Study of DNA damage via the comet assay and base excision repair activities in rat brain neurons and astrocytes during aging

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ABSTRACT

Earlier we have used biochemical approach to assess the number of single (SSBs) and double (DSBs) strand breaks in brain cellular DNA. However, a quick method to obtain a reliable measure of DNA damage in cells was in need for population studies. Therefore, single cell gel electrophoresis technique (popularly known as “comet” assay) has been standardized using the Trevigen protocol. DNA damage was assessed in isolated neurons and astrocytes from the cortex of young (7 days), adult (6 months) and old (2 years). Marked increase is seen in DNA damage in terms SSBs and DSBs in both types of cells by 6 months of age, which increased further by 2 years of age. The number of 8-oxoguanine DNA glycosylase (OGG1) and uracil DNA glycosylase (UDG) sensitive sites also increased in DNA with age with the simultaneous decrease in OGG1, UDG and AP endonuclease (APE1) activities. Thus the comet assay adapted to our lab conditions has proven to be useful for a quick assessment of DNA damage in a large number of samples that constitute our future studies.

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1. Introduction

DNA damage could be defined as any modification of DNA that changes its coding properties therefore in the normal information transfer fidelity (Lindahl, 1993; Rao, 1993). Damage to the native structure of DNA can occur through two main mechanisms: spontaneous damage caused by sources within a cell and damage caused by external sources such as chemicals and radiation. Protracted oxidative, hydrolytic, deamination or alklylation reactions can modify DNA bases, or even sometimes cause a complete loss of bases within DNA, resulting in strand breakage. Similarly, cellular DNA can be damaged by external sources such as ultraviolet or ionizing radiation (X-rays, γ-rays, α particles and cosmic rays) and an array of chemical substances can induce interstrand and intrastrand cross-links, DNA–protein cross-links, bulky DNA adducts, single strand breaks (SSBs) and double strand breaks (DSBs) (Rao, 1990, 2002, 2003; Rao and Loeb, 1992; Reddy and Vasquez, 2005; Martin, 2008). As the multitude of ways in which DNA can be damaged increases, equally diverse repair mechanisms have been evolved by nature (Misteli and Soutoglou, 2009). No matter how and what type of structural modification is induced in DNA and no matter through which type DNA repair pathway this structural alteration is corrected, the initial point of the whole recovery process is to recognize the damage and its nature to activate the appropriate repair pathway. It is therefore, obvious that accurate assessment of damaged DNA, irrespective of its molecular nature, would itself be a valuable information to weigh the health status of the cell or tissue. We have been using the post mitotic brain cells as a model system to examine the relationship between DNA damage, DNA repair and aging. Making use of a biochemical approach Mandavilli and Rao (1994) had assessed the number of SSBs and DSBs through nick translation of free 3′-OH groups in DNA with Escherichia coli polymerase I and addition of nucleotides at the terminal 3′-OH by calf thymus terminal deoxynucleotidyl transferase respectively from young, adult and old rat brain neurons and astrocytes.

Measurement of DNA damage in any cell or tissue is an important pointer for a stable or unstable nature of the genomic apparatus which would reflect physiological health status of a cell and the whole organism. Thus, this parameter could be a valuable one for assessing the genomic stability. However, this biochemical method is time consuming and requires elaborate laboratory facilities thereby making unsuitable for analysis of the large number of

Abbreviations: SSBs, single strand breaks; DSBs, double strand breaks; BER, base excision repair; U, uracil; 8-oxoG, 8-hydroxy-8-oxoguanine; OGG1, 8-oxoguanine DNA glycosylase 1; UDG, uracil DNA glycosylase; APE1, AP endonuclease; pol β, DNA polymerase β; AP site, apurinic/apyrimidinic site; THF, tetrahydrofuran; ALS, alkali-labile sites; TBE, Tris–borate EDTA buffer; CHAPS, [3-[3-cholamidopropyl] dimethylammonio]-1-propane sulfonate).

