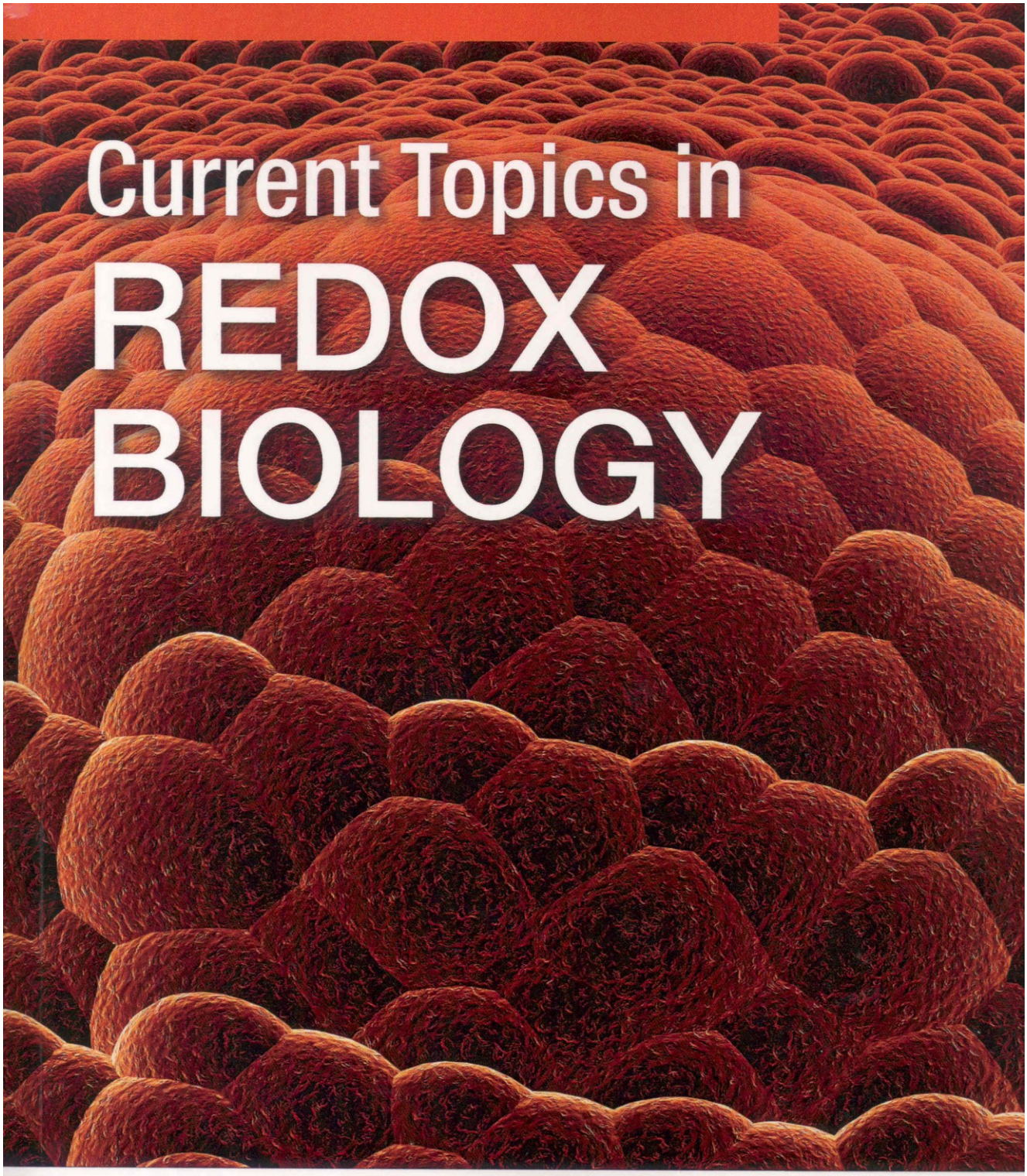
Current Topics in REDOX BIOLOGY



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Protein Carbonylation, a Marker of Oxidative Stress, During Aging and its Modulation by Dietary Restriction

