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# Mitochondrial alterations in aging rat brain: effective role of (−)-epigallo catechin gallate

Ravichandran Srividhya [a], Kamelija Zarkovic [b], Marina Stroser [b], Georg Waeg [c], Neven Zarkovic [d], Periandavan Kalaiselvi [a,*]

[a] Department of Medical Biochemistry, Dr. A.L.M. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai 600 113, India
[b] Division of Neuropathology, Medical Faculty University of Zagreb, Clinical Hospital Centre, Kispaticeva 12, HR-10000 Zagreb, Croatia
[c] Institute of Molecular Biosciences, University of Graz, Heinrichstr. 31, A-8010 Graz, Austria
[d] Laboratory for Oxidative Stress, Rudjer Boskovic Institute, Bijenicka 54, HR-10000 Zagreb, Croatia

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ABSTRACT

Aging is a multi-factorial process which involves deprivation in body's metabolism. Brain mitochondria are prone to oxidative damage owing to their high metabolic rate. The decline in antioxidant system during aging augments the neuronal damage to mitochondrial components like antioxidant system, Kreb's cycle enzymes and electron transport chain complexes. Since brain is an organ rich in fatty acids, lipid peroxidation products like hydroxynonenal are predominant. Those lipid peroxidation products conjugate with amino acids to form adducts which alter their structural and functional properties. Epigallo catechin gallate is a potent antioxidant which is rich in green tea extract. This study elucidated the antioxidant potential of epigallo catechin gallate to counteract the mitochondrial oxidative damage in brain. The study comprised of young (3–4 months old; 150 ± 20 g) and aged (above 24 months; 420 ± 20 g) male albino rats of Wistar strain in Groups I and II. Groups III and IV comprised of young and aged rats supplemented with epigallo catechin gallate (2 mg/kg body weight) for 30 days. Antioxidants, Kreb's cycle enzymes and electron transport chain complexes were assayed in the mitochondrial fraction. Hydroxynonenal expression was carried out using immunohistochemical analysis. Epigallo catechin gallate supplementation decreased the expression of hydroxynonenal in aged brain, up-regulated the antioxidant system and augmented the activities of Kreb's cycle enzymes and electron transport chain complexes in aged brain mitochondria thus proving its antioxidant potential at the level of mitochondria.



## 1. Introduction

The brain accounts for less than 2% of the bodyweight and it consumes 20% of the basal oxygen uptake. High oxygen consumption is linked to leakage of electrons along the respiratory chain with subsequent radical formation. The high amounts of polyunsaturated fatty acids (PUFAs) present in neuronal membranes (Floyd, 1999) also contribute to high metabolic rate.

Mitochondria have been proposed to act as central organelles in the regulation of aging (Wallace, 2005), because they control cellular energy levels, reactive oxygen species (ROS) production/detoxification and apoptosis, all of which are crucially important in determining lifespan. Mitochondria constitute the major source of superoxide ($O_2^{\bullet}-$) and other ROS within cells, generating approximately 85% of total cellular $O_2^{\bullet}-$, via aberrant $O_2$ reactions (Droge, 2002). Reaction of these radicals with double bonds of fatty acids in lipids produces peroxides gives rise to α,β-unsaturated aldehydes including malondialdehyde (MDA), 4-hydroxynonenal (HNE) and acrolein (Adibhatla and Hatcher, 2008). HNE is thought to be the most reactive of these compounds and is, therefore, an important mediator of free radical damage (Esterbauer et al., 1991).

Mitochondrial dysfunction appears to contribute to some of the loss of function accompanying aging (Sastre et al., 1996). Mitochondria from aged tissue use oxygen inefficiently, which impairs ATP synthesis and results in increased oxidant production (Hagen et al., 1997). The steady-state levels of oxidatively damaged molecules are depending both on net ROS formation and clearance of damaged molecules (Trifunovic and Larsson, 2008).

Antioxidant defenses also decline with age (Sanz et al., 1997), making mitochondria even more vulnerable to oxidative injury. The resultant mitochondrial decay may eventually cause inadequate energy production and/or the loss of calcium homeostasis. Such changes could result in unwarranted cellular apoptosis and also lead to the general metabolic decline evident in aging. Our previous studies elucidated the antioxidant potential of epigallocatechin

* Corresponding author. Tel.: +91 44 24480767; fax: +91 44 24926709.
*E-mail address:* pkalaiselvi@yahoo.com (P. Kalaiselvi).

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gallate (EGCG) against oxidative stress induced macromolecular damage in rat brain (Srividhya et al., 2007). The present study focuses on the role of EGCG in combating the oxidative stress mediated mitochondrial deterioration in aged rat brain.

## 2. Experimental procedures

### 2.1. Isolation of mitochondria

Mitochondria were isolated from brain tissues homogenized in 230 mM mannitol, 70 mM sucrose, 1.0 mM EDTA, and 10 mM Tris–HCl, pH 7.40, at a ratio of 10 ml of homogenization medium/g of tissue in a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 700 × *g* for 10 min and the supernatant at 8000 × g for 10 min to pellet mitochondria that were washed in the same conditions to obtain mitochondrial preparations (Navarro et al., 2005). The mitochondria thus obtained, was stored in the same medium. The purity of the mitochondrial preparation was assessed by the activity of succinate dehydrogenase.

### 2.2. Chemicals and reagents

(−)-Epigallo-3-catechin gallate (EGCG) was procured from Sigma–Aldrich, USA. All other routine chemicals and solvents were of analytical grade and were obtained from SISCO Research Laboratory, India.

### 2.3. Animal model

The study involved young (3–4 months old; 150 ± 20 g) and aged (above 24 months; 420 ± 20 g) male albino rats of Wistar strain purchased from Tamil Nadu Veterinary and Animal Sciences University, Chennai, India. The animals were maintained under standard conditions of humidity, temperature (25 ± 2 °C) and light (12 h light/12 h dark). They were fed with standard rat pelleted diet (M/s Pranav Agro Industries Ltd., India) marketed under the trade name Amrut rat/mice feed ad libitum and had free access to water. Experimental animals were handled according to the University and Institutional Legislation, regulated by the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

### 2.4. Experimental design

The rats were divided into four groups consisting of six animals each. Young animals served as Group I that received saline (0.89% NaCl) alone orally for 30 days. Aged animals receiving saline alone orally for 30 days served as Group II. Young animals that were administered EGCG (2 mg/kg body weight/day) dissolved in saline through oral gavage for a period of 30 days served as Group III. Aged animals that were administered with EGCG (2 mg/kg body weight/day) dissolved in saline through oral gavage for a period of 30 days served as Group IV. After the 30 days experimental period, all the animals were killed by decapitation. Brain tissues were excised immediately and immersed in ice-cold physiological saline and immersed in formalin saline (fixative) for immunohistochemical analysis. One hemisphere of the brain was used for preparing tissue homogenate and the other half was used for immunohistochemical analysis. Paraffin wax sections were prepared with the fixed tissues. Tissue homogenate was prepared and suitably diluted using mitochondria isolation medium.

