Abstract. This study investigated mitochondrial DNA (mtDNA) damage in rats with obstructive jaundice (OJ) and to explore its effect on mitochondrial and hepatic function. Forty-eight male Wistar rats were randomly divided into two groups: sham-operated (Sham) and bile duct ligation (BDL). Blood and tissue samples were collected from the two groups on days 1, 4, 7 and 14 following surgery. Hepatic and mitochondrial function were measured. Long and accurate PCR, restriction enzyme digestion and gene sequencing were used to analyze the locations of mtDNA deletions. In addition, quantitative fluorescent PCR was used to measure the relative amounts of total DNA in hepatocytes and mtDNA deletions. Results showed that the hepatic and mitochondrial function was compromised in the BDL group compared to the Sham group. Notably, a novel 11,194-bp mtDNA deletion (nucleotide positions 4101-15294) and fewer mtDNA copies were found compared to the Sham group. With prolonged ligation time, there was a decrease in the copy number, while the ratio of mtDNA deletions to total mtDNA levels increased in the BDL group. These changes were consistent with damage to hepatic and mitochondrial function. A novel 11,194-bp mtDNA deletion and fewer mtDNA copies were detected in hepatocytes of rats with OJ. The mtDNA deletions may therefore be an important factor leading to mitochondrial and hepatic dysfunction.

Introduction

Mitochondria are the only intracellular structures, besides the mammalian nucleus, that possess their own genetic material. Although the mitochondrial genome is small, mitochondrial DNA (mtDNA) self-replicates, controlling the function of the organelle under the direction and regulation of proteins encoded by nuclear genes. Therefore, mtDNA plays a pivotal role in regulating the function of mitochondria. Due to the lack of protective histones, DNA-binding proteins and DNA repair systems, mtDNA has a much higher rate of mutation compared to nuclear DNA (1-3). The majority of alterations are heteroplasmic, as both mutant and wild-type mtDNA coexist within the cells. Mitochondrial dysfunction caused by mtDNA mutations, such as point mutations, insertions and deletions, has been implicated in aging (4,5), mitochondrial respiratory chain disorders such as Kearns-Sayre syndrome (KSS), progressive external ophthalmoplegia, adult-onset diabetes mellitus with deafness, Pearson syndrome (6-9), neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease and Huntington's disease (10), liver cirrhosis (11,12), aggressiveness and tumor progression (13-15).

Biliary obstruction, also known as obstructive jaundice (OJ) and cholestasis, is known to induce numerous pathophysiological changes (16). Bile flow may be impaired anywhere between the liver cell canaliculus and the ampulla of Vater, and may be caused by intrahepatic or extrahepatic factors, such as stones and tumors. Only a few reports are currently available on the mechanism by which OJ affects the morphology and function of mitochondria (17,18). In addition, little is known regarding the correlation between OJ and changes in mtDNA in hepatocytes. Goncalves et al (19) found selective defects in complexes I, III and IV, which were encoded by mtDNA in two siblings who had presented with neonatal cholestasis and early liver insufficiency. Certain investigators (12,20) have found that the mtDNA copy number decreased following bile duct ligation (BDL). We hypothesized that damage to mtDNA results, not only in changes to mtDNA copy number, but also in structural differences that then affect the normal function of mitochondria.

In the present study, we investigated the changes in mtDNA structure and copy number following BDL in rats, and the effects of such changes on mitochondrial and hepatic function. The findings may be helpful in understanding the mechanism of altered hepatic function in OJ.

Materials and methods

Animals and experimental groups. Forty-eight healthy male Wistar rats, provided by the Animal Center of The Third
Military Medical University, weighing 175-330 g, were used for the experiments. All animals were housed in a temperature-controlled room on a normal 12-h light/dark cycle and received a normal diet ad libitum. The animals were fasted for 12 h prior to sacrifice or surgery.

During the surgery, rats were anesthetized lightly with ether. The abdominal skin was washed with soap and sterile water, and then disinfected with iodine tincture. The abdomen was opened through a midline incision. Forty-eight rats were randomized into two groups: i) for BDL, the common bile duct was exteriorized and double ligated close to the liver, and excised just below the confluence of the lobular ducts; ii) for the Sham operation (Sham), the common bile duct was exteriorized, and then the abdomen was closed. Following recovery from anesthesia, each rat was housed in individual cages and received a normal diet ad libitum. Rats were sacrificed by decapitation on days 1, 4, 7 and 14 following surgery.

At each time-point, six live rats were used. Blood samples (3-5 ml) were obtained from the inferior vena cava, and livers were excised and stored in liquid nitrogen at the designated times. The serum was separated by centrifugation and stored at -70˚C until use. All animal procedures were approved by the Animal Care and Use Committee of the Third Military Medical University and conformed to the Guide for the Care and Use of Laboratory Animals obtained from the National Institutes of Health. We ensured that animals did not suffer unnecessarily at any stage of the experiments, whether acutely or chronically.

