Contents lists available at SciVerse ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Short report

Grape seed proanthocyanidin lowers brain oxidative stress in adult and middle-aged rats

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ARTICLE INFO

ABSTRACT

There is growing concern over the increasing instances of decline in cognitive abilities with aging in humans. The present study evaluated the benefits of the natural antioxidant, grape seed proanthocyanidin extract (GSPE) in treating the effects of age-related oxidative stress (OS) and accumulation of lipofuscin (LF) on the cognitive ability in rats. Female *Wistar* rats of 3- and 12-months of age received a daily oral supplement of GSPE until they attained 6- and 15-months of age. During this period, rats were tested for their cognitive ability. At the end of this period, blood glucose and markers of OS were assessed in the hippocampus. GSPE lowered blood glucose, lipid peroxidation, hydrogen peroxide level, and increased protein sulphydryl (P-SH) content in the hippocampus. In addition, GSPE significantly improved cognitive performance in the two age groups. These results demonstrate that the extent of OS-related LF accumulation is reducible by GSPE. They also suggest a critical role for GSPE as a neuroprotectant in the hippocampus and in preventing cognitive loss with aging.

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1. Introduction

During the lifespan of humans and animals, lysosomes in the postmitotic cells in particular, accumulate a lot of lipofuscin, a highly cross-linked organic substance, which they are unable to metabolically degrade and exocytose them. Also an imbalance between prooxidants and antioxidants is known to initiate a chain of molecular events that results in lipofuscin (LF) formation. The brain is particularly vulnerable to oxygen and nitrogen radicals and these radicals are implicated in several disorders of the brain in humans and animals, and are linked to lipid peroxidation(LP) in the hippocampus, the cerebral cortex and several other regions of the brain (Chaimad et al., 2009).

Our earlier studies have shown that the use of antioxidants such as vitamin E, delays or prevents, the storage of lipofuscin-like substances in the cerebral cortex (CC) and the hippocampus (HC) of aging rats in vivo (Asha , 2009; Asha and Kiran, 2004) and in vitro in primary cultures of CC cells of mice (Kan et al., 1991). Lipofuscin is thought to be an innocuous end-product of these oxidations, but its excessive

accumulation results in conglomerates occupying huge areas of cytoplasm that mechanically interfere with the normal functioning of the cells (Brunk and Terman, 2002; Sundelin and Nilsson, 2001).

Grape seed proanthocyanidins are procyanidin mixtures. These are derived from the flavan-3-o1 class of flavonoids, which are widely distributed in red grape seeds that include (±)-catechin and (−)-epicatechin. Flavanols from red wines mainly catechin monomers and procyanidins dimers and trimers have a significant *in-vitro* antioxidant activity in several lipid systems and particularly act against the oxidation of LDL(da Silva Porto et al., 2003). Further, proanthocyanidins are water-soluble and can cross the blood brain barrier more easily than other antioxidants such as tocopherol. Furthermore, their bioavailability is similar to the flavanols derived from other sources that may not be absorbed until metabolized by the microflora of the intestine and metabolized once more in the liver prior to its function as an antioxidant (Scalbert and Williamson, 2000). Considering its neuroprotective function after supplementation in human (Bagchi et al., 2002) and animal subjects (Asha et al., 2006) it is important to know whether it can prevent the loss in cognitive performance in normal aging subjects.

Since long-term and invasive studies of aging in the humans can be difficult and expensive a reliable animal model has been used in the present study to selectively quantify behavioral and biochemical

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changes along with qualitative morphological measures which can serve as correlates to measures of declining cognitive function with age in humans. The purpose was to examine the effects of long-term oral supplementation of GSPE in adult and middle-aged rats on (1) spatial learning and memory of a given task; (2) age-related changes in lipid peroxidation and protein oxidation in the hippocampus and (3) morphological changes in the hippocampus.

The hippocampus was the region of interest since this is the region that coordinates with the various pathways that can make an organism to recall a task that was learnt and consolidate the same in the cerebral cortex.

2. Materials and methods

All procedures involving animals were approved by the Institutional Animal Ethical Committee (IAEC), Bangalore University, Bangalore.