### 2.5. Estimation of protein

The amount of protein in the tissue homogenate was estimated by the method of Lowry et al. (1951). To 0.1 ml of 1/10 diluted rat brain mitochondrial fraction, 0.9 ml of water and 4.5 ml of alkaline copper reagent were added and kept at room temperature for 10 min. Then 0.5 ml of Folin's reagent was added and the color developed was read after 20 min at 640 nm. The levels of protein are expressed as mg/ml.

### 2.6. Enzymic antioxidants

The enzyme superoxide dismutase (SOD) was assayed by the method of Marklund and Marklund (1974). The degree of inhibition of auto-oxidation of pyrogallol, in an alkaline pH by SOD was used as a measure of the enzyme activity. To 1/5 diluted brain mitochondrial fraction, 0.25 ml of ethanol and 0.15 ml of chloroform were added. After 15 min of shaking, the suspension was centrifuged and the resulting supernatant, constituted the enzyme extract. The reaction mixture for auto-oxidation consisted of 2 ml of 0.1 mM Tris–HCl buffer (pH 8.2), 0.5 ml of 2 mM pyrogallol in 0.05 M Tris–HCl buffer (pH 7.4) and 1.5 ml of water. Initially the rate of auto-oxidation of pyrogallol was noted at an interval of 1–3 min. The assay mixture for the enzyme contained 2 ml of the buffer, 0.5 ml of pyrogallol, aliquots of the enzyme preparation and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted at 420 nm using a UV–visible spectrophotometer. The enzyme activity is defined as the enzyme required for 50% inhibition of pyrogallol auto-oxidation/min (U/mg protein).

The activity of catalase was measured at 25 °C by determining the rate of degradation of $H_2O_2$ at 240 nm in 10 mM phosphate buffer (pH 7.0) containing 10 mM $H_2O_2$. To 100 μl of 1/2 diluted mitochondrial fraction, 900 μl of 10 mM phosphate buffer containing 10 mM $H_2O_2$ was added. The extinction co-efficient of 43.6 $mM^{-1}$ $cm^{-1}$ was used for calculation. One unit is defined as 1 nmol of $H_2O_2$ consumed/minute and the specific activity is reported as U/mg protein (Aebi, 1984).

Glutathione peroxidase (GPx) was assayed by measuring the amount of reduced glutathione (GSH) consumed in the reaction mixture according to the method of Rotruck et al. (1973). The reaction mixture consisting of 0.2 ml each of ethylene diamine tetraacetate, sodium azide and $H_2O_2$, 0.4 ml of phosphate buffer, 0.1 ml of 1/5 diluted mitochondrial fraction was incubated at 37 °C at different time intervals. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 0.5 ml dithio nitrobenzoic acid (DTNB) were added and the color developed was read at 412 nm immediately. The activity of GPx is expressed as mmoles of glutathione oxidized/min/mg protein.

### 2.7. Non-enzymic antioxidants

Ascorbic acid is oxidized by copper to form dehydro ascorbic acid, which reacts with 2,3-DNPH to form 2,4-DNPH. This undergoes further rearrangement to form a product with absorption maxima at 520 nm. Thiourea helps to prevent the interference of non-enzymatic chromogens (Omaye et al., 1979). To 0.5 ml of brain mitochondrial fraction, 0.5 ml of water and 1 ml of TCA were added, mixed thoroughly and centrifuged. To 1 ml of the supernatant, 0.2 ml of dinitrophenyl hydrazine–thiourea–copper sulphate reagent was added and incubated at 37 °C for 3 h. Then, 1.5 ml of 65% ice-cold sulphuric acid was added to it, mixed well and the solutions were allowed to stand at room temperature for another 30 min. The color developed was read at 520 nm. The level of ascorbic acid is expressed as mg/mg protein.

α-Tocopherol was estimated by the method of Quaife et al. (1949). To 1.5 ml of mitochondrial fraction, 1.5 ml of ethanol and 1.5 ml of xylene were added and centrifuged at 3000 rpm for 10 min. To 1.0 ml of the xylene layer, 1.0 ml of dipyridyl reagent (120 mg of 2,2′-dipyridyl in 100 ml of n-propanol) was added and mixed well. 1.5 ml of the above solution was taken and absorbance was measured at 460 nm using distilled water as reference blank. To 1.5 ml of the above aliquot, 0.325 ml of ferric chloride reagent (120 mg of ferric chloride in 100 ml of absolute ethanol) was added and the absorbance was measured at 520 nm after 2 min. The level of α-tocopherol is expressed as μg/mg protein.

Glutathione was estimated by the method of Moron et al. (1979). DTNB is reduced by the sulphydryl compounds to form a yellow colored complex which is measured spectrophotometrically at 412 nm. 1 ml of brain mitochondrial fraction was precipitated with 1 ml of TCA and the precipitate was removed by centrifugation. To 0.5 ml of supernatant, 2 ml of DTNB was added and the total volume was made up to 3 ml with phosphate buffer. The absorbance was read at 412 nm. The level of glutathione is expressed as mmol/mg protein.

### 2.8. Lipid peroxidation status

Estimation of lipid peroxidation (LPO) was carried out following the procedure of Hogberg et al. (1974) using thiobarbituric acid (TBA). Malondialdehyde (MDA), formed as an end product of peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with TBA to generate a colored product that absorbs at 532 nm. The extinction co-efficient of MDA–TBA complex at 532 nm, $1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$ was used for calculation. The level of lipid peroxides was expressed as nmoles of MDA released/g tissue.

### 2.9. Protein carbonyls

The protein carbonyl contents were analyzed by 2,4-dinitrophenylhydrazine (DNPH) method as described by Levine et al. (1990). Briefly, 1 ml of mitochondrial fraction containing 0.5 mg protein was pipetted into the tubes, to which 4.0 ml of DNPH in 2.5 M HCl was added. The blank was made by adding with 2.5 M HCl only. Samples were incubated at room temperature for 1 h. Then, protein was precipitated by adding 5 ml of 20% trichloroacetic acid and washed three times with 4 ml of ethanol:ethyl acetate (1:1). Precipitated protein was redissolved in 2.0 ml of 6 M guanidine HCl and insoluble substance removed by centrifugation. Carbonyl content was calculated from the maximum absorbance at 366 nm. The results were expressed as nmoles of carbonyl per mg protein.

