METHODOLOGY
METHODS

1. Animals

All experiments were conducted with healthy albino rats of Charles Foster strain maintained as per the guidelines of the animal ethical committee of the institute. For some experiments, the animals were divided into two groups: adult (4-6 months of age, body weight 120-150gms) and aged (20-24 months old, body weight 250-300gms). The animals were killed by cervical dislocation and the brains dissected out cleanly and collected on petri dishes kept over ice.

2. Generation of hydroxyl radicals (OH) by iron (FeSO₄) and ascorbate

Two different assay procedures were used for this purpose

a) 2-deoxyribose degradation assay

Ascorbate (2mM) plus FeSO₄ (50μM) or FeSO₄ (50μM) plus H₂O₂ (500μM) or H₂O₂ (500μM) were incubated for 1h in 50mM phosphate buffer, pH 7.4 containing 2-deoxyribose (1mM) and other additions (catalase, 50μg/ml or mannitol, DMSO or sodium benzoate at a concentration of 20mM each) as necessary in a total volume of 800μl. At the end of the incubation 1.4 ml of 2.8% TCA and 0.8 ml of 1% thiobarbituric acid (TBA) were added to each tube followed by heating for 10min in a boiling water bath. The tubes were cooled briefly and absorbance were taken at 532 nm [Halliwell and Gutteridge, 1981; Chakrabarti et al, 1990].

b) Benzoate hydroxylation assay

Ascorbate (2mM) and FeSO₄ (50μM) were incubated in 50mM phosphate buffer, pH 7.4 containing sodium benzoate at 100μg/ml and other additions (catalase, 50μg/ml or mannitol and DMSO at a concentration of 20mM each). The mixtures were incubated at 37° for 1h, and fluorescence was measured at 407 nm emission after excitation at 305 nm [Gutteridge, 1987]. Results were expressed in relative fluorescence intensity units.
3. Isolation of rat brain mitochondria

Rat brain mitochondria were isolated by differential centrifugation as adopted from a published method [Berman and Hastings, 1999]. Briefly, the brain from one adult rat was homogenized in 10ml of buffer containing 225mM mannitol, 75mM sucrose, 5mM HEPES, 1mM EGTA, 1mg/ml BSA, pH 7.4. The homogenate was brought to 30ml with the same buffer and centrifuged at 2000g for 3min. at 4°C. After that the supernatant was divided into 2 tubes and centrifuged at 12,000g for 10min. The pellet containing the mixture of synaptosomes and mitochondria was suspended in 10ml of homogenization buffer containing 0.02% digitonin to lyse the synaptosomes followed by centrifugation at 12,000g for 10min. to pellet down both extrasynaptosomal and intrasynaptosomal mitochondria. The mitochondrial pellet was washed twice in the same buffer without EGTA, BSA and digitonin. For some experiments mitochondria were suspended in 50mM phosphate buffer, pH 7.4 and either used immediately or kept frozen in aliquots and used within 3 days. In other experiments, freshly isolated mitochondria were resuspended in isotonic buffer A (145mM KCl, 50mM sucrose, 5mM NaCl, 1mM EGTA, 1mM MgCl₂, 10mM phosphate buffer, pH 7.4).

4. Assay of Citrate Synthase for mitochondrial integrity

The mitochondria suspended in the isotonic buffer A were checked for membrane integrity by assaying citrate synthase activity before and after treatment with Triton X-100 to obtain the latency value of citrate synthase and ratios exceeding 10 were considered indicative of good membrane integrity [Clark et al., 1997]. In this method coenzyme A released during the reaction of oxaloacetate with acetyl coenzyme A catalyzed by citrate synthase present in mitochondrial
sample was measured using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) which reacted with the free-SH groups of coenzyme A.

5. Induction of in-vitro oxidative stress in rat brain mitochondrial fraction

For some experiments mitochondria were suspended in 50mM phosphate buffer, pH 7.4 and either used immediately or kept frozen at -20°C in aliquots and used within 3 days. In other experiments, freshly isolated mitochondria were resuspended in isotonic buffer A (145mM KCl, 50mM sucrose, 5mM NaCl, 1mM EGTA, 1mM MgCl₂, 10mM phosphate buffer, pH 7.4). The mitochondrial pellet was incubated for 1 h at 37°C in 50mM phosphate buffer, pH 7.4 or in isotonic buffer A in the presence or absence of ascorbate (2mM) plus Fe²⁺ (50μM) or Fe²⁺ (50μM) plus H₂O₂ (500μM) or H₂O₂ (500μM) with or without other additions like, catalase (50μg/ml) or mannitol (20mM) or dimethyl sulfoxide (20mM) or sodium benzoate (20mM) or BHT (0.2mM). At the end of the incubation, the samples were directly processed for further experiment or else mitochondria were pelleted down by centrifugation at 4°C for 15min., resuspended in an appropriate buffer and then used for different experimental protocols.

