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Amelioration of age-dependent increase in protein carbonyls of cerebral hemispheres of mice by melatonin and ascorbic acid

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ABSTRACT

Melatonin secreted by the pineal gland acts as a free radical scavenger besides its role as a hormonal signaling agent. It detoxifies a variety of free radicals and reactive oxygen intermediates including hydroxyl radical, peroxynitrite anion and singlet oxygen. Ascorbic acid (Vitamin C), a water soluble vitamin, is a naturally occurring antioxidant and cofactor in various enzymes. Protein carbonyls are formed as a consequence of the oxidative modification of proteins by reactive oxygen species. Oxidative modification alters the function of protein and is thought to play an important role in the decline of cellular functions during aging. In the present study, the effect of melatonin and ascorbic acid on age-related carbonyl content of cerebral hemispheres in mice was investigated. Protein carbonyls of cerebral hemispheres have been found to be significantly higher in 18-month-old mice as compared to 1-month old mice. Administration of a single dose of melatonin (10 mg/kg body weight) and ascorbic acid (10 mg/kg body weight) intraperitoneally for three consecutive days decreases the carbonyl content in 1- and 18-month-old mice significantly. The present study thus suggests that the formation of protein carbonyls in the cerebral hemispheres of the aging mice can be prevented by the antioxidative effects of melatonin and ascorbic acid that could in turn be beneficial in having health benefits from age-related neurodegenerative diseases.

1. Introduction

Aging is a natural biological process characterized by a progressive decline in physiological functions that affect many tissues with a more pronounced effect on brain functions. The "oxidative stress theory" states that reactive oxygen species (ROS) production increases with age leading to functional alterations, increased incidence of diseases and a reduction in lifespan (Hagen, 2003). Oxidative alterations of proteins by ROS or other reactive substances have been implicated in the progression of aging and age-related neurodegenerative disorders. An increased production of ROS in mitochondria of old vs. young animals has been demonstrated in different tissues (Richter, 1995). Oxidation reactions can mediate intra- and intermolecular cross-linking of peptides and proteins and fragmentation of polypeptide chains. Protein carbonyls (PCO) are formed as a consequence of the oxidative modifications of proteins by reactive oxygen species. Carbonyl groups are introduced into proteins by two distinct mechanisms: oxidative (direct) and non-oxidative (indirect). Oxidative mechanisms which are metal catalyzed involve the direct reaction of certain reactive oxygen species (e.g., hydrogen peroxide and lipid hydroperoxides) with protein side chains. Common amino acid targets of direct oxidative processes leading to carbonylation are threonine, lysine, arginine and proline (Adams et al., 2001). Non-oxidative carbonylation of proteins involves the reaction of the nucleophilic centers in cysteine, histidine or lysine residues with reactive carbonyls (RCOs). RCOs are carbonyl-containing malondialdehyde (MDA), acrolein and carbohydrates (e.g., glyoxal, methylglyoxal) (Fu and Dean, 1997). Brain is highly vulnerable to free radical damage because of its high oxygen utilization, high concentrations of polyunsaturated fatty acids and transition metals such as iron and low concentration of cytosolic antioxidants (Reiter, 1995).

Melatonin (*N*-acetyl-5-methoxy-tryptamine), the chief secretory product of the pineal gland, is a derivative of the essential amino acid tryptophan. Several physiological functions of melatonin are related to its hormonal properties. These include, in mammals, the control of seasonal reproduction, immunoresponsiveness, circadian adjustments, vascular regulation and cancer inhibition among others. Many reports have documented protective actions of melatonin in various models of oxidative stress due to its high efficacy as a free radical scavenger and indirect antioxidant (El Missiry et al., 2007). Melatonin is an effective hydroxyl radical scavenger (Vijayalaxmi et al., 2004) and has the capacity to detoxify other reactive oxygen and nitrogen species including singlet oxygen, nitric oxide, peroxynitrite anion as well as its metabolites, peroxynitrous acid and hydrogen peroxide (Tan et al., 2002). In addition to these scavenging actions, melatonin also stimulates a host of antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione

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reductase (GRx); this action further reduces the oxidation state of cells (Barlow-Walden et al., 1995; Antonlin et al., 1996; Pablos et al., 1997; Okatani et al., 2000). Moreover, it is the only antioxidant known to decrease substantially after middle age, and this decrease closely correlates with a decrease in total antioxidant capacity of human serum with age (Benot et al., 1999).

