MODULATORY ROLE OF RESVERATROL-INDUCED DIETARY RESTRICTION AND ENVIRONMENTAL ENRICHMENT ON NEUROBEHAVIOURAL OUTCOME IN YOUNG HEALTHY MICE

By

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DECLARATION

I declare that the work in this thesis, entitled "Modulatory role of resveratrol-induced dietary restriction and environmental enrichment on neurobehavioural outcome in young healthy mice'' has been performed by me in the Department of Human Physiology, Ahmadu Bello University, Zaria under the supervision of Dr. R. A. Magaji and Dr. A. Mohammed.

The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

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CERTIFICATION

This thesis entitled "MODULATORY ROLE OF RESVERATROL-INDUCED DIETARY RESTRICTION AND ENVIRONMENT ENRICHMENT ON NEUROBEHAVIOURAL OUTCOME IN YOUNG HEALTHY MICE" by Muhammad Mustapha Shehu meets the regulations governing the award of the degree of Master of Human physiology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to Allah (S.W.T), the Lord, Cherisher and Sustainer of the universe.

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ABSTRACT

Dietary Restriction (DR) otherwise known as Caloric Restriction (CR) has been generally defined as consumption of nutritious diet that is 30% to 40% less in calories compared to *ad libitum* diet while Environmental Enrichment (EE) is defined as a sustained and progressive increase in cognitive and sensorimotor stimuli with aggregated voluntary physical activity and complex social interactions. The aim of this experiment was to investigate the modulatory role of Resveratrol induced CR and EE on neurobehavioural responses in young healthy mice. Twenty five mice of both sexes were divided into five groups of 5 animals each: group I served as the control and received carboxymethylcellulose (CMC) 50 mg per kg/day orally, group II animals were maintained on every other day feeding, group III animals received Resveratrol 50 mg/kg, suspended in 10 g/L of (CMC) orally per kg/day. Group IV animals received CMC and kept in an Enriched Environment while group V animals received Resveratrol 50 mg/kg and kept in an Enriched Environment. The treatment lasted for the period of four weeks. On day 26, 27 and 28 of the study period, the animals were subjected to neurobehavioural evaluation of motor coordination, motor strength, learning and memory. Brain and plasma samples were evaluated for lipid profile, lipid peroxidation and antioxidant enzymes. The results showed significant increase ($P < 0.05$) in the transfer latency (acquisition) in the every other day feeding group (69.20 ± 9.03 seconds) compared to the control group (36.80 ± 5.58 seconds). A significant increase ($P <0.05$) was observed in the concentration of low density lipoprotein (LDL) in every other day feeding group (1.72 ± 0.12 mg/dl) and environmental enrichment treatment group (1.73 ± 0.16 mg/dl) when compared to the control group (1.11 ± 0.07 mg/dl). The results obtained also showed significant decrease (P

< 0.05) in MDA concentration in the Resveratrol treatment group kept in EE (1.50 ± 0.05 IU/L) compared to the control (1.84 ± 0.09 IU/L) and GPx activity in the Resveratrol treatment group kept in EE (41.00 ± 2.02 IU/L) compared to the control (59.00 ± 2.85 IU/L). In conclusion, the results obtained demonstrated that Resveratrol induced CR and EE have no effects on neurobehavioural responses coupled with some significant alterations in lipid profile, lipid peroxidation and antioxidant enzymes in young healthy mice over a period of four weeks.

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LIST OF ABBREVIATIONS/SYMBOLS

ABCA	ATP-binding cassette transporter
Aβ	β-amuloid
ADP	Adenosine diphosphate
AMP	Adenine 3',5'monophosphate
AMPA	Amino-3-hydroxy-5-methyl-4-isox-azolepropionate
AMPK	AMP-activated kinase
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
CAT	Catalase
Cl⁻	Chloride anion
CMC	Carboxymethylcellulose
CNS	Central nervous system
Con A	Concanavalin A
COX-2	Cycloxygenase- 2
DAT	Dopamine transporter
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
EODF	Every other day feeding

e-1/e-2	Sub unit of N-methyl-D-aspartate
FOXO	Forkhead box O
GDNF	Glia cell-line derived neurotrophic factor
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GRP	Glucose-regulated protein
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HepG2	Human hepatoblastoma cell line
HSP	Heat shock/stress protein
HSP-70	Heat stress protein 70
HSP-78	Heat stress protein 78
HDL	High density lipoprotein
HPA	Hypothalo-pituitary-adrenal
Hprt	Hypoxanthine phosphoribosyl transferase
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
iNOS	Inducible nitric oxide synthase
K^+	Potassium ion
Km	K maximum
LDL	Low density lipoprotein
LH	Luteinizing hormone
LTP	Long-term potentiation

MAPK	Mitogen activated protein kinase
P38 MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MPTP	1-methyi-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger Ribonucleic Acid
N/L	Neutrophil/lymphocyte
Na^+	Sodium ion
NaCl	Sodium chloride
NF-□B	Nuclear factor □B
NGF	Nerve growth factor
NK	Natural killer
NT-3	Neurotrophin-3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
O_2	Oxygen
O_2^-	Superoxide anion
$OH^·$	Hydroxyl radical
6-OHDA	6-Hydroxydopamine
PGC	peroxisome proliferator-activated receptor γ coactivator
PGE_2	Prostaglandin E_2
PKC	Protein kinase C
pH	Potential for hydrogen
PO	Preoptic nucleus

RNA	Ribonucleic acid
PPAR-γ	Peroxisome proliferator-activated receptor- γ
PGC- 1α	Peroxisome proliferator-activated receptor- γ co-activator 1α
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
Sir2	Silent information regulator 2
Sir $^{-/-}$	Sirtuin receptor knock out
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
T_3	Triiodothyronine
T_4	Thyroxine
TAG	Tryacylglycerol
TCHL	Total cholesterol level
TF	Trophic factors
THI	Temperature-humidity index
TNF	Tissue necrosis factor
TSH	Thyroid stimulating hormone
UDS	Unscheduled DNA synthesis
UNECS	United nations economic and social council commission

CHAPTER ONE

1.0 INTRODUCTION

Dietary restriction (DR), otherwise known as caloric restriction (CR) has been generally defined as consumption of nutritious diet that is 30% to 40% less in calories compared to *ad libitum* diet (Anekonda, 2009). In other words, CR can be defined as a simple reduction in caloric intake in the absence of malnutrition (Baur *et al.,* 2006). Caloric restriction has been demonstrated to possess many health benefits. It provides protection against numerous deadly diseases such as cancer, neurological disorders, and obesity, and is found to be the only reliable treatment that extends life span or causes healthy aging consistently in a multitude of organisms ranging from bacteria to monkeys (Obin *et al*., 2000; Lin *et al*., 2004; Wolf, 2006).

However, the mechanism or extent by which CR extends human life span is not well established (Baur, 2010). The most frequently mentioned effect of CR has been its influence on creating a mild stress in the organism and a typical up-regulation of adaptive mechanisms involving stress proteins accompanied by elevated defence or survival molecules (Sinclair, 2005). This response is similar to the hormetic response (Calabrese and Baldwin, 2001). According to the theory of "hormesis," toxins and pollutants generally show biphasic dose response, where a low dose of toxin triggers a positive, adaptive stress response, which may help an organism sustain much higher levels of toxins which otherwise cause harmful effects (Kyarizis, 2005). Caloric restriction (CR) has also been found to retard several aspects of the aging process in mammals, including age-related mortality, tumorigenesis, physiological decline (Weindruch and Walford, 1988) and the

establishment of age-related transcriptional profiles (Lee *et al.,* 1999). The effects of CR have been tested in different laboratories, and its beneficial effects on nearly every age-related change have been documented (Weindruch and Walford, 1988; Masoro, 2005). The overwhelming conclusion is that CR affects something very fundamental to the aging process, as determined by its effects on disparate age-related diseases, as well as detailed analyses of mortality rates (Yen *et al.,* 2008).

Resveratrol (3, 5', 4-trihydroxystilbene), a natural polyphenolic compound found mainly in the skin of grapes and red wine has previously been shown to extend lifespan in yeast; (*Saccharomyces cerevisiae*), nematode worm (*Caenorhabditis elegans*), fruitfly (*Drosophila melanogaster*), and short-lived fish (*Nothobrachius furzeri*) through a Sirtuins 1 dependent mechanism (Howitz *et al*., 2003; Bauer *et al*., 2004; Wood *et al*., 2004; Viswanathan *et al*., 2005; Valenzano *et al*., 2006). However, the effects and mechanisms of resveratrol in life extension in invertebrates are currently not well established. Studies have shown that mice fed with a high fat diet supplemented with high levels of resveratrol were shown to have extended lifespan compared to the control animals, and several metabolic alterations similar to what is observed with CR, thus, resveratrol is said to be caloric restriction mimetic (Baur *et al*., 2006; Lagouge *et al*., 2006).

Environmental enrichment (EE) is defined as a sustained and progressive increase in cognitive and sensorimotor stimuli with aggregated voluntary physical activity and complex social interactions (Anastasia *et al*., 2009). Abundant experimental evidence shows that EE is beneficial in animal models of schizophrenia, amyotrophic lateral sclerosis, epilepsy, stroke, Huntington's disease and Alzheimer's disease

(Nithianantharajah and Hannan 2006; Laviola *et al.,* 2008). Environmental enrichment also induces neuroprotection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity to mice (Bezard *et al.,* 2003; Faherty *et al.,* 2005) and improves motor function after unilateral 6-OHDA injection in rats (Jadavji *et al.,* 2006). The mechanism of EE neuroprotection is also not well understood, but numerous data suggest that synthesis and release of trophic factors (TFs) may play a crucial role (Nithianantharajah and Hannan, 2006). Environmental enrichment alters the expression of TFs and their receptors in several brain areas (Ickes *et al.,* 2000; Spires *et al.,* 2004) and induces astrogliogenesis (Steiner *et al.,* 2006). In the nigrostriatal system, EE-housed animals show increased brain-derived neurotrophic factor (BDNF) expression in the striatum (Bezard *et al.,* 2003) and glia cell-line derived neurotrophic factor (GDNF) mRNA in the substantia nigra (SN) (Faherty *et al.,* 2005).

1.1 Statement of Research Problem

Numerous works have been done on CR and its mimetics such as resveratrol, sulforaphane, etc (Anekonda, 2009; Baur, 2010), and much attention have been lavished on the effect of CR and EE on the aged and age related disorders to the neglect of its possible beneficial role in young animals. The beneficial role of CR on the nervous system have been extensively studied in aged animals (Deng *et al*., 2009). Moreover, several reports showed the physiological, neurological, and behavioural effects of CR and EE in rodents, but the majority of efforts have been focused on the beneficial effects of CR and EE on aging and life span in the past decades, and little attention has been paid to the physiological effects of CR and EE especially in young healthy animals. Furthermore, there are controversial

reports on the effects of CR on cognition in young animals. Some investigators demonstrated that CR enhances learning and memory (Hashimoto and Watanabe, 2005), while others reported that CR had negative effects on cognitive functions (Yanai *et al*., 2004). Although results obtained from some studies shows that resveratrol in high doses increases lifespan in invertebrate and prevent mortality in aged mice fed a high fat diet (Barger *et al.,* 2008) but the question is whether resveratrol can produce the same physiological effects on neurobehavioural outcome in young healthy animals over a short period of time.

1.2 Justification

Studies have shown that CR and its mimetic such as resveratrol improve motor coordination and increase longevity in mice (Baur *et al.,* 2006; Lagouge *et al.,* 2006). This may be related to the improvement in endurance and strength (Baur, 2010). Resveratrol has also been shown to prevent cognitive decline in a number of disease models, and to reduce neurodegeneration *in vivo* (Kim *et al*., 2007). Environmental enrichment has also been found to improved spatial memory in aged mice with no significant effect in young and middle age mice (Harburger *et al*., 2007). Collectively, the available data suggest that both CR and EE exert similar beneficial effects on neurons in the brain, and share a mechanism involving increased neurotrophic factor production (Mattson *et al*., 2001). However, there is paucity of information elucidating the physiological effects of resveratrol induced CR and EE on neurobehavioural responses in young healthy mice. Therefore, this study intends

to determine the modulatory role of resveratrol induced dietary restriction and environmental enrichment on neurobehavioural outcome in young healthy mice over a short period of time.

1.3 Hypothesis

Resveratrol-induced DR and EE have no significant effect on neurobehavioural outcome in young healthy mice.

1.4 Aim and Objectives

The aim of the study was to determine the neurobehavioural responses of young mice to resveratrol-induced caloric restriction and environmental enrichment.

The specific objectives of the study include:

1) To evaluate the effect of resveratrol-induced CR and EE on neurobehavioural parameters.
2) To determine the effect of resveratrol-induced CR and EE on lipid profiles.
3) To determine the effect of resveratrol induced CR and EE on lipid peroxidation and oxidative stress markers.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Background of Caloric Restriction

Extending length of life and slowing or even reversing aging has been a human preoccupation throughout history (Birren, 1996). Over the years, a multitude of different substances have been claimed to prevent aging and extend life. Manipulating lifestyle has been viewed as a means of slowing aging and increasing longevity. Indeed, a tremendous increase in human life expectancy in the developed nations occurred during the 20th century due to improved hygiene as well as other environmental and lifestyle factors and most of this increase was not due to retarding aging rather, advances in nutrition, medicine, living standards, and public health which enabled the population to age by preventing early death (Masoro, 2002).

However, studies carried out early in the 20th century on caloric restriction reported that retarding the growth of rats by nutritional means resulted in an increase in length of life of the animals (Osborne *et al*. 1917). Five years later, Robertson and Ray (1920) found that the rate of growth of mice positively correlates with length of life. Similarly, in a study carried out by McCay *et al.* (1929) on growth retardation in trout due to a low protein diet, they observed that the fish with retarded growth outlived those that grew normally. In 1930, experiments were carried out in white rats to determine the effect of retarded growth on life span in which they allowed one group of rats to grow to maturity at a normal rate and slowed the growth of two other groups by restricting their calorie (McCay *et al*., 1935). The restricted groups did not grow at all and, indeed, began to fail, whereupon the amount of

food was increased just enough to keep the animals alive. Thus the restricted groups underwent long periods of no growth interspersed with spurts of growth. One of the two groups was maintained on the restricted diet regimen for 700 days and the other for 900 days, after which all were allowed to eat *ad libitum.* While the mean length of life of the rats that grew normally was about 600 days, many of the rats on the growth-retarding dietary regimes lived much longer. This first study had involved the reduced intake of all nutrients. In a second study, McCay *et al*. (1939) decreased the intake of calories but not of protein, minerals, or vitamins; and found a similar result to their first study and concluded that the increase in length of life was due to the decrease in rate of growth.

However, most of the research work on CR from the 1940s through the 1960s focused on the age-associated diseases of rats and mice. Saxton and Kimball (1941) reported that CR retarded the progression of chronic nephropathy in rats, where as Saxton *et al*. (1944) found that CR inhibited the development of leukemia in mice. Other investigators carried out extensive research on the influence of CR on longevity and age-associated diseases of rats, with an emphasis on neoplasia and the possible role of specific nutrients (Ross and Bras 1965). They concluded that the reduction in caloric intake increased longevity and retarded disease. Moreover, Berg and Simms (1960) undertook similar studies with rats, in which they proposed that reduction in body fat is responsible for the life extension and inhibition of age-associated diseases seen with CR.

Numerous works are been carried out on caloric restriction and its mimetics on various species ranging from invertebrate to rhesus monkeys and much attention and resources are

spent in order to unveil the probable mechanisms by which CR mediates its physiological responses in aging, diseases and longevity. Yet, no uniform conclusion has been reached.

2.2 Age of Initiation of Caloric Restriction

Most CR studies have been initiated in young rats and mice. However, it has become clear that CR need not begin with young growing rodents to markedly affect longevity (McCay *et al.,* 1939). Weindruch and Walford (1982) showed that CR can be initiated in mice at middle age and still achieve a significant life-extending action. They gradually established CR in 12-month-old mice by decreasing about 30% of their caloric intake and observed that CR increased the maximal life span of these mice by 10-20%. However, with regard to the rat, studies have shown that CR does not increase longevity when initiated at advanced ages. Study has shown that CR begun at 18 months of age in Long-Evans rats did not affect life span nor does it affect longevity when initiated at 18 or 26 months of age in rats (Lipman *et al.,* 1995).

2.3 Cellular and Molecular Functions in Caloric Restriction

Caloric restriction has been found to influence many cellular processes, causing some functions to change almost immediately and slowing age-associated changes in others. One or more of these cellular and or molecular effects is or are likely to play key roles in CR's anti-aging and life-extending activities.