6. Detection of protein carbonyls by immunoblotting technique

Rat brain mitochondria freshly isolated or incubated in appropriate buffer with other additions were solubilised in 6% SDS and the protein carbonyls derivatized by treatment with 20mM DNPHP in 10% TFA for 10 min. [Shaeter et al., 1996]. The derivatized protein carbonyls were detected by immunoblotting using rabbit anti-DNP antibody (1:1000 dilution) as described previously [Laemmli et al., 1970]. The derivatized samples were then neutralized with 2M Tris / 30% glycerol / 19% β-mercapto ethanol [Levine et al., 1994] and then subjected to SDS-poly acrylamide...
gel electrophoresis (SDS-PAGE) by 10% separating and 4.5% stacking gel [Laemmli et al., 1970]. Following electrophoresis, the gel was kept in transfer buffer containing 25mM Tris/192mM glycine/20% methanol. A piece of polyvinyl difluoride (PVDF) membrane was cut to the size of the gel and soaked successively in methanol for 20 sec., in water for 5-7 min. and in transfer buffer for 5-7 min.

Three pieces of blotting papers of the size of PVDF membrane were soaked in transfer buffer and kept over the anode plate of a semidry blotting system. Then the wet PVDF membrane was placed over the stack of blotting papers and the gel was carefully laid over the PVDF membrane ensuring that the membrane remains towards the anode and no air bubbles were present between the gel and the membrane. Three pieces of wet blotting papers were then placed over the stack. A constant current of 2mA per sq. cm. of the gel was passed for 40 min. from a power supply with cross-over facility [Floor and Wetzel, 1998]. At the end of the transfer, the membrane was taken out and incubated in blocking buffer containing 0.5% gelatin/0.1% Tween 20/25mM PBS. The membrane was further incubated with rabbit anti-DNP antibody in 1:1000 dilutions at room temperature with constant shaking for 2h. The membrane was then removed and washed thrice (15mins. each) with the wash buffer containing 0.1% Tween 20/25mM PBS. The PVDF membrane was next incubated for 1h in 1:2000 diluted secondary antibody (goat anti-rabbit IgG-alkaline phosphatase conjugated) followed by two washings (15mins. each) with 0.1% Tween 20/25mM PBS and a final wash with 25mM PBS, pH 7.4. The color development was completed by incubating the membrane with a mixture of BCIP/NBT (substrate for alkaline phosphatase). Appropriate negative control was kept.
7. Assay of lipid peroxides by 2-thiobarbituric acid (TBA) assay

Lipid peroxidation in mitochondrial fraction was measured by the 2-thiobarbituric acid (TBA) assay as described by Ohkawa et al. [Ohkawa et al., 1979]. For this, to each sample 200μl of mitochondrial suspensions in 50mM phosphate buffer pH 7.4 was added in a sequence - 200μl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.85% TBA and 400μl water followed by heating for 15min. in a boiling water bath. After cooling the tubes, the pink color developed was extracted with 5ml of (1:15) n-butanol: pyridine. The organic layer was collected after centrifuging the tubes at 3000rpm for 10 min. The absorbance was read at 532nm and the amount of MDA calculated from the molar extinction co-efficient of MDA-TBA adduct (€= 1.56×10^5 mmol^-1 cm^2).

