise trained plus vitamin E supplemented groups were 13.8±0.7, 15.2±1.4, 13.1±0.5 and 14.2±1.2 nmol/min/mg protein, respectively. There were no significant differences among these four groups though trends to increase were shown in exercise trained and exercise trained plus vitamin E supplemented groups.

Glutathione peroxidase enzyme activity levels of rats in sedentary diabetic, exercise trained diabetic, vitamin E supplemented diabetic and exercise trained plus vitamin E supplemented diabetic groups were 15.2±0.8, 18.0±0.9, 13.5±0.8 and 16.4±0.5 nmol/min/mg protein, respectively. Exercise trained diabetic group has significantly higher glutathione peroxidase activity than sedentary diabetic ($p<0.05$) group.

When compare between nondiabetic and diabetic groups as shown in Figure 13 (lower), it was found that glutathione peroxidase activity of exercise trained diabetic group was significantly higher than exercise trained group ($p<0.05$). Glutathione peroxidase activity of other three diabetic groups also showed a tendency

to increase when compare to corresponding nondiabetic groups but the difference was not significant.

Table 8 Glutathione peroxidase activity (nmol/min/mg protein) of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without STZ - induced diabetes.

	Glutathione peroxidase activity (nmol/min/mg protein)	
Experimental Treatment	Non DM	DM
Sedentary	13.8±0.7 (n=8)	15.2±0.8 (n=8)
Exercise training	15.2±1.4 (n=7)	$18.0\pm0.9^{*+}$ (n=8)
Vitamin E supplementation	13.1±0.5 (n=8)	13.5±0.8 (n=8)
Exercise training plus vitamin E supplementation	14.2±1.2 (n=6)	16.4±0.5 (n=8)

Values are means ± SEM.

$^{*}p<0.05$; significant differences from sedentary diabetic group.

$^{+}p<0.05$; significant differences from exercise non-diabetic group.

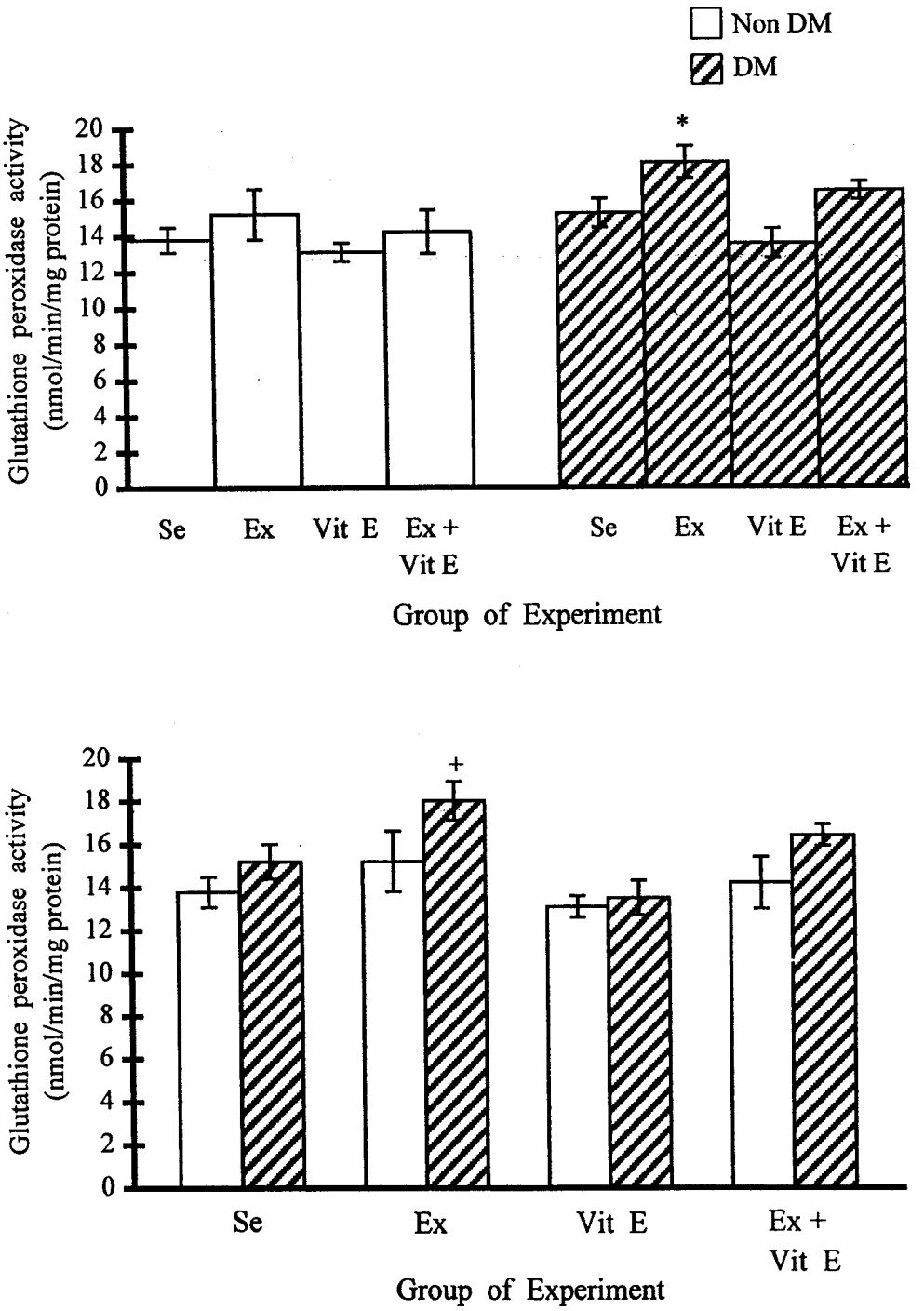


Figure 13 Glutathione peroxidase activity of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without diabetic induction. Values are means ± SEM.

*$p<0.05$; significant differences from sedentary diabetic group.

+$p<0.05$; significant differences from exercise non-diabetic group.

9. Catalase Activity

Values for catalase enzyme activity in eight groups of rats, sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E), exercise trained plus vitamin E supplemented (Ex + Vit E), sedentary diabetic (Se + Dm), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm) and exercise trained plus vitamin E supplemented diabetic (Exs + Vit E + Dm) groups are shown in Table 9 and Figure 14.

Catalase activity levels of rats in sedentary, exercise trained, vitamin E supplemented and exercise trained plus vitamin E supplemented groups were 5.6±0.7, 5.6±0.7, 4.7±0.3 and 4.1±0.3 μmol/min/mg protein, respectively. As illustrated in Figure 14 (upper), there were no significant differences among these four groups of rats. The levels of catalase activity of rats in sedentary diabetic, exercise trained diabetic, vitamin E supplemented diabetic and exercise trained plus vitamin E supplemental diabetic groups were 14.8±1, 15.8±2, 7.0±9 and 9.5±111 μmol/min/mg protein, respectively. Vitamin E supplemented diabetic and exercise trained plus vitamin E supplemented diabetic groups had significantly lower catalase activity than the sedentary diabetic group ($p < 0.001$ and $p < 0.01$, respectively).

As shown in Figure 14 (lower), catalase enzyme activity of rats in the sedentary diabetic group was significantly higher when compared with the sedentary group ($p < 0.001$) and catalase enzyme activity of rats in exercise trained diabetic and exercise trained plus vitamin E supplemented diabetic group were also significantly higher than exercise trained and exercise trained plus vitamin E supplemented group, respectively ($p < 0.001$ and $p < 0.01$). When compared with the vitamin E

supplemented group, the tendency of catalase activity to be increased in vitamin E supplemented diabetic group, on the other hand, was not statistically significant.

Table 9 Catalase activity (μmol/min/mg protein) of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without STZ - induced diabetes.

	Catalase activity (μmol/min/mg protein)	
Experimental Treatment	Non DM	DM
Sedentary	5.6 ±0.7 (n=8)	14.8±1*** (n=8)
Exercise training	5.6 ±0.7 (n=7)	15.8±2*** (n=8)
Vitamin E supplementation	4.7 ±0.3 (n=8)	7.0 ±0.9^{+++} (n=8)
Exercise training plus vitamin E supplementation	4.1 ±0.3 (n=6)	9.5±1.1^{**++} (n=8)

Values are means ± SEM.

$^{**}p<0.01$, $^{***}p<0.001$; significant differences from corresponding non-diabetic group.

$^{++}p<0.01$, $^{+++}p<0.001$; significant differences from sedentary diabetic group.

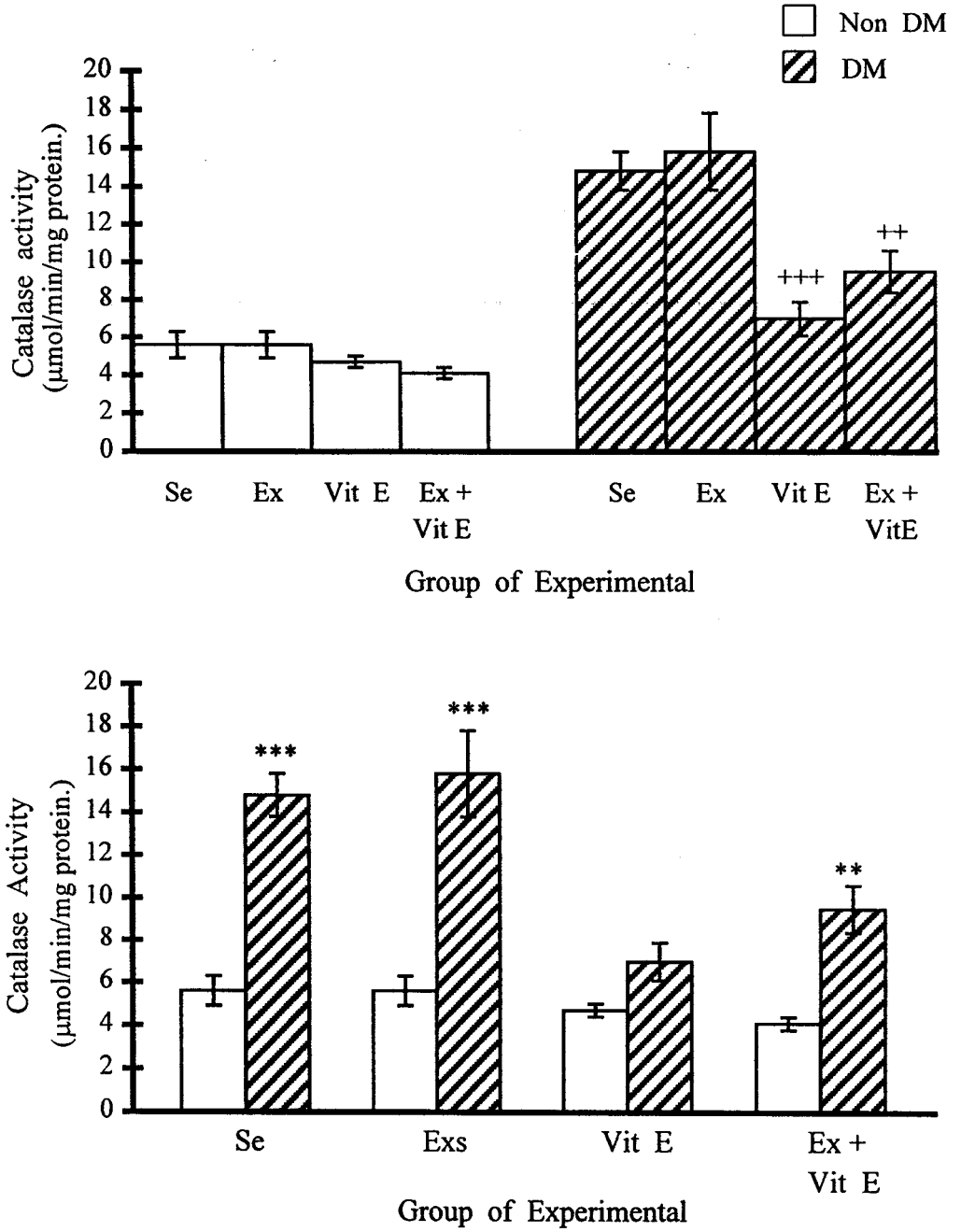


Figure 14 Catalase activity of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) group with and without diabetic induction. Values are means ± SEM.