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ABSTRACT

Aging is a progressive loss of function accompanied by increasing mortality with advancing age. The lifespan of an organism is the sum of deleterious changes and counteracting repair and maintenance mechanisms that respond to damages. There are several causes attributed to the phenomena of aging phenotype, however, free radical theory of aging has gained much attention in these many years. Excessive bioavailability of reactive oxygen species (ROS) generated in the mitochondria during aging leads to oxidative stress. High level of ROS which may be incompletely neutralized by the endogenous antioxidants within the cell results in the oxidation of lysine, arginine, or proline residues in proteins and has been frequently used as a marker for oxidative protein modification termed as carbonylation. We chose to study the level of such oxidative marker and its modulation by long-term dietary restriction in mouse model system. Dietary restriction, a reduction in calorie intake without malnutrition, is known to extend the lifespan in experimental animals and delays the onset of various age-associated diseases. Protein carbonylation in the brain and heart of young and aged mice was investigated. Additionally, the levels of ROS and catalase were also measured in the cerebral hemispheres of young and aged mice in our laboratory. The oxidative stress parameters (ROS and protein carbonylation) were significantly higher in aged animals as compared to their young counterparts. We have also observed reduced catalase expression in the brain of aged mice, indicative of lowered antioxidative system giving rise to an increased oxidative stress in aged animals. However, when aged mice were subjected to a long-term dietary restriction regimen (alternate days of feeding) of 3 months there was a significant lowering of protein carbonylation in aged mice indicating the anti-oxidative, tissue-protective and anti-aging effects of dietary restriction.

Key words: Aging, protein carbonyls, dietary restriction, catalase, ROS, cerebral hemispheres, heart

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INTRODUCTION

Aging is a multifactorial process which is characterized by a progressive decline in biological functions with time, and results in a decreased resistance to multiple forms of stress, as well as an increased susceptibility to numerous diseases. Recently, oxidative stress is viewed as a causative factor in aging and numerous age-related diseases. Various theories have been proposed in causing aging and the free radical theory of aging is one of the most prominent amongst them. This review summarizes the role of free radicals and oxidative stress in aged tissues and their augmentation by dietary restriction strategies.

The free radical theory of aging

The free radical theory of aging (FRTA), proposed by Harman (1956) postulates that increase reactive oxygen species (ROS) accompany aging and accumulation of oxidative damage from endogenous ROS results in cell loss, organ failure and ultimately death. A simplified diagrammatic representation of this theory is illustrated in Fig. 1. A more modern version of this tenet is the "oxidative stress theory" of aging. An imbalance between generation and elimination of ROS by antioxidant defences results in excessive bioavailability of ROS which leads to oxidative stress in a physiological milieu.

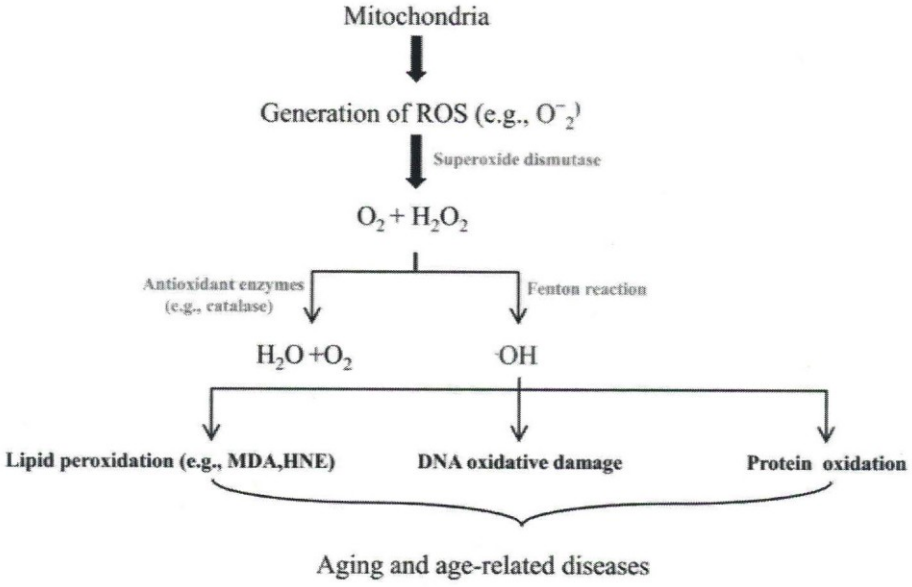

Figure 1: Free radical generation and its endogenous neutralization. Excess of ROS get diverted through Fenton reaction leading to lipid peroxidation, protein oxidation and DNA oxidative damage which gives rise to aging and age-related diseases.

