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Gene-specific oxidative lesions in aged rat brain detected by polymerase chain reaction inhibition assay

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Abstract
An exposure of isolated rat brain genomic DNA to oxidative stress in the form of iron salts (Fe^{2+}) and ascorbate results in gene-specific DNA lesions detectable by a quantitative polymerase chain reaction (PCR) based assay in which PCR amplification efficiency of the affected genes (e.g. β-actin and p53) is grossly impaired. Such oxidative DNA lesions are prevented by hydroxyl radical scavengers like mannitol (20 mM) and sodium benzoate (20 mM) or by the antioxidant enzyme catalase (50 μg/ml) present in the incubation mixture during exposure to Fe^{2+} and ascorbate. When brain DNA isolated from young (4–6 months of age) and aged (20–24 months of age) rats are analyzed similarly by the PCR based method, the amplification levels of β-actin and p53 genes are noticeably decreased in the case of aged rat indicating an accumulation of gene-specific DNA lesions during brain aging.

Keywords: *DNA, aging, brain, reactive oxygen species (ROS), ascorbic acid*

Introduction

Age-related alterations in genomic DNA arise in a large measure from oxidative damage and the latter is particularly important in an organ like brain which is highly vulnerable to oxidative injury [1,2,3]. Oxidative DNA lesions include loss of bases, a variety of base modifications, strand breaks, mismatched base-pairs, protein-DNA cross-links and various DNA adducts which can be detected by a number of analytical techniques [4,5,6,7]. In most of the studies with aged brain, however, the oxidative DNA lesions have been detected by the measurement of 8-hydroxy deoxyguanosine (8-oxodG) by HPLC, although there are scattered reports indicating DNA strand breakage, DNA-protein cross-linking or formation of etheno-DNA-adducts during brain aging [8,9,10,11]. The measurement of 8-oxo-dG in young and aged brain has, however, yielded variable results possibly because of artifactual DNA damage during processing of the brain samples [8,10,12]. Moreover, the measurement of general oxidative DNA damage parameters in aged brain does not reveal whether specific genomic regions responsible for critical neuronal functions are affected. In post-mitotic cells like neurons, such gene-specific oxidative lesions may be reflected primarily in altered transcriptional rate or in ultimate synthesis of non-functional proteins. The process is likely to have important implications in neuronal dysfunctions associated with brain aging. In this study we have utilized a sensitive quantitative PCR based technique to establish DNA damage within specific genes of interest in isolated brain genomic DNA exposed to an oxidative insult and then attempted to examine if similar genomic areas are also affected in the aged brain.

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Materials and methods

Chemicals

DNA isolation kit was purchased from Roche Molecular Biochemicals, USA. Microcentrifugal ultrafiltration device (molecular weight cut-off 10,000 dalton) was from Vivascience AG, Germany. Hot start *Taq* polymerase ("Taq bead"), dNTP mixture, $MgCl_2$ were from Promega, USA. DNA molecular weight markers (100 bp DNA ladder plus, O'GeneRuler^{TM}) were purchased from Fermentas (USA). Mannitol, sodium benzoate, catalase, agarose, ethidium bromide were from Sigma Chemical Co., USA. All other chemicals were of the highest analytical grade available.

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: Young (4–6 months of age, body weight 120–150 gm) and aged (20–24 months old, body weight 250–300 gm). The animals were killed by cervical dislocation and the brains dissected out cleanly and collected on Petri dishes kept over ice.

Genomic DNA isolation from rat brain

About 200 mg of rat brain cortical and cerebellar tissue was utilized to extract genomic DNA as per the protocol of the commercial kit which involved lysis of the brain tissue in the presence of a strong anionic detergent and proteinase K followed by the removal of contaminating RNA by RNase. The proteins were removed from the sample by selective precipitation and centrifugation, and DNA was recovered from the sample by isopropanol precipitation. The precipitated DNA was washed in cold ethanol, dried in air and dissolved in 10 mM Tris, pH 8.0. The quality of purified DNA was checked by the ratio of A_{260} and A_{280} as well as by 0.7% agarose gel electrophoresis.

