Impact of alcohol on hepatic mitochondrial DNA damage in streptozotocin diabetic rats

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ABSTRACT

Mitochondrial dysfunction with increased production of reactive oxygen species (ROS) is a characteristic feature of diabetes which is associated with damage of mitochondrial DNA (mtDNA). Alcohol metabolism generates ROS with enhanced oxidative stress leading to damage of cellular constituents including mtDNA. The aim of the present study is to investigate the impact of alcohol consumption on hepatic mtDNA damage in diabetic rats. MtDNA was isolated from hepatic tissues of non-diabetic and streptozotocin induced diabetic rats after alcohol treatment. Comprehensive screening of mtDNA displacement loop (D-loop) was carried out by PCR-Sanger’s sequencing analysis. MtDNA deletions were analyzed by long-extension PCR. Furthermore, activities of enzymatic and non-enzymatic antioxidants were measured in hepatic tissue of all rats. Our results showed increased frequency of D-loop mutations in alcoholic-diabetic rats when compared to diabetic or alcoholic non-diabetic rats. DNA mfold analysis predicted higher free energy for 15507C, 15560T and 16116C alleles compared to their corresponding wild alleles which represents less stable secondary structures with negative impact on overall mtDNA function. MtDNA deletions were observed in all experimental groups except controls. Markedly decreased activities of antioxidant enzymes viz., GPx, SOD, catalase and GSH content was identified in alcoholic-diabetic rats when compared to remaining groups. In conclusion, decreased GSH content and lowered activity of catalase, SOD and GPx favor the environment for oxidative stress, which might lead to exacerbation of hepatic mitochondrial DNA damage in diabetic rats receiving alcohol.

KEYWORDS: Diabetes; Alcohol; Antioxidant; Mitochondrial DNA; D-loop; Secondary structure
INTRODUCTION

Diabetes is a complex metabolic disease characterized by defects in insulin production and/or action (Kahn et al., 2014). It is an established cause of liver diseases (Tolman et al., 2007). There has been an increase in global prevalence of diabetics consuming alcohol. It is well known that excessive alcohol consumption leads to liver damage resulting in alcoholic liver disease (ALD) which claims millions of deaths every year (Wong et al., 1998; Chan 2006; Cheng and Kong 2011). Mitochondria play a vital role in cellular ATP production along with several other functions such as fat oxidation and intermediary metabolic processes (Chan 2006; Song et al., 2014). Also it plays an important role in both hepatic alcohol metabolism and bioenergetics of the hepatocyte. Mitochondrial dysfunction is associated with a broad range of pathologies including diabetes, ethanol toxicity, metabolic syndrome, and cardiac failure. Alcohol toxicity induced mitochondrial dysfunction has been reported in diabetes mellitus (Zelickson et al., 2011). Mutations and copy number alterations of mitochondrial DNA (mtDNA) have been linked to the pathogenesis of type 2 diabetes (Kucharska et al., 2000; Rolo and Palmeira 2006). Increased mitochondrial ROS production is likely to damage mtDNA, which may affect overall mitochondrial function (Tan et al., 2013).

Mammalian mtDNA is a circular double-stranded DNA molecule of 16.5kb in size (16.313kb in rats) and encodes for 13 polypeptide components of the electron transport chain (ETC) along with 22 tRNAs and 2 rRNAs (Anderson et al., 1981). MtDNA exhibits higher mutation rate than nuclear DNA and is more vulnerable to oxidative damage due to lack of protective histone proteins, limited DNA repair mechanisms and a high rate of ROS generation. Displacement loop (D-loop), the only regulatory region mtDNA, has been shown to be highly susceptible to attack by electrophilic compounds and inflicted with more oxidative damage compared to other regions of mtDNA (Lee and Wei 2005). Numerous studies have demonstrated mtDNA damage in alcoholics as well as in diabetes (Manzo-Avalos and Saavedra-Molina 2010; Lynch et al., 2011). However, there is paucity of information about the effect of alcohol consumption on mtDNA damage in diabetes. This study investigated the impact of alcohol consumption on hepatic mtDNA damage in diabetic rats with a view to understand whether excessive alcohol consumption leads to exacerbation or amelioration mtDNA damage in diabetes.

