DIETARY MANIPULATION OF FAT ON MITOCHONDRIAL DECAY AND ITS IMPLICATIONS ON AGEING AND APOPTOSIS

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2009

CERTIFICATE

This is to certify that the thesis entitled **"DIETARY MANIPULATION OF FAT ON MITOCHONDRIAL DECAY AND ITS IMPLICATIONS ON AGEING AND APOPTOSIS"** submitted in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **VETERINARY PHYSIOLOGY** to the Tamil Nadu Veterinary and Animal Sciences University, Chennai-51, is a record of bonafide research work carried out by **Dr. CORBON GODFREY DAVID, I.** under my guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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ABSTRACT

Title	:	***Dietary manipulation of fat on mitochondrial decay and its implications on ageing and apoptosis***
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An investigation was carried out to study the effect of dietary manipulation of fat and calorie restriction (CR) on the production of ROS in relation to activity of mitochondrial electron transport complexes and its implications on apoptosis in ageing heart and brain.

Eighteen months old male Wistar Albino rats were fed on coconut and corn oil based diets *ad libitium* or at 40 per cent CR up to 24 (short term) or 30 months (long term) of age. At the end of feeding trial rats were sacrificed. Mitochondria from heart and brain were isolated for the estimation of membrane fatty acid composition, H_2O_2 and OXPHOS enzyme activity. Mitochondrial inner membrane surface area was measured stereologically from electron micrographs. Antioxidant enzyme status, markers of oxidative stress measured as TBARS and protein carbonyls and DNA fragmentation were measured in both the tissues.

Myocardial collagen and serum lipid profile were also estimated to correlate the effect of dietary fat and CR on retarding ageing.

Both short and long term feeding of linoleic acid low coconut oil and linoleic acid rich corn oil based diets with CR increased oleic (18:1) linoleic acid (18:2) levels respectively in the heart and brain mitochondrial membranes. Reinforcement of 18:1 levels in membranes increased inner membrane surface area, membrane potential and reduced H2O2 production in rats fed on coconut oil based diet with CR compared to corn oil diet. The oxidative enzyme activities did not show appreciable age related variation in rats fed on coconut oil and corn oil based diets.

Although, short term feeding of both coconut oil and corn oil based diets with CR effectively minimized age related changes in heart and brain, on the long run coconut oil based diet with CR was found effective to reduce oxidative damage to lipids and proteins but not DNA through enhanced antioxidant status. It is concluded that coconut oil based diets with CR reduces age related changes in heart and brain through reinforcement of membrane fatty acids, improved membrane integrity and decreased oxidative stress.

Key words: Calorie restriction- Dietary fat- coconut oil- corn oil- mitochondria – oxidative stress- antioxidant status- apoptosis – ageing- heart – brain.

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LIST OF ABBREVIATIONS

%	:	Per cent
° C	:	Degree Celcius
ABTS	:	2, 2`- azino bis 3-ethyl benzothiazoline 6- sulfonic acid
ADP	:	Adenosine di phosphate
AIN	:	American Institute of Nutrition
APS	:	Ammonium per sulphate
ATP	:	Adenosine tri phosphate
BSA	:	Bovine Serum albumin
C16:0	:	Palmitic acid
C16:1	:	Palmitoleic acid
C18:0	:	Stearic acid
C18:1	:	Oleic acid
C18:2	:	Linoleic acid
C18:3	:	Linoleinic acid
C20: 4	:	Arachidonic acid
C20:5/ EPA	:	Eicosapentaenoic acid
C22:6/ DHA	:	Decosahexaenoic acid
CAT	:	Catalase
Cm	:	Centimeter (s)
Complex I	:	NADH- ubiquinone oxidoreductase
Complex II	:	Succinate – ubiquinone oxidoreductase
Complex III	:	Ubiquinone – cytochrome c oxidoreductase
Complex IV	:	Cytochrome c oxidase

Complex V : Adenosine tri phosphate synthase

Co-Q / Q : Coenzyme Q / Ubiquinone,

CR : Calorie Restriction

Cu/Zn SOD: Copper zinc Super oxide dismutase

DNA : Deoxyribose nucleic acid

DTNB : 5,5`- dithiobis-2-nitrobenzoic acid

e^- : Electron(s)

EDTA : Ethylene diamine tetra acetic acid

EFA : Essential fatty acid

EGTA : Ethylene glycol bis (amino ethyl ether) N,N,N`,N` tetra acetic acid

ELISA : Enzyme linked immunosorbent assay

EM : Electron microscope

EPA : Eicosapentaenoic acid

ET : Electron transport

ETC : Electron transport chain

FA : Fatty acid(s)

FADH2 : Reduced flavin adenosine dinucleotide

g : Gram(s)

GSH : Reduced glutathione

GSH-Px / GPx : Glutathione peroxidase

GSSG : Oxidized glutathione

H_2O_2 : Hydrogen peroxide

HDL : High density lipoprotein

IU	:	International Units
Kg	:	Kilogram
L	:	Liter(s)
LA	:	Linoleic acid
LDL	:	Low density lipoprotein
LPO	:	Lipid Peroxidation
M	:	Mole(s)
mA	:	Milliampere
MDA	:	Malondialdehyde
mg	:	Milligram (s)
Min	:	Minutes
ml	:	Milliliter (s)
mM	:	Millimole (s)
MMP/ $\Delta\varphi m$	:	Mitochondrial membrane potential
Mn-SOD	:	Manganese super oxide dismutase
mRNA	:	Messenger ribonucleic acid
mt DNA	:	Mitochondrial deoxy ribose nucleic acid
MUFA	:	Monounsaturated fatty acid
mV	:	Millivolts
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Reduced nicotinamide adenine dinucleotide
nm	:	Nanometer(s)
nM / nmol	:	Nanomoles(s)
O_2^-	:	Super oxide

•OH	:	Hydroxyl radical
OXPHOS	:	Oxidative phosphorylation
PC	:	Phosphatidylcholine
PE	:	Phosphatidyl ethanolamine
PGI2	:	Prostaglandin I2
PHPA	:	Parahydroxy phenyl acetic acid
PMF	:	Proton motive force
PUFA	:	Polyunsaturated fatty acid
ROS	:	Reactive oxygen species
Rpm	:	Revolutions per minute
RT	:	Room temperature
SDS	:	Sodium dodecyl sulphate
SE	:	Standard error
SFA	:	Saturated fatty acid
SOD	:	Super oxide dismutase
TBA	:	Thiobarbituric acid
TBARS	:	Thiobartituric acid reactive substances
TCA	:	Trichloroacetic acid
TMRM	:	Tetra methyl rhodamine methyl ester
U	:	Units
UFA	:	Unsaturated fatty acid
V	:	Volts
VLDL	:	Very low density lipoprotein
W/v	:	Weight / volume

x g	:	Gravitational force
µg	:	Micro gram(s)
µl	:	Micro liter(s)
µM	:	Micro mole(s)
ω- 3 PUFA	:	Omega-3 polyunsaturated fatty acid
ω- 6 PUFA	:	Omega-6 polyunsaturated fatty acid

To know how to grow old is the master work of wisdom and one of the most difficult chapters is the great act of living.

-Amiel-

CHAPTER 1

INTRODUCTION

Ageing is an increase in intrinsic mortality and a decrease in reproductive rate. This multifaceted physiological process is regulated by the homeostatic and integrative mechanisms of the body. It is the accumulation of the diverse deleterious changes in cells and tissues leading to increased oxidative stress and increased accumulation of products of oxidation. This deterioration is based on the changes in tissues and sub cellular structures over time.

Ageing is a fascinating and highly important topic because of its great social relevance and scientific complexity. One of the most important theories to explain this multifunctional process is the free radical theory, of Harman (1956). According to his theory free radicals produced during aerobic respiration causes cumulative oxidative damages resulting in ageing and death. The electron reduction of O_2 generates super oxide (O_2^-) and hydrogen peroxide (H_2O_2), both of which are generated by numerous routes *in vivo*. In the presence of free transition metals *viz.* Fe and Cu, O_2^- and H_2O_2 together generate the extremely reactive hydroxyl radical (**.**OH), which is responsible for initiating oxidative destruction of macro biomolecules.

There are numerous sites of oxidant generation, four of which have attracted much attention: mitochondrial electron transport chain (ETC), peroxisomal fatty acid (FA) metabolism, cytochrome P-450 reaction and the "respiratory bursts" of the phagocytic cells (Beckman and Ames, 1998). In addition to these there exist numerous other enzymes capable of generating oxidants under pathological conditions in a tissue specific manner as observed by the deamination of dopamine by monoamine oxidase generating H_2O_2 in some neurons.

Mitochondrial electron transport chain is a major source of cellular O_2^- which yields relatively stable H_2O_2 by dismutation and the highly reactive **.**OH species. The pumping of protons into the inter membrane space by complexes I, III and IV during redox cycling, e- flow produces an electrochemical proton gradient across the inner membrane. This controls mitochondrial respiration and ATP synthesis by ATP synthase (complex V), ionic equilibria essential for the mitochondrial stability and cell viability and the production of O_2^-.

Mammals have highly active internal organs and have increased mitochondrial enzyme activity. This increased mitochondrial capacity to produce ATP is due to the elevated rates of tissue metabolism and greater energy demands by cells which pumps ions to maintain cellular ion homeostasis (Else and Hulbert, 1987). At rest when not synthesizing ATP, mitochondria still consumes O_2 and pump protons across the mitochondrial inner membrane (Hulbert *et al.*, 2003) generating more reactive oxygen species (ROS). Oxidative damage may also adversely affect the inner mitochondrial membrane, which is a specialized structure that serves as the site of ATP production. As the vicious cycle continues, the increased level of mutations would adversely affect mitochondrial electron transport and further increase the rate of oxidant production.

Mitochondrial decay may affect the cardiac function, since increased ROS production affects electron transport, reduces the capacity to synthesize ATP. Loss of ATP production would affect both contraction and relaxation characteristics leading to overall loss of cardiac distensibility (Hagen *et al.*, 2002). In the ageing brain, multiple molecular, cellular and functional changes occur. During ageing there is a progressive accumulation of damaged molecules and impaired energy metabolism in brain cells (Mattson *et al.*, 2002).

The normal functioning of the cells require the membrane bilayers to be fluid to facilitate lateral movement of cellular components. The membrane phospholipids maintain a fluid state owing to the presence of two unsaturated acyl chains. This is attributed to the presence of additional desaturase enzyme system that introduces extra double bond into acyl chains, a process which consumes O_2 and e- obtained from the ET system. The absence of desaturases in mammals makes polyunsaturated fatty acids (PUFAs) an essential dietary component, although they can elongate and further desaturate short chain ω-3 and ω-6 polyunsaturates. Nevertheless, the high concentration of unsaturated fats in mitochondria present a special danger of destructive oxidation a certain proportion of saturated fat appears to be necessary for stability of the mitochondria.

The membrane processes are responsible for the maintenance of associated transmembrane gradient. The mitochondrial proton leak varies allometrically with body mass in mammals and is directly related to difference in membrane acyl composition especially DHA (Porter *et al.*, 1996) and indirectly related with the degree of monounsaturation (Brookes *et al.*, 1998).

The level of phospholipid unsaturation has been related to decreased lipid peroxidation and lipoperoxidative tissue damage and has been suggested to be an adaptation to their longevity (Pampalona *et al.*, 1998, 2000). With age, the fatty acid constituents comprising the phospholipids of the inner mitochondrial membrane become altered in composition (Penzes *et al.*, 1988), making them even more prone to oxidative damage thus altering the membrane fluidity and membrane bound protein topography. Alterations to membrane fluidity as well as the oxidation of critical functional groups on proteins could seriously

impair the ability of mitochondria to meet cellular energy demands, leading to apoptosis.

The nature of the modification of fatty acid composition and the physical properties of membranes brought about by dietary changes (Brenner, 1984; Stubbs and Smith, 1984) both influence the activities of membrane enzymes and thus affects cellular metabolic pathways (Wong *et al.*, 1984). This requires the maintenance of a suitable structure for the preservation of adequate functionality.

Calorie restriction (CR) without malnutrition is a well known dietary intervention that consistently increases lifespan by delaying ageing process in a wide variety of animal species (McCay *et al.*, 1935; Weindruch and Sohal, 1997). The beneficial effects of CR can be observed not only when initiated at a young age but also in adulthood. Although, Foster *et al.* (2003) reported that CR could be ineffective or even detrimental if started late in life, various investigations show that positive effects of CR depend on the reduced intake of calories themselves (Masoro *et al.*, 1991). The mechanisms underlying ageing retardation by CR are poorly understood. However, it has been suggested that they may involve a decrease in cellular O_2 consumption and ROS production (Ramsey *et al.*, 2000). The available studies suggest that the lessening of oxidative damage to mitochondrial DNA during CR is mainly due to the decrease in the rate of endogenous damage (Sanz *et al.*, 2005) rather than to greater scavenging (Leon *et al.*, 2001) or repair of the damage already infiltrated (Stuart *et al.*, 2004). CR also brings about changes in the membrane acyl composition of both mitochondria and microsomes resulting in decreased susceptibility of these membrane bilayers to lipid peroxidation (Langaniere and Yu, 1989).

Ramsey *et al.* (2005) showed that the linoleic acid rich corn oil decreased the proton leak compared to DHA rich fish oil which induced

the apoptotic cascade. They concluded that longevity is inversely proportional to membrane content of PUFA and diets differing in their linoleic acid and DHA content would produce difference in proton leak. Dietary fat manipulation can alter the activities of the mitochondrial ETC complex III, IV and V and change the amount of CoQ_9 and CoQ_{10} in mitochondrial membrane.

The link between the free radical and membrane pace maker theories of ageing is missing despite the fact CR also brings about the changes in the membrane linoleic acid content. It was therefore decided to look into the effect of linoleic acid through feeding corn oil and coconut oil in the mitochondrial decay in cardiac and brain tissues. In studies conducted hitherto, replacement of one fat source with other (soy by corn oil), fail to address the effect of linoleic acid on proton leak since both soy bean and corn are rich in linoleic acid. Secondly, there exist quite dubious claims on the effect of CR and other dietary manipulation when started well beyond middle age. Since these results are intriguing, this study was planned to carryout dietary interventions including manipulation of fat and CR in rats from 18 months of age.

It was hypothesized that reduction in free radical formation by dietary manipulation of fat through linoleic acid and calorie restriction would reduce occurrence of apoptosis in the brain and cardio myocytes and retard the ageing process. This study was taken up with the following objectives

1. To study the effect of dietary manipulation of fat and calorie restriction on

    a. the production of ROS in relation to activity of specific mitochondrial electron transport complexes

    b. Apoptosis in ageing heart and brain

2. To unravel the mechanism involved in the retardation of ageing by dietary manipulation of fat and calorie restriction.

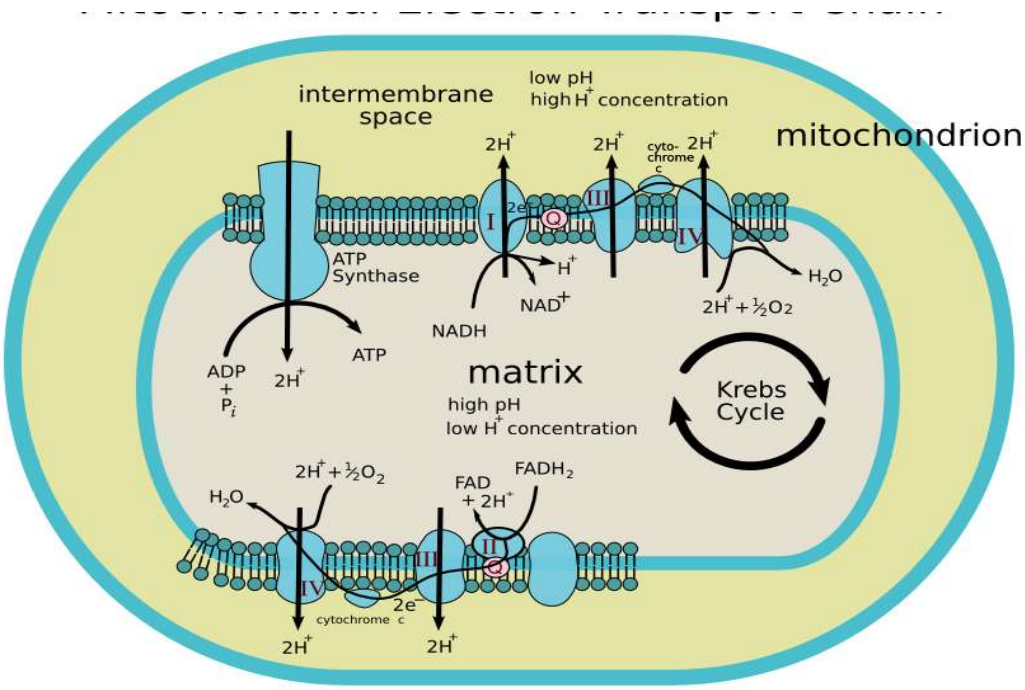

Fig. 1 Electron transport chain in the mitochondria showing arrangement of the respiratory complexes I to V and the movement of electrons between the complexes.

CHAPTER 2

REVIEW OF LITERATURE

Mitochondria are the powerhouses of the cell. The enzymes and enzyme complexes vital for mitochondrial functions are organized in a compact form in the different compartments of the organelle and isolated from the cytoplasmic compartment by a special coupling membrane that is impermeable to most solutes (LaNoue and Schoolworth, 1979). The functions that are common to most, if not all, mitochondria include the synthesis of ATP, terminal oxidation of pyruvate derived from carbohydrate and amino acid catabolism, β oxidation of fatty acids, and oxidation of acetyl Co-A derived from the diet and from ethanol, fatty acid, protein, and carbohydrate oxidations (Tzagolott, 1982).

The efficiency by which dietary calories are converted to ATP is determined by the coupling efficiency of oxidative phosphorylation (OXPHOS). As a toxic by-product of OXPHOS, the mitochondria generate most of the endogenous ROS. If the mitochondrial ROS production rate increases, the rate of cell loss will also increase, resulting in early tissue failure and age-related disease. However, if mitochondrial ROS production is reduced, then the tissue cells will last longer and age-related symptoms will be deferred (Wallace, 1999; 2001).

Dietary manipulations also affect the mitochondrial composition, activities of mitochondrial enzymes, and content of mitochondrial proteins. Variation in carbohydrate diet has a large effect on the enzymes of energy metabolism, especially those related to triglyceride synthesis, and can affect function, at least indirectly, through changes in mitochondrial phospholipid composition (Wander and Berdanier, 1985). Rats receiving a diet containing 20% (w/w) fat in the form of soybean oil had only 70% the monoamine oxidase activity of those receiving the same amount of lard (Crane and Greenwood, 1987).

2.1 Mitochondrial decay

2.1.1 Membrane fatty acid composition

The Arrhenius activation energy of mitochondrial membrane associated enzymes from homeothermic animals increases below the temperature limits of phase transition of the membrane lipids (McMurchie and Raison, 1979), suggested that cellular reactions evolving membrane associated enzymes were regulated by membrane fluidity. There is a reduction in the rate of lipid metabolism and turnover of fatty acids with ageing (Hansford and Castro, 1982).

In homeothermic animals membrane lipid composition is influenced by the dietary lipids. Changes in acyl composition of membrane bilayers can be very rapid occurring within minutes in cultured cells (Chakravarthy *et al.*, 1986). Pan and Storlien (1993) have shown that the acyl composition of the diet significantly alters the metabolic rate of rats.

Diets deficient or low in EFA produce well defined changes in mitochondrial phospholipid with increases in C20:3, C18:1 and C16:1 and decreases in C18:2 and C20:4 fatty acyl chains accompanied by a decrease in the activity of membrane associated enzymes (Haeffner and Privett, 1975). On the other hand diets rich in PUFA increase the proportion of C18:2 fatty acyl chains in mitochondrial phospholipid and decreases the Arehenius activation energy of membrane bound enzymes (McMurchie and Raison, 1979).

Century *et al.* (1963) reported that the lipids of chicken brain mitochondria were uniquely low in linoleic acid and the bulk of EFA content of brain mitochondria was represented by arachidonic acid compared to other tissues including heart where most EFA content consisted of linoleic acid. Rose (1964) showed that ox heart cardiolipin

contained a large amount of 18:2 than the rat liver cardiolipin and connected the difference with the different amounts of linoleate consumed by each organ.

Barzanti *et al.* (1994) reported that the capacity of maintaining the structural homeostasis could prove inadequate during changes in lipid intake (PUFA) and during ageing due to extensive changes in lipid metabolism *viz.* reduction in lipid long chain metabolism and turnover of fatty acids.

Swanson *et al.* (1987) and Yamaoka *et al.* (1988) observed an increase in the Ca-Mg ATPase in cardiac sarcoplasmic reticulum and cardiac mitochondria when fed on corn oil than menhaden oil suggesting that certain membrane enzymes are sensitive to changes associated with different dietary fatty acids. Zuniga *et al.* (1989) reported that long chain PUFA are involved not only in membrane fluidity but also in changing the bio-layer thickness with consequential differential exposure of active enzyme site.

Nalbone *et al.* (1988) suggested an increase in amount of metabolites synthesized from the elongation and desaturation of 20:4n-6 might be the reason for no significant increase of arachidonic acid in phosphatidyl choline (PC) but provoked a drop in arachidonic acid in phosphotidyl ethanolamine (PE) when fed linoleic acid enriched diets. Hoffman *et al.* (1986) observed a higher release of heart PGI_2 in rats fed linoleic acid rich diets than in those receiving linoleic acid poor diets. Leonardi *et al.* (1987) claimed that arachidonic acid may be more efficiently released by a more active cardiac phospholipase A_2.

2.1.2 Membrane surface area

Mitochondria are topologically closed bilayered systems where the synthesis of ATP from ADP and inorganic phosphate occurs via oxidative phosphorylation. The ordered architecture of the mitochondrial inner and

outer membrane contributes a critical topographic arrangement for their energy providing mechanism (Bertoni – Freddari *et al.* 2003). An age related decline in the number and increase in size of mitochondria is reported to be a general trend for mitochondria during ageing. Hence age related cellular metabolic decay rely on both the decline in mitochondrial metabolic competence and their specific reduction in numbers (Bertoni – Freddari *et al*, 2005).

2.1.3 Membrane permeability

The supply of energy by mitochondria depends on the maintenance of proton motive force (PMF) – a chemiosmotic gradient across its inner membrane. PMF has two components the membrane potential ($\Delta\varphi m$) which arises from the net movement of positive charge across the inner membrane and the pH gradient of these two components $\Delta\varphi m$ contributes most of the energy stored in the gradient (Fig. 1). The $\Delta\varphi m$ is the central bioenergetic parameter that controls respiratory rate, ATP synthesis and generation of ROS and is itself controlled by electron transport and proton leak (Nicholls, 2004). Maintenance of $\Delta \varphi m$ is fundamental for the normal performance and survival of cells that have a high energy requirement such as the beating cardiomyocyte (Mathur *et al.*, 2000).

Rottenberg and Wu (1997) monitored $\Delta \varphi m$ in lymphocytes from young and old mice and observed more heterogeneity of potential from the old preparations.

Cavazzoni *et al.* (1999) reported extremely low values of $\Delta \varphi m$ for hepatocytes from both adult and old rats concomitant with enormous increase in peroxide formation with no significant change in levels of super oxide. A 10 mV decrease in $\Delta \varphi m$ from mouse liver mitochondrial preparations compared to young and middle aged animals has been reported by Kokoszka *et al.* (2001).

Delcamp *et al.* (1998) reported a membrane potential of -110 mV in isolated guinea pig ventricular myocyte. Comparable Δ φ m had been recorded by Wan *et al.* (1993) when the mitochondria were utilizing glucose rather than pyruvate as metabolic substrate. These reports delineated the significance of metabolic substrate in measuring Δφm. However, more negative Δφm have been reported in other tissues *viz.* liver (-154mV) by Bevilacqua *et al.* (2004). The differences in Δφm between hepatocytes and caridiomyocytes could be attributed to the actual difference between the tissues or the experimental conditions (Hagen *et al.* 1997).

2.1.4 Mitochondrial ETC activity

Mitochondrial respiration and OXPHOS were gradually uncoupled and the activities of the respiratory enzymes are concomitantly decreased in various tissues upon ageing due to an increased production of ROS and free radicals in the mitochondria through increased electron leak from the ETC (Wei *et al.*, 1998). McMillan *et al.* (1993) reported that decline in myocardial carnitine leads to a significant loss in β oxidation of fatty acids and suggests deprivation of mitochondria of their major source for ATP synthesis. This may be due to changes in the content of cytochrome as well as the impaired activity of complex I of the OXPHOS chain in the ageing cardiac mitochondria (Castellueio *et al.*, 1994).

The activities of complexes III and IV declined or remained unchanged (Hepple *et al.*, 2005) with age. Similarly, the activity of complex II, the succinate dehydrogenase may decline (Telesara and Arora, 1994), remained unchanged (Sugiyama *et al.*, 1993) or increased (Vitorica and Machado, 1981) in senescent rat tissues although the predominant view supported a lack of age related loss in activity for complex II. Heich *et al.* (1994) observed a more drastic decline in the activity of cytochrome C oxidase (complex IV) compared to NADH-cytochrome c reductase and a mild decrease in succinate cytochrome c

reductase activity in ageing rat skeletal muscle. Navarro and Boveries (2004) reported a 29 and 28 per cent decrease in the activities of complex I and IV respectively where as Sohal and Sohal (1991) observed a two fold increase in activities of NADH- ferricyanide reductase, succinate-ubiquinone reductase (complex II) and succinate cytochrome c reductase in old than from young house fly. Similarly, a 38 to 41 per cent reduction in the activities of citrate synthase, complexes I to IV in plantaris muscle mitochondria was observed in 35 month old rats (Hepple *et al.*, 2005).

Despite an overall decline in respiratory chain capacity there was an increase in the complex IV activity in the randomly distributed defective cells in the skeletal muscle and heart and this random pattern could be related to the cellular heterogeity of the ageing process (Muller-Hocher, 1992). Therefore it was postulated that accumulation of respiratory deficient cells that lack cytochrome c oxidase in the human heart with increasing age limits life span of each individual (Kadenbach and Muller-Hocher, 1990).

Powers *et al.* (1993a) reported that there was no age-related difference in the activity of citrate synthase and lactate dehydrogenase in diaphragm of rats depicting a steady state in the oxidative capacity in senescent diaphragm of rats. The age associated impairment in respiratory enzymes not only decrease ATP synthesis but also enhanced the production of ROS through increased e- leakage from the ETC (Wei *et al.*, 2001). On the other hand Chang *et al.* (2007) analyzed the protein pattern using two-dimensional electrophoresis and found the level of one protein to increase whereas three other proteins to decrease in the heart of aged rats. These observations make it vivid that changes in the ETC activity are to some extent tissue specific and also depend up on the genetic make of these animals.

The vicious cycle involving increased proton leak, ROS production and impaired electron transport function could be attributed to the

presence of defective mitochondrial DNA (mtDNA) encoded protein subunits in the respiratory enzymes (Wei *et al.*, 1998). Cassarino and Bennett (1999) stated these toxic consequences of ETC dysfunction lead to further mitochondrial damage including oxidation of mtDNA, proteins and lipids and opening of mitochondrial permeability transition pore an event linked to programmed cell death or apoptosis.

It has been also found that thyroid hormone and exercise stimulate the expression of a nuclear respiratory factor that induces nuclear gene TFAM, which encodes the mitochondrial transcription factor A which in turn controls mitochondrial replication and transcription thereby prevent defective OXPHOS and increased ROS production (Fosslien, 2003). This could be plausible as the levels of thyroid hormones are found to decrease as the animal ages (Lal *et al.*, 2001).

Deletion of mtDNA molecules along with LPO of mitochondrial membranes contributed to the overall decline of mitochondrial function (Gadaleta *et al.*, 1992) due to lower activity of aerobic and anaerobic enzymes and protein content (Kaczor *et al.* 2006).

2.2 Apoptosis and ageing

2.2.1 Apoptosis

Apoptosis, also thought of as cell suicide is a tidy way of removing cells no longer useful, in essence, a form of selective deletion. Apoptosis was triggered by a myriad of stimuli such as steroid hormones, DNA damage, withdrawal of growth factors or receptor mediated as in clonal deletion of immune cells (Johnson *et al.*, 1999). This pathway involves activation of a family of proteases called caspases and nucleases leading to controlled degradation of cellular structures, followed by removal of the membrane bound debris. Warner *et al.* (1997) speculated that dysregulation of programmed cell death might contribute to ageing.

Green and Reed (1998) reported that both apoptotic and necrotic forms of cell death were mediated by mitochondria.

Johnson *et al.* (1999) summarized the logistic mitochondrial events that mediate apoptosis to include release of mitochondrial stores of caspase activators including cytochrome c, disruption of respiratory chain ETC and production of ROS. It has been reported that the activity of ETC appears to decline with age (Lee *et al.*, 1997) and the rate of ROS production increases with age in many mammals (Sohal *et al.*, 1994a).