$^{++}p<0.01$, $^{+++}p<0.001$; significant differences from sedentary diabetic group.

$^{**}p< 0.01$, $^{***}p< 0.001$; significant differences from corresponding non-diabetic group.

10. Glutathione Content

Table 10 and Figure 15 show glutathione levels of rats in eight groups, sedentary (Se), exercise trained (Ex), vitamin E supplement (Vit E), exercise trained plus vitamin E supplemented (Ex + Vit E), sedentary diabetic (Se + Dm), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm), and exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm) group.

The glutathione content of rats in sedentary, exercise trained, vitamin E supplemented and exercise trained plus vitamin E supplemented groups were 359.4±12, 342.9±18, 392.5±12 and 352.5±14 nmol/g tissue, respectively. The vitamin E supplemented group had the highest glutathione content and reached statistical significance when compare with the group with the lowest content, the exercise trained group ($p < 0.05$). Glutathione content of STZ-treated rats, sedentary diabetic, exercise trained diabetic, vitamin E supplemented diabetic and exercise trained plus vitamin E supplemented diabetic groups were 388.12±19, 365.6±10, 398.7±18 and 389.8±17 nmol/g tissue, respectively. Although there was a tendency for glutathione content to be increased in all diabetic groups, as compared to non-diabetic groups, there was no significant difference comparing.

Table 10 Glutathione content (nmol/g tissue) of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without STZ - induced diabetes.

	Glutathione content (nmol/g tissue)	
Experimental Treatment	Non DM	DM
Sedentary	359.4±12 (n=8)	388.1±19 (n=8)
Exercise training	342.9±18 (n=7)	365.6±10 (n=8)
Vitamin E supplementation	392.5±12* (n=8)	398.7±18 (n=8)
Exercise training plus vitamin E supplementation	352.5±14 (n=6)	389.8±17 (n=8)

Values are means ± SEM.

*$p<0.05$; significant differences from exercise trained group.

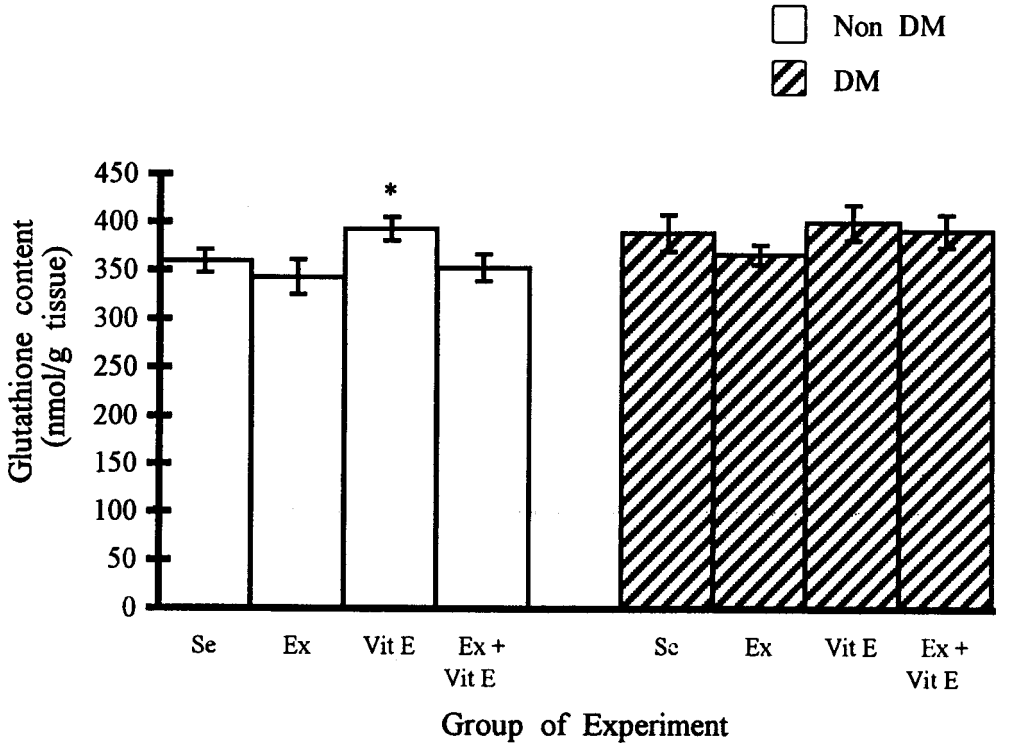


Figure 15 Glutathione content of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) group with and without diabetic induction. Values are means ± SEM.

*$p<0.05$; significant differences from exercise trained group.

11. Malondialdehyde

Table 11 and Figure 16 show levels of malondialdehyde in eight groups of rats, sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E), exercise trained plus vitamin E supplemented (Ex + Vit E), sedentary diabetic (Se + Dm), exercise trained diabetic (Ex + Dm), vitamin E supplemented diabetic (Vit E + Dm), exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm) groups.

As shown in Table 11, malondialdehyde level of rats in sedentary, exercise trained, vitamin E supplemented, exercise trained plus vitamin E supplemented group were 118.5±6. 122.7±3. 108.9±7 and 105.8±5 nmol/100 mg protein, respectively. Although there appeared to be a decreased in malondialdehyde level in the vitamin E supplemented and exercise trained plus vitamin E supplemented groups (Figure 16). Malondialdehyde level of rats in sedentary diabetic, exercise trained diabetic, vitamin E supplemented diabetic and exercise trained plus vitamin E supplemented diabetic group were 142.5±1, 144.1±3, 134.6±5 and 142.3±5 nmol/100 mg protein, respectively. Rats in the vitamin E supplemented diabetic group had the lowest malondialdehyde level among these four groups and was significantly lower statistically, than the sedentary diabetic group ($p < 0.05$).

As seen in Fig 16 (lower), all four groups of diabetic rats had significantly higher malondialdehyde levels than non-diabetic rats. Sedentary diabetic, exercise trained diabetic and vitamin E supplemented diabetic groups were significantly higher in malondialdehyde level than their corresponding non-diabetic groups ($p < 0.01$). This effect was more pronounced when comparing the malondialdehyde level of rats

in the exercise trained plus vitamin E supplemented diabetic groups and non-diabetic group ($p<0.001$).

Table 11 Level of malondialdehyde (nmol/100 mg protein) of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without STZ - induced diabetes.

	Level of malondialdehyde (nmol/100 mg protein)	
Experimental Treatment	Non DM	DM
Sedentary	118.8±6 (n=8)	143.8±1** (n=8)
Exercise training	122.7±3 (n=7)	144.1±3** (n=8)
Vitamin E supplementation	108.9±7 (n=8)	134.6±5** (n=8)
Exercise training plus vitamin E supplementation	105.8±5 (n=6)	142.3±3*** (n=8)

Values are means ± SEM.

** $p<0.01$, *** $p<0.001$; significant differences from corresponding non-diabetic group.

+ $p<0.05$; significant differences from sedentary diabetic group.

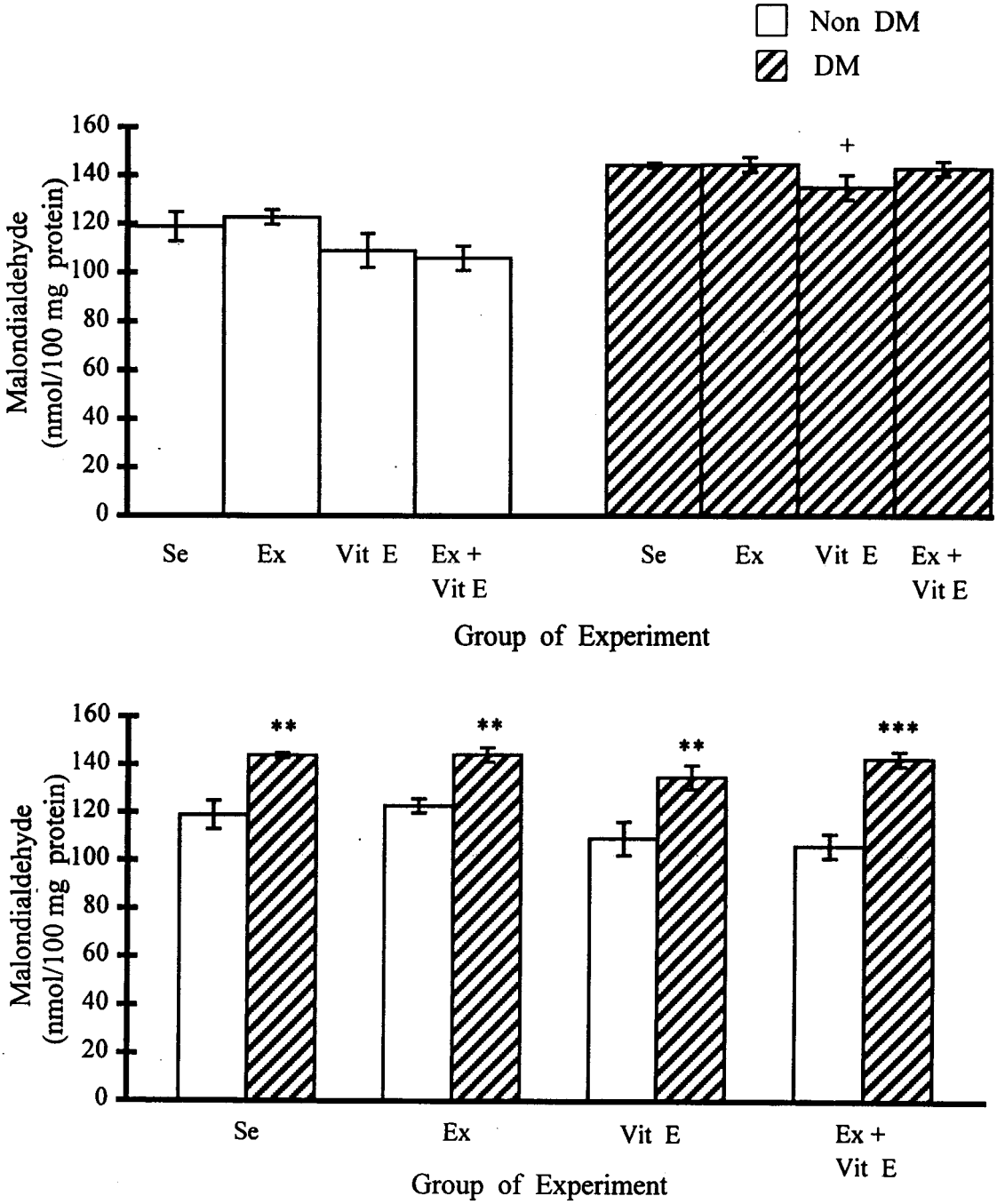


Figure 16 Level of malondialdehyde of rats in sedentary (Se), exercise trained (Ex), vitamin E supplemented (Vit E) and exercise trained plus vitamin E supplemented (Ex + Vit E) groups with and without diabetic induction. Values are means ± SEM.
$^{+}p<0.05$; significant differences from sedentary diabetic group.
$^{**}p<0.01$, $^{***}p<0.001$; significant differences from corresponding non - diabetic group.

12. Histology

Figure 17 Pancreas of the sedentary control rat.

The picture shows both exocrine and endocrine components of the pancreas. Islets appear as clusters of cells embedded within the exocrine pancreatic tissue. The endocrine islet cells are round/polygonal, with lightly stained granular cytoplasm. In contrast, the exocrine or acinar cells surrounding the pancreatic islet are large, triangular in shape and strongly stained.