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samples, e.g., population studies. We, therefore, turned our attention towards the so-called “comet” assay also known as single cell gel electrophoresis. The concept of microgel electrophoresis of dispersed cells was introduced in 1984 by Ostling and Johanson (1984) as a method to measure DNA DSBs that caused relaxation of DNA supercoils. A modified version was published by Singh et al. (1988), which used alkaline conditions. The idea was to combine DNA gel electrophoresis with fluorescence microscopy to visualize the migration of DNA strands from individual agarose-embedded cells. If the negatively charged DNA contained breaks, DNA supercoils are relaxed and broken ends were able to migrate towards the anode during a brief electrophoresis. If the DNA was undamaged, the lack of free ends and large size of the fragments prevent rapid migration. The alkaline denaturation of DNA and electrophoresis at pH > 13 is generally considered capable of detecting DNA SSBS, alkali-labile sites (ALS), DNA–DNA/DNA–protein cross-linking, and SSBS associated with incomplete excision repair sites (Singh et al., 1988; Tice et al., 2000). The comet assay also uses the neutral electrophoresis buffer that allows largely the detection of DSBs (Ostling and Johanson, 1984; Singh et al., 1988; Lemay and Wood, 1999).

Determination of the relative amount of DNA that migrated provided a simple way to measure the number of DNA breaks in an individual cell. Tail moment, a measure of both amounts of DNA in the tail and distribution of DNA in the tail, became a common denominator along with tail length and percentage of DNA in the tail (Olive and Banath, 2006).

The present study was undertaken to assess damage to DNA at the single cell level in isolated neurons and astrocytes from animals of different ages. The results also showed that DNA damage in terms of SSBS, DSBs, 8-oxoguanine DNA glycosylase (OGG1) and uracil DNA glycosylase (UDG) sensitive sites increases in both rat neuronal and astrocyte DNA with advancement of age.

This method is found to be suitable for multiple sample analysis that constitutes our future work.

2. Materials and methods

2.1. Preparation of neurons and astrocytes from young, adult and old rat brains

Cohorts of Wistar strain rats of both sexes in 8- to 12-week generation and maintained in our animal house were used. The three age groups studied were 7 days postnatal, 6 months, and ≥24 months. We designated these three age groups as ‘young’, ‘adult’, and ‘old’ respectively. According to the nuclear and astroglial cell preparations made from rats of these three ages were referred in this paper as young, adult, and old neurons and astrocytes. Rats were maintained in a pathogen-free environment with a 12-h light–dark cycle. Food and water were provided ad libitum. These protocols were cleared by the Animal Ethics Committee, University of Hyderabad.

Neuronal and astroglial cell enriched fractions from the rat cerebral cortex of different ages were prepared essentially as standardized in this laboratory (Lani et al., 1983). In brief, the procedure involves incubation of gray matter with trypsin to dissociate the cells, passage of the dissociated tissue successively through nylon screens of decreasing pore size (106, 84, and 48 μm). Further, separation and purification of neuronal and astroglial cell enriched fractions is achieved by centrifugation on ficoll discontinuous gradient (from bottom to top, 28%, 22%, and 10% ficoll in medium). The neuronal cells were obtained as a pellet and these cells show 80–90% viability and purity by trypan blue exclusion test and morphological integrity and characteristics respectively. Neuronal cells were identified by their characteristic shape, large nucleus, and abundant cytoplasm with some of them still retaining the stumps of axon and dendrites. The neuronal cell preparation was also characterized by immunochemistry using antibodies for neuron specific enolase and glial fibrillary acidic protein (Krishna et al., 2005). The purity of neuronal cell preparation has ranged from 80% to 95% in all isolates from different ages.

