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ORIGINAL ARTICLE

The ultrastructural and biochemical evidences of the beneficial effects of chronic caffeic acid phenethyl ester and melatonin administration on brain and cerebellum of aged rats

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ABSTRACT

Nervous system is highly vulnerable to the deleterious effects of age-related oxidative stress. A large body of researches has consistently confirmed the implication of free radicals both in normal cerebral ageing and ageing-related pathologies. In the present study, in addition to the light and electron microscopic pictures of brain and cerebellum of young, old and antioxidant administered old Sprague–Dawley rats, pro-oxidant status was evaluated in terms of measurements of total glutathione, lipid peroxidation (malondialdehyde) and activities of superoxide dismutase, catalase and glutathione peroxidase. Taking the results together, we suggest that supplemental administration of caffeic acid phenethyl ester and melatonin is beneficial in delaying age-related cellular damage in nervous system.

INTRODUCTION

Ageing is an undesired situation creating extensive, deleterious and to a certain extent irreversible changes in cells, tissues and organism itself. The free radical theory proposes that ageing is the cumulative results of oxidative damage to cells and tissues that arises primarily as a result of aerobic metabolism. Mitochondria are strong producers of both reactive oxygen species (ROS) and reactive nitrogen species (RNS). They are also susceptible to the oxidative damage produced by the action of ROS and RNS on lipids, proteins, and DNA. The phenomenon called 'oxidative stress' is the result of the accumulation of free radicals within the cell. Reactive oxygen species are scavenged by antioxidant enzymes in biological systems. When free radical generation exceeds the defense capabilities of the antioxidant system, molecular damage appears. As this damage accumulates, cellular function gradually declines, eventually leading to death of cells, organs and the organism itself. This is the summary of ageing and death.

Some of the organs such as heart, vessels, brain, ovary and testis are highly vulnerable to the deleterious effects of age-related oxidative stress. Traditionally it is believed that humans lose about 100 000 neurons per day. Apoptosis is the major form of neuronal cell death in ageing of the nervous system. The loss of cortical neurons is viewed as one of the main cause of decline in brain function during ageing. Dementias are more common in elderly people than younger ones.

Brain and nervous system are prone to oxidative stress and are inadequately equipped with antioxidant defense systems to prevent 'ongoing' oxidative damage, let alone the extra oxidative damage imposed by the neurodegenerative diseases [1]. The sensitivity to oxidative stress is probably because (i) neurons have an extended neuron

surface, increasing the possibility of lipoperoxidation, (ii) the oxygen metabolism is raised in cerebral areas and (iii) their glutathione content is low [2]. Oxidative stress has been extensively investigated in neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), memory loss, depression, and mental stress [2,3]. A large body of researches has consistently confirmed the implication of free radicals both in normal cerebral ageing and ageing-related pathologies [4].

In the present study we aimed to investigate ageing-related histological, cellular and biochemical alterations in brain and cerebellum in old rats. We investigated light microscopic picture of brain and cerebellum and detailed electron microscopic features of neurons. Additionally we detected the level of lipid peroxidation, the levels/activities of antioxidant enzymes such as total glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Finally, we evaluated the effects of long term exogenous caffeic acid phenethyl ester (CAPE) and melatonin administration on ageing-induced histological and biochemical alterations. To our knowledge, this is the first study investigating the effect of CAPE against ageing-related neuronal damage.

EXPERIMENTAL PROTOCOL

Animals and experimental protocol

Twenty-eight male Sprague–Dawley rats weighing 200–450 g were used. Animals were fed with standard rat chow and tap water ad libitum. They were maintained on a 12 h light/12 h dark cycle at 21 °C.

Animals were divided into four groups. First group included 4 months of age rats (young group, $n = 7$), second, third and fourth groups included 18 months of age rats (old groups, $n = 7$ each). The third and the fourth groups received 5 mg/b.w./day melatonin (Old+Mel) and 15 mg/b.w./day CAPE (Old+CAPE), respectively; both intraperitoneally for 95 days. Melatonin was dissolved in absolute ethanol and further dilutions were made in saline, with 1% final concentrations of ethanol. CAPE was prepared in the biochemistry laboratory according to a standard method described by Grunberg et al. [5]. Animals form first and second groups were injected equivalent doses of saline. Body weights of all rats at the beginning and at the end of the experiment were measured. At the end of the experiment animals were sacrificed by decapitation.

Animal experiments were performed in accordance with the guidelines for animal research from the National Institute of Health and were approved by the Committee of Animal Research at Inonu University, Malatya, Turkey (2007/47).