In vitro *oxidative stress to rat brain genomic DNA*

DNA isolated from adult rat brain was subjected to *in vitro* oxidative stress. Briefly, the isolated DNA was incubated for up to 1 h at 37°C in 50 mM phosphate buffer, pH 7.4 in the absence (control sample) or in the presence (experimental samples) of ascorbate (2 mM) and $FeSO_4$ (50 μM) with or without catalase (50 μg/ml) or mannitol (20 mM) or sodium benzoate (20 mM). At the end of the incubation, DNA samples were washed with autoclaved water thrice using microcentrifugal ultrafiltration device of 10 KD molecular cut-off to completely remove the small molecular weight reagents from the incubation mixture. The DNA remaining over the membrane in each microcentrifugal tube was collected, made up to a volume equal for each sample and used further for the assessment of oxidative DNA damage by PCR inhibition assay. For some other experiments DNA samples (control and experimental) were directly subjected to 1% agarose gel electrophoresis at the end of the incubation.

PCR inhibition assay for oxidative DNA damage

DNA damage caused by oxygen free radicals or other genotoxic agents can lead to decreased efficiency of PCR amplification of specific genes or genomic regions of the affected DNA [13,14,15].

Simple quantitative PCR assay was used in this study to detect oxidative DNA damage. Because of the complexity of PCR amplification kinetics and possibility of replicate variation, a proper optimization of the method was required. In our assay, the amplification cycle was kept at 25 and the template DNA content varied from 15 to 60 ng and under this condition fluorescence intensity of the product increased nearly linearly with the increase in template DNA concentration. In all our assays, the template DNA content in different samples in a set of experiments was kept constant and within 15–60 ng range, so that the fluorescence intensity of the product indicated PCR amplification efficiency. Two rat gene fragments, e.g. β-actin gene fragment (923 bp) and the p53 gene fragment (1644 bp) were amplified. An aliquot of genomic DNA containing an equal amount of DNA from control (without Fe^{2+}-ascorbate treatment) or each experimental sample (Fe^{2+}-ascorbate treated with or without radical scavengers or antioxidants) as described earlier in the text was added to the PCR mixture of final volume 25 μl containing 1.25 units of *Taq* polymerase, 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton-X 100, 3 mM $MgCl_2$, four dNTPs of 0.2 mM each, 5% DMSO, 2 mM β-mercaptoethanol and 0.80 μM each of the appropriate forward and reverse primers [16,17,18]. The reaction mixture was covered with a layer of mineral oil (Promega, USA) and the PCR tubes were incubated in a preheated (95°C) programmable thermocycler. The amplification regime for β-actin fragment was 1.2 min at 92°C, 2 min at 62°C and 2 min at 72°C for 25 cycles. The amplification protocol for p53 gene fragment was 1.2 min at 92°C, 1.5 min at 61°C and 2 min at 72°C for 25 cycles. After 25 cycles of amplification, the reaction mixture was additionally incubated at 72°C for 5 min. The primer details are given below.

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,

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Gene fragment	Size of fragment (bp)	Primer (24 bp)
β-actin	923	(+)5′-GTTGTCCCTGTAT GCCTCTGGTCG-3′ (Forward)
		(-)5′-TAGAAGCATTTGCG GTGCACGATG-3′ (Reverse)
p53	1644	(+)5′-ATTCACAGTCGGATAT GAGCATCG-3′ (Forward)
		(-)5′-CAGCGTCTCACGACC TCAGTCATG-3′ (Reverse)

Hercules, CA) and quantitation done using Bio-Rad Quantity-1 software.

Agarose gel electrophoresis of rat brain genomic DNA exposed to oxidative stress

Control (without exposure to Fe^{2+}-ascorbate) and experimental (exposed to Fe^{2+}-ascorbate with or without radical scavengers or antioxidants) samples of brain genomic DNA were analyzed by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0). At the end of the electrophoresis, DNA was visualized by UV-transilluminator after staining with ethidium bromide.

PCR inhibition assay for age-related DNA damage

Brain genomic DNA was isolated from young and aged rats as described earlier in the text. The isolated DNA from each group without any *in vitro* treatment was utilized in the PCR based assay as described above using the primers for β-actin or p53.