Reactive oxygen species

ROS which are generated in the mitochondria at complex I and III of the electron transport chain include unstable oxygen radicals such as superoxide radical (O_2^-), hydroxyl radical ($^{\cdot}OH$) and nonradical molecules like hydrogen peroxide (H_2O_2). These ROS which are continually generated as byproducts of normal aerobic metabolism, can also be produced to a greater extent under stress and pathological conditions, as well as taken up from the external environment. Additional examples of intracellular sources of ROS production include reactions involving peroxisomal oxidases (Schrader and Fahimi, 2004), cytochrome *P*-450 enzymes (Zangar et al., 2004), NAD (P)H oxidases (Li et al., 2001), or xanthine-xanthine oxidase (Rieger et al., 2002). At low levels, ROS have important intracellular signaling functions, particularly for the control of ventilation, nerve transmission, and immune regulatory processes (Chung et al., 2006) and are therefore considered "absolutely essential for the regulation of the metabolome" (Linnane et al., 2007). Cellular ROS sensing and metabolism are tightly regulated by a variety of proteins involved in the redox mechanisms. ROS directly interact with critical signaling molecules to initiate signaling in a broad array of cellular processes, such as proliferation and survival (MAP kinases, PI3 kinase, PTEN, and protein tyrosine phosphatases), ROS homeostasis and antioxidant gene regulation (thioredoxin, peroxiredoxin, Ref-1, and Nrf-2), mitochondrial oxidative stress, apoptosis, and aging (p66Shc), iron homeostasis through iron–sulfur cluster proteins (IRE–IRP), and ataxia-telangiectasia mutated (ATM) - regulated DNA damage response. Hence, the initial and direct regulation of signaling molecules by ROS is also referred to as the "oxidative interface" (Ray et al., 2012). Mitochondria in mammalian cells undergo quantal, stochastic bursts of superoxide production, which is visualized as 'mitochondrial flashes' (mitoflashes) by the sensor protein circularly permuted yellow fluorescent protein (cpYFP) in the mitochondrial matrix. The mitoflash frequency is highly sensitive to oxidative stress and is used in monitoring free-radical production for testing mitochondrial theory of aging. Reports indicate that mitoflash activity in pharyngeal muscles of *Caenorhabditis elegans* increases during reproduction phase and when animals started to age. It has been elegantly shown that mitoflash activity of mitochondria is well correlated with the lifespan of C. elegans (Shen et al., 2014).

To protect against the deleterious effects of oxidative by-products of normal cellular metabolism, and also under conditions of oxidative stress, cells have an effective endogenous system of antioxidant enzymes such as superoxide dismutases (SOD), catalase and glutathione peroxidise (GPx). SOD converts superoxide anion into oxygen and hydrogen peroxide, a relatively stable ROS. Catalase and GPx break down hydrogen peroxide, including that released by the SODs, into O_2 and water.

Protein carbonylation

ROS mediated oxidative stress causes several changes in cellular biomolecules giving rise to its molecular effects. One of such changes is the protein oxidation. Oxidative modification of proteins leads to formation of protein carbonyls which are associated with oxidative stress. Primary modification can result from hydroxyl radical attack to the side chains of certain amino acids like proline, arginine, lysine, and threonine—which became oxidized to aldehydes or ketones. Such hydroxyl radicals can be produced by ionizing radiation (Schuessler and Schilling, 1984) or by metal-catalyzed oxidation (MCO), a Fenton reaction of metal cations with hydrogen peroxide (Stadtman and Levine, 2000). Protein carbonyls is also associated with a number of diseases, including amyotrophic lateral sclerosis, Alzheimer's disease, respiratory distress syndrome, muscular dystrophy, cataractogenesis, rheumatoid arthritis, progeria, and Werner's syndrome. It is implicated also in atherosclerosis, diabetes, Parkinson's disease, essential hypertension, cystic fibrosis, and ulcerative colitis (Berlett and Stadtman, 1997). The intracellular level of oxidized proteins reflects the balance between the rate of protein oxidation and the rate of oxidized protein degradation. Oxidative modification thus caused may be inadequately repaired or eliminated and can lead to physiological deterioration and phenotypic changes in the aged and increased incidences of age-related diseases and death, and may be a key determinant of maximum lifespan (MLS) of a species.

