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DIETARY RESTRICTION LOWERS ENDOGENOUS LEVELS OF OXIDATIVE STRESS IN DIFFERENT BRAIN REGIONS OF ADULT MICE

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Abstract

Increase in the cellular burden of oxidative stress is critically involved in various pathological manifestations of aging, including age-related neurological disorders. Dietary restriction can lower reactive oxygen species formation, and thereby lower oxidative damage in the brain. The brain consists of a diverse group of neurons with varying functions. However, attenuating role of dietary restriction on oxidative stress in different regions of brain is not well known. In the present study we demonstrated that by restricting diet intake for a period of six months, mice lowered the endogenous levels of oxidative stress markedly by decreasing lipid peroxidation and protein carbonyl contents in cerebral cortex, hippocampus and striatum regions of the brain. Based on these results we suggest that dietary restriction can significantly reduce oxidative stress in various regions of the brain by virtue of lowering endogenous levels of reactive oxygen species, which might prove beneficial for preserving normal brain function with age.

Key words: Oxidative stress, dietary restriction, lipid peroxidation, protein carbonyl.

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Abbreviations: AD: Alzheimer's Disease; **ANOVA**: Analysis of Variance ; **BSA**: Bovine serum albumin; **CNS**: Central nervous system; **DNPH**: Di nitro phenyl hydrazine; **HSP**: Heat shock protein; **MDA**: Malondialdehyde; **Min**: Minute; **mg**: Milligrams; **NIH**: National Institute of Health; **nm**: Nanometer; **nmol:** Nanomolar; **ROS**: Reactive oxygen species; **RPM**: Revolutions Per Minute; **SDS**: Sodium dodecyl sulphate; **SEM**: Standard error of the mean ; **Sir**: Sirtuins; **SOD**: Super oxide dismutase; **TBA**: Thio barbituric acid; **TBARS**: Thio barbituric acid reactive substance; **TCA**: Tri chloro acetic acid; **DNA**: Deoxyribonucleic acid.

INTRODUCTION

Aging is manifested by changes at anatomical, genetic, molecular and cellular levels that are mediated by various poorly characterized and progressive processes. However, in the brain age-associated increase in the reactive oxygen species (ROS) and oxidative stress have been hypothesized to be critical determinants of aging (22). The oxidative stress that occur during aging is also known to increase the vulnerability of cells as well as organ systems to various diseases including diabetes, cardiovascular disease, and neurodegenerative disorders (7, 19, 33).

Lipid peroxidation and protein carbonyl formation are standard parameters of assessing ROS induced oxidative damage. Lipid peroxidation has been convincingly recognized as a significant factor in the pathophysiology of acute CNS traumatic and ischemic injury (8, 46) and subarchanoid hemorrhage (5). The proteins and lipids involved in various functions in the brain are susceptible to oxidative modifications that may contribute to the dysfunction and degeneration of neurons that occur with aging and in neurodegenerative disorders (31). For example, lipid peroxidation and oxidative modifications of brain ion-motive ATPases,

glucose transporters, and G protein-coupled receptor signaling are implicated in the pathogenesis of Alzheimer's disease (21, 28). Several studies have reported increases in protein oxidation and lipid peroxidation in various regions of aged mammalian brains (23, 25, 35, 47). The toxicity of lipid hydroperoxides in brain is best illustrated by the degeneration of neuronal cells after the treatments of neurotoxicants such as tertiary butyl hydroperoxide (2, 1) and kainic acid (37). Similarly an increase in protein carbonyl content in brain cells also has been proposed to represent protein oxidation (38). Protein oxidative damage is a major contributor to AD-associated neuron death and decline of cognitive abilities (9, 47). In conditions of higher oxidative stress, chemical transformations of amino acid residues in proteins can lead to loss of specific protein functions (14, 48).