Results

DNA damage detection by polymerase chain reaction (PCR) amplification inhibition assay

The amplification of β-actin and p53 gene fragments from rat brain genomic DNA resulted in 923 and 1644 bp bands, respectively (Figures 1–3). Using different amounts of template DNA (15–60 ng) and keeping the amplification cycle number constant at 25, it was observed that the band intensity of PCR amplified product, e.g. β-actin gene fragment increased nearly linearly with the increase in template DNA content (Figure 1). It was shown that the exposure of genomic DNA to Fe^{2+} (50 μM) and ascorbate (2 mM) for varying period of time up to 1 h caused subsequently a remarkable inhibition of β-actin gene amplification by PCR (Figure 2). The time course study also indicated that the PCR inhibition was noticeable just after 5 min incubation with Fe^{2+} (50 μM) plus ascorbate (2 mM) and a very pronounced impairment of PCR amplification occurred by the end of 1 h (Figure 2). The hydroxyl radical scavengers like mannitol (20 mM), or sodium benzoate

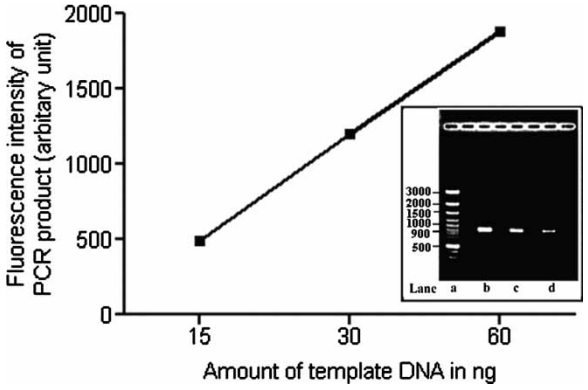

Figure 1. PCR amplification of β-actin gene fragment from rat brain genomic DNA. Genomic DNA isolated from rat brain was used to amplify a 923 bp β-actin gene fragment by PCR as described in the Materials and methods. About 15–60 ng of template DNA was utilized to amplify the β-actin gene fragment and the product quantified by densitometric scanning and analysis by Biorad Quantity 1 software. Inset: Agarose-gel electrophoresis of PCR product. Lane (a): marker DNA (100 bp DNA ladder), lanes (b)–(d): 923 bp β-actin gene fragment amplified from 15 ng (d), 30 ng (c) and 60 ng (b) of genomic DNA.

(20 mM) or the antioxidant enzyme catalase (50 μg/μl) in the incubation mixture significantly prevented the subsequent impairment of β-actin gene amplification in PCR based assay (Figure 2). Quantitatively mannitol, sodium benzoate and catalase provided protection against Fe^{2+}-ascorbate induced damage to the extent of 88, 77 and 71%, respectively, as analyzed in the PCR-based assay (Figure 2). Similarly, impairment of p53 gene amplification from rat brain genomic DNA was noticed when brain DNA was preincubated with Fe^{2+}-ascorbate for 15 min and in this case also mannitol, sodium benzoate and catalase provided significant protection (Figure 3).

Analysis of DNA fragmentation by agarose gel electrophoresis

During *in vitro* incubation with iron–ascorbate a time-dependent fragmentation of rat brain genomic DNA was found as monitored by smearing of genomic DNA on agarose gel which was visible from 15 min onwards (Figure 4). Interestingly, almost complete protection was achieved with catalase (50 μg/ml), mannitol (20 mM) or benzoate (20 mM) in the incubation mixture (Figure 4).

Damage to rat brain genomic DNA during aging

As shown in Figure 5 there was no difference in agarose gel electrophoretic patterns of genomic DNA isolated from young and aged rat brain. Neither any smearing nor the characteristic ladder pattern of DNA fragmentation was noticeable on agarose gel in genomic DNA isolated from aged rat brain indicating no significant DNA fragmentation in aged brain compared to young. However, under the defined