2.3.1 Stability of the nuclear genome

It has long been theorized that nuclear genomic instability is the basis of senescence and genomic DNA damage and mutations are known to increase with age (Bohr and Anson,

1995). Caloric restriction has been found to be associated with the attenuation of certain types of age-associated DNA damage (Haley-Zitlin and Richardson, 1993). It has also been found to slow the age-associated increase in genomic mutations in lymphocytes of mice and rats based on the hypoxanthine phosphoribosyl transferase *(hprt)* gene locus test (Aidoo *et al.,* 1999). In a study carried out by Weraachakul *et al.* (1989) using unscheduled DNA synthesis (UDS) in cells exposed to ultraviolet light as a measure of DNA repair ability; they found that hepatocytes and kidney cells isolated from male rats exhibited a slowing of repair as the age of the rat increased. At all the ages studied, cells from rats on CR had a higher rate of UDS than the control rats of the same age. Other investigators have also concluded that CR enhances DNA repair using various rats and mouse cell types (Srivastava and Busbee, 1992; Tilley *et al*., 1992). Under nutrition has also been found to increase DNA repair in human peripheral lymphocytes (Rao *et al*., 1996). Moreover, some investigators studied the effect of age and CR on nucleotide excision repair, comparing the transcribed albumin gene of isolated hepatocytes and non transcribed DNA of these cells. They found that the rate of repair of the transcribed strand of albumin DNA was 40% less in hepatocytes from 24-month-old rats than 6-month old rats, and concluded that CR prevented this age-associated decrease (Guo *et al*., 1998).

2.3.2 Gene expression

Changes in gene expression have also been viewed as a factor underlying senescence and most studies used the level of mRNA transcript of a gene as an index of the expression of that gene (Vijg, 1996). However, with increase in age, the mRNA transcript level has been found to increase for some genes, decrease for others, and does not change for many and

CR in rodents has been found to blunt the age change in the level of many mRNA transcripts, affecting both those that increase and decrease with increase in age (Van Remmen *et al.,* 1995). Using Reverse Transcription Polymerase Chain Reaction (RT-PCR) analyses, some investigators report that CR resulted in an upregulation of cytoskeleton protein encoding genes and a decrease in the expression of genes involved in mitochondrial bioenergetics in the vastus lateralis muscle of 20-year-old rhesus monkeys (Kayo *et al*., 2001).

Furthermore, Weindruch *et al.* (2001) suggested that transcriptional patterns in muscle indicate that CR retards the aging processes by causing a metabolic shift toward increased protein turn over and decreased macromolecular damage. In another study by Lee *et al.* (2000), it was observed that CR partially or completely prevented many of the perceived age changes in gene expression and appeared to selectively attenuate age changes in genes encoding inflammatory and stress responses. It was however, suggested that aging influences the expression of genes in four major physiologic classes: stress response, biosynthesis, protein metabolism, and energy metabolism and CR appeared to completely or partially suppress the age changes in the expression of 84% of these changes (Weindruch *et al*., 2002).

Using the array technology for the study of gene expression in the liver of mice, some investigators reported age-related increases in the expression of genes associated with inflammation, cellular stress, and fibrosis and decreases in the expression of genes associated with apoptosis and DNA replication and concluded that both long as well as short-term CR reverse most of these age changes in hepatic gene expression (Cao *et al*.,

2001). A conflicting result was reported by Teillet *et al*. (2002) where they found that CR does not influence genes with age-associated changes in expression in the liver of rats, but rather produces a new profile of gene expression that includes genes related to lipid metabolism as well as those in the energetic pathways.

Caloric restriction was found to increase the level of mRNA transcript for superoxide dismutase, catalase, and glutathione peroxidase, and their enzymatic activities in rat liver (Rao *et al.,* 1990). It was reported to cause a decrease in calcitonin-gene-related-peptide and its mRNA level in rat thyroid gland (Salih *et al*., 1993). Although it was reported that aging does not alter the hepatic expression of glucose-regulated protein 78 (GRP78) in mice, CR decreases its expression (Spindler *et al*., 1990). It acts by destabilizing GRP78-mRNA, thereby decreasing its level in liver cells (Tillman *et al*., 1996). It also decreases the expression of glucose-regulated protein 94 (GRP94) and its mRNA in mouse liver (Spindler *et al.,* 1990).

Research carried out on the influence of age and CR on the induction of heat shock protein 70 (hsp70) in cells was promising. This protein, which is induced in cells by heat as well as other cellular stressors, protects cells from the damaging actions of stressors. With increasing age, there is a decrease in the ability of heat stress to promote the induction of hsp protein and its mRNA in rat hepatocytes. Caloric restriction was found to increase the induction of hsp protein and the level of its mRNA in hepatocytes of young and old rats (Heydari *et al*., 1993). However, it is likely that CR enhances the transcription of the *hsp 70* gene by altering the molecular structure of the hepatic HSF1 protein (Masoro, 2002). Caloric restriction was found to blunt the heat-stress induction of proteins hsp 27, 70, and

90 in rat hypothalamus that is usually attenuated with increasing age (Aly *et al*., 1994). It influences hepatic gene expression so as to increase the enzymatic capacity for gluconeogenesis and the disposal of by-products of protein catabolism, as well as decreasing the enzymatic capacity for glycolysis (Dhahbi *et al.,* 1999).

2.3.3 Mitochondrial function

Mitochondria are known to play a central role in cellular energetic, cell death as well as cellular signalling pathways. Each mitochondrion has its own genome that codes for key mitochondrial proteins and many authors believe that alterations in mitochondria and their genomes are intimately involved in organismic aging (Beckman and Ames, 1998). It has been reported that CR increases the respiratory control index in isolated mouse mitochondria, which led to the suggestion that CR increases the efficiency of mitochondrial electron transport and oxidative phosphorylation (Weindruch *et al*., 1980). It prevents the age-associated increase in state 4 (resting) respiration that occurs in mitochondria from *ad libitum*-fed animals as well as it lowers the rate of mitochondrial generation of superoxide and hydrogen peroxide that increases with age (Sohal *et al.,* 1994).

Some investigators observed that CR opposes all age-associated changes in the mitochondrial electron transport chain (ETC) in mouse gastrocnemius muscle (Desai *et al*., 1996) whereas others reported a decrease in the amount of complex I and III of the ETC chain and an increase in the Km of complex III (Feuers, 1995). Based on these, it was suggested that these alterations underlie the age-associated increase in mitochondrial generation of reactive oxygen molecules, and by attenuating these alterations in the ETC, CR decreases the formation of reactive oxygen molecules (Feuers, 1995). Evidences

indicating how CR influences mitochondria in the intact organism reports that CR reduces the level of mitochondrial lipid and DNA damage in the liver and skeletal muscle of old rats (Lass *et al*., 1998). It also attenuates the age-associated increase in mitochondrial DNA deletions in rat liver and skeletal muscle (Kang *et al.,* 1998).

2.3.4 Membrane structure and function

It was proposed that the anti-aging action of CR may be due to the protection of the physiological properties of cellular membranes and CR has been found to retard many of the age-associated changes in these membranes (Yu, 1995). With increasing age, rat membranes exhibit a change in the composition of their lipid components, with polyunsaturated long-chain fatty acids, such as 20: 4, 22: 5, and 22: 6, replacing 18: 2 and 18: 3; CR has been shown to attenuate this age change (Merry, 2000). Thus CR was found to reduce the susceptibility of membrane lipids to peroxidation, and at the same time maintains membrane fluidity (Pieri, 1997). CR has also been found to lessen the age-associated decrease in fluidity of mitochondrial membranes in rat liver, brain, and heart respectively (Choi and Yu, 1995; Lee *et al*., 1999). It was also reported that CR prevents the age-associated increase in the level of dolichol (which is a lipid that resides in cellular membranes) in the liver of male Sprague-Dawley rats suggesting the beneficial role of CR on the functioning of cellular membranes (Cavallini *et al*., 2002).

2.3.5 Protein structure, function, and turnover

Proteins play an important role in an organism's living processes and the functioning of protein species depends on their three-dimensional structure most of which are modified post translationally with increase in age (Gafni, 2001). These modifications are either

covalent (oxidation, glycation, glycoxidation, deamidation) or non-covalent (conformation, aggregation). However, altered functional characteristics often result from such modifications and it is not known whether these changes in protein structure are the cause or the result of aging (Masoro, 2002).

Caloric restriction has been found to be particularly effective in preventing oxidative damage of proteins in some regions of mouse brain such as the striatum, cerebellum, midbrain, and cortex (Dubey *et al*., 1996) and causes a decrease in age associated increase in the carbonyl content of proteins in the cerebellum and cortex of both genders and in the hippocampus of the females rats alone (Askenova *et al*., 1998). It has also been found to prevent the age-associated increase in the carbonyl content of mitochondrial proteins in mouse skeletal muscle (Lass *et al*., 1998). An increase in carbonyl content and decreased sulfhydryl content of proteins in the heart and brain of mice fed *ad libitum* was observed, and CR was found to attenuate these changes (Forster *et al.,* 2000).

Caloric restriction has also been found to attenuate the protein carbonyl content in the liver of *ad libitum-fed* rats that usually increased late in life and also decreases the carbonyl content of proteins in liver mitochondria of 30-month-old rats (Goto *et al*., 2002). It decreases the glycation of haemoglobin, plasma proteins skin and aorta collagen in Wistar rats (Cefalu *et al*., 1995), as well as retards the accumulation of advanced glycation end products in the skin, tail tendon, and aorta collagen of male mice (Reiser, 1994).

However, the accumulation of damaged proteins with age could be due to the decrease in protein degradation (proteolysis) with increasing age and the proteosome, which is a multi

enzymatic proteolytic complex, is known to play a role in the degradation of damaged proteins (Friguet *et al*., 2000). Caloric restriction was observed to enhance proteolysis which may play a role in decreasing the level of damaged proteins (Ward, 1988).

It has been demonstrated that the turnover of body protein is increased by CR in rats and mice which is sustained until old ages (Dhahbi *et al*., 2001). Studies have shown that CR of a few months duration increases protein turnover in old rats (Goto *et al*., 2002). But when old rats were maintained on long-term CR, protein synthesis by liver and kidney cells was found to be greater than that of rats of the same age on an *ad libitum* dietary regime (Ricketts *et al.,* 1985). These increased in protein turnover may contribute to CR's anti-aging action by decreasing accumulation of damaged proteins (Weindruch *et al*., 2001).

2.3.6 Cell proliferation

Decrease in the number muscle fibres underlies some of the functional deterioration of the aging phenotype and the age-associated increase in the number of cells (e.g., hyperplasia and neoplasia) also results in dysfunction (Wolf and Pendergrass, 1999). For optimal function to be obtained, the generation of new cells (cell proliferation) and the death of existing ones should be maintained in a state of equilibrium. It was reported that CR decreases the proliferation of many cell types in young mice (Lok *et al*., 1990). On the contrary, when old mice maintained on a long-term CR regimen, proliferation of hepatocytes, kidney tubule cells, and pancreas acinar cells increases to levels close to those observed in young *ad libitum*-fed mice (Wolf *et al*., 1995). Thus, CR is said to maintain the youthful potential for cell proliferation. Caloric restriction also preserves the proliferation

capacity of mouse lens epithelial cells provides protection against age-associated increase in intestinal and colon cancer (Li *et al.,* 1997).

2.3.7 Apoptosis

Apoptosis refers to genetically programmed cell death, which serves to carry out important physiological functions. With aging, excessive apoptosis can cause an undesirable decrease in the number of a particular cell type, such as the age-associated loss of neurons in particular regions of the central nervous system (Masoro, 2000). On the other hand, too little apoptosis can lead to the accumulation of damaged cells, hyperplasia, and/or neoplasia and CR has been hypothesized to elicit its anti-aging action due to the upregulation of apoptosis (Warner *et al*., 1995; Zang and Herman, 2002). Caloric restriction has also been found to promote apoptosis in the liver, small intestine and colon of aging mice (Holt *et al*., 1998). However, some investigators reported that mild CR and life-long exercise attenuate the age-related increase in oxidative damage and apoptosis (Wohlgemuth *et al*., 2010).

2.3.8 Cellular signal transduction

Several changes occur in receptors and other components of signalling pathways and the influence of age and CR on cellular signal transduction in the central nervous system has been the subject of several studies. There have been conflicting reports on the effect of CR restriction on dopamine receptors in rodents. Some investigators report that CR retards the age-associated decrease in dopamine receptors in the corpus striatum of the rat while others reports that CR does not attenuate this loss in mice (Roth *et al.,* 1984; May *et al.,* 1992). In the cholinergic system, CR was found to increase the level of muscarinic binding sites in

the striata of old rats (London *et al*., 1985). The decreased ability of a cholinergic and dopaminergic agonist to promote phosphoinositide hydrolysis at advanced age in the cortex and corpus striatum of young and old rats was found to be attenuated by CR (Undie and Friedman, 1993). Caloric restriction was also found to prevent the age-associated decline in basal extracellular signal-regulated kinases (ERK) phosphorylation and kinase activity, and it attenuates the reduction in p38 MAPK activity (Zhen *et al.,* 1999).

Caloric restriction has also been found to modulate cellular signalling in the growth hormone-IGF-1 axis of rodents (Sonntag *et al.,* 1999). It delays the progressive impairment in the growth hormone receptor signal transduction pathway that usually occurs with age in mice (Xu and Sonntag, 1996a). It also blunts the age-associated decrease in growth hormone receptor phosphorylation as well as decreases both growth hormone-induced activation and nuclear translocation of Stat-3 (Xu and Sonntag, 1996b). In addition, CR increases the density of type 1 IGF-1 receptors in liver, heart, and skeletal muscle (D'Costa *et al*., 1993). It was also found to retard the age associated loss of beta-adrenergic receptors in rat lung and prevents the age-associated decrease in isoproterenol- and epinephrine-stimulated adenylate cyclase activity (Scarpace and Yu, 1987). It also partially prevents the activation by concanavalin A of Mitogen-activated protein kinase (MAPK) and calcineurin that usually decreases in rat splenic T cells with increase in age (Pahlavani and Vargas, 2000).

2.4 Systemic Effects of Caloric Restriction

Caloric restriction has been found to affect various systems of the body as well as integrated organismic functions. Some of the effects of CR on body systems include the modulation of age-associated changes.

2.4.1 Body composition

The effects of CR on body composition and metabolic function were numerous. Caloric restriction has been observed to reduce body weight in different animal species primarily due to a decrease in total body fat mass (Colman *et al*., 1998). The life span effects of a 40% reduction in food intake on body weight in a spectrum of rat and mouse strains reduced the body weight of the animals to about 40% below that of the animals fed *ad libitum* (Sprott and Austad, 1996). It also reduced the body weight of rhesus monkeys maintained on CR (Colman *et al*., 2009; Mattison *et al*., 2012).

Caloric restriction markedly reduces the body fat content of rats and mice due to a decrease in both the size and number of adipocytes in the fat depots (Garthwaite *et al*., 1986). Plasma leptin concentration has been observed to be reduced by CR and this decreased level plays a key role in the anti-aging action of CR (Shimokawa and Higami, 2001).

Early study on CR by McCay *et al.* (1935) revealed that CR has a harmful effect on bone either due to the extreme level of food restriction in the study, or the likelihood of severe calcium deficiency. Kalu *et al*. (1984) reported that CR prevents senile bone loss and the marked increase in circulating parathyroid hormone, both of which occur late in life in male F344 rats. Long-term CR reduces the bone mass in rhesus monkeys which appears to be

secondary to reduction of lean body mass (Black *et al*., 2001). It attenuate the age associated decline in muscle mass (sarcopenia) and maintain the lean muscle mass of rhesus monkeys on CR regiment when compared to the control (Colman *et al*., 2008; Colman *et al*., 2009).

2.4.2 Nervous system

Over the years, research on the effect of CR on the nervous system has yielded conflicting results. Some investigators have shown that CR markedly retards age-associated changes, while other investigators reported contrary findings. Several studies reported that CR retards or delays age-associated decline of maze and spatial memory performance of rats and mice (Algeri, 1991). It was observed that retardation of the age-associated impairment in spatial memory performance in mice correlates with the influence of CR on the densities of ~-amino-3-hydroxy-5-methyl-4-isox-azolepropionate (AMPA) receptors in the frontal and parietal cortices and the CA1 and CA3 regions of the hippocampus, as well as the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors in the frontal cortex and CA1 region of the hippocampus (Magnuson, 1998). Therefore, CR's enhancement of learning and memory in old mice appears to relate to an increase in expression of the mRNA of the e-1 subunit of the NMDA receptor and of the mRNA of the e-1 or e-2 subunit (Magnuson, 2001).

CR also protects rodents against age-associated deficits in long-term potentiation and retards the age-associated decline in sensorimotor coordination in mice. It improves performance of coping with an avoidance-learning problem in aged mice and motor learning in rats (Eckles-Smith *et al.,* 2000). It prevents the age associated loss in the ability

of rats to both learn and utilize memory (Pitsikas *et al*., 1990). It was also observed that CR does not retard age-associated loss in sensorimotor function as well as influence cognitive decline in male rats (Markowska, 1999). On the other hand, some investigators found that long-term CR improves sensorimotor functions and place discrimination aspect of cognition in old female rats (Campbell and Gaddy, 1987). Moreover, it was observed that motor function coordination declines with increasing age in mice, and CR attenuates this age change by increasing locomotor activity in both rats and mice (Duffy *et al.,* 1997).

Caloric restriction has also been found to modulate age changes in nervous system morphology. In rats, CR was found to protect neurons of the myenteric plexus of the small intestine that undergo extensive age-associated cell death (Cowen *et al.,* 2000). It was also found to decrease the age-associated loss in dendritic spines in the parietal cortex of rats. CR decreases the deposition of lipofuscin in the neurons of the hippocampus and frontal cortex of mice (Moroi-Fetters *et al*., 1989). Studies have shown that CR retards the age-associated decrease in thickness of retinal layers and the decrease in density of retinal cells in rats (Obin *et al*., 2000).