Microscopic examination

Cerebral and cerebellar hemispheres from all animals were taken out quickly and divided into three portions. The first part of the samples were placed in 10% buffered formalin and prepared for routine paraffin embedding. Sections of tissues were cut at 5 μm, mounted on slides, stained with hematoxylin–eosin (H–E). DNA fragmentation was detected by the terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end-labeling (TUNEL) method described by Promega (Colorimetric TUNEL system) (Promega Corporation, Madison, WI, USA). Sections were examined by a Lyca DFC280 light microscope and Leica Q Win and Image Analysis system (Leica Micros Imaging Solutions Ltd, Cambridge, UK). Assessment of tissue alterations in 20 different fields for each section was conducted by an experienced histologist who was unaware of the treatment. TUNEL + cells were counted in cerebral and cerebellar cortex. Thicknesses of cerebral cortex, molecular and granular layers of cerebellar cortex were measured.

The second part of the tissues was processed for electron microscopic examination. For that purpose, samples were fixed in 2.5% gluteraldehyde buffered with 0.2 M NaH_2PO_4 + $NaHPO_4$ (pH 7.2–7.3) and post fixed in 1% OsO_4. After dehydration in acetone, they were embedded in Araldite CY 212. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a Zeiss Libra 120 electron microscope (Carl Zeiss SMT AG Company, Oberkochen, Germany).

Biochemical determination

The other part of tissue samples was stored at −80 °C for the determination of malondialdehyde (MDA), SOD, CAT, GPx, and GSH.

After cutting into small pieces on ice, tissues were homogenized in 1/5 (w/v) phosphate buffer saline. Homogenates were divided into two portions; one part was directly used for MDA measurement. The second was sonicated four times for 30 s with 20 s intervals using VWR Bronson scientific sonicator (VWR Int. Ltd, Merch House Pool, UK). Then, homogenates were centrifuged at 20 000 ***g*** for 15 min in Beckman L-8-70M ultracentrifuge (Rotor SW-28; Beckman L8-70M Ultracentrifuge, München, Germany). Supernatants were separated and kept −40 °C until enzyme activity

measurements were performed. Care was taken to keep the temperature at +4 °C throughout the preparation of homogenates and supernatants.

Protein determination in supernatants was done according to Lowry and Rosebrough [6] using BSA as standard. A Shimadzu 1601 UV/Vis spectrophotometer (Shimadzu, Kiyoto, Japan) with a connected PC and a Grand LTD 6G thermo stability unit adjusted to 37 ± 0.1 °C was employed for all spectrophotometric assays.

Catalase activity was measured in supernatants by the method of Luck [7]. The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm. Specific activity was defined as micromole substrate decomposed per minute per milligram of protein. CAT levels were expressed as micromole per milligram of protein (U/mg protein).

GP_X activity measurements were conducted according to Lawrence and Burk [8]. 1.0 mL of 50 nM PBS solution (pH 7.4) including 5 mM ETDA, 2 μM NADPH, 20 μM GSH, 10 μM NaN_3 and 23 mU of glutathione reductase was incubated at 37 °C for 5 min. Then 20 μL of 0.25 mM H_2O_2 solution and 10 μL of supernatant were added to assay mixture. The change in absorbance at 340 nm was monitored for 1 min. A blank with all ingredients except supernatants was also monitored. Specific activity was calculated as micromole NADPH consumed per minute per milligram of protein (i.e. U/mg protein), using an appropriate molar absorptivity coefficient (6220 M^{-1} cm^{-1}). GP_X levels were expressed a micromole per milligram of protein (U/mg protein).

SOD (Cu, Zn-SOD) activity was measured in the supernatant fraction using xanthine oxidase/cytochrome *c* method according to McCord and Fridovich [9] where 1 unit of activity is the amount of enzyme needed to cause half-maximal inhibition of cytochrom *c* reduction. The amount of SOD in the extract was determined as ng of enzyme per mg protein, utilizing a commercial SOD as standard.

The formation of 5-thio-2-nitrobenzoate (TNB) is followed spectrophotometrically at 412 nm [10]. The amount of GSH in the extract was determined as nmol/mg protein utilizing a commercial GSH as standard.

The level of MDA in tissue homogenate was determined using the method of Uchiyama and Mihara [11]. Half a milliliter of homogenate was mixed with 3 mL H_3PO_4 solution (1% v/v) followed by addition of 1 mL thiobarbituric acid solution (0.67% w/v). Then the mixture was heated in water bath for 45 min. The colored complex was extracted into n-butanol and absorption at 532 was measured using tetramethoxypropane as standard. MDA levels were expressed as nanomol per milligram of protein (nm/mg protein).

Statistical evaluation

Statistical analysis was carried out using the SPSS 10.0 statistical program (SPSS Inc., Chicago, IL, USA). All data are expressed as arithmetic mean ± SE. The differences between mean values of cell counts, zone thicknesses and tissue enzyme levels for each group were analyzed by using one-way analysis of variance (ANOVA) and post hoc Duncan tests. Values of $P < 0.05$ were regarded as significant.