The final preparation of the neuronal and astroglial cells were suspended in extraction medium consisting of 20 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.5% CHAPS, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 1 mM MgCl2, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.5 M KCl. Leupeptin, pepstatin A and PMSF were added just before use. The suspension was sonicated for 5 s, three times with the setting at 5 in a Branson sonifier and incubated on ice for 1 h. The suspension was centrifuged at 100 000 x g for 1 h in a Sorvall ultracentrifuge, model 80L and the clear supernatant was used as the source for enzymes/factors needed for base excision repair (BER). Protein concentration was estimated by Coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, IL, USA)

2.2. Comet (alkaline condition) assay

DNA strand breaks in neurons and astrocytes were evaluated using Trevigen Comet Assay™ kit (Trevigen Inc., Gaithersburg, MD). Cells were suspended in ice cold PBS (Ca2+ and Mg2+ free) to a concentration of 1 x 10⁶ cells/ml. Briefly, an aliquot of 50 μl of cells (1 x 10⁶ cells/ml) was added to 500 μl of molten LMAgrose (15 low-melting agarose) kept at 42 °C. Fifty microliters were immediately pipetted and evenly spread onto an area of the comet slides. The slide was incubated at 4 °C in the dark for 10 min to accelerate geling of the agarose disc and then transferred to prechilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 1 Triton X-100, pH 10) for 60 min at 4 °C. A denaturation step was performed in alkali solution (300 mM NaOH, 1 mM EDTA, pH > 13) at room temperature for 20 min in the dark. The slide was then transferred to prechilled alkaline electrophoresis solution pH > 13 (300 mM NaOH, 1 mM EDTA) and subjected to electrophoresis at 1 V/cm, 300 mA for 40 min in the dark at 4 °C. At the end of the electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris–HCl, pH 7.4) and immersed in ice cold 100% ethanol at room temperature for 5 min and air dried. DNA was stained with 50 μl of SYBR Green I dye (Trevigen, 1:10 000 in Tris–EDTA buffer, pH 7.5) for 20 min in the refrigerator and immediately analyzed using an Olympus digital camera attached to an Olympus BX51 epifluorescence microscope.

2.3. Comet (neutral condition) assay

The comet assay (Trevigen Inc., Gaithersburg, MD) was performed according to manufacturer’s protocol using neutral conditions. The procedure is essentially same as described in Section 2.2 with slight modification. After lysis, the slides were washed once with 1 x Tris-base EDTA buffer solution, pH 8.3 (TBE) for 10 min each. The slides were placed in a horizontal electrophoresis chamber and covered with TBE buffer. Electrophoresis was carried out at the rate of 1.0 V/cm for 20 min. The slides were removed from the electrophoresis chamber, washed in deionized water for 5 min and immersed in ice cold 100% ethanol for 5 min. Subsequently, the slides were air dried. DNA was stained with 50 μl of SYBR Green I dye (Trevigen, 1:10 000 in Tris–EDTA buffer, pH 7.5) for 20 min in the refrigerator and immediately analyzed using an Olympus digital camera attached to an Olympus BX51 epifluorescence microscope.

2.4. Fragment length analysis using repair enzymes (FLARE™) by comet assay

DNA lesions in neurons and astrocytes were evaluated using Trevigen Comet Assay FLARE™ kit (Trevigen Inc., Gaithersburg, MD) using human OGG1 or UDG. Following lysis, the slides were washed three times for 10 min each with 1 x FLARE™ buffer 1, gently blotted dry with tissue paper and covered with 50 μl of FLARE™ reaction buffer (1 x FLARE™ buffer, 0.1 mg/ml BSA) and OGG1 or UDG in the FLARE™ reaction buffer (0.5 units per gel). These slides were incubated in a humidity chamber at 37 °C for 60 min. A denaturation step was performed in alkali solution (300 mM NaOH, 1 mM EDTA, pH > 13) at room temperature for 20 min. After the electrophoresis and comet staining procedure is same as described in Section 2.2.

2.5. Comet analysis

For each slide, 50 randomly chosen comets were analyzed using an Olympus BX51 epifluorescence microscope with an excitation filter of BP 450–480 nm and a barrier filter of 515 nm. Fluorescent images of single cells were captured at 400x magnification and images were scored for comet parameters like the tail length and tail moment (product of percentage of DNA in tail and tail length) using the Tritek CometScore™ Freeware v1.5 image analysis software.