Within the islet, the cells are evenly distributed and appear to be normal and healthy. Surrounding the cell cords, some microvascular branches are present either with or without visible red blood cells. White blood cells are absent. The larger alpha and delta cells are heavily stained at the periphery, while the smaller beta cells, which are the major component of the islets, are in the central region.

Figure 18 Pancreas of the sedentary diabetic rat.

Although the exocrine acinar cells of the pancreas look normal, the reticular fibers bordering the islets and the acinar cells are disrupted. The size of the islet looks smaller than that in the sedentary control rat. Islet cells are disorganized and unhealthy. The beta cells are abnormal and decreased in number. There are spaces among the cell cords. The cells contain irregular nuclei sizes and there is evidence of nuclear pyknosis. Generalized cellular vacuolization and cytoplasmic swelling are also seen. Some microvascular branches can still be observed, some containing white blood cells.

Figure 19 Pancreas of the vitamin E supplemented diabetic rat.

The reticular capsules of the islets are more clearly defined when compare to those of the sedentary diabetic rat. The beta cells are more numerous and evenly distributed. Less pyknotic nuclei are observed.

Figure 20 Pancreas of the exercise trained plus vitamin E supplemented diabetic rat.

The reticular capsule of the islets are well defined. The overall features of the islet cells appear to be a little worse than the vitamin E supplemented group but still much better than the sedentary diabetic rat. Some cellular vacuolization and pyknotic nuclei are present and some white blood cells are seen.

Figure 21 Pancreas of the exercise trained diabetic rats.

The reticular fibers bordering the islets and the acinar cells are disrupted. Size of the islet looks smaller than that in sedentary control rat, spaces among the cell cords can be seen. There are evidence of nuclear pyknosis and cytoplasmic swelling. The overall features of islet cell appear to be same as the sedentary diabetic rat.

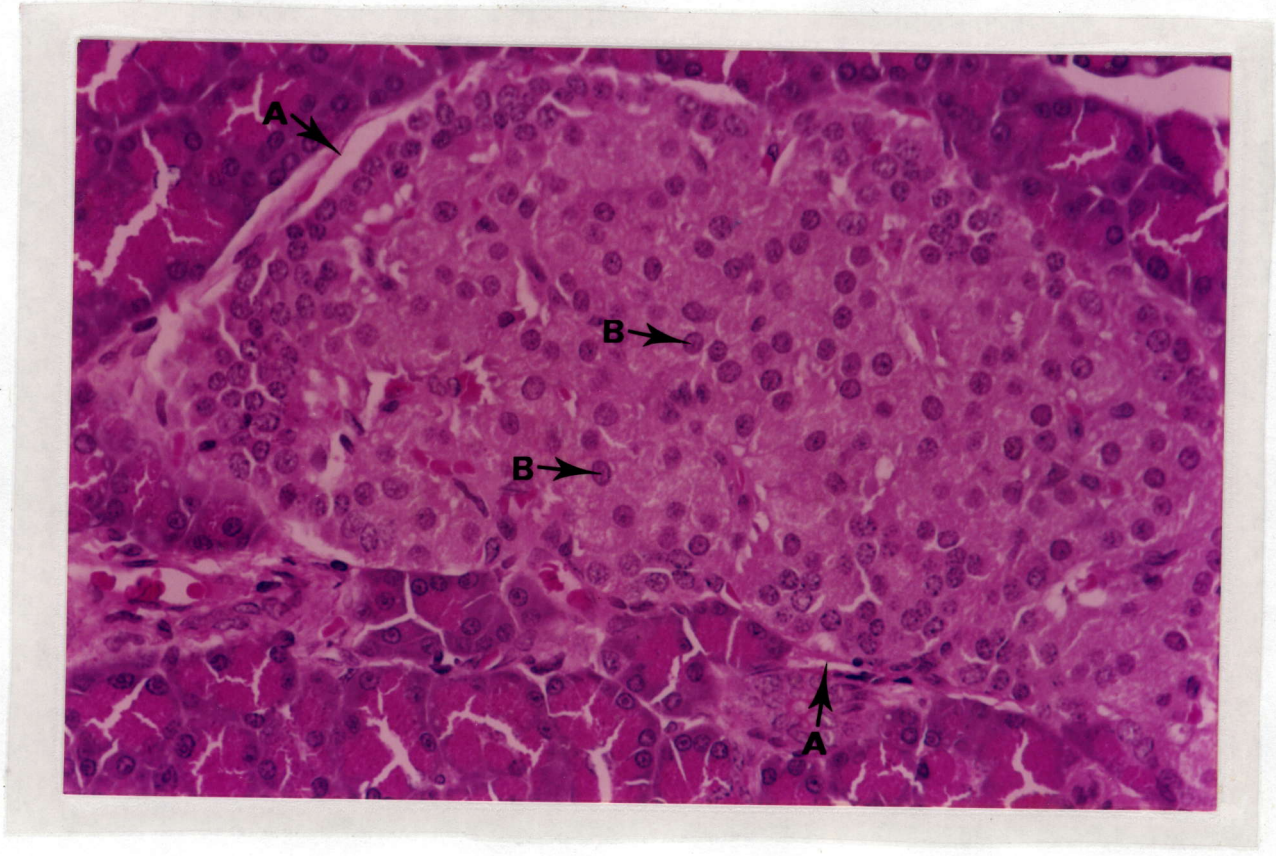


Figure 17. Pancreas of the sedentary **control rat** (Hematoxylin & Eosin, X2260).

A: pancreatic islet in reticular fiber capsules

B: beta cells (cells in the central part of islets)

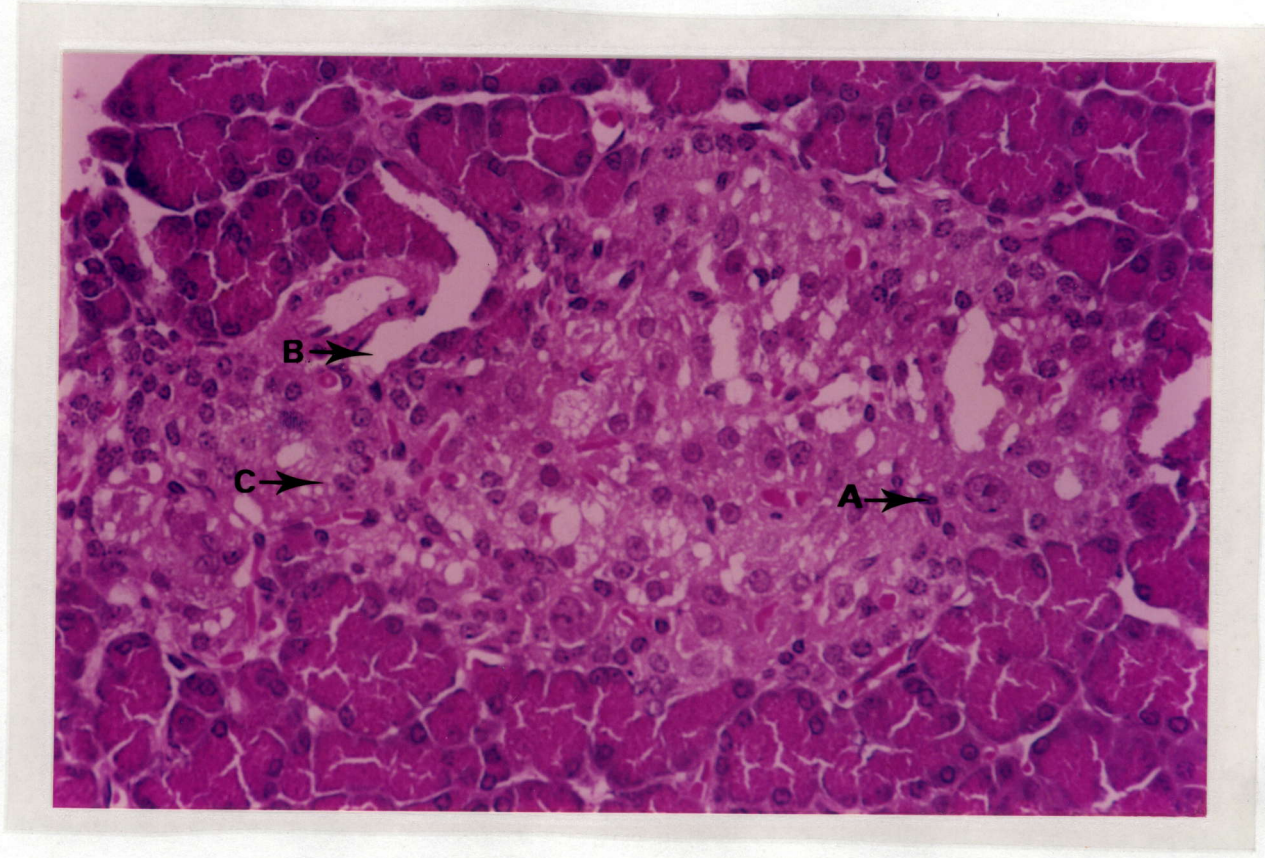


Figure 18. Pancreas of the sedentary **diabetic rat** (Hematoxylin & Eosin, X2260).

A: nuclear pyknosis

B: space between shrunken cells

C: cytoplasmic swelling & vacuolization

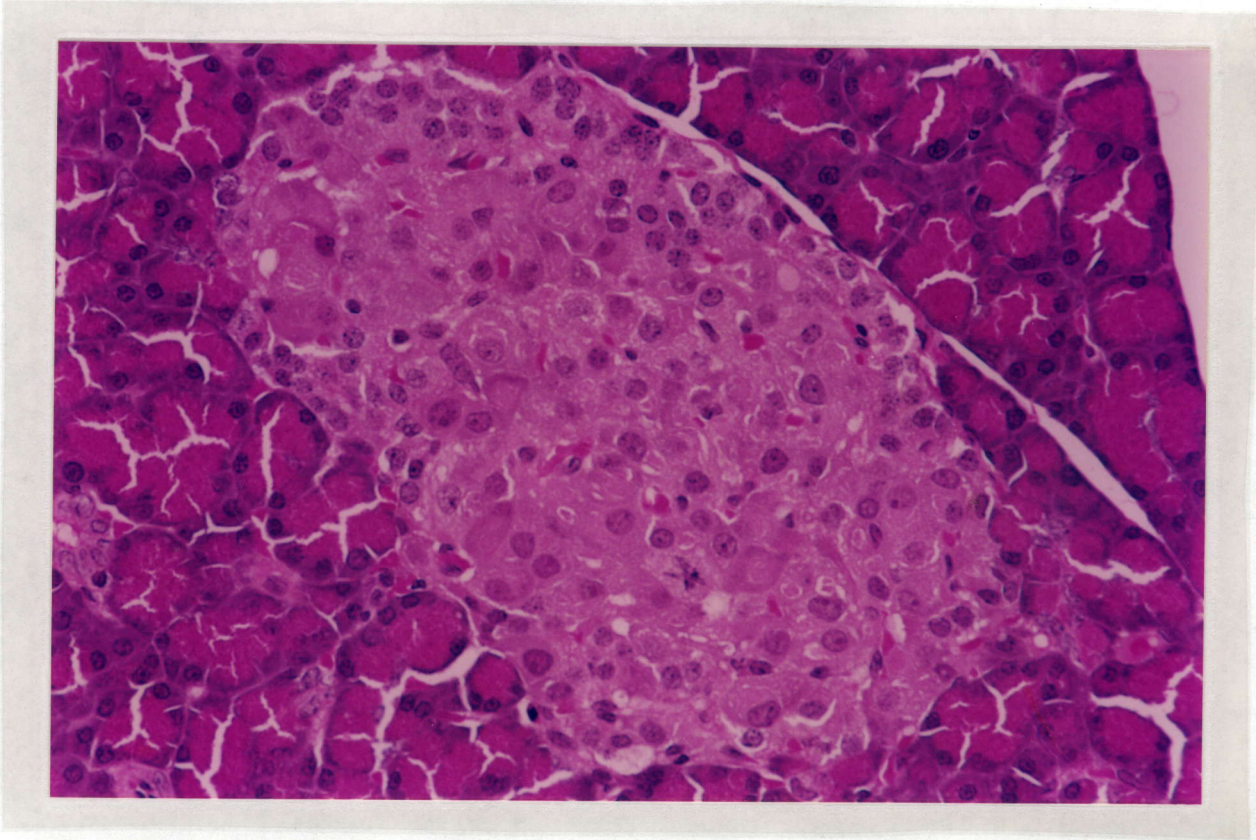


Figure 19. Pancreas of the **vitamin E supplemented diabetic** rat (Hematoxylin & Eosin, X2260).

