Grape Seed Proanthocyanidin Extract (GSPE) and antioxidant defense in the brain of adult rats

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Summary

Background: Proanthocyanidin (PA) is a naturally occurring antioxidant from grape seed extract. The present study aims at assessing the neuroprotective effects of grape seed proanthocyanidin (GSPE) on the cerebral cortex (CC), cerebellum (CB), and hippocampus (HC) in the adult rat brain.

Material/Methods: GSPE was orally administered at 25, 50, and 75 mg per kg body weight daily and for a total period of 9 weeks. Antioxidant enzymes (AOEs), superoxide dismutase (SOD), and catalase (CAT) were analyzed along with malondialdehyde (MDA) and protein carbonyl content (PCC) as markers of lipid peroxidation (LPO) and protein oxidation (PO). The cholinergic system was studied by analyzing choline acetyl transferase (ChAT) and acetylcholine esterase (AChE) activities along with acetylcholine content (ACh).

Results: The results obtained revealed an increased SOD activity in the 75-mg PA-supplemented animals, with a substantial decrease in MDA and PCC. The cholinergic neurotransmitary system analysis showed increased ChAT activity indicative of increased Ach content in the supplemented animals and the increase was more in the 75-mg PA group with a concomitant and moderate decrease in AChE activity. Regional changes were more with reference to HC.

Conclusions: Our study shows that PA intake in moderately low quantity is effective in up-regulating the antioxidant defense mechanism by attenuating LPO and PO. Changes in the cholinergic system, however, indicate an increase in the Ach concentration with a moderate reduction in AChE activity, suggesting further that PA may have a potent role in enhancing cognition in older rats.

key words: brain • catalase • lipid peroxidation • proanthocyanidin • protein oxidation • superoxide dismutase

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Background

The present scenario in the field of antioxidant research is characterized by a focus on the biological, pharmacological, and medical properties of plant-derived polyphenols, which have gained attention as functional food components. In vivo protective effects of natural phytonutrients against reactive oxygen species (ROS) have been attributed to flavonoids, a class of polyphenols [1].

Brain cells are continuously threatened by the damage caused by free radicals and ROS produced during normal oxygen metabolism or induced by exogenous sources [2,3]. The mechanism and the sequence of events by which free radicals interfere with cellular functions are not fully understood, but one of the most important events seems to be lipid peroxidation (LPO), which results in cellular membrane damage, causing a shift in the net charge of the cell, changing the osmotic pressure, and depleting endogenous scavenging compounds, leading to swelling and, eventually, cell death. The loss of motor function due to cell death and region-specific loss of neurons in the mammalian brain with OS is more prominent in the cerebral cortex (CC), hippocampus (HC), and cerebellum (CB). The CC and HC are regions associated with cognition and feed-back control of stress, and the CB is concerned with motor function [4]. In vitro studies on the responses of the CC to administered lipid-soluble antioxidants such as vitamin E have revealed its neuroprotective role in the brain of mice [5]. Green tea catechins have also been reported for their antioxidative property and preventive effects against cerebral ischemic damage in rat brain [6]. Water-soluble flavonoids, that have an additive effect on cellular antioxidant enzymes [7,8], can also have provocative effects on the function of the endogenous antioxidant system (AOS). An increasing number of studies on polyphenols suggests the ability of flavonoids to protect against neuronal cell death [9].

Proanthocyanidin (PA) is a naturally occurring polyphenolic compound widely available in fruits, vegetables, nuts, seeds, and flowers. Grape seed PA, a combination of biologically active flavonoids including oligomeric PA, exhibits a novel spectrum of biological, pharmacological, therapeutic, as well as chemoprotective properties against oxygen free radicals and OS. Epicatechin, a component of PA, has not only been found to be a potent neuroprotector preventing neuronal cell death, but it also improves memory [10]. Though studies have revealed the potential role of PA as a cardioprotector and minimizer of atherosclerosis, little has been done to assess its role as a neuroprotective antioxidant compared with other water-soluble antioxidants [11].

It was our interest to look into the possible role of PA as an effective AO in critical areas of the brain. Therefore the objectives of the present study were framed to study 1) PA as an antioxidant in up-regulating the antioxidant defense mechanism when administered in moderately low quantities, which was done by assessing the superoxide dismutase and catalase activity, the main detoxifying enzymes in the brain, and also through an evaluation of malondialdehyde (MDA) and protein carbonyl content (PCC), and 2) the changes in the cholinergic neurotransmitter system in response to PA by measuring the changes in ChAT and AChE activity, along with ACh concentration in the CC, HC, and CB. These objectives were framed to test the hypotheses that 1) PA intake, when initiated in adults, may equip the endogenous antioxidant defense system to withstand the OS during later years of life and 2) that possible alteration in the cholinergic system in response to PA supplementation may be beneficial in minimizing age-related loss of cognition.