2.1. Animals and experimental design

Female albino rats of *Wistar* strain and of 1–2 months of age were obtained from the Indian Institute of Science (Bangalore) and were maintained till they reached 3(adult) and 12 (middle-age) months in a colony of aging animals. These animals were placed two–three/cage in polypropylene cages fitted with stainless steel wire-mesh bottoms, maintained at a temperature of $28 \pm 1\ ^0$ C, relative humidity of $77.5 \pm 1\%$ and under daily photoperiods of 12-h light and 12-h dark cycle. They had free access to food (Amruth Feeds, Bangalore) and tap water. The feed contained 21.22% of crude protein, 4.12% of fat, 4.23% crude fibre, 1.25% of calcium, 0.76% of phosphorus, 6.75% of total ash. 52% of carbohydrates and 8.4% of moisture. Their calculated energy was 303 kcal/100 g.

2.2. Chemicals

Gravinol (Kikkoman Co.Ltd, Noda, Japan), is a natural substance extracted from grape seeds with ethanol and water as eluants and then purified and condensed, avoiding any use of toxic solvents. Gravinol powder contains 96% proanthocyanidin and other minor components. *p*-hydroxyphenylacetate(PHPA), thiobarbituric acid, pyridine, horseradish peroxidase, 5,5′-dithiobis-2-nitrobenzoate (DTNB), bovine serum albumin(BSA) , sodium dodecyl sulphate (SDS), 1,1,3,3-tetramethoxypropane (TMP) and Triton X-100 were purchased from Sigma Aldrich Chemicals (St. Louis, MO, USA). All organic solvents were obtained from Spectrochem (Mumbai, India) and other chemicals were either of reagent or analytical grade.

2.3. Grouping and supplementation

Ten rats of each age group were selected randomly and were divided into Controls (CON, $n = 5$) and Experimental (PA, $n = 5$). Controls remained on a normal diet with supplements of distilled water. Experimentals received an oral supplementation of 75 mg per kg body weight (BW) of GSPE daily for a total period of 12 weeks. The animals were weighed weekly during the entire maintenance period. During the period of supplementation animals were subjected to behavioral experiments.

2.4. Behavioral studies

2.4.1. Learning
Learning through simultaneous or successive brightness-discrimination task in a T-maze. Experiments were performed in a dimly lit room that was adjacent to the home cage room and were similar to those described in an earlier study (Jolitha et al., 2009). In brief, in this task, the goal (food pellet) was systematically connected with the discriminida (light). The correct arm of the T-maze was dimly illuminated with a zero candle bulb. Rats were initially allowed to explore the apparatus for 5 min with this experimental set up. The lights of both the goal areas were switched on. The start compartment door was raised, the animal was allowed to make a choice, if it entered, of the correct arm; the animal had an access to the food pellet. If a wrong choice was made, the animal was made to stay for 3 min as a punishment. Each animal was given 3–5 min and an inter trial interval of 15–20 min. This task was continued until the animal learnt to perform at least nine correct choices out of 10 trials/day. Therefore the total number of trials was 30 in number.

2.4.2. Memory
Memory was tested after a period of 7 d, 15 d, 21 d and 30 d following the last learning trial as memory retention test. Here the goal was randomly shifted from right to left and vice versa and tested for the number of correct choices.

2.5. Biochemical studies

2.5.1. Blood glucose
Blood glucose was measured by the glucose oxidase method and using glucose kit (Kruise, Ahmedabad, India).

2.5.2. Isolation of mitochondria and hydrogen peroxide assay
The procedure used in this study was a modified version of Bruce and Wings (1984) and have been explained in an earlier paper (Jolitha et al., 2006). The rate of hydrogen peroxide release by the mitochondria was measured fluorometrically (Genway, UK), following Hyslop and Skar (1984), by oxidation of *p*-hydroxyphenylacetate, coupled to enzymatic reduction of hydrogen peroxide by horseradish peroxidase. Release of hydrogen peroxide was expressed in terms of nmol/mg protein/min.

2.5.3. Catalase (CAT, EC 1.11.1.6)
CAT was measured by the method of Aebi (1984). An extinction coefficient of 43.6 M/cm was used to determine CAT activity, one unit of which is equal to the millimoles of hydrogen peroxide degraded per minute per mg protein.