2.6. Oligonucleotides

The sequences of the oligonucleotides used in this study are presented in Table 1. Oligonucleotides containing uracil (U), tetrathydrofuran (THF) (Integrated DNA Technologies, Coralville, IA, USA) and 7.8 dihydro-8-oxoguanine (8-oxoGc) (Trevisen, Gaithersburg, MD) were 5’[2P]-kinased by incubating with [γ32P]-ATP (BRIT, Mumbai, India) in the presence of τα-poly(oligonucleotide kinase (Bangalore Genei, India). Unincorporated free [γ32P]-ATP was separated from the reaction mixtures using Sephadex G-50 spin columns. The 5’-kinased oligonucleotides were then annealed to the complementary strands in the presence of 50 mM NaCl and 5 mM MgCl2 by heating the samples at 70 °C for 10 min and allowing them to cool slowly to room temperature.

2.6.1. OGG1 activity assay

The OGG1 activity in neuronal and astrogial extracts was determined by an oligonucleotide incision assay. Briefly, OGG1 activity was assayed in a reaction volume 20 μl containing 20 mM Tris–HCl pH 8, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 200 fmol of [γ32P]-kinased 8-oxoG containing oligonucleotide duplex (Table 1) and 25 μg protein extract. Reaction mixtures were incubated for 120 min at 37 °C, and terminated with 10 μl of 3 M alkaline loading buffer (300 mM NaOH, 97% formamide and 0.2% bromophenol blue). The denatured samples were subjected to
Table 1
Oligonucleotides used in this studies.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>5'gcaacctgUgctaacgatgcg 3'</td>
</tr>
<tr>
<td>THF</td>
<td>5'cgatggcagtggcgtacg 3'</td>
</tr>
<tr>
<td>8-oxoG</td>
<td>5'gaactagtt8-oxoGatccccggggtgc 3'</td>
</tr>
</tbody>
</table>

Boldface letters represent important modifications.
U: uracil; THF: tetrahydrofuran abasic site analog; 8-oxoG, 7,8 dihydro-8-oxoguanine. All other bases are normal.

20% polyacrylamide sequencing gel electrophoresis with 7 M urea at 2300 V for 3 h. Analysis of the substrate and product was done by autoradiography and quantified using Image J software (NIH, USA). Incision activity was determined as the intensity of the product bands relative to the combined intensities of the substrate and product bands.

2.7. UDG activity assay

The UDG activity in neuronal and astroglial extracts was also determined by an oligonucleotide incision assay. Briefly, the 20 µl reaction mixture contained 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 25 µg/ml bovine serum albumin, 200 fmol of [α-32P]-kinased U containing oligonucleotide duplex (Table 1) and 10 µg of neuronal and astroglial extract. Reaction mixtures were incubated at 37 °C for 20 min and terminated with 10 µl of 3X alkaline loading buffer (300 mM NaOH, 97% formamide and 0.2% bromophenol blue). The samples were heated at 95 °C for 10 min and cooled to 4 °C. The denatured samples were loaded on to 20% polyacrylamide sequencing gel with 7 M urea and analyzed as described in Section 2.6.

2.8. AP endonuclease (APE1) assay

The APE1 activity in neuronal and astroglial extracts was assessed in a 20 µl reaction mixture containing 10 mM HEPES–KOH, pH 7.4, 100 mM KCl, 10 mM MgCl₂, 200 fmol of [α-32P]-kinased THF containing oligonucleotide duplex (Table 1) incubated with 200 ng of neuronal and astroglial extract protein. The reaction mixtures were incubated for 10 min at 37 °C and terminated by the addition of 10 µl of loading dye (95% formamide, 50 mM EDTA, 0.1% of xylene cyanol, 0.1% of bromophenol blue) and heated at 95 °C for 5 min. The denatured samples were loaded on 20% polyacrylamide sequencing gel with 7 M urea and analyzed as described in Section 2.6.

2.9. Statistical analysis

Statistical analysis was performed by Graphpad Prism 5.0.1 software (Graphpad Software Inc., CA, USA). Data were expressed as mean ± standard error of mean (SEM) of three independent experiments. Significance of difference between the groups was analyzed by Student’s t-test and one way ANOVA with Tukey post hoc test. A p-value <0.05 was considered significant for all analysis.