The physiology and biochemistry of neurons and glial cells were found to be influenced by caloric restriction. Caloric restriction protects the synaptic function of neurons by increasing the resistance of synaptic terminals to oxidative impairment of membrane glucose and glutamate transport, enhancing mitochondrial function as well as increasing the local levels of HSP-70 and GRP-78 (Guo *et al.,* 2000). Decrease in creatine kinase activity in the cerebral cortex, cerebellum, and hippocampus of rat and increases the activity of

glutamine synthetase have also been reported (Askenova *et al*., 1998). Life-long CR was found to enhance the rotational and stereotypic responses of old Wistar rats to intrastrial dopamine, amphetamine, and atropine, which suggests that CR preserves the functional integrity of the striatal dopaminergic/cholinergic circuits during aging (Joseph *et al.,* 1983).

Research has revealed that CR protects rodents from experimentally induced neurodegeneration caused by various toxins such as the administration of 1-methyl-4-phenyl-l,2,3,6-tetrahydropyridine (MPTP) which simulates Parkinson's disease in rodents. It was observed that CR protects the dopaminergic neurons of the substantia nigra from the toxic effects of MPTP, specifically MPTP-induced deficits in motor function (Duan and Mattson, 1999).

The hippocampus and striatum of rats are particularly prone to damage with increasing age and CR was found to increase the resistance of hippocampal neurons to excitotoxin-induced degeneration, and protects striatal neurons from degeneration induced by mitochondrial toxins, 3-nitropropionic acid and malonate in young rats (Masoro, 2002). In addition, it also increases the resistance of the young rats to kainic acid-induced deficits in water-maze learning and memory tasks, and at the same time, enables the animals to withstand the impairment in motor function caused by 3-nitropropionic acid (Bruce-Keller *et al*., 1999).

In mice models with a presenilin mutation, CR was found to decrease the death of hippocampal neurons and increase the level of brain-derived neurotrophic factor (BDNF) in hippocampal and cortical neurons (Duan *et al*., 2001). Some investigators proposed that CR's beneficial actions on brain are due to its ability to stimulate the expression of

neurotrophic factors, stress proteins, and stabilizing cellular calcium homeostasis thereby protecting neurons by suppressing reactive oxygen molecule production (Mattson *et al*., 2003).

2.4.3 Locomotion and skeletal muscle

Several studies have demonstrated that aging alters locomotor activity both in humans and animal models. Yu *et al*. (1985) showed that rats maintained on CR for a long period of time, exhibited an increase in locomotor activity when compared to *ad libitum* fed rats. It was also observed that when provided with a running wheel, rats on a CR regimen were less active than those fed *ad libitum* when young, but more active at older ages (Goodrick *et al.,* 1983). Subsequently, some investigators observed that male rats on a CR regimen were found to be more active throughout life than *ad-libitum* fed rats (McCarter *et al.,* 1997). However, studies on the effect of caloric restriction on locomotor activity in rhesus monkeys have yielded conflicting results. Some authors reported that CR improves motor activity while others state that CR has negative effect on motor functions (Moscrip *et al.,* 2000).

The loss of muscle mass and strength is a hallmark of the aging phenotype in both humans and animal models and caloric restriction was found to delay the age-associated skeletal muscle atrophy in mice (Bronson *et al.,* 2000; Colman *et al*., 2008). With increasing adult age, the mitochondria of mouse skeletal muscle exhibit lipid and protein oxidative damage and Caloric restriction was reported to attenuate this damage and decreases the generation of superoxide anion free radicals by the mitochondria (Lass *et al.,* 1998).

2.4.4 Cardiovascular system

A loss in responsiveness to adrenergic stimulation is a major age associated alteration in the functioning of the human heart. Enhancement of responsiveness of rat heart to adrenergic stimulation with increase in age by CR was reported by Kelley and Herlihy (1998). Long time caloric restriction has been found to prolong cardiac contraction and relaxation times and increases the sensitivity of the heart to isoproterenol in male rats (Klebanov *et al.,* 1997). Furthermore, CR also attenuates the age-associated decrease in the ability of the cardiac synaptosomes (adrenergic nerve terminals) of male rats to release norepinephrine and increases the norepinephrine content of cardiac synaptosomes in adult rats by enhancing the ability to sequester norepinephrine (Snyder *et al*., 1998). It also prevents the reduction in myocardial contractility that occurs between 18 and 24 months of age in male rats fed *ad libitum* (Chang *et al.,* 2001).

Caloric restriction was also found to enhance the age-associated shift in the cardiac myosin isozyme profile from the fast V_1 to the slow V_3 isoform (Klebanov and Herlihy, 1997). Long-term Caloric restriction has also been found to decrease the mean arterial blood pressure of old male rats and enhances the baroreflex of both young and old rats (Thomas *et al.,* 1993). Caloric restriction inhibits the age-associated loss of force generation of aortic smooth muscle in rats and attenuates the age-associated increase in oxidative damage in aortic cells of mice (Guo *et al.,* 2001).

2.4.5 Endocrines and metabolism

Contrary to widely held view that caloric restriction retards aging process by decreasing the intensity of energy metabolism, some investigators reports that the anti-aging action of

caloric restriction including the increase in longevity, in male F344 rats can occur without a decrease in metabolic rate per unit of metabolic mass (McCarter and Palmer, 1992). Following long-term studies of CR in rhesus monkeys, researches have shown a decrease in the metabolic rate per kg of lean body mass following the initiation of CR, but rose to that of the non restricted monkeys as the CR regimen was continued while others reported a decreased in the metabolic rate per kg lean body mass after ten years on the dietary regime (Ramsey *et al.*, 1997; De Laney *et al*., 1999).

Caloric restriction also influences protein metabolism by enhancing both hepatic protein synthesis and degradation over most of the life span (Ward, 1988). However, an increased rate of protein turnover was said to play a significant role in slowing the rate of aging by preventing cellular accumulation of damaged proteins (Tavernakis and Driscoll, 2002).

Caloric restriction was reported to markedly decrease plasma triglyceride levels in rats and rhesus monkeys and also delays the age-associated increase in plasma total cholesterol, HDL-cholesterol and phospholipid levels in rats (Verdery *et al*., 1997). However, it does decrease the molecular weight of LDL and the binding of LDL to arterial proteoglycans and lowers the plasma concentration of lipoprotein (a) in male but not in premenopausal female rhesus monkeys (Edwards *et al*., 2001). It was also found to increase the level of HDL2b in rhesus monkeys, the sub-fraction of HDL associated with the protection from atherosclerosis in humans (Verdery *et al.,* 1997).

On the endocrine functions, caloric restriction was shown to lower the anterior pituitary levels of mRNA coding for TSH in young male rats and cause a decrease the plasma levels

of thyroxine (T4) and triiodothyronine (T3) in Sprague-Dawley rats (Han *et al*., 1998). Reports on the effect of caloric restriction on the plasma level of T3 and T4 in rhesus monkeys have been conflicting. Some investigators reported that CR decreases the plasma level of T4 with no significant change in T3 (Ramsey *et al.* 2000) while others reported a contrary finding (Mattison *et al*., 2002). Caloric restriction has also been found to attenuate the age associated increase in the expression of the glucagon receptor in liver and decreases the age-associated increase in glucagon-induced hepatic accumulation of c-AMP in female rats (Teillet *et al*., 2002).

The peak plasma level of melatonin was found to decrease with increasing age in rhesus monkeys, and a 30% decrease in caloric intake over a 12-year-period was found to prevent the age-associated decrease (Roth *et al.,* 2001). Rhesus monkeys were also found to exhibit an age-associated decline in the plasma concentration of dehydroepiandrosterone sulfate (DHEAS), and caloric restriction was reported to slow this decline (Lane *et al*., 1997). These findings and those of several rodent studies have led to the suggestion that decreased levels of DHEA and DHEAS may play a significant role in senescence (Nelson, 1995).

2.4.6 Reproduction

The effects of caloric restriction on reproduction have been widely explored. Caloric restriction has been found to delay the onset of puberty in female rats maintained on a 50% CR since weaning as well as reduces oocyte atresia, resulting in an increase in the number of oocytes per ovary at 42 days of age (Keenan *et al*., 1995). However, CR does not affect the age-associated decrease in male fertility maintained on a 30% caloric restriction for 17 weeks (Chapin *et al*., 1993).

The effects of caloric restriction on reproductive functions in female rats was said to be mediated via increase in the mean plasma luteinizing hormone (LH) concentration and its secretion by the adenohypophysis (McShane and Wise, 1996). It was further suggested that CR's effect on the neuropeptide Y-pituitary axis may be involved and it was observed that the expression of neuropeptide Y, which augments the response of the adenohypophysis to gonadotropin-releasing hormone, was found to be greater in the hypothalamus of rats and sheep on caloric restriction than fully fed animals (McShane *et al.,* 1993). On the other hand, some investigators reported different findings on the effect of long-term CR on reproductive hormones in sexually mature female rats, namely, decreased amplitudes of peak concentrations of LH and estradiol-17fi; decreased serum progesterone level, decreased serum prolactin level; and increased level of serum FSH (Holehan and Merry, 1985). In male rhesus monkeys, it was observed that long-term CR does not influence plasma testosterone levels (Roth *et al.,* 1993).

2.4.7 Body temperature regulation

The effect of caloric restriction on body temperature regulation has been controversial. Some researchers reported that the decrease in body temperature elicited by caloric restriction may play a role in mediating the anti-aging function of CR while others indicates that the decrease in body temperature plays, at most, a minor role in CR's anti-aging action (Masoro, 2002). Lowering the environmental temperature of several species of poikilotherms and thus their body temperature has been found to increase their life span (Finch, 1990). Also, housing mice at 30^0C prevents most of the CR-induced decrease in

body temperature, and attenuates some of the physiological effects of CR, such as slowing cellular proliferation and lymphopenia (Koizumi *et al*., 1993).

2.4.8 Immune function

Many findings have suggested that caloric restriction slows the age associated deterioration of immune function in mice and rats. Caloric restriction was found to influence the production of immunoglobulin and offsets age changes in immunoglobulin production (Jolly *et al*., 1999). However, much effort has been focused on determining the cellular and molecular basis of the effects of CR on age changes in the immune function of mice but the ability of CR to prevent the age-associated decrease in mouse T-cell proliferation does not appear to be linked to enhanced efficiency of transmembrane signaling (Grossman *et al.,* 1990).

2.4.9 Wound healing

The effect of caloric restriction on wound healing has been debatable. While some investigators report that increasing age has been found to impair wound healing in mice, rats and rhesus monkeys and long-term CR does not retard this impairment (Roth *et al.,* 1997), others indicate that CR maintains the youthful capacity to heal wounds into old age, but this effect was manifested only with an abundant intake of energy just prior to and following the wounding (Reed *et al*., 1996). The increased capacity for wound repair appears to relate to an enhancement of collagen biosynthesis and cell proliferation when the caloric restriction animals were provided an abundant dietary source of energy (Masoro, 2002).

2.5 Effect of Caloric Restriction on Age-Associated Disorders

Several studies on a various mouse and rat strains have shown that caloric restriction delays the onset and slows the progression of different age-associated diseases.

2.5.1 Effect of caloric restriction on cancer

The total energy intake has been shown to be an important determinant of carcinogenesis in both mice and rats (Albanes, 1987). Several studies have shown that both caloric restriction and intermittent fasting reduce the incidence of spontaneous tumours and suppress the development and growth of induced cancers such as mammary tumours and hepatocarcinogenesis induced by diethylnitrosamine in mice and Wistar rats, respectively (Berrigan *et al*., 2002; Mattson, 2005). The incidence of cancer increases with age in rhesus monkeys with intestinal adenocarcinoma as the most commonly diagnosed cancer in these animals (Rodriguez *et al*., 2002). Caloric restriction has been shown to reduce the incidence of gastrointestinal adenocarcinoma by 50% in rhesus monkeys undergoing CR as compared to the controls (Mattson *et al*., 2003; Colman *et al*., 2009; Mattison *et al*., 2012). The probable mechanism by which caloric restriction suppresses cancer formation may involve enhancement of apoptosis and inhibition of angiogenesis (Masoro, 2002).

However, many mechanisms were proposed by Weindruch (1989) for the effect of CR on cancer which includes: diminished cellular oxidative damage; retardation of the age-associated decrease in immune function; modulation of hormonal and growth factors; reduced exposure to dietary carcinogens; reduced energy for tumour growth; less activation of carcinogens; and greater maintenance of DNA repair. It has also been suggested that Caloric Restriction retards the occurrence of cancers by decreasing cell division (Rogers *et*

al., 1993) and increasing apoptosis (Warner *et al*., 1995). Caloric restriction has also been found to significantly influence the hepatic enzymes involved in the metabolism of carcinogens in rodents (Manjgaladze *et al.,* 1993). It delays the occurrence of interstitial cell tumours in the testes of male rats and reduces the expression of testicular cytochrome P450 enzymes involved in the activation of carcinogens, a factor that may play an important role in the delayed appearance of these tumours (Seng *et al.,* 1996).

2.5.2 Effect of caloric restriction on type II diabetes

Type II diabetes (non insulin-dependent diabetes mellitus) is a major age related disease in humans associated with excessive weight gain attributed to long-term dietary consumption and energy intakes in excess of requirement (Costacou and Mayer-Davis, 2003). Both caloric restriction and every other day feeding have been found to reduce blood glucose and insulin concentrations and improve glucose tolerance both in rodents and rhesus monkeys (Anson *et al*., 2003; Colman *et al*., 2009; Mattison *et al*., 2012). Caloric restriction has also been shown to prevent the occurrence of type II diabetes in Rhesus monkey (Hansen and Bodkin, 1993). However, increased in insulin sensitivity has been suggested to be the major mechanism responsible for the antidiabetic effects of caloric restriction and intermittent fasting (Anson *et al*., 2003).

2.5.3 Effect of caloric restriction on cardiovascular disease

Studies have shown that caloric restriction reduces the risk of cardiovascular diseases and stroke in humans (Altmann *et al.,* 1987). It protects the heart and the brain cells against ischemic damage (Fontana *et al*., 2004). It also protects the heart against myocardial infarction in rats (Crandall *et al*., 1981). The incidence of cardiovascular disease (e.g

valvular endocardiosis, cardiomyopathy, and myocardial fibrosis) which is also a prevalent age-associated disorder in rhesus monkeys was found to be reduced by 50% in rhesus monkeys subjected to CR as compared to the controls (Colman *et al*., 2009). On the contrary, Mattison *et al*. (2012) reported no significant change in the incidence of cardiovascular disease in rhesus monkeys maintained on caloric restriction when compared to the control.

Caloric restriction has been shown to increase the plasma levels of the HDL_{2b} sub-fraction of high-density lipoproteins (HDL) associated with protection from atherosclerosis in adult rhesus monkeys and decrease the binding of plasma low density lipoproteins (LDL) to proteoglycans isolated from the arterial wall, thereby reducing their atherogenic potential (Verdery *et al*., 1997; Edwards *et al.,* 1998). It also lowers the arterial blood pressure in rhesus monkeys which is in agreement with the report on the human participants of the Biosphere 2 study in which the participants underwent caloric restriction for a period of two years and were observed to exhibit a decrease in both arterial blood pressure and plasma cholesterol level, functional changes that decrease the risk of atherosclerotic disease (Lane *et al.,* 1999; Walford *et al.,* 2002).

2.5.4 Effect of caloric restriction on neurodegenerative disorders

Studies of rats and mice maintained on caloric restriction suggest that a decrease in caloric intake has been observed to slow age-related molecular changes in the brain including increase in levels of glial fibrillary acidic protein and oxidative damage to proteins and

DNA (Finch and Morgan, 1997). Age-related changes in the expression of genes that encode proteins involved in innate immune responses, oxidative stress and energy metabolism were found to be counteracted by caloric restriction and this retardation of aging at the molecular level may underlie retardation of brain aging at the functional level (Lee *et al*., 2000). Caloric restriction has been reported to attenuate age-related deficits in learning and memory ability and motor function in rodents (Stewart *et al*., 1989). It was also found to protect hippocampal and basal forebrain cholinergic neurons against death induced by excitotoxins (Contestabile *et al*., 2004). In humans, caloric restriction has been found to decrease the risk of developing Alzheimer's disease (AD), Parkinson's disease (PD) and stroke (Logroscino *et al*., 1996; Grant, 1997; Mayeux *et al*., 1999). It also promotes neuronal differentiation and survival, synaptogenesis, and synaptic plasticity (Russo-Neustadt, 2003).

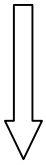
In vitro studies on caloric restriction and fasting diets have provided evidence for two major mechanisms by which caloric restriction elicits its physiological responses both in health and disease conditions (Mattson, *et al*., 2001). One of the mechanisms involves the production and removal of free radicals, while the second involves stress resistance (Merry, 2004). Overeating has been shown to increase oxidative stress in cells throughout the body as indicated by lipid peroxidation and the accumulation of oxidatively damaged proteins and DNA, while caloric restriction has been found to decrease the amount of oxidative damage to cellular proteins, lipids, and nucleic acids in various tissues of rodents (McCay *et al*., 1989). Both caloric restriction and every other day feeding may suppress oxidative stress by reducing the amount of superoxide anion radical produced in the mitochondria

and by stimulating the expression of genes that encode antioxidant enzymes and protein chaperones (Sreekumar *et al*., 2002).