Dietary restriction

Dietary restriction (DR) is a well known intervention to promote longevity of various model organisms. Dietary restriction (DR), a reduction in total calories intake without malnutrition is the only non-genetic intervention which extends both mean and maximal lifespan in a variety of species (Sharma, 2004). DR has been shown to improve a number of health outcomes including reducing several cardiac risk factors (Fontana et al., 2007), improving insulin-sensitivity (Larson-Meyer et al., 2006), and enhancing mitochondrial function (Civitarese et al., 2007). Additionally, prolonged caloric restriction has also been found to reduce oxidative damage to both DNA and RNA, as assessed through white blood cells in humans (Hofer et al., 2008). Fasting periods and intermittent fasting regimens in particular can trigger similar biological pathways as caloric restriction. Hence, there is increasing scientific interest in further exploring the biological and metabolic effects of intermittent fasting periods. The oxidative damage within a tissue represents the equilibrium between the rates of oxidant generation and the rates of oxidant scavenging, repair and turnover processes (Beckman and Ames, 1998). These processes are all modified in a tissue-specific and time-dependent manner by dietary restriction (Merry, 2000).

Cellular and molecular mechanisms of action of dietary restriction

Dietary restriction prolongs the lifespan of yeast, roundworms, rodents and monkeys, even when initiated in midlife (Guarente and Picard, 2005). Age-related deficits in

learning and motor coordination are reduced by dietary restriction in rodents. Various beneficial effects of caloric restriction on the nervous system may result from the activation of adaptive cellular stress responses, in a process called hormesis (Calabrese et al., 2007; Mattson 2008). The dietary restriction imposes a mild stress on cells which results in the activation of stress response pathways including those involving transcription factors such as CREB, Nrf-2 and NF-κB (Mattson and Cheng, 2006). Some of the adaptive stress response proteins are up-regulated in neurons in response to dietary restriction (Martin et al., 2006).

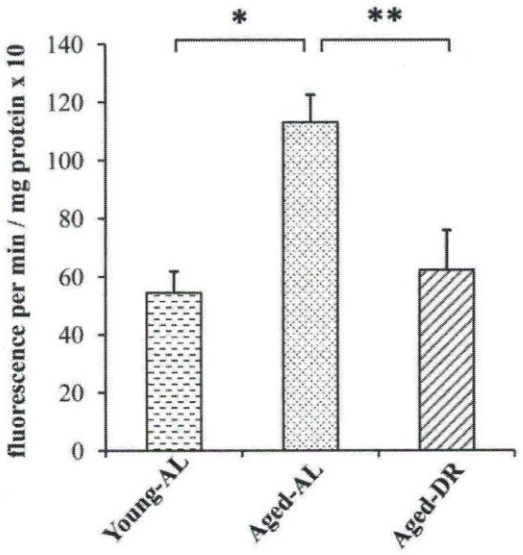

Figure 2: Effect of dietary restriction (DR) on the level of reactive oxygen species from cerebral hemispheres of young (4 week) *ad libitum* mice, aged (84 week) *ad libitum* mice and aged-dietary restricted mice (Aged-DR). Values are expressed as mean ± S.D. (n = 5) in each group. * and ** indicate statistical significance at $p<0.001$ and $p<0.02$, respectively.

Dietary restriction decreases age-related tissue concentrations of peroxidised lipids, protein carbonyls and damaged bases in nuclear and mitochondrial DNA (Merry 2004; Hunt et al. 2006). Several mechanisms have been proposed to explain antioxidant properties of calorie restriction. Some studies suggested that calorie restriction enhanced antioxidant defenses, including superoxide dismutase, glutathione peroxidase and catalase (Agarwal et al. 2005; Rankin et al. 2006; Dkhar and Sharma, 2014). A decrease in the mitochondrial production of reactive oxygen species has been reported specifically at complex I of the respiratory chain during dietary restriction regimen (Sohal et al. 1994; Gredilla and Barja 2005).

