CHAPTER – VI
EXPERIMENT - V

The protective role of selected protein isolates on induced myocardial infarction in mice

6.1. Introduction

The cardiovascular system is susceptible to many chronic diseases such as hypertension and myocardial infarction. Cardiovascular disease is an umbrella term covering disorders of the heart and blood vessels. It includes coronary artery disease, atherosclerosis, and hypertension (high blood pressure).

At any given time, the distribution of blood in the body of a normal human subject is estimated to be approximately 64% in veins, 13% in arteries, 9% in pulmonary vessels, 7% in the heart and 7% in arterioles and capillaries. Although the heart weighs less than 1% of the total body weight, it relentlessly receives nearly 5% of the total blood flow (which may explain why the arteries of the heart can so easily develop problems). Congenital defects and infectious disease can strike anywhere, but by far the most common disease occurs in the arteries: atherosclerosis.

Blockages can occur in veins as well as in arteries, but these tend to be caused by blood clots (thrombi) rather than by atherosclerosis. Clotting of blood in the veins can occur when blood flow is slow or stagnant.

i) Coronary artery disease

In order to supply blood to the whole body, the heart muscle has a requirement for oxygenated blood supply for itself, which is usually obtained from the coronary arteries located on the outside wall of the heart. When one or more of these arteries are narrowed, blood flow is restricted or may be halted altogether. The result is coronary artery disease, the major underlying cause of heart attack.

ii) Atherosclerosis

This is a condition in which fatty deposits build up on the inside of arterial walls. As the arteries narrow, they cannot carry sufficient blood to the tissues and in response to this phenomenon; the heart pumps harder, blood pressure increases, and
many parts of the body are damaged as a result. Everything from poor circulation in the limbs to sudden episodes of heart attack is accounted due to atherosclerosis. Some people are more susceptible to atherosclerosis than others due to familial patterns of the disease. People who are overweight, lead a sedentary lifestyle, and have high cholesterol levels are at greater risk for developing atherosclerosis.

Therefore, the need to address and find ways and means to control the heart related disorders is very urgent. Simple dietary modifications or inclusion of ordinary neutraceuticals are often known to provide profound benefit. Proteins from common foods are, hence, considered in this study as therapeutic molecules in coronary vascular disorders.
6.2. REVIEW OF LITERATURE

6.2.1. Myocardial Infarction

Also known as heart attack occurs when the blood supply to part of the heart is interrupted. This is due to occlusion (blockage) of a coronary artery following rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids and leucocytes in the wall of an artery. As a result it ends up in restriction of blood supply (ischemia) and leads to shortage of blood supply and finally death (infarction) of heart muscle tissue (myocardium). MI is the acute condition of necrosis of the myocardium that occurs as a result of imbalance between coronary blood supply and myocardial demand (De and Boon, 1992). Damage to the myocardial cells arises due to the generation of toxic reactive oxygen species such as super oxide radicals, hydrogen peroxide and hydroxyl radical (Vaage and Valen, 1993). It is well known that CVD are directly or indirectly related to oxidative damage that shares a common mechanism of molecular and cellular damage.

6.2.2. Symptoms

The onset of symptoms in myocardial infarction is usually gradual, over several minutes, and rarely instantaneous. Chest pain is the most common symptom of acute myocardial infarction and is a sensation of tightness, pressure, or squeezing. Pain radiates from the left arm and will radiate to the lower jaw, neck, right arm, back, and it may mimic heartburn (Marcus et al., 2007). Shortness of breath (dyspnea) will occur when the damage to the heart limits the output of the left ventricle, causing left ventricular failure and consequent pulmonary edema.

Other symptoms include diaphoresis (an excessive form of sweating), weakness, light-headedness, nausea, vomiting and palpitations. These symptoms are likely induced by a massive surge of catecholamines from the sympathetic nervous system (Little et al., 1986). This occurs in response to pain and the hemodynamic abnormalities that result from cardiac dysfunction. Loss of consciousness (due to inadequate cerebral perfusion and carcinogenic shock) and even sudden death (frequently due to the development of ventricular fibrillation) occur in myocardial infarction. The most common symptoms of MI in women include dyspnea, weakness,
and fatigue. In women, chest pain may be less predictive of coronary ischemia than in men (Mesweeney et al., 2003).

6.2.3. Indian Scenario

Cardiovascular diseases are major causes of mortality and disease in the Indian subcontinent, causing more than 25% of deaths. It has been predicted that these diseases will increase in India and will be host to more than half the causes of heart disease in the world within next fifteen years (Sangeetha, 2006).

MI is a common presentation of ischemic heart disease. Ischemic heart disease is the leading cause of death in developed countries, but third in developing countries. In India, CVD is the leading cause of death. The deaths due to CVD in India were 32% of all deaths in 2000 and are expected to rise from 1.19 million in 2000 to 2.03 million in 2010 (Ghaffar et al., 2004). Heart attacks are the leading cause of death for both men and women all over the world (WHO, 2004). Important risk factors are previous CVD such as angina, older age (men over 40 and women over 50), tobacco smoking, high blood levels of certain lipids-triglyceride, VLDL, and low HDL, diabetes, high blood pressure, obesity, chronic kidney disease, heart failure, excessive alcohol consumption, abuse of cocaine and chronic high stress levels (Bax et al., 2008).

Coronary heart disease and stroke have increased in both urban and rural areas. Case-control studies indicate that tobacco use, obesity with high waist: hip ratio, high blood pressure, high LDL cholesterol, low HDL cholesterol, abnormal apolipoprotein A-1:B ratio, diabetes, low consumption of fruits and vegetables, sedentary lifestyles and psychosocial stress are important determinants of cardiovascular diseases in India. These risk factors have increased substantially over the past 50 years and to control further escalation it is important to prevent them (Gupta, 2008). Reduction of mortality rate and prevention of myocardial infarction are of utmost importance. There is growing interest in the use of alternative medicine for the long term prevention of heart attack in high risk patients. Medicinal herbs have been used in primary health care over many centuries before the advent of modern medicine.
6.2.4. Soy protein

Consumption of soy protein in the diet is associated with reduction in the risks leading to coronary heart disease. Soy protein diet has low saturated fat and cholesterol content and thus, may be effective in reducing the risk of coronary heart disease by lowering blood cholesterol levels (FDA, 1999; Peter, 2002). This is thought to be at least in part because of its amino acid profile, which differs from those of animal proteins in ways that result in a desirable lowering of circulating LDL cholesterol levels (Costa, 2000).

Ingestion of soy protein resulted in a decrease in total and LDL cholesterol and triglycerides with average values of 9.3%, 12.9% and 10.5% respectively when compared to the control group. The hypocholesterolemic effect appears to be directly correlated with the patient’s cholesterolemia, with minimal or no reductions occurring at a cholesterol level of 6 mmol/L or less, and the most benefit occurring in patients with a cholesterol level that was greater than 7 mmol/L. The method of action by which soy protein is believed to lower cholesterol concentrations is through activation of the LDL receptor pathway (Sirtori, 2001).

Intake of soy protein containing 60 mg isoflavones per day provided protection against oxidative modification of LDL. The oxidative modification of LDL particles is considered to be a prerequisite for the uptake of LDL by macrophages in the artery wall, which is a crucial, initial step in the formation of atheroma. Thus, this may be one of the mechanisms by which soy protein inhibition of atherosclerosis may be caused (Tikkanen et al., 1998).

Other mechanisms for inhibiting cardiovascular disease include lowering diastolic blood pressure, improving vascular and endothelial cell function (Washburn et al., 1999). In atherosclerosis, the arteries not only harden, they narrow so much that hardly any blood can get through. Such narrowed vessels are easily blocked by constriction or objects in the bloodstream. The internal surface of an artery is covered with a single layer of endothelial cells that are pressed against each other like flagstones on a terrace.

Atherosclerosis begins with injury to endothelial cells, exposing portions of the artery surface below the endothelium. Free radicals, chemicals in cigarette smoke and other irritants could be responsible for the injury, as could turbulence and
mechanical force due to high blood pressure. Platelets clump around the injured endothelial cells and release prostaglandins, which cause the endothelial cells to proliferate like in the case of cancer. LDL-cholesterol particles release their fat into the areas made porous by prostaglandins.

Macrophages engorge themselves on oxidized LDL-cholesterol until they become unrecognizable "foam cells" that invade atheromas. Then the atheromas are hardened by fibrin (which forms scar tissue) and finally by calcium patches. A vicious circle often arises with scar tissue attracting more platelets and LDL-engorged macrophages. There is an indication that soy products may also inhibit platelet activation and aggregation and reduce the amount of serotonin in platelets (Williams et al., 1998).

An analysis of 38 controlled human clinical trials using in average 47 g of soy protein daily was reported to significantly reduce the total cholesterol (9%), LDL cholesterol (13%) and triglycerides (11%) (Anderson et al., 1995). Another study indicates that the hypocholesterolemic effects of soy protein may function by influencing lipid metabolism through altering lipid-related gene expression (Sirtori and Lovati, 2001). It has been shown that soy protein administration shows beneficial effects on plasma lipids, especially among patients with the highest plasma cholesterol concentration (Anderson et al., 1995).

Several studies suggest that dietary soy, which contains plant estrogens known as phytoestrogens, may inhibit some forms of cancer and effectively lower the risk of heart disease. Phytoestrogen consumption has been shown to reduce risk factors for cardiovascular disease and atherosclerosis (Jayagopal et al., 2002). These effects result from a reduction of plasma low density lipoprotein cholesterol and triglyceride concentration (Ashton and Ball, 2000). Recent studies suggest that the cholesterol-lowering benefits of soy protein may be mediated through up-regulation of LDL-receptor activity, thus providing a novel mechanism for plasma cholesterol reduction different from currently available diet and hypolipodemic drugs.

It was recently shown that phytoestrogens in a supplement containing 25g of soy protein could significantly lower LDL cholesterol concentration and higher the phytoestrogen content (up to 62 mg/day) the better the response (Washburn et al., 1999). In non-human primates, a phytoestrogen containing soy diet (corresponding to
a human dose of 140 mg/day) significantly lowered LDL and VLDL cholesterol and increased HDL cholesterol compared to placebo. Phytoestrogens was reported to reduce infarct size in an experimental model of myocardial ischemia - reperfusion injury (Honore et al., 1997).

Dietary supplementation with soy phytoestrogens was reported to alter insulin resistance, glycemic control, and serum lipoproteins in postmenopausal women with type II diabetes, thereby improving their cardiovascular risk profile (Jayagopal et al., 2002). Diet containing soy protein rich in isoflavones have been shown to reduce insulin levels. Genistein binds to peroxisome proliferator-activated receptor (PPARγ), and transactivates it, thus increasing adipogenesis and probably eliciting some anti-inflammatory effects. Genistein and 17β-estradiol have comparable anti-apoptotic properties in primary cortical neurons and that these properties are mediated through estrogen receptors.

Somekawa et al. (2001) revealed consumption of soy products is associated with increased bone mass in postmenopausal Japanese women. Genistein binds to estrogen receptor (ERα and ERβ), and has ER-mediated estrogenic effects. In addition, it has antiestrogenic effects as well as non-ER-mediated effects such as inhibition of tyrosine kinase. At low concentrations, genistein acts as estrogen, stimulating osteogenesis and inhibiting adipogenesis. At high concentrations it acts as a ligand of PPARγ, leading to upregulation of adipogenesis and downregulation of osteogenesis.

Genistein concurrently activates two different transcriptional factors ERs and PPARγ, which have opposite effects on osteogenesis or adipogenesis. Phytoestrogens appear to have physiological effects in humans, with the most supportive data being related to the effects of soy protein supplements on cardiovascular, central nervous, endocrine, and immune system function. Based on these properties, soy protein and isoflavones have been suggested as dietary treatment modalities to decrease cardiovascular diseases. However, larger and long-term studies are needed to more thoroughly document their clinical effects.
6.2.5. Garlic protein

It has been found that feeding essential oils of onion and garlic protect against experimentally induced atherosclerosis by preventing the fall in the alpha lipoprotein fraction and by enhancing fibrinolytic activity, as well as by lowering the serum cholesterol and triglyceride levels (Bordia, 1977).

Similarly feeding male rabbits with a cholesterol-rich diet resulted in increased levels of cholesterol in plasma, aorta and liver and total lipids, phospholipids, free fatty acids in aorta and liver. Whereas the addition of garlic protein to this diet suppressed these effects, but their levels were still higher as compared to control rabbits. The plasma fibrinolytic activity which was decreased on cholesterol feeding was considerably increased when this diet was supplemented with garlic. When the atherogenic diet was supplemented with garlic, there was a profound increase in the activity of phospholipase in the cell-free supernatant of aorta and liver, with a concomitant decrease in the activity of NADH dehydrogenase of aorta. Histopathological studies of aorta, liver and heart supported biochemical studies and indicated the effect of garlic in slowing down the development of atherosclerosis (Mirhadi, 1991).