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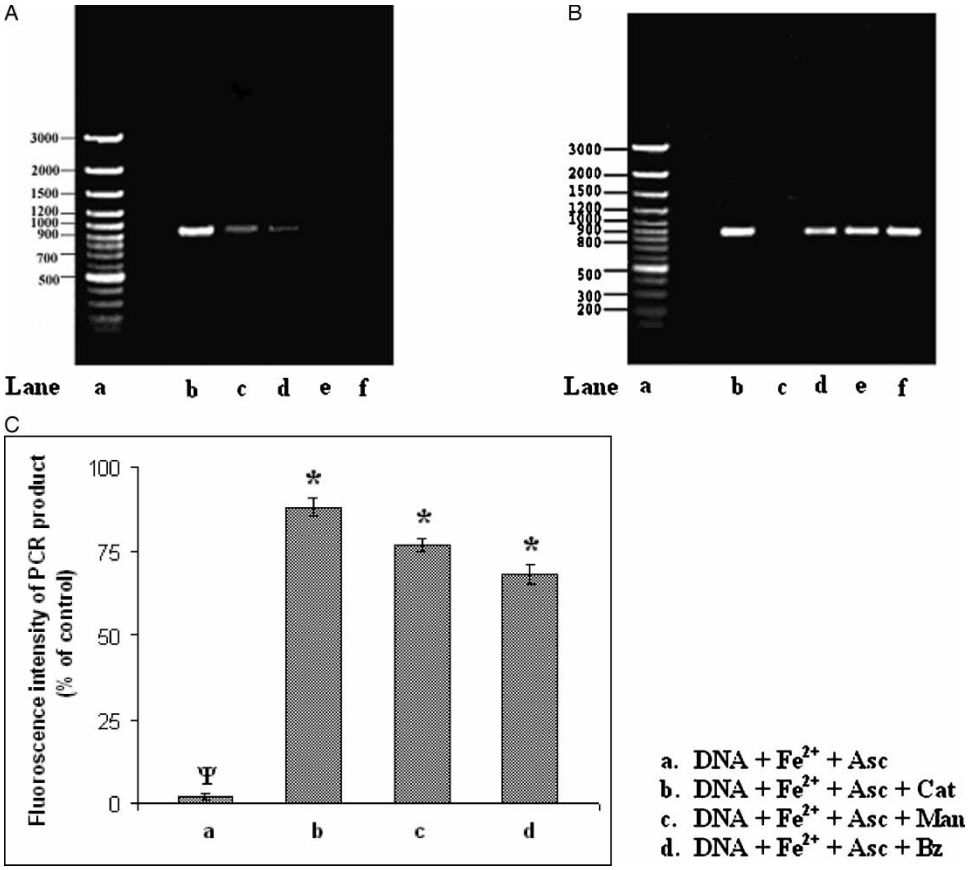

Figure 2. Impaired PCR amplification of β-actin gene from rat brain genomic DNA after *in vitro* oxidative stress Rat brain genomic DNA was incubated without (control) or with Fe^{2+}(50 μM) and ascorbate (2 mM) for varying periods of time up to 60 min in the presence or absence of mannitol (20 mM) or catalase (50 μg/ml) or benzoate (20 mM) and the DNA samples were processed and used for PCR amplification of β-actin gene fragment as described in the Materials and methods. (A) Time course of PCR inhibition. Lane (a): marker DNA (100 bp ladder); lanes (b)–(f): 923 bp β-actin gene fragment amplified from control genomic DNA (b) or genomic DNA exposed to Fe^{2+} and ascorbate for 5 min (c), 15 min (d), 30 min (e), 60 min (f). (B) Effect of antioxidants on PCR impairment by oxidative stress *in vitro*. Lane a: marker DNA (100 bp DNA ladder); lanes (b)–(f): 923 bp β-actin gene fragment amplified from control genomic DNA (b) or genomic DNA exposed to Fe^{2+} and ascorbate (c) or genomic DNA exposed to Fe^{2+} and ascorbate plus mannitol (d) or benzoate (e) or catalase (f). (C) Band intensity of the PCR product (experiment B) in each lane was calculated and analyzed by BioRad Quantity 1 software and expressed as percentage of the control. Ψ Statistically significant, $p < 0.001$ vs. control, *significantly different, $p < 0.001$ vs. DNA + Fe^{2+} and ascorbate, Student's "t"-test, paired.

condition of the PCR amplification inhibition assay, a marked reduction in the intensities of PCR products, e.g. β-actin or p53 gene fragments was noticed with brain genomic DNA from aged rats in comparison to that from young animals, although equal amounts of template DNA (60 ng) were used for both the groups (Figure 5).