Ascorbic acid (Vitamin C), a water soluble vitamin, is a naturally occurring antioxidant and cofactor needed for various enzymes (Sergeev et al., 1990; Meister, 1994). The best-known functions of ascorbate, as an antioxidant and free radical scavenger, are due to its properties as an electron donor. Given its low redox potential, ascorbate is a broad-spectrum radical scavenger that is effective against peroxyl- and hydroxyl-radicals, superoxide, singlet oxygen and peroxynitrite (Vatassery, 1996). Oxidized ascorbate, both semi dehydroascorbate and dehydroascorbate, can be reduced and recycled by glutathione (GSH) and other intracellular thiols (Meister, 1994) and in some cells by a GSH-dependent dehydroascorbate reductase (Rose, 1993; Fornai et al., 1999). Supplementation of ascorbic acid in glutathione deficient mice and rats increases tissue and mitochondrial levels of glutathione (Meister, 1994). In addition to its functions as an antioxidant in the CNS, ascorbate has been shown to be a neuromodulator of both dopamine- and glutamate-mediated neurotransmission (Grünewald, 1993; Rebec and Pierce, 1994). It acts as a cofactor in the enzymatic biosynthesis of collagen, carnitine, catecholamine and peptide neurohormones. Decreased serum vitamin C concentration in humans is associated with neurologic problems and eventually causes scurvy (Richardson et al., 2002). A long-term supplementation of various antioxidants has been found to retard the loss of spatial memory and decrease damage to brain proteins in aged gerbils and rats (Bickford et al., 1992). To protect cells against oxidative damage, therapeutic intervention by antioxidants like melatonin and ascorbic acid could be useful in treating age-associated neurodegenerative disorders. The aim of the present study was to investigate the effect of melatonin and ascorbic acid on protein carbonyls that are reflective of protein damages in aging brain.

2. Materials and methods

2.1. Animals

Swiss albino (Balb/C strain) female mice of two different age groups (1-and 18-month) were used. All animals were maintained on a 12:12 light/dark cycle in an air-conditioned (25 ± 2 °C) room. They were fed with a standard pellet diet and water *ad libitum*. Institutional guidelines were followed during entire experimentation.

2.2. Materials

2,4-Dinitrophenyl hydrazine (DNPH) was purchased from Wako Pure Chemicals, Tokyo. Bovine serum albumin, guanidine hydrochloride, melatonin (*N*-acetyl-5-methoxy-tryptamine), ascorbic acid (Vitamin C), anti-actin antibody were purchased from Sigma Chemical Co., USA. Anti-DNP antibody was purchased from Santa Cruz., Biotechnology Inc., and Precision Plus Prt™ Standards molecular weight markers were from Bio-Rad Co., USA. Other reagents used were of analytical grade.

2.3. Treatments

Trial experiments were undertaken to determine the dose and time response of melatonin and ascorbic acid (Vitamin C) in influencing protein carbonylation in the cerebral hemispheres of mice. Two doses of 5 and 10 mg melatonin/kg body weight were chosen and the time response monitored after 1–2 h treatments. Maximum response was obtained with a single dose of 10 mg/kg body weight in 0.3 ml alcoholic saline, intraperitoneally after 1 h treatment. Control mice received an equal amount of alcoholic saline only. They were sacrificed by cervical dislocation 1 h after the treatment. Similarly, the doses for ascorbic acid ranged from 10 to 50 mg/kg body weight of mice and the time response monitored on the 1st, 2nd and 3rd day. Finally, ascorbic acid was administered at a dose of 10 mg/kg body weight in 0.3 ml water for 3 days, intraperitoneally. Control mice received an equal amount of water only. The control and treated mice were sacrificed 1 h after the last dose.