MATERIALS AND METHODS

Experimental animals

Six to eight week old (120-140g) Albino Wistar rats (n = 8) were procured from Sri Venkateswara Agencies, Bangalore, India. Animals were maintained on a standard pellet diet (M/s. Hindustan Lever Ltd., Mumbai, India) and water ad libitum with 24 h light-dark cycle in the university animal house. After acclimatization for a week, animals were divided into four groups viz., group-I control (C), group-II alcohol (AL), group-III diabetic (DB) and group-IV alcohol-diabetic (AL+DB). Alcohol (20%) was administered at a dose of 5g/kg b.wt/day for 60 days and streptozotocin (STZ) was administered by a single intraperitoneal injection at a dose of 55mg/kg b.wt. Experimentation and animal maintenance were done with prior approval of institutional animal ethical committee. At the end of experimental period the animals were fasted overnight and sacrificed by cervical dislocation. Livers were collected and used for experimentation.
Induction of diabetes in rats

Diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin (55 mg/kg b.w) (Sigma, St. Louis, Mo, USA) in 0.1 M citrate buffer (pH 4.5) to overnight fasted rats. After 1 week of STZ administration, animals with fasting blood glucose levels >200 mg/dl were considered diabetic and included in this study.

Collection of liver samples and DNA isolation

At the end of 8 weeks treatment period, all the rats were sacrificed by decapitation and liver samples were immediately removed, weighed and homogenized in RIPA lysing buffer, pH 7.4. Total DNA was extracted from liver tissues by a standard extraction protocol using proteinase-K and sodium dodecyl sulphate (SDS) according to methods previously described (Govatati et al., 2014). The extract containing both nuclear DNA and mtDNA, was used for PCR and sequencing analysis without further purification.

Comprehensive screening of mtDNA D-loop

The entire mitochondrial D-loop region (np15416-16313) was screened by PCR-Sanger’s sequencing analysis using specific primers (Table 1) as described earlier (Govatati et al., 2015). PCR amplicons of 432 bp (primer set 1) and 519 bp were sequenced by Taq-Dye deoxy-terminator cycle sequencing kit (Applied BioSystems, Foster City, USA) using an automated ABI-3770 DNA sequencer (Applied BioSyst,ems, Foster City, USA).

The mtDNA sequence of wistar rat (Rattus norvegicus) strain BBDP/Rhw was kept as reference (Acc. No. FJ919760) and all the obtained individual mtDNA sequences were compared with the reference sequence. Sequences were aligned using CLUSTAL-X software and mutations were scored as described earlier (Govatati et al., 2012). Impact of identified mutations on D-loop secondary structures was assessed by DNA mfold web server.

Determination of mtDNA deletions

MtDNA deletions were analyzed by PCR method as described previously (Govatati et al., 2016a) using specific primers (Table 2). Whole mtDNA genome was amplified by long extension PCR using Expand Long Template PCR system (Roche). For whole mtDNA amplification, 25 cycles of primary PCR were followed by 25 cycles of nested PCR. The 1st primers set (primary PCR) amplify a 16,007 bp fragment of rat mitochondrial genome while the 2nd primers set (nested PCR) amplify a 15,708 bp fragment. The PCR amplification products were electrophoresed through a 1 % agarose gel stained with ethidium bromide.