3. Results and discussion

3.1. Single and double strand breaks accumulate in brain cells with age

The alkaline version of the comet assay is used to determine the level of DNA strand breaks in isolated neurons and astrocytes prepared from animals of different age groups, and the results are shown in Fig. 1. After SYBR Green I staining, young neuronal nuclei appeared very bright and round (Fig. 1A). Furthermore, very little migration of DNA is evident; suggesting that DNA damage is at its minimum in young neurons. However, with advancement of age (adult and old), there is significant relaxation and migration of DNA from the nucleus, forming a “comet” tail (Fig. 1A). It is also clear that the tail moment values increased with age. The tail moment value, in comparison with that in young neurons increased 4.1 fold in adult and 5.1 fold in old neurons (Fig. 1B). A similar pattern of DNA damage was found in astrocytes as well. The tail moment value of adult and old astrocytes increased 3.6 fold and 4.2 fold respectively in comparison to young astrocytes (Fig. 1B). Closer evaluation of the data reveals that a considerable number of strand breaks accumulated by adulthood itself (6 months) with only a marginal further increase in the strand breaks in old age in both types of cells.

We have also examined neutral version of the comet assay without treatment with alkaline buffer for electrophoresis. Under these conditions, the comet is considered to detect largely, if not exclusively DSBs. The extent of DNA damage measured as tail length under these conditions also increased in neurons with age (Fig. 2A). The tail length value of adult neurons increased 6.2 fold in comparison to that in young while in the case of old neurons, there was a 7.4 fold increase in comparison to young neurons (Fig. 2A and B). A similar pattern of DNA migration of the tail was found in astrocytes preparations (Fig. 2A and B). The tail length value in adult and old astrocytes increased 5.3 fold and 6.1 fold respectively in comparison to young.

A striking feature appears to be that the damage accumulation occurs very rapidly during the first 6 months of life. A similar trend is seen by us in our earlier studies of DNA polymerase β (pol β) activity in rat neurons. Maximum reduction occurred during the first 6 months itself (Rao et al., 2000). Similarly, in our relatively recent studies (Krishna et al., 2005), the gap repair in neurons was also reduced markedly by 6 months of age itself. A somewhat comparable result was noticed in a very early study carried out by Price et al. (1971). It is possible that this phenomenon of significant accumulation of DNA damage coupled with decreased DNA repair, particularly in a post mitotic tissue, may have something to do with the timing of the attainment of reproductive maturity of the animal (Bernstein and Bernstein, 1991). This however remains a speculation at this time. It is also possible that during this rapid growth and reproductive phase of the animal, the repair efficiency is not able to match with the accumulation of damage.

3.2. Age-dependent accumulation of OGG1 and UDG sensitive sites in neurons and astrocytes

Modification of the alkaline comet assay by incorporating a lesion-specific enzyme increases its specificity through the recognition of the damaged base substrate and introduction of additional breaks. Thus additional strand breaks are induced at the location of damaged base substrate, causing DNA relaxation and migration (Collins et al., 2001; Smith et al., 2006). When using lesion-specific enzymes to measure DNA damage, the usual practice is to incubate a slide (two gels) with buffer alone in parallel along with the +enzyme slide, and to subtract the mean comet score of the control (buffer) slide from the mean score of the +enzyme slide (Collins and Duiniska, 2002; Collins, 2009).

In this study, to examine the presence of OGG1 sensitive sites in DNA of neurons and astrocytes, we have introduced human OGG1 in the assay on naked DNA after lysis was performed. Human OGG1 initiates the repair of 8-oxoG bases by excising them and cutting the sugar–phosphate backbone of the DNA molecule. Thus additional strand breaks are induced at the location of oxidized base substrate, causing DNA relaxation and migration (Collins et al., 2001; Smith et al., 2006).