Petrosillo *et al.* (2003) reported that the release of cytochrome c from mitochondria is the central event in apoptosis induction appeared to be mediated by ROS. The release of cytochrome c occurs in two steps consisting of the dissociation from cardiolipin, followed by permeabilization of the outer membrane probably by interaction with voltage dependent anion channel (VDAC). Since cardiolipin is rich in UFA (90% represented by linoleic acid), which appears to be essential for its interaction with cytochrome c in order to anchor the protein to the membrane (Tuominen *et al.*, 2002). Shidoji *et al.* (1999) reported a loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. In addition, it has been found that changes in the cardiolipin content due to oxidative damage (Nomura *et al.*, 2000) or to alteration in its biosynthetic pathway (Ostrander *et al.*, 2001) triggered the release of cytochrome c from mitochondria in the apoptotic process.

2.2.1.1 Heart

Apoptosis and necrosis are two distinctly different forms of cell death and both occur in human and animal heart. In contrast to necrosis, apoptosis was not associated with inflammation due to its intact nature (not rupturing) prior to phagocytosis by macrophage and also the rapidity of phagocytosis process compared to necrosis. These features make apoptosis a valuable component of morphogenesis,

mediation of hormonal and immunological responses and the homeostatic balance between hypertrophy and atrophy or involution.

Olivetti *et al.* (1995) reported that more than one third of ventricular cardiac myocytes were lost during normal human ageing in males, albeit apparently not in females and the loss of cells was accompanied by hypertrophy of the remaining cells. On the other hand, Kajstura *et al.* (1996) observed that in rats majority of the cells underwent necrosis yet a substantial number of cells were lost *via.* apoptosis.

2.2.1.2 Brain

Morrison and Hoff (1997) observed that the loss of neurons with normal ageing may not be very intensive as thought in brain. Pakkenberg and Gundersen (1997) reported that the overall number of cortical neurons declined by 10 % with ageing, although particular brain regions showed higher levels of loss it was not clear that these losses were responsible for functional changes that accompany ageing. The mitochondrial mass of brain remained unchanged in young, adult and old adult and senescent rats, which ranged between 15 to 17 mg protein / g tissue (Navarro and Boveries, 2004).

2.2.2 Ageing

Ageing resulted in a loss of post mitotic cells *viz.* cardiac myocytes and brain, which are attributed to the mitochondria triggered apoptosis caused by age associated increase in oxidant production or increased activation of mitochondrial permeability transition pores. This may be due to tissue specific adaptation of Bcl -2 Bax ratio which may directly influence release of cytochrome c (Pollack *et al.*, 2002).

The cardiac myocytes is the most energy demanding cell in the body contracting constantly and requiring larger supplies of high energy phosphates (Jacobus, 1985). Age related changes in the human and

rodent hearts include a reduction in number of myocytes, myocytes hypertrophy (Olivetti *et al.*, 1991), cardiac fibrosis (Cornwell *et al.*, 1991; Waganat *et al.*, 2002), lipofuscin pigment accumulation (Del Roso *et al.*, 1991) and a reduction in calcium transport across the sacroplasmic reticulum membrane (Jiang and Narayanan, 1990). Aging results in induction of genes involved in structural roles such as extra cellular matrix components, collagen deposition and cell adhesion (Park and Prolla, 2005). There was also transcriptional evidence of a metabolic shift from the oxidation of fatty acids the major energy source in adult heart to carbohydrate oxidation. Park and Prolla (2005) observed the genes involved in FA transport to mitochondria, fatty acid β oxidation in mitochondria, lipid metabolism and protein synthesis were down regulated. Similarly, Bodyak *et al.* (2002) reported a gene expression profile consistent with reduced protection against stress induced injury and impairment of mitochondrial ET system associated with development of contractile dysfunction. These findings suggest that these changes could be an adaptation to reduced mitochondrial function and impaired ATP production and the alteration in the transcriptional status of cardiomyocyte plays an important and perhaps underlying role in functional losses and reduced stress resistance in aged hearts (Park and Prolla, 2005). It is opined variation in longevity among different species was inversely correlated with rate of mitochondrial generation of super oxide anion ion and H_2O_2 (Sohal and Weindruch, 1996). Under identical conditions the mitochondria from animals with low mean lifespan potential relatively release greater amounts of H_2O_2 (Sohal *et al.*, 1990).

2.2.3 Markers of oxidative stress

2.2.3.1 Lipid peroxidation and protein carbonyls

Under normal physiological conditions, the use of O_2 by cells of aerobic organisms, generate potentially deleterious ROS metabolites. The mitochondrial respiratory system is the major intracellular source of the

ROS and free radicals, which are generated as byproduct during the transfer of electron from NADH or $FADH_2$ to molecular O_2. A chronic state of oxidative stress exists in cells because of an imbalance between pro oxidants and antioxidants. An age dependent increase in the fraction of these toxic by-products may escape the defense mechanism of human and animal cell can induce a broad spectrum of oxidative damage to biomolecules in the mitochondria and the cell as a whole. The amount of oxidative damage increases as an organism ages and is postulated to be a major causal factor of senescence (Wei, 1998).

The increased oxidative stress may arise from a variety of factors such as enhanced generation of free radicals, reduced levels of antioxidants available, enhanced consumption, leakage or destruction of antioxidants, reduced protective capacity including antioxidant enzymes, leakage of electrons from the mitochondrial ETC and phagocyte recruitment and activation (Minic-oka *et al.*, 1999). This age related oxidative damages were largely irreversible as evident through cross over studies conducted in mice (Lass *et al.*, 1998).

Reactive oxygen metabolites cause enzymes to loose activity induce mutations and damage cell membranes (Halliwell and Gutteridge, 1999; Sohal and Weindruch, 1996; McCord, 1995), despite other beneficial effects in modulation of cellular redox state, signal transduction, activation of gene transcription and apoptosis (Sen and Parker, 1996; Suzuki *et al.*, 1997). Shifts in the level of oxidative stress probably affect all these cellular functions. It has been widely postulated that damage to macromolecules from reactive oxygen metabolites is a major cause of senescence (Harman, 1956; Stadtman, 1992). Ji *et al.* (1990) reported that LPO was greater in skeletal muscle and liver in aged rats and concluded the age related increase in lipid peroxidation in skeletal muscle was probably due to increased oxygen free radical production and reaction. This variation in the level of LPO depends on the energy

requirement of each tissue and an increased LPO was observed in tissues with high energy demand *viz.* muscle compared to those with low energy demand; LPO was also found to be positively correlated with proportion of 4977 bp mtDNA deletion (Wei *et al.*, 1996).

Comparison among mammals with wide variations in longevity indicates that species with longer lifespan generate super oxide anion and H_2O_2 at lower rates (Ku *et al.*, 1993) accrued less oxidative damage (Sohal *et al.*, 1993; 1995) and resist experimentally induced oxidative stress (Agarwal and Sohal, 1996). On the contrary, it has been observed that birds which have high metabolic rates but are nonetheless quite long lived generate mitochondrial super oxide anions (O_2^-) and H_2O_2 at lower rates and have relatively high activities of SOD and GPx.

Siqueira *et al.* (2005a) observed age related increase in the generation of free radical in hippocampus, hypothalamus and adrenal glands as well as LPO in hippocampus and hypothalamus. Bevilacqua *et al.* (2005) reported that there was an age related 18% increase in H_2O_2 production between 24 month old rats compared with 18 months old rats. This increase in H_2O_2 production may be due to the intermolecular cross linking in the inner mitochondrial membrane (Navarro and Boveries, 2004).

The excess production of ROS may initiate LPO in cell membranes, damage membrane proteins or cause DNA fragmentation. Spiteller (2002) observed the products of LPO to react with glutathione and the resultant product to induce apoptosis. It has been found that cellular components besides mitochondria and synaptosomes were oxidatively modified during brain ageing (Babusikova *et al.*, 2007).

Ageing was associated with accumulation of increased intracellular oxidized protein measured as protein carbonyls and attributed to a decline in proteosomes activity due to the inhibition rendered by oxidized

and cross-linked protein aggregates (Grune *et al.*, 2001). Jacobson (1996) opined that these processes may result in a loss of contractile function of the heart and lead to severe myocardial cell damage. The level of protein carbonyl content in the brain was significantly higher in older animals (Tian *et al.*, 1998). LPO and protein carbonyls increased by 61 to 69 per cent in the brain during the ageing process (Navarro and Boveries, 2004).

Protein carbonyls were linked with the age related decline in antioxidative enzyme activity but LPO was found less sensitive to predict the ageing process as the later levels were not significantly altered between young and older rats (Tian *et al.*, 1998).

2.2.3.2 DNA fragmentation

The DNA due to its central role in life is bound to be implicated in ageing. As early as 1959 Szilard hypothesized that damage accumulation to the DNA caused ageing as cited by Miller *et al.* (1988). DNA mutations and chromosomal abnormalities increase with age in mice (Doll and Virg, 2002). One possibility is that ROS damage DNA and Hamilton *et al.* (2001) showed an increase in oxidative damage to DNA with age. Since, ROS originates in the mitochondria and mitochondria possess their own genome, many advocates of the free radical theory of ageing consider the ROS damage to mtDNA is critical to ageing (Barja and Herraro, 2000; Barja, 2002). Ozawa (1998) demonstrated extreme fragmentation of mtDNA in the cardiac myocytes during senescence. In variance to the above findings, Schriner *et al.* (2000) and Tsuzuki *et al.* (2001) demonstrated that over expression of catalase in the nucleus did not prevent damage to ageing and knocking out the gene responsible for 8 oxo dGTPase failed to accelerate ageing. These results hint that free radical and the DNA damage theories of ageing are not complementary. Despite such controversies regarding the ROS generation and DNA damage in ageing, the free radical theory of ageing still holds good as one of the causal factors in ageing.

2.2.4 Antioxidant enzyme activity

Age associated changes in antioxidant enzyme activities are specific to tissues with increased activity of glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase in skeletal muscle and decreased activity of GPx and SOD and glutathione (GSH) in liver. Powers *et al.* (1993a) reported increased GPx activity increased with age in the diaphragm of rat. These changes may also depend on the concentration of protein in various tissues, since, the protein concentration decreased as age advanced (Powers *et al.*, 1991). A positive correlation has been found to exist with increased activity of catalase in the mitochondria and life span in mice (Beal, 2005). These data suggest that antioxidant enzyme activity depends on the level of production of ROS in different tissues.

2.2.4.1 Heart

Ji *et al.* (1990) postulated that an increased rate of free radical formation or some by product formation are potential stimuli for induction of antioxidant enzymes in heart and skeletal muscle. In this regard, Boveries and Chance (1973) reported that rate of free radical production in isolated pigeon heart mitochondria increased in direct proportion to the rate of mitochondrial oxygen consumption.

Powers *et al.* (1993b) observed moderate or high intensity exercise endurance to up regulate SOD activity in the ventricular myocardium. Das (2002) suggested that the low plasma levels of SOD may directly related to an increased production of super oxide anion and plasma levels of SOD may be envisaged as a potential marker for risk assessment of coronary heart disease.

SOD activity in brain and heart tissues decreased with age whereas catalase levels decreased only in brain (Tian *et al.*, 1998) with GPx activity unchanged in heart and brain tissues (Rao *et al.* 1990a). In

contrast, Hussain *et al.* (1995) observed increase in the levels and activities of SOD, glutathione whereas no significant increase in activity of GPx and the catalase levels were different only in the cerebellum. These reports suggest that the overall enzyme activities tend to increase with age despite variation in level of antioxidant enzymes in different regions of the brain.

The changes in the activity of antioxidant enzymes were paralleled by similar change in the relative level of the mRNAs coding these enzymes. Studies using transgenic fruit flies revealed that over expression of the cytoplasmic form of SOD – Cu/Zn SOD (SOD1) and catalase increased longevity by 34 % and delayed ageing process (Orr and Sohal, 1994). Sohal and Weindruch (1996) reported that over expression of antioxidative enzyme retarded age related accrual of oxidative damage and extend maximum lifespan of transgenic fruit flies. Wei *et al.* (2001) reported a decline in the mitochondrial pool of reduced glutathione and enhanced DNA damage with concomitant increase in the intracellular levels of H_2O_2 and oxidative damage to DNA and lipids with age.

In variance to the above findings Trifunovic *et al.* (2005) in their study using homozygous knock in mice expressing an error prone version of mtDNA polymerase γ (poly A mut) associated with premature ageing and reduced lifespan found no difference in expression of SOD1 and GPx from heart, and liver mitochondrial protein extracts. They concluded that there is no major increase in oxidative stress in mtDNA mutator mice thus ruled out free radical theory as a major cause for ageing.

2.2.4.2 Brain

Geremia *et al.* (1990) reported that the age dependent changes in the antioxidant enzyme activity were at lower levels in neuronal cells

than in glial cells and the Cu/Zn SOD was the most affected antioxidant enzyme system of brain ageing which could be responsible for the increased lipid peroxidation (LPO) in neuronal and glial cells. The age related changes in the various antioxidant enzymes varies between different tissues.

The antioxidant enzyme activity was found to differ between different regions of the brain. Ciriolo *et al.* (1991) found considerable difference in antioxidant activity in various parts of brain. GPx and catalase activities decreased in 3-12 months old rats with increasing activity in hippocampus and decreasing activity in hypothalamus during ageing along with diminished catalase activity in these areas. A region specific activity of the antioxidant enzymes has been reported during ageing with decreasing activity of catalase, SOD and GPx in cerebellum. SOD and GPx activities increased in all regions of brain except hippocampus above 12 months of age with increased glutathione levels in cerebellum and brainstem in 2 year old mice (Hussain *et al.*, 1995). Siqueira *et al.* (2005a) observed increased activity of SOD with decreased activity of catalase in the cerebellum in ageing rats. From a similar study Siqueira *et al.* (2005b) reported reduced GPx activity in the hippocampus and hypothalamus of 6 month old wistar rats but the activities were found to reduce in the adrenal glands by 24 months of age. It is thus concluded that the age related variation of total antioxidant defenses in brain predispose structures to oxidative stress related neurodegenerative disorders.

2.2.5 Myocardial collagen

An age related increase in myocardial collagen has been reported to accompany loss of myocytes and the resultant compensatory hypertrophy (Patiyal and Katoch, 2006). Duncan *et al.* (2000) reported myocardial hypertrophy and necrosis increased myocardial infiltration of

collagen. Increased collagen levels were associated with stiffness of heart (Hagen et al, 2002).

2.2.6 Lipid profile

Plasma cholesterol and triglyceride concentrations were significantly higher in 18 months old than younger animals. (Schneeman and Richter, 1993).

Fernandez and West (2005) reviewed, the mechanism by which dietary fatty acids modulate plasma lipids stating that PUFAs of n-6 family and monounsaturated fatty acids (MUFA) decrease plasma low densisity lipoprotein (LDL) cholesterol concentration unlike saturated fatty acids (SFA) which increases the LDL in plasma by decreasing LDL turnover. The possible mechanisms by which n-6 PUFA decrease plasma cholesterol include up regulation of LDL receptor and increased Cholesterol 7α hydroxylase (CYP7) activity, whereas n-3 PUFA decreases the plasma triglycerides by decreasing lipogenesis and VLDL secretion increasing LDL activity and increasing reverse cholesterol transport.

2.3 Dietary fatty acid

Oleic, linoleic and linolenic acids (18:1, 18:2 and 18:3) are the major unsaturated fatty acids (UFAs) distributed in plants. Oleic acid can be synthesized *de novo* by introducing double bond at Δ9 position into the corresponding saturated fatty acids (SFA), only linoleic and α linolenic acids have to be essentially supplemented through diet (Harper et al., 1998). Amongst these 18:2 is the only UFA that has been shown to be indispensable for the growth of some animals.

Phospholipids in the cell membrane contain UFA important in maintaining membrane fluidity. Century *et al.* (1963) demonstrated that the bulk of essential fatty acid (EFA) content of brain mitochondria was represented not by linoleic acid but arachidonic acid, unlike other tissues where linoleic acid content was high. Several factors determine the extent to which the dietary lipids influence cell structure and subsequent metabolism; these include relative concentration of specific

lipid moieties, the FA composition including chain length, extent of unsaturation, specific metabolic family and FA balance and the presence of non acidic components such as phytosterols and tocopherols (Barzanti *et al.*, 1994). Linoleic acid and more UFA of its series are important constituents of biological membranes and are indispensable for the activity of some of the enzyme systems (Levine *et al.*, 1990). In analyzing the relationship between dietary lipids and enzyme activities more attention has been paid to the effect of PUFA in particular to the effects of varying levels of n-6 and n-3 C18 FAs with available information on the comparative metabolic effects of dietary intake of PUFA (McMurchie *et al.*, 1983; Yamaoka *et al.*, 1988, 1990; Zuniga *et al.*, 1989; Barzanti *et al.*, 1990). Fish oil prevented the conversion of linoleate to arachidonate (Century and Horwitt, 1963).

2.4 Calorie restriction

Calorie restriction (CR) without malnutrition is a well known dietary intervention that consistently increases lifespan by delaying or retarding the ageing process in a wide variety of animals (McCay *et al.*, 1935; Weindruch and Sohal, 1997). In general a level of 30 – 40 % *ad libitum* intake of calories was restricted in almost all CR studies carried out hitherto (Chandrasekar *et al.*, 2001; Sreekumaran *et al.*, 2002; Ramsey *et al.*, 2004; Hagopian *et al.*, 2005). In experiments conducted in mice Cao *et al.* (2001) provided a semi purified diets that yielded 53 Kcal /wk at CR and 95 Kcal/wk on *ad libitum* feeding.

Masoro and Compton (1983) and Masoro *et al.* (1992) suggested that restriction of total energy, not reduced protein or fat energy appears to underlie the major contribution of reduced food intake to extend life. Yu *et al.* (1985) observed that the greatest extension of lifespan is achieved when CR was initiated at early ages and sustained throughout life, although the benefit of CR was observed when initiated at middle age and sustained throughout life.

The mechanisms underlying the retardation of ageing using CR were poorly understood due to the multifaceted regulations brought about by CR on bodily functions. One of the plausible mechanisms may involve a decrease in cellular oxygen consumption and ROS production (Ramsey *et al.*, 2000). Therefore dietary manipulation through CR retards the ageing brought about by both rate of living theory and the free radical theory. In both the theories mitochondria are central to both energy metabolism and ROS production. Accordingly it has been suggested that a decrease in cellular oxygen consumption and ROS production may be involved in bringing out the beneficial effects of CR on ageing (Ramsey *et al.*, 2000).

2.5 Experimental diet and feeding regimen

To study the interrelationships of dietary lipids upon the fatty acid composition of brain mitochondria, erythrocytes and heart tissue Century *et al.* (1963) used an isocalorically equivalent diet containing 1.2 % ethyl linoleate alone and in combinations of other non essential fatty acids or lipids as well as diets containing SFA only or no fat at all to feed chicks for a 21 day period. Yamamoto *et al.* (1965) prepared a fat free diet containing casein, gelatin, sucrose salt mixture, vitamin mixture and choline chloride to which either linolenate or linoleate were added at a rate of 700 mg/day/rat were also included in the diet.

Barzanti *et al.* (1994) prepared an experimental diet with a basal fat free diet containing casein, cellulose, sucrose and starch , salt mixture , vitamin mixture and choline chloride. To this basal fat free diet olive oil (100g/kg), olive oil- blackcurrent – fish oil (5:1:1 v/kg) or blackcurrent –fish oil (1:1 v/kg) were added along with vitamin E in sufficient amounts (120 mg/kg diet) to prevent oxidative stress arising from dietary PUFA.

Ranhotra *et al.* (1995) provided diets containing casein, soyabean oil, pregelatinized corn starch, minerals and vitamin mixture. The negative control diet contained 45 % silica and mineral oil, the positive control diet contained similar levels of sucrose plus shortening. The experimental diet contained 45 % polydextrose – caprenin blends in 2:1, 1:1 or 1:2 ratio.

Power and Newsholme (1997) prepared purified diets comprising a low fat diet containing 25 g/kg fat (corn oil), 495.8 g/kg carbohydrates (Cornstarch plus sucrose), 200 g/kg protein (sodium caseinate) and 2.5 g/kg non digestible material (cellulose). To this basal low fat diet one of the five high fat diets were supplemented with 210 g/kg fat (200 g/kg of hydrogenated coconut oil, olive oil, safflower oil, evening primrose or menhaden oil plus 10 g/kg corn oil). The high fat diets contained the same amounts of carbohydrates, corn oil and protein but the amounts of non digestible material were lowered proportionately to accommodate the increased fat content.

Demaison *et al.* (2000), to study the effect of PUFA and training on contractile function of cardiac myocytes fed wistar rats a semipurified diet containing casein (20 %), commercial grade sucrose (50 %), corn starch (15.3 %), salt mixture (4 %), cellulose powder (2 %) vitamin (1 %), fat (10 %) which was either fish oil or sunflower seed oil. A second group of rats were fed with a commercial diet containing 5 % lipids with large amounts of n-6 PUFA but also n-3 PUFA mainly 20:5n-3 and 22:6n-3 in noticeable proportions.

The modified AIN-93M diet recommended to feed rodents consisted of casein (140 g/kg), L cystine (1.8 g/kg), corn starch (40 g/kg), soy oil (32 g/kg), mineral mixture (35 g/kg), vitamin mixture (10 g/kg), t-burtlhydroquninone (0.008 g/kg), choline bitartrate (2.5 g/kg). In studies conducted to assess the effect of dietary fatty acids using fish oil or vegetable oils menhaden oil was included in fish oil diets (32 g/kg)

replacing corn oil (32 g/kg) and the t-butylhydroquinone levels were decreased to 0.0016 g/kg (Ramsey *et al.*, 2005).

More recently, Pinnotti *et al.* (2006) prepared a UFA rich diet with commercial rodent chow. The UFA source corn oil was included at 220 ml plus 30 ml coconut oil per 1100 g of rat chow. The UFA diet had 13.39 % fat of which 73.57 % UFA and 26.43 % SFA. The linoleic acid content of the UFA diet was 45.04 % and with lower amounts of carbohydrates (44.96 %), protein (16.4 %) and vitamins (4.7%).

2.5.1 Feeding regimen

2.5.1.1 Calorie restriction

Most of the works that studied the effect of calorie restriction on ageing adopted the use of AIN-93M purified diet recommended by Reeves *et al.* (1993). A decrease in corn starch component resulted in an increase in percentage protein, fat, mineral and vitamins in the restricted diets thus achieving equal absolute daily intakes of essential nutrients between control and CR group (Hagopian *et al.*, 2005).

Madapallimattam *et al.* (2001) fed the experimental animals on a liquid diet containing (364 KJ/day), glucose (262.8 g/60ml/day/rat), amino acids (48.8 g/60ml/day/rat) and fat (52.4 g /60ml/day/rat). On the other hand the hypo energetic feed contained energy (92 KJ/day) D-glucose (66 g/60ml/day/rat), amino acids (12.5 g/60ml/day/rat) and fat (13.5 g/60ml/day/rat).

Duffey *et al.* (2002) in their study provided 31% diet restriction when fed on AIN-93M diet containing no additional mineral and vitamins and 40 % diet restriction when feeding NIH-31 diet which contained 1.76 % additional vitamin mixture.

2.6 Effect of dietary fatty acid on growth rate

2.6.1 Body weight and organ weight

Feeding fats of different sources tend to influence the growth performance of rats differently. Yamamoto *et al.* (1965) reported that the growth rate of rats fed linoleate did not vary from those rats which received linolenate, but the control rats maintained on a fat free diet showed retarded growth rate, signifying the importance of linoleic and linolenic acids for growth of animals.

Rafael *et al.* (1984) observed a reduction of 22 % body weight in male rats maintained on an EFA (linoleic acid) deficient diet which further reduced to 25 % when the dietary lack was maintained. However, there was no significant difference in between the groups in the level of feed intake that indicated a significantly different feed efficiency. In variance to the above findings Christon *et al.* (1988) observed no difference in the body weights of rats in the control and EFA deficient groups except that the EFA deficient rats had increased liver weight.

Tomlinson *et al.* (1988) found no significant increase in body weight when the rats were fed with different oils as fat source and compared with rats fed on a fat free diet. Similarly, Nalbone *et al.* (1988) reported that the body weight and heart weight did not differ when the rats were fed with either a low fat diet or corn and salmon oil.

It was found that feeding of female rats with palm oil, olive oil or safflower oil did not significantly alter the maternal body weights, liver weight/body weight or foetal body weight and liver weight/body weight (Haave *et al.*, 1998). Similarly, Herzberg and Rogerson (1988) observed no significant variation in body weight when rats were fed with tallow, corn or marine oil as fat source, but the feed intake was lower in animals fed fructose based diets compared with glucose based diets and rats fed fructose based diets had increased liver weight.

The daily feed intake or EPA/DHA intake did not affect body weight in rats. Similar findings were recorded by Chen *et al.* (1987) in rats fed

either fish oil concentrate, corn oil or menhaden oil compared to lipid free emulsion fed rats. Zuniga *et al.* (1989) observed a reduced body weight gain (30 %) in rats fed commercial non purified diets compared to animals fed purified diet containing menhaden oil, corn or coconut oil.

Demaison *et al.* (2000) compared the effect of feeding 10 % dietary PUFA as fish oil or sunflower seed oil on blood pressure in rats, observed that weight gain of the animals were not modified by diet as well the heart weight of sedentary rats was not affected by dietary PUFA. Zaloga *et al.* (2006) reported that rats fed n-6 and n-3 PUFA *ad libitum* had similar growth rates and cardiac weights. Pinnotti *et al.* (2006) studied the effect of UFA rich diet on myocardial performance found that rats fed UFA rich diet had the same body weight as compared to control, which they attributed to the fact that rats on hypercaloric diet ate less compared to those on commercial chow.

2.7 Effect of dietary fatty acid on mitochondrial decay

2.7.1 Membrane fatty acid composition

The amount of fat in diet can influence tissue or membrane fatty acid accretion (Robblee and Clandinin, 1984; Jones *et al.*, 1995). The levels of EFA in tissues are affected by the intake of different unsaturated and non essential PUFA in a manner that reflects the composition of the dietary fat source (Innis and Clandinin, 1981; Tahin *et al.*, 1981). Addition of 7 % cod liver oil or lauric acid to 1.2 % ethyl linoleate caused extensive substitution of several non essential fatty acids in lipids of brain mitochondria, erythrocytes and whole heart with a decrease in EFA or increased EFA in brain, heart and erythrocytes along with a decrease in monoenes and eicosatrienoic acid respectively suggesting a mechanism by which lauric acid increased effectiveness of linoleic acid in preventing EFA deficiency (Century *et al.* 1963). It has been reported that

the conversion of linoleate to arachidonate in tissues was inhibited by fish oil in the diet (Edwards *et al.*, 1963).

Yamamoto *et al.* (1965) observed an increase in 18:2 and 18:6 levels in mitochondrial cardiolipin and phospholipid fractions cephalin and lecithin. Cardiolipin is an important factor in the respiratory chain of the mitochondrion (Fleicher *et al.*, 1962). Rose (1964) showed that ox heart cardiolipin contained larger amounts of 18:2 than rat liver cardiolipin and connected this difference with the different amounts of linoleate consumed by each organ. Yet feeding of linoleic acid resulted in large amounts of 20:5, 22:5 and 22:6 fatty acids in liver phospholipids.

The fatty acid composition of PC, PE and cardiolipin were influenced by dietary oil in a reversible way (Innis and Clandinin, 1981). They also showed that mitochondrial membrane phospholipids isolated from rats sacrificed before dietary treatment contained a much higher proportion of C_{14} and C_{16} SFA than rats sacrificed later, in which the amount of C_{14} and C_{16} fatty acids in all phospholipids and C_{18} fatty acid in cardiolipin decreased with feeding of different oils. Feeding of PUFA rich soya bean oil altered the composition of PE but not PC, increasing the proportion of ω-6 FA in PE. This report demonstrated for the first time, in the whole animal fed diets adequate in all nutrients that sub cellular membrane lipids readily respond to change in dietary FA balance.

Chen *et al.* (1987) observed that the fatty acid composition of corn oil diet was 26.8% oleic acid, 57.1% linoleic acid and fish oil concentrate and menhaden oil contained relatively high concentration of ω-3 FA namely eicosapentaenoic acid (EPA;14.9 – 15.9 %), dexahexaenoic acid (DHA; 6.9-9.9 %) plus large amounts of mysteric acid (7.1-8.8 %), palmitic acid (16 %), palmiloleic acid (9.6-13.6 %) and oleic acid (14-17 %). Dietary marine oils resulted in large levels of EPA in phospholipids from all neural tissues compared to sunflower oil in rats, and the dietary

treatments did not significantly affected the amount of DHA in PC, PE or PS fractions from these tissues where 16:0, 18:0 and 18:1 were the principal FA in all four phospholipid fractions of the brain (Philbrick *et al.*, 1987). On the other hand Nalbone *et al.* (1988) reported that there was no variation in total phospholipids in rats fat either low fat diet, corn or salmon oil diets but there was a sticking difference in the FA composition of PC and PE. In PC the MUFA was lower in high fat diets than in low fat diet. In the heart PC and PE 22:4n-6 and 22:5n-6 levels were higher in rats fed corn oil diet but arachidonic acid, 20:4n-6 in cardiac PC was not different in rats fed low fat diet but in PE it was higher than in hearts of rats fed low fat diet.