RESULTS

The animals from all groups survived until the end of the experiment. The young and antioxidant administered rats (especially melatonin administered ones) were highly active. The old rats from 2nd group were exhausted. There was a clear dissimilarity between the old and, young and antioxidant administered old rats with respect to posture, hair shine, eye brightness, and action. The young and, old rats form 2nd group somewhat gained weight during the experiment duration (238.28 g at the beginning, 302 g at the end, and 412.42 g at the beginning, 419.14 g at the end, respectively). CAPE and melatonin injected rats somewhat lose weight (368.43 g at the beginning, 367.57 g at the end, and 378.57 g at the beginning, 370.42 g at the end, respectively). Body weights of the animals of all groups are summarized in *Table I*.

The light microscopic pictures of brain and cerebellum from all groups were normal in histological appearance at first glance. However, brain cortex and granular layer of cerebellar cortex of old animals from second group were thinner than those of young animals ($P < 0.001$). Mean thickness of molecular layer of cerebellar cortex was also reduced in old rats but statistical difference was not detected. Melatonin administration restored the thickness of brain cortex and granular layer of cerebellum

Table I Mean body weight of animals from all groups.

Groups	Mean body weight at the beginning	Mean body weight at the end
Group 1 (Young)	238.28 ± 6.0	302.00 ± 10.8
Group 2 (Old)	412.42 ± 4.3	419.14 ± 10.6
Group 3 (Old + Mel)	378.57 ± 16.7	370.42 ± 17.4
Group 4 (Old + CAPE)	368.42 ± 4.4	367.57 ± 7.9

Data are expressed as mean ± SE.

Table II Mean thicknesses of brain cortex and granular and molecular layers of cerebellar cortex.

Groups	Mean thickness of brain cortex (μm)	Mean thickness of granular layer (μm)	Mean thickness of molecular layer (μm)
Group 1 (Young)	1721.3 ± 23.5	290.8 ± 12.3	237.7 ± 16.9
Group 2 (Old)	1612.6 ± 21.4[a]	207.3 ± 10.9[a]	219.0 ± 10.1
Group 3 (Old + Mel)	1684.1 ± 14.2[b]	236.8 ± 8.0[b]	208.4 ± 4.6
Group 4 (Old + CAPE)	1594.13 ± 8.8	228.6 ± 3.7	213.5 ± 5.2

Data are expressed as mean ± SE.
[a]$P < 0.001$ vs. Group 1.
[b]$P < 0.05$ vs. Group 2.

Table III Mean number of TUNEL + cells in brain and cerebellum.

Groups	Mean number of TUNEL + cells in brain cortex	Mean number of TUNEL + cells in cerebellar cortex
Group 1 (Young)	0.14 ± 0.14	0.00 ± 0.00
Group 2 (Old)	7.71 ± 1.10[a]	0.57 ± 0.29
Group 3 (Old + Mel)	1.00 ± 0.30[b]	0.28 ± 0.18
Group 4 (Old + CAPE)	1.28 ± 0.42[b]	0.42 ± 0.78

Data are expressed as mean ± SE.
[a]$P < 0.001$ vs. Group 1.
[b]$P < 0.001$ vs. Group 2.

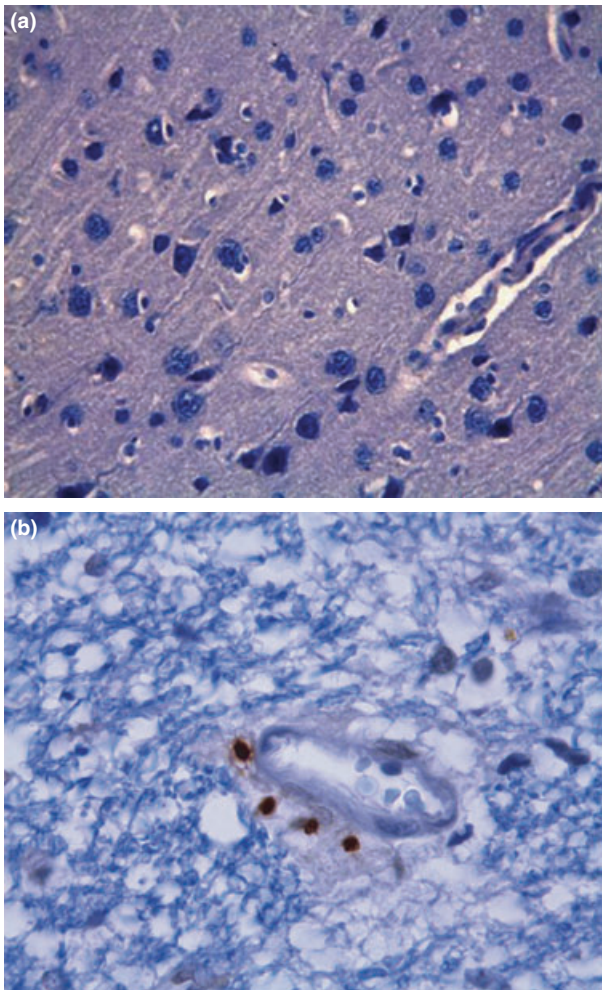

Figure 1 Sections of brain cortex of young and old rats stained with TUNEL method. (a) There is no TUNEL + cell in the brain section of a young rat. ×40, (b) TUNEL + nuclei of apoptotic cells surrounding a vessel is observed in the brain section of an old rat ×100.