2.4. Determination of carbonyl content

Carbonyl content of proteins was determined by the DNPH method as described by Levine et al. (1994) with some modifications (Dkhar and Sharma, 2010).

2.5. Protein determination

Protein was measured by the Coomassie Blue binding method according to Bradford (1976) using bovine serum albumin as standard.

2.6. Assessment of protein carbonylation by Western blotting

Protein carbonyls were assessed according to the procedure described by Levine et al. (1994) with certain modifications of our own (Dkhar and Sharma, 2010). In brief, proteins (40 μg) were incubated with 2,4-dinitrophenyl hydrazine to form the 2,4-dinitrophenyl hydrazone derivatives. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted to nitrocellulose membrane. DNP-containing proteins were then immunostained using rabbit anti-DNP antibody (1:1000). Equal loading of protein was ascertained by probing the replica blot with anti-actin antibody (1:300). Bound primary antibody was detected with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:500) using TMB/H_2O_2 as the substrate. The developed blots were scanned in a HP Scan jet 7400 C scanjet and the images quantified using the Kodak Digital Science 1 image analyzer.

2.7. Statistical analysis

Values were expressed as mean ± SD in each group. Data obtained from different sets were analysed using Student's *t*-test. A *p*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Age-related changes in the levels of protein carbonyls

The level of protein carbonyls in cerebral hemispheres was found to be significantly higher (86.58%) in 18-month-old mice as compared to 1-month-old ($p < 0.001$) (Fig. 1A). It was further ascertained by Western blot analysis of the carbonylated proteins. Comparison of the ratio of the combined densitometry intensity of the carbonylated immunopositive proteins to that of CBB stain of total cytosolic proteins indicated that there was a progressive age-associated increase in carbonylation between 1- and 18-month-old mice ($p < 0.02$) (Fig. 1B).

3.2. Effect of melatonin on the level of protein carbonyls

In melatonin-treated mice, the level of protein carbonyls was significantly decreased in comparison with the age-matched