Determination of liver antioxidant status

Liver tissue was homogenized (10% w/v) in tris buffer (0.1M, pH 7.4), centrifuged (10,000 xg for 20 min at 4°C) and the supernatant was used to assess the activities of enzymatic and non-enzymatic antioxidants. Glutathione-s-transferase (GST) was assayed by the method described earlier (Mannervik and Guthenberg 1981). Total reduced glutathione (GSH) content was measured by Ellman’s method (Ellman, 1959) and the activities of glutathione peroxidise (GPx) (Rotruck et al., 1973), catalase (Aebi 1984) and superoxide dismutase (SOD) (Kakkar et al., 1984) were determined. Protein concentration was estimated by the method of Lowry et al. (1951).
Table 1: Primers used for PCR-sequencing analysis of mitochondrial DNA D-loop

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Primer Sequences</th>
<th>Nucleotide location</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: 5’-CACCATCAACACCCAAAGC-3’</td>
<td>15358 - 15376</td>
<td>432 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GGCCCTGAAGTAAGAACC-3’</td>
<td>15771 -15789</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F: 5’-GGTTCTTACTCAGGCCATC-3’</td>
<td>15772 -15792</td>
<td>519 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GTGGAATTTTCTGAGGGTAGGC-3’</td>
<td>16269 -16290</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Primers used for mitochondrial DNA deletion analysis

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Primer Sequences</th>
<th>Nucleotide location</th>
<th>Amplicon size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: 5’-CATCCTCCGTGAAATCAACCCCG-3’</td>
<td>15671-15696</td>
<td>16,007 bp</td>
<td>93°C for 15 s, 62°C for 30 s, 68°C for 15 min, 25 cycles</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TTTGGGTGTGATGTGGGGAGGTAG-3’</td>
<td>15377-15350</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F: 5’-AAGACATCTCGATGTAAACCGGTC-3’</td>
<td>15826-15849</td>
<td>15,708 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 5’-CCAGAGATTGGTATGAGAATGAGG-3’</td>
<td>15233-15209</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mitochondrial DNA D-loop mutations

A total of 8 mutations were identified in the D-loop region of investigated groups (Table 3; Fig 1 and 2). All the identified mutations were single base substitutions (Y or R transitions). Among them, 3 were present in AL and AL+DB groups, 2 were present in DB and AL+DB groups, 1 was present in AL, DB and AL+DB groups and remaining 2 were present in all experimental groups. In overall, 6 were located in the termination associated sequences (TAS, ETAS), 1 was in the central block (CB) and 1 was located in conserved sequence block 3 (MT-CSB3). Interestingly, frequency of D-loop mutations was significantly higher in AL+DB group when compared to other groups.

Effect of mutations on secondary structure of D-loop

To find out the impact of D-loop mutations on its secondary structure conformation, in silico analysis was performed using DNA mfold web server (Fig 1 and 2). Our results showed lesser free energy for 15483G (ETAS1), 15572G (TAS-A) alleles and higher free energy for 15507C (TAS-D), 15560T (ETAS2), 16116C (MT-CSB3) alleles when compared to their corresponding wild alleles. However, for 15529T/C (TAS-C), 15545T/A (TAS-B) and 15779G/A (CB) variants no considerable difference was observed in free energy levels between wild and mutant alleles.
Table 3: Mitochondrial DNA D-loop mutations observed in the present study

<table>
<thead>
<tr>
<th>Locus (Position in D-loop)</th>
<th>Nucleotide position</th>
<th>Ref sequence</th>
<th>Base change</th>
<th>IUPAC code</th>
<th>Status C</th>
<th>AL</th>
<th>DB</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETAS1 (15446-15503)</td>
<td>15483</td>
<td>A</td>
<td>G</td>
<td>R</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>TAS-D (15497-15511)</td>
<td>15507</td>
<td>T</td>
<td>C</td>
<td>Y</td>
<td>✓</td>
<td>×</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>TAS-C (15520-15531)</td>
<td>15529</td>
<td>T</td>
<td>C</td>
<td>Y</td>
<td>✓</td>
<td>✓</td>
<td>×</td>
<td>✓</td>
</tr>
<tr>
<td>TAS-B (15541-15554)</td>
<td>15545</td>
<td>T</td>
<td>A</td>
<td>R</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>ETAS2 (15511-15572)</td>
<td>15560</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>×</td>
<td>×</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>TAS-A (15571-15584)</td>
<td>15572</td>
<td>A</td>
<td>G</td>
<td>R</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>CB (15673-15979)</td>
<td>15779</td>
<td>G</td>
<td>A</td>
<td>R</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>MT-CSB3 (16116-16133)</td>
<td>16116</td>
<td>T</td>
<td>C</td>
<td>Y</td>
<td>✓</td>
<td>✓</td>
<td>×</td>
<td>✓</td>
</tr>
</tbody>
</table>


