Research Article

Effect of grape seeds extract and -α-lipoic acid on mtDNA deletion and mtDNA copy number in D-galactose-induced aging rats

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Abstract

The mitochondrial common deletion (mtDNA 4834-bp deletion in rats), is the most typical and frequent form of mtDNA damage associated with aging and degenerative diseases, this hypothesis tested in the current study to investigate the effects of grape seeds extract and -α-lipoic acid supplementation on common deletions and copy number of mitochondrial DNA in D-galactose induced aging male rats. Aging was induced by intraperitoneal injection of D-galactose (300 mg/kg dissolved in 1 ml DW) every day for 9 weeks to accelerate senescence and aging induction. The animals were randomly divided into four groups of 7 rats per group. Animals of 1st group (GI) represented negative control group without any treatment, animals of the 2nd group(GII), was D-galactose injected rats daily for 9 weeks and regarded as induced age control group, animals of the 3rd group (GIII) was injected daily with D-galactose(300 mg/kg ) and orally supplemented with grape seed extract(200 mg/kg) daily for 9 weeks, and the animals of the 4th, group(GIV) was daily injected by D-galactose (300 mg/kg) and orally supplemented with α-lipoic acid (100 mg/kg) for 9 weeks. The mtDNA 4834-bp large deletion (mtDNA4834 deletion) and mtDNA content were analyzed by quantitative real-time PCR. Present study revealed that the mitochondrial common deletion and mitochondrial DNA copy number (content) were significantly increased in hepatic tissue of D-Galactose induced aged rats for 9 weeks, as compared with non treated rats of control group. The results obtained showed that the treatment of D-galactose induced aged rats with(300 mg/kg) of grape seed extract and (100 mg/kg) of DL-α-lipoic acid were significantly(p<0.01) reduced the percentages of mtDNA 4834 bp deletion and mtDNA copy number (0.691± 0.205%, 0.724± 0.195%) respectively as compared to its in non treated D-galactose aged group (2.127± 0.675%), also the treatments of D-galactose aged rats with(300 mg/kg) of grape seeds extract and (100 mg/kg) of DL-α-lipoic acid were significantly(p<0.01) reduced in the mtDNA copy numbers (3310±285, 2538±695) respectively as compared to its in non treated D-galactose aged group (7057± 524). Also results suggest that grape seeds extract and α-lipoic acid suppresses mitochondrial DNA damage via increasing cellular and mitochondrial antioxidant capacity. The treatments of D-galactose aged rats with(300 mg/kg) of grape seeds extract and (100 mg/kg) of DL-α-lipoic acid were significantly(p<0.01), increased the superoxide dismutase (Units/mg protein) in the hepatic tissue (7.502 ±1.032 , 5.786 ±1.257) as compared to its activity in non treated D-galactose aged group (2.872± 1.505), an increased catalase (Units/mg protein) in the hepatic tissue (10.12±2.106 , 7.92±2.216) as compared to its activity in non treated D-galactose aged group (4.44± 0.518).In conclusion, this investigation demonstrates that the mtDNA 4834 deletion and relative mtDNA copy number are increasingly accumulated in tissues of the D-gal-induced aging rats resulted by oxidative stress damage effect for DNA and enhanced mtDNA biogenesis.

Keywords: Aging, mitochondrial DNA, mtDNA 4834 deletion, Grape seed, α-lipoic acid.

Introduction

Aging has been associated with mitochondrial DNA (mtDNA) common deletion (CD). Oxidative damage to mtDNA is associated with excessive reactive oxygen species production. Mitochondrial DNA (mtDNA) mutations are key factors in aging and age related diseases. The mtDNA 4834-bp deletion (common deletion, CD) in rats, is the most typical mtDNA damage associated with aging. Therefore, CD has been used as a biomarker for aging (Meissneret al.,2008;Markaryanet al.,2009; Chenetal.,2010; Zhonget al.,2011).

D-Galactose (D-gal) has been used to induce oxidative stress in vivo to mimic natural aging in rats, mice and Drosophila(Song et al.,1999; Cui et al.,2004). Overdose
of D-gal can be catalyzed by aldose reductase into galactitol, which cannot be metabolized but will accumulate in the cell, leading to osmotic stress and generation of ROS (Ho et al., 2003). In addition, accumulated D-gal in animal tissues can react with amino groups of proteins and peptides to form advanced glycation end products (AGE) which has been suggested to accelerate the aging process and linked to the pathogenesis of many age-associated diseases such as diabetes, arteriosclerosis, nephropathy, infection, and Alzheimer’s disease (Song et al., 1999).