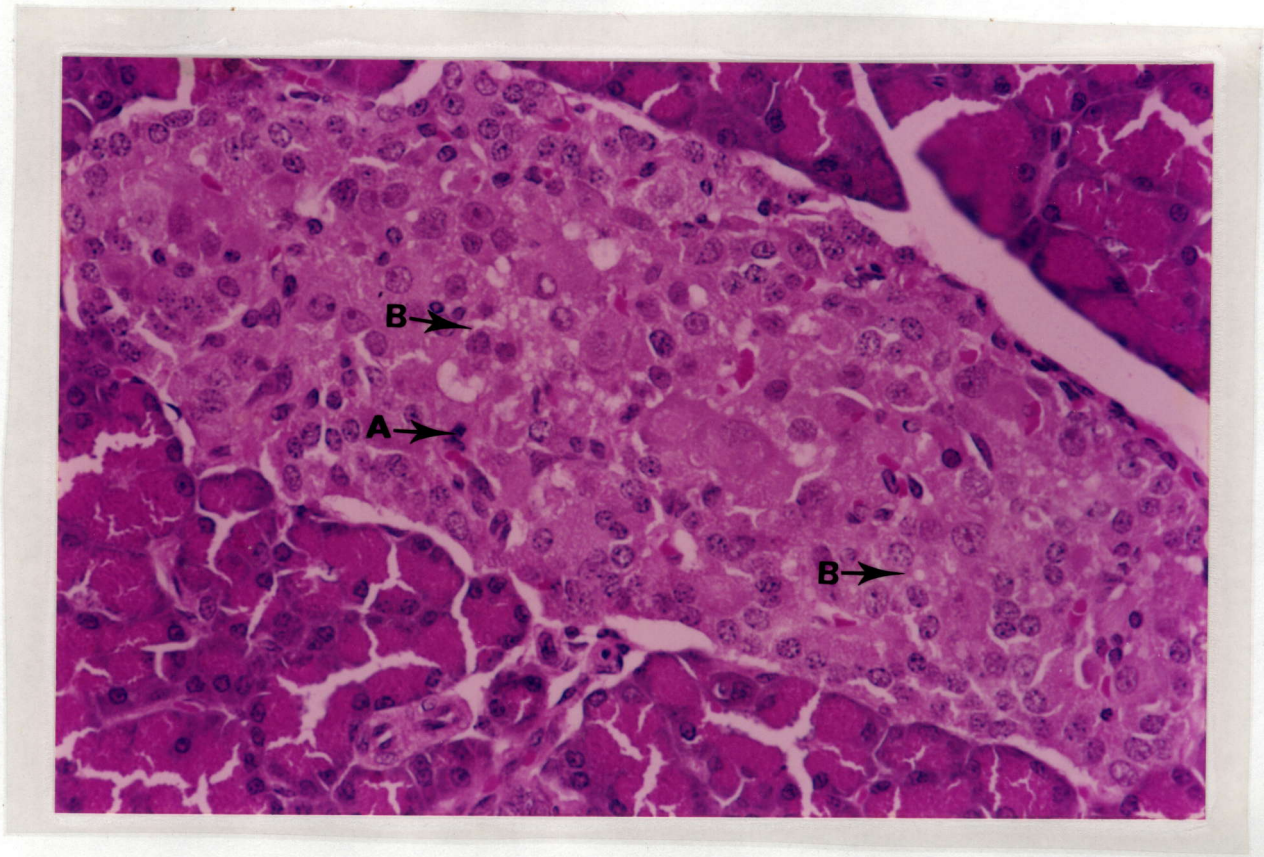


Figure 20. Pancreas of the exercise trained plus **vitamin E** supplemented **diabetic rat** (Hematoxylin & Eosin, X2260).

A: nuclei pyknosis

B: vacuolization

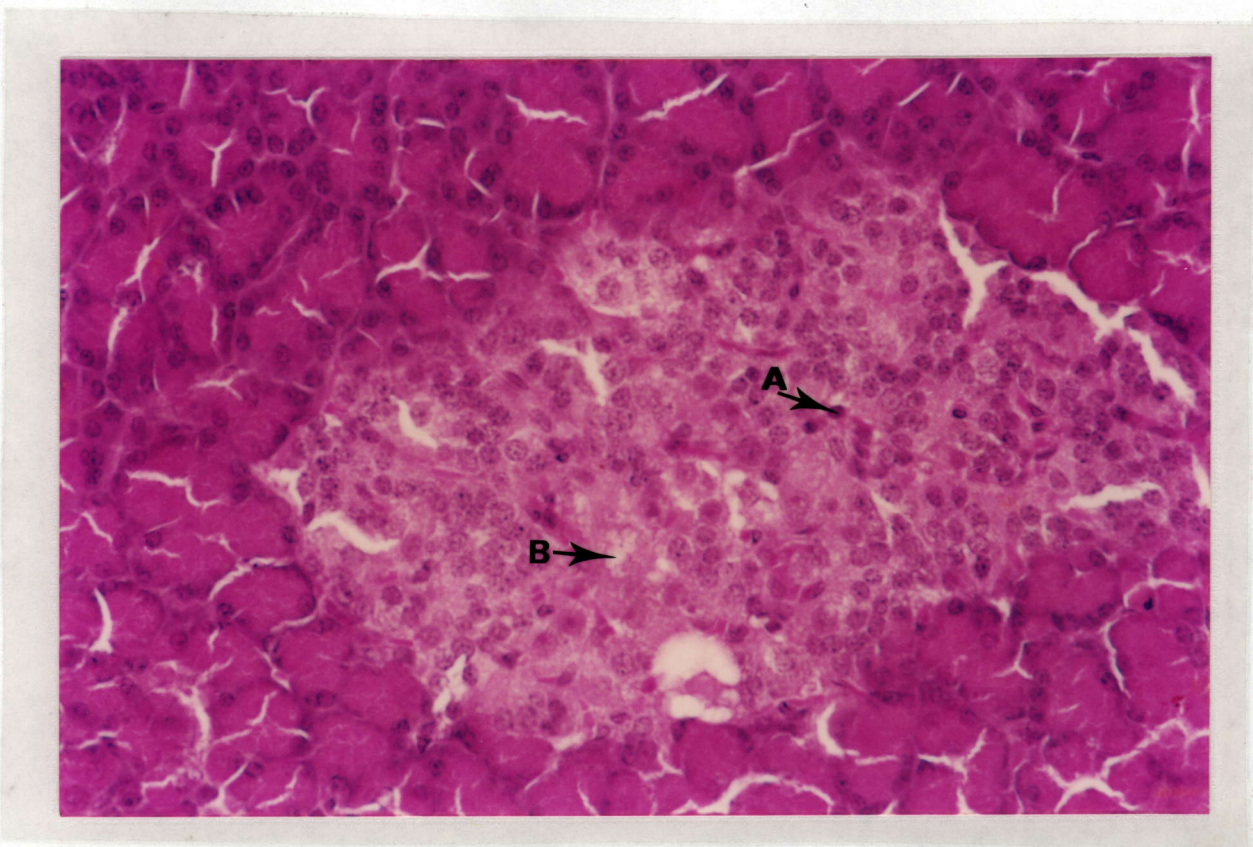


Figure 21. Pancreas of the exercise trained diabetic rat (Hematoxylin & Eosin, X2260).

A: nuclear pyknosis

B: cytoplasmic swelling

CHAPTER V

DISCUSSION

1. Diabetogenic Response to Streptozotocin (STZ)

Since the original discovery that STZ had highly specific cytotoxic action on the beta cells of the Islets of Langerhans (159), this drug has been widely used to induce experimental diabetes mellitus in rats. It is effective in different species-specific doses, ranging from 25 to 200 mg/kg, in rats, dogs, mice, hamsters, monkeys, miniature pigs, pigs and rabbits. However, some evidence suggested that there is a relationship between the dose of STZ injected and the severity of the diabetic state induced (160, 161). In the present study, the relationship between the three doses (25, 35 and 45 mg/kg) of intravenously administered STZ and the diabetogenic response was also investigated in Sprague Dawley rats by measuring the level of blood glucose at 24 hr, 2, 3, 4, 5 and 6 days after administration of STZ. Before STZ was administered, rats in all 3 groups were in the same ranges of age and body weight and were subjected to the same environmental conditions at the Animal Center, Faculty of Science, Mahidol University. After STZ administration, by subjective observation, the groups of rats given a dose of 35 or 45 mg/kg produced a larger amount of urine than those given the dose of 25 mg/kg, which appeared as healthy as before drug treatment. According to the level of blood glucose, we found that the severity of the diabetic state was clearly related to the dose of STZ administered (Figure 6 and Table 1) which is in agreement with several earlier studies (160, 161). In this experiment,

the rats in the group which received 25 mg/kg had the lowest blood glucose level, which remained at about 100 mg% throughout the six days of experiment. This blood glucose level is not considered to indicate diabetes in rats. A similar finding was observated by Junod, et al. (160) in male white Wistar rats; the plasma glucose and weight curve of the animals treated with STZ at the dose of 25 mg/kg, was similar to that of untreated controls. The result of several investigations using the low dose range indeed suggested that the 25 mg/kg dose produced a metabolic state quite similar to that encountered in the so-called "chemical" phase of early diabetes in man. This state of diabetes needs a more sensitive indicator than blood glucose. The slope of the regression line defining the relation between plasma immunoreactive insulin (IRI) and plasma glucose during the first 30 minutes after glucose administration was reported to be an effective means to detect such as early diabetic state (160).

In the present study, we found that hyperglycaemia occurs 24 hr after 35 and 45 mg/kg STZ administration and persists throughout the subsequent days of the experiment. This result was supported by other studies which indicated that after intravenous administration of STZ, the triphasic phenomenon occurs, consisting of an early hyperglycaemic phase, followed by a hypoglycaemic phase and then a permanent hyperglycaemic phase occurring at approximately 4, 7 and 24 hr, respectively (160, 162). The cause of early hyperglycaemia at 4 hr after the STZ administration is due to decreased insulin secretion from betacells, since STZ generates oxygen free radicals which causes some changes in the islet microcirculation. Progressive leakage of plasma constituents from islet microvessels, and islet edema occur. The edema leads to a deterioration of oxygen diffusion capacity resulting in hypoxia (163). Switching

of betacells to a reduced metabolic state would decrease the energy-consuming process of insulin secretion (164). Kai, et al. (162) suggested that hepatic gluconeogenesis is likely to be an important process involved in the production of blood glucose during the early hyperglycaemia induced by STZ, since 48 hr fasted rats showed a lower blood glucose in the early hyperglycaemic phase than nonfasted control rats. Further, CCl_4, which is an inhibitor of hepatic gluconeogenesis showed a dose dependent effect on attenuation of the early hyperglycaemia. The hypoglycaemic phase at 7 hr after STZ injection is due to the releases of stored intracellular insulin from necrotic beta-cells. In the final, permanent hyperglycaemic phase at 24 hr after STZ administration, there is a lack of plasma insulin (163).