8. Estimation of fluorescent-lipid peroxidation products in brain mitochondria

Fluorescent lipid peroxidation products are heterogeneous groups of relatively stable damage products formed by the interaction of aldehyde end products of lipid peroxidation with amino groups present in protein side chains or elsewhere [Dillard and Tappel, 1973; Kikugawa et al., 1989]. The fluorescent lipid peroxidation products have both organic solvent-soluble and water-soluble components having similar emission maxima (420-430nm) when excited at 360nm and the assay method used is based on the methods described by Simasaki [Shimasaki, 1994].

a) Total fluorescent lipid peroxidation products

The mitochondrial pellet was dissolved in 2ml of 15% SDS-PBS solution. The fluorescence intensity of the total fluorescent lipid peroxidation products was measured at excitation 360nm and emission 430nm [Shimasaki, 1994; Chakraborty et al., 2003], and expressed as relative fluorescence units with respect to quinine.
sulfate (1 mg/ml in 0.05 M H₂SO₄).

a) Organic solvent soluble fluorescent products of lipid peroxidation

Mitochondria were subjected to ethanol-diethyl ether extraction (3:1 by volume). The suspension was vortexed and then centrifuged for 10 min. at 3000 rpm. The upper layer was separated and the fluorescence intensity was monitored at excitation 356 nm and emission 426 nm [Meaba et al., 1990; Shimasaki et al., 1990]. and expressed as relative fluorescence unit with respect to quinine sulphate (1mg/ml) in 0.05M H₂SO₄ [Bridges, 1978].

9. Assay of mitochondrial complex I and complex IV activity

Frozen and thawed samples of mitochondria suspended in 50 mM phosphate buffer, pH 7.4 were used for complex I or complex IV assays with or without incubation as per the experimental protocol. Complex I activity was assayed by using ferricyanide as the electron acceptor coupled with NADH oxidation at 340 nm [Hatefi, 1978]. The assay system at 30° contained 0.17 mM NADH, 0.6 mM ferricyanide and Triton X-100 (0.1% V/V) in 50 mM phosphate buffer pH 7.4. The reaction was initiated by the addition of mitochondrial suspension (10-30 μg protein) to the sample cuvette and the rate of oxidation of NADH was measured by the decrease in absorbance at 340 nm [Clark et al., 1997]. Complex I assayed by this method (NADH-ferricyanide reductase) is not rotenone-sensitive since the latter compound acts at the O₂⁻ site and not the substrate site of the flavoprotein from where ferricyanide accepts electrons [Horgan et al., 1968].

The activity of complex IV was measured by following the oxidation of reduced cytochrome c (ferro-cytochrome c) at 550 nm. Reduced cytochrome c was prepared by reducing commercially available oxidized cytochrome c by ascorbic acid followed by the removal of excess ascorbic acid by a passage through Biogel
P-6 column equilibrated with 10mM phosphate buffer. Reduced cytochrome c (50μM) in 10mM phosphate buffer pH 7.4 was added in two 1 ml cuvettes. In the blank cuvette, ferricyanide (1mM) was added to oxidize ferrocytochrome c and the reaction initiated in the sample cuvette by the addition of mitochondrial suspension (10-30 μg protein). The rate of decrease of absorbance at 550nm was measured at room temperature. The activity of the enzyme was calculated from the first order rate constant taking into account the concentration of reduced cytochrome c in the cuvette and the amount of mitochondrial protein added [Wharton and Tzagoloff, 1967; Khan et al., 2005].

10. Mitochondrial MTT reduction ability

The incubation of mitochondria was carried out in isotonic buffer A, pH 7.4 at 37° for 1h with or without the addition of Fe2+ (50mM) plus ascorbate (2mM) in the presence or absence of other additions. At the end of incubation mitochondria were washed and the pellets were resuspended in isotonic buffer A. For other experiments freshly isolated mitochondria from young and aged rat brains were suspended in buffer A and used for MTT reduction assay. An aliquot of mitochondrial suspension was mixed with isotonic buffer A containing 10mM succinate (or 10mM pyruvate or 10mM α-ketoglutarate or 10mM succinate and 10mM pyruvate both) and MTT (0.42mg/ml) and kept at 37° for 10 min. The samples were quenched with 500μl of lysis buffer (45% DMF in 10% SDS, pH 4.7) and the difference in absorbance values at 550nm and 620nm noted [Cohen et al., 1997; Khan et al., 2005].

11. Measurement of mitochondrial reactive oxygen species

Mitochondrial ROS production was measured by incubating freshly isolated
mitochondria (0.5-1mg mitochondrial protein/ml) with H$_2$DCF-DA (ROS specific fluorescent dye, 10µM) in isotonic buffer A containing 10mM each of pyruvate and succinate at 37° for 30min.. H$_2$DCF-DA is a nonpolar compound and gets hydrolyzed within the mitochondria to form a non-fluorescent derivative, which in presence of a proper oxidant converted to a fluorescent product. At the end of the incubation mitochondria were washed with fresh buffer at 15,000g for 10min. at 4° and resuspended in 1ml of the same buffer and the fluorescence was measured spectrofluorometrically ($\lambda_{em}$ 507nm, $\lambda_{ex}$ 530nm) [Luo and Shi, 2005]. For all measurements basal fluorescence was subtracted.