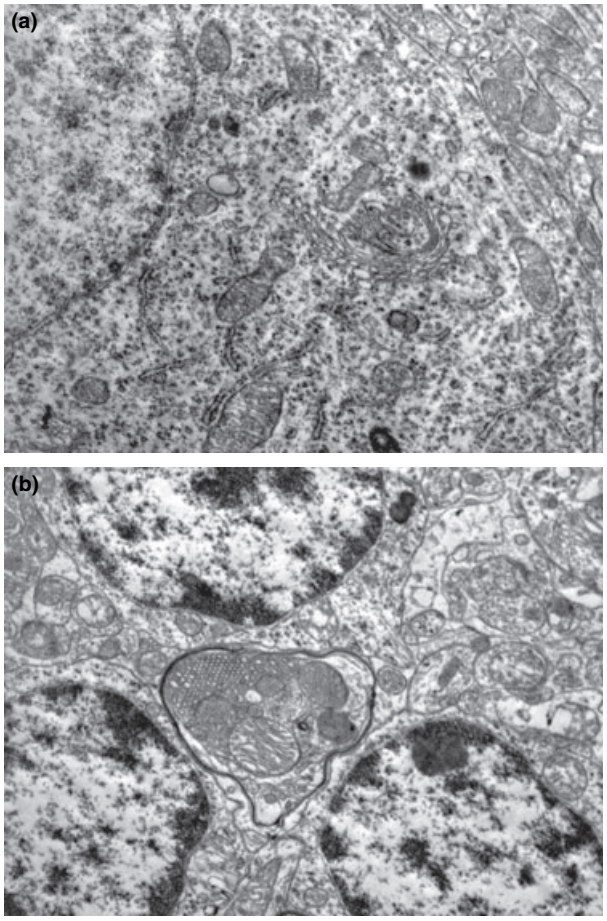

Figure 2 Electron micrographs form brain and cerebellum of young rats. (a) A pyramidal neuron containing many mitochondria, ribosomes, cisternea of endoplasmic reticulum and Golgi apparatus in brain cortex is observed. Nucleus is euchromatic in appearance. (b) The healthy granular cells in granular layer of cerebellum are observed. Uranyl acetate and lead citrate ×16 000, ×10 000, respectively.

($P < 0.05$). The data of main thickness of brain cortex and granular and molecular layers of the animals from all groups are summarized in *Table II*.

Mean number of TUNEL + cells in brain cortex of old animals was statistically higher than that of young animals ($P < 0.001$) (*Figure 1a,b*; *Table III*). Mean number of TUNEL + cells in cerebellar cortex was also increased in old rats but statistical difference was not detected. Melatonin and CAPE administration reduced mean number of TUNEL + cells both in brain ($P < 0.001$) and cerebellum. The data of main number of TUNEL + cells in brain and cerebellum of animals from all groups are summarized in *Table III*.

Neurons and neuroglial cells were healthy in young animals (*Figure 2a,b*). We could not detect apoptotic changes such as semilunar chromatin condensation, nuclear and cellular fragmentation, and the formation of apoptotic bodies by electron microscopic investigation. However, we detected many prominent cellular degenerative changes both in brain and cerebellum of old rats. Cytoplasmic edema, mild endoplasmic reticulum dilatation, prominent mitochondrial degeneration (*Figure 3a*), lysosome accumulation (*Figure 3b*) and peripheral heterochromatin condensation were observed in cerebral neurons of old rats. Many mitochondria were edematous with increased translucence of the matrix, partial or total destruction of cristae and sometimes presence of vacuoles and myelin figures (*Figure 3a*). In cerebellum, both granular cells and Purkinje cells were edematous in appearance, density of cytoplasmic matrix was highly reduced, many degenerated mitochondria (*Figure 3c,d*) and lysosomes (*Figure 3c*) were present within the cytoplasm. Cristae loss, decrease in mitochondrial matrix density (*Figure 3c,d*) and presence of vacuoles and myelin figures were observed. Peripheral heterochromatin condensation was occasionally observed (*Figure 3c*).