In this study, the plasma glucose of rats following the dose of 45 mg/kg was found to be slightly higher than in rats given a dose of 35 mg/kg. The plasma glucose level in rats given 35 mg/kg STZ, in this study were in the range of 279-330 mg%. In animals given 45 mg/kg STZ, plasma glucose was in the range of 353-409 mg% (Table 1). This range was comparable to that reported by Kang, et al (165), who recently reported that the plasma glucose of male Sprague-Dawley rats at 48 hr after 40 mg/kg STZ injection was 335-351 mg%, midway between the values we observed following our 35 mg/kg dose and 45 mg/kg dose.

From these observations, the dose of 35 mg/kg appears to be the minimum intravenous dose which can induce mild diabetes in rats, with effects that persist throughout the experiment, which is in agreement with Ho, et al. (166). Animals given a dose below 35 mg/kg have been reported to tend towards spontaneous recovery (167). So the 35 mg/kg dose was chosen for further use in our experiment.

2. Effect of Long-Term Exercise on Body Weight Gain

Numerous studies have explored the premise that physical activity may have a favorable effect on fat distribution as well as on overall adiposity and weight. There is no doubt that regular exercise reduces body weight in both animals and humans (168-171). In our study, it was also found that both the exercise-trained and exercise-trained plus vitamin E supplemented rats showed significantly lower body weight gain overtime than sedentary and vitamin E supplemented groups (Figure 7, Table 2). This effect was evident from the 4^{th} week of exercise training. These four groups of rats were in the same environment, of the same age and identical in all respects, except for the factor of exercise training. Therefore, the lower body weight gain in both exercise trained groups is a clear indication of the effect of exercise training.

Adipose tissue is an abundant and efficient source of fuel, with a high energy reserve per unit of weight. The utilization of fat for fuel is under coordinated metabolic control and can only occur under aerobic conditions. Approximately 30-50% of the fat that is oxidized during exercise comes from free fatty acids liberated from adipocytes by the hydrolysis of stored triglyceride by hormone-sensitive lipase activity. With regard to reducing total body fat stores, however, the total kilocalorie expenditure during exercise is most important. There is also evidence that the proportion of energy derived from fatty acids steadily increases with increasing duration of a given exercise bout at a fixed work rate.

Several adaptations leading to improved fat utilization during submaximal exercise occur with exercise training. These adaptations include a) increased epinephrine-stimulated hydrolysis from subcutaneous adipose tissue; b) increased

capacity of the trained muscle to oxidizes lipids; c) increased hydrolysis of triglycerides within the trained muscle; d) increased hydrolysis of circulating triglycerides through lipoprotein lipase activity; and e) decreased insulin concentrations, a primary inhibiting factor to lipid mobilization. An increase in the resting metabolic rate has been reported to be another mechanism (172).

Our results also demonstrated that the body weight gain of the rats treated with vitamin E at the dose of 70 IU/kg BW/day was similar to the control rats, indicating that this dose of vitamin E alone did not interfere with normal growth.

3. Effect of Long-Term Exercise Training on Resting Heart Rate

Several adaptations in the cardiovascular system are observable under both resting and exercise conditions following successful exercise training, including a decreased resting heart rate. Our results also showed a significant reduction in heart rate in trained rats after 12 weeks of treadmill running (Table 3, Figure 8). The decrease in resting heart rate or bradycardia in exercise-trained animals could be due to either a decrease in the intrinsic rate of the atrial pacemaker (S-A node), an increase in vagal tone, or both.

The slowing of the S-A node could be caused by:

a) an increased level of acetylcholine (the parasympathetic neurotransmitter) found in atrial tissue following exercise training

b) a decreased sensitivity of cardiac tissue to catecholamines (which are a class of chemicals that includes dopamine, epinephrine, and norepinephrine) or

c) mechanical effect related to a training-induced change in cardiac dimensions (cavity size or wall thickness) (173).

Although the heart is regulated by two components of the autonomic nervous system, the sympathetic, (which leads to an increase in heart rate when stimulated) and the parasympathetic (which leads to a decrease in rate when stimulated), it is the parasympathetic nervous system that predominates at rest. It has been reported to increase after training (174, 175) and is responsible for resting bradycardia.

4. Effect of Exercise Training and Vitamin E Supplementation on Citrate Synthase Enzyme Activity.

Post studies have shown that biochemical adaptation which occurs in skeletal muscle of endurance trained rats favors an increase in the capacity for aerobic metabolism, indicated by rising levels of mitochondrial enzymes such as citrate synthase (176-178). Citrate synthase is the first enzyme in the first reaction of the citric acid cycle or tricarboxylic acid (TCA) cycle; its activity is a marker for aerobic metabolism.

In agreement with the above reports, our data shows that after 12 weeks of treadmill running, there was a 50% increase in citrate synthase activity in both exercise trained and exercise trained plus vitamin E supplemented rats (Table 4 and Figure 9). Goodyear and co-workers (176) reported that treadmill running (1060 min/week) for 8 weeks also increases the level of citrate synthase activity by about 50%. However, Hall and her co-workers (177) found that treadmill running 60 min, 5 days/week for 10 weeks induces various levels of citrate synthase activities in different types of muscle. The increase in citrate synthase enzyme activity is a consequence of increase in size, number and efficiency of mitochondria (124).

No effect of vitamin E supplementation alone was found on skeletal muscle citrate synthase activity in this study. It has also been reported that there was no change in citrate synthase activity in macrophages from mature and aged rats after 6 weeks and 17 months, respectively, of vitamin E supplementation (179). Another study using vitamin E deprivation in the female rat model found that citrate synthase activity in skeletal muscle (red vastus, white vastus, soleus, plantaris, and gastrocnemius) was not impaired (180).

Effect of diabetes on citrate synthase activity.

In the diabetic condition, citrate synthase activity does not appear impaired and is not resistant to the normal effects of training. This finding is supported by the result obtained by Goodyear and co-workers (176), which showed that streptozotocin-induced diabetic condition did not affect citrate synthase enzyme in both sedentary and treadmill trained rats. Hall and co-workers (177) also found a similar result that citrate synthase activity was unaffected by diabetes in the left ventricle and triceps brachii muscles.

Although the exercise program employed in this study in considered to be of relatively low intensity when compared with others which have been reported earlier (177, 181, 182), this program is adequate to induce both physical and chemical adaptations to training indicated by the decrease in resting heart rate, low body weight gain and increase in citrate synthase activity.

5. Effect of Long-Term Exercise Training, Vitamin E Supplementation, and Diabetic Condition on Skeletal Muscle Glucose Uptake

In this study, the skeletal muscle used to study glucose uptake is diaphragm. The choice of using the diaphragm was motivated by the fact that, during exercise, both frequency and depth of breathing increase. This would subject the diaphragm to a training effect, in a parallel manner to other exercising skeletal muscle. Furthermore, the whole piece of diaphragm muscle is thin with high surface area, and easily removed for the preparation of glucose uptake.

The results of present study clearly showed that a 12-week physical training program of treadmill running did not alter the skeletal muscle glucose uptake, in either exercise trained, vitamin E supplemented or diabetic rats (Table 5, Figure 10). The lack of an effect with exercise training in normal nondiabetic rats agrees (176, 183, 184) and disagrees (185-187) with previous reports. There are several factors that may explain the different results obtained from the various studies. The strain and/or sex of the animals studied may have been a factor, since in the present study we used male Sprague-Dawley rats, whereas female Sprague-Dawley rats (176, 183), male Wistar rat (185, 188) and female Wistar rats (184) have been used by other investigators. The types and intensity (speed, % inclination and duration) of the training programs employed were also different among studies. Our animals were subjected to a moderate treadmill training program that produced a 50% increase in muscle citrate synthase activity in both nondiabetic and diabetic animals, which is considered to indicate an effective protocol for inducing physiological adaptation to

training. However, our training was quite different from that of Reaven and Change (189) and Dall'aglio, et al. (190), who trained their animals in wheel cages for 3 weeks. It is possible that this type of spontaneous, low intensity, long-duration exercise is more effective in increasing insulin sensitivity than is the more intense exercise treadmill training that we used. And, unlike the other skeletal muscle, the diaphragm itself may not be subjected to a training effect, especially on glucose uptake. Further study using other muscle types needs to be done.

The other factor which may be the most important is the time elapsed before measurement, following the last bout of exercise. Dall'aglio and co-workers (190) measured insulin-stimulated glucose uptake 6 hr after withdrawal from the exercise wheels. Dela, et al. (191) measured glucose tolerance in humans at 12-18 hr after the last training session. In both of these studies enhancing insulin action was found. In contrast, other studies which measured at a later period after the last bout of exercise (176, 183) did not find any change in insulin sensitivity. Ivy, et al. (184) measured glucose uptake in hindlimb muscle of treadmill running rats at 24 and 46-48 hr after the last training session. They found about a 50% increase in glucose uptake in the trained group at 24 hr, but no change was detectable at 46-48 hr after exercise. It has been suggested that the increase in sensitivity of muscle to insulin is a residual effect of the most recent session, and that training does not result in a long-term adaptation of elevated insulin sensitivity, and thus enhanced muscle glucose uptake.

This study also supported previous finding that skeletal glucose uptake was not affected by STZ treatment (183) or vitamin E supplementation (192).

6. Effect of Vitamin E Supplementation and Long-Term Exercise Training on the Diabetic Condition.

In this study we have demonstrated that vitamin E supplementation significantly improved the diabetic condition when compared with non-supplemented diabetic rats, a result which agrees with several previous reports (193-197).

Free radicals and lipid peroxidation are central to the cytotoxic effect of cytokines on islets in autoimmune diabetes (198), as well as in STZ-induced diabetes (194). Among the free oxygen radical scavengers, vitamin E is known to be a natural, and highly effective chain breaking compound, and can be accumulated in the pancreas. This accumulation is a function of time and the quantity of vitamin administration (196). Hayward, et al. (193) found that a vitamin E supplemented diet (1000 IU/kg diet) decreased the incidence of diabetes in NOD mice. Moreover, Behrens and co-workers also showed that when rats were fed a high vitamin E diet (1.0 g of vitamin/kg diet) for 6 months, there was a decrease in the incidence of diabetes in BB rats. Similarly, Pozzilli, et al. (197), did a one-year prospective study in recent onset diabetic humans using C-peptide levels as an indication of intact insulin production. They demonstrated the preservation and slight increase of C-peptide level at one year with vitamin E treatment (15 mg/kg BW/day) combined with intensive insulin therapy. These effects were similar to nicotinamide treatment, the well-known and more often used compound. Slonim and co-workers (194) indicated that the diabetogenic effect of streptozocin (which is a free radical producing agent) was lessened by vitamin E, administered intraperitoneally 72, 48 and 2 hr before streptozotocin injection. This study also indicated that poor antioxidant capacity will

enhance susceptibility to the diabetogenic action of STZ. A direct method to induce free radical damage, ischemic reperfusion, has been used to prove an actural mechanism by which vitamin E provides protection of the pancreas. Ikeda, et al. (195, 199) reported that the free radical damage during ischemic reperfusion in pancreas can be mitigated by pretreatment with α-tocopherol for 7 days. Protection was indicated by a complete suppression of increase in lipid peroxidation level during reperfusion, and the ability of the pancreas to maintain its endocrine function. The mechanism by which vitamin E may protect against STZ-induced damage includes a direct protective effect the beta cells and reduction of the cytotoxicity mediated by cytokines and their products in the islet infiltrate. However, vitamin E may not be able to interfere with certain steps in the autoimmune process, such as in genetic autoimmune diabetes, shown by its lack of effect on the insulitis process in some studies (193, 200).