12. Assay of mitochondrial cardiolipin content

Cardiolipin content of mitochondria was measured using the cardiolipin specific dye nonyl-acridine orange [Petit et al., 1992]. Rat brain mitochondria (freshly isolated or incubated for in vitro oxidative stress) were suspended in isotonic buffer A pH 7.4, containing 10mM succinate (or 10mM pyruvate or 10mM a-ketoglutarate or 10mM succinate and 10mM pyruvate for some experiments) were incubated at 37° for 45min. in the presence of 5µM nonyl-acridine orange. The excess dye was washed out by centrifugation and the mitochondrial pellet appropriately diluted in buffer A for fluorescence measurements ($\lambda_{ex}$ 485nm, $\lambda_{em}$ 535nm) in a Hitachi spectrofluorometer (model F-4010). The fluorescence values normalized to 1mg in 0.05 M H$_2$SO$_4$, $\lambda_{em}$ 360nm, $\lambda_{ex}$ 457nm) as the reference.

For some experiments nonyl-acridine orange binding was measured in intact mitochondria in the presence of 1mM CN$^-$ while in some other experiments a lysed preparation of mitochondria (4 cycles of freezing-thawing in 10mM phosphate buffer, pH 7.4) was used in the absence of any respiratory substrate for nonyl-acridine orange binding reactions.
13. Measurement of mitochondrial membrane potential

Mitochondrial transmembrane potential was assessed by confocal microscopy using 'Mitosensor' dye from a commercial kit (Mitochondrial membrane sensor kit) as adopted from published procedures [Sen et al., 2004]. 'Mitosensor', a cationic dye, was taken up by the mitochondria in proportion to mitochondrial transmembrane potential and the accumulated dye underwent aggregation in the interior of the mitochondria. The monomeric form of the dye emitted green fluorescence while the aggregated form exhibited intense red fluorescence. In our experiments with freshly isolated mitochondria, the excess dye (monomer) remaining outside was washed off thoroughly and, therefore, only the intense red fluorescence emitted from within the mitochondria was visible.

For a more quantitative assessment of mitochondrial membrane potential in control and experimental samples, a spectrofluorometric measurement was adopted using a mitochondrial membrane potential sensitive carbocyanine dye JC-1 [Reers et al., 1991]. When excited at 490nm, the monomeric form of JC-1 had an emission maximum at 527nm, but the aggregated form (J-aggregates) showed an emission maximum at 590nm. The negative transmembrane potential of mitochondria caused a directional uptake of the cationic dye into the matrix with subsequent formation of concentration-dependent J-aggregates and the fluorescence intensity of J-aggregate at 590nm, therefore, reflected changes in mitochondrial membrane potential [Reers et al., 1991].

Rat brain mitochondria freshly isolated or incubated for in vitro oxidative stress with Fe^{2+}/ascorbate were used for the measurement of membrane potential. Aliquots of mitochondrial suspension, in buffer A were further incubated at 37°C for 30 min. either in the presence of 'Mitosensor' dye (5 μg/ml) for confocal
microscopic experiments or JC-1 (10μg/ml) for spectrofluorometric measurements. After 30 min., the dye loaded mitochondria were collected by centrifugation at 4° with an excess of buffer A, washed once more with the same buffer to remove thoroughly the dye remaining outside and finally resuspended in appropriate dilution in buffer A for confocal microscopy (TCS-SP Leica Confocal Microscope) or spectrofluorometric measurements (Hitachi spectrofluorometer, model F-4010). The spectrofluorometric readings normalized to 1mg/ml protein were expressed as relative fluorescence units as described.