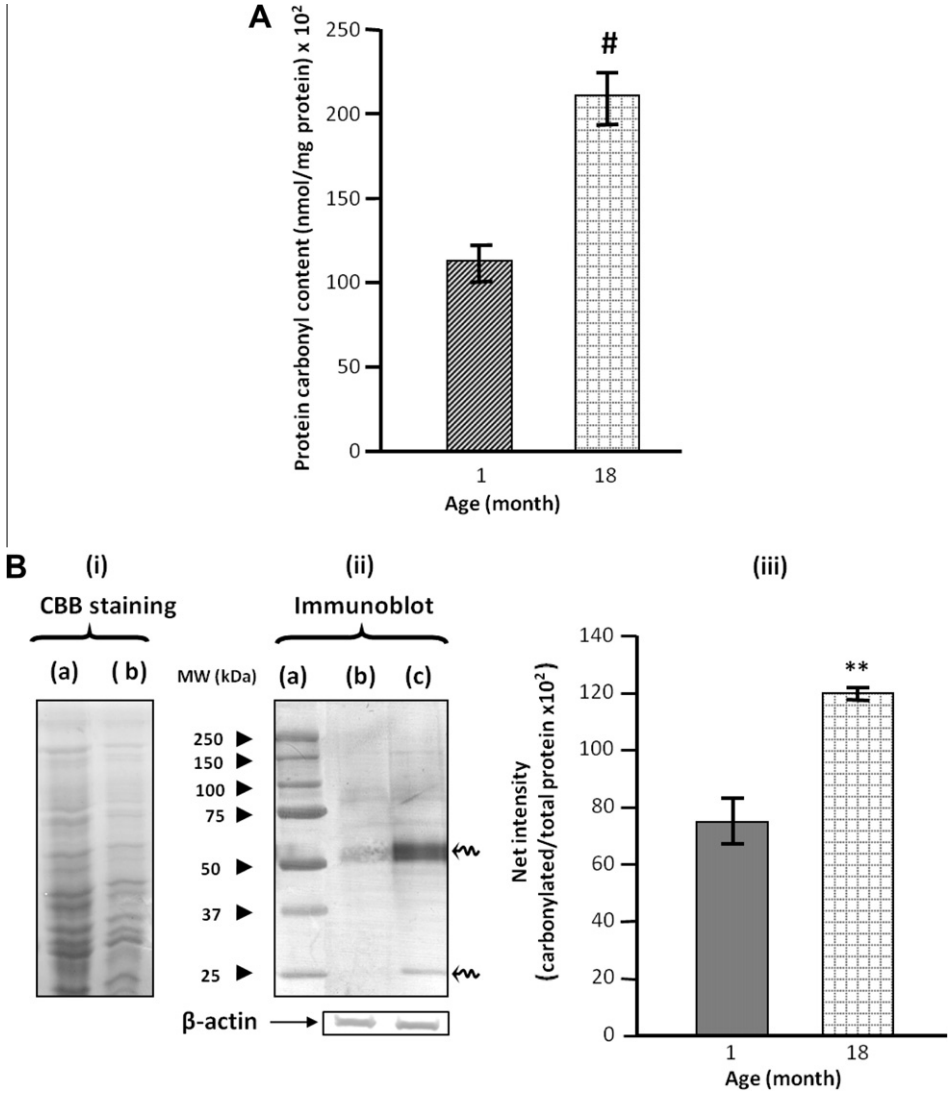

Fig. 1. (A) Normal endogenous level of protein carbonyls in cerebral hemispheres of young (1-) and old (18-month) mice. Values are expressed as mean ± SD ($n = 4$) in each group. #Indicates statistical significance at $p < 0.001$ with respect to the young mice. (B) Immunoblot of protein carbonyls from cerebral hemispheres of young (1-) and old (18-month) mice. (i) Total cytosolic proteins of cerebral hemispheres stained by Coomassie Blue. Lane a: young, b: old. (ii) Immunoblot of protein carbonyls in cerebral hemispheres from young and old mice. Lane a: molecular weight markers b: young, c: old. β-Actin immunoblot is shown as a loading control. (iii) Densitometric analysis of the immunoprobed Western blots for protein carbonyls (b and c) after normalization to actin. The bar graph shows the ratio of the net intensity of the carbonylated bands to that of all the bands on the SDS–PAGE to the factor of 10^2. Data represent the mean ± SD of three separate experiments. **Indicate statistical significance at $p < 0.02$ compared to young mice.

control mice. The percentage of decrease in case of 1-month old mice was 39.81% ($p < 0.001$) while in the case of 18-month-old mice the decrease was 25% ($p < 0.02$) (Fig. 2A). The percent decrease in the level of protein carbonyl by melatonin was also confirmed by immunoblot analysis of total cytosolic proteins. Comparison of Western blots of the protein carbonyls was made between control (alcoholic saline-treated) and melatonin treated mice of 1-month of age (Fig. 2B). The melatonin-treated mice showed a significant decrease in immunodensity in comparison to the control mice ($p < 0.02$) (Fig. 2B(iii)). A comparison of the immunodensity ratio of carbonylated bands of 18-month-old mice is significantly low in melatonin-treated than in the control mice ($p < 0.01$) (Fig. 2C(iii)).

3.3. Effect of ascorbic acid (vitamin C) on the level of protein carbonyls

In ascorbic acid-treated mice, the level of protein carbonyls was significantly decreased in comparison with the age-matched control mice. The percentage of decrease in case of 1-month- old mice was 27.84% ($p < 0.01$) while in the case of 18-month-old mice, it was 46.71% ($p < 0.001$) (Fig. 3A). Similarly, the decrease in the extent of protein carbonylation by ascorbic acid was also ascertained by immunoblot analysis of total cytosolic proteins. Western blots of the protein carbonyls were also compared between normal and ascorbic acid-treated mice of 1-month of age (Fig. 3B(ii)). The immunodensity ratio of proteins showed a significant decrease in protein carbonylation in the ascorbic acid-treated mice in comparison to the control mice ($p < 0.001$) (Fig. 3B(iii)). A significant decrease in the immunodensity ratio of the carbonylated bands of 18-month-old mice was also observed in the ascorbic acid-treated mice as compared to the control mice ($p < 0.01$) (Fig. 3C(iii)).