Christon *et al.* (1988) reported that when rats were fed on a EFA deficient diet containing 2.6 g linoleic acid/kg (18:2 (n-6)) there was no change in either microsomal phospholipid content or the amount of different classes of membrane constitutive phospholipids but the FA levels were changed however, with low levels of most of n-3, n-6 PUFA and higher levels of oleic, palmiloleic with a resultant lower unsaturation index. Since, feeding corn oil resulted in 0.2-0.6 % higher linoleic acid in PC but double the level in PE fractions of phospholipids compared to menhaden oil or coconut oil despite the presence of four times more linoleic acid in corn oil, Zuniga *et al.* (1989) suggested that the large amount of 18:2 (n-6) in corn oil diets had a minimal effect of the n-6 PUFA composition of liver membranes of corn oil fed rats compared to those isolated from rats fed coconut oil.

Ramsey *et al.* (2005) observed a moderate increase in linoleic acid concentration by 2.5 % between rats fed corn oil and fish oil diet.

2.7.2 Membrane surface area

` The effect of dietary fatty acid on the mitochondrial membranes has not been studied elaborately and hence available literatures are

scant. However, the reduction in surface area of mitochondrial inner membrane observed in page rate could be related to effect of dietary fatty acid. (Fattoretti *et al.* 1996).

2.7.3 Mitochondrial membrane potential

Malis *et al.*(1990) demonstrated that fatty acids from fish oil were readily incorporated into renal cortical mitochondria and when exposed to ROS, the mitochondria enriched with n-3 fatty acids had significantly greater uncoupled respiration thus reduced mitochondrial membrane potential (MMP) than those from beef tallow or corn oil fed rats.

Brand *et al.* (2003) reported that phospholipids fatty acyl polyunsaturation was positively correlated and mono unsaturation and negatively correlated with proton conductance and therefore with $\Delta\varphi$ m.

Ramsey *et al.* (2005) observed that the feeding of corn oil did not significantly increase the Δ φ m in the liver of rats compared to those fed on fish oil diet even though the later group showed increase Δ φ m. However it was envisaged that changes in membrane surface area and/or inclusion of dietary fat at higher levels might alter membrane potential.

2.7.4 Mitochondrial ETC activity

Changes that occur in the activity of enzymes during a particular age with dietary manipulation could be associated with the particular fatty acid composition of mitochondrial membrane at that stage through an influence on physico-chemical status (Barzanti *et al.*, 1994). Zuniga *et al.* (1989) documented that the long chain PUFA appeared not only to be involved in the fluidity but also in changing the biolayer thickness with consequential differential exposure of active enzyme sites.

2.7.4.1 Heart

Rafael *et al.* (1984) by studying the effect of EFA deficiency on basal respiration and function of liver concluded that the increased metabolic rate of EFA deficient rats related to the body weight was not explained from impaired functional integrity of the inner mitochondrial

membrane and respiratory chain enzyme activities and amounts of mitochondria in heart were unchanged by EFA deficiency.

Jackson *et al.* (1988) reported that metabolites of arachidonic acid are unlikely to be primary controlling factors of muscle growth or specific mediators of sarcolemmal damage leading to enzyme efflux.

2.7.4.2 Brain

The respiratory chain features depends on the mitochondrial subtype and on their respective brain area (Battino *et al.*, 1991). The brain heterogeneity exists at sub cellular, cellular and cerebral regional levels representing non synaptic free mitochondria, inter synaptic and heavy mitochondria because of the presumably different protein/lipid ratio. Villa *et al.* (1989) reported that the heavy mitochondria had the lowest content of respiratory components and the lowest enzymatic activities both in the respiratory chain and in the matrix. Since the heavy mitochondria fractions represent the oldest mitochondrial population and the physiological continuous damage accumulating at the mitochondrial level as a result of both electron leakage at complex I site, as well as, of the super oxide anion production could be responsible for loss of enzymatic activities and impairment of the typical mitochondrial function (Turrens, 1997).

Battino *et al.* (2002) reported that the presence of higher proportion of 20:4n-6, 22:4n-6 or 22:6n-3 found in heavy mitochondria may be responsible for increasing the loss of enzymatic function. However, Mataix *et al.* (1998) suggested that the correlation between MUFA/PUFA index in membrane and the ability of the latter to withstand LPO and oxidative stress and this index could even be more important than the net PUFA content itself.

Quiles *et al.* (1999) observed that the mitochondrial membranes were able to modify their FA profile to maintain constant membrane

fluidity and has been proposed for partial substitution of SFA in the membrane by PUFA. Battino *et al.* (2002) summarized these changes in the mitochondrial membranes are not enough to counter the accumulated damage but contribute to the same by increasing the oxidizability of the membrane due to ageing imbalances encountered to reestablish mitochondrial fluidity and functioning.

2.8 Effect of dietary fatty acid on apoptosis and ageing

2.8.1 Apoptosis

Hong *et al.* (2002) demonstrated that compared to corn oil rich in 18:2n-6 , fish oil rich in EPA up regulated apoptosis by incorporation of n-3 FAs into mitochondrial phospholipids increasing their susceptibility to per oxidation resulting in decreased MMP, release of cytochrome C into cytosol and activation of caspase-3. With reference to the interaction between cardiolipin and cytochrome C it was evident that loss of linoleic acid caused the disassociation of cardiolipin from cytochrome c triggering apoptotic cascade. This support our hypothesis that strengthening the membrane linoleic acid (LA) content would prevent rapid peroxidation of the membranes due to increased turnover of linoleic acid, which fortified the association between cardiolipin and cytochrome c thus retarding apoptosis unlike with n-3 PUFA.

2.8.2 Ageing

The type of dietary fat whether, monounsaturated or polyunsaturated plays a significant role in the ageing process. Post mitotic tissues were highly prone to oxidative stress and LPO compared to mitotic tissues and α-tocopherol was found to adapt to oxidative stress and were preferentially accumulated during ageing in post mitotic tissues *viz.* heart (Ochoa *et al.*, 2003). Pinnotti *et al.* (2006) reported that feeding UFA caused oxidative stress on the myocardium that was responsible for the cardiac morphological injuries.

2.8.3 Oxidative stress markers

2.8.3.1 Lipid peroxidation and protein carbonyls

Hammer and Wills (1978) reported that diet containing PUFA regulates the fatty acid composition of liver endoplasmic reticulum and this in turn was possibly an important factor controlling the rate and extent of LPO *in vitro* and *in vivo*.

Leibovitz *et al.* (1990) observed increased TBARS in the liver and heart from menhaden oil feeding than corn oil lard feeding in rats due to increase in n-6 fatty acids (37.4 %) and decrease in n-3 fatty acids (3.7 %) in membranes.

Paradies *et al.* (1998) exposed mitochondrial membranes from rat heart to a free radical generating system that resulted in mitochondrial lipid peroxidation and a marked loss of cytochrome c oxidase activity. Shidoji *et al.* (1999) suggested that peroxidation of cardiolipin in mitochondrial inner membrane may disrupt a molecular interaction between the membrane phospholipid and the protein.

Ramsey *et al.* (2005) observed no difference in the markers of protein and lipid oxidative damage when rats were fed with LA rich corn oil when compared with soy oil fed rats.

Pinnotti *et al.* (2006) demonstrated that supplementation of UFA rich diet caused myocardial oxidative stress and mild structural alterations without changing the mechanical functioning of the heart.

Hulbert *et al.* (2006) demonstrated that despite presence of similar amounts of membrane total UFA in mole rat and mice the latter is more susceptible to lipid peroxidation and oxidative stress due to the presence of nine times more DHA in their membrane.

Nalbone *et al.* (1988) studied the effect of dietary salmon oil feeding on rat heart reported that short term feeding (2 months) of fish oil

resulted in moderate accumulation of lipid associated with lipofuscin like material. These may be attributed to presence of long chain monosaturated fatty acids in the composition of fish oil and a degradative form of peroxidized lipids.

The long chain polyunsaturates in membrane bilayer of mitochondria however are very susceptible to damage both chemically and also because of their locations close to the site of ROS production, membrane lipid peroxidation is an autocatalytic chain reaction and many of its products including hydroxynonemal (from ω-6 PUFA) and hydroxyhexanemal (from ω-3 PUFA) are themselves very potent damagers of proteins (Halliwell and Gutteridge , 1999). These findings clearly suggested that membrane phospholipid composition was an important determinant of longevity.

2.8.3.2. DNA fragmentation

Dietary fatty acids are shown to affect the cellular machinery differently owing to the fact of fatty acid gene interaction (Flick *et al.* 1977). The high levels of PUFA in several of the vegetable oils predispose membranes to increased lipoxidation (Hulbert *et al.,* 2003) due to increased production of ROS (Rao *et al.* 1990a), compared to UFA or mono unsaturated fatty acid (Zuniga et al, 1989). Hamilton et al, (2001) reported occurrence of increased DNA fragmentation due to increased production of H2O2 and oxidative stress.

2.8.4 Antioxidant enzyme activity

Venkataraman *et al.* (1994) determined the protective action of n-3 and n-6 lipids and concluded that corn oil fed mice had significantly lower activities and expression of catalase, GPx and SOD with higher peroxidation indices and TBARS generation despite lower

eicosapentaenoic and docosahexaenoic acid levels in hepatic microsomes.

Feeding of long chain PUFA influenced the concentration of 20:4 (n-6) and 22:6 (n-3) in body tissues of rats (Suarez *et al.*, 1996) and reduced endogenous antioxidant levels and increased LPO (Suarez *et al.*, 1999).

Pinnotti *et al.* (2006) observed a significant reduction in the levels of SOD and catalase in cardiac tissue there by lowering the antioxidant activity in the myocardium treated with corn oil.

2.8.5 Myocardial collagen

Direct evidence to effect of dietary fat source on the levels of myocardial collagen are not available. However, the dietary fatty acids are known to affect myocardial collagen by their action on protein oxidation (Zimmerman *et al.* 1993). Collagen accumulation has been reported to reduce coronary blood flow, ultimately affecting ventricular compliance (Fioretto et al, 2002).

2.8.6 Lipid profile

Dietary fat is known to affect serum concentrations of total and lipoprotein cholesterol. However, all components of dietary triglycerides, saturated, monounsaturated, and polyunsaturated fatty acids do not have identical effects on serum cholesterol levels. In contrast, an HDL-lowering action has been related to polyunsaturates (Grundy, 1989). Dietary (n-3) and (n-6) PUFA differ in their effects on serum lipid metabolism which may modulate the mean life span of SAMP8 mice fed each of the different dietary oil. The serum total cholesterol, HDL cholesterol, triglyceride and phospholipid concentrations were significantly lower in the perilla oil group than in the safflower oil group (Umezawa *et al.*, 2000). Feeding of mono- and poly- unsaturated fat diets

lowered the total cholesterol, LDL and HDL cholesterol concentrations relative to the saturated fat diet with concomitant reduction in the hepatic apolipoprotein C-III mRNA concentrations and increase in liver triglycerides (Borasseau *et al.*, 1995).

In contrast to the general dogma of increase in plasma concentrations of cholesterol and triglycerides with age, Ahn *et al.* (1994) observed the plasma triglyceride concentrations to increase with age in both groups, but especially in the *ad libitum* fed rats.

A high ratio of polyunsaturated fatty acid (PUFA) to saturated fatty acids (SFA) in the diet is a major factor in lowering plasma cholesterol concentrations by dietary means is considered beneficial in preventing coronary heart disease. Chen *et al.* (1987) reported that the total amount of fatty acids found in the lymph after marine oil feeding was significantly less than that found after corn oil feeding, which was suggestive of a decrease in digestibility and absorption of menhaden oil and fish oil concentrate compared with corn oil.

Nalbone *et al.* (1988) reported that feeding of either fish oil or corn oil did not show any change in the concentrations of triglycerides and cholesterol as compared with a low fat diet.

Pinnotti *et al.* (2006) observed an increase in LDL-C and decreased triglycerides in cardiac tissue due to the high fat content in UFA enriched diet (`20 %) than control diet.

On the other hand, the acid distribution in the phospholipids of the various tissues was at all ages very different from that of the corresponding diets and their alterations, characteristic for each tissue, may therefore be correlated more with age than with dietary conditions (Desmeth *et al.*, 1981). On the contrary, Malaguarenera *et al.* (1998) reported a significant reduction in total cholesterol, triglycerides and LDL-C concentrations as well as total cholesterol/ HDL-C and LDL-

C/HDL- C ratios and a significant increase in HDL-C, apolipoprotein A1, apolipoprotein B100 and lipoprotein values with age.

2.9 Effect of calorie restriction on growth rate

2.9.1 Body weight

CR rats showed considerable reduction in body weight compared to their *ad libitum* fed counterparts. Bevilacqua *et al.* (2004) showed that following the initial period of adaptation CR animals had reduced body weights (32 %) compared to the ad libitum fed control animals at the end of 6 months feeding regimen. Ramsey *et al.* (2004) observed the body weight to decrease by 12 % and 29.9 % after 1 and 6 months of CR respectively.

Hagopian *et al.* (2005) observed a 35 and 40% reduction in body weight in long term (12 and 18 months) CR rats respectively.

Bevilacqua *et al.* (2005) reported that after 12 months of CR there was a 35.7% decrease in mean body weight between CR and the age matched control group. Sreekumaran *et al.* (2002) reported that the 36 week CR programme resulted in substantially lower body weight in CR rats compared to control rats.

Madapallimattam *et al.* (2001) reported that rats fed on a hypoglycemic diet had lower body weights compared to *ad libitum* fed control rats and the loss in body weight was progressive with the duration of hypo energetic diet with a mean weight loss of 29.2±4.1 % recorded against 17.5 ± 4.0 % in the control rats.

Mai *et al.* (2003) subjected mice to CR and found a relatively lower body weight (75 % of control) compared to those on high fat diet (108 % of control). The difference in body weights were attributed to lower fat, lean mass and were also associated with a reduction in calorie intake.

2.9.2 Organ weight

The reduced body weight following CR was also reflected on the reduction in the organ size and weights. Bevilacqua *et al.* (2004) observed no significant difference in tissue and organ weights after 2 weeks between CR and control rats. A significant decrease in weights of liver (30 %) and heart (13 %) except kidneys were observed after 2 months of CR which increased to 38 %, 17 % in liver and heart including kidneys (20 %) at the end of 6 months.

Ramsey *et al.* (2004) reported that all the organs weighed less after 6 months of CR and liver showed maximum reduction in weight compared to other organs. Hagopian *et al.* (2005) reported that heart (24-28 %) and kidneys (22-28 %) were the only organs next to liver to show weight difference after 12 and 18 months of CR.

2.10 Effect of calorie restriction on mitochondrial decay

2.10.1 Membrane fatty acid composition

Studies have reported long term calorie restriction an intervention that has consistently been shown to increase maximum life span and mitochondrial LA content (Langaniere and Yu, 1989; Ramsey *et al.*, 2004) and decrease the decohexaenoic acid content (Langaniere and Yu, 1989; 1993). A doubling of mitochondrial linoleic acid concentration was seen when rats were subjected to long term CR (Langaniere and Yu, 1993). Faulks *et al.* (2006) reported that changes occurred in the fatty acid composition of phospholipids from heart, brain, liver, kidney and skeletal muscle as early as at one month in mice fed on CR at different levels.

2.10.2 Membrane surface area

Available literature on the effect of CR on surface of mitochondria is negligible. However, in CR rats the observed age related enlargement of the organelles is a morpho-functional compensatory response (Fattoretti *et al.* 1998). The age associated increase in the enlargement in mitochondria did not alter the surface area of the membrane due to

attainment of more spherical form (Safiulina *et al.*, 2006). Improved surface area of mitochondrial inner membrane was related to efficient enzyme complex activities (Schwerzmann et al,. 1989).

2.10.3 Mitochondrial membrane potential

Several studies have reported a negative correlation between linoleic acid and proton leak and a positive correlation between long chain PUFA such as decosopentaenoic acid (22:5n-3) and proton leak (Brand *et al.*, 2003; Porter *et al.*,1996; Brookes *et al.*, 1998). Pehowich (1999) also showed that changes in dietary n-3 FA can alter proton leak in liver and EFA deficiency has been shown to increase mitochondrial proton leak (Fontaine *et al.*, 1996). The increase in proton leak reduces the MMP.

Short term (6 month) or medium and long terms (12 and 18 months) CR incredibly increased the $\Delta \varphi m$ in rat skeletal muscle due to a decrease in proton conductance (Bevilacqua *et al.*, 2004; 2005).

Hagopian *et al.* (2005) reported that despite an increasing trend in proton leak was observed at 12 month of CR , membrane potential were not found to be different between groups at these maximum leak dependent respiration point. However, higher $\Delta \varphi m$ was observed at other points indicative of lower proton leak in CR animals.

Hagopian *et al.* (2005) and Lopez-Lluch *et al.* (2006) reported that CR resulted in a decrease in $\Delta \varphi m$ and lower O_2 consumption and higher proton leak. On the contrary, Lambert and Merry (2004) reported decreased proton leak and higher $\Delta \varphi m$ in CR mitochondria contributing to reduced ROS production.

In variance, Ramsey *et al.* (2004) opined that changes in $\Delta \varphi m$ that tend to increase at 1 month of energy restriction tend to disappear by 6 months of CR.

2.10.4 Mitochondrial ETC activity

CR was found to increase the level of one protein and decreased three proteins in the heart and concluded that the effect of age on mitochondrial proteome appeared to be tissue specific and CR has a major effect on age related protein changes (Chang *et al.*, 2007).

2.10.5 Hydrogen peroxide

The lower mitochondrial free radical generation is related to the mitochondrial proton leak (Bevilaqua *et al.*, 2004; Harper *et al.*, 1998; Lal *et al.*, 2001), a process that is responsible for 20 – 30 % of resting cellular expenditure (Rolfe and Brown, 1997; Brand *et al.*, 2003).

Hydrogen peroxide production from mitochondria is related to the backflow of electrons from complex II to complex I (Chance and Hollunger, 1961) and the difference in between liver, heart and skeletal muscle H_2O_2 production may reflect differences in duration and degree of CR and difference between mitotic and post mitotic tissue.

Drew *et al.* (2003) found that lower level of H_2O_2 production only in isolated gastrocnemius muscle mitochondria following lifelong CR in 26 months old Fischer 344 rats compared with *ad libitum* fed controls but not in heart mitochondria. In variance to the above, Gredilla *et al.* (2002) observed a decrease in H_2O_2 production after 40 per cent CR for 1 year in rat heart mitochondria but not after 6 wks or 4 months. Similarly, Bevilacqua *et al.* (2004) reported that the mitochondrial production of H_2O_2 decreased by 53 % in 2 wks of CR which decreased further to 74 % by 6 months. Lopes-Torres *et al.* (2002) observed a 47 % decrease in H_2O_2 production and a 46 % decrease in oxidative damage to mitochondrial DNA with long term CR for 1 year in rat liver mitochondria. Hagopian *et al.* (2005) reported that there was no difference in H_2O_2 between groups when mitochondria were respiring on succinate in the presence of rotenone unlike that observed when

mitochondria were respiring on pyruvate plus maleate alone or in the presence of antimycin A.

Bevilacqua *et al.* (2005) observed an age related decrease in H_2O_2 production in CR rats at 51 % and 49 % by 18 and 24 months of age, respectively. However, feeding 6 month old rats with 60 % of calculated *ad libitum* intake amount had no change in production of H_2O_2 in liver mitochondria (Ramsey *et al.*, 2005). On the other hand, Faulks *et al.* (2006) reported that there was no effect by CR on the *in vitro* ROS production by liver or skeletal muscle mitochondria at 3 months which significantly decreased after 6 months in liver mitochondria suggesting ROS production is tissue specific responding differently to CR paradigm. These findings clearly state that the age of the animal is directly proportional to the mitochondrial production of H_2O_2 and the difference in mitochondrial H_2O_2 production could also be related to the age at which CR was initiated.

2.11 Effect of calorie restriction on apoptosis and ageing

CR retards ageing in rodents and other animals including non human primates by means of various molecular mechanisms among which oxidative stress appears very relevant. Therefore, mitochondria and their function represent a primary target of studies with CR. CR treatment has been reported to decrease the number of mitochondrial DNA (mtDNA) deleted species in rat skeletal muscle (Aspnes *et al.*, 1997) but very little has been found about other tissues with recent techniques (Kang *et al.*, 1998).

2.11.1 Markers of oxidative stress

A rapidly expanding body of data implicates oxidative stress in the development of Parkinson's disease, Alzheimer's disease, heart failure and other diseases. In animal studies, organs such as brain and heart in which the parenchyma consists of post mitotic cells are susceptible to

oxidative damage. Thus oxidative stress and damage may be causal factors in the attrition of senescence and various diseases associated with ageing and CR may attenuate the damage (Weindruch and Sohal, 1997).

2.11.1.1 Lipid peroxidation and protein carbonyls

Supplementation of dietary n-3 and n-6 lipids were susceptible to lipid peroxidation and feed restriction was beneficial in decreasing lipid peroxidation (Bhattacharya *et al.*, 2003). Gomi and Matsuo (1998) observed difference in lipid peroxidation between control and CR rats only after the animals reached 30 months of age. Similarly, Tian *et al.* (1998) reported that significant age related increase in protein oxidative damage did not occur in the liver from rats until the animals were 24 months old.

Ramsey *et al.* (2004) reported no significant increase in the oxidative markers measured as TBARS and protein carbonyls in 12 months old energy restricted rats following a 6 month feeding period. This may be due to the fact that difference in liver ROS production occurs between control and CR animals only in late middle age (Hagopian *et al.*, 2005). In variance, Bevilacqua *et al.* (2005) observed that there was an age dependent decrease in the ROS measured as lipid peroxidation in CR rats.

2.11.1.2 DNA fragmentation

CR has been shown to minimize oxidative stress due to increased antioxidant defense mechanism (Baek et al, 1999). However, Tsuzuki *et al.* (2001) demonstrated that over expression of catalase in the nucleus did not prevent oxidative damage to DNA. A decreased oxidative damage to DNA decreased DNA fragmentation (Hamilton *et al.* 2001).

The absence of significant difference in DNA fragments with age or with CR reveals that CR might provide neuroprotection to the ageing

brain by preserving DNA repair enzymes and /or up-regulated specific anti apoptotic proteins involved in neuronal cell death (Hiona and Leeuwenburgh, 2004).

2.11.2 Antioxidant enzyme activity

The direction of the influence of CR on free radical detoxification is tissue specific (Feuers *et al.*, 1993) and the benefits of CR on lifespan extension were related to the extent of restriction and the benefits of CR declined with delay in onset of treatment (Speakman and Hambly, 2007).

Baek *et al.* (1999) reported that the age related changes in the antioxidant enzymes *viz.* catalase and GPx were reversible with dietary restriction but SOD showed little change with ageing and dietary restriction in cerebrum, hippocampus and cerebellum.

Lee and Yu (1990) observed the SOD activity to increase in CR than *ad libitum* fed rats providing a possible biochemical mechanism for the anti lipoxidation action on membrane lipid peroxidation during food restriction.

Dietary restriction increased the activities of SOD and catalase at 21 and 28 months of age whereas GPx activities were found to increase by 28 months of age (Rao *et al.*, 1990b).

Piere *et al.* (1992) observed an age dependent decrease in GPx that was attenuated by CR. Luhtala *et al.* (1994) reported that 30 % dietary restriction attenuated the age related increase in skeletal muscle catalase activity but that of GPx was entirely prevented. These changes were more profound in cytosolic and mitochondrial fractions. Stowe *et al.* (2006) reported that CR decreased the age related increase in GPx despite several other factors *viz.* gender, selenium level and litter size that are known to influence GPx levels in dogs. Life long dietary restriction leads to an increase in the activities of GPx through changes in the levels of mRNAs (Semsei *et al.*, 1989).

Chandrasekar *et al.* (2001) found a rapid attenuation of myocardial oxidative stress by CR that was evident by increased expression of Mn-SOD, catalase and GPx mRNAs. Sreekumaran *et al.* (2002) studied the effect of CR on mitochondrial function and gene transcription in rat skeletal muscle using a high density oligonucleotide microarray containing approximately 800 genes and found a significant increase in the gene transcript levels for SOD (287 %), SOD2 (163 %) in CR rats compared with non CR rats. This increased transcription of the ROS scavenger enzyme genes could be one of the reasons for the increased expectancy and reduced oxidative damage in CR animals (Sohal and Weindruch, 1996). On the contrary, Sohal *et al.* (1994b), observed no clear cut overall pattern of age related or dietary related changes in antioxidant defense provided by SOD, catalase, GPx in CR mice.

2.11.3 Myocardial collagen

The effect of CR on myocardial collagen is mediated through its beneficial effect to minimize protein oxidation (Zainal *et al.*, 2000). Boreham *et al.*, (1988) reported that CR resulted in reduction in collagen levels. In contrast Okoshi *et al.*, (2001) reported that dietary restriction resulted in increased collagen concentration with minimally affected mechanical function.

2.11.4 Lipid profile

The effect of calorie restriction on lipid profile is not limited or scant. Food restriction started either at 6 weeks or 6 months of age was also more effective in extending the length of life than food restriction limited to early life (i.e., from 6 weeks to 6 months of age) or than restriction of protein but not calories. Masoro and Compton (1983) observed that food restriction started at 6 months of age was as effective as food restriction started at 6 weeks of age in modulating the age-related

increase in serum concentrations of total cholesterol, HDL-cholesterol and triglyceride, whereas the dietary regimens of restricting food from 6 weeks of age to 6 months of age and of restricting protein but not calories were much less effective.

It has been reported that calorie restriction increase in LA content (Chandrasekar *et al.*, 2001). Therefore, it is plausible that the effect of CR on lipid profile is related to the direct effect of linoleic acid itself. Flick *et al.* (1977) suggested that LA is the component of fat that indirectly affects lipogenic enzyme activities and it has been suggested that some metabolite of LA *viz.* arachidonic acid and most probably an eicosanoid and not LA itself initiate these repressive effects.

CHAPTER 3

MATERIALS AND METHODS

The study on the manipulation of dietary fat on mitochondrial decay and its implications on ageing and apoptosis was conducted at the Department of Veterinary Physiology, Madras Veterinary College, Chennai, Tamil Nadu, over a period of two years from 2006 to 2008. The study included evaluation of feeding dietary fat sources rich and low in linoleic acid with or without calorie restriction on membrane polyunsaturation, its implications on electron transport chain enzyme activity and ROS production in the heart and brain, and suppression of occurrence of apoptosis and ageing in heart and brain.

3.1 MATERIALS

3.1.1 Experimental animals

Adult male Wistar Albino rats of 12 months old were obtained from Centralized Animal House, Sri Ramachandra Medical College and Research Institute, Chennai and utilized in the study. The rats were maintained at the Centralized Laboratory Animal House at the Madras Veterinary College premises. The experimental procedures were approved by the Institutional Animal Ethical Committee.

3.1.2 Experimental diet

mmercial rodent chow was procured from M/s Vetcare India Pvt. Ltd. Bangalore. Purified diets according to American Institute of Nutrition (AIN) requirements were made from starch, cellulose, casein, sucrose, L - cystine, mineral and vitamin mixtures, coconut oil and corn oil were procured from local market, Chennai.

3.1.3 Chemicals

All the chemicals used in the present study were of analytical or molecular biology grade. Chemicals were procured from M/s Sigma Aldrich

(USA), Bangalore Genei (India), Sisco Research Laboratories (India) and other reputed firms.

3.1.4 Diagnostic kits

Diagnostic kits to estimate total cholesterol and high density lipoprotein cholesterol were procured from M/s Span Diagnostics, Chennai and triglycerides estimation kit was procured from M/s Erba Pvt Ltd., Mumbai.

3.1.5 Buffers and reagents

The details of the buffers and other solutions used in this study are given in the Appendixes II to IV. The analytical and molecular biology grade reagents were used for preparation of various buffers and reagents using Millipore nanopure distilled water.