14. Genomic DNA isolation from rat brain

About 200 mg of rat brain cortical and cerebellar tissue was utilized to extract genomic DNA using a commercial kit (Roche Molecular Biochemicals, USA). Briefly the tissue was homogenized at a medium speed using the lysis buffer (2.5ml) supplied with the kit followed by treatment with proteinase K solution at a final concentration of 200μg/ml at 65° for 1h. After proteinase K treatment the solution was cooled mixed gently with RNase solution at a final concentration of 200μg/ml and kept at 37° for 30min. After RNase treatment 1.1 ml of protein precipitating solution, supplied with the kit, was added and the mixture kept on ice for 10min. The mixture was spun down at 26,900g for 20 min. and the supernatant collected and to the supernatant 7 volumes of isopropyl alcohol was added with gently mixing for few minutes to precipitate down the DNA. The precipitated DNA was then collected by centrifugation and washed twice with 70% alcohol followed by air drying. The isolated DNA was finally dissolved in 10mM Tris, pH 7.4 and stored at -20° until use. The purity and quality of the extracted DNA was examined by taking its optical density ratio at 260nm and 280nm. The purified DNA was used for further investigations.
15. Incubation protocol for in-vitro oxidative stress in rat brain genomic DNA

DNA isolated from adult rat (4-6 months old) was used for in vitro experiments. Briefly the isolated DNA was incubated for up to 1h at 37° in 50mM phosphate buffer, pH 7.4 in the presence or absence of ascorbate (2mM) and FeSO₄ (50µM) with or without catalase (50µg/ml) or mannitol (20mM) or sodium benzoate (20mM). At the end of the incubation, DNA was washed with autoclaved water thrice using ultrafiltration centrifugal cartridge of 10KD molecular cut off. The remaining DNA over the membrane was analyzed further for the assessment of oxidative DNA damage.

16. Agarose gel electrophoresis of rat brain genomic DNA

Genomic DNA samples isolated from young and aged rat brains with or without further incubations as per the experimental protocol were analyzed by 1% agarose gel electrophoresis (using TAE buffer: 40mM Tris-acetate, 1mM EDTA, pH for 1.5h at 100V). At the end of the electrophoresis DNA was visualized by UV-transillumination after staining with ethidium bromide and bands were visualized by the Bio-Rad imaging system (Bio-Rad Laboratories, Hercules, CA).

17. Fluorescence-detected alkaline DNA unwinding (FADU) assay to estimate single stranded breakage in rat brain genomic DNA

The modified FADU method of Birnboim and Jevcak [Birnboim and Jevcak, 1981] was used to detect DNA stranded breaks by their effects on the rate of DNA denaturation in alkali, monitored by the fluorescence intensity of an intercalating dye, ethidium bromide. The assay is based on time-dependent alkaline denaturation of DNA under moderate denaturing conditions (pH: 12.2-12.8) were DNA unwinding takes place from the ends as well as all the break points. The
remaining double-strand regions are selectively bound to the fluorescent intercalating dye ethidium bromide (2,7-diamino-10-ethyl-9-phenel-phenanthridium bromide) and fluorescence was read at room temperature in a Perkin-Elmer spectrofluorimeter at 520nm excitation and 590nm emission.

The procedure required 3 sets of tubes:

a) in one set unwinding in alkali was totally prevented; the fluorescence (T) given by double-stranded DNA, plus the background fluorescence given by the unbound dye and the contaminants.

b) in the second set unwinding was maximized by alkalization and sonication; the fluorescence emitted (B) represents background fluorescence only i.e. components other than double-stranded DNA.

c) in the third set, unwinding was allowed to occur in alkali without any manipulation resulting in partial denaturations beginning from the DNA strand breaks; the fluorescence emitted (P) represents double-stranded DNA remaining after partial unwinding.

Most of the earlier studies carried out FADU assay directly on cellular lysate. However, in this study purified genomic DNA was utilized for FADU assay and further pH-dependent unwinding was induced by 10mM Tris buffer pH 13.0.

Briefly, freshly isolated genomic DNA samples from rat brain were distributed to twelve test tubes (each tube contain 10µg genomic DNA) divided in three sets of four. The three sets represented: (1) a ‘total’ sample (T) in which DNA samples were kept in 10mM Tris, pH 7, yielding total fluorescence; (2) a ‘blank’ sample (B) which DNA samples were sonicated immediately after alkalization in a Branson B-15, with tapered microtip 1/8", amplitude 200, 3 sec. in 10mM Tris solution pH 13 and incubated for 30min.; (3) an ‘experimental’ sample (P) in which samples were kept in 10mM Tris, pH 13 for 30min. followed by neutralization by 50mM Tris, pH 53.
10 (to bring the final pH around 11) and immediate sonication. To a total volume of 1 ml of reaction mixture 10μg of ethidium bromide was added and the reading was taken after 10min. Every tube in each set was run in quadruplicate and the mean of four fluorescence readings were calculated for T, B, and P. T-B provided an estimate of the amount of total double-stranded DNA where as P-B provided an estimate of the amount of DNA that still remained double stranded after denaturation. The percentage of double-stranded DNA was measured for each sample by the following formula:

\[ D = \frac{(P-B)}{(T-B)} \times 100 \]