Ultrastructural picture of brain and cerebellum of melatonin and CAPE administered rats was nearly normal in appearance (*Figure 4a*). However, myelin figure formation and cristae loss within the mitochondria (*Figure 4b,c*), lysosome accumulation and peripheral

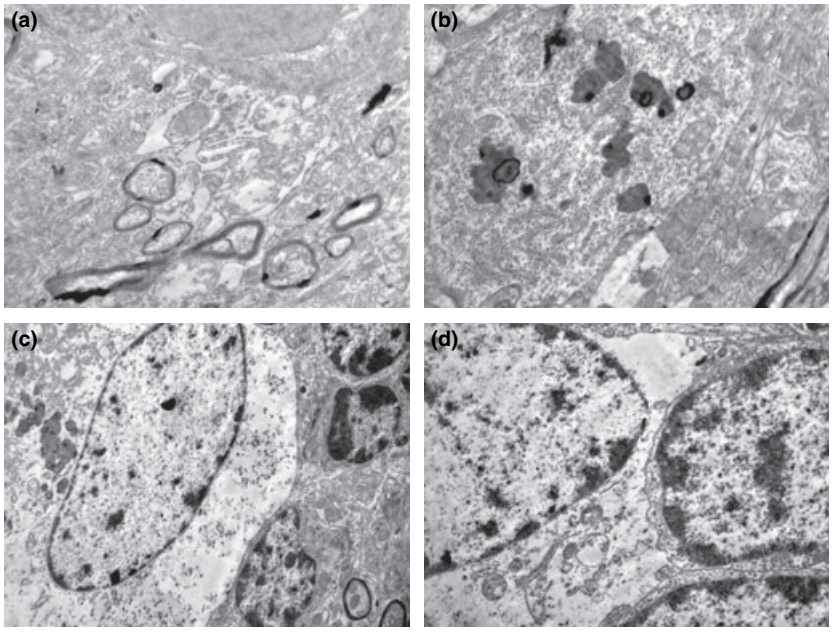

Figure 3 Electron micrographs form brain and cerebellum of old rats from second group. (a) Endoplasmic reticulum dilatation, mitochondrial swelling, vacuole formation, and damage to mitochondrial christae are observed in the cytoplasm of a pyramidal neuron in brain cortex. (b) Lysosome accumulation is clearly observed in the cytoplasm of a pyramidal neuron in brain cortex. (c) Prominent edema, lysosome accumulation and mitochondrial swelling and cristae loss are observed within Purkinje cells in cerebellar cortex. In one of the small granule cells at the right side of the figure, peripheral heterochromatin condensation is seen. (d) Mitochondrial swelling, decrease in the density of mitochondrial matrix, and cristae loss are obvious in one of the granular cells in the cerebellar cortex. The granule cell at the left side of the figure is edematous whereas those at the right side seem normal. Uranyl acetate and lead citrate, ×12 500, ×12 500, ×6300, ×10 000, respectively.

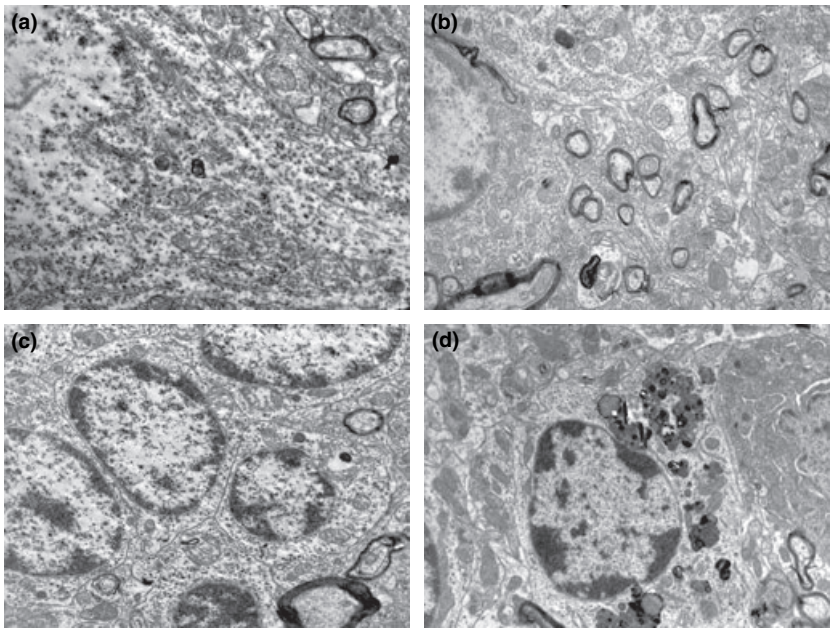

Figure 4 Electron micrographs form brain and cerebellum of antioxidant administered old rats from third and fourth groups. (a) A pyramidal neuron which normal in ultrastructural appearance is observed in cerebral cortex of a melatonin administered old rat. (b) Ultrastructural appearance of neurons are nearly normal, however, some of the mitochondria of left sided neuron show cristae loss in cerebral cortex of a CAPE administered rat. (c) Vacuole and myelin figures are present within the mitochondria of the granule cells in the cerebellar cortex of a melatonin administered old rat. (d) Lysosome accumulation within the cytoplasm and peripheral heterochromatin condensation in the granule cell of the cerebellar cortex of a CAPE administered old rat is obviously seen Uranyl acetate and lead citrate ×12 500, ×10 000, ×8000, ×6300, respectively.

heterochromatin condensation (*Figure 4d*) were rarely detected.