Analyses of gene expression (and levels of the encoded proteins) reveal that CR and EODF increase the production of multiple cell survival–promoting proteins in tissues ranging from liver to brain which includes protein chaperones such as heat-shock protein 70 and glucose-regulated protein 78 (Duan and Mattson, 1999) and growth factors such as brain-derived neurotrophic factor (BDNF) and glial cell line–derived neurotrophic factor (GDNF) (Maswood *et al*., 2004). The ability of cells to activate a stress response is enhanced in animals that have been maintained on CR (de Cabo *et al*., 2003) and such enhanced stress resistance has been proposed as a major mechanism whereby caloric restriction and intermittent fasting increase life span as shown in figure 2.1.

Energy restriction

Intermittent fasting



Signal transduction

Growth factors (IGFs, BDNF, and NDGF)

Neurotransmitters (glutamate, serotonin)

Calcium influx

Gene expression

Stress resistance (HSP-70, GRP-78)

Antioxidants (SODs, GSH)

Energy metabolism enzymes (ETC and glycolysis)

Enhanced function

Disease resistance

Insulin sensitivity

Decreased blood pressure

Improved lipid metabolism

Decreased oxidative damage

Figure 2.1; Mechanisms by which reduced energy intake and meal frequency may protect against disease (Mattson *et al*., 2005).

2.5.5 Effect of caloric restriction on kidney disease

As human life expectancy has increased, a progressive increase in lifetime incidence of end stage renal disease was observed (Stern *et al.,* 2001). Renal lesions begin in young adulthood and progress in severity with age, often ultimately culminating in renal failure. Studies using different rat strains have shown that caloric restriction initiated at weaning or soon after, markedly delays the onset of renal lesions and slows the progression in severity (Davis *et al.,* 1983). Caloric restriction has also been found to retard the development of kidney pathology in rhesus monkeys (Hansen *et al.,* 1999). However, it was suggested that caloric restriction inhibits the occurrence and progression of chronic nephropathy by retarding the early development of glomerular hypertrophy (Masoro, 2002).

2.6 Mechanism of Anti-aging Action of Caloric Restriction

The lifespan of different species of animals used in biomedical research have been increased by up to 50% simply by reducing their caloric intake with maintenance of micronutrient intake (Mattson *et al*., 2001). This has been shown to be the case in many organisms from yeast *Sacchromyces cerevisiae* to rhesus monkeys (Lane *et al.,* 1999; Lin *et al*., 2000). Different mechanisms have been attributed to the lifespan extension and or anti-aging action of caloric restriction in these organisms such as growth retardation, decrease in body fat content, decrease in metabolic rate, lowering ambient temperature and increased in physical activity (Masoro, 2002). Others include enhancement of apoptosis

which increase the elimination of damaged cells and increased in turnover of body proteins, which steps elimination of damaged proteins (Tavernakis and Driscoll, 2002).

However, four mechanisms were profound to play a cardinal role in the anti-aging action of caloric restriction. These mechanisms includes; oxidative damage attenuation; alteration of the glucose-insulin system; alteration of the growth hormone-IGF-1 axis and hormesis (Masoro, 2002).

2.7 Caloric Restriction Mimetics

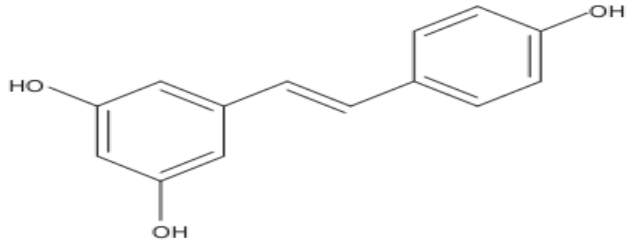
Over the years, serious effort has been made in developing pharmacological agents that could act as calorie restriction mimetic thereby providing the beneficial metabolic, hormonal, and physiological effects of caloric restriction without altering dietary intake or eliciting any potential adverse consequences of excessive restriction (Fontana and Klein, 2007). Several compounds such as plant-derived polyphenolic molecules (resveratrol, quercetin, butein, piceatannol), insulin-action enhancers (metformin) or pharmacological agents that inhibit glycolysis (2-deoxyglucose) have been proposed as potential caloric restriction mimetics (Ingram *et al*., 2006).

Studies has shown that 2-deoxyglucose, a glycolysis inhibitor developed as the first candidate was found to recapitulate the increase in insulin sensitivity and decrease in core body temperature observed in caloric restriction animals, but its toxicity near the therapeutic doses has prevented studies on longevity, or further development of the molecule for use in humans (Lane *et al*., 1998; Baur, 2010). Metformin was also found to mimic a large proportion of the transcriptional changes induced by caloric restriction in

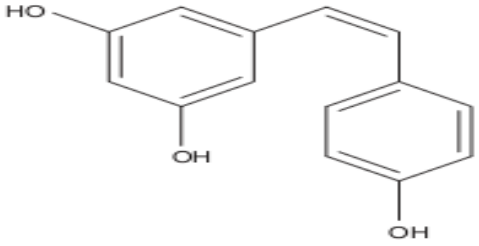
liver (Dhahbi *et al*., 2005). Although the effects of metformin on oxidative metabolism was said to be incompletely understood, activation of AMP-activated protein kinase (AMPK), a sensor of energy stress, was found to play a central role (Leverve *et al*., 2003). Phenformin was shown to extend mouse lifespan, albeit in a tumour-prone strain but the precise role of AMPK in mediating caloric restrictive effects of phenformin has been debated (Gonzalez *et al.,* 2004). Moreover, phenformin has been pulled off the market as a human drug due to fatal cases of lactic acidosis (Kwong and Brubacher, 1998).

2.7.1 Resveratrol as caloric restriction mimetic

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) or 5-[(E)-2(4-hydroxyphenyl)-ethenyl]benzene-13,-diol, is a polyphenolic flavonoid found in the seeds and skins of grapes, red wine, mulberries, peanuts, and rhubarb that exert a diversity of health benefits by activating intracellular pathways, many of which are the same as those activated by caloric restriction, an intervention long known to enhance health and prolong lifespan (Wood *et al* 2004, Markus and Morris, 2008). It is found both in *trans*- and *cis*- forms (Figure 2.2) with the trans-resveratrol having greater biological activity (Baur and Sinclair, 2006).



trans-Resveratrol



cis-Resveratrol

Figure 2.2: Chemical structure of *trans* and *cis*-resveratrol

Resveratrol targets the sirtuin class of nicotinamide adenine dinucleotide (NAD)-dependent deacetylase, of which *SIRT-1* is found to mediate the beneficial effects of resveratrol and caloric restriction on health and longevity (Guarente and Picard, 2005). A number of intracellular pathways were found to be activated by *SIRT-1* (Figure 2.3) and the extent to which the sirtuin-activating actions of resveratrol are direct or indirect have still not been resolved completely (Denu, 2005).

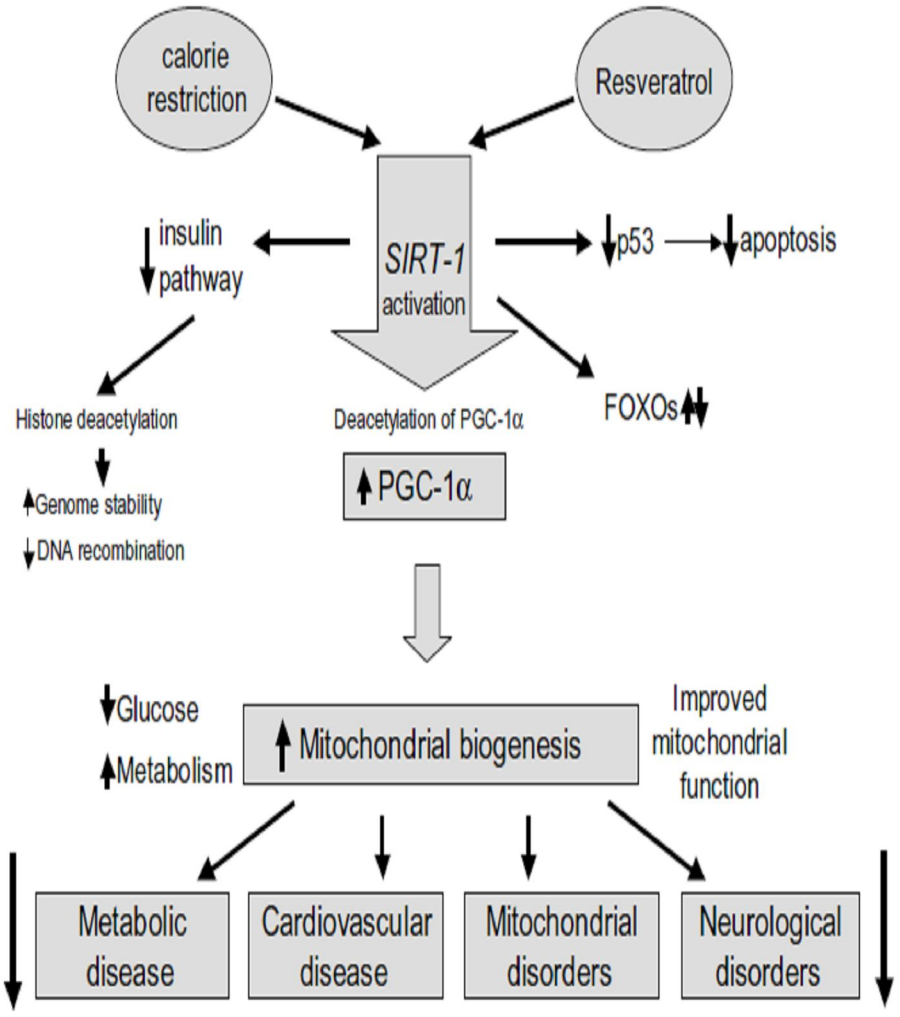


Figure 2.3: Resveratrol and caloric restriction *SIRT-1* activation pathways; Adopted from (Markus and Morris, 2008).

Some of the pathways regulated by sirtuins include gluconeogenesis and glycolysis in the liver, fat metabolism, and cell survival, forkhead box O (FOXO) group of transcription factors which activate or suppress specific genes, leading to a decrease in apoptosis, an increase in antioxidant activities, DNA protection, anti-inflammatory effects, and modulation of various other mechanisms so as to promote the health of the cell, and thus

the organism (Morris, 2008). It may be that sirtuins benefit survival by ramping up stress resistance pathways in cells in times of adversity (Guarente and Picard 2005). SIRT-1 was also found to interacts directly and deacetylates the metabolic regulator and transcriptional co-activator, peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α) thereby improving mitochondrial function, inducing genes for mitochondrial and fatty acid oxidation and increasing mitochondrial membrane potential (Lagouge *et al.,* 2006; Gerhart-Hines *et al.,* 2007; Anderson *et al.,* 2008).

Class IA phosphoinositide 3- kinase (PI3K) have been stated as another direct target of resveratrol that could represent a sirtuin-independent pathway central to the control of lifespan implicating insulin-like signalling through the P13K/protein kinase B cascade and the downstream FOXOs (Frojdo *et al*., 2007; Markus and Morris, 2008). Resveratrol suppresses the expression of mRNA for the senescence-inducer INK4 and modulates the expression of a number of genes such as those involved in the Ras pathway, apoptosis, cell growth, and division, G-protein signalling, cell–cell adhesids, immune system regulation, neuroendocrine signalling, muscle development, transcription, and proteolysis that were mostly expected to affect cell lifespan (Stefani *et al.,* 2007).

2.7.1.1 Effect of resveratrol on cardiovascular disease

The discovery that resveratrol is obtained primarily from red wine in most human diets has prompted extensive research into its potential to explain its cardioprotective effects, a notion that has been termed the 'French Paradox' where a moderate consumption of red wine was found to be associated with lower incidence of cardiovascular diseases in populations with a high fat diet (Renaud and de Lorgeril, 1992). The inhibitory effects of

resveratrol on cardiovascular disease observed to be similar to those of 25% caloric restriction over 6 years in humans include a reduction in platelet aggregation, dilatation of blood vessels, antiatherosclerotic effects, lowering of lipid peroxidation, reduction in endothelin-1, protection of endothelial cells against apoptosis, lowering of blood pressure, oxidative stress and end-organ damage in hypertensive animals, as well as improvement of serum cholesterol profile and triglyceride concentrations (Fontana *et al.,* 2004; Markus and Morris, 2008).

Resveratrol has also been found to elicit its beneficial effect on the cardiovascular system through its antioxidant activity (Baur and Sinclair, 2006). It was found to prevent oxidation of LDL by chelating copper and scavenging reactive oxygen species (ROS) in addition to preventing the increase in lipid peroxidation induced by tumours or ultra-violet irradiation (Frankel *et al*., 1993, Baur and Sinclair, 2006). It further activates the cholesterol efflux genes, ATP-binding cassette transporter 1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), resulting in a more limited cholesterol accumulation by human macrophages (Markus and Morris, 2008).

2.7.1.2 Effect of resveratrol on neurological disorders

Resveratrol was found to inhibit nuclear factor κB (NF-κB) signalling indirectly by activating *SIRT-1* providing protection against microglia-dependent β-amyloid toxicity in a model of Alzheimer's disease (Chen *et al*., 2005). Alzheimer's disease is characterized by the presence of neurofibrillary tangles and extracellular amyloid β plaques in the cortex and hippocampus and resveratrol has been found to reduce the extent of neuronal cell death and neurotoxicity of the mutant proteins (p25, a toxic co-activator of CDK5, and a mutant form

of superoxide) expressed in mouse models of Alzheimer's disease and amyotrophic lateral sclerosis (ALS), respectively (Kim *et al.,* 2007).

The effect of resveratrol was mediated by SIRT-1, via increased deacetylation of p53 and/or PGC-1α and by repressing P53; it may protect neurons from oxidative damage and prevent apoptotic neuronal death (St-Pierre *et al.,* 2006; Markus and Morris, 2008). Another possible pathway involved in promoting neuronal survival involves suppression or activation of FOXO proteins evidenced by studies in Huntington's disease models of both mice and *C. elegans*, where Resveratrol was found to rescue early neuronal dysfunction phenotypes induced by mutant polyglutamines (Parker *et al*., 2005).

2.7.1.3 Effect of resveratrol on lifespan

Sirtuins are conserved family of NAD+-dependent deacetylases (class III histone deacetylases) named after the yeast silent information regulator 2 (Sir2) proteins that are essential for a variety of biological processes related to regulation and maintenance of genes and DNA (Brachman, 1995; Chen and Widom, 2004). Sir2 has been found to be a key regulator of lifespan in yeast (*Saccharomyces cerevisiae*), fruitfly (*Drosophilia melanogaster*), nematode worm (*Caenorhabditis elegans*) and short-lived fish (*Nothorbranchius furzeri*). Resveratrol acting as a sirtuin activating compounds (STACs) was reported to extend the lifespan of these organism by 70%, 29%, 18% and 59%, respectively (Howitz *et al*., 2003; Bauer *et al*., 2004; Wood *et al*., 2004; Viswanathan *et al*., 2005; Valenzano *et al*., 2006).

The role of Sir2 and resveratrol in mediating the lifespan function of caloric restriction in these organisms has become the subject of controversy. Some investigators reported that both Sir-2 and Sir-2.1 are not required for the lifespan extension by caloric restriction in some strains of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* and deletion of all of the sirtuin family proteins does not prevent the response to caloric restriction in *Saccharomyces cerevisiae* (Kaeberlein *et al*., 2004; Tsuchiya *et al.,* 2006; Hansen *et al*., 2007), whereas others could not reproduce the results obtained in C. *elegans* and D. m*elanogaster* after several trials (Bass *et al*., 2007).

However, *in vivo* studies in mice model of obesity demonstrated that resveratrol elicits its physiological potentials in lifespan extension by activating the mammalian SIRT-1; a homologue of the yeast Sirt-2 and other pathways associated with increased insulin sensitivity, reduced insulin-like growth factor-1 (IGF-I) levels, increased AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor- coactivator 1α (PGC-1α) activity, increased mitochondrial number, and improved motor function thereby producing changes associated with longer lifespan (Baur *et al*., 2006; Lagouge *et al*., 2006; Berger *et al*., 2008).

2.7.1.4 *Effect of resveratrol on cancer*

The potential of resveratrol to inhibit the initiation and growth of tumours in different cancer models of both mouse and rat has been investigated by some researchers. It has been shown to prevent colon and prostate cancer as well as increase survival of mice with

subcutaneous neuroblastomas (Tessitore *et al*., 2000; Chen *et al.,* 2004; Harper *et al*., 2007).

The mechanisms by which resveratrol slows tumour development includes; anti-proliferative and pro-apoptotic effects leading to down regulation of cell cycle proteins and an increase in apoptosis of tumour cells, inhibition of angiogenesis, alterations in cell cycle and apoptosis, as well as antioxidant effects and some of these mechanisms modulates their functions by suppressing cyclooxygenases and ornithine decarboxylase expression through inhibition of protein kinase C (PKC), both enzymes that normally promote angiogenesis (Subbaramaiah *et al.,* 1998; Aggarwal *et al*., 2004; Baur and Sinclair 2006). Cytochrome P450 are enzymes involved in drug metabolism and are considered pro-carcinogens. Resveratrol was reported to inhibit the activities of cytochrome P450 enzymes in mice (Zhou *et al.,* 2005; Baur *et al*., 2006).