### 2.10. Tricarboxylic acid cycle (TCA) enzymes

Isocitrate dehydrogenase activity was assayed according to the method of King (1965). To 0.1 ml of 0.1 M Tris–HCl buffer (pH 7.5), 0.2 ml of 0.1 M trisodium isocitrate, 0.3 ml of 0.015 M $MnCl_2$, 0.2 ml of 1/5 diluted mitochondrial fraction, 0.2 ml of 0.001 M $NADP^+$ were added and the volume was made upto 1.0 ml using double distilled water. The above mixture was incubated at room temperature for 1 h. Then, 1.0 ml of 0.001 M 2,4-dinitrophenyl hydrazine was added followed by 0.5 ml of 0.005 M EDTA. The above mixture was incubated at 37 °C for 20 min. 10 ml of 0.4N NaOH was added and the color developed was read colorimetrically at

540 nm. The specific activity of the enzyme was expressed as nmoles of α-ketoglutarate formed/min/mg protein.

Succinate dehydrogenase activity was determined spectrophotometrically by measuring the decrease in absorbance at 600 nm caused by the reduction of 2,6-dichlorophenol indophenol by succinate by the method of Vrbacky et al. (2007) with slight modifications. To 1 ml of phosphate buffer (pH 7.6), 0.1 ml of EDTA, 0.1 ml of BSA, 0.1 ml of sodium succinate, 2.0 ml of water, 0.1 ml of potassium cyanide was added. To the above mixture, 0.2 ml of 1 in 5 diluted mitochondrial suspension was added. 0.1 ml of 2,6-dichlorophenol indophenols was added, mixed well and the absorbance was measure spectrophotometrically at 600 nm immediately against a reagent blank without the mitochondrial fraction and chromogen. The extinction co-efficient of 21 $mM^{-1}$ $cm^{-1}$ was used for calculation. The specific activity of the enzyme was expressed as nmoles of succinate oxidized/min/mg protein.

Malate dehydrogenase activity was assayed by the method of Mehler et al. (1948). To 0.3 ml of 0.25 M Tris buffer (pH 7.4), 0.1 ml of 0.0015 M NADH and 0.0076 M oxaloacetic acid were added and made up to 2.5 ml with distilled water. To the above suspension, 0.1 ml of 1/5 diluted mitochondrial fraction was added and the decrease in absorbance was measured at 340 nm for 5 min at 15 s interval in a spectrophotometer. The enzyme activity was expressed as nmoles of NADH oxidized/min/mg of protein.

Citrate synthase was measured by the method of Srere (1969). Aliquots of mitochondria were added to a medium containing 0.1 mM acetyl-CoA, 0.2 mM dithionitrobenzoic acid and 100 mM Tris–HCl, pH 8.0. Changes in the absorbance at 412 nm were monitored in a spectrophotometer. The reaction was followed initially for 3 min at 15 s interval to obtain the activity of acetyl-CoA deacylase. Then, the enzyme reaction was started with addition of 0.2 mM oxaloacetate and further followed for 3 min at 15 s interval. The extinction co-efficient of 13.6 $mM^{-1}$ $cm^{-1}$.was used for the calculation of enzyme activity. The enzyme activity was expressed as nmoles of dinitrobenzoic acid formed/min/mg of protein.

Fumarase was assayed by following the formation at 25 °C of fumarate from L-malate at 250 nm in the presence of 1% Triton X-100 (Kanarek and Hill, 1964). The extinction co-efficient of 2.4 $mM^{-1}$ $cm^{-1}$ was used for the calculation of enzyme activity. The enzyme activity was expressed as nmoles of fumarate formed/min/mg of protein.

For the assay of aconitase, 0.05 ml of mitochondrial suspension and 100 mM Tris buffer (pH 8.0) were added to 50 mM citrate to give a final volume of 1.0 ml. The reaction was followed for 3 min at 15 s interval in a spectrophotometer at 240 nm (Racker, 1950). The molar extinction co-efficient of cis-aconitate (3.6 $mM^{-1}$ $cm^{-1}$) was used for the calculation. The enzyme activity was expressed as nmoles of cis-aconitate formed/min/mg of protein.

### 2.11. Electron transport chain complexes

Complex I (NADH-CoQ oxidoreductase) activity was assayed by the method of Hatefi and Rieske (1967) with slight modifications. To 880 μl of double distilled water, 50 μl of 1.0 M phosphate buffer (pH 8.0), 50 μl of 1.0 mM CoQ, and 12 μl of 10 mM NADH were added and mixed well. 50 μl of 1/5 diluted mitochondrial fraction was added and the decrease in absorbance was measured at 340 nm for 3 min at 15 s interval. The activity was calculated using the extinction co-efficient of 6.3 $mM^{-1}$ $cm^{-1}$.

Complex II (succinate-CoQ oxidoreductase) activity was measured by the method of Hatefi and Stiggall (1978) with slight modifications. To 20 μl of 1.0 M sodium succinate (pH 7.4), 0.5 μl of 0.2 M EDTA (pH 7.0), 20 μl of 0.1 M sodium azide and 800 μl of 50 mM potassium phosphate buffer (pH 7.4) were added and incubated at 37 °C for 10 min. To 16 μl of 4.65 mM 2,6-dichlorophenol indophenols, 20 μl of 2.5 mM CoQ was added. To the above mixture, 50 μl of 1/5 diluted mitochondrial fraction was added and the activity was measured at 600 nm for 3 min at 15 s intervals. The activity was calculated using the extinction co-efficient of 21 $mM^{-1}$ $cm^{-1}$.

Complex III (CoQ-cytochrome *c* oxidoreductase) activity was measured by the method of Shimomura et al. (1984) with slight modifications. Reduced CoQ ($CoQH_2$) was prepared by adding a small crystal of potassium borohydride to 50 μl of 10 mM CoQ in ethanol. 5 aliquots of 0.1 M HCl was added by gentle mixing until the yellow solution became colorless. $CoQH_2$ was transferred to a fresh tube, avoiding the borohydride crystal followed by the addition of 5 μl of 1 M HCl. The final CoQ concentration was recorded. It was stored in ice during the assay. If the color changed, the reduction process was repeated. To 700 μl of 25 mM phosphate buffer (pH 7.5) containing 25 μM EDTA, 200 μl of 0.1 M sodium azide, 20 μl of 30 mM cytochrome *c* were added. 63 μM $CoQH_2$ was added followed by the addition of 50 μl of 1/5 diluted mitochondrial suspension. The reaction was followed at 550 nm for 3 min at 15 s interval. The increase in absorbance was noted. The extinction co-efficient of 18.5 $mM^{-1}$ $cm^{-1}$ was used for the calculation.