For quantitation purpose, the amplification levels of p53 or β-actin gene fragment in the PCR based assay with brain genomic DNA samples from young or aged rats were calculated relative to the amplification level of the corresponding gene fragment (taken as 100%) under the same PCR assay conditions using a reference template DNA. The reference template DNA used was purified PCR amplified β-actin or p53 gene fragment. This method of calculation compensated for day-to-day variations in PCR assays as well as the sample to sample variations in the quality of template DNA within each group (young or aged). Moreover, PCR assay for each sample was run in duplicate and the mean value of the product intensity calculated. The data in Table I demonstrate that PCR amplification efficiency of β-actin or p53 gene fragment was markedly diminished (approximately 40%) in brain genomic DNA from aged rats in comparison to that from young animals.

Discussion

Under the optimized PCR assay condition used in this study, PCR product formation increases nearly linearly with increase in original template DNA concentrations in the range of 15–60 ng (Figure 1) and, therefore, any decrease in PCR product intensity reflects an impairment of amplification efficiency when equal amounts of template DNA are used for different samples within 15–60 ng range. The decreased PCR amplification efficiency has been

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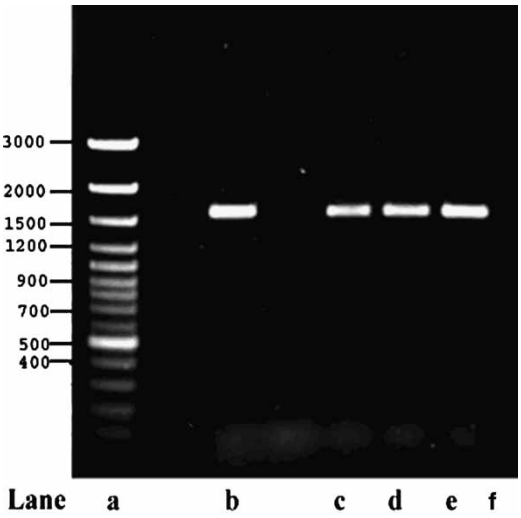

Figure 3. Effect of *in vitro* oxidative stress on PCR amplification of p53 gene fragment from rat brain genomic DNA Rat brain genomic DNA was incubated without (control) or with Fe^{2+} (50 μM) and ascorbate (2 mM) for 15 min in the presence or absence of mannitol (20 mM) or catalase (50 μg/ml) or benzoate (20 mM) and the DNA samples were processed and used for PCR amplification of p53 gene fragment as described in the Materials and methods. Lane (a): marker DNA (100 bp DNA ladder); lanes (b)–(f): 923 bp β-actin gene fragment amplified from control genomic DNA (b) or genomic DNA exposed to Fe^{2+} and ascorbate (c) or genomic DNA exposed to Fe^{2+} and ascorbate plus mannitol (d) or benzoate (e) or catalase (f).

related to template DNA damage. Such PCR based assays have been used by others to study DNA damage by oxidative stress and other genotoxic compounds [13,14,15,19,20].

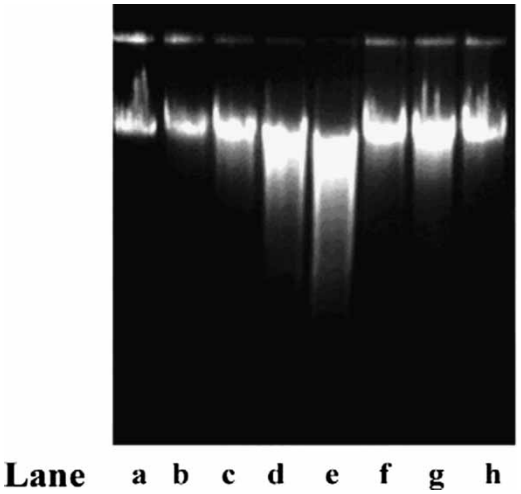

Figure 4. DNA fragmentation after *in vitro* oxidative stress Rat brain genomic DNA was incubated without (control) or with Fe^{2+} (50 μM) and ascorbate (2 mM) for various periods of time (5, 15, 30 and 60 min) in the absence or presence of other additions like benzoate (20 mM) or mannitol (20 mM) or catalase (50 μg/ml) for 1 h at 37°C, followed by agarose gel electrophoresis as described in the text. Lane (a): control DNA; lanes (b)–(e): DNA + Fe^{2+} and ascorbate incubated for 5 min (b), 15 min (c), 30 min (d), 60 min (e); lanes (f)–(h): DNA + Fe^{2+} and ascorbate with mannitol (f) or benzoate (g) or catalase (h).