The interaction of oxygen-derived free radicals with cell membrane lipids and essential proteins contributes to myocardial cell damage, leading to depressed cardiac function and irreversible tissue injury with concomitant depletion of certain key endogenous antioxidant compounds, e.g., SOD, catalase, reduced glutathione, and glutathione peroxidase. Chronic garlic administration was reported to prevent the oxidative stress and the ultra structural changes associated with myocardial injury (Sanjay et al., 2002).

6.2.6. Coconut protein

Mendis and Kumarasunderam (1990) compared the effect of coconut oil and soy oil in normolipdemic young males. The coconut oil resulted in an increase in the HDL cholesterol, whereas the soy oil reduced this desirable lipoprotein. Cleary et al. (1999) fed genetically obese animals with high fat diets of either safflower oil or coconut oil. Safflower oil-fed animals had higher hepatic lipogenic enzyme activities
than did coconut oil fed animals. When the number of fat cells was measured, the
safflower oil-fed animals had more fat cells than their coconut oil-fed counterparts.

Monserrat and colleagues (1995) showed that a diet rich in coconut oil could
protect animals against renal necrosis and renal failure produced by a diet deficient in
choline (a methyl donor group). The animals had less or no mortality and increased
survival time as well as decreased incidence or severity of the renal lesions when 20%
coconut oil was added to the deficient diet. A mixture of hydrogenated vegetable oil
and corn oil did not show the same benefits.

Consumption of coconut is beneficial for individuals with the fatigue and
immune dysfunction syndrome known as CFIDS. Coconut oil is a neutral fat in terms
of atherogenicity. A study conducted in a population shows that dietary coconut oil
does not lead to high serum cholesterol or to high coronary heart disease mortality or
morbidity (Kaunitz and Dayrit, 1992).

Salil et al. (2001) demonstrated that feeding rats with coconut protein results
in lower levels of total cholesterol, LDL, VLDL, cholesterol, triglycerides and
phospholipids in the serum and higher levels of serum HDL cholesterol and thereby,
decreasing malondialdehyde level in the heart and increasing the activity of SOD and
catalase and finally reduction of hyperlipedemia and peroxidative effect induced by
high fat, cholesterol-rich diet.

6.2.7. Milk proteins

Whey, a protein complex derived from milk, is being touted as a functional
food with a number of health benefits. The biological components of whey, including
lactoferrin, beta-lactoglobulin, alpha-lactalbumin, glycomacropeptide, and
immunoglobulins, demonstrate a range of immune-enhancing properties. In addition,
whey has the ability to act as an antioxidant, antihypertensive, antitumor,
hypolipidemic, antiviral, antibacterial, and chelating agent. The primary mechanism
by which whey is thought to exert its effects is by intracellular conversion of the
amino acid cysteine to glutathione, a potent intracellular antioxidant (Keri, 2004).

A number of clinical trials have successfully proved the effect of whey in the
treatment of cancer, HIV, hepatitis B, cardiovascular disease, osteoporosis, and as an
antimicrobial agent. Whey is a popular dietary protein supplement purported to
provide antimicrobial activity, immune modulation, improved muscle strength and body composition, and to prevent cardiovascular disease and osteoporosis (Keri, 2004).

In a study, the effect of dietary whey protein versus casein on plasma and liver cholesterol concentrations was investigated in female, weanling rats. It was shown that at high dietary protein level whey protein significantly lowered plasma and liver cholesterol. The hypocholesteremic effect of whey protein was associated with decrease in VLDL cholesterol (Zhang and Beynen, 1993).

6.2.8. Arginine

Nitric oxide is produced from L-arginine by several classes of enzymes known as nitric oxide synthases (NOS). Three forms of NOS are i-NOS (Inducible), e-NOS (endothelial) and n-NOS (neuronal). Another alternative route of NO synthesis involves L-arginine interactions with free radicals (ROS and superoxide radical) through non-enzymatic synthesis of the same.

Nitric oxide elicits its effects either through action of GC i.e., guanylyl cyclase (with production of c-GMP); or independent of GC involvement. L-arginine is present in the blood typically in millimolar concentrations, whereas the saturation point for NOS is in the micromolar range.

High circulating levels of ADMA (Asymmetric dimethyl L-arginine), a derivative of arginine has been shown to inhibit NOS production (Usui et al., 1998). The levels of AMDA are higher in several pathologic states such as hypertension, diabetes mellitus, aging, hypercholesterolemia and renal failure (Matsuoka et al., 1997). ADMA is formed from the post-translational methylation of peptide-bound arginine in proteins such as heat shock proteins and histones (Boger et al., 1998), and not from free circulating L-arginine. Catabolism of ADMA is through the enzyme dimethylarginine dimethyl aminomethyl hydrolase (Nagase et al., 1997), which is reduced by 40-60% through high-cholesterol feeding in experimental hypercholesterolemic animal models (Ito et al., 1999). In addressing this problem, supplementation of L-arginine competed with ADMA and thus, increased NOS activity.
L-arginine has several roles in human beings, and it helps the human body to mounting a protective response to injury. Several endothelial cell-(EC) derived paracrine factors have been implicated in distinguishing healthy vessels from those affected by CVD. Body fluidity, vascular tone and cell proliferation are important functions of ECs. Impaired vasodilatation is elicited partly through enhanced degradation of NO due to superoxide anion overproduction, reduced availability of NOS cofactors, or impaired NO synthesis due to ADMA accumulation (Vallance et al., 1992). Response to normal physiological stimuli in humans as well as experimental animals has been shown to be impaired in atherosclerosis in humans as well as hypercholesterolemic animal models (Raeger et al., 1990).

Vasodilatory impairment is also correlated with LDL levels and standard risk factors like smoking, hypertension and lipidemias, which can be detected through assessment of the L-arginine/ADMA ratio. Vasomotor dysfunction and vasodilatory responses to cholinergic agonists (Ach, methacholine), through release of EDRF (endothelium-derived relaxing factor) were restored to normal through administration of L-arginine in experimental hypercholesterolemic rabbits and hypertensive, hypercholesterolemic humans.

Cholinergic agonist administration (an endothelial dependent process) shows impaired vasodilator responses and is seen even in young patients just developing the disease (Cooke and Dzau, 1997). Hypertension commonly shows a decrease in endothelium-dependent responses, but endothelium-independent responses have remained intact. NOS inhibitors when administered causes increase in arterial blood pressure by 40% in experimental animals and in human patients; whereas the response is lesser in hypertensive patients (Calver et al., 1992).

Compromised limb flow and exercise tolerance was improved in animals through exercise performance assessment after L-arginine administration. A 6% solution of arginine showed improvement in cholesterol transport out of the blood in apolipoprotein-E deficient mice. An increase in treadmill exercise by 61% was seen (Maxwell et al., 1998), which was blocked by NOS inhibitors; which shows the role of NO produced from L-arginine as being the factor raising exercise tolerance levels. Significant (150%) improvement in the walking distance was seen in patients with peripheral arterial disease (Boger et al., 1998). Also, acetylcholine induced coronary
vascular relaxation was improved by 149%, reducing anginal episodes by 70% (Lerman et al., 1998).

Several complications such as platelet aggregation, leukocyte adhesion, proliferation of vascular smooth muscle cells, oxidized LDL accumulation, and superoxide anion formation enhanced atherosclerotic plaque formation. NO derived from L-arginine inhibits pro-atherogenic factors. Fewer intimal lesions were observed in LDL receptor knockout mice and hypercholesterolemic rabbits upon treatment with L-arginine when compared to the controls. Conversely, treatment with NOS inhibitors accelerates lesion formation. L-arginine is believed to elicit effects similar to lovastatin, a HMG-CoA reductase inhibitor (Boger et al., 1997).

Administering L-arginine can lead to regression of pre-existing intimal lesions in the hypercholesterolemic rabbit, as per (Candipan et al., 1996) and can inhibit myointimal hyperplasia after balloon angioplasty (Schwarzacher et al., 1997).

It is believed that factors involved in the process of angiogenesis depend upon NO for their normal action. Endothelial cells grown in culture, treated with pro-angiogenic factors can increase and up regulate NOS function. Use of NOS inhibitors interferes with this phenomenon, as NOS are sensitive to inhibitory effects of these compounds. In vivo administration of L-arginine speeds up wound healing and increases gastric blood flow in rats subjected to acid-induced ulcerations, a process requiring angiogenesis (Tachibana et al., 1985).

**6.2.9. Isoproterenol**

Isoproterenol [1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride] (ISO) is a synthetic catecholamine and β-adrenergic agonist, which has been found to cause severe stress in the myocardium resulting in infarct-like necrosis of the heart muscles (Wexler, 1978). Catecholamines are important compounds produced under stress conditions and are also administered in conditions of cardiac stress to support
blood pressure and cardiac function in patients. However, due to the generation of ROS, these compounds may contribute to oxidative stress, as evidenced by accumulation of lipid peroxides and production of free radicals, being one of the postulated mechanisms of cardiac damage induced by ISO (Sushama et al., 1989).

ISO-induced MI is accompanied by an increase in cardiac marker enzymes and lysosomal enzymes (Sathish et al., 2003; Acikel et al., 2005). Intracellular release of lysosomal enzymes following myocardial ischemia may cause cell injury and death, either directly or through the activation of complement pathway. The oxidation of hydroxyl groups in catecholamines leads to their conversion into quinones and to the subsequent formation of adrenochromes; which is the most probable reason for the hazardous effects of catecholamines. During this reaction, highly toxic oxygen-derived free radicals are generated which are detrimental to extra and intracellular enzymes and proteins (Thompson and Hess, 1986). Adrenochromes and other oxidation metabolites of catecholamines can cause cell necrosis and contractile failure in the animal’s heart (Yates et al., 1981). Cohen and Heikkila (1974) have reported that the auto-oxidation of catecholamines results in the generation of highly cytotoxic free radicals. Furthermore, free radicals could initiate the peroxidation of membrane-bound polyunsaturated fatty acids, leading to both functional and structural myocardial injury (Thompson and Hess, 1986).

When myocardial cells are damaged or destroyed due to the deficiency of oxygen supply or glucose, the cell membrane becomes permeable or ruptures, resulting in leakage of enzymes. The release of cellular enzymes reflects the alterations in plasma membrane integrity and/or permeability as a response to β-adrenergic stimulation. This might be due to the damage caused to the sarcolemma by the β-agonist that has rendered it leaky. ISO-induction produces free radicals via β-adrenoceptor mechanism and affects the cell metabolism to such a degree that cytotoxic free radicals are formed, producing myocardial necrosis (Sumitra et al., 2001).

**Objective:** The incidence of cardiovascular disease from the perspective of Indian scenario is alarming, especially with the country’s economy booming and the lifestyle of people changing. In this background, the objective of this experiment is to find cheap and easily available preventive/curative biomolecules to treat
cardiovascular disorders. Since, the therapeutic value of protein is increasingly understood, we have hypothesized that protein isolates from selected sources, i.e., soy, garlic, coconut, whey and milk protein will have a curative effect in situations similar to myocardial infarction.
6.3. MATERIALS AND METHODS

6.3.1. Chemicals
Isoproterenol were obtained from M/s. Sigma Chemical Company, St. Louis. MO, USA. Other chemicals used in this study were obtained from Himedia laboratories, Mumbai, India and were of analytical grade.

6.3.2. Animals
Male Swiss albino mice (*Mus musculus*) of body weight in the range of 10-11 g were used for the present study. Mice were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. The animals were housed individually in polypropylene cages with wire mesh bottom and maintained at 25°C ± 2, under standard lighting conditions (12 -hrs light/dark cycle).

All animal experimental procedures were carried out in accordance with the ‘principles of laboratory animal care’ (NIH publication no.85-23, revised 1985) and with the approval of the “Institute’s ethical committee on animal experiments” (Registration no: 738/03/abc/CPCSEA) at St. Joseph’s College (Autonomous), Tiruchirapalli, Tamil Nadu, India.

6.3.3. Diet composition and preparation
The diet fed to mice in these experiments were formulated according to AIN-93G guidelines (Reeves *et al.*, 1993 and Reeves, 1997) and the composition of the diet used is given in Table 5.1. Specific protein isolate diet was prepared by mixing the protein (20%) with salt and vitamin mixtures (as shown in Table 5.1). The diets were prepared once in 2 weeks and were stored in airtight containers in a refrigerator and used. Preparation of protein isolates (see page 39).