Complex IV (cytochrome *c* oxidase) activity was assayed using the method of Wharton and Tzagoloff (1964) with slight modifications. Reduced cyt *c* was freshly prepared before each experiment by adding a few grains of sodium borohydride to a 10 g/l solution of the pigment in 10 mM potassium phosphate buffer (pH 7.0). Addition of 0.1 M HCl stabilized the reduced cyt *c* and excess borohydride was removed by centrifugation at 12,000 × *g* for 4 min. To 2.85 ml of 50 mM phosphate buffer (pH 7.0), 100 μl of reduced cytochrome *c* was added and incubated. To the above mixture, 50 μl of 1/5 diluted mitochondrial suspension was added and the decrease in absorbance was measured at 550 nm for 3 min at 15 s interval. The extinction co-efficient of 21.1 $mM^{-1}$ $cm^{-1}$ was used for calculation.

### 2.12. HNE expression by immunohistochemistry

Immunohistochemical analysis of 4-HNE was performed as desribed before (Zarkovic et al., 1999) using a monoclonal antibody for detection of HNE-modified proteins. It was obtained from the culture medium of the clone derived from a fusion of Sp2-Ag8 myeloma cells with B-cells of a BALBc mouse immunized by HNE-modified keyhole limpet hemocyanine (Waeg et al., 1996). The antibody is specific for the HNE–histidine epitope in HNE–protein (peptide) conjugates. For the immunohistochemical detection of the HNE–protein adducts the immunoperoxidase technique using LSAB KIT (Dako, Glostrup, Denmark). The paraffin-embedded sections (10 μm) were dehydrated in xylene twice each for 5 min and in series of graded alcohols viz., absolute alcohol, 96% alcohol and 70% alcohol for 5 min each. The tissue sections were washed in PBS for 5 min. The tissue slices were washed in primary antibody for HNE for 2 h at room temperature followed by washing with PBS for 1 min. Then, the slides were incubated in 3%$H_2O_2$ in PBS for 20 min in dark followed by washing thrice in TBS for 5 min each. The secondary antibody linked to biotin was added and incubated for 15 min followed by washing thrice in TBS for 5 min each. Streptavidin peroxidase was added and incubated for 15 min followed by washing thrice with TBS for 5 min each. This was followed by the addition of substrate DAB followed by washing with distilled water. Totally, four sections from each animal were processed in a similar way (6 animals per group). The slides were then counter-stained using haematoxylin, dehydrated and visualized.

Immunohistochemical investigation of HNE positivity was determined by experienced neuropathologist not knowing the group assignment of the samples analysed and scored in a semiquantitative way as described before (Zarkovic et al., 1997) (− 0% positive cells, + <5% positive cells, ++ 5–25% positive cells, +++ 25–50% positive cells, ++++ >50% positive cells). The presence of HNE–protein adducts in specific structures (ependim, blood vessels, etc.) was defined as the absence of the HNE–protein adducts (−) or the presence of the HNE–protein adducts (+).

### 2.13. Statistical analysis

The results were expressed as mean ± standard deviation (S.D.) for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS software package for windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test; significance at *p*-values <0.001, <0.01, <0.05 have been given respective symbols in the tables.

## 3. Results

### 3.1. Enzymic antioxidants

Enzymatic antioxidants like SOD, Catalase and GPx showed a decline in the mitochondrial fraction of aged rats ($p < 0.001$) when compared to control young rats. The results are represented in Table 1. Administration of EGCG for 30 days improved the antioxidant status to a significant extent in Group IV rats ($p < 0.01$) on par with aged rats (Group II). There were no significant changes in the enzymic antioxidant status of young rats (Group III) upon supplementation of EGCG.

### 3.2. Non-enzymic antioxidants

Aged animals showed a decline in the non-enzymic antioxidant status when compared to young rats (Table 2). The levels of GSH, tocopherol and ascorbic acid showed a decline by 31%, 43% and 32% respectively in aged rats on comparison with control (Group I) rats. EGCG supplementation resulted in the increment of the non-enzymic antioxidant status to an appreciable extent ($p < 0.01$). Similar to enzymic antioxidants, EGCG did not bring about any change in the non-enzymic antioxidant status in the young rats (Group III).

### 3.3. MDA levels

Fig. 1 shows the MDA levels of control and experimental animals. Aged animals showed a remarkable increase in the MDA levels on comparison with young control animals (28%). EGCG

**Table 1**
Enzymic antioxidant status in young and aged rat brain mitochondria.

| Parameter | Group I (young) | Group II (aged) | Group III (young + EGCG) | Group IV (aged + EGCG) |
|---|---|---|---|---|
| Superoxide dismutase (SOD) | 6.58 ± 0.58 | 4.37 ± 0.49a*** | 6.77 ± 0.55 | 5.43 ± 0.38b** |
| Catalase (CAT) | 9.01 ± 0.72 | 6.64 ± 0.83a*** | 8.54 ± 0.81 | 7.56 ± 0.5b* |
| Glutathione peroxidase (GPx) | 0.9 ± 0.13 | 0.68 ± 0.08a*** | 0.94 ± 0.08 | 0.79 ± 0.07b* |

Values are expressed as mean ± S.D. for 6 animals in each group. SOD: amount of enzyme required to prevent 50% auto-oxidation of pyrogallol/min/mg protein; catalase: nmoles of $H_2O_2$ consumed/min/mg protein; GPx: μmoles of GSH oxidized/min/mg protein. The symbols a and b represent comparative study with Groups I and II respectively.
* Statistical significance at $p < 0.05$.
** Statistical significance at $p < 0.01$.
*** Statistical significance at $p < 0.001$.

**Table 2**
Non-enzymic antioxidant status in young and aged rat brain mitochondria.

| Parameter | Group I (young) | Group II (aged) | Group III (young + EGCG) | Group IV (aged + EGCG) |
|---|---|---|---|---|
| Ascorbic acid | 0.153 ± 0.011 | 0.104 ± 0.011a*** | 0.159 ± 0.014 | 0.138 ± 0.016b*** |
| α-Tocopherol | 2.44 ± 0.36 | 1.39 ± 0.19a*** | 2.45 ± 0.29 | 1.79 ± 0.18b* |
| Glutathione (GSH) | 0.071 ± 0.008 | 0.049 ± 0.007a*** | 0.076 ± 0.005 | 0.064 ± 0.006b*** |

Values are expressed as mean ± SD for 6 animals in each group. Ascorbic acid: μg/mg protein; α-tocopherol: μg/mg protein; GSH- μmol/mg protein. The symbols a and b represent comparative study with Groups I and II respectively.
* Statistical significance at $p < 0.05$.
*** Statistical significance at $p < 0.001$.

administration for 30 days improved the lipid peroxidation status to a considerable extent ($p < 0.001$).