2.7.1.5 Resveratrol and antioxidant system

The role of resveratrol in modulating the antioxidant system either by direct scavenging property or activation of pathways that upregulate cells' natural antioxidant defences has not been well settled. Although resveratrol was reported to behave as a poor scavenger of ROS in *vitro*, it functions as a potent antioxidant *in vivo* (Baur and Sinclair, 2006). Resveratrol was reported to maintain the concentrations of intracellular antioxidants found in biological systems such as glutathione (GSH) in oxidation-stressed peripheral blood mononuclear cells isolated from healthy humans and increased the amount of GSH in human lymphocytes that were activated by hydrogen peroxide as well as several

antioxidant enzymes, including glutathione peroxidase, glutathione-S-transferase and glutathione reductase (Losa, 2003; Yen, 2003).

In DU 145 cells (a prostate cancer cell line), administration of resveratrol was found to inhibit the proliferation that had been accompanied by a reduction in NO production and inhibition of inducible nitric oxide synthase (iNOS) (Kampa *et al*., 2000). It was also reported to inhibit the formation of reactive oxygen species (O_2^-, H_2O_2) produced by macrophages stimulated by lipopolysaccharide (LPS) and lipid peroxidation induced by tumour necrosis factor (TNF) in a wide variety of cells (Mann *et al*., 2000). It also scavenges peroxyl and hydroxyl radicals in the post ischemic reperfused myocardium, thereby lowering malonaldehyde formation, a presumptive marker of lipid peroxidation (Rays *et al*., 2000).

2.7.1.6 Effect of resveratrol in obesity

Obesity has been a major health concern in many developed and developing countries globally. The number of age-related chronic diseases such as obesity and diabetes has been on the increase and the progressive increase in the number of overweight individuals worldwide has reached about 2.1 billion, leading to an explosion of obesity-related health problems associated with increased morbidity and mortality (Li and Heber, 2005). Studies in rodents have provided convincing evidence that resveratrol exerts favourable effects in animals consuming high fat diet. Experimental evidences demonstrates that mice maintained on high calorie diet supplemented with 0.04% of resveratrol incorporated into the animals diet for 15 weeks or 15 months were found to have increase survival rate, improved motor function, decreased total body fat content and decreased depots of

epididymal, inguinal and retroperitoneal white adipose tissue and changes in the expression of numerous genes towards the ones found in animals on a standard diet (Baur *et al*., 2006; Lagouge *et al.,* 2006).

Other numerous beneficial effects of resveratrol were revealed in rats fed a high-fat diet which includes reduced visceral fat index, liver mass index and body weight gain in rats (Aubin *et al*., 2008; Shang *et al*., 2008) and mice (Lagouge *et al*., 2006), but other investigators reported that 10 mg/kg body weight of resveratrol administered for 8 weeks appeared to be ineffective in reducing body weight gain in obese Zucker rats, although a significant reduction in plasma triglycerides, free fatty acids, cholesterol and liver triglycerides coupled with although a slight decrease in body fat content was observed in obese treated animals when compared to the non treated obese Zucker rats (Rivera *et al*., 2009).

The mechanism via which resveratrol elicits its function in obesity involves activation of Sirt1 which catalyzes deacetylation and activation of peroxisome proliferator gamma coactivator-1α (PGC-1α), a cofactor in mitochondrial biogenesis (Rodgers *et al*., 2005). In another study with embryonic fibroblasts obtained from Sirt−/− mice, resveratrol appeared to be ineffective and failed to decrease PGC-1α acetylation and to modulate the expression of PGC-1α target genes (Lagouge *et al*., 2006). Another mechanism results from phosphorylation/activation of 5′-AMP-activated protein kinase (AMPK) which inhibits acetyl-CoA carboxylase enhancing oxidation of fatty acids and decreasing their synthesis (Hardie and Pan, 2002). A substantial increase in AMPK activity induced by resveratrol

was reported in rats fed a high-fat diet as well as obese Zucker rats (Baur *et al*., 2006; Rivera *et al*., 2009).

2.7.1.6 Effect of resveratrol on diabetes

Over the last few years, studies in rodents and *in vitro* experiments provided evidence that resveratrol elicits beneficial effects in the organisms and may be helpful in preventing and treating some metabolic diseases, including diabetes (Szkudelska and Szkudelski, 2010). The management of diabetes involves reduction of blood glucose, preservation of β-cells, and improvement in insulin action. Resveratrol was reported to displace the binding of glibenclamide which is a sulfonylurea drug applied in type 2 diabetes to enhance insulin secretion that blocks ATP-sensitive K^+ channels in β-cells (Hambrock *et al*., 2007). Under physiological conditions, ATP-sensitive K^+ channels are normally blocked as a result of the increase in the ATP/ADP ratio resulting from metabolism of glucose or other fuel secretagogues. The rise in the ATP/ADP ratio induces depolarization of the plasma membrane and triggers secretion of insulin (Henquin, 2000).

However, different results were obtained on the effect of resveratrol in normal, hyperinsulinemic and diabetic animals. Szkudelski (2008) reported that 50 mg/kg of resveratrol given intragastrically in normal non-fasted rats reduced blood insulin at 30 min while Chi *et al*. (2007) demonstrated that Resveratrol (3 or 10 mg/kg body weight) significantly increased blood insulin in overnight fasted rats 90 min after intraperitoneal administration. No effect was shown in rats receiving 5mg/kg body weight of resveratrol orally for 30 days (Palsamy and Subramanian, 2009).

Interestingly, resveratrol incorporated into mice diet with a marked hyperinsulinemia induced by high-fat diet was found to diminish blood insulin compared with animals consuming high-fat diet without resveratrol (Baur et al., 2006; Lagouge et al., 2006). Su *et al.* (2006) and Chi *et al*. (2007) have observed a significant blood glucose-lowering property of resveratrol in normal rats. Su *et al*. (2006) reported that administration of resveratrol at very low doses (0.25–0.75 mg/kg body weight) to overnight starved rats resulted in a decline in glycemia 90 min after treatment. In addition, resveratrol (0.5 mg/kg) also decreased glycemic response to an intravenous glucose challenge (2g glucose/kg) compared with non-treated animals (Su *et al*., 2006). Chi *et al.,* 2007 also reported similar results, who demonstrated resveratrol's blood glucose reducing properties.

In soleus muscle, adipocytes and hepatocytes isolated from streptozotocin-induced diabetic rats and incubated in the presence of resveratrol (0.01–1 μM), but without insulin, glucose uptake was found to be substantially increased (Su *et al*., 2006) and a prolonged (8 hours) incubation of H9C2 cardiac myoblast cells with resveratrol also enhanced glucose uptake in an insulin-independent manner (Penumathsa *et al.,* 2008). Different mechanisms are proposed to be responsible for resveratrol-induced intracellular glucose transport where some investigators suggested the effect of glucose transporters GLUT1 and increased intrinsic activity and phosphorylation of AMPK, expression of GLUT4 (Penumathsa *et al*., 2008; Breen *et al.,* 2008), while others reported the involvement of oestrogen receptor-α as well as P13, the p38/Erk- and p38/Akt-dependent pathways in mediating the early and late phase action of resveratrol, respectively (Deng *et al.,* 2007).

2. 8 Historical Background of Environmental Enrichment

Environmental enrichment defined as a combination of complex inanimate and social stimulation was first described by Hebb (1949), as a research tool for understanding the effects of experience on the brain showing how increasing the complexity of a laboratory rodent's environment from a typical laboratory setting improved its subsequent behaviour in learning tasks. Hebb however brought laboratory rats to his home, where they were treated as family pets. Subsequently, other investigators repeated the basic finding in the laboratory on the basis that a more stimulating rearing environment enhanced performance on complex learning tasks (Bingham and Griffiths 1952; Forgays and Read 1962).

The term "environmental enrichment" was first brought into light or coined by Krech *et al*. (1962) when describing the paradigm, who originally found biochemical changes in the brains of rats reared in a complex housing environment supplemented with daily exposure to novel items in an open field (Krech *et al.,* 1960). Since that time, environmental enrichment, variously implemented, has often been used as a research tool to illuminate how the physiology, anatomy, and behaviour of an organism adapt to and learn from the environment.

2. 9 Environmental Enrichment and the Brain Functions

Numerous cognitive theories on how environmental enrichment affects the brain have been proposed, among which includes the arousal hypothesis, which emphasizes on the 'arousal response' of animals when confronted with novelty and environmental complexity (Walsh and Cummins, 1979), and the 'learning and memory' hypothesis, in which the mediator of

the morphological changes is seen in the cellular mechanisms underlying learning processes (Rosenzweig and Bennett, 1996). Although the learning and memory hypothesis is favoured by many investigators, it is difficult to prove that the neural consequences of the enriched environment are related to learning rather than to increased voluntary motor behaviour (Van Praag, 2000).

An interesting feature of the adult brain is its capacity for structural and functional modification in response to external stimuli and this plasticity of the adult nervous system has been the focus of research efforts for decades (Mohammed *et al*., 2002). The impact of environment on the brain was first brought into lime light by Charles Darwin (1874) in his description of wild rabbits having increased brain size compared to domesticated rabbits. However, many investigators were discordant and stimulated by the primitive view, that the brain is fixed and static at adulthood and therefore not susceptible to modification by environmental influences. These led to the numerous findings suggesting that housing adult rats in an environment rich in sensory stimuli induces measurable changes in gross brain structure and levels of acetylcholinesterase including neuroanatomical changes of finer structures, such as dendritic branching, dendritic length and dendritic spine density (Globus *et al.,* 1973; Diamond *et al*., 1976). These observations stimulated much research on the influence of enriched environment on different aspects of brain function and behaviour which resulted to the discoveries concerning environmentally induced increase in adult neurogenesis (Van Praag *et al*., 2000).

2.9.1 Effect of environmental enrichment on the cortex

Some investigators have observed that differential housing of rats from 25 to 55 days of age resulted in different brain weights between litter-mates housed in Enriched environmental Condition (EC) and Impoverished environmental Condition (IC) with the largest EC-IC difference (9.4%) found with respect to the occipital cortex (Rosenzweig *et al*., 1962; Bennett *et al*., 1969). Some investigators reported that rats from the enriched environment were found to developed thicker and longer cerebral cortices compared to litter mates from the impoverished and standard environments as well as more glial cells in the occipital cortex (Diamond *et al*., 1975).

Dendritic branching in occipital layer II stellate neurones, pyramidal neurones and stellate neurones of the occipital cortex was found to be increased in enriched condition animals than the impoverished condition littermates (Holloway, 1966). Differences in dendritic branching patterns in the visual cortex were also observed predominantly in the basal dendrites (Greenough and Volkmar, 1973).

The neuroanatomical changes in the visual cortex could at least in part be mediated by neurotrophins, as their levels was reported to increased in the visual cortex of enriched conditon rats, and the nerve growth factor inducible (NGF-IA) immediate early gene, which is also induced in enriched condition animals' visual cortex as well as changes in mRNA levels in the brains of enriched condition animals (Torasdotter *et al*., 1998). These neuroanatomical changes are likely to have functional consequences due to the speculations that plastic changes occurring at synaptic sites could be related to long-term memory, thus

increased synaptic density and larger synaptic sites appear to be associated with enhanced capacity for learning, as studies have repeatedly shown that enriched condition animals were found to perform better than impoverished condition animals in a variety of models, such as the Hebb-Williams maze, the Krechevsky hypothesis apparatus, the radial arm maze and the Morris water maze (Mohammed *et al*., 2002).

2.9.2 Effect of environmental enrichment on the hippocampus

Numerous studies have generated convincing evidence of environmentally induced changes in the hippocampus of enriched environmental conditioned rats as compared to impoverished conditioned rats, in a wide array of biological variables. These studies have shown that enriched environmental condition induces hippocampal changes in gene expression and/or protein levels of neurotrophins (Torasdotter *et al.,* 1998), glucocorticoid receptors (Mohammed *et al.,* 1993), serotonin receptors (Rasmuson *et al.,* 1998), AMPA receptor binding (Gag *et al.,* 1998) and neurogenesis (Nilsson *et al.,* 1999). Exposure to a complex environment induces increased spine densities in the hippocampal CA1 pyramidal cells (Moser *et al*., 1994). The hippocampus involvement in plasticity related phenomena, such as learning, has been the focus of intensive investigation. Neurotrophins, for instance, have been of special interest as they are abundantly expressed in the hippocampus and have been implicated as mediators of plasticity in hippocampus and other brain regions (Thoenen, 2000).

Growth factors provide important extracellular signals regulating proliferation and differentiation of stem and progenitor cells in the central nervous system during development. The members of the nerve growth factor family of neurotrophic factors, such

as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are abundantly expressed in the hippocampus, where they are localised in pyramidal cells and dentate granule cells (Barde, 1990). Behavioural studies have revealed both BDNF and NGF to be importantly involved in mediating learning and memory (Mizuno *et al.,* 2000; Woolf *et al*., 2001).

There are numerous studies indicating involvement of these neurotrophins in synaptic plasticity in the hippocampus and other brain regions, such as the somatosensory and visual cortex (McAllister, 2000). Of great interest are observations that NGF regulates cell body size, dendritic arborisation and dendritic spine density, which are changes that are also induced by environmental enrichment (Sofroniew *et al.,* 2001). Similarly there was the intriguing observation that the dendritic growth of pyramidal neurones in the developing visual cortex is dynamically regulated BDNF and NF-3, where BDNF stimulates growth in layer IV, while NT-3 stimulates growth in layer VI (McAllister *et al*., 1997). It was also observed that treatment with NGF was found to increase dendritic branching in the pyramidal neurones in layer V of the cortex, and increased dendritic spines in rats (Kolb *et al.,* 1997). These findings suggest that neurotrophins play an important role in mediating changes in dendritic morphology of enriched rats.

2.9.3 Effect of environmental enrichment on the cerebellum

The cerebellum which is well known for its functions in maintaining motor coordination and learning has been found to display plastic properties in response to environmental influences (Glickstein, 1993). The cerebellum of enriched environment conditioned rats have been found to have a higher density of IGF-1 receptors than that of impoverished

environment conditioned animals and since the enriched environment conditioned animals are actively engaged in exploring, climbing and balancing, it is reasonable to expect changes in the cerebellum of these animals (Mohammed *et al*., 2002). Complex motor skill learning leads to an increase in synapse number in the cerebellar cortex and changes in morphology of cerebellar Purkinje cells in response to environmental stimulation have been observed (Kleim *et al*., 1998). More extensive spiny branchlets of Purkinje cells have also been observed in monkeys reared in a stimulating environment, in contrast to isolated environment (Floeter and Greenough, 1979).

2.9.4 Effect of environmental enrichment on the amygdala

The amygdala is a key brain area thought to play a critical role in generating behavioural responses to emotionally arousing stimuli and the amygdaloid complex consists of several nuclei which differ in their architecture, and in their intra- and extra-amygdaloid connectivities (Pitkanen *et al*., 1997). Studies employing classical and instrumental conditioning paradigms have highlighted the differential roles of various sub-structures of the amygdaloid complex in emotional learning and the amygdala has been found to show plastic properties in response to environmental influences (Mohammed *et al*., 2002). Morphological changes in both synapses and neurones in the medial amygdaloid nucleus were observed following manipulation of the pheromonal environment (Ichikawa *et al*., 1993).

Functional activity in different brain regions was studied by employing expression of the immediate early gene c-Fos and its protein product c-Fos and studies on aversive learning have revealed differential expression of c-Fos in some amygdaloid nuclei (Kaczmarek and

Chaudhuri, 1997). However, it was hypothesised that animals differ in emotion and behaviour and differentially housed rats respond differently in fear conditioning as revealed by c-Fos expression in brain regions known to be important in mediating emotional behaviour (Mohammed *et al*., 2002). Detailed analysis of c-Fos immuno-labeling of various amygdala subnuclei in enriched environment condition and impoverished condition animals revealed significant differences in their responses to the conditioning procedure and the most pronounced c-Fos expression was observed within the medial amygdala and amygdaloid-striatal transition area, in which the impoverished environment condition animals displayed higher c-Fos levels than the enriched environment condition animals (Nikolaev *et al*., 2001).

Synaptogenesis has also been reported to be increased in enriched animals as indicated by an increase in dendritic spines, possibly indicating enhanced synaptic contacts. Subsequent studies found that enrichment reduced neuronal density but increased synapse-to-neuron ratios, synaptic disc diameter and sub-synaptic-plate perforations (Beaulieu and Colonnier, 1987). Similar morphological changes have been observed within the hippocampus for dentate granule neurons as well as pyramidal cells in areas CA1 and CA3 and enriched female rats were found to have more dendrites per neuron in the dentate gyrus than control the (Juraska *et al*., 1985).

2.10 Effect of Environmental Enrichment on Electrophysiological Changes

Researchers have demonstrated the possible relationship between enriched living and electrophysiological changes. Hippocampal slices taken from enriched or individually

housed rats and excitatory postsynaptic potential (EPSP) slopes in the dentate gyrus were found to be greater in the slices from enriched rats (Foster *et al*., 2000). Similarly, exposing rodents to a new, complex environment has been found to enhance hippocampal field potentials (Sharp *et al.,* 1985). Extensive studies have documented the relationship between physical and neuronal activity, and the discharge frequencies of hippocampal pyramidal cells and interneurons have been shown to increase with increased wheel running velocity in rats (Czurko *et al*., 1999). In another study, EPSP amplitudes and long-term potentiation (LTP) were compared in hippocampal slices from running and control mice and EPSPs were found to be unchanged in both groups but LTP amplitude was selectively enhanced over the controls in the dentate gyrus in slices from running mice (Van Praag *et al*., 1999).