6.3.4. Experimental setup
Weaned mice of 10-11 g of body weight were used for the study. The animals were divided into the following seven groups, of six mice each.

| Group I  | - | Control |
| Group II | - | Isoproterenol (Experimental control) |
| Group III | - | Soy protein |
Figure 6.1: Schematic representation of the study using different protein sources on induced myocardial infarction (MI)

Weaned mice

Groups

I Control
II Isoproterenol
III Soy protein
IV Garlic protein
V Coconut protein
VI Whey protein
VII Casein

Diet from day 1 to 60

Normal
Soy protein
Garlic protein
Coconut protein
Whey protein
Casein

Saline
Isoproterenol
Isoproterenol
Isoproterenol
Isoproterenol
Isoproterenol

Day 60

Sacrificed
Sacrificed
Sacrificed
Sacrificed
Sacrificed
Sacrificed

After 24 hours

Sacrificed
Sacrificed
Sacrificed
Sacrificed
Sacrificed
Sacrificed
Group IV  -  Garlic protein
Group V  -  Coconut protein
Group VI  -  Whey protein
Group VII  -  Casein

The first group, the control and the second group ISO (Experimental control) were fed with the normal protein diet and water *ad libitum* throughout the study. The groups from III to VII were given protein from different sources as given above, but equal in amount to the normal diet. The control and ISO animals were continued on the normal diet and the groups III to VII were fed their respective diets till the 60th day. A schematic representation is given in Figure 6.1.

After 60 days of pretreatment with five protein isolates, isoproterenol (150 mg/kg body) was administered intraperitoneally to groups II to VII to induce myocardial infarction.

Twenty four hours after the administration of ISO, mice from all the groups were anaesthetized and killed by cervical decapitation. Blood was collected in two test tubes (i.e.) one with anticoagulant for plasma separation and the other without anticoagulant for serum separation. Plasma and serum were used for the assay of different biochemical indices. Immediately after killing, the rats were dissected; the heart was removed and washed in ice cold saline. About 100mg of the tissue was weighed and homogenized in chilled 0.1 M Tris-HCL buffer (pH: 7.4) in Potter Elvejham homogenizer. The homogenate was used for the estimation of various biochemical parameters namely cardiac markers (CK and LDH), cell lysis marker enzymes (AST and ALT), Antioxidant enzymes (SOD and CAT) and lipid peroxidation. In addition to the above biochemical parameters histology of heart tissue were carried out.

### 6.3.5. Creatine Kinase

Creatine kinase activity was determined using kit, LAB KIT, Barcelona, according to manufacturer’s instructions.
Principle

Creatine kinase catalyses the reversible transfer of a phosphate group from phosphocreatine to ADP. This reaction is coupled to those catalysed by hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH):

\[
\text{Phosphocreatine} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP} \\
\text{ATP} + \text{Glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glucose-6-phosphate} \\
\text{G6P} + \text{NADP}^{+} \xrightarrow{\text{G6P-DH}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^{+}
\]

The rate of NADPH formation, measured photometrically, is proportional to the catalytic concentration of CK present in the sample.

Reagents Composition

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Imidazol pH 7.0</td>
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<td>100 mmol/L</td>
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<tr>
<td>R₁</td>
<td>Glucose</td>
<td>20 mmol/L</td>
</tr>
<tr>
<td>Buffer</td>
<td>Magnesium acetate</td>
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<tr>
<td></td>
<td>EDTA</td>
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<tr>
<td></td>
<td>ADP</td>
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<td></td>
<td>AMP</td>
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<tr>
<td></td>
<td>di-Adenosine-5-pentaphosphate</td>
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<tr>
<td></td>
<td>NADP⁺</td>
<td>2 mmol/L</td>
</tr>
<tr>
<td>R₂</td>
<td>Hexokinase</td>
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<td>Substrate</td>
<td>Glucose-6-phosphate dehydrogenase (G6P-DH)</td>
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<td></td>
<td>N-acetyl cysteine</td>
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<tr>
<td></td>
<td>Creatine phosphate</td>
<td>30 mmol/L</td>
</tr>
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</table>

Reagent Preparation

Working reagent: Dissolve 1 Tablet of R.2 in 2.5 mL of R.1

Procedure

1ml of working reagent and 40 µl of sample/standards were taken in a cuvette and incubated for 2 minutes at 25°C. Later the absorbance at minute interval thereafter for 3 minutes was taken using 340 nm.
Calculations

25 – 30°C; ΔA/min x 4176 = U/L of CK

Units: One international Unit (1U) is the amount of enzyme that transforms 1 µmol of substrate per minute. In standard conditions: The concentration is expressed in units per litre of sample (U/L).

6.3.6. Lactate Dehydrogenase

Lactate dehydrogenase activity was assayed using kit from Biosystems, Spain, as per the manufacturer’s instructions.

Principle

Lactate dehydrogenase catalyses the oxidation of lactate by NAD⁺, to form pyruvate and NADH. The catalytic concentration is determined from the rate of increase of NADH, measured at 340nm.

\[ \text{Lactate} + \text{NAD}^+ \xrightarrow{LDH} \text{Pyruvate} + \text{NADH} \]

Reagents

A. N-Methyl -D-glucamine 0.046 mol/L, lactate 62.5 mmol/L, pH 9.4
B. NAD⁺ 50 mmol/L

Working reagent: Contents of A and B were mixed well and later used for the assay.

Procedure

25 µl of sample and 1ml of working reagent were taken in a cuvette, contents were mixed well and the cuvette was inserted into the photometer. After 30 seconds, the initial absorbance and at 1 minute intervals thereafter for 3 minutes, the absorbance was recorded. The average absorbance per minute (A/min) was calculated.

\[ \frac{V_t \times 10^6}{\varepsilon \times l \times V_s} = U/L \]

Molar absorbance (\(\varepsilon\)), Vt- total reaction volume, (l) - light path, Vs-sample volume

6.3.7. Alanine Transaminase

ALT activity was assayed in serum and heart tissue using a kit purchased from Qualigens, as per manufacturer’s instructions.
Principle

Alanine transaminase catalyzes the transfer of an amino group from L-alanine to \( \alpha \)-ketoglutarate with the formation of pyruvate and glutamate as products of the reaction. The pyruvate thus formed is allowed to react with 2,4 dinitrophenyl hydrazine (DNPH) to produce 2, 4 dinitrophenyl hydrazone derivative which is brown coloured in alkaline medium. The absorbance of hydrazone derivative is correlated with alanine transaminase activity by plotting a calibration curve using the values obtained for pyruvate standard.

\[
\text{L-alanine} + \alpha \text{-ketoglutarate} \xrightarrow{\text{ALT}, \text{pH 7.4}} \text{Pyruvate} + \text{2,4 DNPH} \rightarrow \text{2,4 dinitrophenyl hydrazone (Brown coloured)}
\]

Reagents

1. Buffered substrate (pH 7.4): 1.78 g of DL-alanine and 30 mg of \( \alpha \)-ketoglutarate were dissolved in 20 ml of phosphate buffer containing 1.25 ml of 0.4 M NaOH and the solution was made up to 100 ml with buffer and adjusted to pH 7.4 upon sensing the necessity for doing so.
2. DNPH colour reagent: 200 mg of 2, 4 dinitrophenyl hydrazine (2, 4 DNPH) was dissolved in hot 1M HCl and made up to 1 liter with the same.
3. Sodium hydroxide (4 N): 16 g of sodium hydroxide was dissolved in about 800 ml of distilled water and then accurately made up to 1 liter with distilled water. (1:10 dilution with DW before use)
4. Pyruvate standard (2 mM): 220 mg of sodium pyruvate was dissolved in phosphate buffer and made up to 100 ml. 10 ml of this solution was diluted to 100 ml with phosphate buffer to obtain a working standard solution containing 2 m mol of pyruvate per ml.

Procedure

500 µl of buffered substrate was taken, both in blank test tube and sample test tubes and then, the mixture was incubated at 37°C for 3 minutes. 100 µl of serum/tissue homogenate was taken in sample tubes. The contents were mixed well and the test tubes were incubated at 37°C for 30 minutes. 500 µl of DNPH colour reagent was added to all test tubes, and they were mixed well and allowed to stand at
room temperature for 20 minutes. 0.1 ml of distilled water was added to the blank following 500 µl of working sodium hydroxide, which was added to all tubes, mixed well and allowed to stand at room temperature for 10 minutes and then, the absorbance was taken for each of the tubes at 505 nm. Suitable aliquots of standard solution were taken and treated in a similar manner to obtain a standard curve for comparison.

6.3.8. Aspartate Transaminase

AST activity was assayed in serum and heart tissue using a kit purchased from Qualigens, as per manufacturer’s instructions.

Principle

Aspartate transaminase catalyzes the transfer of an amino group from L-alanine to α-ketoglutarate, which culminates in the formation of oxaloacetate and glutamate as products of the reaction. The oxaloacetate so formed, is allowed to react with 2, 4 dinitrophenyl hydrazine (DNPH) to produce a derivative of 2,4 dinitrophenyl hydrazone, which is brown-coloured in an alkaline medium. The absorbance of hydrazone derivative is correlated with aspartate transaminase activity by plotting a calibration curve using pyruvate standards.

\[
\text{L-alanine} + \text{α-ketoglutarate} \xrightarrow{\text{AST}} \text{oxaloacetate} + \text{L-glutamate}
\]

\[
\text{Oxaloacetate} + 2,4 \text{ DNPH} \xrightarrow{\text{alkaline medium}} 2,4 \text{ dinitrophenylhydrazone (Brown coloured)}
\]

Reagents

1. Buffered substrate pH 7.4: 2.66 g DL- aspartic acid and 30 mg α -keto glutarate were both dissolved in 20.5 ml of 1 M NaOH. The pH was adjusted to 7.4 by adding 1 M NaOH drop-wise while stirring the solution.
2. DNPH colour reagent: 200 mg of 2, 4 dinitro-phenylhydrazine (2,4 DNPH) was dissolved in hot 1 M HCl and made up to 1 litre with 1 M HCl.
3. Sodium hydroxide 4 N: 16 g sodium hydroxide was dissolved in about 800 ml of distilled water and made up to 1 litre using the same.
4. Pyruvate standard 2 mM: 220 mg of sodium pyruvate was dissolved in phosphate buffer and made up to 100 ml. 10 ml of this solution was further made up to 100
ml with phosphate buffer to obtain a working standard solution containing 2 m
mol of pyruvate per ml.

**Procedure**

500 µl of buffered substrate was taken, both in blank test tube and sample test
tubes and then, the mixture was incubated at 37°C for 3 minutes. 100 µl of
serum/tissue homogenate was taken in sample tubes. The contents were mixed well
and the test tubes were incubated at 37°C for 30 minutes. 500 µl of DNPH colour
reagent was added to all test tubes, and they were mixed well and allowed to stand at
room temperature for 20 minutes. 0.1 ml of distilled water was added to the blank
following 500 µl of working sodium hydroxide, which was added to all tubes, mixed
well and allowed to stand at room temperature for 10 minutes and then, the
absorbance was taken for each of the tubes at 505 nm. Suitable aliquots of standard
solution were taken and treated in a similar manner to obtain a standard curve for
comparison.

**6.3.9. Super Oxide Dismutase**

SOD activity was assayed in serum and heart tissue using a kit purchased from
Cayman (USA), as per manufacturer’s instructions.

Superoxide dismutases are metalloenzymes that catalyze the dismutation of
the superoxide anion to yield molecular oxygen and hydrogen peroxide, and thus form
a crucial part of the cellular antioxidant defense system.

\[
2O_2^- + 2H^+ + SOD \rightarrow H_2O_2 + O_2
\]

The Cayman chemical kit for superoxide dismutase assay utilizes a
tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase
and hypoxanthine. One unit of SOD is defined as the amount of the enzyme needed to
exhibit 50% dismutation of the superoxide radical.

**Pre-assay preparation**

1. Assay buffer (10X): 3 ml of assay buffer concentrate was diluted with 27 ml of
HPLC grade water. This assay buffer (50 mM Tris-HCL, pH 8.0, containing 0.1
mM diethylenetriaminepenta-acetic acid and 0.1 mM hypoxanthine), was used to
dilute the radical detector.
2. Sample buffer (10X): sample buffer concentrate was diluted with 18 ml of HPLC grade water. This final sample buffer (50 mM Tris-HCL, pH 8.0) was used for the preparation of SOD standards and for diluting xanthine oxidase.

3. Radical detector: Solution of tetrazolium salt.

4. SOD standard: Bovine erythrocyte SOD.

5. Xanthine oxidase: 50 µl of enzyme source was diluted with 1.95 ml of the sample buffer.

Sample preparation

Tissue homogenate: Heart tissue was perfused with phosphate buffered saline, pH 7.4, containing as little as 0.16 mg/ml heparin to remove any RBC and clots that may interfere with the assay. The tissue was homogenized in 5-10 ml of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EDTA, 210 mM mannitol and 70 mM sucrose per gram tissue. The homogenate was centrifuged at 1,500 x g for 5 minutes at 4°C. Supernatant was used for the assay.