Overeating is a major modifiable risk factor for several age-related diseases, including neurodegenerative disease, cardiovascular disease and type 2 diabetes mellitus (32*)*. Studies showed that the aging process (49) and disease pathogenesis (29) can be slowed or halted by dietary restriction. Thus dietary restriction can delay pathological and physiological changes associated with aging (27). The prophylactic effects of dietary restriction against previously mentioned disease conditions appear to result from reduced energy intake and reduced burden of cellular oxidative stress. Importantly, dietary restriction can attenuate age-related deficits in brain function and can protect neurons against dysfunction and death in animal models of Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke (15**,** 16, 30). However, the mechanism(s) by which dietary restriction protects brain cells during aging are not well known. One major mechanism by which dietary restriction retards the aging process is its remarkable ability to reduce oxidative damages (50). Dietary restriction has been shown to lower the rate of production of ROS in mitochondria and protect cells against oxidative stress (3, 13, 41, 52). The objective of the present study was to investigate the effect of dietary restriction on free radical-mediated oxidative damage in cerebral cortex, hippocampus and striatum regions of brain of adult mice.

MATERIALS AND METHODS

Animals and Feeding

Swiss albino mice (Mus Musculus albinus) purchased from the college of veterinary science and Animal Husbandry, Mhow, MP, India were used for the experiment. All animals were 3 months aged and acclimatized at room temperature with relative humidity on a 12 hrs light: 12 hrs dark cycle. Animals were fed on a standardized normal pellet diet purchased from Hindustan Foods Pvt. Ltd. India. The composition of food was equivalent to the fortified formulation of NIH-31 diet. Water was provided *ad libitum.*

Experimental Design and Treatment

Animals were divided into two groups with ten mice in each. For the study involving dietary restriction, average daily food intake was measured over one week before the beginning of the dietary restriction period. Dietary restricted animals were given 50% (what is the average daily consumption per animal) less food (2.5 g each per day) than control group and water *ad libitum.* Mice of control group were provided standardized normal pellet diet and water *ad libitum.* Animals were housed singly in cages (30 x 20 x 12 cms) and the bedding used for mice was sterile paddy husk which was changed every week. All mice weighed 25.21 ± 0.64 g and the weight of mice was also recorded at a specified interval during the period of study. Animals were sacrificed by decapitation after 6 months of dietary restriction experiments. Brain from each group of animals were dissected and kept in normal chilled saline to remove adhering fat and blood. Different regions of brain were isolated and weighed.

Tissue Homogenization

Subsequent to dissection and weighing, the different brain regions were homogenized in phosphate buffer (pH 7.7) at a speed of about 1000-3000 rpm using tissue homogenizer (Remi, India) equipped with an electric motor while the test-tube was manually raised and lowered during the process of homogenization.

Lipid Peroxidation Assay

The lipid peroxidation in brain homogenates was determined by measuring the release of thiobarbituric acid reactive substance (TBARS) using a molar extinction coefficient of 1.56 x 10^5/min/cm as described by Ohkawa et al. (34). Briefly, the homogenate was centrifuged at 3000 g for 15 minutes and the supernatant was used for assay. Samples of 0.1ml homogenate were taken and mixed with 0.2 ml of 8.1% SDS, 1.5 ml 20% glacial acetic acid and 1.5 ml of 0.8% TBA. Following these additions, tubes were mixed and heated to 95 ºC for one hour on a water bath and cooled under tap water before mixing 1 ml of distilled water and 5 ml mixture of n-butanol and pyridine (15:1). The mixture was centrifuged at 2200 g for 10 min. The amount of MDA formed was measured by the absorbance of upper organic layer at a wave length of 532 nm in Perkin Elmer Spectrophotometer using appropriate controls. The results were expressed as nmol TBARS/mg protein.

Protein carbonyl content assay

The protein carbonyl content of the brain homogenates was assayed by the method used by Levine and coworkers (24). Briefly 100 ml of brain homogenates were incubated with 0.5ml 2,4-dinitrophenylhydrazine (DNPH) for 60 min. The protein was subsequently precipitated from the solution with the addition of 20% trichloroacetic acid (TCA). The pellet was centrifuged (3400 g) and washed three times with ethyl acetate and ethanol (1:1 v/v ratio) mixture to remove excess of DNPH. The final protein pellet was dissolved in 1.5ml of 6M guanidine hydrochloride. The carbonyl content was evaluated using Perkin Elmer Spectrophotometer at a wave length of 370nm. A standardization curve of bovine

serum albumin (BSA) was included in each assay to determine linearity and measure the extent of derivatization. The results were presented as nmol/mg protein.