### 3.4. Protein carbonyls

Protein carbonyl levels (Fig. 2) showed an increase by 45% in the case of aged rats (Group II) when compared to young control rats. EGCG supplementation brought back the carbonyl levels to an appreciable extent ($p < 0.001$). EGCG did not alter the carbonyl levels in Group III rats.

### 3.5. TCA cycle enzymes

The activities of TCA cycle enzymes in young and aged animals are showed in Table 3. The activities of these enzymes showed a decline in the aged (Group II) animals on par with young control animals ($p < 0.001$). EGCG administration to the aged animals improved the activities of succinate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase, citrate synthase, acontiase and fumarase by 30%, 24%, 19%, 22%, 19% and 11% respectively in Group IV animals when compared to Group II aged animals.

### 3.6. Electron transport chain complexes

Respiratory chain complex activities are shown in Table 4. Group II animals showed a significant decrease in the respiratory chain complex activities ($p < 0.01$) when compared to Group I control animals. Administration of EGCG (Group IV) resulted in an augmentation of the enzyme activities ($p < 0.05$) on comparison with aged (Group II) animals.

### 3.7. 4-Hydroxynonenal

Immunohistochemistry results of 4-HNE in the Purkinje cells and choroid plexus of the brain are shown in Figs. 3 and 4 respectively. In young animals no HNE was detected in Purkinje

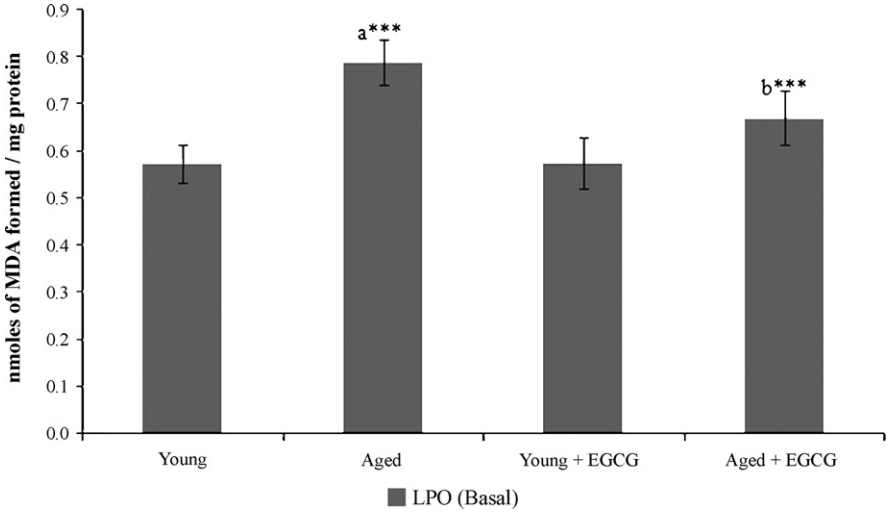


**Fig. 1.** Effect of EGCG on the levels of malondialdehyde in young and aged rat brain mitochondria. Group I: young; Group II: aged; Group III: young + EGCG; Group IV: aged + EGCG. Values are expressed as mean ± S.D. for 6 animals in each group. The symbols a and b represent comparative study with Groups I and II respectively. Statistical significance at ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ respectively.

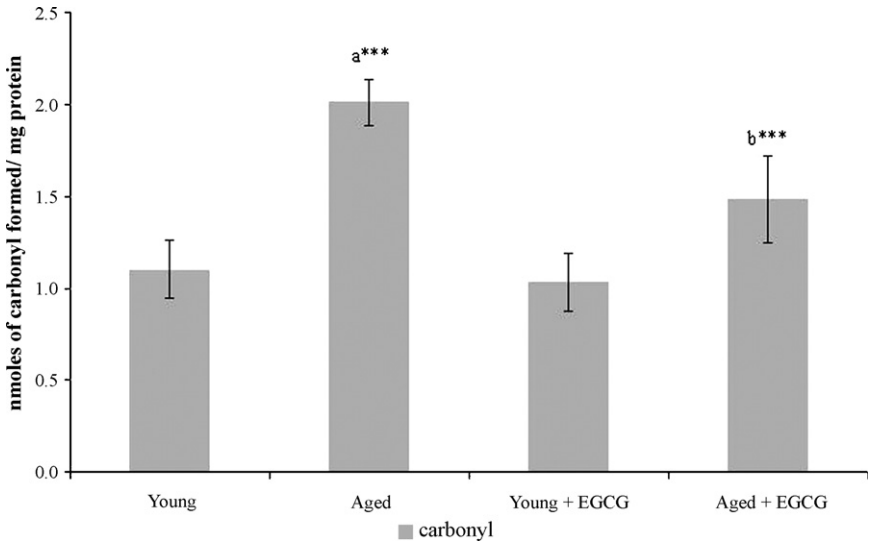


**Fig. 2.** Effect of EGCG on the levels of protein carbonyls in young and aged rat brain mitochondria. Group I: young; Group II: aged; Group III: young + EGCG; Group IV: aged + EGCG. Values are expressed as mean ± S.D. for 6 animals in each group. The symbols a and b represent comparative study with Group I and II respectively. Statistical significance at $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ respectively.

cells and choroid plexus while aged animals showed immunopositivity in the nuclei of the Purkinje cells and in numerous cells of the choroid plexus. Treatment with EGCG (Group IV) eliminated HNE from the Purkinje cells of the aged animals. Administration of EGCG (Group IV) also resulted in the reduction of 4-HNE when compared to Group II aged animals. (Fig. 4, the only immunopositivity for HNE in Group IV is associated with the remaining erythrocytes in the blood as indicated in the figure). In other structures of the brain of both young and aged animals, irrespective of the treatment with EGCG, there were no prominent HNE–protein adducts determined.

## 4. Discussion

Ames et al. (1995) postulated that mitochondrial oxidants are the main source of the oxidative damage that accumulates with age and that these oxidation products are major contributors to cellular, tissue, and organism aging. Alterations in mitochondrial function occur with age as a consequence of increased oxidative damage (Van Remmen and Richardson, 2001). Hence an effective anti-aging drug should be targeted to alleviate mitochondrial deterioration. The protective role of EGCG in counteracting oxidative stress is well established. Age induced oxidative stress