18. Polymerase chain reaction based detection of oxidative DNA damage in rat brain genomic DNA

DNA damage caused by oxygen free radicals or other genotoxic agents leads to inhibition of polymerase function including Taq polymerase which results in decreased efficiency of PCR amplification of specific genes of the affected DNA [Ponti et al., 1991; Jennerwein and Eastman, 1991; Kalinowski et al., 1992; McCarthy et al., 1996; McCarthy et al., 1997; Ploskonosova et al., 1999; Jenkins et al., 2000; Milano and Day, 2000; Grimaldi et al., 2002; Liang and Godley, 2003; Santos et al., 2006]. Oxidative damage in the form of strand breakage, abasic sites, base modifications such as tandem repeats of 8-oxodG etc. are specifically important in causing impaired PCR amplification of affected genes [Ploskonosova et al., 1999; Milano and Day, 2000; Dizdaroglu et al., 2002; Liang and Godley, 2003; Sikorsky et al., 2004]. Simple semiquantitative PCR assay was used in this study to detect oxidative DNA damage. Since kinetics of PCR amplification process is often complex and replicate variation are well known, a proper optimization of the method is required. In our assay, the amplification cycle was kept at 25 and the
templet DNA content varied from 5 to 60ng and under this condition fluorescence intensity of the product increase nearly linearly with the increase in templet DNA concentration and a calibration curve could be drawn. In all our assays, the templet DNA content in different samples in a set of experiments was kept constant, so that the fluorescence intensity of the product indicated PCR amplification efficiency.

Other conditions of PCR assays were as follows: Two rat gene fragments were amplified β-actin gene fragment (923bp) and the p53 gene fragment (1644). For amplification of either of the gene fragments, an aliquot of genomic DNA from each sample (control or oxidatively stressed) containing an equal amount of DNA was added to the PCR mixture of final volume 25μl containing 1.25 units of Taq polymerase (‘Taq bead’, Promega, USA), 50mM KCl, 10mM Tris-HCl, 0.1% Triton-X 100, 3mM MgCl₂, four dNTPs of 0.2mM each, 5% DMSO, 2mM β-mercapto ethanol and 0.75μM each of the appropriate forward and reverse primers [Nudel et al., 1983; Hulla and Schneider, 1993]. The reaction mixture was covered with a layer of mineral oil (Promega, USA) and the PCR tubes were incubated in a preheated (95°) programmable thermocycler (MJ Research, USA). The amplification regime for β-actin fragment was 1.2 min. at 92°, 2 min. at 62° and 2 min. at 72° for 25 cycles. The amplification protocol for p53 gene fragment was 1.2 min. at 92°, 1.5 min. at 61° and 2 min. at 72° for 25 cycles. After 25 cycles of amplification, the reaction mixture was additionally incubated at 72° for 5min.

The primer details were as follows:

<table>
<thead>
<tr>
<th>Gene fragment</th>
<th>Size of fragment (bp)</th>
<th>Primer (24 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>923</td>
<td>(+)5'-GGTGTCCTGTATGCGCTTGGTCG-3' (Forward)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-)5'-TAGAAGATTTCGTCGACGATG-3' (Reverse)</td>
</tr>
<tr>
<td>p53</td>
<td>1644</td>
<td>(+)5'-ATTCACAGCGGATATGAGCATCG-3' (Forward)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(-)5'-CAGGGTCCTAGCCACGTCATG-3' (Reverse)</td>
</tr>
</tbody>
</table>
Similar PCR based assay was utilized to detect oxidative damage in young and old rat brain genomic DNA. PCR amplification products were analyzed by 1% agarose gel electrophoresis followed by ethidium bromide staining and visualization over an UV-transilluminator. The band intensities were analyzed in a Bio-Rad imaging system (Bio-Rad Laboratories, Hercules, CA) and quantitation done using Bio-Rad Quantity-1 software.