3.1.6 Equipments

Weighing Balance (Sartorius, Germany; Adams, Germany), Magnetic stirrer (Remi equipments, India), Digital pH meter (Digisun Electronics Systems, India), Microcentrifuge 5810R (Eppendorf, USA), Centrifuge (Remi equipment, India), water bath (Precision Scientific, USA), Orbital shaker (Labline Instruments, USA), deep freezer, -20˚C (Blue star, India), Gel electrophoresis apparatus for BN PAGE and Second dimension (Biorad, USA ; Bangalore Genei), India), -80˚C freezer (Haereus, Germany), Autoclave (Scientronic India), Hot air oven (Scientronic India), Tissue Homogenizer (Heidolph, Germany), Electron Microscope (Philips Ltd.,), Fluorescent specptrophotometer (Hitachi, Japan), Gas Chromatography-Mass Spectroscopy Shimadzu 2010 (Shimadzu, Japan), were used in the study.

3.2 METHODS

3.2.1 Experimental design

A trial was conducted to study the effect of late onset of feeding corn oil containing diet and CR on activity of specific ET complexes and

mitochondrial decay on the production of ROS and its implications on ageing and apoptosis in heart and brain.

Adult male Wistar Albino rats (18 months old) were used in this study to look into the effect of late onset of dietary manipulation on longevity. The rats were housed in individual cages and fed on semi purified commercial rat chows until they reached 18 months of age. During this period the rats had free access to water and feed.

The rats were maintained in a natural environment with exposure to prevailing environmental conditions throughout the study period and to a photoperiod controlled condition (12L: 12D) with lights from 0600 - 1800 hrs. The rats were fed one hour before the beginning of dark period throughout the period of study.

3.2.2 Experimental diets

3.2.2.1 Control diet – Low linoleic acid

The control diet was prepared using 20 per cent coconut oil added to starch, casein, cellulose, sucrose, L-cystine, mineral and vitamin mixtures. This diet supplied the necessary linoleic acid to prevent essential fatty acid deficiency, hence low linoleic acid diet.

3.2.2.2 Experimental diet – Linoleic acid rich

The experimental diet was prepared using 20 per cent corn oil added to starch, casein, cellulose, sucrose, L-cystine, mineral and vitamin mixtures. Corn oil is a rich source of linoleic acid hence linoleic acid rich diet. The compositions of the experimental diets along with their proximate analysis are presented in Appendix 1.

3.2.3 Feeding regimen

The rats were fed on commercial rat chows until they reached 18 months of age. The feeding of purified diets containing coconut oil (linoleic acid low) or corn oil (linoleic acid rich) began when the rats

attained 18 months of age and continued for a period of 12 months. Both the rations were fed at *ad libitum* and at 60% of *ad libitum* intake to study the effect of CR.

Groups	**Treatment**	**18 month**	**18-24 months**	**18 - 30 months**
I *	Commercial Chow	7	-	-
II	Low LA diet (coconut oil ; control)		7	7
III	Low LA diet + CR		7	7
IV	LA rich diet (corn oil)		7	7
V	LA rich diet + CR		7	7

*Includes animals to be sacrificed at the start of the trial to assess the normal changes that has occurred due to conventional feeding

To prevent the loss of mineral and vitamin intake in calorie restriction diet, they were additionally supplemented in diet.

3.2.4 Sacrifice of experimental animals

Six animals from each group were periodically sacrificed under chloroform anesthesia at 18, 24 and 30 months. One animal was exclusively maintained and sacrificed for EM studies.

3.2.5 Body weight and organ weight

Body weight and body weight gains of the experimental animals were recorded at monthly intervals. Following sacrifice of the animals heart and brain were removed and weighed using an electronic balance.

3.2.6 Collection of samples

Upon sacrifice of the animals the heart and brain were immediately removed and stored in ice cold isolation buffer for heart and brain (Appendix-II) respectively and processed for isolation of mitochondria. The samples for electron microscopic (EM) studies were collected from

heart and brain in gluteraldehyde and ultra thin sections were made separately to study the surface area of the mitochondria.

Blood samples were collected at sacrifice, centrifuged, serum separated and stored at -20°C until processed for lipid profile.

3.2.7 Isolation of mitochondria

3.2.7.1 Heart mitochondria

Mitochondria from rat heart were isolated following the method described by Palmer *et al.* (1977).

The hearts were removed and immediately placed in cold Buffer A (pH 7.4; Appendix- II). After removal of extra ventricular tissue, the hearts were rinsed 5 to 10 times in buffer A and blotted dry. The tissues were finely minced and brought to a final concentration of 10 g tissue/100 g of Buffer A plus 2 mM EGTA and 0.2 % BSA. The tissue suspension was homogenized in a polytron homogenizer for 30 seconds at 6500 rpm. Collagenase was added to the homogenate at a final concentration of 5 mg/g wet tissue, diluted 2-fold with Buffer B (pH 7.4; Appendix- II) and immediately centrifuged at 5000 x g for 5 minutes.

The high speed pellet remaining after collagenase treatment was resuspended in original volume of Buffer B and sedimented 500 x g to yield nuclear pellet. The supernatants were collected separately and the nuclear pellet washed twice in Buffer B plus 2mM EGTA and 0.2 % BSA and the supernatants added to nuclear supernatant. The supernatants were centrifuged at 3000 x g for 10 minutes. The resultant pellet was washed twice and then resuspended in Buffer B plus 0.5 mM EGTA to a final concentration of 25 mg /ml

3.2.7.2 Brain mitochondria

Mitochondria from the brain of rat was isolated following the procedure described by Sciamanna et al. (1992).

The brain was collected in ice cold MSETB buffer (pH 7.4; Appendix-II). The meninges were removed and the grey matter scrapped from cortices using a blunt spatula.

The tissue was homogenized using a tissue homogenizer in the MSETB buffer (1g/10ml) at 4°C. The homogenate was centrifuged at 2000 x g for 3 minutes and the supernatant was centrifuged at 12000 x g for 8 minutes. The pellet was resuspended with MSETB buffer and centrifuged at 12000 x g for 10 minutes twice. The mitochondrial suspension obtained by suspending the final pellet with SET buffer (Appendix-II) at 4°C and stored at -20 °C for analysis of enzymes.

3.3 Mitochondrial bioenergetics and oxidative stress

3.3.1 Mitochondrial membrane fatty acid composition

Fatty acid profile of the mitochondrial membranes was estimated following the method of Lepage and Roy (1986) following the chromatographic conditions described by Pampalona *et al.* (1998).

Direct transestrification of the samples

To 100 µl of the isolated mitochondria in buffer an internal standard consisting of 100 µg of tridecanoic acid (C13:0) dissolved in methanol: benzene (4:1) was added. 200 µl of acetyl chloride was slowly added to this mixture while stirring over a period of 1 minute. Tubes were tightly closed with Teflon lined caps and subjected to methanolysis at 100°C for 1 hr. After tubes were cooled in water, 5 ml of 6 per cent K_2CO_3 solution was slowly added to stop the reaction and neutralize the mixture. The tubes were then shaken and centrifuged and an aliquot of the benzene upper phase was injected into the chromatograph.

Gas chromatography

Fatty acids were chromatographed as methyl esters on a 30-m fused silica column with an internal diameter of 0.32 mm using Gas

Chromatography-Mass Spectrometer (GC-MS). The mass spectrometer was used as a detector in the electron impact ionization mode. GC-MS conditions were: injector and detector port temperatures 220°C and 250°C respectively. Column temperature ranged from 100°C with an increase of 10°C/min from 200°C to 240°C at 5°C/min and a final hold of 12 minutes. Identification of methyl esters were made by comparison with authentic standards. The molar per cent of the fatty acids were calculated from multiplying the area of each peak by arithmetic factor molecular weight correction factor for each fatty acid.

3.3.2 Mitochondrial inner membrane surface area

The mitochondrial volume densities and mitochondrial surface areas in the heart and brain were determined by electron micrographs using stereological technique described by Weibel (1969).

Preparation of tissues

Upon sacrifice of the animals heart and brain were removed and quickly placed in cold gluteraldehyde fixative in 0.1M cacodylate buffer and 0.175M sucrose (pH7.2) in which they were diced to squares of less than 0.25 mm` and fixed for 4 hours. The samples were then washed in cacodylate buffer for 16 hours then fixed using osmium tetroxide 2 % in 0.1 M cacodylate buffer for 4 hours, rinsed with sodium acetate and bulk stained using 2 % uranyl acetate. The tissue blocks were then dehydrated in an ethanol series (30 -100% dry) for 3 hours, transferred to 100 % acetone infiltrated with 1:1 acetone/resin for 1 hour and then 1:9 acetone resin for 12 hours and finally cured at 60°C for 24 hours in fresh 100 % low viscosity epoxy resin. Ultra thin sections (60-150nm) were cut with glass knives on an LKB 8800 III ultra microtome.

Electron microscopy

Electron micrographs at x 23000 and x 49000 were taken with electron microscope. The electron micrographs at x 49000 were taken at

random from one specific corner of the squares of the supporting 200 mesh copper grids and were used for determining relative mitochondrial volume densities and inner mitochondrial membrane surface areas.

Stereological analysis

Stereological analysis was carried out to measure mitochondrial inner membrane surface area. Inner membrane surface area was measured using point counting method. Stereological measurements were done using Analysis MCID 7.0 software.

3.3.3 Mitochondrial membrane potential

The mitochondrial membrane potential was estimated fluorimetrically following the method described by Scaduto and Grotyohann (1999). This method employed the use of TMRM - a mitochondria specific dye to estimate membrane potential.

The fluorescence intensity at one excitation wavelength was determined before the addition of mitochondria. This measurement was used as an indication of total dye concentration using a standard cure made by serial dilution of TMRM in incubation media. Mitochondria (0.25 mg/ml) was incubated in the presence of 0.33 μM TMRM in the medium (pH 7.0) in the presence of 10 mM of succinate. After a minute, the fluorescence of the supernatant of the suspension at both excitation wave lengths (546 and 573 nm) and at 590 emission wave length was measured and the contents of the cuvette were immediately centrifuged at 11,000 x g for 10 minutes to pellet the mitochonria. Results were calculated according to Nernst equation and expressed in mV using a standard curve.

3.3.4 Mitochondrial OXPHOS enzyme activity

The activities of the complexes I-IV were measured spectrophotometrically following the procedure described by Rouslin (1983).

3.3.4.1 Activity of complex I

Principle: The activity of complex I was measured by following the decrease in absorbance due to the oxidation of NADH at 340 nm with reference set at 425 nm.

Procedure

40 µg of isolated mitochondria were disrupted by freezing and thawing 3 times in potassium phosphate buffer (20 mM/L; pH7.2), and then they were added to a buffer containing 25 mM potassium phosphate/L, 5mM $MgCl_2$/L, 2mM KCN/L, 2 mg antimycin A/ml, 2.5 mg fat free BSA/ml, 0.13 mM NADH/L and 65 µM ubiquinone/L. The complex I activity was measured for 3 minutes and then for another 3 minutes after the addition of 2 mg rotenone.

The decrease in NADH activity, after rotenone was added is the blank slope and was subtracted from the NADH slope before adding rotenone. An extinction coefficient of 6.22 mM L^{-1} Cm^{-1} was used to calculate absolute changes.

3.3.4.2 Activity of complex II

Principle: Complex II activity was measured following the reduction of 2, 6, dichlorophenol indophenol at 600 nm.

Procedure

The mitochondria (40 µg) were disrupted by freezing and thawing 3 times in potassium phosphate buffer (20mM/L; pH 7.2), and pre-incubated in a buffer containing 25mM potassium phosphate buffer/L, 5mM $MgCl_2$/L and 20mM succinate/L at 30°C for 10 minutes to activate the enzyme.

After pre-incubation, 10mM sodium azide/L (to inhibit complex IV), 2 mg antimycin A/ml (to inhibit complex III) , 2 mg rotenone/ml (to

inhibit complex I) and 50 mM 2,6 dichlorophenol inodophenol /L were added and base line changes were recorded.

16 mg ubiquinone was then added and the reduction was measured. An extinction coefficient of 19.1 mM L^{-1} Cm^{-1} was used to calculate absolute changes.

3.3.4.3 Activity of complex III

Principle: Complex III activity was measured by following the reduction of cytochrome C Fe^{3+} by decyl ubiquinol.

Procedure: 10µg of mitochondria were disrupted and solublized in 0.1 % triton X-100 and were added to an incubation mixture containing 100 µM EDTA/L, 0.2 % fat free BSA, 3mM sodium azide/L and 60 µM cytochrome C Fe^{3+}/L in 50mM potassium phosphate buffer (pH8.0) in a reaction volume of 1 ml. The mixture was pre-incubated for 3 minutes at 30°C. The reaction was started by addition of 10 µl decyl ubiquinol and the decrease in absorbance at 550 mM was recorded. The blank was measured by adding 10 mg antimycin A to the above mixture to inhibit complex III. An extinction coefficient of 21 nmol L^{-1} cm^{-1} was used to calculate absolute changes.

3.3.4.4 Activity of complex IV

Principle: The complex IV activity was measured by following the oxidation of cytochrome C Fe^{3+}.

Procedure: The mitochondria were disrupted by freezing and thawing them 3 times in potassium phosphate buffer (20 mM/L; pH 7.2), and were extracted in 1.5 % dodecyl maltoside.

The extracted mitochondrial protein was added to 1 ml of reaction mixture containing 50 mM phosphate buffer (pH7.2) and 0.05 per cent dodecyl maltoside. The reaction was started by adding cytochrome C Fe^{3+} to a final concentration of 15 µM/L. The reaction was followed at an

absorbance of 550 nm with the reference at 580 nm for 2 minutes. After 2 minutes the remaining unoxidized cytochrome C Fe^{3+} in the reaction mixture was completely oxidized by adding saturated potassium ferricyanide. The optical density was again measured after complete oxidation. The first order rate constant for oxidation was calculated from the initial rate and total cytochrome C.

3.3.4.5 Citrate synthase

Citrate synthase was measured following the method described by Zheng *et al.* (1990).

Principle: This method is based on the chemical coupling of the reduced form of Co enzyme A (CoASH) released from acetyl CoA during the enzymatic synthesis of citrate, to Ellman's reagent 5,5' dithiobis(2-nitrobenzoic acid) (DTNB). The release of the absorbing mercaptide ion is recorded at 412 nm with 530 nm as the reference.

Procedure: The mitochondrial membrane was disrupted by freezing and thawing 3 times in 200 mM potassium phosphate buffer (pH 7.2) to facilitate access of NADH to its binding sites on the inner mitochondrial membrane. Mitochondria were then further solublized by adding an equal amount of 0.2 % triton X–100.

10 µg of the solublized mitochondria was added to a reaction mixture containing 0.1 M Tris HCl buffer/L (pH8.0), 0.25 mM oxaloacetate /L, 0.1 mM DTNB/L and 0.05mM acetyl CoA/L. The reaction was carried out at 25°C and followed for 3 minutes. An extinction coefficient of 13.6 mM L^{-1} Cm^{-1} was used to calculate absorbance changes.

3.3.4.6 Electrophoretic analysis of oxidative phosphorylation Complexes

The OXPHOS complexes were isolated and quantified using two dimensional electrophoresis according to the method described by Schagger and Von Jagow (1991).

First dimension electrophoresis

The isolated heart and brain mitochondria were processed prior to subjection to first dimension electrophoresis using BN-PAGE. The Isolated mitochondria were centrifuged for 20 minutes at 20000 x g and supernatant discarded. 1M amino caproic acid and 50 mM Bis Tris-HCl pH 7.0 was added and homogenized by twirling with a tiny spatula. 10% dodecyl maltoside was added and the tubes were centrifuged for 15 minutes at 100,000 x g and supernatant collected. Serva G (5% in 1M amino caproic acid) was added and the whole volume was applied to 10 x 1.6 mm gel wells.

Gel dimensions of 13 x 14 x 0.16 cm and combs with 5 to 10 mm teeth were used. Gradient separation gels were cast at 4°C and maintained at RT for polymerization. Essentially linear 5-13% polyacrylamide gradient gels overlaid by 4% sample gel were cast as summarized in Appendix III.

After casting the 4% gel at RT and removal of combs, gels were overlaid with gel buffer (1x) and stored at 4°C.

Electrophoresis

Blue native PAGE using cathode buffer A (Appendix-III) was performed at 4°C in a vertical apparatus. After sample application the electrophoresis was started at 100 V until the sample has completely entered the sample gel and continued with voltage and current limited to 500 V and 15mA. For a better detection of faint bands cathode buffer A was removed after one third of the run and electrophoresis continued with cathode buffer B (Appendix III) containing 10 fold lower dye concentration (0.002%).

Second dimension: Tricine- SDS- PAGE

Following BN-PAGE, individual lanes were excised laid on a glass plate at the sample gel position and soaked with 1 % SDS, 1 % mercaptoethanol for 2 hrs. Spacers thinner than those used for first dimension were then positioned and the second glass plate was put on top and mercaptoethanol was removed thoroughly.

The second dimension SDS gel was then cast in three steps by passing acrylamide mixtures sequentially through the gaps between the native gel and spacers. First a 16.5 % acrylamide –Tricine-SDS gel was cast leaving a 2 cm gap over the native gel and overlaid with water. After polymerization a 10 % acrylamide-Tricine- SDS mixture was added. After polymerization another 10% acrylamide-Tricine-SDS gel was used to surround the first dimension gel without covering it.

Electrophoresis of 14 x 14 x 0.16 cm gels in the second dimension was performed at 120 V over night at RT.

3.3.5 Hydrogen peroxide

Mitochondrial H_2O_2 production was determined using fluorimetric assay described by Hyslop and Sklar (1984) with minor modifications. The p-hydroxyphenyl acetate (PHPA) used as a fluorescent probe was replaced by ABTS and the green colour developed was measured in an ELISA plate reader at 405 nm.

Procedure: Freshly isolated mitochondria were incubated in 10 mM potassium phosphate buffer (pH 7.4) containing KCl, EGTA and $MgCl_2$ with 500µg ABTS, 4U horse raddish peroxidase, 5 mM rotenone and 10mM succinate. H_2O_2 production was measured using ELISA plate reader at 405 nm. H_2O_2 production rate was calculated from a standard curve.

3.4 Apoptosis and ageing

3.4.1 Markers of oxidative stress

3.4.1.1 Sample collection

Heart and brain tissue samples were collected from all control and experimental group rats at the end of the 18th, 24th and 30th months of trial and stored at -20°C till the following assays were carried out.

3.4.1.2 Preparation of samples

Heart and brain tissues were processed for estimation of antioxidant enzyme activity following the method of the Bruce and Baudry (1995).

Heart and brain tissues were minced and homogenized in 0.05 M ice cold phosphate buffer (pH 7.4; 1:10 W/V) by using a teflon homogenizer. 0.2 ml of the homogenate was used for estimation of LPO measured as Thiobarbituric Acid Reactive Substances (TBARS). The remaining portion of the homogenate was divided in to two parts. One part was mixed with 10 % TCA (1:1), centrifuged at 5000 x g (4° C, for 10 min.) and the supernatant was used for GSH estimation. The remaining portion of the homogenate was centrifuged at 15000 x g at 4° C for 60 min. and the supernatant was used for protein estimation and SOD, catalase and GPx assay.

3.4.1.3 Lipid peroxidation

Lipid peroxidation in heart and brain were estimated by the method of Yagi (1976). The brilliant pink colour formed by the reaction of 2-thiobarbituric acid with breakdown product of lipid peroxidation to form MDA was read spectrophotometrically at 532 nm, and expressed as µmoles of MDA/g tissue.

3.4.1.4 Protein carbonyls

Protein carbonyls were analysed in heart and brain using the method described by Levine *et al.* (1990).

The protein solution was obtained by precipitating the tissue homogenate using 20 per cent TCA. To each tube 500 µl of 2, 4 dinitrophenylhydrazine in 2 M HCl was added and allowed to stand at RT for 1 hr. with vortexing every 10 to 15 minutes. 500 µl of TCA was added and the tubes were centrifuged for 3 minutes at 11000 x g. The precipitate was washed three times with 1 ml of ethyl acetate-ethanol (1:1) to remove the free reagent, allowing the samples to stand for 10 minutes before centrifuging and discarding the supernatant each time. The precipitate was redissolved in 0.6 ml guanidine solution and incubated at 37°C for 15 minutes. Blank was prepared by treatment with 2 M HCl instead of 2, 4 dinitrophenylhydrazine.

The absorbance was read at 366 nm and the protein carbonyl levels were calculated using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}.

3.4.1.5 DNA fragmentation

Nuclear DNA fragmentation was demonstrated using agarose gel electrophoresis as described by (Nakamura *et al.*, 2000).

Nuclear DNA was extracted from fresh-frozen myocardial and brain tissues. Tissues were homogenized and lysed in lysis solution (Appenndix – IV) and digested in 0.5 mg/ml proteinase K at 56°C for 16 hours. RNase A was added to each sample to achieve a final concentration of 0.2 mg/ml of RNase A and the samples were incubated at 37°C for 2 hours. DNA was extracted using phenol /chloroform (1:1) twice and DNA was precipitated using 10 mM sodium acetate and cold ethanol and dissolved in TE buffer.

DNA samples (10 µl) were subjected to electrophoresis on 2 % agarose gel in TAE buffer (Appendix-IV) at 80 V for 45 to 60 minutes, stained with ethidium bromide and photographed under ultraviolet illumination.

3.4.2 Antioxidative enzyme status

3.4.2.1 Estimation of protein

The protein content was estimated by the method of Lowry *et al.* (1951). Reduction of Folin Ciocalteau reagent by alkaline copper-treated protein formed blue colour which was measured at 660 nm.

3.4.2.2 Enzymatic antioxidants

Glutathione peroxidase was estimated by the method of Rotruck *et al.* (1973), catalase by the method of Caliborne (1985) and superoxide dismutase by the method of Marklund and Marklund (1974).

3.4.2.3 Non-enzymatic antioxidant

Reduced glutathione was estimated by the method of Moron *et al.* (1979).

3.4.3 Myocardial collagen

Collagen content was estimated by analyzing the hydroxy proline content in heart muscle as described by Neuman and Logan (1950) with minor modifications.

Preparation of tissues

One hundred milligrams of dry myocardial tissue was hydrolyzed with 1 ml of 6N HCl at 105°C in a sealed tube overnight. The hydrolysates were neutralized with NaOH solution and filtered. The volume was made up to 10 ml with distilled water.

Estimation of hydroxyproline

To 1 ml of the neutralized hydrolysate, 1ml of 0.1N $CuSO_4$, 1 ml of 2.5N sodium hydroxide and 1 ml of 6 per cent hydrogen peroxide were added. The solution was mixed thoroughly and kept at 80°C in a water bath for 5 minutes. The contents were cooled in ice and then 4 ml of 3N

sulphuric acid followed by 2 ml of freshly prepared 5 per cent p-dimethyl amino benzldehyde solution in n-propanol were added. The contents of the tubes were thoroughly mixed incubated in a water bath (70°C) for 15 minutes and cooled under tap water. The intensity of pink colour was read at 540 nm. The hydroxyproline content was calculated with the help of the standard graph and expressed as mg/g of tissue.

The concentration of collagen was derived from hydroxyproline using a multiplication factor 7.46 and expressed as percentage.

3.4.4 Lipid profile

Total cholesterol and HDL-C were estimated in serum using the method described by Wybenga and Pilegi (1970). Triglycerides were estimated using diagnostic kits following prescribed method. LDL-C and VLDL were calculated using Friedewald's formula (Friedewald *et al.*, 1972).

3.5 Statistical analyses

The data is presented as mean ± S.E. Analysis of variance was used to find the significance of study parameters and Post-hoc LSD test was used to find the pair wise significance using SPSS version 15.0 software for windows to find out the effectiveness of the treatment. The statistical significance was fixed at $P < 0.05$. Student's t test was used to compare the significance between the 18 months old rats was compared with those of 24 and 30 months old rats.

CHAPTER 4

RESULTS

4.1 Effect of dietary fat and calorie restriction on growth rate

The mean ± S.E of body weight at sacrifice, weights of heart and brain from untreated 18 months old rats and from rats fed on coconut oil and corn oil based diets with or without CR were compared and presented in Tables 1 and 1a.

4.1.1 Body weight

Comparison of body weights from 18 months old rats with 24 months old rats fed on coconut and corn oil based diets *ad libitum* or CR revealed that coconut oil feeding significantly increased ($P<0.05$) and decreased ($P<0.05$) the body weight of rats fed *ad libitum* and on CR respectively. However, rats fed on corn oil based diets *ad libitum* and CR showed a non significant ($P>0.05$) increase and decrease in body weights. Long term feeding of coconut oil and corn oil based diets *ad libitum* up to 30 months of age resulted in a significant ($P<0.05$) increase in body weight. CR rats showed a significant ($P<0.05$) decrease in body weight when fed on coconut oil diet alone but not with rats fed with corn oil based diet (Table 1).

Among the treatment groups, both coconut and corn oil based diet fed on CR showed a significant ($P<0.05$) decrease in body weight compared to their *ad libitum* fed counterparts of 24 and 30 months of age. Body weights of *ad libitum* fed rats with either coconut or corn oil diets did not vary significantly ($P>0.05$) between 24 and 30 months old rats (Table 1a).

4.1.2 Organ weight

4.1.2.1 Heart

Twenty four months old rats fed on coconut and corn oil based diets from 18 months onwards *ad libitum* and on CR showed a non significant ($P>0.05$) increase and decrease, respectively in the weights of

heart compared to those rats of 18 months of age. However, long term feeding up to 30 months of age resulted in significant ($P<0.05$) increase in coconut oil diet *ad libitum* fed rats. On the other hand, short and long term feeding of rats on corn oil diet with CR did not show any significant ($P>0.005$) changes compared to rats of 18 months of age (Table 1).

Short term feeding of rats on CR with both coconut and corn oil based diets resulted in a significant ($P<0.05$) decrease in the weights of heart compared to when both the diets were fed *ad libitum*. The decrease was more significant ($P<0.05$) when fed on corn oil diet than on coconut oil diet. However, long term feeding of the experimental diets showed significant ($P<0.05$) increase in weights of hearts only with corn oil diet fed *ad libitum* and a significant decrease ($P<0.05$) in coconut oil diet fed on CR (Table1a).

4.1.2.2 Brain

Short term or long term feeding of rats with coconut or corn oil based diets fed *ad libitum* or CR did not show significant ($P>0.05$) changes in the weight of brain compared to the untreated counterparts of 18 months of age (Table 1).

Among the treated rats, the weight of brain in corn oil fed on CR up to 24 months of age alone showed a significant ($P<0.05$) decrease compared to other groups. However, a non significant ($P>0.05$) increase was observed in rats fed on coconut oil based diet *ad libitum* in both short and long term feeding (Table 1a).

4.2 Effect of dietary fat and calorie restriction on mitochondrial bioenergetics

4.2.1 Mitochondrial membrane fatty acid composition

The various fatty acid (FA) in heart and brain mitochondrial membrane 18:1, 18:2, 20:0, 20:3, 22:0, 22:1 and 24:0 were analyzed using GC-MS in 24 and 30 months of in rats fed on coconut oil and corn oil based diets *ad libitum* or on CR. The results obtained are compared with untreated 28 months old rats (Table 2 and 3).

4.2.1.1 Heart

Short term feeding up to 24 months resulted in a significant increases ($P<0.05$) in 18:1 levels only in rats fed on coconut diet with CR compared to 18 months old rats (Table 2).

Rats fed on corn oil based diets *ad libitum* and CR showed a significant ($P<0.05$) increase in 18:2 acid.

Fatty acid fractions of 20:3, 22:0 and 22:1 were found to increase only in rats fed on corn oil based diet with CR compared to 18 months old pretreatment rats.

The other fractions (16:0, 18:0, and 20:0) did not show significant ($P>0.05$) variation in their incorporation into the mitochondrial membrane compared to 18 months old rats.

Long term feeding of the experimental diets up to 30 months resulted in a significant ($P<0.05$) increase in 18:1 fraction in rats fed on coconut oil based diet with CR alone compared to those of 18 months of age.

A significant ($P<0.05$) increase was observed in 20:3 levels in rats fed on corn oil based diet *ad libitum* and CR.

Rats fed on corn oil based diet *ad libitum* resulted in a significant ($P<0.05$) decrease in 20:0 fraction compared to untreated 18 months old rats.

Among the treatments, a significant (P<0.05) increase was observed in 18:0, and 18:1 FA in the membranes of 24 month old rats fed on coconut oil based diets *ad libitum* and CR compared to those fed on corn oil based diets for shorter duration.

On the other hand, feeding of corn oil based diets *ad libitum* and CR resulted in a significant (P<0.05) increase in 18:2 fraction compared to rats fed on coconut oil based diets. A significant (P<0.05) related decrease was observed in rats fed on corn oil diet *ad libitum.*However, a significant (P<0.05) increase was observed in 20:3 FA only in rat fed on corn oil based diet with CR.

Short term feeding of the experimental diets did not show significant (P>0.05) variation in their level of incorporation of 16:0, 20:0 and 22:0 in the mitochondrial membranes.

Long term feeding of coconut and corn oil based diets *ad libitum* and CR up to 30 months resulted in significant variation in the level of incorporation of FA into the mitochondrial membrane (Table 2a).