**Table 3**
Mitochondrial enzymes in young and aged rat brain.

| Parameter | Group I (young) | Group II (aged) | Group III (young + EGCG) | Group IV (aged + EGCG) |
|---|---|---|---|---|
| Succinate dehydrogenase | 10.59 ± 1.02 | 6.21 ± 0.43a*** | 10.99 ± 1.25 | 8.9 ± 0.87b*** |
| Isocitrate dehydrogenase | 16.06 ± 0.93 | 11.22 ± 0.95a*** | 16.77 ± 0.97 | 14.7 ± 1.48b*** |
| Malate dehydrogenase | 1.2 ± 0.18 | 0.79 ± 0.12a*** | 1.19 ± 0.13 | 0.97 ± 0.09b* |
| Citrate synthase | 137.22 ± 17.13 | 90.02 ± 12.31a*** | 140.07 ± 15.11 | 115.15 ± 14.04b** |
| Aconitase | 119.85 ± 7.75 | 88.19 ± 7.14a*** | 121.11 ± 10.71 | 108.77 ± 6.27b*** |
| Fumarase | 730.68 ± 70.97 | 598.59 ± 38.42a*** | 714.54 ± 52.20 | 672.92 ± 41.56b* |

Values are expressed as mean ± S.D. for 6 animals in each group. Succinate dehydrogenase: nmoles of succinate oxidized/min/mg protein; isocitrate dehydrogenase: nmoles of α-ketoglutarate formed/min/mg protein; malate dehydrogenase: μmoles of NADH oxidized/min/mg protein; citrate synthase: nmoles of DNBA formed/min/mg protein; aconitase: nmoles of cis-aconitate fomed/min/mg protein; fumarase: nmoles of malate formed/min/mg protein. The symbols a and b represent comparative study with Groups I and II respectively.

* Statistical significance at $p < 0.05$.
** Statistical significance at $p < 0.01$.
*** Statistical significance at $p < 0.001$.

**Table 4**
Electron transport chain complexes in young and aged rat brain mitochondria.

| Parameter | Group I (young) | Group II (aged) | Group III (young + EGCG) | Group IV (aged + EGCG) |
|---|---|---|---|---|
| Complex I | 124.93 ± 15.49 | 95.34 ± 11.54 a*** | 120.02 ± 9.18 | 115.03 ± 13.67 b* |
| Complex II | 27.38 ± 3.48 | 17.53 ± 2.20 a*** | 29.85 ± 2.02 | 22.27 ± 2.73 b** |
| Complex III | 182.66 ± 14.46 | 146.63 ± 10.11 a*** | 180.86 ± 14.37 | 168.02 ± 11.47 b** |
| Complex IV | 45.87 ± 6.67 | 35.26 ± 3.28 a** | 44.99 ± 6.54 | 43.07 ± 3.42 b** |

Values are expressed as mean ± S.D. for 6 animals in each group. Complex I: nmoles of NADH oxidized/min/mg protein; complex II: nmoles of dichlorophenol indophenol oxidized/min/mg protein; complex III: nmoles of cytochrome *c* reduced/min/mg protein; complex IV: nmoles of cytochrome *c* oxidized/min/mg protein. The symbols a and b represent comparative study with Groups I and II respectively.

* Statistical significance at $p < 0.05$.
** Statistical significance at $p < 0.01$.
*** Statistical significance at $p < 0.001$.

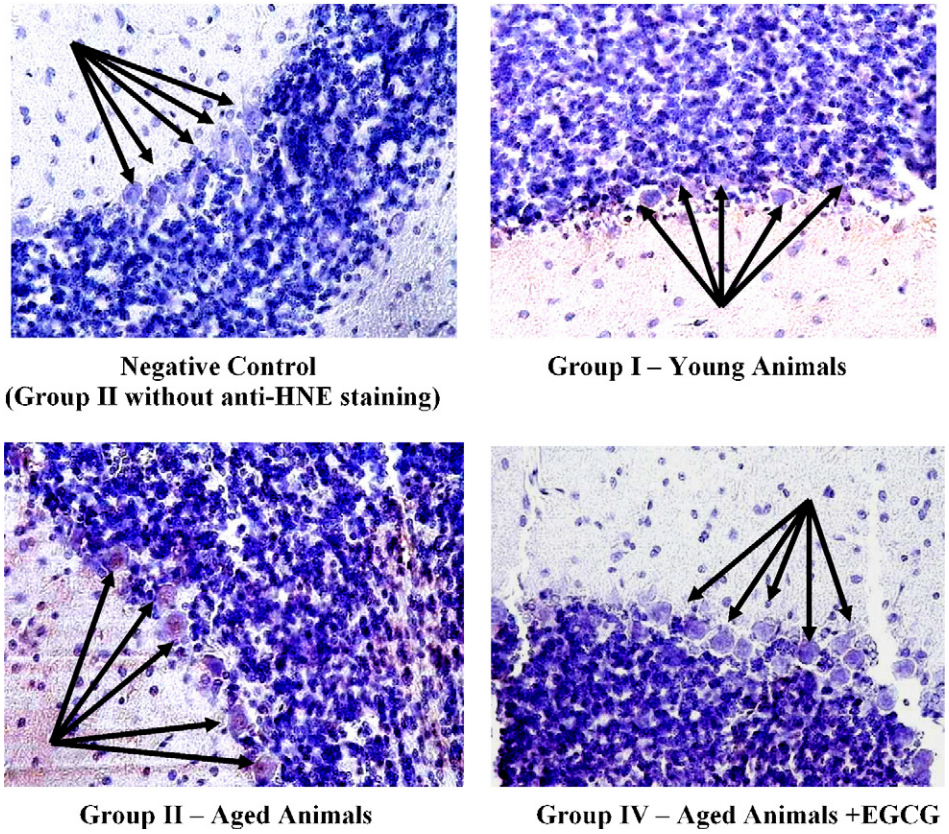


**Fig. 3.** Effect of EGCG on the expression of HNE in Purkinje cells in young and aged rat brains. Negative control: without antibody; Group I: young; Group II: aged; Group IV: aged + EGCG. Purkinje cells are indicated by arrows (magnification 200×), the HNE-immunopositivity can be seen as darker brown/violet color in comparison to the light-blue hematoxylin contrast staining in the cells that do not contain HNE. Since majority of the Purkinje cells in the Group II were positive, the presence of HNE-protein adducts in the cells was classified as strongly positive (++++). In the other three groups HNE was absent in the Purkinje cells therefore these cells were classified as negative (−). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

has been dampened by EGCG in neurons (Srividhya et al., 2007). Although, the effect of EGCG in respect to its ability to cross the blood–brain barrier and restore the mitochondrial alterations in age-associated degeneration remains unexplored. However, it should be mentioned that the lipid peroxidation product HNE which was found in the brain cells of older animals in our study is known to increase permeability of the blood–brain barrier (Zarkovic et al., 1997; Mertsch et al., 2001). Many previous workers have demonstrated the antioxidant property of catechins in several oxidative stress models (Chen et al., 2003; Sastre et al., 2002). Henceforth, this study was put forward to evaluate the functions of the major catechin, EGCG in fighting the oxidative damage to mitochondria during brain aging. Therefore, we could assume that aging was associated with the development of HNE that could increase permeability of the blood–brain barrier and consequently increase bioavailability and the effectiveness of EGCG, in particular, in aged animals as was noticed in our study. This possibility will be further studied.