Total protein Assay

Total protein content of tissue homogenates was assayed by Folin-Phenol reaction as described by Lowry et al. (26). The standard curve of bovine serum albumin (BSA) was included in each assay to determine linearity and measure the extent of derivatization.

Statistical analysis

Statistical comparison of markers of oxidative stress between the groups and between the different brain areas was made using student t test and one-way analysis of variance (ANOVA). The significance of statistical differences was set at $p < 0.05$. Barograms in the figures represent mean ± standard error of the mean (SEM). All computations were performed using PlotiT (Scientific Programming, Hazlet, MI).

RESULTS

To assess the effect of dietary restriction on oxidative damage in various regions of brain, the present study measured the lipid peroxidation as TBARS and protein carbonyl content in cerebral cortex, hippocampus and striatum. TBARS is the accurate marker of oxidative damage of lipids. We found clearly considerable endogenous levels of markers of oxidative stress in cortex, hippocampus and striatum in mice that were fed *ad libitum* (Fig. 1). These endogenous levels of TBARS in hippocampus were found to be significantly higher than in cortex and striatum regions of the brain ($p<0.05$, one-way ANOVA). Dietary restriction significantly attenuated oxidative damage in all the brain regions examined in the present study. Namely, the endogenous level of TBARS declined in cerebral cortex from 6.80 ± 0.54 to 3.90 ± 0.25 nmol/mg protein, hippocampus from 15.2 ± 0.44 to 7.59 ± 0.67 nmol/mg protein and striatum from 6.98 ± 0.32 to 5.80 ± 0.16 nmol/mg protein.

Protein carbonyl formation is the oxidative marker of protein oxidation and certain oxidized proteins could play an active role in degeneration of neurons. Similar to our observations with lipid peroxidation, we found evidence of considerable levels of endogenous protein carbonyl in all brain regions studied when the mice were kept on the *ad libitum* diet schedule (Fig. 2). Likewise, we also observed significant decreases in endogenous levels of protein carbonyls in all regions of brain included in the study from the group put on dietary restriction. The protein carbonyl content was found to decline in the cerebral cortex from 133.00 ± 12 to 112.22 ± 4 nmol/mg protein, hippocampus from 100.00 ± 9 to 52.42 ± 8.56 nmol/mg protein and striatum from 55.15 ± 5.4 to 15.17 ± 1.32 nmol/mg protein.

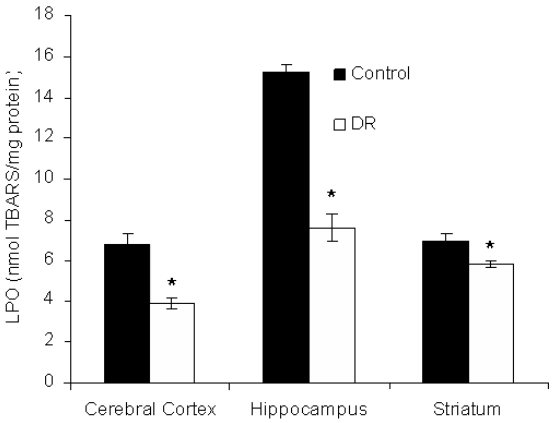

Figure1. Showing lipid peroxidation (LPO; nmol TBARS/mg protein) in cerebral cortex, hippocampus and striatum regions of brain of normal mice (control) and dietary restricted mice. *Significantly different from normal (control) animals at $p<0.05$.

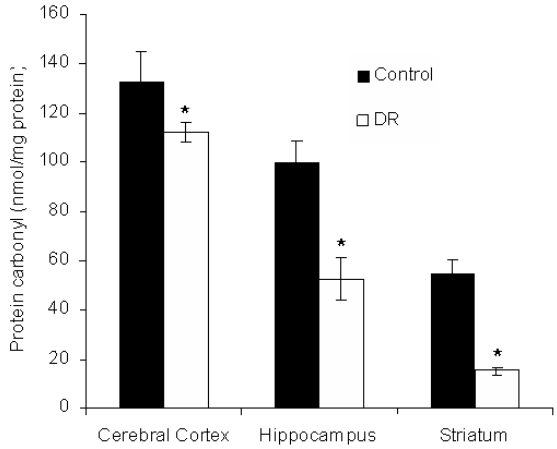

Figure 2. Showing protein carbonyl content (nmol/mg protein) in cerebral cortex, hippocampus and striatum regions of brain of normal mice (control) and dietary restricted mice. *Significantly different from normal (control) animals at $p<0.05$.