2.5.4. Lipid peroxidation (LP)
Malondialdehyde (MDA), a product of lipid peroxidation was measured by the method of Ohkawa et al. (1979) using TMP as a standard. MDA was expressed in terms of nmol/mg protein.

2.5.5. Protein oxidation
The concentration of protein sulphydryl content was measured spectrophotometrically(ELICO, SL159, India) following Habeeb (1972). The concentration of –SH– was calculated from extinction coefficient of $13{,}600\ M^{-1}\ cm^{-1}$ and expressed as μmol of DTNB/mg protein.

2.5.6. Protein measurement
Total protein content of mitochondria was measured by the method of Lowry et al.(1951) using BSA as a standard.

2.6. Tissue preparation for qualitative microscopy studies

At the end of the memory tests and at the ages of 6 months and 15 months, the animals were etherized mildly, perfused with phosphate buffered saline (PBS), and the brains were carefully removed, weighed and the isolated hippocampus was placed in ice-cold PBS for biochemical studies and in appropriate fixatives for histological and ultrastructural studies.

2.6.1. Light microscopy
Dorsal hippocampal tissue was fixed in 10% formalin and embedded in paraffin. With these specimens, three-μm sections

were cut serially with a rotatory microtome for morphological and histochemical studies.

2.6.1.1. Hematoxylin and eosin staining. Paraffin sections were deparaffinized in xylene, rehydrated in alcohol grades and stained with hematoxylin, differentiated in 1% acid alcohol, blued in 0.2% ammonia water, and rinsed in 95% alcohol (Pearse, 1961). Finally these sections were counterstained with eosin, dehydrated in grades of alcohol, cleared and mounted in DPX. All sections were visualized under a light microscope blinded to the brain sections.

2.6.1.2. Sudan Black B staining. Five-μm paraffin sections were deparaffinized with xylene and serial gradient concentrations of alcohol were used to dehydrate them. Slide-mounted sections were incubated with Sudan black B dye for 3 h and excess of dye was removed by washing in 70% alcohol. These sections were counterstained with neutral red, washed and mounted in glycerin jelly and cover slipped for visualization of neurons with lipofuscin pigment granules (Pearse, 1961). They were observed under an inverted microscope (Olympus IX, Japan) and images were captured using cool snap CCD camera.

2.6.2. Electron microscopy

For ultra structural studies the dorsal hippocampus was fixed with buffered 3% glutaraldehyde in phosphate buffer (pH 7.3) for 24 hours followed by post fixation in 1% osmium tetroxide and subjected to the conventional procedure as described by Palay and Chan-Palay (1974). Tissue blocks were cut under Leica Ultramicrotome. Initially 1 μm thick sections collected on a plane glass slide were stained using 1% toluidine blue and viewed under light microscope to find interested areas and to study light microscopic features. Later randomly selected 400–500 Å thick ultrathin sections were stained using uranyl acetate & lead citrate and were scanned under the transmission electron microscope (Tecnai G2 Spirit Bio-twin) and representative digital images were captured using a *Megaview-III* CCD camera.

2.7. Statistical analyses

All the data are expressed as mean ± S.E. and are analyzed within a two factor analysis of variance (ANOVA) between age and groups. When a significant F ratio was found, Tukey's post-hoc tests were used to assess the differences between group means. Probability values $(P) < 0.05$ were considered significant. Learning and memory were analyzed by two-way ANOVA between trials and groups followed by a post-hoc test. Only statistically significant data are described in the results section.