Mitochondrial DNA deletions
Whole mitochondrial genome from all the investigated groups was analyzed by PCR amplification using Long-extension PCR. Intact wild type mtDNA was observed in control group (C) rats, while rats from remaining three groups (AL, DB and AL+DB) showed large scale (5 kb) mtDNA deletions (Fig 3).

Activities of enzymatic and non-enzymatic antioxidants in liver
Data depicted in Figure 4 shows information on antioxidant status in AL, DB and AL+DB groups in comparison with controls. Decrease in GSH content and activities of other defense enzymes such as GPx, GST, SOD and catalase in AL, DB and AL+DB groups in comparison with controls was observed in this study and the decrease is more pronounced in AL+DB group.

DISCUSSION
Diabetes and ethanol toxicity are associated with increased production of free radicals and/or impaired antioxidant defense system playing a central role in the onset, progression, and pathological consequences of diabetes (Edwards 2013). Alcohol increases ROS generation in hepatic mitochondria leading to multiple hepatic mitochondrial DNA deletions (Zhang et al., 2006) which affects the integrity of mtDNA. Oxidative damage can cause structural modifications of the nucleotide bases by cross-linking which leads to mtDNA mutations and damage. Furthermore, high glucose exposure of cells leads to diminished mtDNA replication and increased mtDNA damage (Sivitz and Yorek 2010). Accumulation of mtDNA mutations/damage may lead to the disturbance of mitochondrial bioenergetics (Raza et al., 2011).
Mitochondrial DNA D-loop, the key regulating site of mtDNA replication and transcription, is highly vulnerable to oxidative damage (Tan et al., 2013). Thus, D-loop sequence alterations/mutations might affect the overall mitochondrial function by altering mitochondrial replication, transcription and/or biogenesis. Numerous studies have reported association of D-loop mutations with the increased risk of several complex diseases (Tipirisetti et al., 2014; Govatati et al., 2013, 2016b). Recent studies have demonstrated that the D-loop region was highly susceptible to attack by electrophilic compounds and inflicted with more oxidative damage when compared with the other regions of mtDNA (Lee and Wei 2005). We are in opinion that alcohol metabolism linked production of ROS may result in an additive effect on mtDNA damage in diabetes. As expected we identified highest frequency of D-loop mutations in alcohol-diabetic group compared to alcohol and diabetic groups individually (Table 3). This could be due to over production of ROS by alcoholic and diabetic stress.
It is evident that DNA secondary structures can influence the molecular processes such as replication, transcription and recombination (Seffens and Digby 1999; Katz and Burge 2003). In general, hairpin or cruciform structures serve as binding sites for several transacting elements (Wright et al., 2003; Hoede et al., 2006). Hence, local intra-strand DNA secondary structures have a key role in replication and transcription processes. As key regulatory site of mtDNA replication and transcription, D-loop mutations can influence overall mtDNA stability. Therefore, we analyzed impact of identified mutations on D-loop secondary structure. Our results showed higher free energy for 15507C (TAS-D) 15560T (ETAS2) and 16116C (MT-CSB3) alleles compared to their corresponding wild alleles (Fig. 1 and 2). Higher free energy represents less stable secondary structures which may have negative impact on overall mtDNA function. The 15507C (TAS-D) and 16116C (MT-CSB3) variants were observed alcoholic rats and 15560T (ETAS2) variant was observed in diabetic rats; but interestingly all the three variants were present in alcoholic-diabetic rats. This could be due to elevated oxidative stress by both alcohol and diabetes. However, further studies are warranted to clarify the underlying molecular mechanisms involved in our findings.
We found lesser free energy for 15483G (ETAS1) and 15572G (TAS-A) alleles when compared to their corresponding wild alleles (Fig. 1 and 2). Lesser free energy represents more stable secondary structures. Interestingly, both of these variants were present in all groups of rats, hence they can be considered as single nucleotide polymorphisms rather than mutations. The remaining 3 variants [15529T/C (TAS-C); 15545T/A (TAS-B) and 15779G/A (CB)] showed no much difference in free energy levels between wild and mutant alleles.