Aging and various neurodegenerative disorders are characterized by increased levels of several inflammatory mediators (Chung et al. 2002; Sarkar and Fisher, 2006). NFκB, a

central component of this inflammatory process, can be triggered by several sources of injury such as reactive oxygen or nitrogen species or amyloid Aβ and causes enhanced transcription of interleukins (IL1β, IL2, IL4, IL6), tumor necrosis factors (TNFα and TNFβ) and the pro-inflammatory enzymes cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in various tissues, including the brain (Gloire et al., 2006; Valerio et al., 2006). Calorie restriction reduced NFκB levels (probably a Sirt1-dependent process), blocked the synthesis of interleukins and TNFα and suppressed the activity of COX-2 and iNOS in animal models and in humans (Ugochukwu and Figgers, 2007).

Our present investigation focuses on the effects of three months of DR (alternate day of feeding) on carbonyl levels in the post-mitotic tissues (cerebral hemispheres, heart) of aged mice as well as ROS level and catalase activities in the cerebral hemispheres. The results indicate that protein carbonyl content increased with age in the cerebral hemispheres and heart (Figs. 3 & 4 A&B). These tissues are also the targets of several age-related degenerative disorders in which oxidative stress has been implicated. The age-related accumulation of oxidized protein may be due to either or both increased protein oxidative damage and decreased oxidized protein degradation and repair. Age-

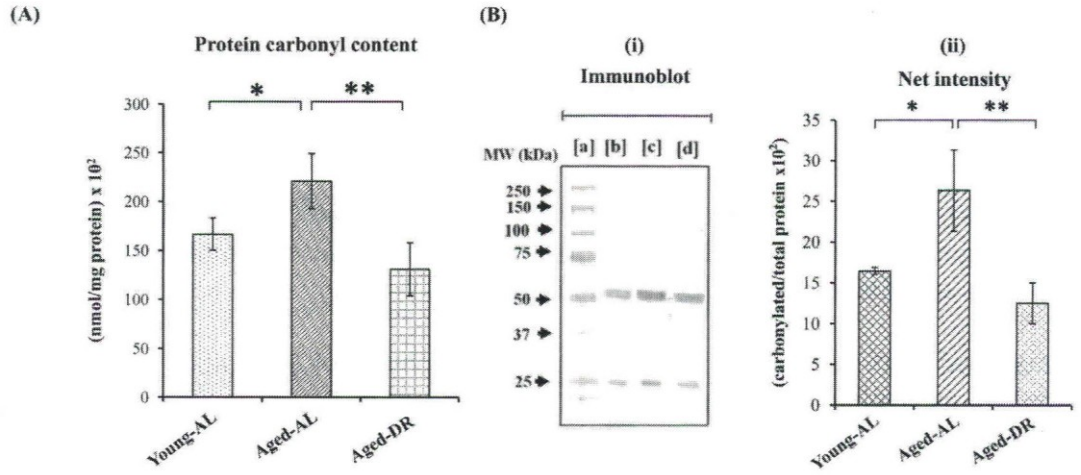

Figure 3(A): Effect of dietary restriction (DR) on protein carbonyls in cerebral hemispheres of young (4 week) *ad libitum* mice, aged (84 week) *ad libitum* mice and aged-dietary restricted mice (Aged-DR). Values are expressed as mean ± S.D. (n=6) in each group. * and ** represent statistical significance at $p<0.05$ and $p<0.02$, respectively [Dkhar and Sharma, 2014].

Figure 3(B): (i) Immunoblots of protein carbonyls in cerebral hemispheres of *ad libitum* (AL) *fed* (4 week), aged (84 week) and aged-dietary restricted mice (Aged-DR). Lane a: molecular weight markers, b:Young-AL, c: Aged-AL, d: Aged-DR. **(ii)** The immunoblots (b, c and d) were quantified by densitometry. The data shown are presented as arbitrary values of net intensity and represent the mean ± SD of three separate experiments. * and ** represent statistical significance at $p<0.05$ [Dkhar and Sharma, 2014].