Long term feeding of corn oil diet resulted in a significant increase in level of 16:0.

Feeding of coconut oil based diets *ad libitum* and CR for longer duration resulted in a significant (P<0.05) increase in the level of 18:0 FA compared to those fed on corn oil based diets. On the other hand feeding of coconut oil based diet with CR alone resulted in a significant (P<0.05) increase in incorporation of 18:1 FA into the membranes. However, feeding of coconut oil based diet *ad libitum* resulted in an age related significant (P<0.05) decrease in the level of 18:1 in the membranes.

Long term feeding of corn oil based diets fed on *ad libitum* or with CR resulted in a significant (P<0.05) increase in 18:2 levels. However, coconut oil based diet fed *ad libitum* resulted in an age related decrease in 18:2 levels.

There was no significant (P>0.05) in variation in the level of incorporation of other fatty acid fractions into the mitochondrial membranes.

4.2.1.2 Brain

Comparison of the level of incorporation of various FA in the brain mitochondrial membrane between 24 months old rats fed on coconut or corn oil based diets *ad libitum* or CR and 18 months old pretreated rats revealed the following changes in one of the treated groups (Table 3).

A significant decrease in levels of 16:0 fraction was observed in all the groups except in rats fed on coconut oil based diet with CR.

Coconut oil based diets with CR significantly (P<0.05) increased 18:1 fraction whereas, corn oil based diets fed *ad libitum* and on CR resulted in a significant (P<0.05) decrease.

Highly significant (P<0.001) increase in the level if 18:2 FA was observed in rats fed on corn oil based diets *ad libitum* and on CR.

On the other hand 18:0 and 22:0 FA did not show appreciable changes in their levels of incorporation into the brain mitochondrial membranes.

Long term feeding of the experimental diets up to 30 months of age resulted in the following changes compared to those of 18 months old (Table 3).

A significant (P<0.05) increase in 18:1 fraction was observed in rats fed on coconut oil based diets with CR and a significant (P<0.05) decrease was observed in corn oil based diets fed *ad libitum* and CR.

On the other hand feeding of corn oil based diets *ad libitum* and on CR resulted in a significant (P<0.05) increase in the level of 18:2 FA.

Among the different treatments a significant (P<0.05) increase in the levels of 16:0, was observed in rats fed on coconut oil based diets with CR followed by coconut oil diet fed *ad libitum.* However no significant (P>0.05) variation was observed in rats fed on corn oil based

diets for longer duration. A significant (P<0.05) age related increase was observed in rats fed on corn oil based diet *ad libitum* (Table 3a).

A significant (P<0.05) increase was observed in the levels of 18:0 in rats fed on coconut oil based diets when compared to those fed with corn oil based diets. A significant (P<0.05) age related increase was observed in rats fed with corn oil based diets.

Rats fed on coconut oil based diet with CR showed a significant (P<0.05) increase in the levels of 18:1 followed by coconut oil based diet fed *ad libitum* compared to those fed on corn oil based diets for shorter duration (Table 3a).

Short term feeding with corn oil based diets fed *ad libitum* and on CR resulted in a significant (P<0.05) increase in the level of 18:2 FA in the brain mitochondrial membrane.

However, short term feeding up to 24 months did not significantly (P>0.05) modulate levels of other FA fractions in mitochondrial membrane.

Among the treatment groups a significant (P<0.05) decrease in 18:0 FA was observed in rats fed on coconut oil diet *ad libitum* alone up to 30 months of age compared to other groups. However, rats fed on corn oil based diets *ad libitum* and CR showed a significant (P<0.05) age related increase in 18:0 levels.

On the other hand, long term feeding of rats with coconut oil based diets *ad libitum* and on CR resulted in a significant (P<0.05) increase in level of incorporation of 18:1 in the brain mitochondrial membrane.

Long term feeding of corn oil based diets *ad libitum* and CR showed a significant (P<0.05) increase in 18:2 FA despite a significant (P<0.05) age associated decrease in its level of incorporation was also observed.

Except for a non significant (P>0.05) increase in the level of 16:0, a significant (P<0.05) influence of age was seen only in rats fed on coconut oil based diets *ad libitum.*

None of the other FA showed significant (P>0.05) variation in their levels of incorporation into the mitochondrial membranes.

4.2.2 Mitochondrial inner membrane surface area

The surface area of the heart and brain mitochondrial inner membrane measured stereologically in 24 and 30 month old rats fed on coconut and corn oil based diets *ad libitum* and on CR were compared with those of 18 month old untreated rats. The mean ± S.E of mitochondrial surface area are presented in Table 4,4a, 5 and 5a.

4.2.2.1 Heart

Among treatment groups a non significant (P>0.05) increase in the surface area of the mitochondrial inner membrane was observed in 24 and 30 month old rats fed on coconut oil based diets (*ad libitum* and CR) (Table 4).

In rats fed on coconut oil diet (*ad libitum* and CR) up to 24 and 30 months of age had significantly higher (P<0.05) surface area in comparison with the rats fed on corn oil diets *ad libitum* (Table 4a) .

4.2.2.2 Brain

Rats fed on coconut and corn oil diets *ad libitum* and on CR showed a significant (P<0.001 and P<0.05, respectively) increase in surface area of mitochondrial inner membrane compared to 18 months old rats. Long term feeding revealed a significant (P<0.05) increase in the surface area. Coconut oil diet with CR significantly (P<0.05) increased the surface area of the inner membrane when fed up to 30 months of age (Table 5).

Feeding of rats with coconut oil based diets with CR up to 24 and 30 months of age resulted in a highly significant (P<0.001) increase in surface area of the mitochondrial inner membrane, whereas short term feeding of corn oil based diets (*ad libitum* and CR) showed significant (P<0.05) increase in the surface area of the mitochondrial membrane.

Long term feeding with corn oil based diet *ad libitum* revealed highly significant (P<0.001) decrease in the surface area of the mitochondrial membrane. On the other hand rats fed with corn oil showed significant (P<0.05) decrease in the surface of the mitochondrial membrane (Table 5).

Among treatment groups, coconut oil based diets with CR had highly significant increase (P<0.001) in surface area in short term feeding, whereas short term feeding rats fed with corn oil based diets *ad libitum* revealed significant (P<0.05) decrease in the surface of the mitochondrial membrane.

Similarly, rats fed on corn oil based diet *ad libitum* showed a significant (P<0.05) reduction in the surface area. The reduction in the surface area observed with corn oil diet fed on CR was significantly (P<0.05) lower to rats fed on coconut oil diet with CR but not with other groups when fed on the experimental diets up to 30 months of age (Table 5a).

4.2.3 Mitochondrial membrane potential

Mitochondrial membrane potential in heart and brain mitochondria of rats fed on coconut oil or corn oil based diets either *ad libitum* or with CR for short term (up to 24 months) and long term (up to 30 months) and compared with untreated 18 months old rats. The Mean ± S.E are presented in Tables 6, 6a, 7, and 7a.

4.2.3.1 Heart

Comparison of both short and long term feeding of coconut oil and corn oil based diets with and without CR revealed that coconut oil and corn oil fed rats at both levels had a highly significant (P<0.001) increase in MMP compared to 18 month old untreated rats (Table 6)

Amongst the various treatments rats fed on coconut oil at both levels showed significant (P<0.05) elevation in MMP compared to the corresponding corn oil fed rats for shorter and longer duration. Feeding

of coconut oil based diet with CR showed a significant ($P<0.05$) increase in MMP compared to corn oil diet fed *ad libitum* up to 30 months of age (Table 6a).

4.2.3.2 Brain

Comparison of feeding of coconut oil and corn oil based diets with and without CR revealed that coconut oil fed rats at both levels, had a significant ($P<0.05$ and $P<0.001$, respectively) higher MMP compared to 18 months old untreated rats. However, rats fed on corn oil did not show any significant ($P>0.05$) difference in MMP (Table 7).

Amongst the various treatments, rats fed on coconut oil at both levels showed significant ($P<0.05$) elevation in MMP compared to the corresponding corn oil fed rats for shorter duration (24 months of age). Long term feeding of coconut oil with CR significantly ($P<0.05$) increased MMP compared to when fed on corn oil based diets. However, long term feeding of coconut oil with CR tend to increase significantly ($P<0.05$) the MMP. A significant ($P<0.05$) age related decrease was observed in rats fed on corn oil based diets *ad libitum* (Table 7a).

4.2.4 Production of oxidants

4.2.4.1 Hydrogen peroxide

The production of H_2O_2 in the heart and brain mitochondria is an indicator of level of oxidant produced by these tissues and the Mean ± SE of level of H_2O_2 in 24 and 30 months old rats fed on coconut or corn oil based diets with or without CR are compared with 18 months old rats and presented in Tables 6, 6a, 7 and 7a.

4.2.4.1.1 Heart

Comparison of levels of H_2O_2 produced by 18 months old rat heart mitochondria with 24 and 30 months old rats fed with various dietary treatments revealed that H_2O_2 levels were highly significantly decreased ($P<0.001$) in coconut oil based diets (*ad libitum* and CR) and in corn oil based diet with CR, whereas in rats fed on corn oil based diet *ad libitum* recorded highly significant ($P<.0.001$) increase in H_2O_2 level when compared to other groups (Table 6).

The levels of H_2O_2 were significantly ($P<0.05$) decreased in 24 months old rats fed either on coconut or corn oil based diets with CR compared to *ad libitum* feeding. Long term feeding of these diets also revealed the same trend.

Among the treatment groups, short term feeding of coconut oil and corn oil based diet with CR had significantly ($P< 0.05$) decreased H_2O_2 levels in comparison with *ad libitum* fed groups.

Long term feeding of rats with coconut oil based diet (*ad libitum* and CR) revealed significantly ($P<0.05$) lower levels of H_2O_2 production when compared to rats fed with corn oil *ad libitum*.

An age related significant ($P<0.05$) decrease with coconut oil based diet *ad libitum* and a significant increase was observed in rats fed on corn oil based diets CR (Table 6a).

4.2.4.1.2 Brain

Comparison of levels of H_2O_2 produced by 18 months old rat brain mitochondria with 24 and 30 months old rats fed with various dietary treatments revealed that H_2O_2 levels were significantly decreased in rats fed on corn oil *ad libitum* ($P<0.05$) or with CR ($P<0.001$) (Table 7).

The levels of H_2O_2 were highly significantly ($P<0.001$) decreased in rats fed on corn oil based diets (*ad libitum* and CR) for a shorter duration compared to coconut oil diets.

Long term *ad libitum* feeding of coconut oil diet significantly ($P<0.05$) increased the H_2O_2 production in comparison corn oil based diet *ad libitum*. However, CR feeding of both the diets significantly ($P<0.05$) lowered the levels of H_2O_2 in comparison with those fed on *ad libitum*

The changes in the levels of production by corn oil *ad libitum* were significantly ($P<0.05$) influenced by age of the animal (Table 7a).

4.2.5 Mitochondrial OXPHOS enzyme activity

The mean ± S.E of the mitochondrial OXPHOS enzyme complexes I to IV and citrate synthase activity in coconut oil and corn oil based diets fed *ad libitum* or on CR were compared with untreated rats of 18 months of age and between the treatment groups following six months (24 months old rats) and 12 months (30 months old rats) of feeding are presented in Tables 8, 8a, 9 and 9a.

4.2.5.1 Heart

Short term feeding of rats from 18 months on wards for six or twelve months with coconut oil or corn oil based diets *ad libitum* or on CR did not bring about any significant ($P>0.05$) variation in the activity of OXPHOS complexes I to IV and citrate synthase compared to those of 18 months old rats fed *ad libitum* with commercial chows. However, long term feeding of the experimental diets resulted in a significant ($P<0.05$) decrease in the activity of complex II and III in rats fed on coconut and corn oil based diets *ad libitum* (Table 8).

Among the treated rats, the mitochondrial OXPHOS complexes I to IV and citrate synthase activities were found to be slightly increased in rats fed on both the diets with CR compared *ad libitum* feeding for shorter and longer duration. The enzyme activities followed an age related decrease in all the treatment groups. However, these changes were statistically non significant ($P>0.05$) (Table 8a)

4.2.5.2 Brain

Short term as well as long term feeding of rats from 18 months on wards for six or twelve months with coconut oil or corn oil based diets *ad libitum* or on CR did not show significant (P>0.05) variation in the activity of OXPHOS complexes I to IV and citrate synthase in the brain was compared to those of 18 months old rats fed *ad libitum* with commercial chows (Table 9).

In short term feeding, complex revealed a non significant (P>0.05) reduction in rats fed on coconut oil with CR in comparison with *ad libitum* feeding, whereas non significant (P> 0.05) increase in activity was observed in rats fed on corn oil with CR when compared to *ad libitum* feeding.

In long term feeding complex III showed non significant (P>0.05) increase in CR groups in comparison with *ad libitum* fed groups. Non significant (P>0.05) reduction in the enzyme activities were observed between two age groups.

Among the treated rats, the mitochondrial OXPHOS complexes I to IV and citrate synthase activities were slightly increased in rats fed on both the diets with CR compared *ad libitum* feeding for shorter and longer duration. The enzyme activities followed an age related decrease in all the treatment groups. However, these changes were not statistically significant (P>0.05) (Table 9a).

Blue negative electrophoretic analysis of the isolated mitochondrial enzyme complexes I – 1V revealed that dietary treatment with coconut oil or corn oil based diets fed on *ad libitum or* on CR did not change the electrophoretic pattern of occurrence of the complexes I – IV separated based on their respective weights (1000, 130,490,200 KDa, respectively) on 3.5 to 13 per cent acrylamide gradient gel, (Fig 2 and 3) in both 24 and 30 months old rats.

Second dimension of the first dimension gel resulted in the isolation of the complexes I – IV into their respective sub units ranging between 15 – 75 KDa. The electrophoretic pattern of occurrence did not vary between the different treatments in both brain and heart mitochondria of 24 and 30 month old rats. A representative electrophoretic pattern is presented in Fig (4 and 5).

4.3 Effect of dietary fat and calorie restriction on apoptosis and ageing

4.3.1 Markers of oxidative stress

4.3.1.1 Lipid peroxidation

Mean ± SE of TBARS a product of measurement of LPO in the heart and brain of 24 and 30 months old rats treated with coconut or corn oil based diets and compared with their 18 months old untreated counterparts are presented in Tables 10, 10a, 11 and 11a.

4.3.1.1.1 Heart

The TBARS levels were significantly ($P<0.05$) reduced in rats fed on corn oil with CR in 24 and 30 months of age compared to 18 months old rats fed on a commercial rodent chow (Table 10).

Amongst the treatments, it was observed that coconut and corn oil base diets with CR significantly ($P<0.05$) decreased TBARS production when compared to short term *ad libitum* feeding. On the other hand, long term feeding of coconut oil and corn oil based diets fed *ad libitum* up to 30 months of age significantly ($P<0.05$) increased TBARS production compared to respective CR groups (Table 10a).

4.3.1.1.2 Brain

The TBARS levels were significantly ($P<0.05$) reduced in *ad libitum* fed corn oil rats of 24 and 30 months of age compared to 18 months old rats fed on a commercial rodent chow (Table 11).

Amongst the treatments, no significant ($P>0.05$) decrease in TBARS production was observed in rats fed on coconut or corn oil based diets *ad*

libitum up to 24 months of age in comparison with respective CR groups. On the other hand, long term feeding of coconut and corn oil based diets on CR up to 30 months of age non significantly (P>0.05) decreased TBARS production compared when fed *ad libitum*. However, age significantly potentiated the production of TBARS when fed with corn oil based diets fed either *ad libitum* (P<0.001) or with CR (P<0.05) (Table 11a).

4.3.1.2 Protein carbonyls

The mean ± SE of protein carbonyls a product of measurement of oxidative damage to proteins in the heart and brain of 18, 24 and 30 months old rats fed on commercial chows, coconut and corn oil based diets, respectively with or without CR are compared and presented in Tables 10, 10a, 11 and 11a.

4.3.1.2.1 Heart

Comparison of protein carbonyls in untreated 18 months old rats with 24 and 30 months old rats fed on coconut or corn oils based diets *ad libitum* or with CR revealed a highly significant (P<0.001) increase in *ad libitum* feeding of coconut oil based diet and corn oil based diet (*ad libitum* and CR) and a significant (P<0.05) increase in coconut oil based diet with CR when fed for 6 months period. However, long term feeding of corn oil based diet *ad libitum* showed highly significant (P<0.001) increase in protein carbonyl levels.

Long term feeding of corn oil *ad libitum* resulted in significant (P<0.05) increase in protein carbonyl level (Table 10).

Among the treatment groups, it was observed that protein carbonyl levels were significantly (P<0.05) increased in rats fed on coconut oil diet on CR when fed up to 30 months of age in comparison with other groups (Table 10a).

4.3.1.2.2 Brain

Comparison of protein carbonyls in untreated 18 months old rats with 24 and 30 months old rats fed on coconut oil *ad libitum* or corn oils based diets *ad libitum* and with CR revealed a highly significant ($P<0.001$) increase in protein carbonyls when fed for 6 months period. On the other hand, short term feeding of rats on coconut oil with CR showed significant ($P<0.05$) increase in protein carbonyl level in comparison with 18 months old rats. However, long term feeding of coconut oil *ad libitum* highly significantly ($P<0.001$) increased protein carbonyl level in the rodent brain compared to other groups. Long term feeding of corn oil based diets (*ad libitum* and CR) revealed significant ($P<0.05$) increase in protein carbonyl level (Table11).

Among treatments it was found that feeding of rats with coconut oil based diets with CR significantly ($P<0.05$) decreased production of protein carbonyls compared to *ad libitum* feeding of coconut and corn oils in rats fed for shorter and longer duration. Age has shown to significantly ($P<0.05$) decrease the protein carbonyl production coupled with CR regimen in both the dietary groups compared with *ad libitum* feeding (Table 11a).

4.3.1.3 DNA fragmentation

The fragmentation pattern of DNA isolated from heart and brain in different dietary groups were performed on agarose electrophoresis and the DNA fragments were compared among the different groups and presented in Figure 6,7,8 and 9.

Feeding of coconut and corn oil based diet *ad libitum* or on CR did not decrease the DNA fragmentations that were observed in rats of 18 months of age. The migration of DNA fragments were markedly enhanced and were not altered by the different treatments. In all the cases, these fragments were of high molecular weight ranging between 200 to 750 kb pairs.

4.3.2 Antioxidant profile

Antioxidant levels in the heart and brain of 18 months old rats and from 24 and 30 months old rats fed on coconut or corn oils fed *ad libitum* and CR for 6 or 12 months period from 18 months onwards the mean ± SE values of the antioxidants are presented in Tables 12, 12a, 13, and 13a.

4.3.2.1 Heart

4.3.2.1.1 Superoxide dismutase

Comparison of the levels of SOD revealed that short term feeding regimen showed a significant increase ($P<0.05$) in rats fed on coconut oil with CR and corn oil based diet both *ad libitum* and with CR compared to 18 months old rats. Long term feeding up to 30 months significantly increased ($P<0.05$) SOD levels with both coconut and corn oils with and without CR. This vividly indicated the effect of age over treatment in these animals (Table 12).

Among the treatment groups, a significant ($P<0.05$) increase in SOD levels in rats fed on coconut oil with CR compared to *ad libitum* feeding of coconut oil and corn oil diets was observed when fed for shorter and longer duration (Table 12a).

4.3.2.1.2 Glutathione peroxidase

GPx levels were increased significantly ($P<0.05$) in both 24 and 30 months old rats fed on coconut or corn oil with CR compared to rats fed on commercial chows up to 18 months of age (Table 12).

Both short term as well as long term feeding of coconut oil and corn oil diets with CR significantly ($P<0.05$) increased GPx levels in the heart of rats (corn oil with CR > coconut oil with CR). Among the treatment groups age did not significantly ($P>0.05$) modulated GPx levels attributing to the positive effect of CR over age of the animal (Table 12a).

4.3.2.1.3 Reduced glutathione

Comparison of levels of GSH revealed that 24 and 30 months old rats fed on coconut oil and corn oil with CR had significantly ($P<0.05$) higher concentrations than their 18 months old untreated counterparts Table 12).

Among treatment groups, concentration of GSH was significantly ($P<0.05$) increased when rats were fed on coconut oil or corn oil with CR for shorter and longer duration when compared to coconut oil based diet fed *ad libitum*. (Table 12a).

4.3.2.1.4 Catalase

The levels of catalase were influenced strongly with age. Short term feeding of the four diets up to 24 months significantly decreased catalase when the rats were fed on coconut oil *ad libitum* ($P<0.001$) and corn oil ($P<0.05$) *ad libitum* . On the contrary, CR of both the diets did not significantly ($P>0.05$) reduce catalase levels. However, a highly significant ($P<0.001$) decrease in the level of catalase was observed when the both the diets were fed *ad libitum* or with CR for longer duration up to 30 months of age (Table 12).

Amongst the treatments the short term feeding of coconut and corn oil diets with CR significantly increased ($P<0.05$) catalase levels compared to *ad libitum* feeding of the diets. However, long term feeding of coconut oil diets with CR alone significantly ($P<0.05$) increased catalase levels compared to *ad libitum* feeding. However, long term feeding of coconut and corn oil based diets fed *ad libitum* or CR showed a significant ($P<0.05$) reduction in catalase levels indicating the influence of age over the type of dietary regimen on catalase levels (Table 12a).

4.3.2.2 Brain

4.3.2.2.1 Superoxide dismutase

Comparison of the levels of SOD revealed that short term feeding regimen showed a significant decrease ($P<0.05$) in rats fed on corn oil diet *ad libitum* compared to 18 months old rats. Long term feeding up to 30 months significantly decreased ($P<0.05$) SOD levels with both coconut and corn oil diet fed *ad libitum* (Table 13).

Among the treatment groups, short term feeding of the diets resulted in a non significant increase ($P>0.05$) in rats fed on coconut and corn oil diets with CR. Howbeit, both diets significantly ($P<0.05$) increased SOD levels when fed with CR for longer period up to 30 months of age. Among the treatment groups age did not show significant ($P>0.05$) variation on the levels of SOD this vividly indicated the effect of age over treatment in these animals (Table 13a).

4.3.2.2.2 Glutathione peroxidase

GPx levels were increased significantly ($P<0.05$) in 24 months old rats fed on coconut or corn oil with CR compared to rats fed on commercial chows up to 18 months of age. No significant ($P>0.05$) modulation in the enzyme levels in the *ad libitum* fed rats demonstrated a positive effect of CR over the source of dietary fat for short duration (Table 13).

Long term feeding of coconut oil and corn oil diets with CR and coconut oil *ad libitum* significantly ($P<0.05$) increased GPx levels in the brain of rats.

Among the treatment groups both the diets when fed with CR alone resulted in a significant ($P<0.05$) increase with short term feeding. Long term feeding of coconut oil with CR alone resulted in significantly higher ($P<0.001$) level of GPx. Among the treatment groups, age did not significantly ($P>0.05$) modulated GPx levels except when fed on coconut

oil diet *ad libitum* attributing to the positive effect of CR over age of the animal (Table 13a).

4.3.2.2.3 Reduced glutathione

Comparison of levels of GSH revealed that 24 months old rats fed on corn oil diet *ad libitum* and CR had significantly higher (P<0.05) levels, whereas a significant (P<0.05) decrease was observed with feeding of coconut oil diet *ad libitum*. Corn oil with CR and coconut oil diet *ad libitum* alone resulted in a significant (P<0.05) increase and a significant (P<0.05) decrease respectively in GSH levels when fed up to 30 months of age than their 18 months old untreated counterparts.

Among treatment groups, highly significant (P<0.001) increase in GSH level was recorded in rats fed on corn oil diet with CR for shorter duration, whereas coconut oil with CR showed a significant (P<0.05) increase in GSH level compared to coconut oil diet fed *ad libitum*. Long term feeding of both the diets with CR significantly increased (P<0.005) GSH levels. Both short and long term feeding of corn oil with CR revealed significantly higher (P<0.001) age decrease in GSH levels (Table 13a).

4.3.2.2.4 Catalase

The levels of catalase were found to be influenced strongly due to age. Short term feeding of coconut oil and corn oil with CR up to 24 and 30 months significantly (P<0.05) increased catalase activity. However, a highly significant (P<0.001) increase in the level of catalase was observed in rats fed on coconut oil based diets with CR and a significant (P<0.05) increase with corn oil based diets fed on CR (Table 13).

Amongst the treatments, short and long term feeding of coconut oil with CR significantly (P<0.05) increased catalase levels compared to corn oil based diets and coconut oil based diets fed *ad libitum* (Table 13a).

4.3.3 Myocardial collagen

The collagen levels were estimated in the heart ventricular muscle from rats fed on coconut and corn oil based diets *ad libitum* and on CR and compared with those from untreated 18 months old rats. The mean ± SE values of collagen are presented in Tables 14 and 14a.

The collagen levels were significantly ($P<0.05$) decreased in 24 months old rats fed on coconut and corn oil diets with CR compared to 18 months old rats. However, rats fed on corn oil diet with CR showed a significant ($P<0.05$) decrease in collagen when fed upto 30 months compared to 18 months old rats fed on commercial chows (Table 14).

Among the treated rats, the levels of collagen showed a significant ($P<0.05$) increase in 24 months old rats fed on coconut oil diet *ad libitum*, whereas long term feeding of corn oil with CR showed significant ($P<0.05$) decrease in collagen level (Table 14a).

4.3.4 Lipid Profile

The changes in the lipid profile in serum of rats fed on coconut oil and corn oil based diets either *ad libitum* or CR were compared with the untreated rats fed on commercial chows up to 18 months of age are presented in Tables 15 and 15a.

4.3.4.1 Total Cholesterol

Total cholesterol levels showed no significant ($P>0.05$) variation between different groups of rats fed up to 24 months or 30 months from that of 18 months old rats fed on commercial chows (Table 15).

The changes observed between short and long term feeding were not influenced by different feed regimen and age of the animals (Table 15a).

4.3.4.2 HDL Cholesterol

Short term feeding of coconut oil based diets *ad libitum* was found to significantly ($P<0.001$) decrease HDL-C followed by corn oil fed *ad libitum* and coconut oil with CR ($P<0.05$) fed rats compared to 18 months

old counterparts. Long term feeding of both coconut and corn oil diets fed *ad libitum and* with CR resulted in non significant (P>0.05) variation in HDL-C levels compared to 18 months old rats (Table 15).

In short term feeding, of coconut oil and corn oil diets with CR significantly (P<0.05) increased HDL - C in comparison with *ad libitum* feeding of coconut oil and corn oil diets.

Long term feeding of corn oil based diets with CR is found to significantly (P<0.05) increased HDL- C compared to coconut oil based diets fed *ad libitum* in 30 months old rats. It was found that the HDL- C levels were significantly decreased following coconut oil based diets *ad libitum* (P<0.001) and CR (P<0.05) with age of the animal (Table 15a).

4.3.4.3 LDL Cholesterol

The serum LDL- C revealed non significant (P>0.05) variation in LDL-C levels in 24 and 30 months old rats fed on corn and coconut oil based diets (*ad libitum* and CR) compared to 18 months old untreated rats.

Higher levels of LDL – C were observed with short term feeding of corn oil *ad libitum* and long term feeding of coconut oil *ad libitum*, which were statistically non significant (P>0.05) (Table 15a).

Among the different treatments LDL-C showed a non significant (P>0.05) variation between short and long term feeding (Table 15a).

4.3.4.4 VLDL

Short term feeding resulted in a significant decrease in rats fed on corn oil based diets *ad libitum* (P<0.05) or CR (P<0.001). The levels were significantly higher (P<0.001) in rats fed up to 30 months with corn oil based diet *ad libitum* (Table 15).

Feeding of corn oil diets *ad libitum* and corn oil with CR showed a significant (P<0.05) decrease in VLDL compared to coconut oil based

diets *ad libitum* or CR in 24 months old rats. Feeding the same diets up to 30 months of age resulted in a significantly ($P<0.05$) higher VLDL levels in rats fed on coconut oil diet *ad libitum.*

It was observed that the changes in VLDL levels were highly significantly ($P<0.001$) influenced by age of the animal fed with corn oil diets *ad libitum* and with CR (Table 15a).

4.3.4.5 Triglycerides

In short term feeding the triglycerides were significantly decreased in rats fed on corn oil diet with CR ($P<0.001$) followed by *ad libitum* feeding with corn oil diet ($P<0.05$). Long term feeding of coconut oil and corn oil diets *ad libitum* resulted in a significant ($P<0.05$ and $P<0.001$, respectively) increase in the levels of circulating triglycerides.

Among the treatment groups, long term feeding of corn oil based diet both *ad libitum* and CR revealed significantly ($P<0.05$) higher and lower triglyceride levels respectively in comparison with other groups (Table 15a).