Mitochondrial DNA is highly susceptible to reactive oxygen species ROS induced damage, because of its close proximity to the sites of ROS generation and its paucity of protective histones (Druzhyna et al., 2008). The accumulation of mtDNA deletions in single cells may lead to permanent mitochondrial dysfunction followed by cell death, when the proportion of mutant mtDNA exceeds a certain threshold level (Yin et al., 2007). mtDNA biogenesis is then promoted, as evidenced by increased mtDNA copy number in rat liver and rat hippocampus (Suliman et al., 2003; Gutsaeva et al., 2006). Under oxidative stress, both nuclear and mt gene expressions work in concert to regulate mtDNA biogenesis so as to compensate for defective mtDNA (Liu et al., 2003).

Grape seeds extract (GSE), is a natural extract from the seeds of Vitis vinifera contain several active components including flavonoids, polyphenols, anthocyanins, proanthocyanidins and procyanidines, GSE contain 70% - 95% standardized proanthocyanidins (Ferreira and Li, 2000). These flavonoids have demonstrated a marked spectrum of biological, pharmacological, therapeutic, and chemoprotective properties against oxygen free radicals and oxidative stress. Grape seeds proanthocyanidin extract (GSPE) has more powerful antioxidative activity than other well-known antioxidants, including vitamin C, vitamin E, and gallic acid (Ariga, 2004). GSPE has various biological functions including antiaging, potent phytochemical antioxidants, antibacterial, antiviral, anti-inflammatory, anti-allergic, and vasodilatory actions. Various reports have shown that long term dietary supplementation of polyphenols improved the cognitive performance in aged rats (Alia et al., 2003; Balu et al., 2006; Abdelgawadet et al., 2012).

α-lipoic acid has been described as a potent biological antioxidant, adetoxification and antidiabetic agent, and a diabetes medicine; it has been used to improve age-associated cardiovascular, cognitive, and neuromuscular deficit. α-lipoic acid is a short chain of fatty acid containing two sulfur atoms. Lipoic acids directly eliminate ROS and regenerate oxidized intrinsic antioxidant enzymes in the process of redox coupling with dihydrolipoic acid showing potent anti-oxidants in inhibiting apoptosis of hippocampus (Packer et al., 2001). Lipoic acid (LA) is a thiol compound found naturally in plants and animals. Lipoamide dehydrogenases, found only in mitochondria, reduce free LA to dihydrolipoic acid, which is a potent antioxidant. Thus, LA supplementation may increase cellular and mitochondrial antioxidant status, thereby effective lyattenuating any putative increase in oxidative stress with age (Arivazhagan et al., 2001, 2002; Suher et al., 2001). Cellular defense mechanisms against superoxides include a series of linked enzyme reactions which remove the toxic radicals and repair radical induced damage. The first of these enzymes is superoxide dismutase (SOD) which converts superoxide anion to hydrogen peroxide. Hydrogen peroxide, also toxic to cells, is removed by catalase (Muller et al., 2006). This study aimed to test the hypothesis that antiaging and protective potential effect of both grape seeds extract and lipoic acid on changes in the mtDNA deletion and mtDNA copy number in liver tissues of D-galactose induced aging rats which linked to oxidative stress.

Materials and Methods

Experimental Animals

Young male albino Wistar rats of 4 months age and 280-290 gm body weight, were procured from experimental animal laboratory of College of Veterinary of Baghdad University, Irq. Animals were housed for 10 weeks at the experimental animal housing in polypropylene cages of College of science, Salahdeen University. The rats were housed at a constant temperature of 25±1°C, humidity of 55%, and 12 hr light /dark cycle. The animals were fed standard chow and given tap water ad libitum throughout the experimental periods. After an acclimation period of one week, 28 rats were divided randomly into four groups (7 rats in each group). The rats of the 1st group (G I) served as negative control group without any treatment. The rats of the 2nd group (G II), were intraperitoneally injected daily with D-galactose (300 mg/kg BW; Lu et al., 2006;) (Sigma Chemical Company, St. Louis, Missouri, USA). The rats of the 3rd group (GIII), were injected with D-galactose (300 mg/kg BW/day) and administrated orally by GSE (200 mg/kg BW/day) for 9 weeks. The rats of the 4th group (G IV), were injected with (300 mg/kg BW/day)
of D-galactose and treated daily with single dose of α-lipoic acid (100 mg/kg BW/day) for 9 weeks.