Procedure

20 µl of SOD standard was diluted with 1.98 ml of sample buffer and aliquots comprising a series of working standard in the range 20-200 µl were prepared and used. 10 µl of standards /serum sample and 200 µl of diluted radical detector were added to the designated wells on the plate. Reactions were initiated by the addition of 20 µl of diluted xanthine oxidase to all the wells that were used. The plate was incubated at room temperature for 20 minutes and finally, the absorbance was read at 450 nm using a plate reader.

Superoxide dismutase is expressed as U/mg tissue.

6.3.10. Catalase

Catalase (EC. 1.11.1.6) enzyme activity was assayed according to the method of (Sinha, 1972).

Principle

This enzyme catalyses the reaction

\[ \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]
Unreacted H$_2$O$_2$ is measured by its ability to reduce dichromate in acetic acid solution.

**Reagents**

1. Dichromate acetic acid solution: 5% potassium dichromate in glacial acetic acid
2. Hydrogen peroxide 0.2 M
3. Phosphate buffer (pH 7.0): 0.01M

**Procedure**

To 0.1 ml of sample, 1 ml of buffer and 0.4 ml of distilled water was added. The reaction was initiated by the addition of 0.5 ml of H$_2$O$_2$, and the reaction mixture was incubated at 37°C for 1 minute. The reaction was terminated with the addition of 2.0 ml of dichromate-acetic acid reagent. Standard H$_2$O$_2$ solution in the range of 4-20 µ moles was taken and treated in the same manner. The tubes were heated in a boiling water bath for 15 minutes, cooled and then the absorbance was read using a 510 nm filter.

Catalase activity is expressed as µ moles of H$_2$O$_2$ utilized/min/mg protein.

**6.3.11. Lipid peroxidation**

The lipid peroxide assay was carried out using kit purchased from Cayman (USA), according to manufacturer instructions.

**Principle**

This assay kit measures the hydro-peroxides by directly utilizing redox reactions with ferrous ions

\[
\text{ROOH} + \text{Fe}^{2+} \rightarrow \text{RO}^+ + \text{Fe}^{3+}
\]
\[
\text{RO}^- + \text{Fe}^{2+} + \text{H}^+ \rightarrow \text{ROH} + \text{Fe}^{3+}
\]
\[
\text{Fe}^{3+} + 5\text{SCN} \rightarrow \text{Fe} (\text{SCN})_5^{2-}
\]

Hydro peroxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromogen.
**Preparation of reagents**

1. Reagent a: contains 4.5 mM ferrous sulfate in 0.2 M hydrochloric acid.
2. Reagent b: contains 13% methanolic solution of ammonium thiocyanate.
4. Extract R: contains a crystalline solid used for extraction of samples. About 100mg of the solid was taken into a test tube, 15 ml of methanol was added and vortexed thoroughly for about 2 min.
5. Triphenylphosphine: Contains crystalline triphenylphosphine. About 2.6 mg of triphenylphosphine was dissolved in 1 ml of chloroform-methanol solvent mixture to prepare a 10 mM solution of the same.

**Sample Preparation**

Lipid hydroperoxides were extracted from the sample into chloroform before performing the assay. 500 µl of plasma was taken in a glass test tube and an equal volume of Extract R saturated methanol was added and vortexed and 1 ml of cold chloroform was added to each tube and mixed thoroughly. The mixture was centrifuged at 1,500 x g for 5 minutes at 0°C. The chloroform layer at the bottom of the tube was collected carefully by inserting a Pasteur pipette along the side of the test tube. The chloroform layer was transferred to another test tube and stored on ice.

**Procedure**

500 µl of the chloroform extract of each sample and 450 µl of the chloroform-methanol solvent mixture was added to the sample test tubes. 50 µl of the freshly prepared chromogen was prepared by mixing equal volumes of reagent a and b and vortexed, and then the assay tubes were incubated at room temperature for 5 min. Standard hydro-peroxide (HP) solution in range 10-100 µl was taken and made upto 1000 µl using chloroform-methanol such that a final concentration of 0.5 to 5 nmol (HP) was obtained. 300 µl of above sample from each tube was added into the 96 well plates and the absorbance was taken using a 500 nm filter from a 96 well plate reader. The volume of the extract used for the assay changed depending upon the concentration of hydro-peroxides.
6.3.12. Histology

Heart tissue was fixed in 10% formalin, routinely processed and embedded in paraffin. Paraffin sections (5 µm) were cut on glass slides and stained with hematoxylin and eosin (H and E), periodic acid Schiff (PAS) reagent and examined under light microscope.

6.3.13. Statistical Analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Bonferroni’s test using Statistical Program for the Social Sciences (SPSS) software version 13.0. Results were expressed as mean for six mice in each group. Values of P <0.05 were considered significant.
6.4. RESULTS AND DISCUSSION

The weaned mice, just introduced to solid food were taken for the present study. As already explained they were divided into groups and provided with protein isolates from different dietary sources. Their growth was recorded in terms of body weight at regular time intervals. Animals from groups III to VII were treated with five different protein isolates for 60 days and isoproterenol was injected to all groups on the same day to all animals except the control group and after 24 hours they were sacrificed and different parameters were analyzed in serum and heart tissue.

In our results and discussion the following order is followed, first the body weight is analyzed. Then the cardiac marker enzymes; creatine kinase and lactate dehydrogenase are taken and the data pertaining to the serum and heart tissue are analyzed and discussed. This is followed by an analysis and discussion of the data on the cell lysis marker enzymes (AST and ALT). Similarly the antioxidant enzymes and the lipid peroxide are dealt with separately.

6.4.1. Body Weight

The body weight of mice fed with various protein diets is represented in Table 6.1 and Figure 6.2. Significant (P<0.05) decrease in final body weight was observed in whey protein-fed animals when compared to the animals that belonged to control group. The final body weight of animals pretreated with soy protein was found to be lower than the garlic protein, coconut protein and casein treated animals.

The increase in body weight in the control, garlic and coconut protein fed animals were almost the same. It was around 134% of the initial weight after being fed different diets for 60 days. However, the increase in body weight was reduced in soy protein fed animals to 105% and to a drastic level in whey protein fed animals (76%).

Soy-protein fed rats gained slightly less weight than their casein-fed counterparts. The body weight was nearly identical in both groups until 20 days of pretreatment with the same protein isolates. The soy-fed rats gained less weight, which led to a significant (P<0.05) difference in absolute weight at the end of experiment.
Table – 6.1: The body weight (g) of mice fed the various protein diets in Myocardial infarction

<table>
<thead>
<tr>
<th>Age in weeks</th>
<th>Control</th>
<th>DEN Induced</th>
<th>Soy protein</th>
<th>Garlic protein</th>
<th>Coconut protein</th>
<th>Whey protein</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>Initial body weight</td>
<td>10.29 ± 0.78</td>
<td>10.52 ± 0.54</td>
<td>10.74 ± 1.17</td>
<td>10.78 ± 1.05</td>
<td>11.30 ± 1.42</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>20th day</td>
<td>13.14 ± 1.28</td>
<td>12.90 ± 1.33</td>
<td>12.65 ± 0.90</td>
<td>13.04 ± 1.13</td>
<td>13.32 ± 1.20</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>40th day</td>
<td>22.78 ± 0.86</td>
<td>22.96 ± 0.90</td>
<td>20.66 ± 1.12</td>
<td>23.41 ± 0.99</td>
<td>24.52 ± 1.74</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Final body weight (60th day)</td>
<td>24.02 ± 1.06 (133 %)</td>
<td>24.84 ± 0.60 (136 %)</td>
<td>22.10 ± 1.43 (105 %)</td>
<td>25.36 ± 1.90 (135 %)</td>
<td>26.42 ± 1.47 (133 %)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD for 6 animals.

a $P<0.05$ significantly different compared with control animals.
b $P<0.05$ significantly different compared with ISO alone treated animals.

(%) As percent of control group
Figure 6.2: Body weight of mice at various stages of the study
Reza et al. (2001) observed that rats fed with soy protein diet had a significantly lower weight gain than those on casein diet and also inferred that food intake could not possibly account for these differences.

Ingestion of soy protein has been shown to hold antiobesity or anorectic properties (Erdmann et al., 2008) and that may be the cause for the tendency to gain less body weight than the casein, garlic and coconut protein fed animals.

In this study the whey protein diet had a profound influence on body weight gain of mice as reported by Belobrajdic et al. (2003), despite there being no measurable effect on feed intake. The final body weight of whey fed groups was much lower than all other protein isolate fed groups. Belobrajdic et al. (2003) reported that final body weight of rats fed whey protein group was less than rats fed red meat. A similar observation was seen in our study (Figure 6.2).

Although high protein intake reduces energy intake and adiposity, whey protein is more effective than red meat in reducing body weight gain and increasing insulin sensitivity (Belobrajdic et al., 2004). However, there are tall claims of whey protein increasing muscle mass in body builders. Whey protein, in human studies resulted in enhancing satiety and decrease food intake relative to casein (Hall et al., 2003).

Body weight in rat was lower (-21%) on the whey protein isolate diet relative to casein, with a non-significant influence associated with glycomacropeptide (GMP) inclusion (-30%). Renal and carcass fat mass and also serum insulin were reduced by the inclusion of a high concentration of GMP. Glycomacropeptide, the glycosylated fraction of caseinomacropeptide is a C-terminal fragment of κ-casein released by endopeptidase chymosin and is present in whey (Brody, 2000) and this may be responsible for the weight reduction in animal studies (Belobrajdic et al., 2003). GMP may stimulate the release of cholecystokinin (Yvon et al., 1994), thus regulating satiety and food intake (Kissileff et al., 1981; Liverse et al., 1994).

**Isoproterenol induced cardio toxicity**

Intraperitoneal administration of isoproterenol, a synthetic catecholamine and β-adrenergic agonist into adult rats was reported to bring about biochemical and morphological alterations in the heart tissue of experimental animals, similar to
observations that have been made in human myocardial infarction (Ravichandran et al., 1990; Mohanty et al., 2004).

Altered mitochondrial function and free radical mediated tissue damage may be the basis for the pathological events in ISO-induced cardio toxicity. Depending on the dose of ISO, the mitochondrial function is diminished by stimulating the endogenous phospholipase. The phospholipase breaks down the membrane phospholipids which is the supporting components in maintaining the activities of a number of enzymes (Konda et al., 1987).

Many ISO induced studies in animals have confirmed significant increase in mitochondrial lipid peroxidation products (thiobarbituric acid reactive substances and lipid hydroperoxides) and significant decrease in mitochondrial antioxidants (Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and reduced glutathione). Significantly decreased activities of TCA cycle enzymes such as isocitrate, succinate, malate and α-ketoglutarate dehydrogenase and respiratory chain marker enzymes such as NADH-dehydrogenase and cytochrome C- oxidase were observed in heart mitochondria of myocardial infarcted rats (Devika and Prince, 2008).

Large doses of ISO irreversibly reduced mitochondrial function by activating endogenous phospholipase, which breaks down membrane phospholipids, the essential components in maintaining the activities of NADH-cytocrome C reductase and cytochrome C oxidase (Konda et al., 1987).

**Cardiac markers**

The cardiac markers provided a measure of the intensity of the damage to heart muscle tissue caused due to ISO administration and hence are evaluated after pretreatment with five different protein isolates. The parameters measured in this study may be categorized into, one, the cardiac marker enzymes, namely the creatine kinase and lactate dehydrogenase(CK and LDH) and two, the cell lysis marker enzymes (AST and ALT) and three, the antioxidant enzymes (SOD and CAT) and four, the lipid peroxidation(LPO).
6.4.2. Creatine kinase - Results

Table 6.2 represents the activity of cardiac marker enzyme in serum and heart of control and experimental animals. CK catalyzes the reversible transfer of phosphate groups between creatine and phosphocreatine as well as between ATP and ADP. This enzyme is extensively studied in relation to heart related diseases, hence used as a marker enzyme to study MI.

**Serum CK:** The creatine kinase enzyme activity was significantly (P<0.05) increased in isoproterenol treated mice when compared to those of control mice (Figure 6.3). The level was two and a half times higher than the control animals. However, we observed a significant decrease of CK activities in the animals pretreated with various protein isolates; the extent of decrease varied with different protein isolates. The serum concentration of CK was, of course higher than the control in all the groups. Among the various groups the least concentration of CK was observed in soy protein group (130% as compared to control), and it gradually increased in the following order; garlic protein (167%), coconut protein (191%), whey protein (226%) and casein treated (229%). However there was no significant difference in the values between control and soy fed group.