2.11 Effect of Environmental Enrichment on Neurotransmitters

The early studies of enriched environments reported an increase in neurotransmitters such as acetylcholine and enhanced expression of the gene for serotonin1A receptor (Rosenzweig *et al*., 1969). Some investigators reported that physical activity can change the activity of several neurotransmitter systems in the brain and exercise was found to influence cholinergic parameters, affecting choline uptake in hippocampus and cortex (Fordyce *et al*., 1991). Furthermore, monoamines such as noradrenaline and serotonin were activated by running or physical activity some of which have been reported to influence learning, synaptic plasticity and neurogenesis in the adult brain (Soares *et al*., 1999).

2.11 Effect of Environmental Enrichment on Visual System

Environmental enrichment is widely used for investigating the influence of sensory experience on brain and behaviour, giving the animals the opportunity for voluntary physical activity and social interactions. Rearing animals in an enriched environment has profound effects on the adult organism, leading to anatomical changes in dendritic arborisation, spine density and synapses per neuron that are associated with enhanced neural plasticity, improved learning and memory and reorganization of cortical somatosensory maps (Polley *et al.,* 2004). Some investigators reported that post weaning environmental enrichment prevents dark rearing effects on rat visual cortical development while environmental enrichment from birth in mice results in a conspicuous acceleration of visual system development at behavioural, electrophysiological and molecular level, hence improving the animals visual acuity in adult life (Bartoletti *et al*., 2004; Cancedda *et al*., 2004).

Huang *et al*. (1999) proposed that higher BDNF levels accelerate the development of the inhibitory GABAergic system, which, by affecting receptive field development and synaptic plasticity, could explain both the faster maturation of visual acuity and the precocious decline of cortical plasticity in BDNF over expressing mice. Other mechanisms involve the effect of BDNF on the dendritic growth of pyramidal neurons in the visual cortex in an activity dependent manner and spine formation in the hippocampus (Tyler and Pozzo-Miller, 2003). Therefore, an action on dendritic maturation and spine plasticity could be a possible cellular mechanism of enriched environment on visual cortical development and plasticity.

2.12 Effect of Environmental Enrichment on Psychiatric and Neurodegenerative Disorders

Much progress has been made in understanding how genetic factors and other molecular mediators contribute to various brain disorders, and less is known about how environmental factors and associated experience-dependent plasticity modulate pathogenesis and disease progression (Laviola *et al*., 2008). The plastic response of phenotypic traits to environmental change is a common research focus in several disciplines ranging from ecology, evolutionary biology to physiology and molecular genetics (Callahan *et al*., 1997). There is growing evidence that environmental stimulation induces long term adjustments of neurobehavioral systems involved in learning, memory and defensive responses and environmental enrichment studies using transgenic mouse models of neurodegenerative disorders have provided important insights into gene–environment interactions and experience dependent plasticity in brain disease (Laviola *et al*., 2008). It has also been demonstrated that environmental enrichment has beneficial effects in animal models of brain injury and neurodevelopmental disorders (Nithianantharajah and Hannan, 2006).

2.12.1 Effect of environmental enrichment on huntington's disease

Huntington's disease (HD) is an autosomal dominant brain disease involving cognitive deficits, psychiatric disorders and motor symptoms, due to progressive neurodegeneration, particularly in the cerebral cortex and striatum caused by a trinucleotide (CAG) repeat mutation, encoding an expanded polyglutamine tract in the huntingtin protein (Spires *et al*., 2004). The neuropathology of HD includes neuronal death in the corpus striatum and neocortex, preceded by neuronal dysfunction (Davies and Ramsden, 2001). The

polyglutamine tract appears to confer a toxic gain of function in the N-terminus of the mutant huntingtin protein, leading to a range of molecular and cellular changes, including altered protein-protein interactions, gene expression, molecular traficking, inter- and intra-neuronal signaling, synaptic plasticity and adult neurogenesis (Spires and Hannan, 2007).

Studies in transgenic mouse models of HD revealed that home-cage environmental enrichment delays the onset and progression of motor symptoms (Spires *et al*., 2004) as well as cognitive deficits (Nithianantharajah *et al*., 2008). Investigations into the mechanisms mediating these experience-dependent effects have identified spatiotemporally regulated molecular and cellular changes in response to both environmental enrichment (Spires *et al*., 2005) and voluntary physical exercise on running wheels (Pang *et al*., 2006). Symptomatic mouse model of HD housed in an enriched environment showed increased proliferation and neuronal maturation, as well as neuronal morphological changes that could underlie some of the beneficial effects of enrichment (Lazic *et al*., 2006). These findings indicate that the modulatory effects of environmental enrichment are mediated by experience-dependent changes in transcription of specific genes, synaptogenesis and adult neurogenesis, some of which may be mimicked by a newly proposed class of therapeutics, enviromimetics (Hannan, 2004; Nithianantharajah and Hannan, 2006).

The mechanism via which environmental enrichment elicits its functions may involved increase BDNF levels in the striatum which might promote cell survival and up regulate genes that are transcriptionally disrupted in HD, thus protecting against neuronal dysfunction (Spires *et al*., 2004).

2.12.2 Effect of environmental enrichment on parkinson's disease

Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized by a progressive loss of dopamine neurons in the nigrostriatal system (Dauer and Przedborski, 2003**)**. Parkinson's disease patients have been observed to show significant improvement in motor function as well as increased life span following physical therapy (Kleim *et al*., 2003).

Some investigators reports that enhancing the frequency and intensity of physical activity before or shortly after exposure to a dopaminergic neurotoxin improved behavioural outcome and prevent dopamine terminal degeneration (Kleim *et al.,* 2003; Tillerson *et al.,* 2003). Environmental enrichment was also shown to be beneficial in an animal model of PD as mice raised in an enriched environment were shown to be 200% more resistant to MPTP compared to mice raised in a standard environment due to decreased loss of DA neurons by down regulating the expression of DAT (Bezard *et al*., 2003).

The neuroprotective effects of environmental enrichment in Parkinson's disease was said to be mediated through the synthesis and release of trophic factors (BDNF, GDNF,NGF) and their receptors through reactive astrocytes (astrogliogenesis) which probably participate in endogenous cell repair or neuroprotective mechanisms triggered at very early times following exposure to toxins (Nithianantharajah and Hannan 2006; Steiner *et al*., 2006).

2.12.3 Effect of environmental enrichment on alzheimer's disease

Alzheimer's disease (the major cause of adult-onset dementia), is associated with progressive loss of memory and severe cognitive decline whose clinical features are associated with deposition of β-amyloid (Aβ) peptides and neuronal loss in the cerebral

cortex and hippocampal formation (Lazov *et al*., 2005). Neurodegeneration in AD predominantly affects the cerebral cortex, and a number of genes have been implicated in familial forms of AD, in particular amyloid precursor protein (APP) and the presenelins, which support the hypothesis that amyloid-mediated mechanisms are pivotal in pathogenesis of AD (Masters *et al*., 2006).

However, other neuropathological and genetic studies have also implicated proteins such as tau and apolipoprotein E (ApoE) in AD pathogenesis, and identified various molecular and cellular mechanisms using transgenic mouse models. Environmental enrichment has also been found to alter behavioural, cellular and molecular aspects of pathogenesis in a range of transgenic AD mouse models (Arendash *et al*., 2004). Amyloid precursor protein (APP)-23 transgenic mice raised in an enriched environment has been shown to improve water maze performance, an up-regulation of hippocampal neurotrophin-3 and brain-derived neurotrophic factor and increased hippocampal neurogenesis (Wolf *et al*., 2006). Environmentally enriched transgenic mice expressing both human mutant presenilin-1 and the amyloid precursor protein was also found to outperform mice in standard housing, and were behaviourally indistinguishable from non-transgenic mice across multiple cognitive domains (Costa *et al.,* 2007). Mutant APP transgenic AD mice housed in a "complete enrichment" environment showed protection against cognitive impairment, decreased brain β-amyloid deposition, and increased hippocampal synaptic immunoreactivity (Cracchiolo *et al*., 2007).

2.12.4 Effects of environmental enrichment on drug addiction

Environmental factors (family and peers) have been considered as one of the major risk factor involved in drug addiction in adolescent humans (Swadi, 1999). Drug addiction was found be more frequent in people living in degraded areas or in people that undergo difficult experiences during their childhood (UNECS,1999), while positive family relationships, involvement and attachment appear to discourage drug use and prevent drug addiction (Jessor and Jessor,1980). Little attention has been dedicated to environmental manipulations that could provide protection against drugs' effects and may mimic positive life experiences. An efficient way to provide such a positive environment in laboratory settings is by raising animals in enriched environments and the neurochemical, cellular and molecular modifications induced by environments in areas known to play a cardinal role in drug addiction such the mesolimbic dopaminergic system are known to play important function (Laviola *et al*., 2008).

Environmental enrichments have been shown to decrease the activating effects of drugs such as amphetamine, nicotine in rats (Green *et al*., 2003). Mice raised in enriched environments were found to show less locomotor activity in response to an injection of cocaine than those raised in an impoverished environment (Bezard *et al.,* 2003). On the other hand, at the cellular and molecular levels, enriched mice were found to show lower levels of DAT (main molecular target of cocaine), and higher striatal levels of BDNF than mice bred in an impoverished environment (Laviola *et al*., 2008). In addition, cocaine-induced expression of immediate early genes such as c-fos was different between standard and enriched mice suggesting that plasticity processes may also be altered in relation to the

environment (Laviola *et al*., 2008). Striatal cDNA arrays showed that mice reared during adolescence in an enriched environment have several alterations in the levels of mRNA coding for proteins involved in cell proliferation, cell differentiation, signal transduction, transcription and translation, cell structure and metabolism (Thiriet *et al*., 2005). These findings suggest that environmental enrichment may play a vital role in determining resistance to drugs of abuse such as cocaine.

2.13 Effects of Environmental Enrichment on Obesity

Inhospitable environments which are found to be stressful to animals have been linked to abnormal behaviour and brain development (Wurbel, 2001). The Otsuka Long-Evans Tokushima fatty (OLETF1) rat is an animal model of obesity due to lack of cholecystokinin (CCK1) receptors leading to hyperphagia from birth which eventually lead to obesity in the animals (Schroeder *et al*. 2007). The OLETF male rats were also reported to develop non insulin-dependent diabetes mellitus late in life and have been the focus of many studies that show the development of morbid obesity with complications of the metabolic syndrome (Moran and Bi, 2006). However, behavioural interventions to reduce OLETF rats' obesity such as environmental enrichment have shown significant success in moderating males' obesity when performed in adulthood and long-lasting reductions in obesity when performed early in life (Moran, 2008; Schroeder *et al*., 2010a), while OLETF female rats appear to be less responsive to behavioural interventions than males and usually retain their body weight and adiposity (Schroeder *et al*., 2010b).

However, the probable mechanism via which environmental enrichment elicits the adiposity-reducing effect on the OLETF males rats was said to be mediated through Brain-derived neurotrophic factor (BDNF) which plays important role in the hypothalamic pathway that controls body weight, energy homeostasis and regulates energy metabolism in peripheral organs (Schroeder *et al.,* 2011).

2.14 Effects of Environmental Enrichment on Immune System

The immune system which is a regulatory system that suffers severe age-related alterations and dysfunction in immunity along with the aging process exerts a great influence on age-related morbidity and mortality (Wayne *et al*., 1990). Several age-related changes in immune functions have been correlated with increased mortality, such as low lymphoproliferative response to mitogens and natural killer (NK) cytotoxicity (De la Fuente *et al*., 2009). The proposed oxidative–inflammatory theory of aging suggests a key role for the immune system in accelerating the aging rate of the organism by persistent oxidative–inflammatory stress affecting leukocytes, leading to damage of leukocytes (De la Fuente *et al*., 2009). The nervous and immune systems were said to coordinate their activities to preserve homeostasis and health, hence factors influencing the nervous system could eventually lead to immune modulation (Besedovsky and Del Rey, 1996).

In a study to determine the effect of enriched environment on immune system for the period of 8-16 weeks in rats, Arranz *et al*. (2010) found that environmental enrichment plays a significant role in improving immune function (macrophage chemotaxis and phagocytosis, lymphocyte chemotaxis and proliferation, natural killer cell activity, interleukin-2 and tumor necrosis factor-a levels) and decreasing the oxidative–inflammatory stress (lowered

oxidized glutathione content, xanthine oxidase activity, expression of Toll-like receptors 2 and 4 on CD4 and CD8 cells, and increased reduced glutathione and glutathione peroxidase and catalase activities) of immune cells. These positive effects of enriched environment were found to be pronounced in the animals at older ages. These elucidate the importance of maintaining active mental and or physical activity aimed at improving health and quality of life in terms of immunity.

2.15 Effects of Environmental Enrichment on Gene expression and other disease Conditions

Environmental enrichment has been found to play significant role in mediating the genetic over expression of hypothalamic brain-derived neurotrophic factor (BDNF) and its upregulation there by reducing tumour burden (Cao *et al*., 2009). The hypothalamic BDNF was reported to cause the down regulation of leptin production in adipocytes via sympathoneural β-adrenergic signalling suggesting the genetic or environmental activation of BDNF/leptin axis in mediating the therapeutic potential for cancer in mice (Cao *et al.,* 2010). Environmental enrichment has also been found to increased numeric synaptic density in parietal cortex, and induced structural changes in synaptic junctions after transient focal cerebral ischemia in rats (Xu *et al*., 2009). It was also reported to enhanced migration and survival of both endogenous and transplanted stem cells toward the region of injury or infarct after stroke and also increased the number of endogenous progenitor cells in the subventricular zone of transplanted animals (Hicks *et al*., 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Drugs/ Chemicals

Resveratrol (1000 mg) of analytical grade was purchased Candlewood Stars Incorporated, Danbury, USA (Batch No: MR120718). Carboxymethylcellulose CMC (10 g) (Product no: 27929, BDH Chemicals LTD, Poole England) was obtained from Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria.

3.1.2 Equipment

Elevated plus maze, beam walk and hang test apparatus, toys, dissecting sets, syringes and needles, mortar and pestle, canula, enrichment and impoverished cages, digital weighing balance and biochemical assay kits.

3.1.3 Animal housing and management

The enriched cage (66 cm long × 46 cm wide × 38 cm high) as described by Harburger *et al*. (2007) was used in the study. The cage contains tubes, ramps, stairs, and different 'toys' (hard plastic balls, cubes, cones, and sticks). The toys were changed twice a week to continuously encourage exploration of the environment. The complexity (number of objects) of the housing facility was increased progressively: every 2 days, two to four objects were added to the environment. Ten days after housing animals in the EE, the complexity of the cage is expected to be maximal but the positions of the objects were changed continuously after every 2 days (Anastasia *et al,*. 2009). Five (5) mice were

housed together to allow social interactions. The impoverished condition consists of cages made without objects or running wheels, housing five (5) animals per cage.

Animals were kept in EE housing for four weeks while receiving the appropriate treatment, as described by Steiner *et al.* (2006). The control animals were given carboxymethylcellulose (CMC) and kept under good housing condition. Neurobehavioural study was carried out for the period of three (3) days during the last phase of the experiment after which the animals were sacrificed and the blood and brain samples were collected for the biochemical assessment.

3.1.4 Animals and management

A total of twenty five (25) Swiss albino mice of both sexes, 4 weeks of age and weighing 15-22g were used for this study. The animals were purchased from the Institute of Veterinary Research, Vom, Jos Plateau State. The animals were fed with standard laboratory animal feed and water *ad libitum*. The mice were allowed to acclimatize to the environment for the period of one week before commencement of the experiment.

3.1.4.1 Animal groupings

The animals were divided into five (5) groups each comprising of five (5) mice per group.

Group I: Animals served as the control group receiving caboxymethylcellulose (CMC) 50mg/kg per body weight

Group II: Animals were maintained on every other day feeding (EODF-CR Group) with standard laboratory animals diet

Group III: Animals received resveratrol 50mg/kg orally for four weeks (CR Group)

Group IV: Animals received CMC and kept in environmental enrichment housing

(EE) for four weeks

Group V: Animals received resveratrol (CR) 50mg/kg orally + EE housing (for 4 weeks)

Neurobehavioural studies were carried out for the period of three (3) days during the last phase of the experiment and motor co-ordination, motor strength, learning and memory were assessed.

3.2 Methods

3.2.1 Resveratrol induced caloric restriction

The maximum tolerated dose of resveratrol in mice was found to be 4g/day/kg body weight for 28 days (Gadducci *et al*., 2002). In this study, the animals were treated with resveratrol 50mg/kg orally for four (4) weeks as described by Blanchet *et al.* (2008). Neurobehavioural studies were carried out for three days during the last phase of the experiment and the animals were sacrificed and the blood and brain samples were collected for biochemical assays.