4. Discussion

Cellular damages arising from the oxidative stress have been implicated in neuronal degeneration associated with normal aging (Davies et al., 2001). In the present study, protein carbonyl level

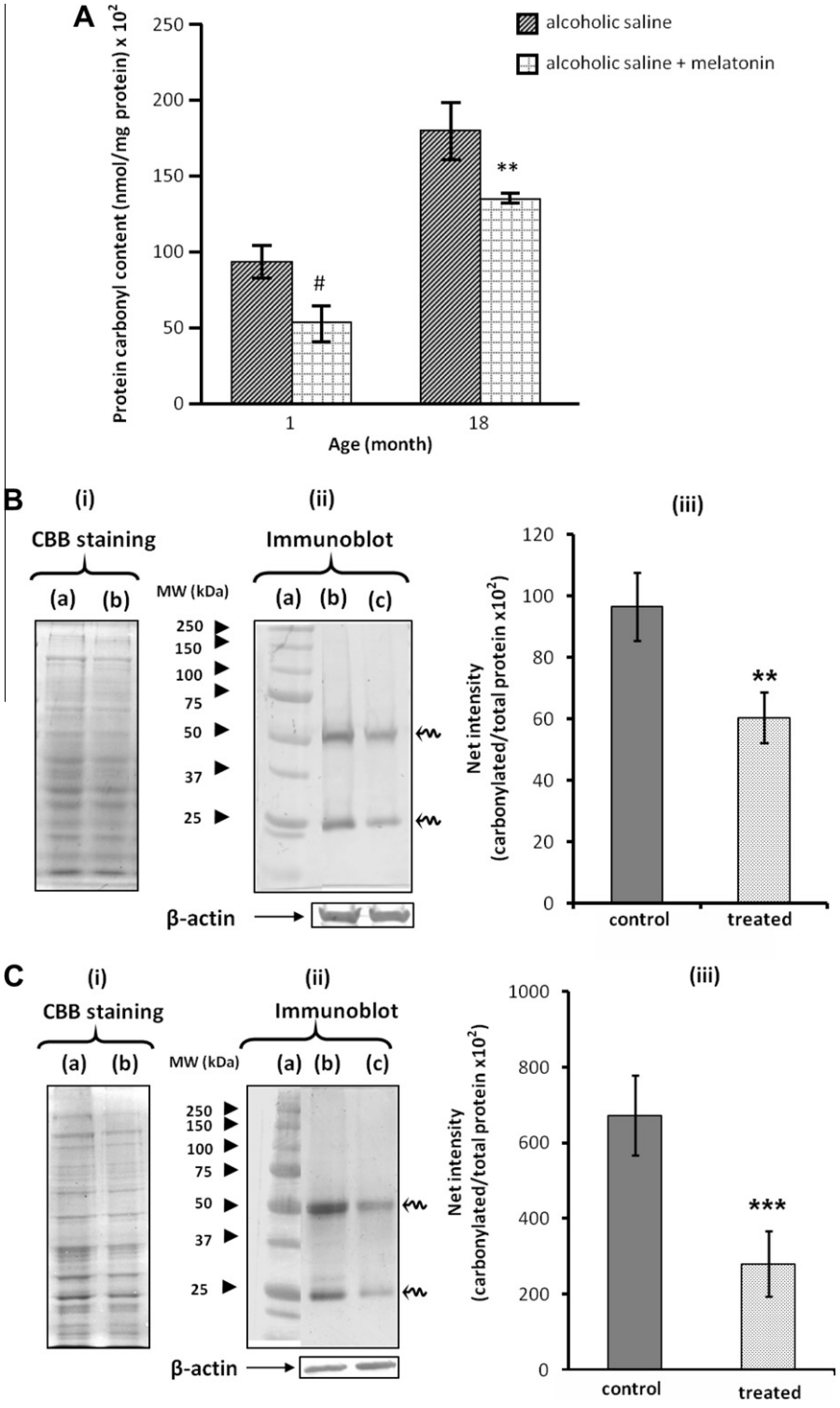

Fig. 2. (A) Effect of melatonin on protein carbonyls in cerebral hemispheres of young (1-) and old (18-month) mice. Values are expressed as mean ± SD ($n = 4$) in each group. # and ** represent statistical significance at $p < 0.001$and $p < 0.02$, respectively as compared to the control group. (B) Immunoblot of protein carbonyls from cerebral hemispheres of young (1-month) mice treated with melatonin. (i) Total cytosolic proteins of cerebral hemispheres stained with Coomassie Blue. Lane a: alcoholic saline treated b: alcoholic saline + melatonin treated. (ii) Immunoblots of protein carbonyls in cerebral hemispheres from young mice. Lane a: molecular weight markers b: alcoholic saline treated c: alcoholic saline + melatonin treated. β-Actin immunoblot is shown as a loading control. (iii) Densitometric analysis of the immunoprobed Western blots for protein carbonyls (b and c) after normalization to actin. The bar graph shows the ratio of the net intensity of the carbonylated bands to that of all the bands on the SDS–PAGE to the factor of 10^2. Data represent the mean ± SD of three separate experiments. **Indicate statistical significance at $p < 0.02$ compared to young mice. (C) Immunoblot of protein carbonyls from cerebral hemispheres of old (18-month) mice treated with melatonin. Other conditions are similar as given for (B).

was measured as an index of oxidative damage in the brain based on the hypothesis that the formation of reactive carbonyl groups represents a major manifestation of oxidative modifications of proteins during aging. The brain produces more reactive oxygen species per gram of tissue than any other organ (Reiter, 1995). The presence of iron in an oxygen-rich environment can further