Table 2 shows tail moment values of isolated neurons and astrocytes prepared from the young, adult and old rat cerebral cortex. When neurons were incubated with only buffer, an increase of DNA migration in the tail was observed with increasing age of the animal (Table 2). This increase in tail movement is taken to indicate increasing DNA damage that occurs due to aging. When the cells were incubated with buffer containing human OGG1,
there was a further increase in the tail movement, which must be due to the removal of 8-oxoG residues accumulating in DNA with age. Incubation of the brain cells with human OGG1 represents the increase in the number of OGG1 sensitive sites with age. The pattern of results is the same with either neurons or astrocytes. Table 2 shows the actual fold increase of OGG1 sensitive sites with age in brain cells. For example, in neuronal DNA the content of OGG1 sensitive sites between the young and adult ages has increased by 5.2 fold and between young and old ages by 7.4 fold. Similarly, adult and old astrocytes accumulated 3.2 and 4.9 fold respectively in comparison to young astrocytes. Closer observation of the data revealed that accumulations of OGG1 sensitive sites occurs continuously through adulthood and old age unlike the general damage due to overall strand breaks which occurred mostly by adulthood itself with only a marginal further increase between adult stage and old age (Table 2).

Similarly, the UDG sensitive sites in neurons and astrocytes at these three ages were assessed using a FLARE™ comet assay kit by the introduction of UDG, which removes uracil and creates abasic sites that are cleaved to result in strand breaks during the alkali processing. When neurons were incubated with only buffer, increase of DNA migration in the tail was observed with age of the animal (Table 3). This increase in tail movement is taken as increased damage occurring due to aging. When the cells were incubated with buffer containing the UDG, there was a further increase in tail movement, which must be due to the presence of uracil residues accumulating in DNA with age. Essentially the enhanced tail movement due to the incubation of the brain cells with UDG denotes the increase in the number of uracil or other UDG sensitive sites with age. The pattern of results is the same either with neurons or astrocytes. Table 3 shows the actual fold increase of such sensitive sites with age in brain cells. For example, in neuronal DNA the content of UDG sensitive sites between the young and adult ages has increased 3.3 fold and between young and old ages by 6.5 fold. Similarly adult and old astrocytes accumulate 3.3 and 6.6 fold respectively.
in comparison to young astrocytes. Keen observation of the data suggests that accumulation of UDG sensitive sites in neurons and astrocytes is a gradual process with age (Table 3).

3.3. Age-dependent decline of some BER enzyme activities (OGG1, UDG and APE1)

As the first step in the BER pathway, excision of damaged base in DNA requires the activity of at least two repair enzymes, a specific glycosylase that can recognize and cleave the damaged bases, and an AP endonuclease that incises the sugar-phosphate backbone at the remaining abasic residue. DNA glycosylase activities were measured using double-stranded DNA oligonucleotides containing specific base damages and APE1 activity was determined using a THF (an abasic site analogue) containing oligonucleotide (Table 1). OGG1 is one of the most important repair enzymes that is involved in BER to eliminate oxidative damage from mammalian DNA.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Tail moment</th>
<th>Net amount of OGG1 sensitive sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Buffer/O GG1</td>
</tr>
<tr>
<td>Young neurons</td>
<td>14.46 ± 1.42</td>
<td>22.75 ± 2.96</td>
</tr>
<tr>
<td>Adult neurons</td>
<td>94.76 ± 6.68</td>
<td>138.18 ± 11.04***</td>
</tr>
<tr>
<td>Old neurons</td>
<td>114.01 ± 3.00</td>
<td>175.64 ± 7.77***</td>
</tr>
<tr>
<td>Young astrocytes</td>
<td>20.04 ± 2.10</td>
<td>30.74 ± 3.47</td>
</tr>
<tr>
<td>Adult astrocytes</td>
<td>100.42 ± 5.22</td>
<td>135.05 ± 6.12**</td>
</tr>
<tr>
<td>Old astrocytes</td>
<td>116.59 ± 3.26</td>
<td>169.49 ± 7.72***</td>
</tr>
</tbody>
</table>

Two different experiments have been conducted and at least 50 comets were scored in each experiment. Superscript letters a and b are significantly different from the corresponding values at young. Superscript letter c is significantly different from the corresponding values at adult. For other details please see Section 2.