**Heart CK:** A significant decrease (P<0.05) in the level of CK was observed in heart tissue of isoproterenol treated mice when compared to animals (Table 6.2). Pretreatment with different protein isolates for 60 days had varied impact on the activity of CK. Unlike in the serum, the CK activity in animals pretreated with soy protein and garlic remained unaffected and their values were similar to the control group. The creatine kinase activity was significantly less in coconut protein (83%), whey protein and casein treated (82% and 80%) respectively compared to the control.

6.4.3. Creatine Kinase - Discussion

Creatine kinase isoenzymes are found in cells with intermittently high energy requirements. They are specifically located at places of energy demand and energy production and are linked by a phosphocreatine/creatine circuit.

The enzyme creatine kinase catalyses the reversible transfer of the N-phosphoryl group from phosphocreatine to ADP to regenerate ATP, thus playing a
Table-6.2: Levels of creatine kinase in serum and heart of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Creatine kinase (IU/L)</th>
<th>Heart Creatine kinase (µmoles of phosphorus liberated min/mg/protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.71 ± 0.28 b</td>
<td>12.46 ± 0.46 b</td>
</tr>
<tr>
<td>ISO administered</td>
<td>4.63 ± 0.48 a (270%)</td>
<td>6.6 ± 0.42 a (53%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>2.24 ± 0.26 b (130%)</td>
<td>12.84 ± 0.82 b (103%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>2.86 ± 0.18 a,b (167%)</td>
<td>12.55 ± 0.51 b (100%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>3.27 ± 0.31 a,b (191%)</td>
<td>10.40 ± 0.45 a,b (83%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>3.88 ± 0.14 a,b (226%)</td>
<td>10.30 ± 0.46 a,b (82%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>3.92 ± 0.10 a,b (229%)</td>
<td>10.07 ± 0.27 a,b (80%)</td>
</tr>
</tbody>
</table>

* Pretreated and ISO administered

Results are expressed as mean ± SD for 6 animals.

a P<0.05 significantly different compared with control animals.
b P<0.05 significantly different compared with ISO alone treated animals.

(%) As percent of control group.

Figure 6.3
key role in the energy homeostasis of cells with intermittently high, fluctuating energy requirements, such as skeletal and cardiac muscle.

Assay of the activity of CK is important in the detection of myocardial infarction because of the marked abundance of this enzyme and virtual absence from most other tissues, considering also its consequent sensitivity. Measuring CK isoenzyme activity is useful not only as an index of early diagnosis of MI, but also generally as an indicator for assessing any kind of myocardial injury. Isoproterenol-induced myocardial infarction serves as a well standardized model to study the beneficial effects of many drugs on cardiac function (Ithayarasi and Devi, 1997).

Our studies recorded a significant increase in CK activity in serum of isoproterenol induced animals and similar observations were made by others as well (Mini and Rajamohan, 2002; Anuraj and Rajamohan, 2003). The increased activity of serum CK might be due to cardiac damage induced by it (Ahmed et al., 2004).

The proteins from different food sources have an ameliorative influence. Soy protein and garlic protein seem to have a highly protective influence on the heart tissue. Although the serum level of CK was higher than control indicating the leakage of the enzymes from the heart, the level of the enzymes in the cardiac tissue remained unchanged.

The whey and casein protein fed animals had, although had a significant decrease in serum and increase in heart CK values, when compared with the induced ISO group, this restoration was in no way closer to the animals treated with soy, garlic and coconut protein.

Low-density lipoproteins have a central role in the atherosclerotic process. LDLs penetrate the walls of blood vessels, where they are oxidized by free radicals and accumulate in the form of gruel-like material that blocks the blood vessel lumen; this material leak into the blood vessel to cause thrombosis. High density lipoprotein (HDL) cholesterol has a protective effect and acts in preventing LDL oxidation and in the removal cholesterol of that accumulates in the blood vessel wall (Messina, 1995).

A significant decrease in serum CK activity following soy protein pretreatment has been reported by Chukwu et al. (2007). Soy protein exerts several antiatherogenic effects. First, it decreases LDL cholesterol levels significantly. Secondly, it tends to increase HDL cholesterol levels (Anderson et al., 1995).
Thirdly, soy isoflavones have antioxidant properties which protect LDL from oxidation (Hutchins et al., 1995). Fourthly, soy isoflavones have favorable effects on blood vessel function (Wei et al., 1995). Soy by virtue of its antiatherogenic effects and antioxidative property might have prevented ISO induced myocardial infarction.

Coconut protein is found to have high content of arginine (Table 2.1) and is reported to prevent ISO induced damage and thereby restored the activities of all cardiac markers to near normal levels compared to ISO treated animals. The other three protein isolates, in the order coconut, whey and casein had lowered cardiac injury and hence had a relatively a lower serum CK values than the ISO induced animals.

In our studies, garlic protein pretreatment was equally effective in preventing ISO induced myocardial infarction. Most of the animal proteins which were proven to be hypercholesterolemic had a lysine:arginine ratio near about 2. The plausible mechanism by which garlic protein might have acted is through its low lysine:arginine ratio of 0.77.

Even though whey protein and casein was not very effective, had augmented CK values compared to ISO treated group. Several biologically active antithrombotic, antioxidant peptides have been reported in these proteins. We speculate that these peptides in whey and casein would have prevented ISO induced alteration in cardiac markers and thereby prevented myocardial infraction.

6.4.4. Lactate dehydrogenase - Results

The levels of lactate dehydrogenase in serum and heart tissue of control and experimental animals are shown in Table 6.3.

**Serum LDH:** A significant increase (P<0.05) in serum LDH activity was observed in ISO alone treated mice when compared to control animals. The level was nearly twice higher than the control animals. Animals pretreated with soy protein, garlic protein, coconut protein, whey protein and casein isolates had a significantly (P<0.05) less activity of LDH when compared to ISO alone treated mice. The extent of decrease varied with different protein isolates. The level of LDH enzyme activity in soy protein, garlic protein and coconut protein ranged between 3.63 U/L to 3.93 U/L.
Table-6.3: Levels of lactate dehydrogenase in serum and heart of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum lactate dehydrogenase (U/L)</th>
<th>Heart lactate dehydrogenase (nmoles of pyruvate liberated/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$2.90 \pm 0.53^b$</td>
<td>$119.96 \pm 1.68^b$</td>
</tr>
<tr>
<td>ISO administered</td>
<td>$6.75 \pm 0.34^a$ (232%)</td>
<td>$70.56 \pm 0.92^a$ (58%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>$3.63 \pm 0.42^b$ (125%)</td>
<td>$118.17 \pm 1.34^b$ (98%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>$3.81 \pm 0.32^{a,b}$ (131%)</td>
<td>$120.18 \pm 0.74^b$ (100%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>$3.93 \pm 0.15^{a,b}$ (135%)</td>
<td>$112.49 \pm 1.39^{a,b}$ (93%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>$4.15 \pm 0.47^{a,b}$ (143%)</td>
<td>$100.38 \pm 1.38^{a,b}$ (83%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>$4.62 \pm 0.46^{a,b}$ (159%)</td>
<td>$91.77 \pm 1.19^{a,b}$ (76%)</td>
</tr>
</tbody>
</table>

*Pretreated and ISO administered

Results are expressed as mean ± SD for 6 animals.

$^a P<0.05$ significantly different compared with control animals.

$^b P<0.05$ significantly different compared with ISO alone treated animals.

(%) As percent of control group.

Figure 6.4
On the whey protein and casein it was 4.15 U/L and 4.62 U/L respectively. There was no significant difference between soy protein treated and control diet fed groups.

**Heart LDH:** A significant decrease (P<0.05) in heart LDH activity was observed in ISO treated mice when compared to normal control animals. Following pretreatment with various protein isolates for 60 days a significant (P<0.05) increase in the activity of LDH was observed in all five groups when compared to ISO treated mice. The extent of increase varied with different protein isolates. Among the various groups the activity of LDH was unchanged in the garlic protein and soy protein and it gradually decreased in the following order; soy protein (98%), coconut protein (93%), whey protein (83%) and casein treated (76%).

### 6.4.5. Lactate dehydrogenase: Discussion

Lactate dehydrogenase is a cytosolic enzyme which is essentially present in all tissues that participate in glycolysis and exists in five different isoforms designated from LDH1-LDH5. In cardiac tissue, LDH1 and LDH2 predominate. Hence, the detection of elevated concentrations of this enzyme released into the bloodstream from the damaged tissue has become a definitive diagnostic and prognostic criterion for myocardial infarction. A study of its isoenzymes has found importance in specifically locating tissue damage (Plaa and Zimmerson, 1997).

Intraperitoneal administration of isoproterenol, a synthetic catecholamine and β-adrenergic agonist into adult rats was reported to bring about biochemical and morphological alterations in the heart tissue of experimental animals, similar to observations that have been made in human myocardial infarction (Ravichandran et al., 1990; Mohanty et al., 2004).

Similarly, in our study, isoproterenol administered mice showed a significant decrease in the activity of LDH in the heart, with subsequent increase in serum, when compared to the pattern seen in control animals (Table 6.3). An increase in the activities of total LDH in serum with a subsequent decrease of the same was observed in the myocardium of ISO-induced rats (Rajamohan et al., 2007). A similar increase in serum activities of LDH was reported (Mini and Rajamohan, 2002; Anuraj and Rajamohan, 2003)
In isoproterenol treated rats, the increased activity of the serum marker enzymes accompanied by their concomitant reduction in the heart homogenate confirms the onset of myocardial necrosis. An increase in the activities of marker enzymes in serum could be due to the leakage of enzymes from the heart as a result of isoproterenol-induced necrosis (Manjula et al., 1993) and the amount of these enzymes appearing in serum varies with proportion to the number of necrotic cells identified (Ithayarasi et al., 1996). A significant decrease in serum LDH activity following soy protein pretreatment (Chukwu et al., 2007), coconut protein (Mini and Rajamohan, 2002) was reported.

Thus in our study pretreatment with soy and garlic protein maintained the activities of marker enzymes in serum and heart close to near normal levels. Also, pretreatment with coconut protein, whey protein and casein showed a moderate effect on the activities of these enzymes in both serum and heart homogenate of the animals from their respective groups. As already discussed soy protein by its hypolipidemic action would have prevented MI, coconut which has a high content of arginine, garlic by virtue of its low lysine:arginine ratio and whey protein and casein possessing antithrombotic peptides might have prevented ISO induced MI.

Cell Lysis Marker Enzymes

6.4.6. Aspartate transaminase - Results

Table 6.4 depicts the activity of aspartate transaminase in serum and heart of control and experimental animals. Aspartate transaminase catalyses the transfer of amino group from L-alanine to α-ketoglutarate with the formation of oxaloacetate and glutamate. AST is found in every tissue of the body, including red blood cells, liver and is particularly high in the cardiac muscle. AST measurements are useful in the diagnosis and monitoring of patients with myocardial infarction.

**Serum AST:** ISO alone treated mice showed a significant (P<0.05) increase in serum AST enzyme activity when compared to those from the control group. The level was two and a half times higher than the control animals. A significant (P<0.05) decrease in the enzyme activity was observed in groups pretreated with various protein isolates. However, the extent of reduction varied with the different protein isolates. The serum concentration of AST in soy protein group was (117% as
### Table 6.4: Levels of Aspartate transaminase in serum and heart of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Aspartate transaminase (U/L)</th>
<th>Heart Aspartate transaminase (nm) of pyruvate liberated/min/mg/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.81 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.38 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISO administered</td>
<td>40.56 ± 1.38&lt;sup&gt;a&lt;/sup&gt; (241%)</td>
<td>17.68 ± 0.42&lt;sup&gt;a&lt;/sup&gt; (49%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>19.80 ± 1.77&lt;sup&gt;b&lt;/sup&gt; (117%)</td>
<td>32.16 ± 0.57&lt;sup&gt;a,b&lt;/sup&gt; (90%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>20.42 ± 1.92&lt;sup&gt;b&lt;/sup&gt; (121%)</td>
<td>30.12 ± 0.56&lt;sup&gt;a,b&lt;/sup&gt; (85%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>21.91 ± 2.67&lt;sup&gt;a,b&lt;/sup&gt; (130%)</td>
<td>27.31 ± 0.67&lt;sup&gt;a,b&lt;/sup&gt; (77%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>33.21 ± 3.29&lt;sup&gt;a,b&lt;/sup&gt; (197%)</td>
<td>25.24 ± 0.61&lt;sup&gt;a,b&lt;/sup&gt; (71%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>35.01 ± 2.82&lt;sup&gt;a,b&lt;/sup&gt; (208%)</td>
<td>22.21 ± 0.46&lt;sup&gt;a,b&lt;/sup&gt; (62%)</td>
</tr>
</tbody>
</table>

* Pretreated and ISO administered

Results are expressed as mean ± SD for 6 animals.