Although several previous studies indicated that physical exercise can dramatically attenuate the severity of diabetes in subjects with insulin deficiency by enhancing insulin action (185, 188, 190), exercise training in normal subjects seem to not to improve glucose homeostasis. Physical training in normal subjects shows a beneficial effect by increasing insulin sensitivity. At the same time a diminished basal insulin level also occurs. Tancrede, et al. (185) reported that physical training for 10 wks induced a decrease in the basal insulin level in normal rats. In addition, Galbo, et al. (201) reported a lower rate of insulin secretion from isolated islets of trained rats, incubated at various glucose concentrations.

The present study (Figure 11, Table 6) shows that, at 24 hr after STZ injection, the plasma glucose levels of exercise trained rats, both with and without

vitamin E supplementation, were not significantly different from sedentary diabetic rats. Because at this time the adaptation effect from the last exercise session still existed, although the insulin sensitivity is increased in exercise trained as well as vitamin E supplemented plus exercise trained rats, the decrease in insulin secretion in these groups counteracted this effect. Thus a similar plasma glucose/diabetic condition was seen at this time. After 24 hr to the 6^{th} day after STZ, the plasma glucose level of exercise trained rats is relatively the same as the control group whereas the vitamin E supplemented plus exercise trained rats had a lower plasma glucose than control. The diabetic condition, as indicated by the level of plasma glucose at this time, reflects the level of beta cell destruction by STZ only (without the effect of training). Our data implied that exercise pretraining had no benefical or detrimental effect on pancreatic betacell destruction which is in agreement with Galbo, et al. (201), who showed that 12 weeks of swimming had no effect on the number of beta cells, as indicated by their DNA content, which was similar to that of islets from untrained rats. Nobel and Farrell (202) demonstrated that 11 weeks of treadmill running did not alter the pancreatic insulin concentration in rat islets.

Trend to decrease in glucose level at day 2 – 6 were seen in vitamin E supplemented plus exercise trained rats when compared to controls diabetic, but the plasma glucose level of this group is still significantly higher than rats given vitamin E supplementation alone. This may be because the protective role of vitamin E on beta cells is attenuated by exercise, since exercise itself can increase the production of free radicals in the body, "using up" vitamin E to scavenge those free radicals.

7. Effect of Long-Term Exercise Training, Vitamin E Supplementation, and Diabetic Condition on the Activity of Antioxidant Enzymes.

Effect of exercise training

Exercise is associated with a large increase in oxygen consumption (138) and rate of oxidative phosphorylation, occurring in the muscle performing the work. This also results in an increased rate of free radical formation (139), which is potentially harmful to muscle cells. Therefore, exercise itself might trigger adaptation in antioxidant enzyme systems in these tissues, secondary to oxidative stress during training. Salminen and Vihko (203) reported that susceptibility of skeletal muscle homogenates to lipid peroxidation was decreased in a 3-week treadmill trained mice.

It is believed that the antioxidative defense system is in a dynamic equilibrium, so that a decrease in one of the factors will to a certain extent be compensated through increased capacity of other antioxidants. This implies that evaluating the capacity of the antioxidative defense system requires determination of the whole antioxidant status at the time (204).

In present study, we measured the level of three scavenging enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT), in gastrocnemius muscle of rats. SOD activity (Table 7, Figure 12) in our study is comparable with Criswell, et al. (205), who studied the effect of running training in male Sprague-Dawley rats. As expected, the specific activity of SOD is significantly greater in exercise trained rats after 12 weeks of treadmill running. Our results are in agreement with those of Higuchi, et al. (138), who reported 20-30% greater SOD

activity in the soleus and red vastus lateralis muscle of running trained Wistar rats. Leenwenburgh and co-worker (206) has also measured higher SOD levels in the skeletal muscles of running rats. Moreover, as recently as 1997, Ørtenblad, et al. (204) indicated that SOD activity was markedly elevated in trained humans. However, Alessio and Goldfarb (135) demonstrated that SOD activity was unaffected by either acute or chronic exercise. Laughlin, et al. (201) also found no effect of exercise training on SOD activity of exercise trained rats. It might be suggested that these different results are due to the differences in experimental conditions, such as type, intensity, and duration of training and differences in exercise protocol.

The effects of training on CAT activity in skeletal muscle also produced conflicting results. Our data indicate that exercise training does not change CAT activity in skeletal muscle (Table 9, Figure 14). These results are in agreement with those of Leeuwenburgh, et al. (206), who found unchanged CAT activity in deep vastus lateralis muscle in response to training. Ørtenblad, et al (204) and Higuchi, et al. (138) also reported that training did not produce any change in skeletal muscle CAT activity. Whereas Alessio and Goldfarb (135), Laughlin, et al. (207) found that CAT activity was lower in the muscle of trained rats. Toskulkao, et al. (208) indicated that endurance exercise training increased the activity of CAT in heart and gastroenemius muscles. Thus available data indicate that effects of training on CAT activity in skeletal muscle are not uniform which might be due to the differences among muscle type, muscle composition, and strain of animal used.

Observations with regard to GPX are more consistent, showing that endurance training elevates the activity of GPX in skeletal muscle.

In the present study, the level of GPX activity in control rat is comparable to those of Laughlin, et al. (207) and Toskulkao (208), who measured the level of GPX in gastrocnemius muscle of male Sprague-Dawley and Wistar rats, respectively. We found that GPX activity trended to be increased in gastrocnemius muscle of trained rats after 12 weeks of treadmill running (Table 8, Figure 13). Earlier studies in both rodents (136, 139, 206-209) and human (204) reported that GPX activity was increased by training, which is in agreement with our result.

The antioxidant enzyme adaptations to endurance exercise training indicated by a significant increase in SOD activity and trend to increase GPX activity found in this study, indicate that SOD and GPX are important antioxidant enzymes in the response to exercise training. This supports an increasing number of studies reporting elevated levels of these two antioxidant enzyme activities in endurance trained skeletal muscle (204, 206, 209).

Effect of vitamin E supplementation

In the present study, an increase in SOD activity was found while there were no change in GPX and CAT activities after 12 weeks of Vitamin E supplementation. These finding seem to be consistent with study of Lopez-Torres et al. (210), who demonstrated that SOD is the only one scavenging enzyme which increased after topical administration of α-tocopherol for 24 hr while GPX activity remained unchange and CAT activity decrease in dermis of hairless mice. The result of present study suggests that Vitamin E supplementation is capable of enhancing antioxidant capacity in rat skeletal muscle. However, the available evidence for effects of Vitamin E supplementation on antioxidant enzyme activity is not consistant. Li et al.

(211) determined the effect of Vitamin E on *in vitro* antioxidant by incubation human cardiomyocytes with Vitamin E for 14 days. They found increase in only GPX. Durak, et al. (212), who measured the level of antioxidant activity in myocadium of guinea pigs found significant increases in SOD, GPX and CAT enzyme activity after 6 days of Vitamin E supplementation, while Manthan, et al. (213) demonstrated a decrease in blood SOD, GPX and CAT after Vitamin E supplementation.

Effect of diabetic condition

Our results show that after 7 days of diabetic induction, all diabetic groups, except sedentary diabetes, had significantly low SOD activity when compared with those without diabetic induction (Table 7, Figure 12). The sedentary diabetes is the only one group in which SOD tended to decrease, but did not reach statistical significance. The decrease in SOD activity found in this study is in agreement with Matkovics, et al. (60).

SOD enzyme activity has been reported to be inactivated by high concentration of H_2O_2 (214, 215). As the utilization of glucose is lessened in diabetes, the oxidation of fatty acid is a dominantly pathway for the formation of energy. β-oxidation of fatty acids, is a metabolic pathway that operates in both peroxisomes and mitochondria in animal tissues. The first dehydrogenation step of peroxisomal β-oxidation involves the reduction of O_2 to H_2O_2 (216, 217). Thus, endogenous production of H_2O_2 may be increased in diabetes, and these elevated concentrations of H_2O_2 can reduce SOD activity.

At the same time, endogenous production of H_2O_2 in diabetes can activate CAT enzyme activity, an adaptive response, since CAT is the most important means

to deal with high concentration of H_2O_2 (216). This idea is consistent with the observed large increase in CAT activity found in our study with diabetes (Table 9, Figure 14) and previously studies as well (60, 218). Moreover, CAT activity has also been reported to increase in starvation-induced hypoinsulinemia, with associated β-oxidation of fatty acid, resulting in increased production of H_2O_2 (219). In addition to that, we found a corresponding change in this enzyme activity with the severity of diabetes. This suggests that increase in CAT activity may be an important adaptive response to conditions of increased peroxidative stress.

Because of the large compensatory increase in CAT activity which found in the sedentary diabetic and exercise trained diabetic group, the potential to remove H_2O_2 may also be much higher in severe diabetes. Then, the inhibition of SOD activity by H_2O_2 is relatively lower in these groups.

In this study, although the diabetic condition seems to have no effect on GPX activity (no significantly change), there is the tendency to increase occurring in all groups, reaching significance in the exercise trained diabetic group (Table 8, Figure 13).

Since GPX and CAT enzyme co-operate with each other to remove H_2O_2 *in vivo* (216), GPX should be affected by these conditions, but less than CAT. However, in exercise trained diabetic rats with the highest plasma glucose level, the largest compensatory increase in both GPX and CAT activity was observed.

8. Effect of Long-Term Exercise Training, Vitamin E Supplementation, and Diabetic Condition on Glutathione Content.

Effect of exercise training

Glutathione (GSH) is one of the most important nonezymatic antioxidants. It is believed to participate in protecting cells against reactive oxygen species, generated during strenous exercise (209). GSH reduces hydrogen or organic peroxides via a reaction catalyzed by the enzyme glutathione peroxidase (GPX), and is capable of non-enzymatic radical scavenging (220, 221).

In the present study, skeletal muscle GSH concentration of control rats was comparable to that reported by Toskulkao and co-workers (208), who also measured GSH content in gastrocnemius muscle of rats. The data indicate that training does not alter GSH status in gastrocnemius muscle of rats (Table 10, Figure 15). This finding is consistent with previous authors, who reported no significant training effect on skeletal muscle GSH in rats (208, 222).

GSH homeostasis is regulated by both synthesis and utilization of GSH. GSH is synthesizsed primarily by the liver and transported to extrahepatic tissue via the circulation (209), whereas the utilization of GSH is controlled by the GPX and glutathione reductase (GR) redox cycle. Previous studies have reported that acute prolonged exercise can attenuate GSH level in plasma and extrahepatic tissue (223, 224), although there was no apparent decrease in GSH in our treadmill-trained animals. Therefore, the preservation of GSH level, in the trained state, as shown in the present study, leads us to speculate that exercise training resulted in a greater capacity for glutathione synthesis.

Effect of vitamin E supplementation

Both GSH and Vitamin E have shown to provide a protection against oxidative stress (225). It has also been shown that strenuous exercise can increase lipid peroxidation and enhance GSH (226, 227) and Vit E (228, 229) utilization.

In the present study, as expected, there was no change in GSH content in the exercise trained group, although a tendency to decrease was seen. The exercise plus vitamin E supplemented group had an overall higher GSH content than the exercise trained group, and a level similar to the sedentary control group. In addition, the vitamin E supplemented group tended to have a higher GSH level than the sedentary control, and significantly higher than the exercise trained group. This result suggested that exercise training increases oxidative stress, causing an increase in GSH utilization. Vitamin E supplementation participated in protecting against the depletion of GSH. This speculation is supported by the study of Pascoe, et al. (230), which showed that GSH depletion, induced by incubation of isolated rat hepatocytes cells with calcium-free medium (which contributed to the observed oxidative stress), can be minimized by supplementation with Vitamin E.

Effect of diabetic condition

Since the oxidation of fatty acids is the major pathway for energy formation in diabetes, H_2O_2 as a by-product is produced in quantity in this condition (217). Our data (Table 9, Figure 14) as well as previous investigations (60, 218) found that CAT is the most important enzyme in the detoxification of this H_2O_2, whereas GPX activity was hardly changed (Table 8, Figure 13).