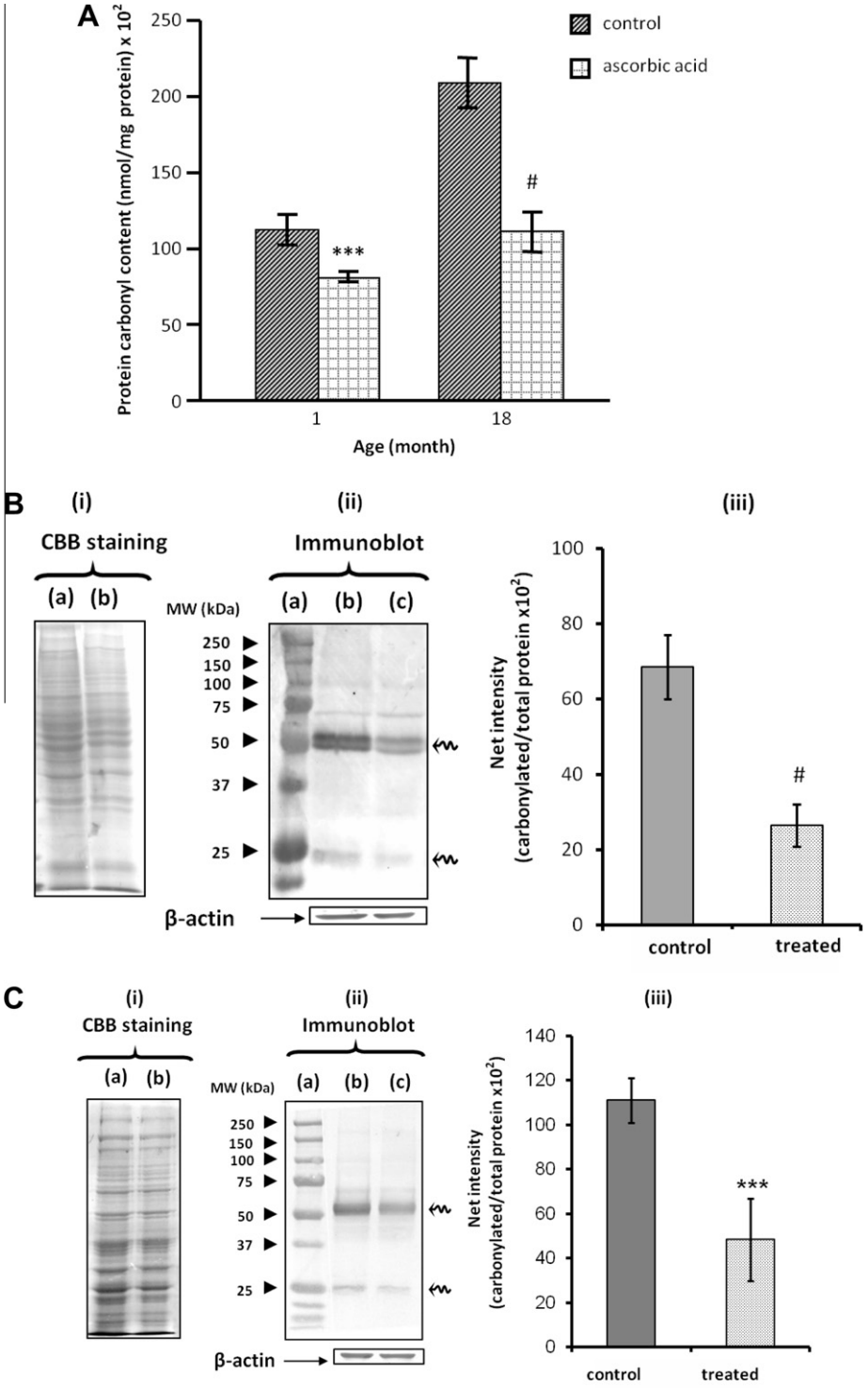

Fig. 3. (A) Effect of ascorbic acid (Vitamin C) on protein carbonyls in cerebral hemispheres of young (1-) and old (18-month) mice. Values are expressed as mean ± SD ($n = 4$) in each group. # and *** represent statistical significance at $p < 0.001$and $p < 0.01$, respectively as compared to the control group. (B) Immunoblot of protein carbonyls from cerebral hemispheres of young (1-month) mice treated with ascorbic acid. (i) Total cytosolic proteins of cerebral hemispheres stained with Coomassie Blue. Lane a: control b: ascorbic acid treated. (ii) Immunoblots of protein carbonyls in cerebral hemispheres from young mice. Lane a: molecular weight markers b: control c: ascorbic acid treated. β-Actin immunoblot is shown as a loading control. (iii) Densitometric analysis of the immunoprobed Western blots for protein carbonyls (b and c) after normalization to actin. The bar graph shows the ratio of the net intensity of the carbonylated bands to that of all the bands on the SDS–PAGE to the factor of 10^2. Data represent the mean ± SD of three separate experiments. #Indicate statistical significance at $p < 0.001$ from the control group. (C) Immunoblot of protein carbonyls from cerebral hemispheres of old (18-month) mice treated with ascorbic acid. Other experimental conditions are the same as given for (B).