Our data convincingly show that an exposure of rat brain DNA to Fe^{2+}-ascorbate leads to damage to β-actin and p53 genes as evident from impaired PCR amplification of those genes (Figures 2 and 3). Although, the incurred DNA damage is likely to be a generalized phenomenon, the experimental method used here helps us to identify such damage within specific genes of interest. The oxidative nature of such gene damage induced by Fe^{2+}-ascorbate is evident from the fact that OH radical scavengers and the antioxidant enzyme catalase can prevent such damage (Figures 2 and 3). The combination of Fe^{2+}-ascorbate is a potent source of reactive oxygen species (ROS), especially OH radicals, as has been shown in cell-free systems by 2-deoxyribose degradation assay for OH radicals [21]. The oxidation of Fe^{2+} leads to the formation of superoxide radicals which undergo spontaneous non-enzymatic dismutation to produce H_2O_2 [22]. The decomposition of H_2O_2 by Fe^{2+} generates OH radicals through Fenton's reaction and the reducing agent like ascorbate promotes the latter reaction by converting Fe^{3+} to ferrous state [22]. Thus, iron–ascorbate or copper–ascorbate mixture has been used in a number of *in vitro* systems to induce oxidative damage to lipid, protein or DNA [21,22,23,24]. Although the combination of Fe^{2+} and ascorbate can induce lipid peroxidation and protein damage in tissue preparations by a different mechanism without direct involvement of oxygen free radicals [21,22], it appears from the present study that in case of DNA damage OH radicals generated by Fenton's reaction are the damaging species and expectedly such damage is prevented by OH radical scavengers as well as catalase (Figures 2–4). It has been suggested that oxidative lesions in the form of strand breakage, abasic sites, base modifications (e.g. tandem repeats of 8-oxodG), etc. can cause impaired PCR amplifications of affected genes [15,19,25].

It is evident that detection of oxidative damage to specific genes or genomic regions is much more important than simple measurement of general oxidative damage parameters of DNA like 8-oxodG, DNA-protein cross-linking, DNA strand breaks, etc. since the former can then be directly related to a functional alteration. Moreover, oxidative damage in specific genes as detected by PCR-amplification inhibition assay is highly sensitive as can be seen from the time-course study where even an exposure to oxidative insult for 5 min results in a noticeable decrease in PCR amplification efficiency (Figure 2), but DNA strand breaks as measured by agarose gel electrophoresis is only recognizable after 15 min of incubation (Figure 4). Thus, the method may be ideal to look in to subtle changes in genes caused by oxidative stress.

Our results on PCR-amplifications efficiency of β-actin and p53 genes with brain genomic DNA from young and aged rats are of potential implications in this

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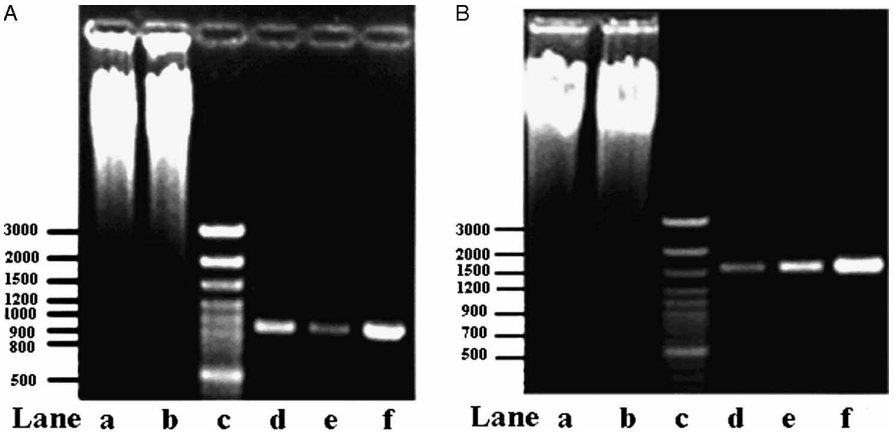