Material and Methods

Animal maintenance and care

The present study was approved by the Institutional Animal Ethics Committee (IAEC), Bangalore University, Bangalore, India. Male albino rats of the Wistar strain weighing about 290 grams and 4 months (4-mo) of age were obtained from the Central Animal Facility, IISc, Bangalore. These were housed 3 per cage in polypyrrole-fitted steel mesh-bottom cages and were maintained at a temperature of 28±1°C, relative humidity of 77±1%, and were exposed daily to a 12/12-hour light/dark cycle. The animals had free access to feed (Amruth feeds, India), and tap water was supplied ad libitum. They were categorized into three experimental groups, PA1, PA2, and PA3, of five animals, receiving an oral supplementation of 25, 50, and 75 mg of PA per kg body weight (BW), respectively. Controls (C) remained on normal diet with supplements of distilled water. Weekly changes in body weights were recorded to assess the effect of PA supplementation in the experimental groups.

Chemicals

Gravinol (Kikkoman Co. Ltd, Noda, Japan), received as a generous gift from Prof. Naokata Ishii, Tokyo, is a natural substance extracted from grape seeds with ethanol and water as eluents and then purified and condensed, avoiding any use of toxic solvents [12]. Gravinol powder typically contains (by weight) 40% proanthocyanidin, 2.4% monomeric flavonoids, 2.4% water, 3.7% crude protein, 0.5% ash, 7.8% glucose, 2.7% other sugars, 16.7% citric acid, 5.1% other acids, and very minor components. Epinephrine, thiobarbituric acid, triton X-100, 1, 1, 3, 3 tetramethoxy propane, and BSA stock were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals were either of reagent or analytical grade.

Tissue preparation

Animals were mildly etherized and decapitated and the brain was immediately removed, rinsed in ice-cold saline, and weighed. The cerebral cortex (CC), hippocampus (HC), and cerebellum (CB) were isolated, weighed, and homogenized in 50 mM phosphate buffer (pH 7.0), containing 0.1 mM EDTA to obtain a 5% homogenate for the catalase (CAT) and malondialdehyde (MDA) tests. SOD was assayed in the supernatant obtained after centrifugation of the 5% homogenate at 600 x g for 10 min at 4°C, while protein carbonyl content (PCC) was assayed in the supernatant obtained after centrifugation at 12,000 x g for 10 min at 4°C (RV/FM Superspin, Plastocraft, India.)
**BIOCHEMICAL ANALYSES**

**Antioxidant enzymes**

CAT (E.C. 1.11.1.6) activity was determined in the homogenate as described by Aebi [15]. Briefly, 100 µl of the tissue supernatant was incubated with an equal volume of absolute alcohol for 30 min at 0°C followed by the addition of triton X-100. A known volume of this tissue reaction mixture was taken in an equal volume of 0.066 M H₂O₂ in phosphate buffer and absorbance was measured at 240 nm for 30 s in a spectrophotometer (ELICO, Model SL 159). An extinction coefficient of 43.6 M/cm was used to determine enzyme activity, which was expressed in terms of mM of H₂O₂ degraded/min/mg protein. SOD (E.C. 1.15.1.1) activity was measured by the method of Misra and Fridovich [14]. 0.05 M carbonate buffer (pH 10.2), containing 0.1 mM EDTA, followed by 30 mM epinephrine in 0.05% acetic acid were added to the tissue extract and the change in activity was measured at 480 nm for 4 min. Activity was expressed as the amount of enzyme that inhibited the oxidation of epinephrine by 50%, which is equivalent to one unit and is expressed in terms of units/mg protein.

**Lipid Peroxidation (LPO)**

Malondialdehyde (MDA) was determined by the method of Ohkawa [15] using 1, 1, 3, 3 tetramethoxy propane as standard. In brief, 8.1% SDS was added to the tissue homogenate and incubated for 10 minutes at room temperature (RT), followed by boiling with 20% acetic acid and 0.6% thiobarbituric acid for 60 minutes in a water bath. On cooling, butanol: pyridine (15: 1 v/v) was added and centrifuged at 600 x g for 5 min. Absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was expressed in terms of nM/mg protein.