lead to enhanced production of superoxide radicals and ultimately to a cascade of oxidative events. Our studies showed an increase in protein carbonyl content in the cerebral hemispheres of 18-month-old mice as compared to the 1-month old mice which indicated that there is increased protein damage with age. Higher levels of carbonyl formation may be due to age-dependent changes

in the rate of oxidized protein degradation (Stadtman and Levine, 2000). Decreased proteolytic activity has also been reported in the aging brain (Carney et al., 1991; Goto et al., 2007). Furthermore, the aged rat brain is much more susceptible to oxidative damage than the brain of young ones (Viani et al., 1991). Our data support the idea that protein carbonyls may serve as a biomarker relevant to aging. The age-related accumulation of protein carbonyls has been demonstrated by many laboratories and fits with the free radical theory of aging (Goto et al., 2007; Dkhar and Sharma, 2010). The changes in cellular homeostasis are able to disturb both pro- and antioxidant processes (Martin et al., 1993) leading to an age-dependent increase in cellular oxidation products. The degradation of the bulk of oxidatively modified proteins is mainly accomplished by the 20S core proteasome (Goto et al., 2007; Breusing and Grune, 2008). However, there is evidence from in vitro studies that oxidized proteins, if cross-linked, are themselves able to inhibit the proteasome leading to a reduced proteolysis in older animals (Friguet and Szweda, 1997; Sitte et al., 2000).