19. Apoptosis in young and aged rat brain: detection by TUNEL assay

Detection of apoptotic DNA damage was performed with the help of DeadEnd, Colorimetric TUNEL assay system, commercially available from Promega. Brain from young and rats were dissected out cleanly after 15 min. transcardial perfusion with PBS and immediately fixed in 10% buffered formalin. A saggital section was made from each cerebral hemisphere 2mm lateral to the midline and the tissue embedded in paraffin. Serial 5 μm sections were cut from the paraffin embedded brain tissue in such a fashion to obtain the regions of cerebral cortex, hippocampus and cerebellum in one section. The sections were deparafinized by successive washing in fresh xylene and 100% ethanol and then rehydrated by sequential washing in graded ethanol (95%, 85%, 70% and 50%) as per the standard protocol [Deng et al., 2005]. The sections were washed successively in 0.85% NaCl for 5 min. and PBS for 5 min. at room temperature. Each tissues section was then covered with 100μl of the 20μg/ml Proteinase K solution and kept for 15 min. to permeabilize the tissue. This was followed by washing in PBS for 5 min. at room temperature and then refxation in 10% buffered formalin in PBS for 15 min. and final washing in PBS.

The Tunel assay was subsequently performed as per the manufacturer's protocol. A positive control was prepared to confirm the staining specificity by
treating a section with DNase I (100μl containing 10unit/ml) for 10 min. at room temperature following refixation. DNase I was removed by washing the slides several times in deionized water and finally with PBS for 5 min. at room temperature.

All the sections were covered with 100μl equilibration buffer for 5-10mins. prior to the assay. The sections were then covered with 100μl of rTdT reaction mixture containing biotinylated nucleotide mixture and terminal deoxytransferase enzyme and at 37° for 60 min. inside a humidified chamber to allow the end-labeling reactions to occur. The negative control was run in parallel omitting the terminal transferase reaction. At the end of the incubation the reactions were terminated by SSC buffer (sodium chloride-sodium citrate buffer (20X) containing 3 M sodium chloride and 0.3 M sodium citrate, pH 7.0) for 15 min. at room temperature followed by three washings in PBS for 5 minutes each at room temperature. Next the endogenous peroxidases were destroyed by treating the sections with 0.3% hydrogen peroxide in PBS for 5 min. at room temperature followed by three washings in PBS 5 mins. each. The sections were incubated with 100μl of streptavidin-HRP solution for 30 min. at room temperature. The slides were washed by immersion in PBS for 5 min. twice at room temperature. Then 100μl of supplied DAB solution was added to develop the color. At the end the slides were rinsed several times in deionized water. The sections were later counterstained with hematoxiliæ-eosine and examined under light microscope [Arsene and Ardeleanu, 2006].

20. Assay of Caspase 3 and 9 in rat brain by spectrofluorometry

Brain tissues (cortex and cerebellum) from young and aged rats were quickly removed and homogenized with a Teflon-glass homogenizer in two
volumes of extraction buffer containing 20mM HEPES-adjusted to pH 7.5 with KOH, 10mM kCl, 1.5mM MgCl2, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 250mM sucrose. The homogenate was centrifuged at 13,000g at 4° for 30 min. and the supernatant used for caspase 3 and caspase 9 assays. An aliquot of cytosolic extract (200-400µg of protein in 200µl of caspase assay buffer) was mixed with 50µM substrate (Ac-DEVD-AFC for caspase 3 and Ac-LEHD-AFC for caspase 9) in the assay buffer containing 10mM HEPES pH 7.4, 5mM EGTA, 2mM MgCl2 and 5mM DTT and incubated for 1h at 37° [Usha et al., 2000]. The reaction was terminated by 800µl ethanol and after centrifugation at 15000xg, for 10min at 4°, the fluorescence of the supernatant was estimated in a Perkin-Elmer spectrofluorimeter (model no.- LS-55) at excitation 400nm and emission 505nm.

Caspase 3 and 9 activities were expressed as relative fluorescence intensity using quinine sulfate (1mg/ml in 0.05M H2SO4) as the reference.