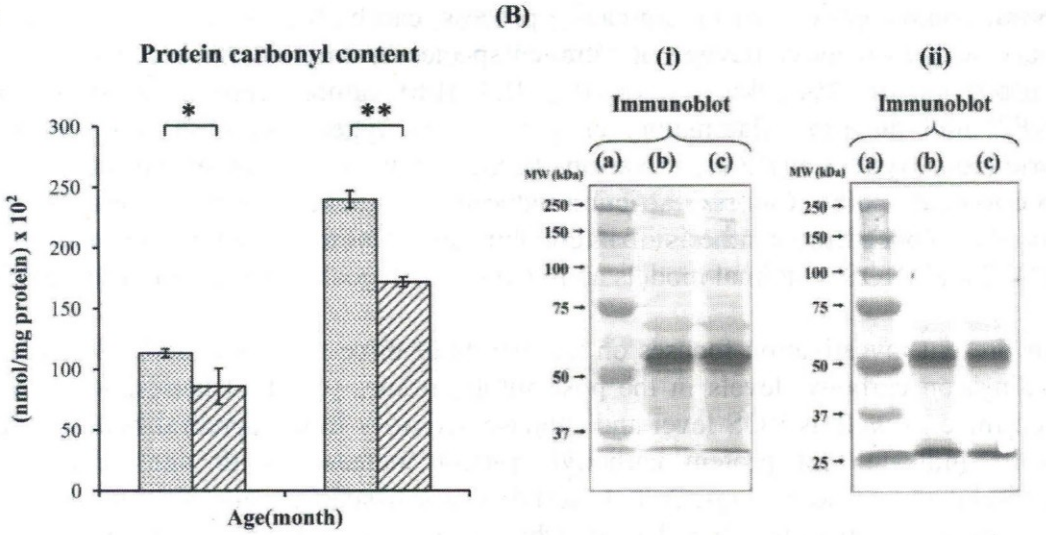

Figure 4(A): Effect of dietary restriction (DR) on protein carbonyls in heart of young (1-) and aged (18-month) mice. Values are expressed as mean ± S.D. (n=6) in each group. * and ** represent statistical significance at $p<0.05$ and $p<0.001$, respectively as compared to the control experiment.

Figure 4(B): Immunoblots of protein carbonyls in heart from **(i)** young mice and **(ii)** aged mice subjected to DR. Lane a: molecular weight markers, b: age-matched control, c: dietary-restricted mice. The data shown is a representative blot of three separate experiments.

related impairment of proteasome, the main intracellular proteolytic pathway, in a wide range of organs and cell types has been found to promote the accumulation of oxidized protein with age (Davies et al., 2001; Goto et al., 2007).

The increased ROS generation in the cerebral hemispheres of aged mice have also been reported by us (Dkhar and Sharma, 2010). Increased ROS generation was also reported during aging from Fischer 344 rats (Radak et al., 2004). Our findings showed that DR attenuates the age-dependent accretion in ROS generation (Fig. 2) The up-regulation of enzymatic and non-enzymatic antioxidants by DR also results in ROS reduction (Sohal et al., 1994; Sanz et al., 2006). An increase in proton leak, reduced state IV mitochondrial membrane potential and reduced ROS generation was observed (Lambert and Merry, 2004) in post mitotic tissues under the DR regime. Our study also showed that there is a reduction in the catalase level in the cerebral hemispheres with age which can be reversed by DR regimen (Dkhar and Sharma, 2014). Age- and DR-related changes in the catalase level have been shown in Fig. 5.