3. Results and discussion

The present study as well as the earlier study (Asha et al., 2006) on female rats acquires special significance in the context of the detailed report on the failure of combined estrogen and progestin therapy in preventing cognitive loss in post-menopausal women (Shumaker et al., 2003). In this study, we did not observe any significant differences in body weight between normal control rats and experimental rats during the 12 week-GSPE supplementation period (Supplementary Fig. S1). To evaluate the changes in cognitive ability in response to GSPE, we examined learning in terms of acquisition of a defined task in the T-maze and by the number of trials that was required to make 80% to 90% accurate choices (Fig. 1). Among the adults, supplemented controls showed a gradual progress in learning during the initial three trials (58%, 74%, and 73%), and further training to make correct choices enhanced maximal learning capacity (85%) by the fourth trial (Fig. 1A). Considerable retention in the learned task was noticed after an interval of 7 days (79%). The percentage of retention was still significant in these animals even after 14, 21 and 30 days (74%, 59% and 56%) compared to what was seen in the unsupplemented ones (Fig. 1B). Among the middle-aged, supplemented controls showed a better learning potential of 42%, 74%, and 96% during the first, second and third trials respectively. During the fourth trial, supplemented animals showed 98% learning ability (Fig. 1C). GSPE was effective in enhancing the memory retention as evident by the per cent retention capacity by 78%, 76% 75% and 66%, which were higher than those of unsupplemented counterparts (58%, 58%, 50% and 34%) at 7, 14, 21 and 30 days respectively (Fig. 1D). The results showed an overall significance between the two groups and the number of trials conducted, between the groups. The improvement in learning and memory especially in the middle-aged animals suggest that grape seeds like proanthocyanidin-rich blue berries may increase the rate of hippocampal cells formation and develop receptors for neurotransmitters (Casadesus et al., 2004; Bastianetto and Quiron, 2002). We reported previously that AChE activity was reduced in GSPE-supplemented female adult rats (Asha et al., 2006). Our results also suggest that the age-associated decline in learning and memory may be related to the altered metabolism in response to increased oxidative stress experienced by the hippocampal neurons.

GSPE was effective in reducing the blood glucose level to that of the adults in the middle-aged rats, and this decrease was by 15.6% when compared to that of the controls. In the adult, however, there was no significant effect of GSPE on their glucose level (Supplementary Fig. S2). In the control animals, glucose increased by 11% in the middle-aged (130 mg/dL) as compared to the adult (110 mg/dL). Interestingly our results have shown that GSPE is effective in reducing the middle-aged glucose value by 15.6% compared to of the control. The current data on improved memory retention because of GSPE in the middle-aged rats suggest that GSPE could impact the hippocampal neurons either in terms of improved regulatory mechanisms for glucose uptake or by altered storage mechanism through chemical or structural components. Interestingly, blood glucose regulation is also possible by berry extracts which can inhibit α-glucosidase and α–amylase (McDougall et al., 2005) and this method is an accepted means of regulating post-prandial glucose levels in patients suffering from non-insulin-dependent diabetes mellitus (Battino et al., 2009).

Hydrogen peroxide increased by 2.2-fold in the middle-aged when compared to the adult animals. GSPE was more effective in reducing hydrogen peroxide generation in the middle-aged (46%) than in the adult animals (34%). Catalase activity increased by 29% in the middle-aged when compared to the adult animals (Table 1). GSPE reduced this activity by 38% and 31% in the adult and middle-aged when compared to respective controls.

Among the controls, MDA level was 36% higher in the adults and the middle-aged. It is known that the brain, predominantly composed of fat undergoes increased lipid peroxidation with age (Bagchi et al., 2002). MDA in the hippocampus also decreased by 41% and 34.5% in GSPE supplemented adult and middle-aged animals when compared to their respective controls, indicating reduced oxidative stress. On the other hand, protein sulphydryl content, which is a non-enzymatic cellular antioxidant defense, increased in response to GSPE in the adult (84.18%) and the middle-aged (50.85%) animals (Table 1).