Grape seeds extract administration

Grape seeds extract prepared according to Balu et al., (2006) and dissolved in double distal water and was daily supplemented orally by gavage 200 mg/kg at the same time of the day for 9 weeks.

Alfa-lipoic administration

DL-α-lipoic acid was purchased from Sigma Chemical Company (St. Louis, Missouri, USA) and was daily supplemented orally by 100 mg/kg daily for 9 weeks (Arivazhagan et al., 2001).

Mitochondrial DNA extraction

After being anaesthesized by intramuscular injection of 0.2 ml/100 gm of a 1 ml ketamine (50 mg) and 1 ml of xylazine (20 mg) solution, the animals were weighed. Animals were sacrificed 48 h after the last dose of the treatment, liver, and brain were quickly excised, and stored at -80°C until analysis. Total DNA was extracted from 25 mg of frozen liver using the gSYNC Mini Kit (Geneaid Co. USA). The extract, containing both nuclear DNA and mtDNA, was used for real-time polymerase chain reaction (real-time PCR) analysis. The DNA concentration of each sample was assayed with the gene quant pro DNA/RNA calculator (BioChrom, Cambridge, UK).

Analysis of mtDNA4834 in rat tissues

The quantity of the mtDNA4834 deletion was determined by coamplifying the mtDNA displacement-loop (D-loop) and mtDNA4834 deletion in a real-time PCR assay. Primers for each were previously described by Brandaet et al., (2002). The degree of mtDNA4834 deletion was quantified with a deletion probe a 5'-VIC reporter and a 3' TAMRA quencher dye and D-loop primer. The cycling conditions included an initial phase of 2 min at 50°C; followed by 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The fluorescence spectra was monitored by the LightCycler Detection system with Sequence Detection software version 4 (LightCycler, Roche Diagnostics) (Chen et al., 2010).

Quantification of mtDNA4834 deletion

The cycle at which a significant increase in normalized fluorescence was first detected designated as the threshold cycle number (Ct). The difference in Ct values was used as the measurement of relative abundance; Ct (Ct deletion – Ct D-loop) was used to calculate the abundance of the mtDNA4834(bp deletion; a smaller Ct indicates more deletions. The; and the percentage of the mtDNA deletion was calculated as 2^(-ΔCt) * 100% (Chen et al., 2010).

Mitochondrial DNA copy number assay

The content of mtDNA (abundance) relative to nuclear genomic DNA was determined by coamplifying the mt D-loop and the nuclear-encoded β-actin gene by real-time PCR assay. Primers for each were previously described by Brandaet et al., (2002). The amount of β-actin gene was quantified by a fluorescent probe [DYXL 5'-CGGTCGCCCTACCG-TTCCAGTT(3325)-3' BHQ1]. PCR amplification was carried out in a 50 µl reaction volume consisting of TaqMan Universal Master mix (20 µl), 200 nmol/L each β-actin primer, 100 nmol/L each D-loop primer, and 100 nmol/L each β-actin and D-loop probe primer. The cycling conditions included an initial phase of 2 min at 50°C; followed by 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The fluorescence spectra was monitored by the Light Cyrculer Detection system with sequence detection software version 4 (LightCycler, Roche Diagnostics). The ratio of mtDNA to genomic DNA content was calculated with Ct (mt Ct D-loop – nuclear Ct β-actin), where the mean amount of mtDNA per cell = 2^(-ΔCt), to account for the two copies of the β-actin gene in each cell nucleus (Janice et al., 2004).

Antioxidant enzyme activity assays

Spectrophotometric assay kits were used to measure SOD (SOD Assay Kit-WST, Technical manual-s311), catalase activity assay kit (Northwest Life Science Sepcilities, LLC-CAT01), in liver homogenates.

Statistical analysis

All the results were expressed as mean ± standard deviation (SD). Data was analyzed using one-way ANOVA followed by using Duncan's multiple range
tests using SAS "Statistical Analysis System" Institute, (9.4version). Differences with a P-value <0.05 were considered as statistically significant.