CHAPTER 5
DISCUSSION

Ageing is a fascinating topic because of its great social relevance and scientific complexity. Mitochondria play a central role in cellular and organismal ageing. There is tantalizing evidence that mitochondrial decay to be the primary factor in ageing process. The free radical theory of ageing and membrane pacemaker theory both lead to mitochondrial decay owing to the production of ROS, oxidative stress and lack of membrane homeostasis.

Dietary manipulations of fat and calorie restriction are the two paradigms aimed to minimize mitochondrial decay and retard ageing. Source of dietary fat is directly related to membrane unsaturation which modifies the fluidity and predisposes the membranes to peroxidation. CR alters the fuel use in most active structures and minimizes oxidative stress. These factors collectively retard the age related changes in the post mitotic tissues.

5.1 Effect of dietary fat and calorie restriction on growth rate

5.1.1 Body weight

Feeding different sources of fats tend to influence the growth performance differently. In the present study, feeding of rats with coconut and corn oil based diets *ad libitum* up to 30 months increased the body weight compared to rats fed on commercial chows up to 18 months of age. This is in accordance with the findings of Zuniga *et al.* (1989) and of Bevilacqua *et al.* (2004), observed increased body weight in rat fed *ad libitum* on corn oil or coconut oil purified diets for 6 months period. On the contrary Tomlinson *et al.* (1988), Herzberg and Rogerson (1988), and Demaison *et al.* (2000) reported that feeding of different fat sources did not have any significant effect on the body weight of rats fed for short to long duration.

In the present study, rats fed on CR with either corn oil or coconut oil showed significant decrease in their body weights. Similar observation has been reported by Ramsey *et al.* (2004) and Hagopian *et al.* (2005). It was found that the decrease in body weight was proportional to the duration of CR feeding when fed on coconut oil diet compared to corn oil diet. This may be due to the presence of UFA in coconut oil which might have attributed to reduced consumption (Pinnotti *et al.*, 2006). Secondly, the high digestibility of corn oil (100%) compared to coconut oil, could have attributed to variation between rats on two different diets. The difference in body weight with CR is attributed to lower fat, lean mass and a reduction in calorie intake (Mai *et al* 2003).

5.1.2 Organ weight

5.1.2.1 Heart

Short term feeding of either coconut oil or corn oil, *ad libitum* did not result in significant (P>0.05) variation in weight of heart. This is in accordance with the findings of Chen *et al.* (1987) and Zaloga *et al.* (2006). However, long term feeding up to 30 months of age significantly increased weight of heart in rats fed on coconut oil diet *ad libitum* alone, which may be due to the 100 per cent digestibility of corn oil compared to coconut oil (Chen *et al.,*1989).

The reduced body weight following CR was also reflected on the reduction in weight of heart, in rats fed on both coconut and corn oil diets, up to 24 and 30 months from 18 months of age. Similarly reduction in weight of heart was reported by Bevilacqua *et al.* (2004) and Ramsey *et al.* (2005) in rats fed on CR after 2 months onwards.

In the present study, rats fed on corn oil based diets *ad libitum* up to 30 months of age showed significant increase in weight of the heart compared to coconut oil diet with CR, which may be due to the increased digestibility of corn oil (Chen *et al.,* 1989) compared to coconut oil or to the reduced intake of UFA rich coconut oil diet (Pinnotti *et al.* , 2006).

5.1.2.2 Brain

In the present study, short and long term feeding of coconut oil and corn oil based diets *ad libitum* or CR did not result in any significant change in the weight of brain compared to 18 month old rats. Short term feeding of corn oil diet with CR resulted in a significant ($P<0.05$) reduction in weight of the brain, which may be attributed to the variation that exist between the organs in responding to CR regimen with liver, heart and kidney to show weight difference after 12 to 18 months of CR (Hagopian *et al.,* 2005).

5.2 Effect of dietary fat and calorie restriction on mitochondrial Bioenergetics

5.2.1 Mitochondrial membrane fatty acid composition

Changes in the fatty acid composition brought about by dietary supplementation of fatty acid might reflect the selectivity of a particular fatty acid function and metabolic pathways.

5.2.1.1 Heart

Feeding of either coconut oil or corn oil diets *ad libitum* or CR resulted in variation in the mitochondrial membrane fatty acids profile. In this study, feeding of coconut oil diets increased the 18:1 level and corn oil diets increased 18:2 levels in 24 months old rats, whereas, CR regimen of both the diet alone increased, respectively 18:1 and 18:2 levels when fed up to 30 months of age. Corn oil diet with CR also resulted in a significant increase in 20:3 levels when fed up to 24 and 30 months of age. This is in agreement with the findings of Barzanti et *al.* (1994) and Demaison *et al.* (2000) that feeding of corn oil diets increased 18:2 levels in mitochondrial membranes. There is a scarcity of literature on effect of feeding coconut oil on mitochondrial membranes. Zuniga *et al.* (1989) observed an increase in 18:1 FA levels in heart mitochondrial membranes supporting to the findings of this study. In the present study, the absence of 22:0, 22:1 and 24:0 fatty acids in coconut oil fed

rats could be due to a reduction in rate of turn over of fatty acids (Hansford and Castro, 1982).

It was evident that long term feeding of coconut oil and corn oil based diets increased the levels of 18:0 and 18:2 respectively. This increase could be attributed to the rate of turn over of fatty acids. However, an age related increase in 18:0 and 18:2 with a concomitant decrease in 18:1 was observed in rats fed on coconut oil based diets *ad libitum* between 24 and 30 months old rats which may be due to age related modulation in lipid digestion (Barzanti *et al.*, 1994).

Calorie restriction on the other hand increased the levels of 18:1 in coconut oil diet fed rats which decreased drastically with corn oil feeding. Similarly, corn oil diets fed on CR significantly increased the levels of 16:0, 18:2 and 20:3 fatty acids. This is in agreement with the findings of Langaniere and Yu (1989) and Ramsey *et al.* (2004). In the present study, the decrease in levels of 18:1, 18:2 in long term feeding of corn oil diet with CR up to 30 months of age may be due to the general age related extensive change in lipid metabolism. Secondly, the higher activities found at 24 months (after 6 months of dietary treatment) could be associated with the particular fatty acid composition of the mitochondrial membrane at that stage through an influence on physico - chemical status.

5.2.1.2 Brain

Short term feeding of coconut oil based diets increased 16:0,18:0 fatty acids whereas, long term feeding of the experimental diets increased the above fractions. Short term and long term feeding of coconut oil and corn oil resulted in increase in 18:1 and 18:2 fatty acids respectively, despite for an apparent increase when fed up to 30 months of age. This is in agreement with previous studies (Innis and Clandinin, 1981; Ramsey *et al.*, 2005) which showed that mitochondrial membrane composition can be altered in a manner that reflects the composition of

the dietary source. The decrease in 18:1, observed in corn oil fed rats against 18:2 in coconut oil based diets, may be due to the interaction of these two in incorporating into tissues (Century *et al.*, 1963).

Calorie restriction on the other hand significantly increased the levels of 18:1 (in coconut oil diet) and 18:2 (in corn oil diet) in the mitochondrial membranes. In this study, a moderate increase was observed in 18:2 between the *ad libitum* and CR rats fed on corn oil diets. This is in contrast to the doubling of 18:2 levels reported in other study (Langaniere and Yu 1993). The discrepancy of results may be due to the time of onset of CR in the two studies, age of the animals etc. The moderate elevation in 18:2 may be also related to the effect of environment as in this study the rats were exposed to varied environmental temperature and relative humidity throughout the experimental period unlike other controlled studies. This underlines the fact that rodents adapt differently to linoleic acid and other PUFA according to the prevailing environmental conditions.

An age related difference in the levels of various fatty acids especially 16:0, 18:1 and 18:2 in corn oil based diets was noticed. This might be due to age associated decrease in lipid metabolism.

However, in both heart and brain the increase in 18:1 levels in coconut oil diets fed rats could possibly reinforce the mitochondrial membrane integrity as evident in studies with reptiles (Hulbert *et al.* 2002). On the other hand increasing the level of membrane 18:2 as well as 20:3 in corn oil fed rats invariably is likely to increase membrane peroxidation (Halliwell and Gutteridge, 1999). On the contrary, Ramsey *et al.* (2005) reported no significant variation in rats fed linoleic acid rich corn oil and soy oil**.** This may be due to the fact that since, both corn oil and soy oil are the rich sources of 18:2, may have nullified the effect of 18:2 on membrane integrity as they are incomparable with respective fatty acid composition. However, in the present study since two different

levels of 18:2 are used, the effects are entirely attributed to the level of 18:2 and/or 18:1 in the mitochondrial membrane.

5.2.2 Mitochondrial inner membrane surface area

Mitochondria are double membrane enclosed eukaryotic organelle with a central role in numerous cellular functions. The ultra structure of mitochondria varies considerably between tissues, organisms and physiological states of the cells. Alterations and remodeling of the inner membrane structures are evident in numerous mitochondrial disorders and during apoptosis.

5.2.2.1 Heart

The mitochondrial cristae surface area showed an apparent age related variation in rats fed on coconut oil based diets *ad libitum* or CR. This increase in surface area is related to the beneficial effect of dietary fatty acids. The cristae surface area showed age related variation in rats fed on coconut oil and corn oil based diets *ad libitum* or CR. However, Sachs *et al.* (2009) reported an age related decrease in area of mitochondrial inner membrane. In the present study, feeding of corn oil *ad libitum* resulted in mitochondria with foamy, vacuolated matrix and scanty cristae compared to those fed on coconut oil diets (Plate 1). Similar ultra structure has been reported in aged rat liver mitochondria (Wilson and Franks, 1975). The variation in mitochondrial ultra structure between the different dietary fat sources could be related to predisposition of linoleic acid rich corn oil to LPO and reinforcement of 18:1 in membranes of rats fed on coconut oil. The beneficial effects of CR on inner membrane surface area may be attributed to the enhanced antioxidant status that resulted from corn oil feeding and the increase in 18:1 levels of coconut oil would have contributed to the increased membrane integrity of inner membrane (Hulbert *et al.*, 2003).

5.2.2.2 Brain

In the present study, both short and long term feeding of coconut oil based diet with CR increase the inner membrane surface area. This was followed by feeding of coconut oil diet *ad libitum* and corn oil diet with CR. The increase in the inner membrane surface area may be due to the level of incorporation of MUFA and PUFA. The ultra structural changes in mitochondria of rats fed on coconut oil diets and corn oil based diets with CR showed an increase the inner membrane surface area. Therefore an age related decrease in inner membrane surface area observed in other studies was not evident in this study. This is attributed to the positive effect of coconut oil and corn oil on membrane integrity. On the contrary, Fattoretti *et al.* (1996) reported a decrease in number of efficient organelles and/or reduction in membrane surface area in old rats. Age related enlargement of the organelles was found to be a morphofunctional compensatory response (Fattoretti *et al.*, 1996). In the present, study similar changes were observed. However, this enlargement did not alter the surface area due to attainment of more spherical form (Safiulina *et al.*, 2006).

The improved surface area of the mitochondrial inner membrane may be responsible for unaltered enzyme complex activity as observed in this study (Schwerzmann *et al.*, 1989).

5.2.3 Mitochondrial membrane potential

The mitochondrial membrane potential (proton motive force) is the central bioenergetic parameter that controls respiratory rates, ATP synthesis and the generation of reactive oxygen species and it itself controlled by election transport and proton leak.

5.2.3.1 Heart

In the present study, feeding of coconut oil based diets altered the MMP significantly compared to corn oil based diets. This is in accordance with the finding of Brand *et al.* (2003), stating the relationship between phospholipid fatty acyl saturation with proton conductance and proton

motive force. Rottenberg and Wu, (1997) and Cavazzoni *et al.* (1999) reported that MMP decreased with age. However, in the present study, a steady increase in the MMP was observed in 24 and 30 months of age. This increase could be attributed to the influence of dietary fat source on MMP in rats (Ramsey *et al.*, 2005). Increase in MMP observed in this study in rats fed on coconut oil based diet could be due to difference in the level of polyunsaturation. It has been proposed, polyunsaturation to be negatively and monounsaturation to be positively correlated with $\Delta \psi$ m (Brand *et al.*, 2003). This has been demonstrated in this study that rats fed on corn oil had increased levels of 18:2 than 18:1 in their membranes and vice versa in rats fed on coconut oil based diets.

Feeding of coconut oil and corn oil based diets on CR, did not significantly alter $\Delta\psi$m in rats of 24 and 30 months of age. This observation was contradictory to several earlier studies (Bevilacqua *et al.*, 2004; Hagopian *et al.*, 2005). This variation may be due to the conditions under which the rats were reared in both the studies. Lack of controlled environment in this study would have caused circadian changes in environmental temperature and relative humidity which might have influenced mitochondrial oxygen consumption, proton leak thus affecting $\Delta\psi$m. More appropriately, the lack of variation between *ad libitum* feeding and CR could have been due to stabilization in proton conductance by 6 months of CR following an initial increment period of 1 month (Ramsey *et al.*, 2005). These results vividly indicated that the changes in $\Delta \psi$ m are more related to the degree of mono unsaturation with coconut oil feeding.

Electron micrographs support the claims of these results that feeding rats with coconut oil with and without CR had intact mitochondrial membranes, dense matrix and increased cristae and showed no vacuolation in the mitochondrial matrix space. On the other hand, rats fed corn oil tend to improve membrane morphology but

yielded mitochondrial with less dense matrix, fewer cristae and more vacuolation suggestive of degenerative changes of the organelle.

5.2.3.2 Brain

Mitochondrial membrane potential did not vary in rats fed on corn oil based diets when compared to their control. This may be due to variation in the fatty acid composition of brain which is rich in 18:2 20:4 acids unlike heart (Century *et al.*, 1963). Since, dietary fat source have been shown to influence $\Delta\psi m$ prospectively (Brand *et al.*, 2003), it is envisaged that feeding of corn oil further enhanced 18:2 in the membrane thus, increasing polyunsaturation when fed for longer duration. The increased 18:2 levels in the membrane could be attributed to the altered lipid metabolism and increased fatty acid turn over that accompany ageing (Barzanti *et al.*, 1994). Secondly, a low level of 18:1 in corn oil diet preferentially increased tissue levels of 18:2 (Century et al., 1963) which reflected on decreasing $\Delta\psi m$ through increased proton conductance (Brand *et al.*,2003).

CR of coconut oil based diet alone maintained improved $\Delta\psi m$ in 30 months old rats, but not corn oil based diets. In contrary, earlier studies have revealed CR but not the corn oil based diets to improve $\Delta\psi m$ in the liver and skeletal muscle as they are high seats of metabolism (Bevilacqua *et al.*, 2005). However, considering the membrane fatty acids to play pivotal role in maintaining membrane potential, the inability of corn oil diets with CR to increase $\Delta\psi m$ is more likely to be related to possibility of enhanced 18:2 fatty acids towards loss of membrane integrity thus reducing $\Delta\psi m$. On the other hand, the ability of coconut oil diet with CR to improve $\Delta\psi m$ is solely attributed to mono unsaturation of membranes with 18:1 thereby improving membrane integrity and proton conductance (Hulbert *et al.*, 2002; Brand *et al.*, 2003).

Electron micrographs supported the claims of these results that revealed that feeding rats with coconut oil and corn oil *ad libitum* had less intact mitochondrial membranes, less dense matrix and moderate cristae with vacuolation in the mitochondrial matrix space. On the other hand, rats fed coconut oil on CR tend to improve membrane morphology but yielded mitochondrial with dense matrix, intact cristae and less vacuolation suggestive of lower degree of degenerative changes of the organelle. The changes in the mitochondria of rats fed corn oil diets with CR were more pronounced compared to coconut oil with CR fed rats.

5.2.4 Production of oxidants

5.2.4.1 Hydrogen peroxide

Hydrogen peroxide, produced by the spontaneous dismutation of superoxide diffuses across the membrane due to its non ionized nature, participation of H2O2 in the production of more reactive **.**OH radicals the importance of its role has been placed on the initiation of free radical cyto-toxity rather than its chemical reactivity. Post mitotic cells *viz.* heart and brain are more vulnerable to mitochondrial ROS mediated cellular damages.

5.2.4.1.1 Heart

Hydrogen peroxide production was found to increase in the heart of 24 months old rats fed on coconut oil and corn oil based diets *ad libitum*. This is in accordance with the reported observation of Bevilacqua *et al.* (2005). This increase in H2O2 production may be due to inter molecular cross linking with the inner mitochondrial membrane (Navarro and Boveries, 2004). Long term feeding of corn oil diets increased the production of $H2O_2$ may be attributed to a loss in membrane integrity and increased production of ROS with age (Bevilacqua *et al.*, 2004: Drew *et al.*, 2003). In the present study, the significant age related decrease was observed in rats fed with coconut oil diet *ad libitum.* On the other hand, comparatively reduced H2O2 levels observed in coconut oil fed rats

is attributed to the level of incorporation of 18:1 and mono unsaturation in stabilizing mitochondrial membrane integrity and thus, ROS production (Boveris and Chance 1973).

The decrease in H2O2 production in short term and long term fed rats on CR with coconut oil and corn oil based diets is attributed to the reduction in ROS production. Similar finding has been reported by Bevilacqua *et al.* (2004) and Hagopian *et al.* (2005) in skeletal muscle. The finding of the study is contradictory to Drew *et al.* (2003) who reported no significant change in production of H2O2 in heart. In the present study also, CR maintained a reduced H2O2 production only in rats fed coconut oil based diets in the long term. This may be attributed to the level of mono unsaturation of membrane brought about by coconut oil diets (Ku *et al.*, 1993).

5.2.4.1.2 Brain

In the present study, corn oil based diets decreased H2O2 production in the brain of 24 month old rats. Long term feeding of the experimental diets resulted in a significant increase in H2O2 production only in rats fed on corn oil based diet *ad libitum.* The age matched variation between heart and brain is likely due to the differences in organ response to dietary manipulation. However, Hagopian *et al.* (2005) reported a lower H2O2 production in the skeletal muscle of rats fed on corn oil diets. In this study, long term feeding of corn oil based diets *ad libitum* resulted in an age related increase in production of H2O2. Similarly, coconut oil feeding resulted in a non significant decrease in H2O2 production. These changes may be attributed to the level of membrane poly unsaturation (Pampalona *et al.*, 2002) and the age related variation in the fatty acid turn over (Barzanti *et al.*, 1994).

Feeding of coconut oil and corn oil based diets on CR resulted in decreased production of H2O2 when feeding up to 30 months, despite the short term decrease in 24 months old rats, long term feeding of corn

oil caused a non significant increase in production of H2O2. This could be attributed to the age of the animal, fatty acid turn over and lipid metabolism. Pampalona *et al* (2002) reported that occurrence of fatty acid unsaturation of mitochondria depends on duration of CR and organ. Similarly, Sohal and Weindruch (1996) reported that response to CR as organ specific and observed 24 per cent decrease in H2O2 production in brain compared to heart (45%) in rats fed on long term CR.

These results suggest that H2O2 production is organ specific and long term feeding of coconut oil CR is effective in reducing H2O2 production in brain and heart compared to corn oil diet.

5.2.5 Mitochondrial OXPHOS enzyme activity

5.2.5.1 Heart

The heart has a continuous requirement for energy in the form of ATP. Mitochondria are the primary ATP provider via the conversion of food fuels to usable energy.

In the present study, no variation in the activity of mitochondrial complexes I to IV and in citrate synthase was observed by feeding coconut oil and corn oil diets *ad libitum* in 24 and 30 months old rats. Similar observation has been reported for complex II (Sugiyama *et al.*, 1993) III and IV (Hepple *et al.*, 2005). The age related changes in the enzyme complexes are resulting out of impaired myocardial carnitine content (McMillan *et al.*, 1993) or cytochrome C content (Castellueio *et al.*, 1994). However, in this study, the activities of the complexes could not be correlated with mitochondrial carnitine and cytochrome C levels. On the other hand, the inconsistent variation in the enzyme activity with the diets may be due to the reinforcement of mitochondrial membrane by mono unsaturated oleic acid (Hulbert *et al.*, 2003), on to the protection of β oxidation of fatty acids due to enrichment of carnitine with linoleic acid (Hagen *et al.*, 2002). The sustained activity of the mitochondrial complexes I to IV has been documented on one and two dimension

electrophoresis which revealed that dietary manipulation could have combated the ROS mediated oxidative damage of enzyme complexes encoded by mtDNA through enhanced antioxidative defense mechanism (Sharma *et al.*, 1998). Further, normal activity of the enzyme complexes improves ATP synthesis and decrease the mitochondrial production of ROS through reduced electron leakage from ETC (Wei *et al.*, 2001). It was evident through electron micrographs that the dietary treatments resulted in varying degrees of intact mitochondrial membranes which would restore the level of cytochrome –C in the mitochondria (Kadenbach and Muller – Hocher, 1990) (plate 1).

Direct evidence of CR on the activity of enzyme complexes and citrate synthase could not be traced and therefore the results obtained in the present study could not be compared. However, it could be related to the beneficial effects of CR in reinforcing membrane integrity and therefore function (Quiles *et al.*, 1999) and through improved antioxidant defense mechanism which could have possibly minimized oxidative damage to mitochondrial OXPHOS complexes (Wei *et al.*, 1998).

5.2.5.2 Brain

Central nervous system depends so heavily on ATP production that the inhibition of oxidative phosphonylation will affect the system before any other system. CNS requires a large amount of energy for the transmission of impulses long the neural pathway; thus mitochondrial functional impairment will result in neurodegeneration and loss in neuronal metabolic control. Such age dependent changes may be a cause of ageing itself and to a certain extent age associated degenerative diseases.

In the present study, short term and long term feeding of coconut oil and corn oil based diets did not show age related changes in the activity of complexes I-IV and citrate synthase. The activity of mitochondrial enzymes complexes have been shown to respond

differently with age (Hepple *et al.*, 2005; Vitorica and Machado, 1981). One probable reason for unaltered enzyme activity found in this study, could be attributed to the fact that brain mitochondria are very heterogenous with respect to their enzyme contents and that the enzyme activities differ among brain regions (Cardozo-Palaez *et al.*, 2000), and since the enzyme activities were measured from pooled mitochondria isolated from brain.

In this study, a lack of age related change in enzyme complexes may be due to the late occurrence of age related changes as beyond 30 months of age as reported by Benzi *et al.* (1992) in mice and rats. In the present study, the sustained activity of complex activities as demonstrated by first dimension blue native page and second dimension tricine SDS page which did not reveal any marked changes in the level of complex-IV. This may be attributed to GSH mediated antioxidant defense in combating peroxynitnite and LPO (Haripriya *et al.*, 2004). This can be related to the protective effect of antioxidant defense in combating mtDNA mutations. This could have possibly prevented coding of defective mitochondrial complexes.

In the present study, lack of age related changes in complex II and III is in accordance with the reports of Sharma *et al.* (1998). This may be due to the fact that subunits of complex II and III were not coded by mitochondrial DNA. Secondly, the activities of enzymes complexes were related to the fluidity of the mitochondrial membranes (Battino *et al.*, 2002). The effectiveness in reinforcing membrane functioning with SFA as reported by Quiles *et al.* (1999) is well documented in the present study (Plate 2).

Effects of CR on enzyme activity are not comparable due to the paucity of related works in this area. However, CR has been shown to increase mitochondrial proteins (Chang *et al.*, 2007). The effects of CR could be mediated through improved antioxidant defense mechanism in

minimizing oxidative damages to the mitochondrial enzyme complex activity.

5.3 Effect of dietary fat and calorie restriction on apoptosis and ageing

5.3.1 Markers of oxidative stress

5.3.1.1 Lipid peroxidation

Lipid peroxidation is a direct indicator of oxidative damage in ageing. The peroxidation of lipids disrupts the cohesive lipid bilayer arrangement and structural organization.

5.3.1.1.1 Heart

Feeding of coconut oil and corn oil based diets *ad libitum* increased LPO in heart of 24 and 30 months of old rats. This increase in LPO is partly due to increased oxidant production that occurs with ageing (Wei and Lee, 2002) and partly due to increase in n-6 fatty acids in the membranes (Leibovitz *et al.*, 1990). Significant feeding of different sources of fatty acid has been shown to differently affect membrane peroxidation (Nalbone *et al.*, 1988, Pinnotti *et al.*, 2006). In this study, *ad libitum* feeding of corn oil diet produced comparatively less LPO to coconut oil diets. Similar effect of corn oil diet has been reported by Ramsey *et al.* (2005). On the other hand, feeding of mono unsaturated rich coconut oil diet to result in increased LPO in heart might be due to the difference that exists in lipid metabolism with different fat sources. This warrants further investigation.

In the present study, CR has effectively reduced LPO in heart of rats fed on coconut oil and corn oil diets. This beneficial effect of CR has been reported in various studies (Ramsey *et al.*, 2004; Bevilacqua *et al.*, 2005). In this study, the beneficial effect of CR is evident even after short term feeding up to 6 months. In contrast, Gomi and Matsuo (1998) observed significant difference in LPO only in 30 month old animals. If such is the case, the variation in LPO observed in this study is likely due to the time of onset of CR and age of the animals (24 and 30 months).

The reduced LPO in CR rats possibly enrich the mitochondrial membrane integrity as evident from EM studies which would have contributed indirectly to decrease in out flux of apoptotic factors from mitochondria thus retarding ageing (Plate 3).

5.3.1.1.2 Brain

Feeding of coconut oil and corn oil diets *ad libitum* and CR altered LPO in brain, in a non significant manner. This may be attributed to the increasing levels of phospholipids present in brain. Secondly, the late onset of dietary manipulations might not have combated the increase in LPO that occur with age in this study. Similar, decrease in LPO due to CR was reported by Chipalkatti *et al.* (1983) and Xia *et al.* (1995) in the brain of rats.

The increasing LPO that occur with age in their study, LPO was found to increase with age in all the groups but was more significant in rats fed on corn oil diets. This is in accordance with the findings of Navarro and Boveries (2004), reporting an age related increase in LPO secondary to increased free radical production (Sies, 1985).

The decrease in LPO in rats fed on coconut oil based diet on CR is attributed to the reduction in peroxidation as a result of decrease in level of unsaturation and their by preserving the mitochondrial membrane architecture (Herrero *et al.*, 2001).

The TBA reactive substance in the heart and brain were reduced by CR regimen of both the diets. In addition it was observed that appearance of lipofuscin as dense bodies on electron micrographs was less frequent in both the tissues compared to corn oil diet fed *ad libitum* (Plate 3). Lipofuscin is believed to be an end product of lipid peroxidation (Rao, *et al.*, 1990a) and the accumulation of lipofuscin in post mitotic cells is one of the most universal changes observed with ageing. The reduction of TBA reactive substance and lipofuscin in heart and brain

strongly suggests that free radical damage is reduced in these organs with coconut oil or corn oil fed on CR.

5.3.1.2 Protein carbonyls

The increasing demand for energy for ATP synthesis by the post mitotic cells of the heart and brain results in increased production of ROS by the mitochondria. The extremely high reactivity of ROS especially of H2O2 results in oxidative damage of proteins resulting in production of protein carbonyls.

5.3.1.2.1 Heart

Protein carbonyl levels were increased in rats fed on coconut oil with CR based diets. This may be attributed to the increased production of H2O2 in rats fed *ad libitum*. This is in accordance with the findings of Grune *et al.* (2001). However, the levels of protein carbonyls were significantly ($P<0.05$) different among the different groups until 24 or 30 months of age. This may be due to the occurrence of age related increase in protein oxidative damage late in life (Tian *et al.*, 1998).

Long term feeding of rats with corn oil and coconut oil based diets *ad libitum* showed decreased protein oxidative damage as measured by protein carbonyls. Similar reduction in protein carbonyls was observed by Lass *et al.* (1998) and Lal *et al.* (2001) in corn oil fed rats. In this study, late onset of CR (after 18 months of age) resulted in reduced protein oxidative changes, since it has been reported that age associated changes in protein carbonyls were evident only in animals beyond 28 months. Nagai *et al.* (2000) reported that late onset of dietary restriction was able to reduce accumulated oxidative damage.

5.3.1.2.2 Brain

The protein carbonyl levels were decreased in 30 months old rats fed on coconut oil diet with CR compared to those of other groups. However, the changes were evident in both short term and long term feeding regimen unlike in heart. This may be related to the composition of brain tissue comprising predominatingly of phospholipids and the increasing demand and production of ATP which increases free radical production this increases oxidative damage of proteins and increased protein carbonyls formation difference in response to oxidative stress by various post mitotic tissues. (Wei and Lee, 2002) reported that post mitotic tissues respond differently to oxidative stress. This increased unsaturation of the membranes seen with feeding PUFA diets further increases the susceptibility to peroxidation and oxidative damage to proteins (Sohal and Weindruch, 1996).

The comparatively lower level of protein carbonyl associated with feeding coconut oil based diet with CR may be attributed to the lower degree of unsaturation of membranes (Herrero *et al.*,2001).