Mean tissue MDA levels were increased in both brain and cerebellum of old rats in comparison to young rats, but statistical importance was not detected (0.57 ± 0.04 to 0.71 ± 0.06; 3.00 ± 0.24 to 3.70 ± 0.26, respectively). Mean tissue SOD, CAT, GP_x activities and GSH levels ($P < 0.005$) of brain and SOD activity and GSH levels of cerebellum of old animals were decreased. Melatonin reduced tissue MDA levels, but increased tissue SOD ($P < 0.001$), CAT, GP_X activities ($P < 0.05$) and GSH levels ($P < 0.05$) in the brain of old animals. CAPE also reduced tissue MDA levels ($P < 0.005$), increased tissue SOD ($P < 0.05$) and GP_X activities and GSH levels in the brain of old animals. Melatonin reduced MDA levels whereas increased SOD ($P < 0.05$) and GP_X activities ($P < 0.05$), and GSH levels in the cerebellum of old rats. Moreover, GP_X activity of melatonin administered rats was statistically higher than that of the young rats ($P < 0.05$). CAPE also reduced MDA levels ($P < 0.005$) but increased SOD ($P < 0.005$) and GP_X activities ($P < 0.05$), and GSH levels. Mean levels of tissue MDA and GSH, mean activity of tissue SOD, CAT and GP_x of brain and cerebellum of the animals from all groups are summarized in *Tables IV* and *V*, respectively.

DISCUSSION

This study adds evidences for the role of oxidative stress in the cerebral and cerebellar ageing process. Oxidative stress was associated with an increase in lipid peroxidation and decreases in antioxidative enzyme activities/levels of the tissues. Statistical differences between the data of young and old animals were rarely detected. However, all of the differences are highly important because this is a chronological ageing study, as a matter of fact that any dramatic change is expected.

It has been reported that human brain cortex undergoes more extensive changes with age than the cerebellum, which regulates basic processes such as heartbeat, breathing and balance. There are two possible explanations; one is that cerebellum has its own aging pattern distinct from that in brain cortex; the other is that it has far fewer genes changing expression with age than

Table IV Mean levels of tissue MDA and GSH, mean activity of tissue SOD, CAT and GP_x in brain cortex of the animals from all groups

Groups	MDA (nm mg/protein)	SOD (U/mg protein)	CAT (U/mg protein)	GP_X (U/mg protein)	GSH (nm/mg protein)
Group 1 (Young)	0.57 ± 0.04	9.35 ± 1.03	2.10 ± 0.17	11.25 ± 0.69	16.81 ± 0.73
Group 2 (Old)	0.71 ± 0.06	7.64 ± 0.61	2.09 ± 0.08	9.57 ± 0.45	13.67 ± 0.71[f]
Group 3 (Old + Mel)	0.59 ± 0.04	12.6 ± 0.78[c,d]	2.19 ± 0.11	11.99 ± 0.22[e]	16.62 ± 0.51[e]
Group 4 (Old + CAPE)	0.51 ± 0.01[a,b]	10.4 ± 0.74[b,e]	1.84 ± 0.15[e]	12.44 ± 1.01	15.08 ± 0.74

Data are expressed as mean ± SE.
[a]$P < 0.005$ vs. Group 2.
[b]$P < 0.05$ vs. Group 3.
[c]$P < 0.05$ vs. Group 1.
[d]$P < 0.001$ vs. Group 2.
[e]$P < 0.05$ vs. Group 2.
[f]$P < 0.005$ vs. Group 1.

Table V Mean levels of tissue MDA and GSH, mean activity of tissue SOD, CAT and GP_x in cerebellar cortex of the animals from all groups.

Groups	MDA (nm/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GP_X (U/mg protein)	GSH (nm/mg protein)
Group 1 (Young)	3.00 ± 0.24	21.84 ± 1.63	1.92 ± 0.15	4.21 ± 0.31	36.44 ± 2.83
Group 2 (Old)	3.70 ± 0.26	20.97 ± 0.84	3.04 ± 0.21[d]	4.42 ± 0.44	33.80 ± 2.50
Group 3 (Old + Mel)	3.41 ± 0.23	27.60 ± 1.08[c]	1.87 ± 0.12[a]	6.00 ± 0.36[c,d]	35.73 ± 1.90
Group 4 (Old + CAPE)	2.40 ± 0.26[a,b]	27.32 ± 1.19[a]	2.34 ± 0.18[b,c]	6.19 ± 0.45[c,d]	35.37 ± 1.62

Data are expressed as mean ± SE.
[a]$P < 0.005$ vs. Group 2.
[b]$P < 0.05$ vs. Group 3.
[c]$P < 0.05$ vs. Group 2.
[d]$P < 0.05$ vs. Group 1.

cerebral cortex does [12]. We detected important electron microscopic and biochemical alterations in both cerebral and cerebellar cortex in old rats. In both areas, cellular edema, mitochondrial degeneration and lysosome accumulation were obvious. Moreover, mean TUNEL + cell number was higher both in brain ($P < 0.001$) and cerebellum sections of old animals then those of young animals. Certainly both organs undergo some aging-induced degenerative changes, but probably cerebral neurons are more susceptible to death. Not only cell death but also cellular degeneration results in organ failure. Thus, all of degenerative alterations are valuable since nervous system is one of the most important systems that undergo age-related discomfort and diseases.