Fig. 2A and D are sections stained with hematoxylin and eosin and demonstrate the morphology of the hippocampal cell layers in the adult and the middle-aged rats. Using Sudan Black B, we were able to identify lipofuscin-like dark-brown deposits in the adult (Fig. 2B) and middle-aged (2E) control rats. The deposits were visible as brown pigments in the cytoplasm, and a qualitative observation showed more neurons with such deposits in the middle-aged rats than in the adult. However, these deposits were less visible in the neurons of GSPE supplemented animals (Fig. 2C and F).In order to verify these observations, electron microscopic sections were prepared, which confirmed lipofuscin deposition. The ultrastructural study of hippocampal neurons of adult control animals showed lipofuscin as small and homogenous deposits resembling primary lysosomes (Fig. 3A and
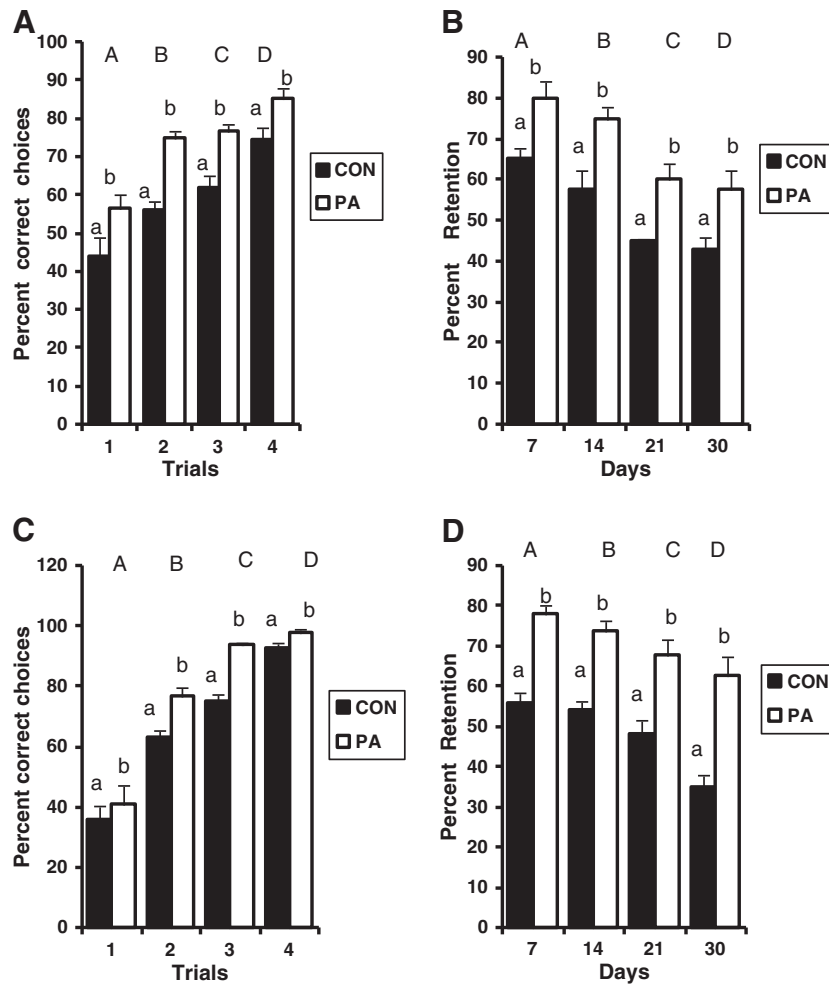
Fig. 1. Percent correct choices during learning (1A, 1C) and memory retention (1B,1 D) in 3- and 12-mo-olds. CON, control, PA, proanthocyanidin. Values are means ± S.E. ($n = 5$ animals/group). Learning test was analyzed by two-way ANOVA between trials and groups followed by Tukey Kramer's multiple comparisons test and considered significant at $P < 0.05$. Significance between trials is represented in upper case (A,B) and between groups in lower case (a,b). Those not sharing the same upper and lower cases are significantly different at $P < 0.05$. Changes were significantly different between the two ages and between the two groups.

B) on the outer periphery of the nucleus. Lipofuscin in the rats from the GSPE-supplemented group was less when compared with the control (Fig. 3C). The neuronal cell bodies in the middle-aged controls showed numerous large lipofuscin deposits that were distributed uniformly all round the nuclear periphery (Fig. 3D). At certain sites, lipofuscin was seen as fused non-homogenous and vacuolated deposits with a more complex structure (Fig. 3E). However, in the GSPE supplemented animals, lipofuscin deposition was minimal (Fig. 3F).