It is well known that oxidative stress is associated with accumulation of mtDNA deletions (Ikushima et al., 2002; Murphy et al., 2005). Large scale mtDNA deletions (5kb) have been detected in various complex diseases including diabetes (Zhang et al., 2006; Tipirisetti et al., 2013; Govatati et al., 2016a). Altered mtDNA replication and/or repair system may be responsible for mtDNA deletions (Krishnan et al., 2008; Sadikovic et al., 2010). In the present study, we identified mtDNA deletions in alcohol, diabetic and alcohol-diabetic groups while control group rats showed no detectable mtDNA deletions. This could be attributed to elevated oxidative stress by alcohol and/or diabetes.

Antioxidant enzymes, SOD, CAT and GPx stand in the first-line of defense against oxidative damage (Maturu et al., 2012). These antioxidants play an important role in scavenging ROS, reduction of hydrogen peroxide and maintaining redox balances in biological system. GSH, an important non-enzymatic antioxidant biomolecule in tissues, is the substrate for GPx and glutathione S-transferase (GST), and also involved in the removal of free oxygen species, such as H₂O₂, superoxide anions, alkoxyl radicals, and maintenance of membrane protein thiols (Padmavathi et al., 2000). Our results revealed reduced GSH content followed by decreased activities of GST, GPx, SOD and catalase in hepatic tissue of AL, DB and AL+DB groups. Interestingly, the effect is more pronounced in alcohol-diabetic (AL+DB) rats.
GSH, a non-enzymatic antioxidant, plays a central role in coordinating the body’s antioxidant defense processes. SOD and catalase remove radical’s in vivo rendering protection to cells and membranes. Decreased catalase activity accounts for less hydrogen peroxide decomposition, consequently the possible overproduction of hydroxyl radicals via fenton reaction. Decreased GSH content and lowered activity of catalase, SOD and GPx favor the environment for oxidative stress which leads to mtDNA damage. Exacerbation of mtDNA damage and excessive oxidative stress in terms of reduced GSH content and lowered activities of defense enzymes in diabetic rats receiving alcohol is evident from the results of present study. Deterioration of anti-oxidantcum defense mechanisms in alcohol diabetic group might result in exacerbation of mtDNA damage. However, further studies are warranted to clarify the underlying molecular mechanisms.

CONCLUSIONS

In the present study we identified exacerbation of mitochondrial DNA damage in STZ diabetic rats receiving alcohol. Decreased GSH content and lowered activity of catalase, SOD and GPx favor the environment for oxidative stress which might lead to exacerbation of hepatic mitochondrial DNA damage in diabetic rats receiving alcohol. To the best of our knowledge, this is the first report demonstrating the effect of alcohol on mtDNA damage in
diabetes. Further investigation is warranted to explore the molecular mechanisms involved in our findings.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest. We wish to disclose that the study was carried out as part of Ph.D. dissertation work of Ms. Swarnalatha Kodidela, and no funds were received either from University or Govt. of India.

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