Free radicals are in fact potent deleterious agents causing cell death or other forms of irreversible damage, e.g., free radicals appear to modify ≈10 000 DNA base pairs every day [13]. Free radicals mainly derived from mitochondria result in damage to cellular proteins, lipids and DNA throughout the cell. The number of oxidative lesions occurring to the mitochondrial DNA is some ten times higher than found in the nucleus and this damage also accumulates more quickly with age [14]. Moreover DNA repair is much efficient in mitochondria than in the nucleus [15]. The resulting damage has been implicated as a cause of ageing. Arrays of antioxidant defenses and repair systems have evolved within the cell to protect itself against oxidative injury. These defenses include some enzymes such as SOD, CAT, GSH, etc. The data of the present study support the hypothesis that the efficiency of these protective enzyme systems decreases with age. In the ageing process, the oxidative damage is mostly found in parallel with the declined capacities of antioxidant systems [16]. Several studies focus on the changes of antioxidative enzyme activity in different organs and species in the process of ageing, although there have been no conclusive results so far. Since Meng et al. [17] found a significant decrease in SOD activity in the brain of old Ficher 344 rats, they suggested that SOD is likely to be highly responsible to the ageing process in brain. In addition to cellular antioxidative enzyme system, various nonenzymatic free radical scavengers including ascorbic acid, vitamin E, melatonin and CAPE

have been shown to be effective in cell protection. The main goal of the anti-ageing strategies is the elimination of harmful agent, in other words, free radicals from the environment. Antioxidative agents and free radical scavengers are currently of great interest in order to protect organs from ROS-related pathologies and aging [18–20].

Melatonin is a highly ubiquitous free radical scavenger and indirectly an antioxidant. It is well documented that it protects macromolecules from oxidative damage of ROS and RNS in all subcellular compartments. Recently, it has been shown that melatonin increases the efficiency of the electron transport chain, thus reduces the generation of free radicals [21]). It has been reported that melatonin supplementation increases both melatonin levels and melatonin binding in aging brain [22]. CAPE is an active component of propolis. It has many biological and pharmacological activities including antioxidative capabilities [23,24]. The stimulatory effect of both agents on the antioxidative enzyme activities has also been reported [25–27]. Melatonin and CAPE increased tissue SOD and GP_x activities and GSH levels in both cerebral and cerebellar tissues in our study. Melatonin administration resulted in a significant increase in SOD ($P < 0.001$) and GP_x activities ($P < 0.05$) and GSH levels ($P < 0.05$) in brain and, in SOD ($P < 0.05$) and GP_x activities ($P < 0.05$) in cerebellum. CAPE administration also increased cerebral SOD activity ($P < 0.05$) and cerebellar SOD ($P < 0.005$) and GP_x activities ($P < 0.05$). Moreover, SOD ($P < 0.05$), CAT and GPx activities of cerebral cortex and SOD and GPx ($P < 0.05$) activities of cerebellar cortex of melatonin administered rats were higher than those of young rats. SOD and GPx activities of cerebral cortex and SOD, CAT and GPx ($P < 0.05$) activities of cerebellar cortex of CAPE administered rats were also higher than those of young rats. These results represent the potent supportive effects of melatonin and CAPE on cellular antioxidative enzyme systems. Recently CAPE has been reported to block free radical production and neurotoxicity [28,29].

The cells do not die without challenging by activating their defensive systems. The levels of antioxidative defense system can be increased by cell itself if it is subjected to oxidative stress. Cell injury may be defined as a failure of the cell, on challenge, to maintain itself within homeostatic tolerance limits. Injury results first in some biochemical and molecular events, followed subsequently by impairment of function. Morphological alterations occur relatively late in this sequence.

Any cellular molecule can be the target of damaging agent. Membrane systems, energy metabolism, and protein synthesis are the most important cellular vital systems that are under threat of oxidative stress. Impairment of energy-dependent pump mechanism of membranes results in swelling of membranous organelles such as endoplasmic reticulum and mitochondria, and cell itself. Dilatation of endoplasmic reticulum in pyramidal neurons and swelling of mitochondria of pyramidal neurons, granular cells and Purkinje cells we observed, represent harmful effect of ageing related oxidative stress on membranes. Impairment of mitochondrial oxidative metabolism and energy generation is the common effect of many harmful agents. The functional events often result in an early stage of mitochondrial condensation and increased electron density, followed by progressive swelling which can ultimately result in mitochondrial rupture [30]. We observed mitochondrial swelling, crista loss and vacuole and myelin figure formation in many neurons both in brain and cerebellum of old rats. Mitochondrial membrane is subjected to oxidative damage, which results in a decreased mitochondrial membrane potential. Such mitochondrial dysfunction is the principal underlying event in ageing [31]. Lysosome accumulation in old animals is an expected circumstance since lysosomes sequester the damaged organelles by autophagocytosis. It has been reported that slowly developing apoptotic form of neuronal injury is associated with substantial increases in the number of mature lysosomes [32]. Autophagy is likely the primary mechanism by which long-lived, stable proteins are degraded, and is only mechanism by which entire organelles such as mitochondria and peroxisomes are recycled [33]. Furthermore it has been suggested that autophagy may play a protective role in early stages of programmed cell death [34].