Our results on increased protein oxidation along with increased lipofuscin accumulation may be explained in terms of possible age-related elevation in iron-catalyzed oxidative processes in the hippocampus. Iron atoms in the lysosome produce abundant reactive oxygen species (ROS), which chemically crosslink the abnormal structures into larger masses in the cytoplasm. Further, it is known that lipofuscin sensitizes lysosomes and neurons to generate oxidative stress. Such damage in the neurons is shown to result in defective mitochondria, oxidatively modified cytosolic proteins and intralysosomal lipofuscin (Terman and Brunk, 2006). Lysosomes are lined with hydrogen peroxide derived from mitochondria and other organelles as well as with iron from digested membranes (Brunk and Terman, 2002). The reaction between Fe (11) and H_2O_2 also results in the production of toxic

Table 1
Oxidative stress parameters as a function of age and grape seed proanthocyanidin supplementation in female albino rats.

Age (months)	Oxidative stress parameters							
	MDA		CAT		P-SH		H_2O_2	
	CON[a]	PA[b]	CON[a]	PA[b]	CON[a]	PA[b]	CON[a]	PA[b]
3[A]	1.57 ± 0.10	0.93 ± 0.15	29.78 ± 1.0	18.41 ± 1.5	10.0[A] ± 1.0	18.4 ± 0.5	0.49 ± 0.05	0.32 ± 0.04
12[B]	2.14 ± 0.15	1.47 ± 0.25	38.40 ± 1.5	26.43 ± 2.0	10.3[A] ± 0.5	24.6 ± 1.0	1.12 ± 0.03	0.61 ± 0.02