**Protein Oxidation (PO)**

Protein carbonyl content (PCC) was measured according to the procedure of Levine [16]. Briefly, 10 mmol DNPH in 2.5 M HCl was added to the tissue homogenate and incubated in the dark for 60 min at RT. This was followed by vortex mixing, addition of 20% TCA (w/v), and subsequent washing thrice with ethanol: acetyl acetate (1:1 v/v) mixture. Precipitated proteins were then redissolved in 6 M guanidine HCl in 20 mM phosphate buffer (pH 6.5). Insoluble substances were removed by centrifugation and absorbance of the supernatant was read at 370 nM. An extinction coefficient of 22,000 M/cm was used to determine the protein carbonyl content and expressed as mM/mg protein.

**Cholinergic system**

**Choline acetyl transferase (ChAT) (E.C. 2.3.1.6)**

ChAT activity was determined by the method of Nachmansohn and Wilson [17]. Briefly, 0.05 M choline chloride, 0.0005 M acetyl Co-A, 12 µg/ml eserine, and 0.001 M EDTA and 0.05 M MgCl₂ in 0.01 M phosphate buffer (pH 7.0) were incubated for 30 min at RT along with tissue homogenate. The reaction was stopped by the addition of 12% TCA and centrifuged at 1500 x g. Nine mM of 5, 5’ dithiobis in 1M phosphate buffer (pH 7.0) was added and the decrease in absorbance was recorded at 412 nm for 3 min. An extinction coefficient of 1.36×10⁻⁴/cm was used to express the enzyme activity in µM of ACh synthesized/hour/100 mg protein.

**Acetylcholine (ACh)**

Tissues were homogenized in 0.02 M ice-cold phosphate buffer (pH 7.0) containing 3 mg/ml eserine. ACh was estimated by Hestrin’s method [18]. Briefly, to 0.004 M buffered substrate, tissue homogenate was added and incubated for 1 min at room temperature, followed by the addition of hydroxylamine hydrochloride, 4 N HCl and 0.37 M FeCl₃. After 1 min, the absorbance was recorded at 540 nm. Standards were prepared with acetylcholine chloride and the levels of ACh were expressed as µg/g tissue.

**Acetylcholine esterase (AChE) (E.C.3.1.1.7)**

AChE was determined by Hestrin’s method. 0.004 M buffered substrate was added to the tissue homogenate and incubated for 1 min followed by the addition of hydroxylamine hydrochloride, 4 N HCl, and 0.37 M FeCl₃. Absorbance was recorded a 540 nm against reagent blank. AChE activity was expressed as µM of ACh hydrolyzed/min/mg protein.

**Statistical analyses**

All the data were expressed as mean ±SE. A two-way ANOVA was conducted to assess the effect of proanthocyanidin supplementation on SOD, CAT, LPO, and PO between the regions and the groups PA₁, PA₂, PA₃, and control. When a significant F ratio was found, Duncan’s multiple range test (DMRT) was used to assess the significance at p<0.05. The paired t-test was conducted for CAT/SOD ratios between the control and the supplemented groups. Results were considered significant at p<0.001.

**RESULTS**

**Antioxidant Enzymes AOE, LPO, and PO**

CAT activity did not reveal any significant changes in all the experimental groups (Figure 1A). SOD activity was considerably up-regulated in all the experimental groups, with PA administration of 75 mg group revealing an increase by 70% in the HC. However, when analyzed between the supplemented groups, the effect was less pronounced in PA₃ animals (Figure 1B). CAT/SOD ratio, evaluated as a marker of tissue H₂O₂ content, decreased in the HC and CB of all the supplemented groups, but more prominently in the PA₃ group. MDA, a product of LPO, was reduced in the CC and HC of PA₃ animals by 70 and 58%, respectively (Table 1). Our results showed greater LPO in PA₂ than in the PA₁ and PA₃ groups. A decreased PCC by 78 and 86% were noticed in the HC and CB, respectively, of PA₃ group. Regional changes were noticeable, with CC revealing a higher extent of PO (Figure 1D).

**Cholinergic system**

The cholinergic system, when studied in the different regions of the brain, revealed higher ChAT activity in the PA₃ group.
group (Figure 2A), with a substantial increase in ACh content (Figure 2B). AChE activity was considerably reduced in all the supplemented groups. Regional variations revealed a smaller AChE activity in the CC than the other two regions (Figure 2C).