Test of significance: control vs others a; ISO administered vs others b

<sup>a</sup> P<0.05 significantly different compared with control animals.

<sup>b</sup> P<0.05 significantly different compared with ISO alone treated animals.

(%) As percent of control group.

---

**Figure 6.5**

![Serum-Aspartate transaminase](image1.png)

![Heart-Aspartate transaminase](image2.png)
compared to normal), and it gradually increased in the following order; garlic protein (121%), coconut protein (130%), whey protein (197%) and casein (208%). The serum level of AST in soy protein, coconut protein and garlic protein treated ranged between 19.80 U/L to 21.91 U/L, activity close to the normal control animals. However, the AST activity of soy protein and garlic protein treated mice, though slightly higher than control, was not significantly different.

**Heart AST:** A significant ($P<0.05$) decrease in heart AST enzyme activity was observed in ISO alone treated animals when compared to those from the control group. The level was more than twice lower than the control animals (Figure 6.5). A significant ($P<0.05$) increase of AST activity was observed in all groups treated with various isolates but the percentage of increase varied within groups in the following order; soy protein (90%), garlic protein (85%), coconut protein (77%), whey protein (71%) and casein treated (62%) compared to control animals.

6.4.7. Alanine transaminase -Results

Table 6.5 depicts the level of ALT in serum and heart of control and experimental animals.

**Serum ALT:** The serum ALT activity was much higher in ISO alone treated mice compared to controls. A significant decrease ($P<0.05$) in ALT activity was observed in all the protein isolates treated groups when compared to ISO alone treated groups. The ALT activity in soy protein, coconut protein and garlic protein treated ranged between 13.50 U/L to 15.76 U/L and 17.34 U/L, 28.36 U/L in whey protein and casein treated respectively. However, the ALT activity of soy protein treated mice, though slightly higher than control, was not significantly different.

**Heart ALT:** A significant decrease ($P<0.05$) in heart ALT activity was observed in ISO treated mice when compared to control animals (Figure 6.6). Following pretreatment with various protein isolates for 60 days a significant ($P<0.05$) increase in the activity of ALT was observed in all five groups when compared to ISO treated mice. The extent of increase varied with different protein isolates. Among the various groups the near normal restoration of ALP activity was recorded in the soy protein treated group (83% as compared to control), and it
Table 6.5: Levels of alanine transaminase in serum and heart of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Alanine transaminase (U/L)</th>
<th>Heart Alanine transaminase (nm) of pyruvate liberated/min/mg/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.58 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.43 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISO administered</td>
<td>32.45 ± 3.17&lt;sup&gt;a&lt;/sup&gt; (257%)</td>
<td>12.60 ± 0.36&lt;sup&gt;a&lt;/sup&gt; (46%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>13.50 ± 0.75&lt;sup&gt;b&lt;/sup&gt; (107%)</td>
<td>22.41 ± 0.41&lt;sup&gt;a,b&lt;/sup&gt; (83%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>15.94 ± 1.60&lt;sup&gt;a,b&lt;/sup&gt; (126%)</td>
<td>21.45 ± 0.45&lt;sup&gt;a,b&lt;/sup&gt; (79%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>15.76 ± 1.67&lt;sup&gt;a,b&lt;/sup&gt; (125%)</td>
<td>18.67 ± 0.40&lt;sup&gt;a,b&lt;/sup&gt; (69%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>17.34 ± 0.67&lt;sup&gt;a,b&lt;/sup&gt; (137%)</td>
<td>18.02 ± 0.46&lt;sup&gt;a,b&lt;/sup&gt; (67%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>28.36 ± 1.38&lt;sup&gt;a,b&lt;/sup&gt; (225%)</td>
<td>16.30 ± 0.64&lt;sup&gt;a,b&lt;/sup&gt; (60%)</td>
</tr>
</tbody>
</table>

* Pretreated and ISO administered

Results are expressed as mean ± SD for 6 animals.
<sup>a</sup> P<0.05 significantly different compared with control animals.
<sup>b</sup> P<0.05 significantly different compared with ISO alone treated animals.

(%) As percent of control group.

Figure 6.6
gradually decreased in the following order; garlic protein (79%), coconut protein (69%), whey protein (67%) and casein treated (60%).

6.4.8. Alanine transaminase and Aspartate transaminase - Discussion

The two transaminases aspartate amino transferase and alanine amino transferase are useful in the diagnosis and monitoring of patients with myocardial infarction. AST is found in every tissue of the body, including red blood cells, and is particularly high in the cardiac muscle; ALT is present in moderately high concentration in cardiac and skeletal muscle and in other tissues.

Intraperitoneal administration of isoprenaline, a synthetic catecholamine and β-adrenergic agonist into adult rats led to biochemical and morphological alterations in the heart tissue of experimental animals. An increase in the activities of AST and ALT in serum and simultaneously a decrease was observed in the myocardium of ISO-induced animals was recorded in our studies as well as by others in experimental studies (Mini and Rajamohan, 2002; Anuraj and Rajamohan, 2003; Rajadurai et al., 2007).

The increase in the activities of marker enzymes in serum could be due to the leakage of enzymes from the heart as a result of isoproterenol-induced necrosis (Manjula et al., 1993) and the amount of these enzymes appearing in serum varied with proportion to the number of necrotic cells identified (Ithayarasi et al., 1997). In isoproterenol-treated rats, the increased activity of the serum marker enzymes accompanied by their concomitant reduction in the heart homogenate confirms the onset of myocardial necrosis.

In the present study, we observed that soy protein isolate fed mice were effective in upholding the activities of AST and ALT both in serum and heart tissue and ameliorated ISO induced myocardial infarction (Table 6.4 and 6.5). Similarly a significant decrease in cell lysis marker enzymes in serum following soy protein pretreatment (Chukwu et al., 2007), coconut kernel protein (Mini and Rajamohan, 2002) was reported.

Although pretreatment with soy, garlic and coconut protein maintained the activities of marker enzymes in serum and heart close to near normal levels. Whey
protein and casein showed a moderate effect on the activities of these enzymes in both serum and heart homogenate.

The AST and ALT values (as percent of control) both in serum and heart tissue suggest that the rate of recovery from myocardial infarction was impressive in the ISO mice administered soy, garlic and coconut protein treated groups. The results of this study indicate a cardio protective effect of these three protein isolates. The protective role may be attributed to high arginine content in these protein isolates (Table 2.1), because L-arginine administration (at 2%) afforded protection against myocardial infarction induced by ISO (Mini et al., 2002).

Thus the consumption of soy, garlic and coconut may cause fewer incidences of MI and the major factor responsible for the cardio protective effect is attributed to high arginine content.

Antioxidant enzymes

6.4.9. Superoxide dismutase - Results

The levels of superoxide dismutase in serum and heart tissue of control and experimental animals are shown in Table 6.6.

**Serum SOD:** A significant decrease (P<0.05) in serum SOD activity was observed in ISO alone treated mice when compared to control animals. The level was nearly twice lower than the control animals. Following pretreatment with various protein isolates for 60 days a significant (P<0.05) increase in the activity of SOD was observed in all groups when compared to ISO alone treated mice. The extent of restoration in SOD activity varied with different protein isolates. Among the various groups the highest activity of SOD was recorded in the soy protein treated group (97% as compared to control), and it gradually deceased in the following order; garlic protein (89%), coconut protein (77%), whey protein (51%) and casein treated (49%). However, the SOD activity of soy protein treated mice, though slightly lower than control, was not significantly different.

**Heart SOD:** A significant decrease (P<0.05) in the activity of SOD was observed in heart tissue of ISO alone treated mice when compared to control animals. The activity was nearly twice lower than the control animals. Following pretreatment with soy protein, garlic protein, coconut protein, whey protein and casein isolates for
Table 6.6: Levels of superoxide dismutase in serum and heart of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Superoxide dismutase (Units/ml)</th>
<th>Heart Superoxide dismutase (U/mg) protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.97 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.94 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISO administered</td>
<td>0.81 ± 0.06&lt;sup&gt;a&lt;/sup&gt; (41%)</td>
<td>1.73 ± 0.16&lt;sup&gt;a&lt;/sup&gt; (43%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>1.92 ± 0.07&lt;sup&gt;b&lt;/sup&gt; (97%)</td>
<td>3.65 ± 0.26&lt;sup&gt;b&lt;/sup&gt; (92%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>1.76 ± 0.08&lt;sup&gt;a,b&lt;/sup&gt; (89%)</td>
<td>3.34 ± 0.38&lt;sup&gt;a,b&lt;/sup&gt; (84%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>1.52 ± 0.08&lt;sup&gt;a,b&lt;/sup&gt; (77%)</td>
<td>2.97 ± 0.06&lt;sup&gt;a,b&lt;/sup&gt; (75%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>1.02 ± 0.10&lt;sup&gt;a,b&lt;/sup&gt; (51%)</td>
<td>2.53 ± 0.41&lt;sup&gt;a,b&lt;/sup&gt; (64%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>0.97 ± 0.04&lt;sup&gt;a,b&lt;/sup&gt; (49%)</td>
<td>2.16 ± 0.17&lt;sup&gt;a&lt;/sup&gt; (54%)</td>
</tr>
</tbody>
</table>

* Pretreated and ISO administered

Results are expressed as mean ± SD for 6 animals.

<sup>a</sup> P<0.05 significantly different compared with control animals.

<sup>b</sup> P<0.05 significantly different compared with ISO alone treated animals.

(%) As percent of control group.

Figure 6.7
60 days a significant (P<0.05) increase in the activity of SOD was observed in all these groups when compared to ISO alone treated mice. The extent of restoration in SOD activity varied with different protein isolates. Among the various groups the highest activity of SOD was recorded in the soy protein treated group (96% as compared to control), and it gradually decreased in the following order; garlic protein (86%), coconut protein (81%), whey protein (75%) and casein (71%). However, the SOD activity of soy protein treated mice, though slightly lower than control, was not significantly different (Figure 6.7).

6.4.10. Catalase - Results

The levels of catalase in serum and heart tissue of control and experimental animals are shown in Table 6.7.

**Serum CAT:** A significant decrease (P<0.05) in serum CAT activity was observed in ISO alone-treated mice when compared to control animals. The level was nearly twice lower than the control animals. Following pretreatment with various protein isolates for 60 days, a significant (P<0.05) increase in the activity of CAT was observed in four groups except casein treated group. The extent of increase in the activity of CAT observed in soy protein treated was (100% as compared to control), and (102%) in garlic protein, coconut protein (87%), whey protein (89%) and casein (75%) respectively. However, the CAT activity in all protein isolates treated mice, though slightly lower than control, was not significantly different.

**Heart CAT:** A significant decrease (P<0.05) in heart CAT activity was observed in ISO alone treated mice when compared to control animals (Figure 6.8). We observed a significant increase of CAT activity in all animals pretreated with various protein isolates; the extent of increase in activity varied with different protein isolates. The maximum level of CAT activity was recorded in soy protein treated group (96% as compared to control), and it gradually decreased in the following order; garlic protein treated (86%), coconut protein treated (81%), whey protein treated (75%) and casein treated (71%). However, the CAT activity in soy protein treated mice, though slightly lower than control, was not significantly different.
## Table-6.7: Levels of catalase in serum and heart of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Catalase (IU/L)</th>
<th>Heart Catalase (µmol of H₂O₂ consumed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.27 ± 0.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.68 ± 1.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISO administered</td>
<td>1.56 ± 0.30&lt;sup&gt;a&lt;/sup&gt; (47%)</td>
<td>10.81 ± 0.13&lt;sup&gt;a&lt;/sup&gt; (52%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>3.28 ± 0.30&lt;sup&gt;b&lt;/sup&gt; (100%)</td>
<td>19.95 ± 1.76&lt;sup&gt;b&lt;/sup&gt; (96%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>3.36 ± 0.39&lt;sup&gt;b&lt;/sup&gt; (102%)</td>
<td>17.96 ± 1.01&lt;sup&gt;a,b&lt;/sup&gt; (86%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>2.86 ± 0.51&lt;sup&gt;b&lt;/sup&gt; (87%)</td>
<td>16.78 ± 1.27&lt;sup&gt;a,b&lt;/sup&gt; (81%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>2.93 ± 0.21&lt;sup&gt;b&lt;/sup&gt; (89%)</td>
<td>15.53 ± 0.55&lt;sup&gt;a,b&lt;/sup&gt; (75%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>2.47 ± 0.39&lt;sup&gt;b&lt;/sup&gt; (75%)</td>
<td>14.70 ± 0.75&lt;sup&gt;a,b&lt;/sup&gt; (71%)</td>
</tr>
</tbody>
</table>

* Pretreated and ISO administered

Results are expressed as mean ± SD for 6 animals.