DISCUSSION

ROS are by-products of normal cellular physiology but various endogenous and exogenous triggers may stimulate their overproduction and accumulation, which may lead to oxidative damage of macromolecules within the tissue (6). Oxidatively damaged proteins, lipids, and nucleic acids play a key role in processes that mediate aging. In the present study we found evidence for accumulation of oxidative damage to proteins and lipids in different regions of the brain in animals that were put on an *ad libitum* diet. More importantly our

data suggests that dietary restriction in mice was effective in attenuating the endogenous oxidative stress-mediated cell damage in various regions of the brain.

The present study showed that the accumulation of oxidative damage to lipids in different regions of brain was a function of increased endogenous ROS production and lipid peroxidation. Lipid peroxidation proceeds by a free radical chain reaction mechanism and yields lipid hydroperoxides as major initial reaction products. Decomposition of lipid hydroperoxides is known to generate various breakdown products that display a wide variety of damaging actions (45). A growing body of evidence causally associates aldehydes that are the end products of lipid peroxidation with most of the pathophysiological consequences of oxidative stress in cells and tissues of brain (44).

In addition to lipids, cellular proteins are also susceptible to oxidative damage by ROS. The present study found that different regions of brain have varying tendency to accumulate protein carbonyl with unrestricted dietary food intake. ROS are known to convert amino groups of protein to carbonyl moieties (10, 39). Oxidative modification of proteins leads to increased recognition and degradation by proteases and loss of enzymatic activity (12, 42). Oxidative modification of different intracellular proteins, including key enzymes and structural protein can lead to formation of neurofibrillary tangles and thereby lead to subsequent degeneration of neurons in the Alzheimer's disease (4). In addition, the oxidative modification of protein could also result in secondary impairment of biomolecules, for instance inactivation of DNA polymerases in replicating DNA is capable of provoking autoimmune responses (18). Indeed, lipid peroxidation and protein carbonyl formation found in the various regions of brain in our study may serve as marker of tissue damage.

Dietary restriction in mice resulted in reduced levels of markers of oxidative stress in various regions of brain (17). Previous studies have found that dietary or calorie restriction promoted the survival of neurons in adult mice (43, 15) by regulating a homeostatic balance between endogenous levels of oxidative stress and antioxidants (27). Our findings in the present study suggest that dietary restriction alone may mediate the mitigation of oxidative stress and cellular damage by virtue of decreasing lipid peroxidation and protein carbonyl formation in different brain regions of mice. Previous studies have shown anti-aging effects of dietary restriction which involve reduction of oxidative stress and increased expression of stress resistance proteins such as HSP-70 (51). However, an important issue in considering antioxidative effect of dietary restriction is whether ROS can be lowered to manageable levels, which do not compromise the normal physiological functions of ROS. One major mechanism by which dietary restriction is thought to retard the aging process is by virtue of its remarkable ability to reduce oxidative damage as found in the present study. However, the mechanism by which dietary restriction lead to reduction in oxidative stress is not well understood. Previous studies have shown that dietary restriction results in deacetylation and activation of superoxide dismutase-2 (SOD2), presumably by upregulating SirT3 (40). The decline in oxidative stress in the brain can delay the onset of various age related disease and cell deterioration (27). Therefore, the present findings are clearly consistent with the earlier studies (11, 20, 36) in showing that dietary restriction can be an effective and viable antioxidative strategy in mitigating brain oxidant injury, thereby slowing the progression of aging. Our study specifically provides strong evidence implicating *ad libitum* diet or overeating as a major factor that could result in accumulation of damaging endogenous levels of ROS and the resulting products of lipid peroxides and carbonylated proteins in the cortex, hippocampus and striatum regions of brain. On the contrary, dietary restriction can serve as a prophylactic measure against oxidative stress by enhancing antioxidant activity and also by lowering the accumulation of indicators and/or mediators of oxidative stress, namely, lipid and protein oxidation products. These findings suggest that the dietary restriction regulate the oxidative stress pathway that plays a fundamental role in reducing the oxidative damage in different regions of brain.

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