Figure 5. Amplification of β-actin and p53 gene fragments from DNA isolated from young and aged rat brain Rat brain genomic DNA was isolated from young (4–6 months) and aged (20–24 months) rats and used for PCR amplification of 923 bp fragment of β-actin gene or 1644 bp fragment of p53 gene as described in the Materials and methods. (A) β-actin gene amplification. Lane (a): intact genomic DNA of young rat; lane (b): intact genomic DNA of aged rat; lane (c): marker DNA; lane (d): β-actin gene fragment (young rat); lane (e): β-actin gene fragment (aged rat); lane (f): reference DNA (PCR amplified). (B) p53 gene amplification. Lane (a): intact genomic DNA of young rat; lane (b): intact genomic DNA of aged rat; lane (c): marker DNA; lane (d): p53 gene fragment (aged rat); lane (e): p53 gene fragment (young rat); lane (f): reference DNA (PCR amplified).

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context. A significant impairment of PCR-amplification efficiency of p53 and β-actin genes in aged rat brain genomic DNA as presented in Figure 5 clearly indicates age-related DNA damage in those genes. In agreement with our data from *in vitro* oxidative damage to rat brain, it is presumable that age-related alterations in β-actin and p53 genes also represent oxidative lesions (inflicted by ROS) and this in part may result from enhanced ROS production ability of aged brain as reported by others [26]. It will be however pertinent to measure the amount of DNA oxidation in aged rat brain using other parameters such as 8-oxodG measurement or detection of DNA strand breakage or abasic site formation to corroborate our results obtained from PCR based assays. Moreover, it would be interesting to study if the administration of potent radical scavengers like the nitrone based spin traps (e.g. α-phenyl-tert-butylnitrone) can prevent such age-related alterations in specific genes in the brain. The neuroprotective actions of such spin trapping antioxidants in experimental neurodegenerative models appear to be encouraging [27]. We have chosen a constitutive gene (β-actin) and an inducible gene (p53) from rat brain genomic DNA for our study on age-related and oxidative stress related alterations in specific genes. Since the amplification efficiency in PCR based assay of both the genes in aged rat brain is diminished by nearly the same extent, it is evident that such age-related oxidative lesions are independent of transcriptional activity of the genes.

Table I. Amplification levels of β-actin and p53 genes from young and aged rat brain genomic DNA

Sample	β-actin gene fragment amplification (percentage of reference DNA)	p53 gene fragment amplification (percentage of reference DNA)
Young (6)	53 ± 5.2	52 ± 5.5
Aged (6)	32 ± 2.6⋆	30 ± 2.9⋆

Brain genomic DNA was isolated from young (4–6 months) and aged (20–24 months) rats and used for PCR amplification of 923 bp fragment of β-actin gene and 1644 bp fragment of p53 gene as described in the Materials and methods. Band intensities of PCR-products were measured and analyzed by BioRad Quantity-1 software. Amplification levels of β-actin or p-53 gene fragments were calculated as percentage of amplification of the corresponding gene fragment under same PCR conditions but from a reference template DNA, as described in the text. The numbers in the parentheses indicate the number of animals used. Values are expressed as the means ± SEM. Statistical significance was calculated by Student's "t"-test, unpaired. ⋆$p < 0.001$ vs. young.

Alterations in gene expressions during brain aging are being investigated actively and impaired expressions of genes responsible for synaptic plasticity, vesicular transport and mitochondrial functions have been reported in human brain after the age of 40 [28,29,30]. On the other hand, ROS responsive genes and those responsible for inflammatory response show increased expression in aged brain [31]. Although, the detailed mechanisms of such altered gene expression during brain aging are not established, our data indicate that impairment of transcriptional activity of some genes may result from gene specific oxidative damage that may be detected by PCR-amplification inhibition assay.

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