### 4.1. Lipid peroxidation

Mitochondrial components are susceptible to LPO and were found to be pronounced in rat brain during aging (Kumar et al., 2008). HNE, a major bioactive marker of lipid peroxidation (Zarkovic, 2003a), is thought to be the most reactive and an important mediator of free radical damage. HNE–modified proteins have been identified in animal and human tissues under various pathological conditions, suggesting an involvement of HNE modification in the pathophysiology of degenerative diseases and cellular aging in particular in the brain (Zarkovic, 2003b).

In our studies, the increase in the HNE in aged animals (Group II) could be correlated to the increased MDA levels in these animals. Chance et al. (1979) have reported that accumulation of peroxidation products in mitochondria leads to a decrease in ATP production and compromises the maintenance of cellular homeostasis. Similarly, HNE modifications lead to the decreased activities of TCA cycle enzymes (Palaniappan and Dai, 2007). The reactivity of HNE with key mitochondrial enzymes may be important in the age-dependent loss in energy generation and enhanced susceptibility of neurons to apoptosis (Floyd and Hensley, 2002).

We observed major difference in the levels of HNE between young and aged animals in the Purkinje cells suggesting that this important cerebellar layer may be mostly affected by LPO in aging of the Wistar rats. Similar findings in humans were also observed by Yamashita et al. (2000) indicating in particular the relevance of HNE in olivopontocerebellar atrophy. The possibility that HNE could be of particular relevance for Purkinje cells was also determined by comparison of the development and aging human brains (Itakura et al., 2002). Therefore, the finding of the absence of the HNE protein adducts in the Purkinje cells of the aged rats observed in our study supports potentially beneficial effects of EGCG for the aging brain.

The finding of abundant HNE-protein adducts in the choroid plexus of the aged brain supports further the difference in the extent of lipid peroxidation between young and the aged animals and resembles our previous findings of the HNE presence in baboons (Schlag et al., 1997). Although the reasons for the accumulation of the aldehyde in choroid plexus have yet to be clarified it is likely that this could be related to the aging and the mitochondrial disorders

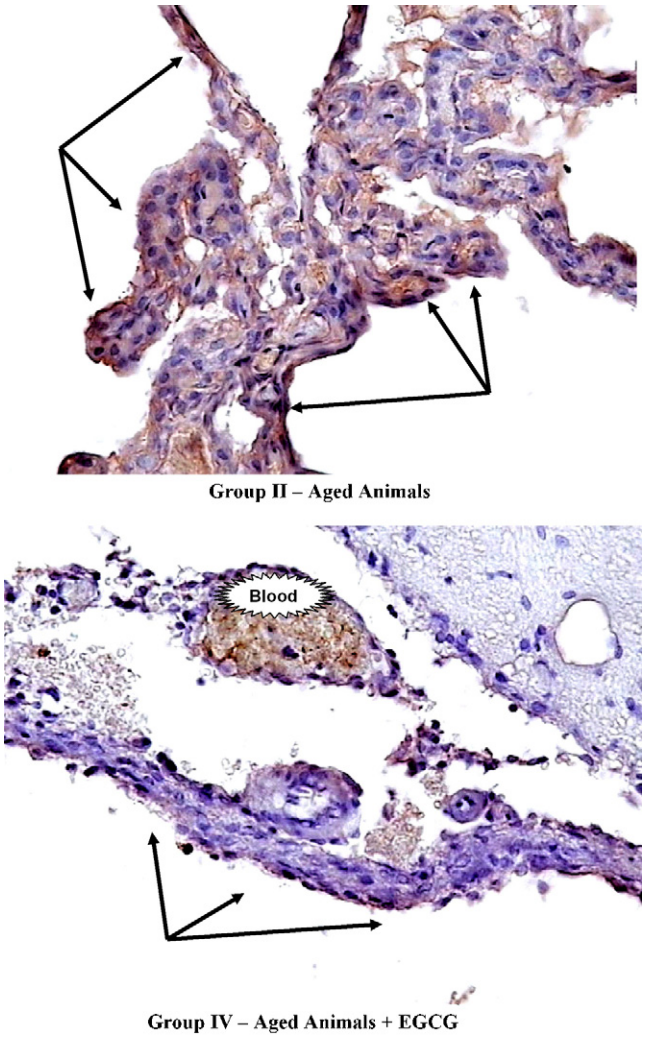


**Fig. 4.** Effect of EGCG on the expression of HNE in choroid plexus in young and aged rat brains. Group II: aged; Group IV: aged + EGCG. Strongly HNE-positive cells (++++) in the choroid plexus of the Group IV (brown color) and HNE-negative cells (−) in the choroid plexus of the Group II (blue color) are indicated by arrows (magnification 400×). The HNE-immunopositivity can be seen also in the blood cells in the lumen of the intact blood vessels of the Group II (indicated as "Blood") further supporting the absence of HNE in the choroid plexus of the same animals. Younger animals (Group I and Group III) did not show apparent HNE presence in choroid plexus and are therefore not presented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Cottrell et al., 2001; Calabrese et al., 2005). A possibility that EGCG could attenuate this process, as observed in our study, further supports the beneficial effects of EGCG.

The superoxide and hydroxyl radical scavenging activity of catechins (Nanjo et al., 1996) would have played essential roles to curtail lipid peroxidation products such as MDA and HNE in Group IV rats when compared to young control rats. Raza and John (2007) have shown that catechins inhibited the ROS formation and thereby oxidative carbonylation of subcellular proteins induced by HNE. In a recent study by Feng et al. (2008), caffeic acid, a polyphenol, effectively protected the isolated brain mitochondria against the peroxidative damage. Similarly, the present study reveals that EGCG can also extend its antioxidant action to mitochondria.

### 4.2. Non-enzymic antioxidants

Glutathione, a major endogenous antioxidant, is found in two intracellular pools viz. in the cytoplasm and the mitochondria (Muyderman et al., 2004). The levels of GSH were found to be low in the aged brain mitochondria (Group II) in unison with other reports (Palaniappan and Dai, 2007). The increase in MDA and HNE levels would have affected the levels of GSH in aged brain mitochondria (Lee et al., 2006; Raza and John, 2006). The antioxidant potential of EGCG would have proven beneficial in augmenting the GSH levels in the brain mitochondria (Group IV). EGCG which has the capacity to quench the peroxyl and hydroxyl radicals would have proven effective in counteracting the lipid peroxidative damage which otherwise depletes the mitochondrial GSH levels.