<sup>a</sup> P<0.05 significantly different compared with control animals.

<sup>b</sup> P<0.05 significantly different compared with ISO alone treated animals.

(%) As percent of control group.

**Figure 6.8**
6.4.11. SOD and CAT - Discussion

Free radicals are molecules having an unpaired electron in the outer orbit of the electron shell. There are two facets to free radicals in biology; they serve as signaling and regulatory molecules at physiologic levels, but as highly deleterious and cytotoxic oxidants at pathologic levels. Free radicals, formed in the body and in the environment due to a number of factors, react with cellular components and cause damage.

Free radicals are known to be highly reactive species that have been implicated in the pathogenesis of many diseases and in myocardial infarction. During myocardial infarction, reactive oxygen species like superoxide, hydrogen peroxide and hydroxyl radicals are produced in enormous amounts (McCord, 1988), which contributes to myocardial tissue injury. Cardiovascular actions of isoproterenol have been shown to initiate processes culminating in cardiac necrosis (Stanton and Schwartz, 1967).

The removal of free radicals is achieved through enzymatic and non-enzymatic reactions. The effects of free radicals are controlled enzymatically by a wide range of antioxidant enzymes such as SOD, glutathione peroxidase and catalase. Super oxide dismutases are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide, and thus, form a crucial part of the cellular antioxidant defense mechanisms (Malstrom et al., 1975). Catalase converts hydrogen peroxide to molecular oxygen and water.

During myocardial infarction, super oxide radicals generated at the site of damage modulate SOD and CAT, resulting in the loss of activity and accumulation of super oxide radical, which damages the myocardium. In our study, a significant decrease of SOD and CAT activity was observed in serum and heart tissue of ISO administered mice. The results of the present study (Table 6.6 and 6.7) are in accordance with the observations made through previous work (Manjula and Shymala, 1994), who showed that SOD and CAT activities were decreased in isoproterenol administration. The decrease in SOD and CAT may be due to the involvement of superoxide and $H_2O_2$ free radicals in myocardial cell damage mediated by ISO (Guarnier et al., 1980).
In vivo, there is a high degree of interaction among endogenous and exogenous antioxidants depending on the order of their corresponding redox potentials; it is common for one antioxidant to regenerate another one from its oxidized species. Antioxidants destroy free radicals in vivo and supplementation of antioxidant rich foods offers increased protection against oxidative damage (Fang et al., 2002).

Diets rich in natural antioxidants are associated with reduced risk of heart diseases. Soy protein, garlic protein and coconut protein pretreatment has been found to prevent the loss of the activity of SOD and CAT followed by ISO administration and also been able to scavenge superoxide radicals and thus reduce myocardial damage caused by free radicals. Pretreatment with whey protein and casein showed a minimal increase in the activities of SOD and CAT.

In the present study administration of garlic protein caused a significant increase in basal SOD and catalase activities in mice serum and heart, which was associated with a concomitant decrease in basal lipid peroxidation both in plasma and heart which parallels with the view of Banerjee et al. (2002). Simultaneous increase in SOD and catalase activities has special importance of being more beneficial than increase in SOD activity alone, because without a concomitant increase in catalase activity, increased SOD activity may lead to intracellular accumulation of H$_2$O$_2$ with detrimental effects (Yim et al., 1990).

In conclusion, the present results indicate that the protective effect of various protein isolates in ISO induced myocardial like situation in mice could be related to its antioxidant defense system.

6.4.12. Lipid peroxidation - Results

The levels of LPO in plasma and heart tissue of control and experimental animals are shown in Table 6.8.

Plasma LPO: In the present study, the level of MDA in plasma were significantly (P<0.05) increased in isoproterenol administered animals, when compared with control animals. Whereas feeding of animals with soy protein, garlic protein, coconut protein and whey protein significantly (P<0.05) decreased the MDA levels. Pretreatment with soy protein and garlic protein reduced the MDA levels.
### Table 6.8: Lipid peroxidation in plasma and heart of control and experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma Lipid peroxidation (nmol / mL)</th>
<th>Heart Lipid peroxidation (nm) of MDA/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.48 ± 0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.55 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISO administered</td>
<td>4.85 ± 0.30&lt;sup&gt;a&lt;/sup&gt; (195%)</td>
<td>7.24 ± 0.34&lt;sup&gt;a&lt;/sup&gt; (159%)</td>
</tr>
<tr>
<td>Soy protein*</td>
<td>2.70 ± 0.26&lt;sup&gt;b&lt;/sup&gt; (108%)</td>
<td>4.70 ± 0.69&lt;sup&gt;b&lt;/sup&gt; (103%)</td>
</tr>
<tr>
<td>Garlic protein*</td>
<td>2.84 ± 0.21&lt;sup&gt;b&lt;/sup&gt; (114%)</td>
<td>4.81 ± 0.29&lt;sup&gt;b&lt;/sup&gt; (105%)</td>
</tr>
<tr>
<td>Coconut protein*</td>
<td>3.42 ± 0.43&lt;sup&gt;a,b&lt;/sup&gt; (137%)</td>
<td>5.64 ± 0.41&lt;sup&gt;a,b&lt;/sup&gt; (123%)</td>
</tr>
<tr>
<td>Whey protein*</td>
<td>4.14 ± 0.23&lt;sup&gt;a,b&lt;/sup&gt; (166%)</td>
<td>5.62 ± 0.42&lt;sup&gt;a,b&lt;/sup&gt; (123%)</td>
</tr>
<tr>
<td>Casein*</td>
<td>4.25 ± 0.52&lt;sup&gt;a&lt;/sup&gt; (171%)</td>
<td>6.75 ± 0.34&lt;sup&gt;a&lt;/sup&gt; (148%)</td>
</tr>
</tbody>
</table>

*Pretreated and ISO administered

Results are expressed as mean ± SD for 6 animals.

<sup>a</sup> P<0.05 significantly different compared with control animals.

<sup>b</sup> P<0.05 significantly different compared with ISO alone treated animals.

(%) As percent of control group.

### Figure 6.9

![Plasma-Lipid peroxide](image1)

![Heart-Lipid peroxide](image2)
significantly from ISO induced animal values and the values were comparable to the control, whereas casein treated animals were similar to ISO induced. Among the various groups, the concentration of plasma MDA was comparable to the control in the soy protein group (108%) and it gradually increased in the following order; garlic protein (114%), coconut protein (137%), whey protein (166%) and casein treated (171%). Results were expressed as nmol/ml and nmoles of MDA/mg protein respectively. However, the level of MDA in soy protein treated and garlic protein treated mice, though slightly higher than control, was not significantly different.

**Heart LPO:** Isoproteronol treated mice showed a significant \((P<0.05)\) increase in the heart MDA level when compared to control mice. The level was nearly twice higher than the control mice. The pattern of MDA level in the cardiac tissues in animals treated with various isolates was similar to that of the plasma MDA pattern. The percentage of values varied within groups in the following order soy protein treated (103%), garlic protein treated (105%), coconut protein treated (123%), whey protein (123%) and casein treated (148%) compared to control animals. However, the MDA level in soy protein and garlic protein treated mice, though slightly higher than control, was not significantly different. The MDA level of casein treated group was not significantly different from ISO induced group (Figure 6.9). Casein and whey protein treated animals had MDA values significantly less than the ISO induced. This is suggestive of a moderate impact of these two protein isolates in influencing lipid peroxidation.

**6.4.13. Lipid peroxide - Discussion**

Formation of reactive oxygen species is a normal consequence of a variety of essential biochemical reactions. It is a proved fact that oxygen radicals could be formed in excess in chronic diseases. Oxygen radical production, which increases with clinical progression of diseases, involves increased lipid peroxidation, as a result of which, there is cellular membrane degeneration and extensive DNA damage.

Lipid peroxidation is an important pathogenic event in myocardial infarction and the accumulated lipid peroxide levels reflects the various stages of the disease and its complication (Goli Kov et al., 1989). The extent of lipid peroxidation could be determined by estimating the amounts of final lipid peroxidation products -
malondialdehyde and 4-hydroxynonenal; compounds known to produce protein cross-linking through Schiff’s base with DNA and thus lead to DNA damage (Sharma, 2001).

Quantification of lipid peroxidation is essential to assess the role of oxidative injury in pathophysiological disorders (Halliwell et al., 1996). Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free radicals of oxygen (Valenzuela, 1990). MDA formed during oxidative stress is considered to be an indicator of lipid peroxidation (Neilsen et al., 1990).

We observed a significant increase in the level of LPO in plasma and in heart tissue of ISO treated animals (Table 6.8). Similarly it was reported that rats induced with ISO (150 mg/kg body weight) for 2 days resulted in a marked elevation in lipid peroxidation, serum marker enzymes and led to a significant decrease in the activities of endogenous antioxidants (Saravanan and Jayaprakash, 2004; Devi and Vijayaraghavan, 2007).

In the present study, soy protein, garlic protein, and coconut protein treated animals showed a significant decrease level of lipid peroxide. Therapeutic influence of soy and casein protein in atherosclerosis progression has been documented by Nagilu et al. (2001). Soy protein isolate in comparison with casein was found to decrease lipid peroxide. In our studies not only soy protein, but garlic protein was also observed to be equally effective.

6.4.14. Histology - Result

Histological examination of the myocardium of animals showed clear integrity of myocardial cell membrane. Endocardium and pericardium were seen with normal limits. No inflammatory cell infiltration was seen (Plate 12). Heart tissues from isoproterenol treated mice showed widespread myocardial structure disorder and subendocardial necrosis with capillary dilation and leukocyte infiltration as compared to control group (Plate-13).

Soy protein pretreated animals showed the heart sections to have normal histoarchitecture similar to that of normal mice (Plate 14). Animals pretreated with coconut protein showed mild myocytic necrosis with moderate infiltration of leucocytes (Plate 16). Garlic protein pretreated mice exhibited decreased degree of
necrosis (mild) and less infiltration of inflammatory cells. Occasional areas of focal myonecrosis were observed (Plate 15).

Animals pretreated with whey protein showed less area of infarction with splitting of cardiac muscle fibers with less inflammatory cells (Plate 17). Casein pretreated mice showed myocardial fibers with coagulative necrosis with edema and less inflammatory cells (Plate 18).

6.4.15. Histology - Discussion

In the present study, normal architecture of the myocardium was observed in control diet fed animals. Animals treated with ISO had histological changes such as mononuclear cellular infiltration, interstitial edema, fragmentation and segmentation of muscle fibers, vacuolar degeneration, hemorrhage, and congestion of the myocardium. Similar observations were reported in ISO administered animals (Rajadurai and Stanely, 2006; Devika and Stanely, 2007; Vandana et al., 2008).

In soy protein pretreated group, the heart sections showed more or less normal architecture to that of control animals. In this group of animals histological findings parallels the results of various biochemical parameters such as cardiac marker enzymes, cell lysis marker enzymes, antioxidant enzymes and lipid peroxide in which the ISO induced changes were restored with soy protein treatment.

A microscopically cellular change in garlic protein pretreated group was less as compared to ISO treated group. The histological changes such as mononuclear cellular infiltration, fragmentation and segmentation of muscle fibers, vacuolar degeneration were reduced with garlic protein pretreatment. The myocardium regained its histological architecture resembling near normal. Changes like hemorrhage, vacuolar degeneration, edema were absent in coconut protein fed animals.

In garlic protein and coconut protein treated group the extent of damage brought about by ISO administration were found to be less compared to whey and casein fed groups. Treatment with tender coconut water prevented from myocardial necrosis with tissue architecture similar to normal animals (Anurag and Rajamohan, 2004).
Plate 12: Heart section of control mice
(Stained with hematoxylin and eosin)

Highlights
1) Normal myocardial cell architecture
2) Endocardium and pericardium are normal
Plate 13: ISO administered mice heart section

N-Nucleus, LI-Leukocyte infiltration, SM-Segmentation of muscle fiber

Highlights
1) Myocardial structure disorder
2) Subendocardial necrosis
3) Segmentation of muscle fibers
4) Interstitial edema
5) Vacuolar degeneration
6) Haemorrhage
Plate 14: Soy protein treated mice heart section

Highlights
1) Likeness of normal myocardial cell
2) Normal endocardium and pericardium
3) Absence of inflammatory cells
Plate 15: Garlic protein treated mice heart section

Highlights
1) Likeness of normal myocardial cell
2) Absence of inflammatory cells
3) Mild necrosis
4) Reduced vacuolar degeneration
Plate 16: Coconut protein treated mice heart section

Highlights
1) Moderate infiltration of mononuclear cells
2) Mild myocytic necrosis
3) Less segmentation of muscle fibers
Plate 17: Whey protein treated mice heart section

![Image of heart section with annotations]

N-Nucleus, S-Segmentation

Highlights
1) Segmentation of cardiac muscle fibre
2) Mononuclear cell infiltration
3) Vacuolar degeneration
4) Edema
5) Coagulative necrosis
Plate 18: Casein protein treated mice heart section

Highlights
1) Segmentation of muscle fibers
2) Coagulative necrosis
3) Extensive edema
4) Vacuolar degeneration
Animals pretreated with whey protein exhibited splitting of cardiac muscle fibers, mononuclear cellular infiltration, and fragmentation of muscle fibers with vacuolar degeneration. Changes like splitting of cardiac muscle fibers, vacuolar degeneration, coagulative necrosis with extensive edema was observed in casein protein fed animals.