GSH acts as free radical scavenger by being a substrate for GPX enzyme (216). Thus, the lack of GSH depletion is puzzling. Saleh, et al. (218) reported the increase in GSSG reductase in all tissue samples (heart, liver, pancreas and kidney) after 12 weeks of STZ induced diabetes. This increase in GSSG reductase may in part the maintained GSH concentration. Because muscle GSSG reductase activity was not determined in this study, this could not be confirmed.

9. Effect of Long-Term Exercise Training, Vitamin E Supplementation, and Diabetic Condition on Lipid Peroxidation.

Effect of exercise training

During exercise, when oxygen consumption is elevated 10- to 15-fold above resting levels, free radicals are produced to a greater extent compared to normal (231). The increased levels of free radicals within cells have been shown to result in the accumutation of lipid peroxidation byproducts, which may jeopardize cell membrane integrity and can lead to cell death (232). Although the beneficial effect of regular exercise in lowering the lipid peroxidation level, compared to sedentary subjects after acute exercise, have been reported (135-137), it has been observed that the same level of lipid peroxidation occurs in trained and untrained subjects at rest.

In this study (Table 11, Figure 16), the occurrence of lipid peroxidation is detected by measuring its byproduct, malondialdehyde (MDA). The resting level of MDA found in this study was in a range comparable with that found by other investigators using the same TBAR method.

Our data show no difference in MDA level when comparing sedentary and 12 weeks treadmill running rats. The training protocols employed in the present study were of sufficient intensity to produce significant increases in gastrocnemius mitochondrial content, as indicated by the increase in citrate synthase activity (Table 4, Figure 9). Since mitochondria are a major source of free radicals (233), the trained rat seems more prone to greater lipid peroxidation. On the other hand, the effect of training on increased antioxidant capacity also occurs, as indicated by the significant increase in SOD (Table 7, Figure 12) and tendency to increase GPX activity (Table 8, Figure 13). In balance, these high antioxidant properties can cope and maintain the level of lipid peroxidation in trained subjects at levels similar to sedentary subjects. This finding is supported by the study of Venditti and Meo (57) who investigated the effect of 10 weeks swimming on susceptibility to damage induced by exhaustive exercise. They found no difference in the resting MDA level in skeletal muscle of trained and untrained rats. Starnes et al. studied the effect of 6 months of treadmill exercise on lipid peroxidation by determining the level of MDA at basal, 2- and 24-hr after incubation in the presence of iron and ascorbate. They found that the trained animals were no different from their age-matched controls regarding the amount of *in vitro* lipid peroxidation occurring at the basal, 2- and 24-hr periods. Moreover an increase in total peroxidizable lipid was observed in the exercised animals, which can be explained at least partially by the result of increased mitochondrial content.

Effect of vitamin E supplementation

Few studies have examined the effect of Vitamin E supplementation on resting lipid peroxidation in subjects with sufficient Vit E. Brady, et al. (235)

demonstrated that resting skeletal muscle lipid peroxidation, as indicated by TBARS, was not altered by Vitamin E supplementation. Similarly Goldfarb, et al. (236), found that Vitamin E supplementation did not affect resting TBARS in red fast-twitch muscle. Our finding was in agreement with those earlier studies. The supplementation with Vitamin E was unrelated to resting skeletal muscle lipid peroxidation level, as indicated by TBARS or MDA content. However, Sumida, et al. (237) reported a lower blood MDA level in Vitamin E supplemented humans at rest. Moreover, skeletal muscle MDA was reported to be significantly lower, when compared with controls in the study of Sen, et al. (238).

The conflicting results may be due to the fiber type used for muscle sampling. In the study of Goldfarb, et al. (236), they measured MDA content in three muscle types, white fast-twitch, red fast-twitch and red slow-twitch. They found significantly lower MDA levels in Vitamin E supplemented rats but only in the red slow-twitch fibers while the other two types showed no change. This finding is consistent with our results. Our study used the left gastrocnemius muscle as the location to measure MDA, since the gastrocenmius is known to be a mix of white and red fast-twitch fibers. In addition to fiber type, the level of Vitamin E supplementation may be another factor. As demonstrated in the study of Sen, et al. (238), who also measured MDA content in red gastrocnemius but used higher levels of Vitamin E supplementation (750 IU/kg body wt), they found significantly lower resting MDA, although using a shorter period of supplementation (8 weeks).

Effect of the diabetic condition

In the present study, the increase in MDA level was found to parallel the degree of hyperglycemia. This finding is consistent with other reported in the literature (235). In diabetes, insulin deficiency leads to a drastic drop in glucose utilization. As a consequence, β-oxidation of fatty acids is promoted, resulting in H_2O_2 formation (217). H_2O_2 itself can diffuse readily between cells and convert to the most reactive oxygen species, hydroxyl radical, by the meta-catalytic process (240). This reactive oxygen species is capable of causing oxidative damage to tissues, especially to membrane lipids, commonly referred to as the lipid peroxidation process. Then the increased product of lipid peroxidation can be observed. However, as recently as 1994, Habib, et al. (241) ound that exhaled ethane, a hydrocarbon by-product of lipid peroxidation, was increased in both STZ induced diabetic and intra peritoneal dextrose administration rats. This study suggested that hyperglycemia alone (as in the dextrose load condition) and not just insulin deficiency was associated with increased lipid peroxidation. Further, vitamin C uptake by the tissues can be competitively inhibited by increased plasma glucose concentration and diabetic animals are known to have lowered stores of vitamin C (239, 241). Thus, alteratons in micronutrient status may be important in any possible mechanistic explanation for the increased lipid peroxidation by-product during diabetes.

10. Histological Study

The morphological characteristic of pancreas from sedentary control (Se), sedentary diabetic (Se + Dm), vitamin E supplemented diabetic (Vit E + Dm),

exercise trained plus vitamin E supplemented diabetic (Ex + Vit E + Dm), and exercise trained diabetic rats were shown in Figures 17-21, respectively.

Pancreas of sedentary control rat (Figure 17) appears to be normal, indicated by healthy-looking islet cells with lightly stained granular cytoplasm and intact reticular fibers bordering the islets. The plasma glucose level of the rat is also normal (≈ 80-100 mg%) and comparable with that of others.

Dramatical morphopathological changes indicating beta cell damage is found in the pancreas of the sedentary diabetic rat. The result is consistent with the persistently high plasma glucose level found in this group (≈ 340-410 mg%). This result also indicated that the dose of STZ used to induced diabetes in this study (35 mg/kg BW) is sufficient to induce beta cell damage.

Vitamin E supplemented diabetic rats show less beta cell damage which indicates protective role of vitamin E against beta cell destruction. This is consistent with the significantly lower plasma glucose concentration in the vitamin E treated-diabetic rat (≈ 220-280 mg%) when compare to non-treated diabetic rat.

No beneficial effect of exercise training is evident in the pancreas of exercise trained diabetic rat, when compared to the sedentary diabetic rat. This could be due to several possibilities: Firstly, exercise training is not effective in boosting the capacity of the pancreatic antioxidant system. Secondly, the intensity and duration of exercise protocol used in this study is not sufficient to enhance the pancreatic antioxidant system to increase its capacity. Thirdly, the positive role of exercise in enhancing the pancreatic antioxidant pool counteracted a negative effect of exercise, by adding oxidative stress to the pancreas.

Moreover, the physiological level of antioxidant enzymes in the pancreas is reported to be notably low. This may result in a reduced effectiveness of exercise in enhancing the pancreatic antioxidant system. Our result, showing that exercise has no beneficial effect on pancreatic beta cell is consistent with the study of Noble and Forrell (202), who found the same degree of beta cell damage in both trained and untrained spontaneous diabetic rat (BB/Wor rat).

When the rats are pretreated with vitamin E and long-term exercise training, the protective effect of vitamin E against beta cell damage is found to be reduced. In exercise trained plus vitamin E supplemented diabetic rat, the degree of beta cell damage is smaller than in the sedentary diabetic rat, but greater than in vitamin E supplemented diabetic rat. This result is supported by the higher plasma glucose concentration in exercise trained plus vitamin E supplemented rats (≈ 320-350 mg%) when compared to sedentary vitamin E supplemented rats (≈ 220-280 mg%).

This result suggested that 12 weeks of vitamin E supplementation is sufficient to cause protective effect on pancreatic beta cells, while exercise training alone had no beneficial or detrimental effect. When pretreatment with vitamin E and exercise training, the protective effect of vitamin E is found to be reduced.

CHAPTER VI

CONCLUSION

In the present study, the effects of exercise training and vitamin E supplementation on onset and severity of diabetes induced by STZ, and scavenging enzymes (SOD, GPX, CAT) activities were studied in rats. Male Sprague-Dawley rats were subjected to treadmill running and/or vitamin E administration for 12 weeks before diabetic induction by using STZ. Plasma glucose concentration, as an indicator of onset and severity of diabetes, was measured for 6 consecutive days after STZ injection. The scavenging enzyme activities were measured in gastrocnemius muscle at before and on day 7 of diabetes.

From the results of this study, the following conclusions can be drawn:

1. The dose of 35 mg/kg BW STZ is suitable for this age of Sprague-Dawley rat, since all animals became diabetic within 24 hr without returning to normal condition throughout the 6 days following STZ administration.

2. The exercise training program employed in this study (1 hr/day, 5 days/week, 12 weeks) is sufficient to cause physiological adaptations indicated by a decrease in resting heart rate and an increase in skeletal citrate synthase activity by the 12^{th} week of experiment.

3. This training program is effective in enhancing the antioxidant system capacity in skeletal muscle, indicated by a significant increase in SOD activity and tendency for GPX activity to increase after 12 weeks of treadmill running.

4. Vitamin E supplementation 70 IU/day for 12 weeks significantly increases SOD activity in skeletal muscle.

5. Diabetic condition induced by STZ causes antioxidant system compensation by significant increase in CAT and tendency to increase GPX activity.

6. 70 IU of daily intragastric vitamin E administration for 12 weeks is enough to provide the protective effect against pancreatic damage by STZ, as indicated by improved plasma glucose concentration and histopathology study.

7. The exercise training program need in this study has no beneficial or detrimental effect on pancreatic beta cells destruction induced by STZ.

8. The exercise training program employed in this study reduced the beneficial effect of vitamin E in protect in against beta cell damage by STZ.

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APPENDIX I

LIPID PEROXIDE CONTENT

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-butanol 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 refrigerator. The level of lipid peroxide is expressed as nmole of MDA (malondialdehyde). Prepare 10^3 nmol/ml of stock TMP solution by pipetting 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 nmole/ml.

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

Mix 0.1 M K_2HPO_4 in 0.1 M KH_2PO_4 to make phosphate buffer pH 7.4 add 1.15 g of KCl to 100 ml 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 (pH 3.5)	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-bath 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 vigorously 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 terms of nmole MDA/ml.

Calibration Curve

1. Prepare a series of tube containing TMP stock standards in water in the following concentrations: 5.0 nmole/0.5 ml, 10.0 nmole/0.5 ml, 15.0 nmole/0.5 ml, 20.0 nmole/0.5 ml, 25.0 nmole/0.5 ml, 30.0 nmole/0.5 ml, 35.0 nmole/0.5 ml, and 40.0 nmole/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 nmole 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 II

SUPEROXIDE DISMUTASE

Reagents

1. 0.1 M EDTA (ethylene diaminetetraacetic acid)

   3.72 g of EDTA is dissolved in 100 ml distilled water.

2. 1.5 mg KCN (potassium cyanide) in 100 ml distilled water

   1.5 mg of KCN is dissolved in 100 ml distilled water.

3. 1.5 mM NBT (nitroblue tetrazolium)

   123 mg of dissolved NBT in 100 ml distilled water.

4. 0.12 mM Riboflavin

   14.5 mg of Riboflavin dissolved in 100 ml distilled water.

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. After washing in ice-cold 0.9% NaCl, muscle tissue is homogenized in 0.1 M Phosphate buffer pH 7.4 at a ratio of 1 g of wet tissue in 9 ml of buffer.