3.2.2 Neurobehavioural assessments

3. 2.2.1 Beam walking assessment for motor coordination

The beam walk apparatus consist of a beam, ruler, goal box, and an elevated wooden stand. The beam is made of wood, 8 mm in diameter, 80 cm long and elevated 30 cm above the bench by a wooden support. Mice were allowed to walk from a start platform along a ruler (80 cm long and 3 cm wide) elevated 30 cm above the bench by a wooden supports to the goal box (enclosed hamster house). Several trials were performed for each mouse and it was designed such that the mice tested were aware that there is a goal box that should be

reached. A ruler was used to train the mice and once the mice find it easy to cross, they were moved immediately to the beam (Stanley *et al*., 2005). The mice were placed on the beam at one end and allowed to walk to the goal box. Mice that fall from the beam were returned to the position they fell from with a maximum time of 60 seconds allowed on the beam. The measurements that were taken were time on the beam, the number of foot slips (one or both hind limbs slipped from the beam) and the number of falls. After each trial, the maze was wiped with a cotton wool dipped in 70% ethyl alcohol and allowed to dry to remove any olfactory clue or odour. The beam walking assay was performed during the last phase of treatment with either CMC, EODF, EE, resveratrol or both resveratrol and EE respectively.

3.2.2.2 Hang test

The Hang test was used to assess motor or neuromuscular strength in mice as described by (Mohanasundari *et al*., 2006). The apparatus consists of a horizontal grid (grid 12 cm^2 opening 0.5 cm^2). The grid was mounted 20 cm above a hard surface, to discourage falling or injury in case of falling. The apparatus was equipped with a 3-inch wall to prevent animals from transversing to the upper side of the grid. The mice were placed on the horizontal grid and supported until they held the grid. The grid was then inverted so that the mice were allowed to hang upside down. The mice were allowed to stay on the grid for 30 s and 10 chances were given with 1 min interval and the best maximum hanging time was recorded. The percentage of success was recorded as maximum time hanging/30 s × 100 (Tillerson *et al*., 2002).

3.2.2.3 Elevated plus-maze

The elevated plus maze was used to evaluate short-term spatial memory (Itoh *et al.,* 1990). The elevated plus maze for mice consist of two perpendicular open arms (21.5 x 7.5 cm) and two closed arms (21.5 x 7.5 x 20 cm) which extends from a central 7.5 x 7.5 cm platform. The platform and floor were made from wood, and the lateral walls of the closed arms were made of wood painted black. The maze was elevated 38 cm above the floor. On the 1st day (training), each animal was placed at the end of one open arm, facing away from the central platform. The transfer latency of the mouse to move from the open to the enclosed arms was recorded within 90s. Following entry into the arm, the animals were allowed to explore the apparatus for 30s. Twenty-four hours later, the second trial (retention test) was performed and the animals were observed for 90s. After each trial, the maze was wiped with a cotton wool dipped in 70% ethyl alcohol and allowed to dry to remove any olfactory clue or odour.

3.2.3 Biochemical assessment

The blood and brain samples were collected using cardiac puncture and surgical incision, respectively and the following biochemical assays were carried out in the Department of Chemical Pathology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

3.2.3.1 Assessment of lipid profiles

These were assessed spectrophotometrically, using enzymatic colometric assay kits (Randox, Northern Ireland), while low-density lipoprotein-cholesterol (LDL-C) was calculated according to the protocol of Friedewald *et al.* (1972).

Determination of serum total cholesterol:

The serum level of total cholesterol was quantified after enzymatic hydrolysis and oxidation of the sample as described by method of Stein (1987). 1000µl of the reagent was added to each of the sample and standard. They were incubated for 10 minutes at 20-25 ^{0}C and the absorbance of the sample (A_{sample}) and standard ($A_{standard}$) were measured against the reagent blank within 30 minutes. The value of TC present in serum was expressed in the unit of mg/dl.

TC concentration = A *sample* /A *standard x* concentration mg/dl

Determination of serum triacylglycerol:

The serum triacylglycerol level was determined after enzymatic hydrolysis of the sample with lipases as described by method of Tietz (1990). 1000µl of the reagent was added to each of the sample and standard. This was incubated for 10 minutes at 20-25 ^{0}C and the absorbance of the sample (A_{sample}) and standard ($A_{standard}$) were measured against the reagent blank within 30 minutes at 546nm.The value of Triacylglycerol present in the serum was expressed in the unit of mg/dl.

TGL concentration = A *sample*/A *standard x* concentration mg/dl

Determination of serum high-density lipoprotein cholesterol:

The serum level of HDL-C was measured by the method of Wacnic and Albers (1978). Low-density lipoproteins (LDL and VLDL) and chylomicron fractions in the sample were precipitated quantitatively by addition of phosphotungstic acid in the presence of magnesium ions. The mixture was allowed to stand for 10 minutes at room temperature and centrifuged for 10 minutes at 4000 rpm. The supernatant represented the HDL-C fraction.

The cholesterol concentration in the HDL fraction, which remained in the supernatant, was determined. The value of HDL-C was expressed in the unit of mg/dl.

Determination of serum low-density lipoprotein cholesterol:

The serum level of (LDL-C) was calculated according to the protocol of Friedewald *et al.* (1972) using the equation below:

LDL-C =TC- (TGL/5 + HDL-C)

The value was expressed in the unit of mg/dl.

3.2.3.2 Assessment of lipid peroxidation

The level of thiobarbituric-acid reactive substance, Malondialdehyde (MDA), as an index of lipid peroxidation was evaluated in brain sample. Quantitative measurement of lipid peroxidation of MDA was assessed using Mouse Malondialdehyde (MDA) ELISA Kit (WKEA MED SUPPLIES CORP) according to the manufacturer's instructions. The principle is based on the reaction of MDA with thiobarbituric acid (TBA); forming an MDA-TBA2 adduct that absorbs strongly at 532 nm (Janero, 1990).

3.2.3.3 Assessment of anti-oxidants enzymes

Superoxide dismutase activity:

The activity of Superoxide Dismutase enzyme (SOD) in the mice brain sample was determined using Mouse SOD ELISA assay kit (Product NWK-SOD02, Specificity: Cu/Zn, Mn and Fe Superoxide Dismutase, Sensitivity: 5 U/mL). The SOD assay was carried out according to the manufacturer's instructions. The assay kit is based on the principle of superoxide inhibition of autooxidation of hematoxylin as described by Martin *et al*. (1987).

Catalase activity:

Catalase (CAT) activity was assessed using Mouse CAT activity assay kit (NWLSS). CAT activity was assessed using NWLSS CAT activity assay kit (Product NWK-CAT01, Specificity: Catalase, Sensitivity: 6.0 U Catalase/mL). Catalase enzyme activity was measured based on the principle of catalase consumption of H_2O_2 substrate at 240 nm (Beers and Sizer, 1952).

Glutathione Peroxidase Activity:

GPx activity was assessed using NWLSS^{TM} cGPx (GPx1) ELISA assay kit (Product NWK-GPX02, Specificity: Glutathione peroxidase, Sensitivity: 12.5 pg/ml). The NWLSS™ cGPx assay is based on a sandwich Enzyme-Linked Immunosorbent assay (ELISA), where sample GPx concentration is determined by comparing the 450 nm absorbance of sample wells to the absorbance of known standards (Takebe, 2002).

3.2.4 Statistical analysis

Data obtained were expressed as Mean ± SEM. Statistical analysis was carried out using SPSS version 17 and all analysis was done using one way ANOVA followed by Tukey's post-hoc test. Values with $P < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Neurobehavioural Assessment

4.1.1 Beam walk test for the assessment of motor coordination

Results of the assessment of the motor coordination using beam walk test presented in table 4.1 showed the latency and number of foot slips in the Every Other Day Feeding (EODF), Resveratrol (RESV), Environmental Enrichment (EE), and Resveratrol (RESV) plus Environmental Enrichment groups (EE) respectively when compared to the control group. However, none of the animal fell observed during the experiment. There was no significant statistical difference observed between the various treatment groups when compared to the control.

4.1.2 Hang test for the assessment of motor strength

Results of the assessment of motor strength using hang test are presented in table 4.2. Results obtained shows no significant statistical difference in the various treatment groups when compared to the control.

4.1.3 Elevated plus maze for learning and memory assessment

The results of memory and learning assessment using the elevated plus maze is presented in table 4.3. Result obtained shows no significant statistical difference ($P > 0.05$) in the transfer latency (memory) between the various treatment groups when compared to the control.

On the other hand, there was a significant increase ($P < 0.05$) in the transfer latency (acquisition) in EODF group (69.20 ± 9.03 secs) when compared to the control group (36.80 ± 5.58 seconds).

TABLE 4.1: Effect of resveratrol-induced caloric restriction and environmental enrichment on motor coordination using beam walk test in Swiss albino mice

Groups	**Number of Foot Slips**	**Time taken to complete the task (Seconds)**
Control	1.20 ± 0.73	10.20 ± 2.13
Every Other Day Feeding (EODF)	1.80 ± 0.66 ^{ns}	9.20 ± 1.96 ^{ns}
Resveratrol (50mg/kg)	1.60 ± 0.24 ^{ns}	10.40 ± 1.96 ^{ns}
Environmental Enrichment (EE) only	0.40 ± 0.40 ^{ns}	9.20 ± 1.66 ^{ns}
Resveratrol	0.00 ± 0.00 ^{ns}	5.60 ± 0.87 ^{ns}

(50mg/kg) treated animals in an Enriched Environment (EE)	

[ns] Not statistically significant

TABLE 4.2: Effect of resveratrol-induced caloric restriction and environmental enrichment on motor strength in Swiss albino mice using hang test

Groups	**Percentage Hanging Time**
Control (CMC)	77.98 ± 10.03
Every Other Day Feeding (EODF)	61.98 ± 4.54 [ns]
Resveratrol (50mg/kg) only	51.98 ± 16.61 [ns]
Environmental Enrichment (EE) only	66.00 ± 15.07 [ns]
Resveratrol (50mg/kg) treated group in an Enriched Environment	66.02 ± 10.98 [ns]

[ns] Not statistically significant

TABLE 4.3: Effect of resveratrol-induced caloric restriction and environmental enrichment on learning and memory using elevated plus maze in Swiss albino mice

Groups	Transfer latency (Acquisition) (Seconds)	Transfer latency (Retention) (Seconds)
Control	36.80 ± 5.58	13.20 ± 2.39
Every Other Day Feeding (EODF	69.20 ± 9.03*	10.40 ± 1.03
Resveratrol (50mg/kg)	44.20 ± 8.15	13.00 ± 2.07
Environmental Enrichment (EE) only	25.60 ± 4.12	8.00 ± 0.89
Resveratrol (50mg/kg) treated group in an enriched Environment	18.00 ± 3.03	10.00 ± 1.61

* Significance ($p < 0.05$)

4.2 Determination of Lipid profile

The concentration of lipid profile in the treatment and the control groups was presented in table 4.4. There was a significant increase ($P < 0.05$) in the Low Density Lipoprotein Cholesterol concentration (LDL) in the EODF (1.72 ± 0.12 mg/dl) and EE groups (1.73 ± 0.16 mg/dl) respectively when compared to the control group (1.11 ± 0.07 mg/dl).

4.3 Assessment of Lipid Peroxidation

4.3.1 Malondialdehyde (MDA) assay

The concentration of MDA in the various treatment groups and the control was presented in figure 4.1. A significant decrease ($P < 0.05$) was observed in the RESV + EE group (1.50 ± 0.05 IU/L) when compared to the control group (1.84 ± 0.09 IU/L).

4.4 Antioxidant Enzyme Analysis

4.4.1 Assessment of superoxide dismutase activity

The activity of Superoxide Dismutase enzyme (SOD) in the treatment and the control groups was presented in Figure 4.2. There was no any significant statistical difference observed in SOD activities in the various treatment groups when compared to the control group.

4.4.2 Assessment of catalase activity

The activity of Catalase (CAT) in the treatment and the control group was presented in figure 4.3. There was no significant statistical difference observed in the CAT activities in the various treatment groups when compared to the control group.

TABLE 4.4: Effect of resveratrol-induced caloric restriction and environmental enrichment on lipid profile in Swiss albino mice in the study

Groups	**Total Cholesterol (mg/dl)**	**Triacylglycerol (mg/dl)**	**High Density Lipoprotein-Cholesterol (mg/dl)**	**Low Density Lipoprotein-Cholesterol (mg/dl)**
Control	2.24 ± 0.09	0.76 ± 0.07	0.98 ± 0.09	1.11 ± 0.07
Every Other Day Feeding	2.54 ± 0.09	1.08 ± 0.09	0.60 ± 0.09	1.72 ± 0.12*
Resveratrol (50mg/kg)	2.12 ± 0.08	0.72 ± 0.15	0.68 ± 0.09	1.29 ± 0.07
Environmental Enrichment only	2.44 ± 0.10	0.94 ± 0. 07	0.62 ± 0.09	1.73 ± 0.16*
Resveratrol (50mg/kg) + Environmental Enrichment	2.56 ± 0.10	0.80 ± 0.15	0.94 ± 0.13	1.46 ± 0.08

* Significant ($P < 0.05$)

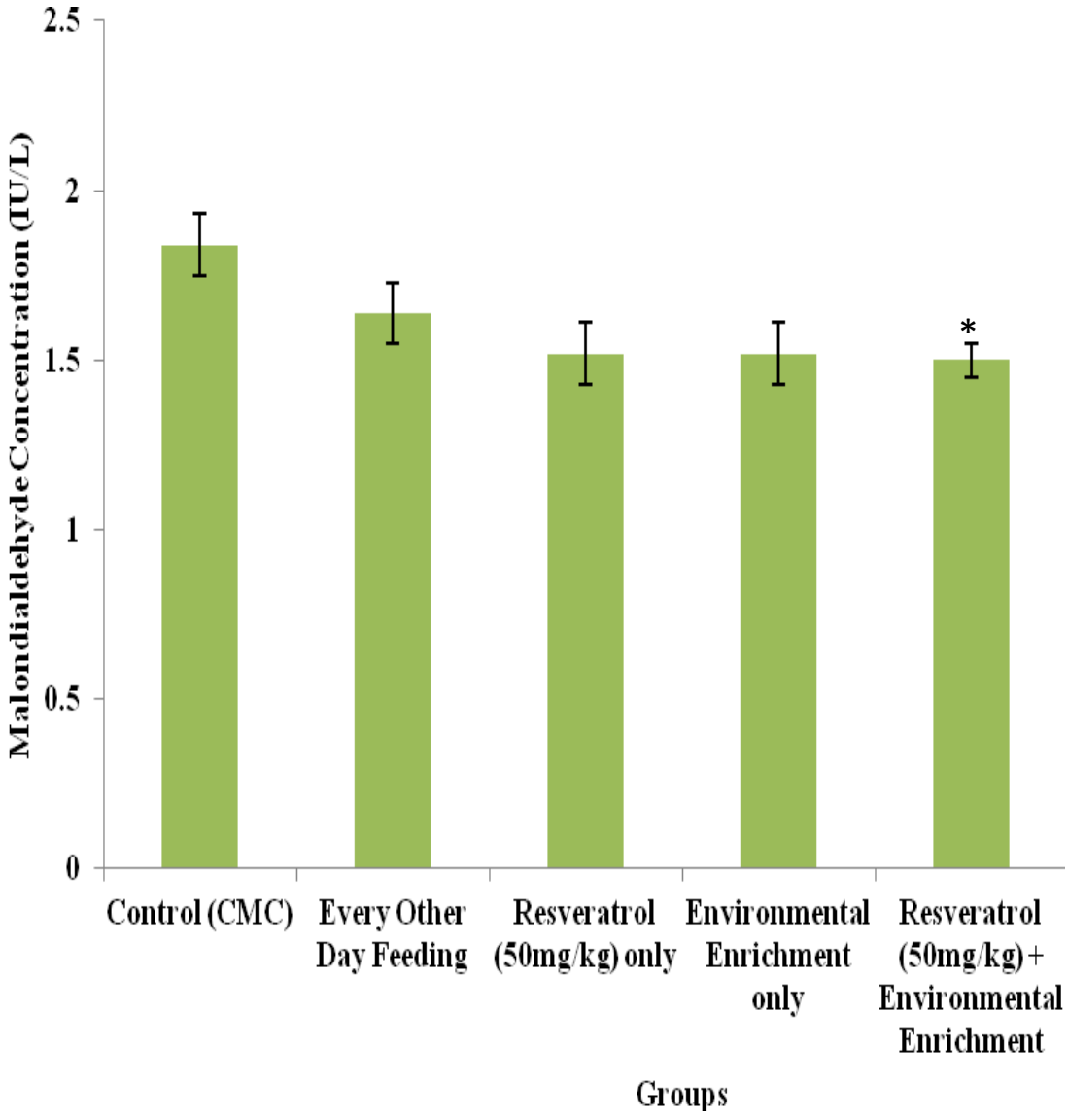


Figure 4.1: Effect of Resveratrol-induced caloric restriction and environmental enrichment on malondialdehyde concentration in Swiss albino mice

* Significance ($p < 0.05$)