**DISCUSSION**

During the last decade, nutrition and antioxidant nutrients have been a focus of health promotion and disease prevention. The focus of concern has shifted from issues of deficiency to health maintenance, throughout all phases of life cycle. It is recommended that healthy adults should consume at least 5–7 servings/week of fruits and vegetables to curtail the free radical damages occurring to vital organs during the later stages of life. Grape products such as wine are especially well known for their protective role in maintaining a healthy heart [19], and studies have indicated that grape products have the highest antioxidant capacity of any other fruit [20], and this may be mediated by neutralizing certain free radicals [21] or by minimizing oxidative attack on membranes [20,22]. Studies have established a “no-observed-adverse-effect” level of grape seed extract for up to 1410 mg/kg BW in male and 1501 mg/kg BW in female Fischer 344 rats [23].

The main objective of the present study was to test the effect of GSPE supplementation in reducing LPO and PO and in strengthening the antioxidant defense and cholinergic neurotransmitter system in the brains of adult rats. The cerebral cortex, hippocampus, and cerebellum were the regions of interest since they are known to be the most...
integrative parts affected during aging and various neurodegenerative disorders. The main detoxifying enzymes in OS are SOD and CAT. SOD destroys free-radical superoxide by converting it to peroxide, which in turn is destroyed by CAT and GPx. CAT is more significant in protecting against severe OS, whereas the glutathione redox cycle, comprising glutathione reductase, glutathione transferase, and GPx, is the major source of protection against low levels of oxidative stress. Proanthocyanidins are seen to inhibit CAT activity while enhancing glutathione transferase activity [24].

True to our hypothesis, the endogenous defense mechanism was strengthened in the PA-fed rats. A relatively higher SOD activity and an unaltered CAT activity signify the role of GSPE against H2O2-induced oxidative damage. Earlier studies from our lab showed increased SOD activity by 30–35% in the HC of adult rats supplemented with 50 IU of vitamin E/kg b.w. [25], signifying PA as a better AO than vitamin E in up-regulating the AO defense system. Our results also revealed a decrease in the CAT /SOD ratio, a marker of H2O2 content in the tissue. This is also substantiated by findings that GSPE provided neuroprotection in H2O2-treated glial cells [26,27]. The detoxification process of H2O2 is a cumulative function of CAT and GPx. The concentration of GPx, the alternate H2O2 scavenging enzyme, is relatively low in the brain compared with CAT. Furthermore, the CAT/SOD and GPx/SOD ratios are better indicators of antioxidant cellular responses than individual enzyme activities [28]. Our findings of a significant decrease in the CAT/SOD ratio in the HC and CB of all three supplemented groups may reflect an inhibited CAT activity and, perhaps, an up-regu-
lated GPx activity by PA. The possible increase in the ratio of GPx/SOD may be speculated. An earlier study demonstrated an increase in GPx activity in the CC and HC of the adult rat brain with no increase in CAT activity in response to vitamin E supplementation [25].

As we hypothesized, a reduction in the LPO was found in the regions of the brain supplemented with PA and more so in response to 75-mg supplementation. GSPE contains high levels of resveratrols and polyphenols, especially catechin, gallic acid, epicatechin, and the like, that effectively scavenge the peroxyl radicals that initiate LPO.

An oral dosage of PA at a higher concentration was found to be effective in minimizing the accumulation of PO end products. However, lower doses of PA failed to reduce carbonyl content in all the three regions analyzed. The inefficiency of PA doses at a lower concentration in attenuating protein damage can be explained through the dinitrophenyl hydrazine (DNPH) reactivity of proteins. It has been suggested that non-enzymatic glycation and reaction with aldehydes generated from peroxidized lipids can give rise to carbonyl groups in proteins [29]. Hence there is possible involvement of glycation of aldehydes in the generation of protein carbonyls. Therefore, protein products detected by the DNPH technique may not necessarily be direct oxidation products, but rather those formed by glycation or secondary reactions from LP [30]. Thus only higher doses of PA, at 50 and 75 mg, proved to be more effective in minimizing the protein oxidation.

Cholinergic neurotransmitter enzymes responded considerably to PA supplementation, as was hypothesized. Our results substantiate the idea that regular intake of fruits and vegetables rich in polyphenols enhance cognition. We have reported an increased synthesis of ACh with a moderately decreased AChE activity, a situation favorable to enhanced cognition. The changes were more evident in the CC and HC regions involved in learning and memory.

**Conclusions**

In summary, our results on the neuroprotective effect of GSPE at a dose of 75 mg/kg BW/day in terms of an effective reduction in the LPO as well as PO while enhancing antioxidant enzymes support our suggestion that moderate quantities of GSPE consumption from an earlier age can influence combating free radical-induced damage in the critical areas of the brain at later stages of life. This could form an interesting area for future research towards an evaluation of the mechanisms of PA action as a functional food in curtailting free-radical damage in the brain and therefore warrants further studies on antioxidant therapy for minimizing disorders of the brain.

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