**Result**

**Mitochondrial DNA 4834 bp accumulation and Mitochondrial DNA proliferation in D-gal-treated rats**

By real-time PCR analysis, the proportion (%) of the total mtDNA common deletions and mtDNA content were calculated in hepatic tissues. The results in table (1), were showed significant(P< 0.01) increase in mtDNA CD% and mtDNA copy number per cell of the D-gal-aged rats group (2.127 ± 0.675%, 7057 ±705) respectively as compared to non treated control rats group (0.164 ± 0.059%; 1862± 524) respectively.

The treatments with grasp seeds extract and α-Lipoic acid caused significant decrease (P < 0.01) in hepatic mtDNA*4834*bp deletion % (0.691 ± 0.205%; 0.724 ± 0.195%) as compared with the D-galactose aged non treated rats group(2.127 ± 0.675%). Also the treatments with grasp seed extract and α-Lipoic acid were caused significant decrease (P < 0.01) in hepatic mtDNA copy number (3310± 285,2538± 695 ) as compared with the content in D-galactose induced aging non treated rats group(7057 ± 705).

Hepatic antioxidant enzyme activities of D-galactose induced aged rats and those treated withgrasp seeds extract and α-Lipoic acid were showed in (Table 2).As shown in this table superoxide dismutase and catalaseactivities in hepatic tissues of the D-gal-treated rats group were significantly higher (2.872± 1.722 ; 4.44±0.518) respectively as compared to the control non treated rats group (8.505± 1.829; 11.23± 1.671 respectively). The activity of superoxide dismutase was increased significantly by treatments with both grasp seeds extract and α-Lipoic acid (7.502 ±1.032, 5.786 ±1.257) respectively as compared to its activity in rats of D-galactose induced age group (2.872± 1.722), also catalyse activity was increased significantly by treatments with both grasp seeds extract and α-Lipoic acid(10.12±2.106, 7.92±2.216) respectively as compared to its activity in rats of D-galactose induced age group(4.44± 0.518).

**Discussion**

According to Harman’s free radical theory of aging, ROS continuously generated by the mitochondrial electron transport chain, the main contributors of age-related accumulation of oxidative damage to mtDNA(Harman(1992). Accelerated aging induced by chronic exposure to D-Gal was associated with oxidative stress, overdose of D-Gal will allow aldose reductase to catalyze the accumulated D-Gal into galactitol, which cannot be metabolized but will accumulate in the cell, resulting in osmotic stress and excessive ROS production. Moreover, some studies have indicated that decreased activity of antioxidant enzymes, advanced glycation end-product formation, mitochondrial dysfunction, neurotoxicity and apoptosis are also involved in the accelerated aging of D-Gal-treated animals (Nicklaset *et al.,* 2004; Lei *et al.,*2008).

Mitochondrial DNA (mtDNA) deletion is an important biomarker of ageing, the most frequent and best characterized age-related mtDNA mutation is CD. The results of the current study showed significantly increased levels of the mitochondrial common deletion in liver of rats with D-Gal-induced aging were agree with pervious observation, reported a high frequency of CD has in heart, brain, liver, skeletal muscles, cochlea, andother tissues of aged individuals(Markaryan *et al.,* 2009;Markaryan *et al.,*2006).Mitochondrial DNA deletions can accumulate with aging in postmitotic tissues with high energetic demands, therefore, mtDNA deletions have been considered to represent an important molecular marker during aging. Among numerous deletions, the mitochondrial common deletion (4834 bp in rats) is the most typical and frequent form of mtDNA damage associated with aging(Nicklaset *et al.,* 2004 ;Meisner*et al.,*2008).

The increased in the percentage of the mtDNA deletion of the total mitochondrial sequences for for D-galactose induced aging group in the current results support pervious results of several investigations (Gadalaeta*et al.,1992;Zenet*et al.,*1999; Janice *et al.,*2004; Jose *et al.,*2006;Chen *et al.,*2010;Ameur*et al.,*2011;Zhong*et al.,*2011 and Kazachkova*et al.,*2013. This sub lethal redox stress resulted by such ROS production could lead to loss of damage mtDNA molecules or to deletions or a loss of function by base modification in the mtDNA(Suliman*et al.,*2002; Suekat*et al.,*2003). It has also been hypothesized that mtDNA large deletions could arise by slip-replication, in which replication is stalled by oxidative damage, allowing slipped mispairing between repeated sequences or by erroneous RNA splicing (Fang *et al.,*2007).