We concluded that both melatonin and CAPE reduced ageing-induced ultrastructural alterations. Biochemically, lipid peroxidation was monitored by measuring MDA, an end product of lipid peroxidation in membrane components of cells. In both brain and cerebellum, MDA levels were higher in old animals than those of the young ones. Melatonin and CAPE reduced MDA levels of both organs. The beneficial effect of CAPE was significantly higher than that of melatonin in brain and cerebellum ($P < 0.05$). CAPE was reported to be effective in reducing cerebral MDA levels in rats with diabetes mellitus [35].

Apoptosis is a well known process that has been associated with ageing [36,37]. It is believed that the

neurons die through apoptotic mechanism. In relation to ageing, apoptosis has a primary negative effect by destroying essential and often irreplaceable cells, but it also acts to eliminate dysfunctional cells and protect the organs against cancer or hypertrophy [38]. Although we detected increased number of TUNEL + neurons in old rats, unfortunately we could not observe any typical apoptotic nuclear change, such as hemilunar chromatin condensation, apoptotic body formation etc., except peripheral heterochromatin condensation in any of the sections during our electron microscopic examination. Total surface area that is examined by electron microscope is certainly extremely smaller than that of examined by light microscope. So probably we could not coincide with any of the apoptotic cell which undergoes late stage apoptotic changes. Angleda et al. [39] found morphological characteristics of apoptosis, such as cell shrinkage and chromatin condensation in approximately 2% neurons in human substantia nigra during normal aging. However, not to detect even one apoptotic cell in any of the sections of all groups is an unexpected incident. Shimada et al. [40] has reported an age-related increase in number of TUNEL + cerebral neurons in a mouse model of accelerated senescence. Since they have not detected the morphological features of apoptosis in these TUNEL + cells, they have suggested that DNA become damaged with advancing age through a mechanism other than apoptosis. In other words, TUNEL + cells we detected may not represent apoptosis but only represents DNA damage. We report here that both melatonin and CAPE reduces DNA damage in brain ($P < 0.001$) and cerebellum of old rats. As a matter of fact that antiapoptotic effect of melatonin and CAPE on neurons has been demonstrated [41,42]. ROS are very probable initiators of the process of apoptosis, and mitochondria are believed to play crucial role in apoptotic death, by releasing apoptogenic signals (i.e. caspases). In the current specialized literature, we can find quite a number of convincing evidences concerning melatonin and its anti-apoptotic effects. Melatonin might counteract the proapoptotic action of singlet oxygen in primary cultures of cerebellar neurons [43,44].

Numerous studies have documented the relationship between morphological change and functional decline in the central nervous system. A number of age-related morphological changes in the brain, including loss of brain weight and volume, enlargement of ventricles and subarachnoid space, and alterations in cell numbers, among other, have attracted the attention of neurobiologists for many years. The loss of cortical thickness and neurons is viewed as the main cause of decline in brain function during ageing [45]. It has been shown that in senile individuals there is a decline in the thickness of cerebellar cortex, in the number of neurons, and in the volume of cerebellum [45–49]. Zhang et al. [45] reported a decrease by 7.05% in the total cortical thickness and that of the molecular layer by 10.93% and the density of neurons of all the layers of cortex in old cats. We also demonstrated decreases in the thicknesses of molecular and granular layers ($P < 0.001$). The decrease is caused probably largely by neuron loss. The results of a human study of Renovell et al. [46] demonstrated that in the human aging a notable loss exists in quantitative (38%) as well as qualitative (cells are smaller) of the granule cells. Although we did not calculate the neuron numbers, increase in TUNEL + cells represents DNA damage and indirectly neuron death. Large amount of neuron loss in cerebellar cortex has been reported in previous studies [45–47,50]. The reduction in the number of neurons leading to global motor behavior impairment has been proved [47,51,52].

In conclusion, our detailed microscopic and biochemical data indicate that both CAPE and melatonin are highly effective in protecting brain and cerebellum against age-related oxidative damage. To our knowledge melatonin is used world wide in order to avoid form aging related pathologies. We notice here that supplemental administration of CAPE is also beneficial in delaying age-related degenerative conditions in nervous system.

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