p < 0.01

p < 0.001.
Table 3
Measurement of UDG sensitive sites in young, adult and old rat brain neurons and astrocytes by FLARE™ comet assay.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Tail moment (Mean ± SEM)</th>
<th>Net amount of UDG sensitive sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
<td>UDG</td>
</tr>
<tr>
<td>Young neurons</td>
<td>14.96 ± 1.70</td>
<td>22.77 ± 2.15</td>
</tr>
<tr>
<td>Adult neurons</td>
<td>115.71 ± 4.35</td>
<td>141.78 ± 9.23&quot;***&quot;</td>
</tr>
<tr>
<td>Old neurons</td>
<td>134.70 ± 5.91</td>
<td>185.34 ± 10.55&quot;***&quot;</td>
</tr>
<tr>
<td>Young astrocytes</td>
<td>18.80 ± 2.23</td>
<td>24.83 ± 2.38</td>
</tr>
<tr>
<td>Adult astrocytes</td>
<td>121.92 ± 5.07</td>
<td>141.72 ± 8.11&quot;***&quot;</td>
</tr>
<tr>
<td>Old astrocytes</td>
<td>135.72 ± 5.54</td>
<td>175.44 ± 8.72&quot;***&quot;</td>
</tr>
</tbody>
</table>

Two different experiments have been conducted and at least 30 comets have been scored in each experiment. Superscript letters a and b are significantly different from the corresponding values at young. Superscript letter c is significantly different from the corresponding values at adult. For other details see Section 2.

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Fig. 3. 8-oxoguanine DNA glycosylase activity (OGG1) in 'young', 'adult' and 'old' rat neuronal and astroglial extracts. Neuronal and astroglial extracts were incubated with 200 pmol of 5'[^32]P]-labeled 24-mer DNA oligonucleotide duplex containing 8-oxoG at position 10, in the assay buffer for 120 min at 32°C, generating the specific 9-mer cleavage product. The samples were then processed as described in Section 2. A typical autoradiogram from three different experiments is shown. (A) Lanes 1–3, neuronal extracts from young brain (Y, 7 days postnatal), adult brain (A, 6 months) and old brain (O, ≥ 2 years). Lanes 4–6, astroglial extracts from young, adult and old brains. Lane 7 is 0.2 units of pure human OGG1. Lane 8 is without any extracts (enzyme blank). One Unit of OGG1 catalyzes the cleavage of 1 pmol of a [^32]P]-oligonucleotide probe in 1 h at 37°C, at an 8-oxoG:C base pair within a duplex oligo. (B) Quantitative analysis of OGG1 activity in neuronal and astroglial extracts as the function of age, determined by densitometric measurements on autoradiogram from three independent experiments. The incision activity was calculated as the relative amount of 9-mer product to 24-mer substrate (product/substrate + product). Values are expressed in mean ± SEM, a and b are significantly different from the corresponding values at young. "***p < 0.001; "p < 0.05.

To examine the age-associated changes in OGG1 activity in brain cell extracts, the radio labeled 24-mer oligoduplex containing 8-oxoG at position 10 (sequence shown in Table 1) was incubated with 25 μg of protein for 120 min resulting in the incision of the radio labeled oligomer, generating a specific 9-mer product (Fig. 3A, lanes 1–6). This cleavage product is also seen in the reaction containing pure OGG1 protein (lane 7). The OGG1 activity in neuronal extracts of adult and old rats has decreased significantly when compared to young rats (Fig. 3B, p < 0.001). However, the decrease seen in adult and old astroglial extracts is of a lesser magnitude, although statistically significant (Fig. 3B, p < 0.05).

A similar oligonucleotide incision assay was performed to examine the activity of UDG. Table 1 shows the sequences of the oligodeoxyribonucleotide used in the incision assay. The 21-mer oligonucleotide containing U at position 8 was 5'-labeled with [^32]P]-ATP. Complementary oligo was annealed to form DNA oligoduplex. A representative autoradiogram is shown in Fig. 4A. The results indicate that incubation of brain cell extracts with the DNA oligoduplex containing U for 20 min resulted in an incision of the radio labeled oligomer, generating a specific 7-mer product (Fig. 4A, lanes 1–6). This cleavage product is also seen in the reaction with pure UDG protein (Fig. 4A, lane 7). The UDG activity in neuronal extracts of adult and old rats has decreased significantly when compared to that in young rats (Fig. 4B, p < 0.05). However, the decrease seen in adult and old astroglial extracts, once again as in the case of OGG1, is of lesser order, although statistically significant (Fig. 4B, p < 0.01).