5.3.1.3 DNA fragmentation

Apoptosis is the predominant form of cell death observed in a variety of physiological and pathological conditions. The typical nuclear changes taking place in apoptotic cells include extensive condensation of chromatin and inter nucleosomal DNA fragmentation into units of 200 base pairs.

In the present study, it was evident that both coconut and corn oil fed rats *ad libitum* and on CR resulted in DNA fragments. No appreciable variation was observed between the ages or between the treatment regimens. This may be due to the occurrence of oxidative stress with both the diets to a certain extent as well as could be related to age (Hamilton *et al.*, 2001).

In the heart the evidence of DNA fragments along with streaking of DNA may be due to the simultaneous occurrence of physiological

necrosis and apoptosis leading to age associated loss of myocytes (Wei and Lee, 2002).

DNA fragmentation in the brain also did not show significant variation between the different treatments. Variation in DNA fragmentation pattern may be due to different diets in reducing the oxidative stress along with enhanced antioxidant enzyme activity brought about by the type of dietary fat. In contrast, Tsuzuki *et al.* (2001) demonstrated that over expression of catalase in nucleus did not prevent oxidative damage to DNA.

5.3.2 Antioxidant profile

Antioxidants combat the various ROS produced via different metabolic pathways in the cell and protect the cellular micro and macro molecules from oxidative damage. This protective mechanism is shown to decrease with age, and is modulated through dietary manipulation. In this study, the effect of dietary manipulation of fat and CR has shown to prevent age associated oxidative stress due to mitochondrial decay.

5.3.2.1 Heart

5.3.2.1.1 Superoxide dismutase

Dietary manipulation with CR paradigm resulted in a significant increase in SOD levels in rats fed on both coconut oil (in 24 months and 30 months old rats) and corn oil based diet (in 30 months old rats). This is in accordance with the finding of Lee and Yu (1990), where CR provided a possible biochemical mechanism for anti-lipoxidation action on membrane lipid peroxidation. This is attributed to the positive effect of both oil sources. It has been demonstrated that feeding of corn oil increases SOD levels in liver (Iritani and Ikeda, 1982). It has been proposed that in rats fed corn oil diets, linoleic acid undergoes lipid peroxidation which simultaneously activates the defense mechanism for peroxide elimination. On the other hand, the effect of coconut oil may be attributed to the lower degree of unsaturation which minimizes the predisposition of membranes to lipoxidation. This might improve the

SOD turn over in ageing rats, by minimizing consumption, leakage or destruction of SOD (Bhakuni *et al.*, 2005).

SOD levels were decreased in 24 and 30 months old rats fed on coconut oil and corn oil based diets *ad libitum*. Rao *et al.* (1990b) reported CR increased SOD with CR is related to an increase in the levels of the mRNAs coding SOD, resulting from changes in transcription, mRNA processing or mRNA turnover.

5.3.2.1.2 Glutathione peroxidase

Glutathione peroxidase levels showed an age related decrease in rats fed coconut oil and corn oil diets *ad libitum* this age related decrease was attenuated by CR. This is in accordance with those of Semsei *et al* (1989) Rao *et al.* (1990b) and Piere *et al.* (1992). CR increased the GPX despite other influential factors *viz.* gender, level of selenium (Stowe *et al.*, 2006). Rapid attenuation of myocardial oxidative stress by CR was evident by increased expression of GPX mRNA (Semsei *et al.*, 1989; Chandrasekar *et al.*, 2001). On the Contrary, to the findings of this study, Sohal *et al.* (1994b) reported GPX levels were not modulated by age or diet related changes.

The changes observed in GPX were also dependent on the type of dietary PUFA. In this study, the relatively higher level of GPX observed with corn oil feeding could be attributed to the increased susceptibility of membranes to oxidation that concomitantly increased GPX activity (Nalbone *et al.*, 1989).

5.3.2.1.3 Reduced glutathione

GSH levels were not modulated by age this study. However, coconut oil based diet fed *ad libitum* decreased GSH levels, compared to corn oil based diet. Nevertheless, a decrease in GSH content with age also has been evidenced in different other tissues (Christon *et al.,* 1995; Sohal and Weindruch 1996). The levels of GSH are related to their utilization, since glutathione –S- transferase activities are found to be

altered by age (Sastre *et al.*, 1992). The enhanced levels of GSH in corn oil fed rats could be related to the increased LPO associated with PUFA rich corn oil diets which trigger GSH mediated detoxification process by enhancing *de novo* synthesis of GSH in the cells through availability of cysteine, or reduction of oxidized glutathione using NADPH and glucose-6-phosphate dehydrogenase (Meister, 1991).

Calorie restriction increased GSH levels in both coconut oil and corn oil fed rats. However, both coconut oil and corn oil feeding did not significantly alter the GSH levels between the two groups. It could be hypothesized that the increasing membrane LPO by corn oil diet triggers GSH (Christon *et al.*, 1995). Secondly, the changes associated with coconut oil feeding may be related to the degree of saturation of membrane in maintaining GSH pool within the cell (Rao *et al.*, 1990b).

5.3.2.1.4 Catalase

The catalase activity was decreased in rats fed on coconut oil and corn oil based diets *ad libitum*. This is in accordance with the findings of Venkataraman *et al.* (1994) and Pinnotti *et al.* (2006) reporting similar age related decrease in corn oil fed rats in liver and heart, respectively. This decrease in catalase actively is attributed to increased lipid peroxidation resulting from corn oil feeding. This altered activity would have combated the increased LPO in corn oil fed group, which increases the consumption or destruction of available catalase (Bhakuni *et al.*, 2005).

On the other hand, CR beneficially increased catalase activity in rats fed on coconut oil and corn oil diets. This is in accordance with the findings of Rao *et al* (1990a), which they attributed to an increase in mRNAs coding catalase gene. This could be one reason for increased expectancy and reduced oxidative damage in CR animals (Sohal and Weindruch 1996). On the contrary to the findings of the present study,

Sohal *et al.* (1994a) observed no clear cut overall pattern of age or diet related changes in the antioxidative defense provided by catalase.

In the present study, catalase activity followed stringent age associated decline. This is in contrast to earlier studies where in dietary manipulations restore catalase activity (Xia *et al.*, 1995). A comprehensive study of expression of catalase mRNA could possibly reveal the disparity observed and this warrants further investigation.

5.3.2.2 Brain

5.3.2.2.1 Superoxide dismutase

Antioxidant enzymes including SOD, GPX and catalase remove excess superoxide converting it into H2O2 and O2 which in turn is converted to H2O by catalase. Thus, cellular levels of superoxide are under stringent regulation.

In the present study, the age related decrease in the levels of SOD was observed in rats fed on coconut oil and corn oil based diets *ad libitum*. This is in accordance with the reports of Geremia *et al.* (1990) and is attributed to the increased lipid peroxidation observed in brain tissue due to their biochemical composition. Considerable variation in SOD levels have been reported from various regions of the brain, with a predominant decrease in SOD levels to occur with age (Ciriolo *et al.*, 1991).

CR paradigm significantly increased the levels of SOD in brain tissue. This is in accordance with the findings of Xia *et al* (1995), that CR rats had higher levels of SOD. On the contrary, Baek *et al.* (1999) reported that age related decrease in SOD levels were not reversible with CR. The findings of this study are also contradictory to the opinion that beneficial effects of CR declined with delay in onset of treatment (Speakman and Hambly, 2007).

The levels of SOD also showed an age related decrease in responding to the dietary treatment. This may be related to the general

concept of antioxidant enzymes decrease with increasing age. This increase might have caused increased LPO in brain tissue (Ciriolo *et al.*, 1991). The improved levels of SOD resulting from CR feeding of coconut oil may be due to reduced degree of poly unsaturation compared to 18:2 rich corn oil which triggers further LPO. It has been suggested that increased levels of SOD in CR rats is manifested through increased SOD mRNA transcription (Rao *et al.*, 1990a).

5.3.2.2.2 Glutathione peroxidase

Feeding of coconut oil and corn oil based diets *ad libitum* reduced the GPX levels to a greater extent with coconut oil compared to corn oil diet. This could be related to the general dogma of decreasing antioxidant levels with advancing age (Xia *et al.*, 1995). On the contrary, Rao *et al.* (1990b) reported that GPX levels were not affected by age. This has been shown in the present study, that GPX levels remained unaltered between the two periods of analysis, except in coconut oil fed groups. This highlights the beneficial effects of coconut oil based diet on enhanced GPX status. This enhancement is attributed to the high degree of unsaturation seen in corn oil but not with coconut oil, which could have increased oxidation of macromolecules membranes thereby increasing antioxidant consumption, leakage or destruction within the cell (Bhakuni *et al.*, 2005).

Short term CR paradigm with both coconut oil and corn oil increased GPX levels but with long term coconut oil with CR sustainable increased GPX levels in brain. The beneficial effects of coconut oil are related to amount of 18:1 present which might have restored membrane integrity and diminishes LPO. Reduced LPO was able to maintain glutathione pool thus enhancing GPX level (Christon *et al.*, 1995). Secondly, fatty acid interaction to bring out increased expression of GPX mRNA and mRNA transcription plays a pivotal role in enhancing GPX levels in rats fed coconut oil diet on CR (Rao *et al.* 1990a).

5.3.2.2.3 Reduced glutathione

Short term and long term dietary manipulation caused a decrease in GSH levels in rats fed on coconut oil *ad libitum*. Similar age related decrease has been reported by Sohal and Weindruch (1996). This decrease in GSH could be attributed to the occurrence LPO in brain as GSH is primarily aimed to reduce LPO (Tateishi, 1986).

CR regimen was found to effectively reverse the age related decline in enzyme activity. It was found that rat fed on CR of corn oil diets had higher enzyme activity which decreased with increasing age. This may be attributed to the increasing LPO associated with corn oil diets that might have decreased the glutathione pool (Christon *et al.*, 1995).

5.3.2.2.4 Catalase

Catalase levels did not show appreciable age related changes in the rats fed on coconut oil or corn oil diet *ad libitum* up to 24 and 30 months of age. This is contrary to the reported observations of Tian *et al.* (1998) and Siqueira *et al.* (2005a). This may be due to, the heterogeneity of activity of catalase in different regions of the brain (Ciriolo *et al.*, 1991).

In the present study, the increased catalase activity observed in rats fed on coconut oil based diet with CR regimen may be attributed primarily to the fatty acid unsaturation between the two diets and to the effect of CR to increase mRNA expression and transcription (Rao *et al.,* 1990a).

In this study, it was evident that dietary manipulation with CR primarily increased the activities of SOD, GPX and catalase which are the first line of antioxidant defense. However, the increase in GSH could be supplementary to increased activity of GPX.

5.3.3 Myocardial collagen

Collagen is a non contractile protein which plays an important role in regulating myocardial contractility and hence it is instrumental in controlling many hemodynamic functions.

An apparent age related increase in the levels of collagen in 24 and 30 months old rats has been observed in all the groups except when fed on corn oil diet with CR. This may be attributed to the reduction in total myocytes (Olivetti *et al.*, 1991) and the accompanying hypertrophy (Patiyal and Katoch, 2006) that accompany ageing. Apoptosis and physiological necrosis may be the major factor contributing to age associated loss of cardiac myocyte (Wei and Lee, 2002). Myocardial hypertrophy and necrosis are shown to increase collagen infiltration (Duncan *et al.*, 2000). Collagen accumulation can lead to a pathophysiological state characterized by reduced coronary blood flow, ultimately affecting ventricular compliance (Fioretto *et al.*, 2002).

Corn oil based diet fed with CR regimen has been shown to decrease the level of collagen in heart muscles, as observed in short term feeding. The decrease in collagen was significant in short term CR feeding of both the diets but on the long run corn oil diet with CR alone was found to decrease collagen levels. Boreham *et al.* (1988) also reported reduction in collagen levels with CR. This reduction may be due to reduced protein oxidation and cross linking as reported by Zainal *et al.* (2000). The response of myocardial collagen to different dietary lipids warrants further investigation. Since long term feeding of both coconut oil and corn oil diets were found to reduce the myocardial collagen levels to a certain extent.

5.3.4 Lipid profile

5.3.4.1 Total cholesterol

In the present study, feeding of coconut and corn oil based diets *ad libitum* or CR did not show any significant variation in total cholesterol levels. The non significant decease observed with coconut oil feeding in 24 months old rats which may be attributed to the presence of medium chain fatty acids in coconut oil (St-Onge *et al.*, 2003). The hypocholesterlemic effect of feeding corn oil based diets with CR may be

related to the linoleic acid content (Chandrasekar *et al.*, 2001) which indirectly affects lipogenic enzymes (Flick *et al.*, 1977).

5.3.4.2 HDL cholesterol

The HDL-C levels were increased in short and long term feeding of coconut and corn oil based diets with CR. This is in accordance with the reports of Choi *et al.*, (1988). In the present study, feeding of coconut oil based diets at both levels increased HDL-C in an age dependent manner. This is in agreement with the reports of Masoro and Compton (1983) who observed increase in HDL-C in old rats compared to young rats. Comparatively, lower levels of HDL-C concentration in coconut oil fed rats may be attributed to the enhanced catabolism due to unchanged levels of apo A-I mRNA (Ahn *et al.*, 1994). The increase in HDL-C is inversely related to decrease in triglycerides in CR animals (Hayek *et al.*, 1993). In addition the HDL-C increasing effect of CR may be attributed to body weight, basal metabolic index and lean body mass which proportionately decreased in CR rats (Verdery *et al.*, 1997).

5.3.4.3 LDL cholesterol

In the present study, LDL-C showed a non significant increase in 24 and 30 months old rats fed on coconut or corn oil based diets *ad libitum* or on CR. This increase in LDL-C may be related to increased LDL turnout or a down regulation of LDL receptors that occur frequently with age or by feeding SFA or PUFA (Fernandez and West, 2005).

5.3.4.4 VLDL

Feeding of corn oil based diet with CR at 24 and 30 months of age decreased VLDL and decreased the same in 30 month old rats. The decrease observed in VLDL may be attributed to the reduced body weight and concomitant increase in HDL-C as reported by Hayek *et al.* (1993). Another possible explanation could be the effect of n-6 PUFA on the hepatic enzymatic machinery to cause the production of VLDL (Fernandez and West, 2005). On the other hand, increase in VLDL at 30

months may be due to age related increase in HDL-C as reported by Masoro and Compton (1983). The increase in VLDL levels in rats fed on coconut oil based diets may be related to increased triglycerids levels (Ahn *et al.*, 1994).

5.3.4.5 Triglycerides

An age related increase in levels of triglycerides was observed in rats fed on corn oil based diets compared to coconut oil diets *ad libitum* and on CR. This may be attributed to the decreased lipolytic activity due to lower apo C-II and apo C-III ratio due to higher hepatic apo C-III mRNA concentration than those fed on poly unsaturated fat diet (Carison and Ballantyne, 1976). An increase in plasma free fatty acid may feed back negatively on the expression of apo C-II and apo C-III (Ahn *et al.*, 1994). The significantly lower triglyceride levels observed by feeding PUFA rich corn oil diet is attributed to decreased expression of apo A-II along with enhanced conversion of HDL2 to HDL3 (Ahn *et al.*, 1994).

To conclude, dietary manipulation of fat was shown to minimize mitochondrial decay and maintain its structural homeostasis by improving membrane integrity, mitochondrial membrane potential and unaltered change in the respiratory enzyme activity with advancing age. Nevertheless both coconut and corn oil based diet positively reduced the age related changes coconut oil based diets on CR was more efficient in restoring mitochondrial morphofunctional integrity through enhanced 18:1 levels thereby increasing robustness of the membrane against lipoxidation. In addition, the age related imbalance in the oxidant production and antioxidant status can be effectively combated by dietary manipulation of fat and CR. Short and long term feeding of coconut and corn oil based diets with CR effectively minimized the oxidative damage to lipids and proteins and enhanced antioxidant levels to combat oxidant production. However, further investigation on the interaction of different sources of dietary fat on mitochondrial carnitine, cytochrome C content, matrix substances including mtDNA and apoptotic proteins and DNA

repair enzymes that play a central role in apoptosis will facilitate the confirmation of the efficacy of coconut oil based diet with CR in retarding ageing process in the post mitotic tissues of heart and brain.

SUMMARY

An investigation was carried out to study the effect of dietary manipulation of fat and CR on the production of ROS in relation to activity of mitochondrial electron transport complexes and its implications on apoptosis in ageing heart and brain.

Eighteen month old male Wistar Albino rats were fed on coconut and corn oil based diets *ad libitium* or at 40 per cent CR up to 24 (short term) or 30 months (long term) of age. At the end of feeding trial, rats were sacrificed, mitochondria from heart and brain were isolated for the estimation of membrane fatty acid profile, H_2O_2 and OXPHOS enzyme activity. Mitochondrial inner membrane surface area was measured stereologically from electron micrographs. Antioxidant enzyme status, markers of oxidative stress measured as TBARS and protein carbonyls were measured in both the tissues. Myocardial collagen and serum lipid profile were also estimated to correlate the effect of dietary fat and CR on retarding ageing.

Short term feeding of coconut oil (low in linoleic acid) and corn oil (rich in linoleic acid) based diets with CR was found to increase oleic acid (18:1) and linoleic acid (18:2) levels respectively in the mitochondrial membranes. Reinforcement of 18:1 levels in the mitochondrial membranes effectively maintained membrane integrity. This was reflected through an increased MMP and reduced H_2O_2 production in rats fed on coconut oil based diets with CR compared to corn oil feeding. The OXPHOS enzyme activities did not show significant ($P>0.05$) age related variation in rats fed on coconut and corn oil based diets. This highlights the beneficial effect of dietary manipulation of fat and CR in maintaining the enzyme activity to pre-treatment levels.

The reduced oxidant production evident in rats fed on coconut and corn oil based diets on CR significantly ($P<0.05$) decreased the oxidative damage to lipids and proteins as documented through the presence of lipofuscin pigments in both the tissues.

Estimation of SOD, GPx, GSH and catalase in the heart and brain revealed a significant ($P<0.05$) increase in rats fed on coconut and corn oil based diets on CR compared to *ad libitum* feeding.

Myocardial collagen levels were significantly ($P<0.05$) decreased in rats fed on corn oil based diets compared to coconut oil based diets on CR. However, the different treatment regimen did not show any appreciable variation in DNA fragments.

In the present study, long term feeding of coconut oil based diet with CR resulted in reduced oxidant production due to improved mitochondrial morphofunctional architecture compared to corn oil based diets with CR. This also decreased oxidative damage of proteins primarily and lipids and reduced the myocardial collagen content. The lipid profile was not affected adversely since coconut oil based diet with CR increased HDL-C and decreased triglycerides. Despite the existing disparity on oxidative damage to DNA the findings of this study highlights the beneficial effect of coconut oil based diet with CR over corn oil based diet.

Further investigation on the interaction of different sources of dietary fat on mitochondrial carnitine, cytochrome C content, matrix substances including mtDNA and apoptotic proteins and DNA repair enzymes that play a central role in apoptosis will facilitate the confirmation of the efficacy of coconut oil based diet with CR in retarding ageing process in the post mitotic tissues of heart and brain.

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Table 8a: Comparison of short and long term feeding of coconut oil and corn oil based diets on mitochondrial respiratory chain enzyme activity in the heart of rats

Mitochondrial respiratory chain enzyme (nM/min/mg protein)	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
Complex I	24	6.64 ±1.06	6.78 ±0.86	8.58 ±1.56	8.40 ±1.24	F=0.930; P=0.444NS
	30	6.44 ±1.06	6.05 ±0.81	8.22 ±1.09	7.4 ±1.10	F=1.242; P=0.321NS
	P value	0.914	0.552	0.859	0.588	
Complex II	24	88.44 ±6.16	85.96 ±8.57	107.13 ±8.60	94.23 ±9.57	F=1.290; P=0.305
	30	67.49 ±7.45	77.66 ±6.15	97.12 ±12.34	91.13 ±6.93	F=2.407; P=0.097+
	P value	0.056	0.450	0.523	0.798	
Complex III	24	2653.14 ±184.93	2578.93 ±257.06	3213.87 ±258.09	2826.87 ±287.05	F=1.290; P=0.305NS
	30	2024.66 ±223.64	2329.88 ±184.64	2913.72 ±372.77	2733.91 ±208.01	F=2.407; P=0.097+
	P value	0.056	0.450	0.523	0.798	
Complex IV	24	2839.60 ±110.00	2865.50 ±205.80	3065.71 ±105.12	3149.88 ±134.72	F=1.155; P=0.352
	30	2662.60 ±214.83	2562.50 ±1.75	2830.86 ±230.73	2764.37 ±134.72	F=0.422; P=0.739NS
	P value	0.480	0.228	0.376	0.058	
Citrate synthase	24	2075.94 ±153.25	2110.95 ±65.91	2283.00 ±272.23	2187.96 ±178.63	F=0.252; P=0.859NS
	30	2040.63 ±219.77	2007.00 ±170.51	2087.26 ±217.08	2060.06 ±214.04	F=0.027; P=0.994NS
	P value	0.898	0.582	0.586	0.658	

Means having different superscripts within a row differ significantly ($P<0.05$).

Table 9a: Comparison of short and long term feeding of coconut oil and corn oil based diets on mitochondrial respiratory chain enzyme activity in the brain of rats

Mitochondrial respiratory chain enzyme (nM/min/mg protein)	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
Complex I	24	320.39 ±29.22	304.24 ±58.15	332.48 ±11.61	308.41 ±37.28	F=0.113; P=0.952NS
	30	297.22 ±11.24	250.27 ±39.94	318.99 ±11.20	301.93 ±10.95	F=1.759; P=0.187NS
	P value	0.914	0.552	0.859	0.588	
Complex II	24	213.46 ±6.16	213.35 ±8.57	206.44 ±8.60	222.23 ±9.57	F=0.104; P=0.957
	30	191.02 ±18.07	204.62 ±18.52	198.07 ±16.15	220.38 ±9.39	F=0.617; P=0.612
	P value	0.056	0.450	0.523	0.798	
Complex III	24	2561.57 ±281.90	2560.22 ±192.44	2477.30 ±300.76	2666.74 ±158.26	F=0.104; P=0.957NS
	30	2292.22 ±216.86	2455.41 ±222.29	2376.88 ±193.79	2644.56 ±112.65	F=0.617; P=0.612NS
	P value	0.056	0.450	0.523	0.798	
Complex IV	24	2089.55 ±129.74	2075.27 ±107.66	2315.48 ±151.35	2114.63 ±120.30	F=0.767; P=0.526
	30	2013.49 ±95.24	1993.34 ±119.55	2214.97 ±184.51	2057.18 ±189.26	F=0.433; P=0.732NS
	P value	0.480	0.228	0.376	0.058	
Citrate synthase	24	2022.94 ±101.35	2160.91 ±231.41	2340.46 ±261.23	2230.44 ±256.12	F=0.252; P=0.859NS
	30	1993.41 ±171.58	2050.87 ±170.11	2190.12 ±298.43	2034.85 ±90.80	F=0.187; P=0.904NS
	P value	0.898	0.582	0.586	0.658	

Means having different superscripts within a row differ significantly ($P<0.05$).

Table 2a: Comparison of short and long term feeding of coconut oil and corn oil based diets on fatty acid profile of the heart mitochondrial membrane in rats

Fatty acids (molar %)	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
16:0	24	24.70[a] ±12.17	32.96[a] ±2.28	21.64[a] ±3.04	20.10[a] ±5.05	P=0.600
	30	23.75[a] ±3.17	29.75[ab] ±1.45	18.77[a] ±3.95	31.48[b] ±0.59	P=0.081+
	P value	0.947	0.363	0.623	0.134	
18:0	24	39.38[a] ±5.16	19.45[b] ±2.80	48.72[a] ±1.40	17.05[b] ±0.13	P=0.004*
	30	46.80[a] ±4.61	31.05[b] ±0.79	54.89[a] ±1.16	27.23[b] ±2.87	P=0.006*
	P value	<0.001**	0.057	0.077+	0.071	
18:1	24	14.23[a] ±1.87	6.46[b] ±1.43	20.17[a] ±1.30	2.51[b] ±2.50	P=0.009*
	30	10.57[b] ±1.77	4.97[c] ±1.02	16.91[a] ±1.29	5.32[c] ±0.22	P=0.006*
	P value	<0.001**	0.485	0.233	0.382	
18:2	24	4.68[b] ±0.51	27.08[a] ±5.46	3.47[b] ±0.23	22.42[a] ±0.27	P=0.008*
	30	5.06[a] ±0.70	13.75[a] ±1.96	7.46[a] ±1.43	14.87[a] ±3.92	P=0.087+
	P Value	0.002*	0.142	0.111	0.195	
20:0	24	1.37[a] ±0.15	0.41[a] ±0.41	0.97[a] ±0.18	0.82[a] ±0.82	P=0.063+
	30	1.28[a] ±0.46	0.38[a] ±0.38	1.38[a] ±0.12	0.51[a] ±0.17	P=0.185
	P value	0.464	0.239	0.303	0.073	
20:3	24	0.98[b] ±0.89	3.13[b] ±0.26	0.24[b] ±0.02	19.84[a] ±5.48	P=0.020*
	30	-	13.44[b] ±1.12	-	19.96[a] ±1.42	P<0.001**
	P value	<0.001**	0.012*	0.423	0.985	
22:0	24	-	0.72[a] ±0.01	0.85[a] ±0.28	0.96[a] ±0.72	P=0.127
	30	0.70a ±0.28	0.93[a] ±0.46	0.48[a] ±0.17	0.12[a] ±0.12	P=0.350
	P value	0.171	0.700	0.376	0.083+	
22:1	24	-	-	-	4.46 ±1.40	-
	30	2.54[a] ±1.29	-	1.92[a] ±1,92	-	P=0.391
	P value	0.203	-	0.423	-	
24:0	24	-	0.81[a] ±0.27	-	0.13[a] ±0.13	P=0.051+
	30	0.33[a] ±0.33	1.34[a] ±1.34	0.23[a] ±0.14	0.66[a] ±0.66	P=0.745

	P value	0.226	0.736	0.239	0.517	

Means having different superscripts within a row differ significantly (P<0.05).

Table 3a: Comparison of short and long term feeding of coconut oil and corn oil based diets on fatty acid composition of the brain mitochondrial membrane in rats

Fatty acids (molar %)	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
16:0	24	19.62[a] ±0.81	15.77[b] ±0.85	21.86[a] ±0.18	17.34[b] ±0.96	P=0.018*
	30	21.56[a] ±2.74	31.12[a] ±1.22	26.56[a] ±7.74	27.90[a] ±3.23	P=0.325NS
	P value	0.568	0.009*	0.606	0.088+	
18:0	24	34.82[a] ±0.75	11.35[b] ±1.11	34.54[a] ±2.54	13.51[b] ±1.17	P<0.001**
	30	26.01[a] ±3.15	35.24[b] ±0.50	32.57[b] ±1.65	34.22[b] ±1.01	P=0.079+
	P value	0.113	0.003*	0.572	0.006*	
18:1	24	15.84[a] ±1.89	1.10[c] ±0.15	24.19[b] ±2.68	2.93[d] ±0.83	P=0.002*
	30	9.77[a] ±1.89	0.77[b] ±0.77	6.36[a] ±6.36	0.85[b] ±0.08	P=0.329
	P value	0.225	0.715	0.123	0.130	
18:2	24	3.71[a] ±0.90	61.78[b] ±3.50	5.37[a] ±1.05	56.82[b] ±5.54	P=0.001**
	30	10.78[a] ±3.21	13.48[b] ±2.82	5.78[a] ±3.21	16.57[b] ±3.10	P=0.181
	P value	0.714	0.009*	0.912	0.024*	
20:0	24	-	0.41[a] ±0.41	-	0.82[a] ±0.82	P=0.067+
	30	-	0.55[a] ±0.55	-	0.27[a] ±0.27	P=0.011*
	P value	-	0.860	-	0.592	
20:3	24	-	2.28[a] ±0.61	-	4.17[a] ±1.61	P=0.584
	30	-	6.82[a] ±1.50	-	5.38[a] ±1.01	P=0.584
	P value	-	0.107	-	0.591	
22:0	24	2.50[a] ±0.18	3.61[a] ±1.43	1.79[a] ±0.17	4.63[a] ±3.28	P=0.712
	30	1.53[a] ±0.52	1.74[a] ±1.74	1.02[a] ±1.02	0.87[a] ±0.87	P=0.936
	P value	0.219	0.494	0.540	0.385	
22:1	24	-	0.26[a] ±0.26	-	0.52[a] ±0.52	P=0.584
	30	5.41[a] ±1.57	1.91 [a]±1.91	3.50[a] ±3.50	0.95[a] ±0.95	P=0.559
	P value	0.075+	0.482	0.423	0.726	
24:0	24	-	1.11[a] ±0.01	-	1.17[a] ±0.14	P=0.181

	30	-	$2.03^{a}\pm0.07$	-	1.02^{a} ±1.02	P=0.122
	P value	-	0.007*	-	0.893	

Means having different superscripts within a row differ significantly (P<0.05).