Depletion in GSH levels would have had an impact on the cellular tocopherol levels, which is the one of the major hydroperoxide scavengers. GSH, tocoperol and ascorbate which form an antioxidant network to recycle lipid hydroperoxides, get affected by the increase in HNE and MDA levels in the mitochondria. As ascorbate which recycles tocopherol by scavenging its radical form gets depleted, it brings down the overall antioxidant capacity of the cell in the aged neuronal mitochondria. EGCG, a well known superoxide and hydroxyl radical scavenger, acts effectively to quench those radicals and also by regenerating ascorbate, preserves the mitochondria from lipid peroxidation end products and thus saves the antioxidant capacity from deprivation.

### 4.3. Protein carbonyl

Mitochondrial oxidative stress generates free radicals which are capable of catalyzing fully reversible modifications to protein (Humphries et al., 2006). HNE modification arises from covalent cross-links with proteins via Michael addition to lysine, cysteine, and histidine residues (Uchida et al., 1994). Oxidative damage to proteins has been postulated to be of key importance in the aging process. Age-related accumulation of altered protein can be due to an increase of free radical-mediated damage, a loss of protease activity, or the combination of both mechanisms. Our studies have clearly indicated that the levels of protein carbonyls increased substantially during aging. EGCG, by its free radical scavenging activity (Yin et al., 2008) shields the proteins from carbonylation and thus restores the activities of many enzymes which are essential for the mitochondrial processes.

### 4.4. Enzymic antioxidants

Under normal physiological circumstances, cellular $H_2O_2$ as well as other ROS are scavenged by the various cellular antioxidants, particularly catalase and the glutathione (GSH) system (Chan, 1996). However, these defense systems may not be capable of counteracting the pathologically enhanced ROS generation resulting from acute or chronic mitochondrial dysfunction.

Superoxide is a reactive molecule but it can be converted to hydrogen peroxide by Mn-superoxide dismutase (mitochondrial matrix) and then to oxygen and water by catalase intramitochondrially (Radi et al., 1991) or glutathione peroxidase (mitochondrial matrix). SOD and catalase are prone to age-associated oxidative damage due to Fenton's reactions (Jouihan et al., 2008). Some reporters have emphasized the importance of mitochondrial catalase in scavenging the $H_2O_2$. Catalase deprivation in aged rats would increase the burden of $H_2O_2$ scavenging to GPx whose activity depends on a non-enzymic antioxidant GSH.

The glutathione pool in the mitochondrial matrix plays a major role in the maintenance of reduced protein thiols and in the detoxification of $H_2O_2$ via glutathione peroxidase, which is exclusively localized in the mitochondrial matrix (Cadenas and Davies, 2000). A decrease in the GSH concentration would have been a causative factor for the decline in the GPx activity in aged mitochondria (Group II). EGCG would have up-regulated the

concentration of GSH thus enhancing the activity of GPx in Group IV animals. In a recent study by Fu et al. (2008), EGCG's capacity to enhance the levels of cytoplasmic and mitochondrial GSH had been attributed to the increase in gene expression of the catalytic subunit GCLc, which is the rate-limiting enzyme in the de novo synthesis of GSH.

### 4.5. TCA cycle enzymes

Since neurons utilize glucose as their primary energy source, it follows that the effective functioning of the TCA cycle is essential. Impaired functioning of key enzymes of the cycle has been described in models of aging-related mitochondrial dysfunction. Succinate dehydrogenase is the membrane-bound component of the citric acid cycle and also a component of the electron transport chain. Since SDH is also an iron–sulphur containing enzyme, it is prone to inactivation by the action of superoxide radicals (Gardner et al., 1994). SDH activity has been reported to be declined during neurotoxin induced oxidative stress (Kamboj et al., 2008). EGCG's capability to scavenge the superoxide radicals would have played a major role in preventing the inactivation and thus augmenting the SDH activity in Group IV rats.

ICDH has been assumed to play a major role in the oxidative decarboxylation of isocitrate in the tricarboxylic acid cycle. The HNE-modification would have resulted in the adduct formation thereby inactivating the enzyme's active sites (Benderdour et al., 2003). EGCG was found to be neuroprotective in cerebral ischemia model, preserving the mitochondrial components from oxidative insults (Sutherland et al., 2005). EGCG acts as a good antioxidant and exerts its protective activity against lipid peroxidative damage (Murase et al., 2002) that would have protected the enzyme from lipid peroxidation thus paving a way for an increment in the ICDH activity and the mitochondrial metabolism.

Aconitase catalyzes the interconversion of citrate and isocitrate in the citric acid cycle, a reaction essential to normal metabolic function. It is the most sensitive enzyme to $H_2O_2$ in the TCA cycle since it contains Fe–S clusters in its active site (Tretter and Adam-Vizi, 2000). Thus lipid peroxidative damage would be high in the presence of reactive metal ions like iron, leading to the inactivation of the critical amino acid residues involved in enzyme activity. EGCG's antioxidant potential and metal-chelating activity would have served useful in preserving the activity of aconitase.

Similarly, oxidative modifications of fumarate and citrate synthase resulted in the decrease in their activities. The free radical scavenging effect of EGCG prevented these oxidative modifications and restored the enzyme activities to near normalcy.

### 4.6. Electron transport chain complexes

Previous studies have emphasized the decrease in the activities of respiratory chain enzymes during aging (Modi et al., 2008). The increase in the lipid peroxidation products MDA and HNE and the decline in the activities of those critical enzymes involved in respiratory chain could be co-related (Navarro et al., 2002). The level of lipid peroxidation would have imposed an effect on the functional capacities of the proteins, thereby affecting their activities in aged animals. Since the electron transport chain complexes are membrane-bound and sensitive to the lipid microenvironment (Keller et al., 1997), oxidative damage to the inner mitochondrial membrane would have an adverse impact on the electron transport chain activities. Previous workers (Bolaños et al., 1996) have attributed the decrease in brain GSH to be a major cause of the decrease in mitochondrial respiratory chain complex activity and signified their sensitivity to cellular antioxidant status. EGCG was found to be protective against lipid peroxidative damage to membranes and preserved the membrane bound enzyme activities in a previous study (Saffari and Sadrzadeh, 2004). Thus, the capacity of EGCG to restore the membrane components from oxidative insults would have proven essential in preserving these enzymes against lipid peroxidative damage and deterioration.

Many recent researchers have identified the anti-apoptotic potential of EGCG using *in vivo* and *in vitro* models (Schroeder et al., 2008; Meng et al., 2008; Yao et al., 2008). Our study has oriented the role of EGCG to restore and replenish the antioxidant stores in neuronal mitochondria and the importance of HNE-induced lipid peroxidative damages in mitochondrial dysfunction. We conclude that the anti-aging effect of EGCG may be attributed to its free radical scavenging activity as evidenced by the low levels of HNE in EGCG treated aged rats.

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