Values are mean ± SE of five animals/group. Catalase(CAT) activity is expressed as mmol of hydrogen peroxide(H_2O_2) degraded/min/mg protein, malondialdehyde(MDA) as nmol/mg protein, protein sulphydryl level(P-SH) is as micromoles/mg protein and H_2O_2 in terms of nmol/mg protein. CON, control; PA, proanthocyanidin. Significance between group means of two age groups was analyzed by two-way ANOVA followed by Tukey Kramer's multiple comparisons test and considered significant at $P < 0.05$.Values between the control and experimental are represented in lower case (a,b) and between the age groups in upper case (A,B). Changes were considered insignificant different between the ages and between the groups whenever they shared the same letters.
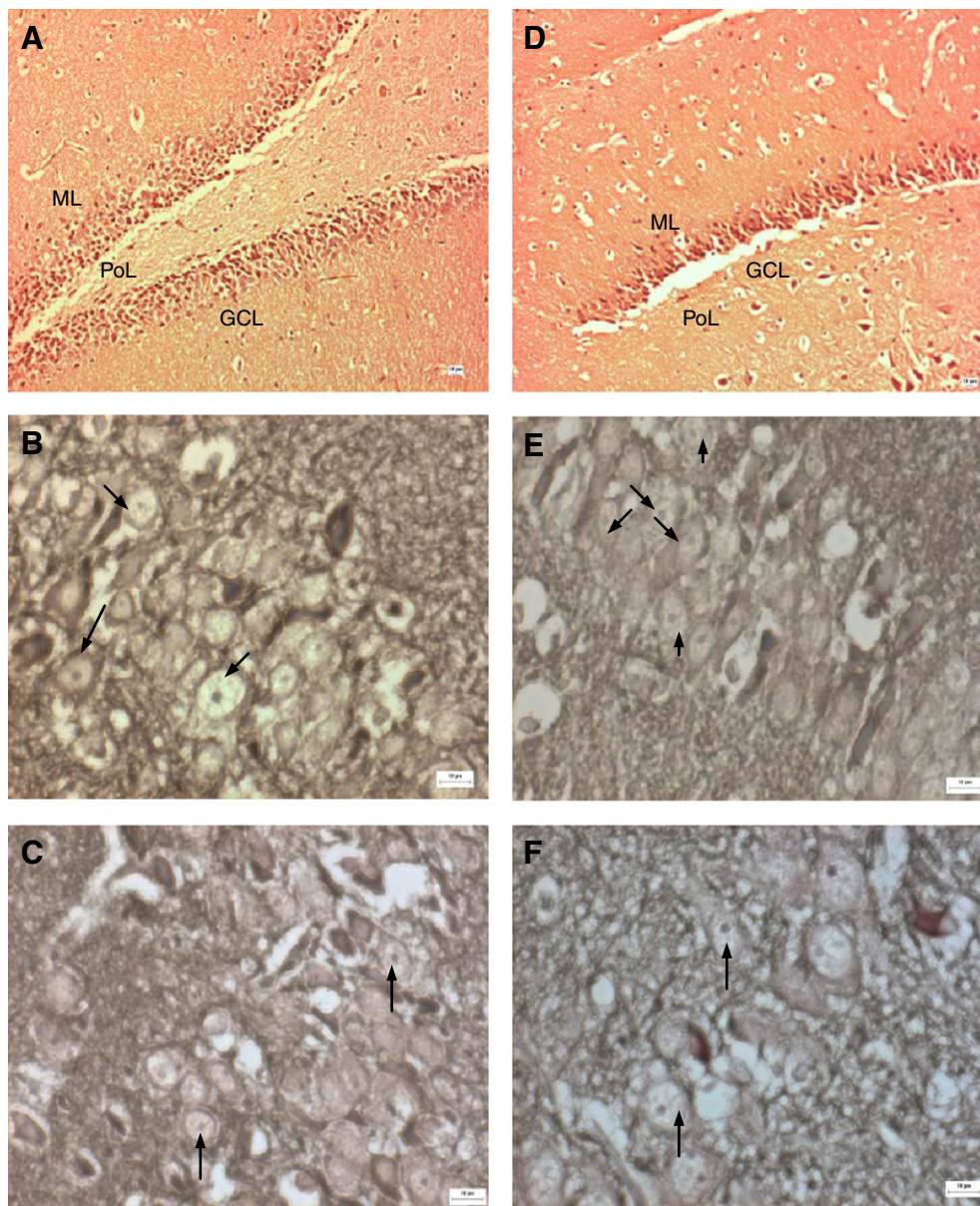
Fig. 2. Photomicrographs of rat hippocampus. Hematoxylin-stained sections from (A) 3- and (D) 12-mo-old rat hippocampus displaying normal architecture (scale = 5 μm). GCL, granular cell layer. ML, molecular layer. PoL, polymorphic layer. (B) Granular cells showing very few Sudan black-B lipofuscin-like deposits at the outer nuclear periphery (arrows) in the 3-mo-old control rat. (C) Cells from GSPE supplemented rats showing lesser number of cells with lipofuscin-like deposits. (E) Neurons from 12-mo-old control. Intense accumulation of lipofuscin-like deposits is seen at the periphery of control neurons. (F) Fewer deposits appear in the nuclear periphery in the GSPE supplemented animals (scale = 10 μm).