Table 1: Effect of short and long term feeding of coconut oil and corn oil based diets on body weight at sacrifice, weights of heart and brain in rats

	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
Body weight (g)	270.55 ±13.99	323.33* ±15.85	304.00 ±11.21	225.17* ± 5.16	250.22 ±12.85	322.69* ±9.40	313.58* ±7.93	222.50* ±5.98	253.48 ±7.24
Weight of heart (mg)	853.83 ± 89.95	1024.00 ±26.50	982.00 ±73.70	817.83 ±45.66	787.60 ±60.92	1144.67* ±76.64	1196.00 ±124.93	873.83 ±72.75	891.33 ±95.40
Weight of brain (mg)	1885.33 ±91.13	1918.50 ±30.58	1693.17 ±109.18	1796.33 ±74.40	1562.50 ±139.72	1880.17 ±89.48	1758.33 ±173.07	1691.67 ±62.18	1616.00 ±102.49

t- test: * significant

Table 1a: Comparison of short and long term feeding of coconut oil and corn oil based diets on body weight at sacrifice, weights of heart and brain in rats

	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
Body Weight (g)	24	323.33[a] ±15.85	304.00[a] ±11.21	225.17[b] ±5.16	250.22[b] ±12.85	F=14.707; P<0.001**
	30	322.069[a] ±9.40	313.58[a] ±7.93	222.50[b] ±5.98	253.48[b] ±7.24	F=38.656; P<0.001**
	P value	0.973	0.502	0.743	0.830	
Weight of heart (mg)	24	1024.00[a] ±26.50	982.00[a] ±73.70	817.83[b] ±45.66	787.60[b] ±60.92	F=4.648; P=0.013*
	30	1144.67[ab] ±76.64	1196.00[a] ±124.93	873.83[c] ±72.75	891.33-[bc] ±95.40	F=3.132; P=0.048*
	P value	0.168	0.171	0.529	0.378	
Weight of brain (mg)	24	1918.50[a] ±30.58	1693.17[a] ±109.18	1796.33[a] ±74.40	1562.50[b] ±139.72	F=2.681; P=0.074+
	30	1880.17 ±89.48	1758.33 ±173.07	1691.67 ±62.18	1616.00 ±102.49	F=0.959; P=0.431NS
	P value	0.694	0.573	0.306	0.764	

Means having different superscripts within a row differ significantly (P<0.05).

Table 2: Effect of short and long term feeding of coconut oil and corn oil based diets on fatty acid profile of the heart mitochondrial membrane in rats

Fatty acids (Molar %)	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
16:0	35.01 ±1.54	24.70 ±12.17	32.96 ±2.28	21.64 ±3.04	20.10 ±5.05	23.75 ±3.17	29.75 ±1.45	18.77 ±3.95	31.48 ±0.59
18:0	44.27 ±2.08	39.38 ±5.16	19.45 ±2.80	48.72 ±1.40	17.05 ±0.13	46.80 ±4.61	31.05 ±0.79	54.89 ±1.16	27.23 ±2.87
18:1	7.14 ±2.01	14.23 ±1.87	6.46 ±1.43	20.17* ±1.30	2.51 ±2.50	10.57 ±1.77	4.97 ±1.02	16.91* ±1.29	5.32 ±0.22
18:2	5.38 ±2.06	4.68 ±0.51	27.08* ±5.46	3.47 ±0.23	22.42* ±0.27	5.06 ±0.70	13.75 ±1.96	7.46 ±1.43	14.87 ±3.92
20:0	0.93 ±0.36	1.37 ±0.15	0.41 ±0.41	0.97 ±0.18	0.82 ±0.82	1.28 ±0.46	0.38* ±0.38	1.38 ±0.12	0.51 ±0.17
20:3	2.03 ±0.36	0.98 ±0.89	3.13 ±0.26	0.24 ±0.02	19.84* ±5.48	-	13.44* ±1.12	-	19.96* ±1.42
22:0	0.64 ±0.12	-	0.72 ±0.01	0.85 ±0.28	0.96* ±0.72	0.70 ±0.28	0.93 ±0.46	0.48 ±0.17	0.12 ±0.12
22:1	1.18 ±0.17	-	-	-	4.46* ±1.40	2.54 ±1.29	-	1.92 ±1.92	-
24:0	0.84 ±0.12	-	0.81 ±0.27	-	0.13 ±0.13	0.33 ±0.33	1.34 ±1.34	0.23 ±0.14	0.66 ±0.66

t- test: * significant; – not detected in chromatograph

Table 3: Comparison of short and long term feeding of coconut oil and corn oil based diets on fatty acid profile of the brain mitochondrial membrane in rats

Fatty acids (Molar %)	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
16:0	26.74 ± 0.22	19.62* ±0.81	15.77* ±0.85	21.86 ±0.18	17.34* ±0.96	21.56 ±2.74	31.12 ±1.22	26.56 ±7.74	27.90 ±3.23
18:0	29.35 ±3.67	34.82 ±0.75	11.35 ±1.11	34.54 ±2.54	13.51 ±1.17	26.01 ±3.15	35.24 ±0.50	32.57 ±1.65	34.22 ±1.01
18:1	11.64 ±1.28	15.84 ±1.89	1.10* ±0.15	24.19* ±2.68	2.93* ±0.83	9.77 ±1.89	0.77* ±0.77	6.36 ±6.36	0.85* ±0.08
18:2	7.22 ±1.27	3.71 ±0.90	61.78* ±3.50	5.37 ±1.05	56.82* ±5.54	10.78 ±3.21	13.48* ±2.82	5.78 ±3.21	16.57* ±3.10
20:0	0.01 ±0.01	-	0.41 ±0.41	-	0.82 ±0.82	-	0.55 ±0.55	-	0.27 ±0.27
20:3	4.06 ±1.18	-	2.28 ±0.61	-	4.17 ±1.61	-	6.82 ±1.50	-	5.38 ±1.01
22:0	10.64 ±3.00	2.50 ±0.18	3.61 ±1.43	1.79 ±0.17	4.63 ±3.28	1.53 ±0.52	1.74 ±1.74	1.02 ±1.02	0.87 ±0.87
22:1	1.01 ±0.06	-	0.26 ±0.26	-	0.52 ±0.52	5.41 ±1.57	1.91 ±1.91	3.50 ±3.50	0.95 ±0.95
24:0	-	-	1.11 ±0.01	-	1.17 ±0.14	-	2.03 .±0.07	-	1.02 ±1.02

t- test: * significant - not detected in chromatogram

Table 6: Effect of short and long term feeding of coconut oil and corn oil based diets on the levels of MMP and H_2O_2 in the heart of rats

	18 months	24 months				30 months			
	Rat chow	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**
MMP (mV)	99.03 ±1.93	126.21** ±7.37	104.15** ±1.51	128.56** ±3.46	110.73** ±4.39	123.50** ±1.95	110.25** ±2.42	133.63** ±5.50	112.96** ±4.27
H_2O_2 (nM/mg protein)	34.65± 2.54	30.74** ±1.61	35.19** ±3.43	20.61** ±1.42	20.91** ±1.82	20.74** ±.3.55	36.51** ±2.05	25.34** ±2.49	28.17** ±2.44

t- test: ** highly significant

Table 6a: Comparison of short and long term feeding of coconut oil and corn oil based diets on the levels of MMP and H_2O_2 in the heart of rats

	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
MMP (mV)	24	126.21^{a} ±7.37	104.15^{b} ±1.51	128.56^{a} ±3.46	110.73^{b} ±4.39	F=6.403; P=0.008*
	30	123.50^{b} ±1.95	110.25^{c} ±2.42	133.63^{a} ±5.50	112.96^{bc} ±4.27	F=7.888; P=0. 001*
	P value	0.453	0.723	0.730	0.058+	
H_2O_2 (nM/mg protein)	24	30.74^{a} ±1.61	35.19^{a} ±3.43	20.61^{b} ±1.42	20.91^{b} ±1.82	F=5.663; P=0.006*
	30	20.74^{b} ±.3.55	36.51^{a} ±2.05	25.34^{a} ±2.49	28.17^{b} ±2.44	F=6.060; P=0.001**
	P value	0.028*	0.828	0.130	0.039*	

Means having different superscripts within a row differ significant (P<0.05).

Table 7: Effect of short and long term feeding of coconut oil and corn oil based diets on the levels of MMP and H_2O_2 in the brain of rats

	18 months	24 months				30 months			
	Rat chow	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**
MMP (mV)	114.59 ±2.53	128.42* ±2.54	115.13 ±2.59	131.10** ±1.32	112.17 ±3.72	121.87* ±1.68	104.18 ±3.11	130.79* ±1.83	110.20 ±3.18
H_2O_2 (nM/mg protein)	31.87 ± 3.13	33.58 ±3.62	20.56* ±1.78	27.96 ±2.28	15.29** ±1.54	32.29 ±2.07	29.36 ±2.13	22.17 ±3.80	17.24** ±1.21

t- test: * significant; ** highly significant

Table 7a: Comparison of short and long term feeding of coconut oil and corn oil based diets on the levels of MMP and H_2O_2 in the brain of rats

	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
MMP (mV)	24	128.42^{a} ±2.54	115.13^{b} ±2.59	131.10^{a} ±1.32	112.17^{b} ±3.72	F=12.42; P=0.001**
	30	121.87^{b} ±1.68	104.18^{c} ±3.11	130.79^{a} ±1.83	110.20^{c} ±3.18	F=21.77 P<0. 001*
	P value	0.057+	0.022*	0.896	0.695	
H_2O_2 (nM/mg protein)	24	33.58^{a} ±3.62	20.56^{b} ±1.78	27.96^{a} ±2.28	15.29^{b} ±1.54	F=10.900; P=0.001**
	30	32.29^{a} ±2.07	29.36^{b} ±2.13	22.17^{c} ±3.80	17.24^{c} ±1.21	F=7.557; P=0.001**
	P value	0.764	0.010*	0.221	0.342	

Means having different superscripts within a row differ significantly (P<0.05).

Table 8: Effect of short and long term feeding of coconut oil and corn oil based diets on the mitochondrial respiratory chain enzyme activity in the heart of rats

Mitochondrial respiratory chain enzyme (nM/min/mg protein)	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
Complex I	7.30 ±1.10	6.64 ±1.06	6.78 ±0.86	8.58 ±1.56	8.40 ±1.24	6.44 ±1.06	6.05 ±0.81	8.22 ±1.09	7.4 ±1.10
Complex II	111.30 ±10.04	88.44 ±6.16	85.96 ±8.57	107.13 ±8.60	94.23 ±9.57	67.49* ±7.45	77.66* ±6.15	97.12 ±12.34	91.13 ±6.93
Complex III	3338.91 ±301.35	2653.14 ±184.93	2578.93 ±257.06	3213.87 ±258.09	2826.87 ±287.05	2024.66* ±223.64	2329.88* ±184.64	2913.72 ±372.77	2733.91 ±208.01
Complex IV	3023.72 ±172.22	2839.60 ±110.00	2865.50 ±205.80	3065.71 ±105.12	3149.88 ±134.72	2662.60 ±214.83	2562.50 ±1.75	2830.86 ±230.73	2764.37 ±134.72
Citrate synthase	2026.98 ±189.62	2075.94 ±153.25	2110.95 ±65.91	2283.00 ±272.23	2187.96 ±178.63	2040.63 ±219.77	2007.00 ±170.51	2087.26 ±217.08	2060.06 ±214.04

t- test: * significant

Table 9: Comparison of short and long term feeding of coconut oil and corn oil based diets on mitochondrial respiratory chain enzyme activity in the brain of rats

Mitochondrial respiratory chain enzyme (nM/min/mg protein)	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
Complex I	344.58± 57.99	320.39 ±29.22	304.24 ±58.15	332.48 ±11.61	308.41 ±37.28	297.22 ±11.24	250.27 ±39.94	318.99 ±11.20	301.93 ±10.95
Complex II	228.82± 10.52	213.46 ±6.16	213.35 ±8.57	206.44 ±8.60	222.23 ±9.57	191.02 ±18.07	204.62 ±18.52	198.07 ±16.15	220.38 ±9.39
Complex III	2745.83 ±126.28	2561.57 ±281.90	2560.22 ±192.44	2477.30 ±300.76	2666.74 ±158.26	2292.22 ±216.86	2455.41 ±222.29	2376.88 ±193.79	2644.56 ±112.65
Complex IV	2141.45 ±166.22	2089.55 ±129.74	2075.27 ±107.66	2315.48 ±151.35	2114.63 ±120.30	2013.49 ±95.24	1993.34 ±119.55	2214.97 ±184.51	2057.18 ±189.26
Citrate synthase	2259.68 ±124.01	2022.94 ±101.35	2160.91 ±231.41	2340.46 ±261.23	2230.44 ±256.12	1993.41 ±171.58	2050.87 ±170.11	2190.12 ±298.43	2034.85 ±90.80

Table 10: Effect of short and long term feeding of coconut oil and corn oil based diets on the levels of markers of oxidative stress in the heart of rats

Markers of oxidative stress	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
TBARS (nM of MDA/ mg protein)	1.50 ±0.11	2.45.38 ±0.14	2.02 ±0.42	1.19 ±0.25	0.79* ±0.25	2.95 ±0.55	2.32 ±.30	1.09 ±0.25	0.76* ±0.17
Protein Carbonyls (nM/mg protein)	6.98 ± 1.13	21.78** ±1.94	18.28* ±1.75	27.94** ±4.90	17.44* ±1.43	17.11* ±3.38	14.19** ±1.24	27.83* ±4.93	18.19* ±3.08

t- test: * significant; ** highly significant

Table 10a Comparison of short and long term feeding of coconut oil and corn oil based diets on the levels of markers of oxidative stress in the heart of rats

Markers of oxidative stress	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
TBARS (nM of MDA/mg protein)	24	2.45^{a} ±0.14	2.02^{a} ±0.42	1.19^{b} ±0.25	0.79^{c} ±0.25	F=5.438; P=0.008*
	30	2.95^{a} ±0.55	2.32^{a} ±0.30	1.09^{b} ±0.25	0.76^{b} ±0.17	F=8.699; P=0. 001*
	P value	0.479	0.584	0.778	0.901	
Protein Carbonyls (nM/mg protein)	24	21.78 ±1.94	18.28 ±1.75	27.94 ±4.90	17.44 ±1.43	F=2.483; P=0.090+
	30	17.11^{b} ±3.38	14.19^{b} ±1.24	27.83^{a} ±4.93	18.19^{b} ±3.08	F=2.964; P=0.047*
	P value	0.257	0.102	0.988	0.507	

Means having different superscripts within a row differ significantly (P<0.05).

Table11: Effect of short and long term feeding of coconut oil and corn oil based diets on the levels of markers of oxidative stress in the brain of rats

Markers of oxidative stress	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
TBARS (nM of MDA/ mg protein)	1.36 ± 0.26	1.19 ±0.25	0.79 ±0.21	1.27 ±0.20	1.10 ±0.25	2.25 ±0.46	2.05 ±0.06	1.34 ±0.22	1.70 ±0.29
Protein Carbonyls (nM/mg protein)	12.13 ± 1.29	35.28** ±3.38	35.55** ±1.51	22.54* ±3.52	29.30** ±1.39	29.25** ±5.34	27.60* ±3.58	17.71 ±2.38	21.44* ±2.68

t- test: * significant; ** highly significant

Table 11a: Comparison of short and long term feeding of coconut oil and corn oil based diets on the levels of markers of oxidative stress in the brain of rats

Markers of oxidative stress	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
TBARS (nM of MDA/mg protein)	24	1.19 ±0.25	0.79 ±0.21	1.27 ±0.20	1.10 ±0.25	F=0.864; P=0.416
	30	2.25 ±0.46	2.05 ±0.06	1.34 ±0.22	1.70 ±0.29	F=1.817; P=0.177
	P value	0.164	0.001**	0.260	0.025*	
Protein Carbonyls (nM/mg protein)	24	35.28^{a} ±3.38	35.55^{a} ±1.51	22.54^{b} ±3.52	29.30^{ab} ±1.39	F=4.584; P=0.013*
	30	29.25^{a} ±5.34	27.60^{a} ±3.58	17.71^{b} ±2.38	21.44^{a} ±2.68	F=4.164; P=0.019*
	P value	0.363	0.153	0.043*	0.026*	

Means having different superscripts within a row differ significantly (P<0.05).

Table 12: Effect of short and long term feeding of coconut oil and corn oil based diets on the levels of antioxidants enzymes in the heart of rats

Antioxidant enzymes (U/mg protein)	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
SOD	12.23 ± 0.62	18.08 ±3.94	19.87* ±0.76	38.05* ±8.35	26.51* ±4.16	20.79* ±3.26	18.93* ±2.72	35.90* ±5.01	30.69* ±3.14
GPx	8.22 ±0.43	7.01 ±1.59	7.75 ±0.90	16.90* ±3.28	25.96* ±9.52	8.73 ±1.47	7.99 ±0.65	19.73* ±4.16	23.10* ±2.61
GSH	10.04 ± 0.90	9.23 ±1.12	13.37 ±1.53	19.72* ±3.03	19.67* ±3.21	9.17 ±0.48	13.82 ±1.66	19.38* ±2.01	21.45** ±1.61
CAT	1.03 ±0.51	0.53** ±0.06	0.56* ±0.12	0.83 ±0.16	0.92 ±0.08	0.21** ±0.03	0.20** ±0.02	0.35** ±0.05	0.28** ±0.02

t- test: * significant; ** highly significant

Table 12a: Comparison of short and long term feeding of coconut oil and corn oil based diets on the levels of antioxidants enzymes in the heart of rats

Antioxidant enzymes (U/mg protein)	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
SOD	24	18.08[b] ±3.94	19.87[b] ±0.76	38.05[a] ±8.35	26.51[b] ±4.16	F=3.170; P=0.047*
	30	20.79[b] ±3.26	18.93[b] ±2.72	35.90[a] ±5.01	30.69[a] ±3.14	F=5.182; P=0.008**
	P value	0.607	0.674	0.830	0.440	
GPx	24	7.01[c] ±1.59	7.75[c] ±0.90	16.90[b] ±3.28	25.96[a] ±9.52	F=11.171; P<0.001**
	30	8.73[b] ±1.47	7.99[b] ±0.65	19.73[a] ±4.16	23.10[a] ±2.61	F=8.844; P=0.001**
	P value	0.447	0.771	0.605	0.554	
GSH	24	9.23[b] ±1.12	13.37[ab] ±1.53	19.72[a] ±3.03	19.67[a] ±3.21	F=4.569; P=0.047*
	30	9.17[b] ±0.48	13.82[ab] ±1.66	19.38[a] ±2.01	21.45[a] ±1.61	F=11.242; P<0.001**
	P value	0.969	0.848	0.929	0.632	
CAT	24	0.53[b] ±0.06	0.56[b] ±0.12	0.83[a] ±0.16	0.92[a] ±0.08	F=3.056; P=0.052+
	30	0.21[b] ±0.03	0.20[b] ±0.02	0.35[a] ±0.05	0.28[ab] ±0.02	F=3.711; P=0.029*
	P value	<0.001**	0.012*	0.018*	<0.001**	

Means having different superscripts within a row differ significantly ($P<0.05$).

Table 13: Effect of short and long term feeding of coconut oil and corn oil based diets on the levels of antioxidants enzymes in the brain of rats

Antioxidant enzymes (U/mg protein)	18 months	24 months				30 months			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
SOD	16.96 ±1.38	13.22 ±2.51	11.85* ±0.74	20.80 ±5.71	19.46 ±1.55	11.31* ±3.26	11.52* ±1.21	19.35 ±1.88	17.23 ±2.27
GPx	8.26 ±0.34	7.08 ±1.35	7.66 ±0.87	13.86* ±2.26	13.20* ±1.30	11.76* ±1.32	7.51 ±0.65	18.08* ±4.16	11.14* ±0.68
GSH	9.31 ±0.49	7.95* ±0.51	9.72 ±0.84	12.08* ±0.72	15.70* ±1.08	7.78* ±0.80	9.05 ±0.72	10.71 ±1.21	11.43* ±0.86
CAT	0.44 ±0.94	0.61 ±0.19	0.33 ±0.08	1.75* ±0.32	0.75* ±0.12	0.57 ±0.16	0.35 ±0.04	1.26** ±0.15	0.71* ±0.10

t- test: * significant; ** highly significant

Table 13a: Comparison of short and long term feeding of coconut oil and corn oil based diets on the levels of antioxidants enzymes in the brain of rats

Antioxidant enzymes (U/mg protein)	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
SOD	24	13.22 ±2.51	11.85 ±0.74	20.80 ±5.71	19.46 ±1.55	F=1.898; P=0.162NS
	30	11.31[a] ±3.26	11.52[a] ±1.21	19.35[b] ±1.88	17.23[b] ±2.27	F=5.761; P=0.005**
	P value	0.504	0.820	0.813	0.435	
GPx	24	7.08[b] ±1.35	7.66[b] ±0.87	13.86[a] ±2.26	13.20[a] ±1.30	F=5.452; P=0.007**
	30	11.76[b] ±1.32	7.51[b] ±0.65	18.08[a] ±4.16	11.14[b] ±0.68	F=6.025; P=0.004**
	P value	0.033*	0.956	0.291	0.190	
GSH	24	7.95[d] ±0.51	9.72[cd] ±0.84	12.08[bc] ±0.72	15.70[a] ±1.08	F=16.959; P=0.001**
	30	7.78[b] ±0.80	9.05[b] ±0.72	10.71[a] ±1.21	11.43[a] ±0.86	F=3.216; P<0.045*
	P value	0.858	0.956	0.353	0.011*	
CAT	24	0.61[b] ±0.19	0.33[b] ±0.08	1.75[a] ±0.32	0.75[b] ±0.12	F=9.469; P=0.001**
	30	0.57[b] ±0.16	0.35[b] ±0.04	1.26[a] ±0.15	0.71[b] ±0.10	F=10.143; P=0.001**
	P value	0.897	0.792	0.202	0.777	

Means having different superscripts within a row differ significantly ($P<0.05$).

Table 14: Effect of short and long term feeding of coconut oil and corn oil based diets on the levels of per cent collagen in the heart of rats

18 months	24 months				30 months			
Rat chow	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**
1.81± 0.18	1.81 ±0.12	1.51 ±0.09	1.42* ±0.06	1.28* ±0.12	1.70 ±0.16	1.50 ±0.06	1.44 ±0.40	1.22* ±0.14

t- test: * significant

Table 14a: comparison of short and long term feeding of coconut oil and corn oil based diets on the levels of per cent collagen in the heart of rats

Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
24	1.81^{a} ±0.12	1.51^{b} ±0.09	1.42^{b} ±0.06	1.28^{b} ±0.12	F=5.163; P=0.008*
30	1.70^{a} ±0.16	1.50^{a} ±0.06	1.44^{a} ±0.40	1.22^{b} ±0.14	F=2.547; P=0.085+
P value	0.567	0.942	0.879	0.748	

Means having different superscripts within a row differ significantly (P<0.05).

Table 15: Effect of short and long term feeding of coconut oil and corn oil based diets on serum lipid profile in rats

Serum lipids (mg/dl)	18 month	24 month				30 month			
	Rat chow	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR
Cholesterol	131.63 ± 9.93	138.60 ±10.68	156.28 ±11.58	122.42 ±9.01	125.92 ±12.30	155.29 ±4.85	142.39 ±11.12	141.13 ±6.47	129.79 ±12.70
HDL-C	45.30 ±5.22	20.56** ±1.50	27.22* ±7.30	30.54* ±2.33	37.23 ±4.99	34.02 ±2.61	38.87 ±5.88	40.45 ±3.67	50.45 ±4.51
LDL-C	51.75 ±10.50	79.44 ±10.39	88.07 ±13.11	56.48 ±11.41	75.98 ±2.13	82.98 ±5.65	64.40 ±1.75	67.70 ±3.91	56.39 ±2.13
VLDL	34.59 ±1.67	37.49 ±6.41	28.25* ±1.86	32.43 ±2.65	20.44** ±1.64	48.46 ±5.65	51.24** ±1.75	39.95 ±3.90	30.17 ±2.13
Triglycerides	172.96 ±8.37	164.37 ±28.57	149.36* ±4.24	136.22 ±20.85	100.15** ±8.44	242.30* ±28.26	256.19** ±8.75	199.75 ±19.52	156.35 ±7.20

t- test: * significant; ** highly significant

Table 15a: Effect of short and long term feeding of coconut oil and corn oil based diets on serum lipid profile in rats

Serum lipids (mg/dl)	Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
Cholesterol	24	138.60 ±10.68	156.28 ±11.58	122.42 ±9.01	125.92 ±12.30	F=1.958; P=0.153NS
	30	155.29 ±4.85	142.39 ±11.12	141.13 ±6.47	129.79 ±12.70	F=1.242 ;P=0.321NS
	P value	0.185	0.406	0.123	0.831	
HDL-C	24	20.56^{b} ±1.50	27.22^{b} ±7.30	30.54^{a} ±2.33	37.23^{a} ±4.99	F=4.058; P=0.021*
	30	34.02^{b} ±2.61	38.87^{b} ±5.88	40.45^{b} ±3.67	50.45^{a} ±4.51	F=2.530; P=0.086+
	P value	0.001**	0.129	0.046*	0.078+	
LDL-C	24	79.44 ±10.39	88.07 ±13.11	56.48 ±11.41	75.98 ±2.13	F=1.554; P=0.232NS
	30	82.98 ±5.65	64.40 ±1.75	67.70 ±3.91	56.39 ±2.13	F=1.026; P=0.402NS
	P value	0.091+	0.301	0.341	0.177	
VLDL	24	37.49^{a} ±6.41	28.25^{ab} ±1.86	32.43^{a} ±2.65	20.44^{b} ±1.64	F=3.837; P=0.025*
	30	48.46^{ab} ±5.65	51.24^{a} ±1.75	39.95^{bc} ±3.90	30.17^{bc} ±2.13	F=6.577; P=0.003*
	P value	0.228	0.001**	0.143	0.001**	
Triglycerides	24	164.37^{a} ±28.57	149.36^{a} ±4.24	136.22^{a} ±20.85	100.15^{b} ±8.44	F=2.247; P=0.114
	30	242.30^{ab} ±28.26	256.19^{a} ±8.75	199.75^{bc} ±19.52	156.35^{c} ±7.20	F=6.225; P=0.004*
	P value	0.081+	0.001**	0.05*	0.001**	

Means having different superscripts within a row differ significantly (P<0.05).

Table 4: Effect of short and long term feeding of coconut oil and corn oil based diet on the levels of mitochondrial inner membrane surface area (m^2/cm^3) in the heart of rats

18 months	24 months				30 months			
Rat chow	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**
13.76 ±1.81	23.67* ±2.72	14.17 ±1.01	26.67* ±3.21	19.50 ±2.69	22.33* ±3.05	17.33 ±1.09	29.67* ±2.78	22.50* ±1.09

t- test: * significant

Table 4a: Comparison of short and long term feeding of coconut oil and corn oil based diets on the levels of mitochondrial inner membrane surface area (m^2/cm^3) in the heart of rats

Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
24	23.67^{ab} ±2.72	14.17^{c} ±1.01	26.67^{a} ±3.21	19.50^{bc} ±2.69	F=5.148; P=0.008*
30	22.67^{ab} ±2.91	17.00^{b} ±1.51	28.50^{a} ±3.13	22.17^{ab} ±2.82	F=3.109; P=0.044*
P value	0.661	0.414	0.879	0.068+	

Means having different superscripts within a row differ significantly (P<0.05).

Table 5: Effect of short and long term feeding of coconut oil and corn oil based diets on the levels of mitochondrial inner membrane surface area (m^2/cm^3) in the brain of rats

18 months	24 months				30 months			
Rat chow	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**	**Coconut oil *ad libitum***	**Corn oil *ad libitum***	**Coconut oil with CR**	**Corn oil with CR**
11.22 ±1.26	20.83** ±1.30	14.17* ±1.11	27.67** ±1.09	19.67* ±2.26	22.33* ±3.05	17.33* ±1.09	29.67** ±2.78	22.50* ±1.09

t- test: * significant; ** highly significant

Table 5a: Comparison of short and long term feeding of coconut oil and corn oil based diet on the levels of mitochondrial inner membrane surface area (m^2/cm^3) in the brain of rats

Age (Months)	Coconut oil *ad libitum*	Corn oil *ad libitum*	Coconut oil with CR	Corn oil with CR	Significance
24	20.83^{b} ±1.30	14.17^{c} ±1.11	27.67^{a} ±1.09	19.67^{b} ±2.26	F=13.353; P=0.001*
30	22.33^{ab} ±3.05	17.33^{b} ±1.09	29.67^{a} ±2.78	22.50^{ab} ±1.09	F=2.953; P=0.050*
P value	0.661	0.414	0.879	0.068+	

Means having different superscripts within a row differ significantly (P<0.05).