21. Immunohistochemical analysis of Bcl2 expression in rat brain sections

The dissection and initial processing of brains from young and aged rats were performed as described for Tunel assay and paraffin embedded sections (5µm) were used for detecting bcl-2 immunoreactivity [Zhang et al., 2006]. Reactive lymphyd follicle in appendiular tissue were taken as positive and negative control. The sections were de-waxed and antigen retrieval was done by ‘heat treatment’ (in pressure cooker for 15min. using citrate buffer, pH 6.2). The slides were washed 3 times (5 mins. each) in PBS at room temperature and incubated for 5 min. at room temperature in 3% hydrogen peroxide in phosphate-buffered saline, pH 7.6 (PBS) to quench endogeneous peroxidase activity. The sections were incubated overnight at 4° with primary polyclonal IgG anti bcl-2 antibody (1:100) in moist chamber followed by 3 washes with PBS. The sections were next incubated with biotinylated
anti-IgG (1:200) for 1h, washed thrice with PBS and further treated with 
streptavidin-HRP conjugate for 1h at room temperature. The detection was done by 
DAB reagents containing 0.03% diaminobenzidine hydrochloride in 0.1 M Tris 
buffer. All sections were counter stained with hematoxylin-eosine and dehydrated, 
cleared, and mounted. Sections from all groups were simultaneously processed in 
the same tray.

21. Measurement of protein content

The protein content was determined by the method of Lowry after solubilizing 
the protein in 1% SDS and using bovine serum albumin as the standard [Lowry et 
al., 1951]. For some samples, protein estimation was performed using Bradford 
reagent (Bio-Rad, Hercules, CA) [Vik et al., 1989].

22. Statistical analysis

All the experiments were checked for reproducibility and statistical 
significance worked out. Qualitative experiments e.g. immunoblotting, confocal 
microscopic experiments, immunohistochemistry and Tunel assay were repeated at 
least five times for each group and a representative figure from a set of 
experiments producing similar results was presented in each case. All values were 
presented as means ± standard error of mean (SEM), using following formula:

\[
\text{SEM} = \frac{SD}{\sqrt{N}} \\
SD = \sqrt{\frac{\sum (X-\bar{X})^2}{N-1}} \\
\bar{X} = \frac{\sum X}{N}
\]
Where, SEM: Standard error of mean

SD : Standard deviation.

X : Mean value

\( \bar{X} \) : Individual value

N : Number of observation

Statistical significance of unpaired and paired observation were assessed Student’s ‘t’-test.

(a) t-test for determining significance of difference between means of two unpaired observation:

\[
SEM_1 = \frac{SD_1}{\sqrt{N_1}} \quad ; \quad SD_1 = \sqrt{\frac{\sum (X_1 - \bar{X}_1)^2}{N_1 - 1}}
\]

\[
SEM_2 = \frac{SD_2}{\sqrt{N_2}} \quad ; \quad SD_2 = \sqrt{\frac{\sum (X_2 - \bar{X}_2)^2}{N_2 - 1}}
\]

\[df = (N_1 + N_2 - 2)\]

\[t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{SEM_1^2 + SEM_2^2}}\]

Where \( X_1 \) and \( X_2 \) are the raw values of two separate groups, \( \bar{X}_1 \) and \( \bar{X}_2 \) are their sample means, \( N_1 \) and \( N_2 \) are the number of observations of two different groups, \( SD_1 \) and \( SD_2 \) are their corresponding standard deviations, \( SEM_1 \) and \( SEM_2 \) are their standard error of mean and \( df \) is the degree of freedom.

(b) t-test for paired observation:

\[D = X_2 - X_1\]

\[\bar{D} = \frac{\sum D}{N}\]

\[t = \frac{D}{SEMD}\]

Where \( D \) is the difference between the paired observations.
\[ \text{SEMD} = \frac{SDD}{\sqrt{N}} ; \quad SDD = \frac{\sum (D - \overline{D})^2}{N - 1} \]

\[ \text{df} = N - 1 \]

Where, \( X_1 \) and \( X_2 \) are the paired values of each set of experiment, \( D \) is the difference between the observation of each paired values, \( \overline{D} \) is the mean of difference, SDD is the standard deviation of difference between paired observation, SEMD is the standard error of mean difference, \( N \) is the number of paired observation, df is the degree of freedom.

Level of significance of unpaired and paired 't'-test were obtained from the Fisher’s table of 't' distribution at corresponding degree of freedom and compared with the computed 't' values to consider the probability (\( P \)) [Das, 1981; Leaverton, 1995].