Aging seems to affect adversely the APE1 activity also as can be seen from the results presented in Fig. 5A. Incubation of brain cell extracts with the radio labeled 21-mer oligoduplex containing THF at position 14 (sequence shown in Table 1) for 10 min resulted in an incision of the radio labeled oligomer, generating a specific 13-mer product (Fig. 5A, lanes 1–6). Such cleavage was confirmed through the action of pure APE1 enzyme (Fig. 5A, lane 7). The age related decline in APE1 activity in both types of brain cells (Fig. 5B, p < 0.01 and p < 0.05) is quite apparent.

Aging is regarded as an elusive and inevitable phenomenon occurring in all higher organisms. Many theories have been proposed to explain this process. One such theory which enjoys considerable logic and rationale is the DNA damage and repair theory. DNA repair potential of an organism could play a vital role in the maintenance of genomic integrity, the failure of which could result in disease and among many other things, aging as well (Gensler and Bernstein, 1981; Hart and Setlow, 1974). The possible interrelationship between DNA repair potential and the aging process has been the subject of intense research and debate (Rao and Loeb, 1992). There seems to be a consensus among scientists in the field that accumulation of DNA damage and a decrease in DNA repair capacity contribute to the phenomenon of aging and related disorders, including neurologi- cal disorders (Gorbunova et al., 2007; Subba Rao, 2007; Schumacher et al., 2008; Vijg, 2008).
The relationship between aging and DNA repair has a special significance in an organ like brain. DNA repair is affected in mammalian cells through four main different pathways (Rao, 2002, 2003, 2007). The BER pathway, which is seen in almost all the tissues and responsible for repairing the simple alterations in DNA structure at the base level, e.g., modifications of bases through methylation, ethylation, oxidation and spontaneous deamination or spontaneous loss of bases resulting in the formation of AP sites (apurinic/apyrimidinic sites), leading to SSBs in DNA. This pathway is one of the most conserved and takes care of the minor but important damages that occur to bases in DNA. Uracil in genomic DNA mainly arises from deamination of cytosine residues and 8-oxoG is abundantly produced in DNA by reactive oxygen species. BER is an important tool for the brain to correct such type of DNA damage.

The present study represents a single attempt to assess various DNA damage parameters like SSBs, DSBs and also the presence of OGG1 and UDG sensitive sites in aging brain cells through a simple technique of comet assay. It is demonstrated that there is an accumulation of SSBs, DSBs, OGG1 and UDG sensitive sites in neurons and astrocytes with age. Furthermore, the results provide evidence that decrease in activity of repair enzymes that are important components of the BER pathway. Indeed the present observations through comet assay substantiate and extend a number of reports appeared in previous years regarding the general decline of BER activity and the activities of various factors/enzymes involved in BER pathway. For example, Chen et al. (2002) showed age dependent decline of DNA repair activity for oxidative lesions in rat brain mitochondria. Similarly, Intano et al. (2003) reported 85% decline in repair activity coupled with decreased abundance of pol β in brain nuclear extracts. While reviewing BER in central nervous system Wilson and McNeill (2007) highlighted the possible deleterious effects that could result in nervous system if BER or its components are compromised in their function. Defective BER is also reported in brains from individuals with Alzheimer's disease (Weissman et al., 2007) and also reduced BER and pol β in brains of old mice as compared to young (Xu et al., 2008). Thus the present observations support the hypothesis that accumulation of DNA damage and a decrease in DNA repair capacity appear to be hallmarks of aging phenomenon and related disorders. What is to be noted is the fact that most of the DNA damage seems to accumulate by the time the animal attains...
adulthood (6 months in the present case). There was of course a further increase in the DNA damage between the ages of 6 and 24 months. However, the magnitude of this increase was less as in comparison to that between young and adult ages. This may suggest a possible time point of initiation of the aging process. More significantly, the present investigation highlights the utility of the comet assay for a quick measurement of DNA damage in a discriminatory manner in a large number of samples like in population studies.

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