2. The supernatant fraction that obtained after centrifugation of the hemogenate at 10,000 rpm and 4°C for 30 minutes (Refrigerator Kontron Centrifuge-A24-24) is subjected to centrifuge at 40,000 rpm and 4°C for 60 minutes (Beckman

Ultracentrifuge-Roter 60 Ti). The resultant supernatant fraction is then collected for enzyme assay.

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

0.2 ml of 0.1 M EDTA

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

0.1 ml of 1.5 mM NBT

0.05 ml of 0.12 mM riboflavin (added last)

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

4. The tube containing no extract served a control for each run. The tubes were then illuminated with a light box for 12 minutes at room temperature (25°C).

5. Optical density was measured at 560 nm.

Calculation

Results were expressed as units of superoxide dismutase per mg protein of tissue. One unit is defined for a particular system as that the amount of enzyme causing half the maximum inhibition of NBT reduction. The percent inhibition of NBT reduction versus the amount of SOD extract was plotted on a linear scale. The 50% of maximum inhibition thus determined was used in the following equation.

$$E = \frac{1000}{\text{ml of SOD extract x mg protein}}$$

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

Percentage inhibition is calculated using the following formula:

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

Reference

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

APPENDIX III

CATALASE

Reagents

1. 67 mM Phosphate buffer pH 7.0

Dissolve 3.522 g KH_2PO_4 and 7.268 g $Na_2HPO_4.2H_2O$ in distilled water, to a final volume of 1,000 ml.

2. H_2O_2-Phosphate buffer (1.25×10^{-2} M H_2O_2)

Dilute 0.12 ml 35% hydrogen peroxide to 100 ml with phosphate buffer (solution 1). Make freshly before each use. The optical density of this solution should be about 0.5000 ± 0.015 at 240 nm, with a 1 cm light path.

Procedure

1. Prepare the supernatant fraction as described in procedural step 1-3 in Appendix II, superoxide dismutase assay.

2. Into cuvettes, pipette each solution as follows:

Solutions	Blank cuvette (ml)	Experimental cuvette (ml)
Phosphate buffer (solution 1)	2.9	-
H_2O_2-phosphate buffer (solution 1)	-	2.9
Enzyme fraction	0.1	0.1

* The appropriate amount of enzyme fraction should be chosen to produce a linear O.D. curve.

3. Mix this solution with a plastic rod.

4. The decrease of O.D. of the system is measured against that of the blank at 240 nm.

Calculation

$$E = \frac{\Delta \text{ OD/min x T.V. x Dilution factor}}{0.071 \text{ x EV x mg protein}}$$

where:

E = Specific enzyme activity in µmole/mg protein/min

TV = Total reaction volume (ml)

EV = enzyme volume (ml)

0.071 = molar extinction of H_2O_2

Reference

Luck H. Catalase. Method for Enzymatic Analysis, Vol. 3. Edited by Han-Ulrich Bergmeyer. New York and London: Academic Press; 1965: 885-8.

Lew H, Quintanilha A. Effect of endurance training and exercise on tissue autoxidative capacity and acetaminophen detoxification. Eur J Drug Metab Pharmacokinet 1991; 16(1): 59-68.

APPENDIX IV

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.

Then mix ethylenediaminetetraacetic â (EDTA) 37.225 mg in 50 mM tris buffer pH 7.6 1,000 ml and keep in refrigerator.

2. Stock solution

Add 9.603 mg (0.25 mM) glutathione (reduced from), 12.5 mg (0.12 mM) NADPH and glutathione reductase (1 unit of R/ml) in 125 ml tris buffer pH 7.6 (solution 1). 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 of distilled water and mix, this solution should be freshly prepared before use.

Procedure

1. The enzyme fraction is prepared from muscle in same method as superoxide dismutase extract.

2. Add the solution into each tube which is immersed in ice as follows:

Solutions	Blank tubes (ml)	Experimental tubes (ml)
Stock solution	1.55	1.55
Enzyme fraction	0.10	0.10

* The enzyme fraction should be appropriate diluted to give a linear O.D. curves.

3. Mix and incubate in the water bath 37°C for 5 minutes.

4. Then this mixture solution is set zero absorbance at 340 nm.

5. Add 0.05 ml of camene hydroperoxide into the cuvette in sample cell, rapidly mix and measure.

Calculation

The decrease in optical density of the reaction mixture at 340 nm as the conversion of NADPH to NADP. The activity of glutathione peroxidase is calculated from the change in O.D. in 1 minute.

$$E = \frac{\Delta \text{ OD/min x T.V. x Dilution factor}}{6.22 \text{ x EV x mg protein}}$$

where:

E = Specific enzyme activity in μmole/mg protein/min

TV = Total reaction volume (ml)

EV = Enzyme volume (ml)

0.071 = Molar extinction coefficient for NADPA at 340 nm

Reference

Tapple AL. Glutathione Peroxidase and hydroperoxide In: Methods in Enzymology, Vol II. Edited by Sidney F, Lester P. New York: Academic Press; 1978: 506.

APPENDIX V

GLUTATHIONE

Reagents

1. 0.1 M Phosphate buffer

Mix 0.1 M $NaHPO_4$ solution and 0.1 M KH_2PO_4 solution to make phosphate buffer pH 7.0, 7.4, 8.0

2. Stock standard glutathione "Freshly prepared"

10 mg of glutathione (reduced form) is dissolved in 10 ml of 0.1 M phosphate buffer pH 8.0

3. Color reagent "Freshly prepared"

39.6 mg of 5-5-dithiobis (2-nitrobenzoic acid) (DTNB) is dissolved in 10 ml of 0.1 M phosphate buffer pH 7.0.

4. 4% Sulfosalicylic acid

4 g of sulfosalicylic acid is dissolved in 100 ml distilled water.

PROCEDURE

1. After washing in ice-cold 0.9% NaCl, muscle is homogenized in 4 volume of 0.1 M phosphate buffer pH 7.4. Then an equal volume of 4% sulfosalicylic acid is added.

2. The mixture is centrifuged at 4,500 rpm for 30 minute (Refrigerator Kontron Centrifuge-A24-24). The supernatant is subjected to assay for glutathione content.

3. Add solutions into each tube (immersed in ice bath) as follows:

Solutions	Standard tubes (ml)					Unknown tubes (ml)	Blank tube (ml)
	10 µg	15 µg	20 µg	25 µg	30 µg		
0.1 M Phosphate buffer pH 8.0	1.49	1.485	1.48	1.475	1.47	1.0	1.5
Supernatant	-	-	-	-	-	0.5	-
Standard GSH	0.01	0.015	0.02	0.025	0.03	-	-
Distilled water	1.5	1.5	1.5	1.5	1.5	1.5	1.5

4. Mix and add 25 µl DTNB solution into this mixture, mix well and allow the color to develop for 20 minute at room temperature.

5. The optical density is measured at 410 nm.

6. The glutathione level is expressed as µmole/g wet weight.

Calibration Curve

Prepare series of tubes containing GSH in the following concentration: 10 µg/3.0 ml, 15 µg/3.0 ml, 15 µg/3.0 ml, 20 µg/3.0 ml, 25 µg/3.0 ml and 30 µg/3.0 ml. Then plot a calibration curve showing optical density (OD) as a function of µg of GSH.

References

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

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

APPENDIX VI

CITRATE SYNTHASE

Reagents

1. 1 M Tris-HCl buffer pH 8.1

12.1 g of Trismabase is dissolved in 100 ml distilled water and adjusted to pH 8.1

2. 100 mM Tris-HCl buffer pH 8.1

Dilute 1 M Tris-HCl pH 8.1 with distilled water.

3. 1 mM 5,5-dithiobis-(2-nitrobenzoate) (DTNB)

Dissolve 3.9 mg of DTNB (mw 396.4) in 10 ml of 1 M Tris-HCl pH 8.1

4. 0.3 mM Acetyl-coenzyme A (Lithium salt)

Dissolve 24.29 mg of Acetyl-CoA (mw 809.6) in 100 ml distilled water.

5. 0.5 mM Potassium-oxaloacetate (oAA) "Freshly prepared"

Dissolve 0.66 mg of oAA in 10 ml of 0.1 M Tris-HCl pH 8.1

Procedure

1. Muscle is homogenized in 9 volumes of a hypotonic medium containing 10 mM potassium phosphate buffer (pH 7.4) and 1 mM EDTA to rupture the mitochondrial membranes.

2. The homogenate is centrifuged at 1,000 rpm for 10 minutes to remove fibrous tissue, and the supernatant fraction is subjected to analysis of enzyme activity.

3. The following solutions are measured into cuvettes:

Solutions	Blank cuvette (ml)	Experimental cuvette (ml)
1 mM DTNB	0.1	0.1
0.3 mM Acetyl CoA	0.03	0.03
Distilled water	0.75	0.7
Supernatant	0.12	0.12

4. Mix well and set zero absorbance at 412 nm.

5. Add 0.05 ml of oAA to experimental cuvette to start enzymatic reaction at a total volume of 1.0 ml, mix immediately.

6. Follow increasing optical density for 3 min by double beam spectrophotometer.

Calculation

$$E = \frac{\Delta \text{ OD/min x dilution factor x TV}}{1.36 \times 10^4 \text{ x EV x mg protein}}$$

where

E = Enzyme activity in µmole/min/mg protein

TV = Total volume (ml)

1.36×10^4 = extinction coefficient

EV = Enzyme volume (ml)

References

Srere PA. Citrate Synthase In: Methods in Enzymology, vol 13. Edited by Lowenstein JVV. New York: Academic Press; 1969: 3-5.

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

APPENDIX VII

PROTEIN

Reagents

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

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

2. 4% NaK Tartate

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

3. 2% Copper sulfate

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

4. Folin Reagent

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

5. Standard protein solution

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

6. Lowry E

Freshly 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 (ml)						Experimental tubes (µl)
	0	10	200	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 to stand for 30 minutes at room temperature.
3. The optical density is measured at 770 nm.

Calculation

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

Reference

Lowry OH, Rosebrough NJ, Farr AL, Randall J. Protein measurement with Folin phenol reagent. J Biol Chem 1951; 193: 265-75.

APPENDIX VIII

STANDARD PARAFFIN AND STAINED TECHNIQUE

Procedure

1. The organs were fixed in 10% neutral buffered formalin for at least 48 hrs.

2. The fixed tissues were trimmed.

3. The trimmed tissues were dehydrated, cleared and embedded in paraffin as shown in detail by the following steps.

   a. Rinse in 70% ethanol, for 30 min.

   b. Soak in 70% ethanol, for 30 min.

   c. Soak in 80% ethanol, for 30 min.

   d. Soak in 90% ethanol, for 30 min.

   e. Soak in 100% ethanol, for 30 min.

   f. Soak in 70% ethanol and xylene (1:1, V:V), for 30 min.

   g. Xylene for 30 min and 1 hr, respectively.

   h. Immerse in soft, medium hard and hard paraffins, each for 30 min, respectively, at 58°C under vacuum.

   i. The tissues were embedded in paraffin in the plastic holders.

4. The blocks of tissues were cut to provide sections 5 microns in thickness.

5. The sections were mounted on glass slides with permount and dried at room temperature overnight; then they were stained with Hematoxylin & Eosin.

HEMATOXYLIN & EOSIN STAINING

a. Absolute ethanol, 2 min.

b. 95% ethanol, 2 min.

c. 80% ethanol, 2 min.

d. 70% ethanol, 2 min.

e. Distilled water, 2 min.

f. Harris hematoxylin, 8 min.

g. Distilled water, 2 min.

h. 80% ethanol, 2 min.

i. Eosin, 5 min.

j. 95% ethanol, 2 min.

k. Absolute ethanol, 2 min, twice.

l. Xylene, 2 min, twice.

BIOGRAPHY

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