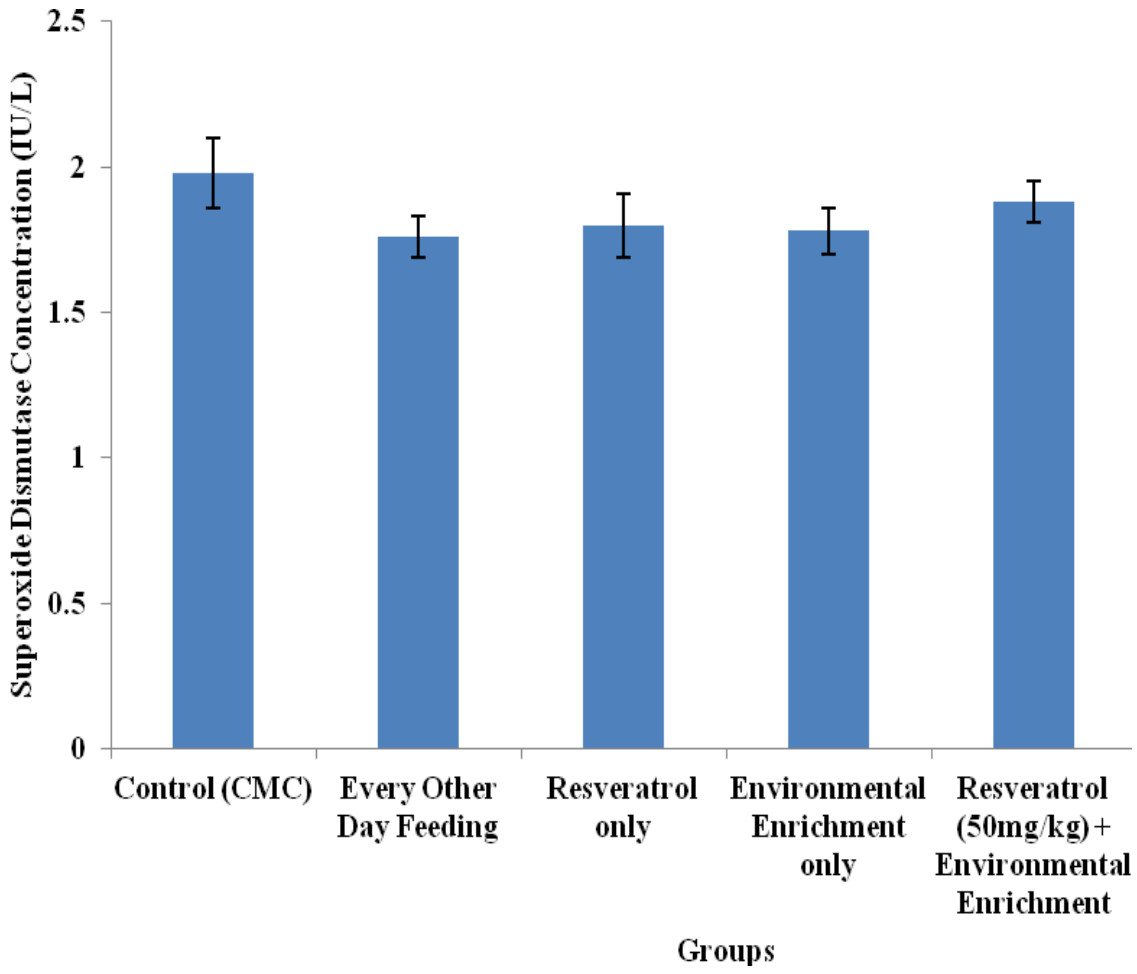


Figure 4.2: Effect of Resveratrol-induced caloric restriction and environmental enrichment on superoxide dismutase activity in Swiss albino mice

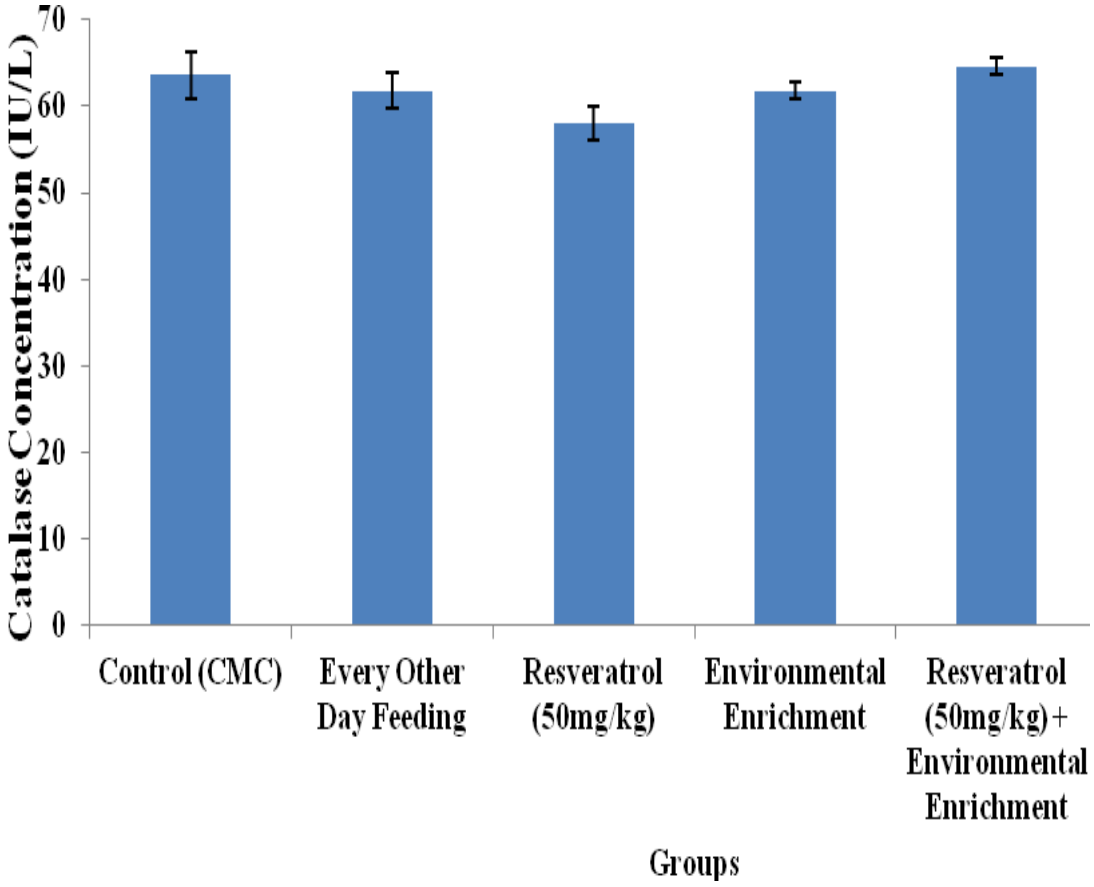


Figure 4.3: Effect of Resveratrol-induced caloric restriction and environmental enrichment on catalase activity in Swiss albino mice

4.4.3 Assessment of glutathione peroxidise activity

The activities of Glutathione Peroxidase enzyme (GPx) in the treatment and the control group was presented in figure 4.4. A significant decrease ($P < 0.05$) in the activities of GPx was observed in the RESV + EE (41.00 ± 2.02 IU/L) when compared to the control group (59.90 ± 2.85 IU/L).

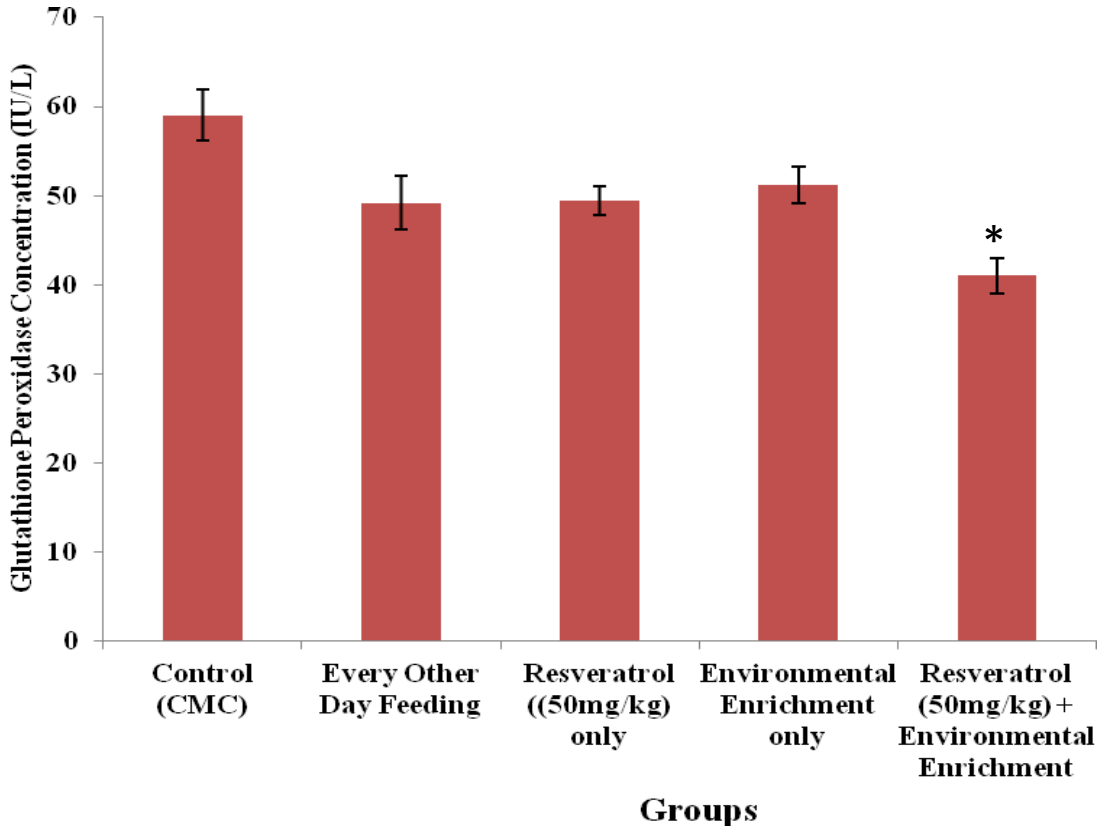


Figure 4.4: Effect of resveratrol-induced caloric restriction and environmental enrichment on glutathione peroxidase activity in Swiss albino mice

* Significance ($p < 0.05$)

CHAPTER FIVE

5.0 DISCUSSION, SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Resveratrol has been shown to poses a neuroprotective effect in both *in vivo* and *in vitro* models of Parkinson's disease (Mukherjee *et al*., 2010). Results obtained from this study demonstrated an improvement in motor coordination in the environmental enrichment, and resveratrol treated group in an enriched environment using beam walk test, although the decrease in number of foot slips and latency were not statistically different when compared to the control group. The results of motor strength using hang test were also not significantly different between the groups. This is contrary to the findings of Anandhan *et al.* (2010) who reported that resveratrol improves motor strength using hang test in MPTP challenged mice. Both Resveratrol and Environmental Enrichment have also been reported to extend lifespan and improved motor function in drosophila and rat models of Parkinson's disease respectively by activating sirtuins gene (Anastasia, *et al*., 2009; Long *et al*., 2009).

Caloric Restriction and Environmental Enrichment have been found to attenuate age-related deficits in learning and memory (Takahashi *et l.,* 2006; Anastasia *et al*., 2009), and increase resistance of neurons to excitotoxic, oxidative, and metabolic insults and improve behavioural outcomes in experimental models of Alzheimer's and Huntington's disease (Duan *et al*., 2001). The result obtained from this study using the elevated plus maze for memory though not statistically significant showed a slight decrease in the transfer latency (retention) across the groups when compared to the control with the least mean value obtained in the environmental enrichment group. This is in agreement with the findings of

Harburger *et al*. (2007) who found no significant change in spatial memory of young male mice when compared to the control after exposing them to enriched environment for a period of one month. These difference was however, attributed to the age as well as the duration of exposure of the animals to Enriched Environment (Harburger *et al*., 2007).

The result obtained on the effect of resveratrol-induced caloric restriction on memory of mice was also in corroboration with the findings of Deng *et al.* (2009) who reported no significant change in short time memory of mice using the passive avoidance test for memory after subjecting mice to a 20% caloric restriction for period of one month. However, no uniform conclusion has been reached on the effect of caloric restriction on cognitive functions in young animals. Some investigators suggested that caloric restriction enhanced learning and memory in young animals (Wu *et al*., 2003; Hashimoto and Watanabe, 2005; Roberge *et al*., 2007), while others reported the negative effects of caloric restriction on cognitive functions (Yanai *et al*., 2004; Tucci *et al*., 2006). This discrepancy was attributed to several factors including differences in Caloric Restriction treatment, duration of caloric restriction, species or strain of animal and type of behavioural test (Deng *et al.,* 2009).

On the other hand, a significant increase was observed in transfer latency (acquisition) in every other day feeding group when compared to the control group. This might probably result from exhaustion caused by food deprivation or starvation in the group which may results to the increase in the transfer latency during the training or acquisition period.

Numerous studies on calorie restriction (CR) in various organisms have shown several beneficial effects of not only decreased body fat and blood pressure, increased insulin sensitivity, and improved lipid profile but also improved endothelial function, decreased oxidative damage by reducing energy flux and metabolism, and decreased fat accumulation (Choi *et al*., 2011). In the present study, a significant increase in low density lipoprotein-cholesterol (LDL) was observed in the every other day feeding (EODF) and environmental enrichment (EE) groups, respectively. This might probably be due excessive dietary consumption resulting from starvation in the EODF group and increased in metabolic activity caused by enrichment in the EE group which may consequently results to increase in the concentration of LDL concentration in both groups when compared to the control. This result was in conflict with most of the findings on the effect of CR on lipid profile where CR was observed to cause a decrease in the concentration of LDL and a proportionate increase in concentration of High Density Lipoprotein (HDL) both in rodents and humans (Bhat *et al.,* 2001; Mansi *et al*., 2007).

The results obtained for the MDA and antioxidant status demonstrates a significant decrease in Malondialdehyde (MDA) and Glutathione Peroxidase (GPx) concentration in the Resveratrol plus Environmental Enrichment (RESV + EE) group when compared to the control. This finding was contrary to the results obtained by Robb *et al*. (2008) on the effect of administration of dietary Resveratrol on MnSOD expression and activity in mouse brain and that of Xu *et al.* (2003) on the modulations by dietary restriction on antioxidant enzymes and lipid peroxidation in developing mice. The decrease in antioxidant activity in the RESV + EE group might probably be due to the scavenging of free radicals by

Resveratrol which prevails over the up-regulation of antioxidant enzymes (Bour and Sinclair, 2006).

Caloric restriction has been consistently found to increase life span of experimental animals and this has been attributed to decreased mitochondrial respiration and resultant decrease in free radical production (Casadesus *et al*., 2002). Superoxide dismutase (SOD) which serves as a first line of defence against the detrimental effects of ROS scavenge superoxide anions (O_2^-) and catalyzes the dismutation of superoxide radical (O_2^-) to oxygen and hydrogen peroxide (H_2O_2), while Catalase and Glutathione Peroxidase (GPx) detoxifies H_2O_2 to water (H_2O) and oxygen (O_2) and consequently removes lipid peroxides (Ugochukwu *et al*., 2004).

Although resveratrol has been reported to behave as a poor scavenger of ROS *in vitro*, it however functions as a potent antioxidant *in vivo*. The *in vivo* antioxidant property of resveratrol probably arises from its ability to increase nitric oxide (NO) synthesis, which in turn functions as an *in vivo* antioxidant, scavenging superoxide radicals (Das and Maulik, 2006). Nitric oxide (NO) behaves as a potent antioxidant *in vivo* due to the presence of unpaired electron and Resveratrol was reported to induce NO synthesis and lowers oxidative stress in the ischemic reperfused heart, brain, and kidney (Hatori *et al*., 2002). The affinity of NO for O_2– was observed to be far greater than the affinity of superoxide dismutase (SOD) for O_2–, hence, NO may compete with SOD for O_2–, thereby removing O_2– and sparing SOD and other endogenous or primary antioxidant enzymes for other scavenging duties (Das and Maulik, 2006).

5.2 Summary

In summary, the results obtained from this study demonstrated that resveratrol induced-caloric restriction and environmental enrichment have no significant effects on neurobehavioural responses coupled with some significant biochemical alterations in lipid profile, lipid peroxidation and antioxidant enzymes activities in young healthy mice over a period of four weeks.

5.3 Conclusion

In conclusion, resveratrol induced-caloric restriction and environmental enrichment have no effect on neurobehavioural responses in young healthy Swiss albino mice in vivo over a period of four weeks.

5.4 Recommendation

Based on the findings of this study, we make the following recommendations;

i. The need to carry out a long term study on the modulatory role of resveratrol-induced caloric restriction and environmental enrichment on neurobehavioural responses in young healthy mice.
ii. The need to understand the mechanism through which resveratrol-induced caloric restriction and environmental enrichment elicits their physiological responses in young healthy mice *in vivo*.
iii. The need to carry out a dose dependent effect of resveratrol induced caloric restriction on neurobehavioural responses in young healthy mice.

REFRENCE

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APPENDICES

Appendix 1:

TABLE 4.2: Effect of Resveratrol induced Caloric Restriction and Environmental Enrichment on Lipid Peroxidation and anti-oxidant enzymes in Mice

Groups	**SOD**	**CAT**	**GPx**	**MDA**
Control (CMC)	1.98 ± 0.12	63.60 ± 2.66	59.00 ± 2.85	1.84 ± 0.09
EODF	1.76 ± 0.07	61.80 ± 1.98	49.20 ± 3.01	1.64 ± 0.09
Resveratrol only	1.80 ± 0.11	58.00 ± 1.87	49.40 ± 1.57	1.52 ± 0.09
EE only	1.78 ± 0.08	61.80 ± 1.07	51.20 ± 2.03	1.52 ± 0.09
Resveratrol + EE	1.88 ± 0.07	64.60 ± 0.93	41.00 ± 2.02*	1.50 ± 0.05*