The increase in mtDNA copy number in the liver of rats with D-Gal-induced aging in this study suggested that replication of mtDNA is relatively active in the liver.
Table 1. Mitochondrial common deletion and total mtDNA in the hepatic tissue in D-Gal. aged rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>mtDNA CD(%)</th>
<th>mtDNA copy number per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (G I)</td>
<td>0.164 ± 0.059% b</td>
<td>1862 ± 524 b</td>
</tr>
<tr>
<td>D-Gal. group (G II)</td>
<td>2.127 ± 0.675% a</td>
<td>7057 ± 705 a</td>
</tr>
<tr>
<td>D-gal. +GSE(GIII)</td>
<td>0.691 ± 0.205% b</td>
<td>3310± 285 b</td>
</tr>
<tr>
<td>D-gal. +lipoic acid group(G IV)</td>
<td>0.724 ± 0.195% b</td>
<td>2538± 695 b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. different letters are statistically significant (P<0.01).

Figure 1. a-Amount of total mtDNA in the hepatic tissue, b-The copy number of the mitochondrial DNA. Values are expressed as mean ± SD. different letters are statistically significant (P<0.01).

Table 2. Superoxide dismutase (Units/mg protein) and Catalase (Units/mg protein) in the hepatic tissue.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg)</th>
<th>Catalase (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (G I)</td>
<td>8.505± 1.829a</td>
<td>11.23± 1.671a</td>
</tr>
<tr>
<td>D-Gal. group (G II)</td>
<td>2.872± 1.722c</td>
<td>4.44±0.518c</td>
</tr>
<tr>
<td>D-gal. +GSE(GIII)</td>
<td>7.502± 1.032ab</td>
<td>10.12± 2.106ab</td>
</tr>
<tr>
<td>D-gal. +lipoic acid group(G IV)</td>
<td>5.786± 1.257b</td>
<td>7.92± 2.216b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. different letters are statistically significant (P<0.01).
exposed to D-Gal, although the liver is a postmitotic tissue. In addition to mitochondrial base excision repair (BER) deficiency, the relatively active mtDNA replication induced by D-Gal exposure, which probably provides more opportunities for replication errors and clonal expansion of mutation events, may partially explain the increased mutation load in the liver. This result supports the hypothesis of the accumulation of deleted mtDNA molecules in aging and mtDNA copy number increases with age. Deleted mtDNA molecules along with changes caused by lipid peroxidation of mitochondrial membranes might contribute to the overall decline of mitochondrial function.

Nine weeks of treatments with grape seed extract and α-lipoic acid, reduced in relative mtDNA deletion and mtDNA copy number. This reduction suggested that grape seed extract and α-lipoic acid restores mitochondrial function and prevents damage of mtDNA copy number. Grape seed extract and α-lipoic acid are a mitochondria targeted antioxidant playing important roles on eliminating ROS. This results indicate free radicals scavengers and protective hepatic mtDNA in rats tissues by high antioxidant capability in mitochondria.

Under the light of the results, concluded that mtDNA deletions generation are due to oxidative stress and direct DNA damage by accelerates ROS production are the main reason contribute to the overall decline of mitochondrial function in aging tissues. Grape seed extract and α-lipoic acid are a useful anti-aging therapy, especially for controlling oxidative damages, they are considered as a potent protective agent against mtDNA damage by oxidative stress and act as free radicals scavengers.

In the present study, the hepatic antioxidant enzyme levels of superoxide dismutase and catalase showed a tendency toward reduced in D-gal-treated rats, however, superoxide dismutase and catalase concentration increased significantly in the groups.
supplemented with grape seed extract and lipoic acid. These antioxidant enzymes are efficient for protecting tissues and cells from oxidative stress. Anhet et al., (2002) also reported that increased SOD and catalase activities are observed in liver tissue after feeding grape seed extract. It has been observed that α-lipoic acid supplementation of D-galactose aged rats resulted in the positive effect of increasing superoxide dismutase and catalase concentration in liver. These results indicate that the α-lipoic acid treatments to aging-induced rats have a greater effect on elevation superoxide dismutase and catalase concentration in liver. According to the results, grape seed extract and lipoic acid supplementation could lead to more defensive antioxidant enzymatic activities such as increased superoxide dismutase catalase.

References


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