hydroxyl radicals, which leads to lipid peroxidation. Our results on reduced accumulation of lipofuscin in the GSPE-fed rats may be attributed to the powerful iron-chelating property of the GSPE (Wu et al., 2010). Grape products are known for their highest antioxidative capacity because they act by neutralizing certain free radicals or by minimizing oxidative injury on membranes (Jamroz and Beltwoski, 2001).

Finally, the results of the present study support the possibility of utilizing GSPE in alleviating oxidative stress-related lipofuscinogenesis with age in the hippocampus. We have demonstrated improved cognitive ability and reduced blood glucose levels in response to GSPE supplementation in the middle-aged rats. This is interesting since aging is accompanied by oxidative stress-related lipid and protein oxidations and hydrogen peroxide generation all leading to increased lipofuscino-genesis in the hippoc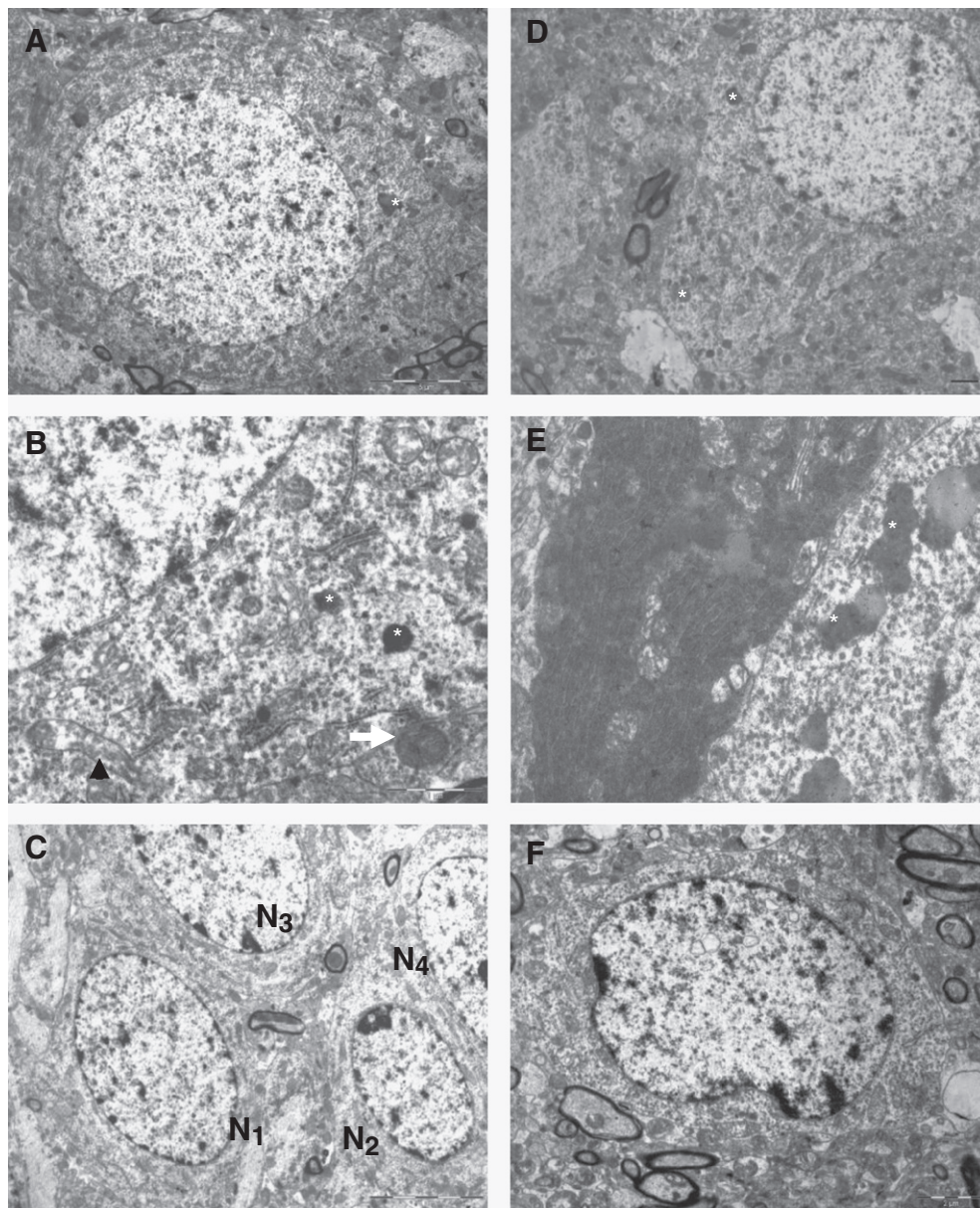ampal neurons and decline in learning and memory ability. Currently our laboratory is identifying specific metabolites of GSPE in the hippocampus since understanding these mechanisms may be useful for developing anti-ageing natural products and managing cognitive deficits with aging.

Fig. 3. Ultrastructure of the neuron hippocampus of 3-mo-old control rat. (A) The neuronal cell body shows lipofuscin granules as dark deposits (asterisk, ×4800). (B) An enlarged neuron showing normal mitochondria (arrow) and a mitochondria that has undergone degenerative changes with vacuolations(arrow head). Lipofuscin deposits were seen as single deposits in the cytoplasm (asterisk, ×18,500). (C) Four neurons (N_1, N_2, N_3 and N_4) in GSPE-treated rats. Lipofuscin granules were hardly evident (×4800). (D) Neuron of 12-mo-old control rat with lipofuscin (×4800). (E) lipofuscin granules showing heterogeneity: dark and light vacuoles (×11,000). (F) Neuron from GSPE treated rats are characterized by fewer sites of lipofuscin deposition (×6800).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10. 1016/j.exger.2011.08.006.

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