The results of biochemical studies are thus, confirmed by the histological observations.

6.4.16. Candidate Proteins - A General Discussion

Cardiovascular diseases such as atherosclerosis and myocardial infarction are a significant public health problem worldwide. Attempts to prevent cardiovascular disease often imply modifications and improvement of causative risk factors. In addition to numerous preventive and therapeutic drug regimens, there has been increased focus on identifying dietary compounds that contribute to cardiovascular health in recent years. Food derived proteins and bioactive peptides, in particular peptides released during gastrointestinal digestion or food processing from a multitude of plant and animal proteins, especially milk, soy, fish and other proteins represent such source of health enhancing components.

Biologically active peptides exert physiologically hormone like effects in humans. They are inactive within the sequence of their parent protein and are released by enzymatic hydrolysis during gastrointestinal digestion. They usually contain 2-20 aminoacids per molecule. Depending on the sequence of aminoacids, these peptides exhibit diverse activities. Certain studies have shown that biologically active peptides have opiate like immunomodulatory, antimicrobial, antioxidant, antithrombotic and anti-hypertensive actions. Antihypertensive inhibitory peptides, antioxidative peptides, antioxidant peptides, antithrombotic peptides, hypocholesterolemic peptides and antiobesity peptides derived from milk, soy, egg, fish, wheat and meat have also been reported by Erdmann et al. (2008).

In the present study, dietary protein isolates from five sources tended to prevent myocardial infarction. Various mechanisms have been proposed to explain as to how proteins might influence cardiovascular diseases.
i) Soy protein

In our study, administration of isoproteronol led to a significant alteration in cardiac marker enzymes, cell lysis marker enzymes, antioxidant enzymes and lipid peroxide levels. Pretreatment with various protein isolates were found to restore the altered levels of various parameters both in serum and heart tissues. The effect varied with different protein isolates, soy protein being the most effective among the five different sources. Several mechanisms have been proposed for soy protein preventing cardiovascular diseases.

Various factors have been cited for myocardial infarction, of which hyperlipidemia is believed to be a high risk factor that leads to myocardial infarction. There is increasing evidence that consumption of soy protein in place of animal protein lowers blood cholesterol levels and provides cardiovascular benefits. Clinical studies by Anderson et al. (1995) showed that soy protein significantly lowered total cholesterol, LDL cholesterol and triglyceride without affecting HDL cholesterol. Furthermore it suggested that the effects were greater in subjects with higher base line cholesterol values.

Soy protein contains all of the essential aminoacids in sufficient quantities to support human life and is therefore complete protein. Hypocholesterolemic effects of soy protein were attributed to its trypsin inhibitors, phytic acid, saponins and isoflavones. Trypsin inhibitor exerts hypocholesterolemic effect by increasing the secretion of cholecystokinin. This then stimulates bile acid synthesis from cholesterol and helps eliminate cholesterol through gastrointestinal tract.

Phytic acid and myoinositol hexaphosphate present in soy protein chelates zinc strongly in intestinal tract, thus decreasing its absorption. High ratio of zinc to copper resulted in a rise of blood cholesterol. It is reported that soy protein foods contain both copper and phytic acid and therefore lowers cholesterol levels by decreasing the ratio of zinc to copper. Saponins present in soy protein lowers cholesterol by increasing bile excretion (Sidhu, 1986).

Soybean protein was reported to accelerate fatty acid beta oxidation and suppress triglyceride synthesis in rat and mice thus acting as a hypocholesterolemic agent and preventing cardiovascular disease risk factors.
Animal studies have shown that amino acids lysine and methionine raise cholesterol levels whereas arginine has opposite effects (Kurowska and Carroll, 1994). Soy protein, compared with animal protein sources, has a higher ratio of arginine to lysine and methionine. The higher arginine to lysine ratio of soy protein decreases insulin and glucagon secretion thereby inhibits lipogenesis (Potter, 1995). Sirtori et al. (2001) reported that consumption of soy protein up regulates LDL receptors in humans. Cholesterol lost from the body in the form of bile shifts the liver toward providing more cholesterol for increased bile acid synthesis and increases LDL receptor activity. Thus, the end result is increased LDL removal from the blood. Thus soy protein was found to lower cholesterol by enhanced bile excretion. Thus soy protein consumption effectively prevents hyperlipidemia and reduces the risk of myocardial infarction.

Soy protein in addition to yielding health effects mediated through its amino acid content is also reported to contain a series of estrogen-like compounds termed isoflavonoids (Figure 6.10). Scientific studies offer evidence suggesting that these isoflavonoids, especially genistein and daidzein, exist as components of soy protein that possess antioxidant properties and are involved in the regulation of circulating lipid levels (Van, 2000). An isoflavone rich extract by itself was shown to have no hypocholesterolemic activity, and therefore; it may be important to consume a soy protein isolate that contains at least a minimal amount of isoflavones (Sirtori, 2001).

Evidence suggest that isoflavones ingested in soy proteins undergo biotransformation by intestinal microflora and are absorbed into the circulation where they affect endogenous estrogen levels. These phytoestrogens and their metabolites have been reported to have several hormonal and non hormonal activities (Setchell, 1998). Mechanisms by which soy isoflavones inhibit atherosclerotic development, independent of their effects on circulating lipoproteins, include antioxidant effects, antiproliferative and antimigratory effects on smooth muscle cells, on thrombus formation and in the maintenance of normal vascular reactivity (Anthony et al., 1998). It has been reported that the consumption of soy protein specifically increases circulating levels of genistein; which inhibits the activity of tyrosine kinase, an enzyme associated with plaque development (Wilcox et al., 1995).
Elevated blood pressure is yet another major independent risk factor for CVD. Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of blood pressure as it promotes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II as well as inactivates the vasodilator bradykinin (Figure 6.11).

Food proteins have been identified as sources of ACE inhibitory peptides. Soy protein is reported (Kodera and Nio, 2006) to have ACE inhibitory peptide glycinin (NWGPLV sequence), antioxidant peptide β-conglycinin (LLPHH) (Chen et al., 1995) and thus prevents oxidant induced damage to cardiovascular system. In conclusion, we may say that soy protein rich in isoflavones, having antihypertensive peptide and antioxidant peptide may have protected ISO induced damage.

ii) Garlic protein

It has been reported that garlic protein isolate protect the blood vessels from the deleterious effects of free radicals and exerts a positive influence on blood lipids and thereby increases capillary flow and lowers elevated blood pressure and prevents the development of arteriosclerosis. Garlic antiatherosclerotic effects are based on the reduction of thrombocytes adhesiveness and aggregation. The tendency of the platelets to aggregate and to form thrombi is significantly decreased by the effective constituents of garlic and as a result fibrinolysis is enhanced, resulting in more rapid dissolution of coagulated blood, plaques and clots and thus preventing myocardial infarction (Popov et al., 1994).

Plant proteins have been shown to be less cholesterolemic than animal proteins in both experimental animals and in humans. Biju et al. (1996) reported that the concentration of cholesterol and triacylglycerol were significantly lower in serum and tissues of the rats treated with garlic protein. Also the concentration of VLDL, LDL fractions of serum was significantly lower in rats treated with garlic protein. The plausible mechanism of action of garlic protein is attributed to the aminoacid composition of protein and the non-protein substances accompanying these proteins. It was reported that the ratio of lysine: arginine of protein affect atherogenicity (Kirtchevesky et al., 1981). Casein with a lysine: arginine ratio of 2 was found to be significantly more atherogenic than soy protein with lysine: arginine ratio of 0.84. Most of the animal proteins which were proven to be hypercholesterolemic had a
Figure 6.10: Structure of dietary isoflavonoids genistein and daidzein compared with estradiol (Peter, 2002)
Figure 6.11: Role of ACE inhibitor peptides on blood pressure regulation

- Angiotensinogen
- Renin
- Angiotensin I
- Bradykinin
- ACE
- Angiotensin II
- Inactive Fragments
- Vasoconstriction
  Blood pressure↑

ACE inhibitors
ACE inhibitory peptides

*Vasodilatory*
lysine/arginine ratio near about 2. The low lysine/arginine ratio of 0.77 in garlic protein may contribute to its hypolipidemic action.

Small glycopeptides released from garlic protein, as products of digestion was absorbed and had effect on lipid metabolism in liver (Block, 1992). It is evident that cysteine derivatives (S-methyl or allyl cysteine sulfoxides) that belong to the free aminoacid of garlic protein showed hypocholesterolemic effect (Itokawa et al., 1973). Therefore, garlic protein having hypocholesterolemic effect and low lysine: arginine ratio could have effected protection against ISO induced damage and protected myocardium, thus restoring altered levels of various cardiac markers.

iii) Coconut protein

Even though pretreatment with various protein isolates were found to restore the altered levels of various parameters both in serum and heart tissues, coconut protein was equally effective compared to garlic protein.

The coconut kernel has 5-6% proteins (Mini et al., 2002); the major fraction being globulins (Padmakumaran et al., 1998) and it is found to significantly lower cholesterol levels in rats fed free cholesterol. Analysis of coconut protein was reported to contain 2.13% lysine and 24.5 % arginine, giving a low lysine /arginine ratio of 0.86. Experimental studies have shown that lysine /arginine ratio of the protein significantly influence on cholesterol metabolism (Kirtchevsky et al., 1981).

Casein diet being atherogenic, inclusion of arginine to a casein diet resulted in significant lowering of cholesterol levels (Rajamohan and Kurup, 1986)), while the addition of lysine had the opposite effect (Muraleedharakurup, 1984). Oral administration of L-arginine was found to reduce myocardial infarction (Casino et al., 1994) cardio protective effect in isoproteronol induced myocardial infarction (Sreepriya et al., 2005).

The results of our study confirm the cardio protective effect of coconut protein. Also these results indicate that the major factor responsible for the cardio protective effect is higher arginine content. It has been reported that cardio protective effect of L-arginine was mediated by L-arginine nitric oxide pathway (Prenow and Wang, 1999). Nitric oxide is synthesized in vascular endothelial cells from L-arginine
by the enzyme nitric oxide synthase. Increased nitric oxide synthase activity improves cardiac function, enhances myocardial blood flow and may be beneficial in the treatment of acute myocardial infarction.

Feeding coconut protein increased nitric oxide synthase activity in the heart, which may improve cardiac function (Mini and Rajamohan, 2002). Nitric oxide functions in the regulation of blood pressure, platelet adhesion, neutrophil aggregation as well as synaptic function in the brain. Its chemical and biological properties are well suited for its non physiological and pathophysiological functions. It also helps in the dilation of blood vessel correcting the inadequate blood supply to the heart muscle. From these effects, it is clear that the L-arginine nitric oxide pathway plays a protective role in cardiac function against the deleterious effects caused by the isoproteronol administration. It is evident from the present results that consumption of coconut protein may reduce the incidence of myocardial infraction.

iv) Whey protein and casein

Similarly whey protein and casein were found to prevent isoproterenol induced myocardial infarction, but they were not very effective as compared to other protein isolates. The mechanism by which these proteins may have prevented MI is through the presence of antioxidant peptide (origin casein-sequence YFYPEL), antithrombotic (K-casein MAIPPKKNQDK (casoplatelin) and smaller fragments and lactoferrin (KRDS-sequence) and ACE inhibitory peptides in it (Jolles et al., 1986; Raha et al., 1988; Suetsuna et al., 2000).

In the present study, of all the five protein isolates studied soy protein, coconut protein and garlic protein were found to be more effective than whey and casein in promoting recovery from myocardial infarction. And we may attribute the beneficial effect to the high content of arginine and also to the presence of antithrombotic, angiotensin converting enzyme inhibitory peptides in them.

Thus it is obvious that soy protein, garlic protein and coconut protein are good dietary regimens for the prevention of CVD because they show minimal indications of tissue damage and changes in levels of biochemical markers of disease. Other proteins are not as efficient as the aforementioned protein isolates.
Although the protein part of coconut is generally used as animal feed after the extraction of oil, its importance as a therapeutic molecule should be highlighted and recommended. People are aware of the soy protein and it is promoted as neutraceuticals. In a similar fashion, the garlic and coconut protein may be recommended as a result of present study.