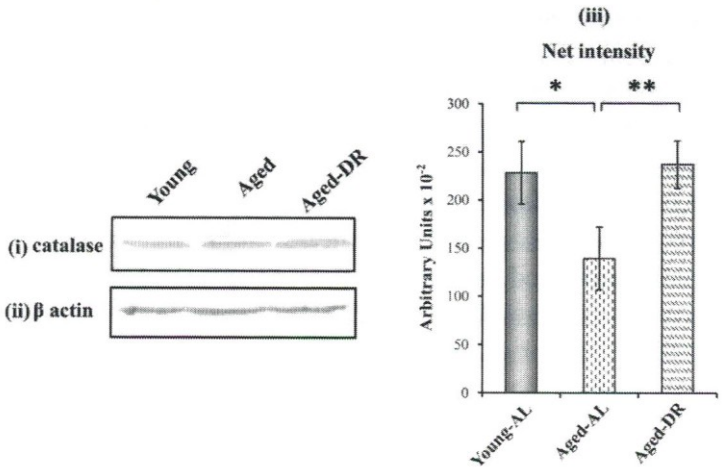

Figure 5: Effect of dietary restriction (DR) on the endogenous level of catalase from cerebral hemispheres of young (4 week) *ad libitum* mice, aged (84 week) *ad libitum* mice and aged-dietary restricted mice(Aged-DR) and **(i)** Immunoblot of the same. Lane a: molecular weight markers, b:Young-AL, c: Aged-AL, d: Aged-DR. **(ii)** β-actin is shown as a loading control. **(iii)** Densitometric analysis of the immunoprobed Western blots for catalase level (b, c and d) after normalization to actin. The bar graph shows the net intensity of the bands to the factor of 10^{-2}. Data represent the mean ± SD of three separate experiments. * and ** indicate statistical significance at $p<0.05$ and $p<0.02$ respectively [Dkhar and Sharma, 2014].

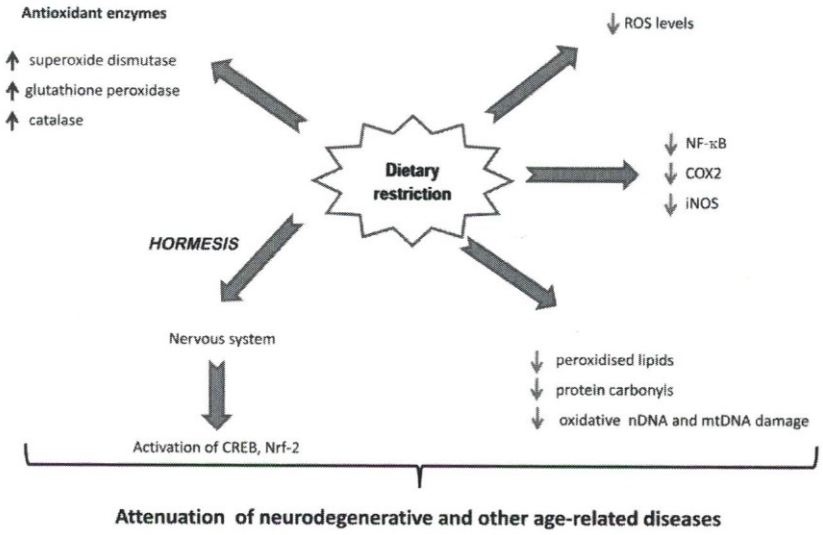

Figure 6: Impact of dietary restriction on reducing free radical load and giving rise to beneficial effects in attenuating neurodegenerative and other age-related diseases. [NF-κB: Nuclear Factor kappa B; COX2-cyclooxygenase-2; iNOS: inducible nitric oxide synthase; CREB: cAMP–responsive element binding protein; Nrf-2: Nuclear Factor-E2-Related Factor 2].

Under the dietary restriction regime, the age-related increase in protein carbonyl levels of cerebral hemispheres and heart was significantly reduced compared to the age-matched control indicating that dietary restriction decreases the age-related increase in protein carbonyl content (Figs. 3 & 4 A&B)). Tissue oxidative damage biomarkers such as peroxidation of membrane lipids, oxidative damage to DNA bases and strand breaks in nDNA and mtDNA and protein carbonyl formation, (Merry, 2000; Hamilton et al., 2001; Dkhar and Sharma, 2014) have been shown to undergo reduction by DR. At the cellular level, the basic mechanism proposed to explain the benefits of DR is the resistance to oxidative insults that occur as a response to the cellular stress in experimental animals.

CONCLUSION

Our findings implicate that there is a ROS-associated age-dependent increase in protein carbonylation in post-mitotic tissues of mice. DR has been found to attenuate the measured oxidative stress parameters in the study suggesting that DR can act as a modulator of oxidative stress and thereby extend lifespan with its anti-oxidative and anti-aging properties (Fig. 6).

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