The results of the present study clearly showed that melatonin administration reduced the protein carbonyl levels in the cerebral hemispheres of both 1-month as well as 18-month-old mice. Many reports have documented protective actions of melatonin in various models of oxidative stress due to its high efficacy as a free radical scavenger and indirect antioxidant (El Missiry et al., 2007). The small size and chemical nature of melatonin allow it to cross biological membranes and reach all compartments of the cell including the mitochondria and nucleus (Reiter et al., 2000). Melatonin exhibits redox properties because of the presence of electron-rich aromatic ring, which allows the indoleamine to easily function as an electron donor. Moreover, because of its *O*-methyl and *N*-acetyl residues, melatonin is amphiphilic in nature. These properties have been suggested as the molecular basis for the widely documented antioxidant protection afforded by melatonin at the level of various subcellular compartments, both in vitro and in vivo models (Reiter, 1997). In addition, melatonin stabilizes mitochondrial inner membrane thereby improving electron transport chain activity (Acuna-Castroviejo et al., 2001). Melatonin reportedly increases the activity of the brain mitochondrial respiratory complexes I and IV in a time-dependent manner whereas the activities of complexes II and III are not affected (Martin et al., 2002). The redox potential (0.94 V) of melatonin suggests its interaction with the complexes of the electron transport chain and may donate and accept electron thereby increasing electron flow, an effect not possessed by other antioxidants (Tan et al., 2000). Retinoic acid receptor-related orphan receptor alpha (ROR-α), considered as a mediator of nuclear melatonin signaling, is also an oxidative stress-related transcriptional factor (Li et al., 2004). Recently, it has been found that ROR-α1 overexpression protects neurones against oxidative stress-induced cell damage (Boukhtouche et al., 2006). Hence, melatonin might quench such free radicals to reduce the protein carbonylation in proteins.

In ascorbic acid (vitamin C) treated mice, the age-related increase in protein carbonyl level was significantly reduced compared to the age-matched control indicating that ascorbic acid also ameliorates the age-related increase in protein carbonyl content. Ascorbic acid is highly water-soluble, and its ability to act as a one- or two-electron reductant for a wide variety of biological oxidizing species has led to a great deal of interest in its role as a major anti-oxidant. The transport, metabolism and antioxidant functions of ascorbate have been detailed in several recent reviews (Padayatty and Levine, 2001; Duarte and Lunec, 2005; Linster and Van Schaftingen, 2007). Lipid peroxidation and oxidative modification of low density lipoproteins (LDL) are implicated in development of atherosclerosis (Steinbrecher et al., 1990). Ascorbic acid protects against oxidation of isolated LDL by different types of oxidative stress, including metal ion dependent and independent processes (Frei, 1997). The antioxidant properties of ascorbate have been used to treat various conditions such as type 2 diabetic macular degeneration, where oxidative stress is involved in the pathogenesis (Heitzer et al., 2001; Evans and Henshaw, 2008). Ascorbic acid suppresses the activation of a major redox transcription factor, NF-κB and inhibits the activation of HIF-1α and p38 MAPK pathways leading to the downregulation of antiapoptotic, cell proliferative, invasive and angiogenic gene products (Kyaw et al., 2001; Juan et al., 2002; Mandl et al., 2009). Dehydroascorbic acid, the oxidized form of ascorbic acid, was also reported to cross the blood brain barrier by means of facilitative transport and was suggested to offer neuroprotection against cerebral ischemia by augmenting antioxidant levels of brain (Huang et al., 2001). We suggest that such an influence of ascorbic acid may reduce the oxidative load, thereby help preventing protein carbonylation in the treated animals.

In conclusion, treatments with melatonin and ascorbic acid significantly reduce the age-related increase in the protein carbonyl levels compared to the age-matched controls. The present study indicate that aging is associated with a shift towards oxidized milieu and clearly demonstrates that melatonin and ascorbic acid attenuate the age-related increase in protein carbonyl formation in the cerebral hemispheres of mice which could be useful in several age-related oxidative neuropathophysiological conditions.

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