Analysis of changes in mitochondrial function. To determine the effect of damaged mtDNA on mitochondrial function, a Clark oxygen electrode was used to measure changes in the mitochondrial respiratory control rate (RCR) and the phosphorus/oxygen (P/O) value (21). In addition, the ATP content in the liver was determined using the method of Kimmich et al (22).

Analysis of hepatic function. Following surgery, blood samples were collected from the two groups at each time-point and levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum total bilirubin (TBIL) and albumin (Alb) were measured to assess hepatic function.

Extraction of total liver DNA. Total DNA was extracted from samples (30 mg liver tissue) from all rats in each group according to standard protocols. DNA extraction kits were purchased from Tianwei Biotech Co., Ltd. (Beijing, China). In addition, a search for deletions was performed on rats, according to the manufacturer's instructions.

Localization of mtDNA deletions by amplification of the full-length mitochondrial genome. The entire mitochondrial genome was amplified according to the long and accurate PCR (LA-PCR) method (23). Primers were designed and synthesized according to the full-length sequence of mtDNA (gi: 5835177). The following oligonucleotides were used: the forward primer P1 (nucleotide positions 709-736) was 5'-AGG CAC TAA AGT AAG CAC AAC AAA C-3', and the reverse primer P2 (nucleotide positions 16256-16283) was 5'-TTT CTG AGG GTA GGC AGG TAA AGA GGG T-3'. The product length was 15,575 bp.

A biphasic hot-start amplification was employed using 50 µl PCR gems (Takara LA PCRTM kit). The bottom phase of the PCR mixture (30 µl) contained 23 µl of H₂O, 6 µl of dNTPs (300 µM) and 0.5 µl of P1 and P2 primers (final concentrations were 0.2 µM). A PCR gem™ (the top phase) was melted at 80°C for 5 min and placed on top of the bottom phase. The top phase consisted of 13.5 µl of H₂O, 5 µl of 10X LA PCR™ buffer (Mg²⁺, 2.5 µM), 1 µl of genomic DNA as the target (150 ng) and 0.5 µl of LA Taq™ (2.5 units). The PCR profile consisted of an initial 2 min denaturation at 94°C, followed by two-step PCR for 30 cycles using 20-sec denaturation at 94°C, 15 min annealing/extension at 68°C and a final extension of 10 min at 72°C in a PE2700 thermal cycler (Perkin-Elmer, Waltham, MA, USA). PCR products (5 µl) were directly loaded onto 0.8% agarose gels containing ethidium bromide. Following electrophoresis, bands were visualized by UV light, and the results were recorded with a video camera.

Restriction enzyme digestion. PCR product (10 µl) was digested with restriction endonucleases SacI, Apal (Takara Bio, Kyoto, Japan) and SaeI (Dingguo Biotech Co., Ltd., China) for 90 min at 37°C, electrophoresed through 0.8% agarose gels for 1 V/cm and analyzed under a UV light.

Localization of mtDNA deletions. Primers were designed and synthesized according to the primary location of mtDNA deletions and the full-length sequence of mtDNA (GI: 5835177). The following oligonucleotides were used: the upstream primer P3 (nucleotide positions 3878-3900) was 5'-CCC TTC CCG TAC TAA TAA ATC CA-3', and the downstream primer P4 (nucleotide positions 15668-15690) was 5'-GTT GAT TTC ACG GAG GAT GTG AG-3'. The PCR reaction mixture (50 µl) consisted of 38.5 µl of H₂O, 6 µl of dNTP (300 µM), 0.5 µl of each primer (0.2 µM), 5 µl of 10X LA PCR buffer (Mg²⁺, 2.5 µM), 1 µl of genomic DNA as the target (150 ng) and 0.5 µl of LA Taq (2.5 units). The PCR profile consisted of an initial 2 min denaturation at 94°C, followed by PCR for 40 cycles using 20-sec denaturation at 94°C, 30 sec annealing at 55°C, 1-min extension at 72°C and a final extension of 10 min at 72°C using the GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). Under these conditions, the extension time was too short to synthesize a product of >11.8 kb in length, and no product was generated. If a 1.1-kb mtDNA deletion existed, a DNA fragment of 600-700 bp in length would have been obtained. Following the PCR reaction, the products (10 µl) were electrophoresed through 1.0% agarose gels for 30 min at 5 V/cm and analyzed under UV light.

Quantitation of mtDNA deletions. Real-time quantitative PCR (LightCycler, Roche, Indianapolis, IN, USA) was used to quantify the relative ratio of the specific mtDNA deletion and total mtDNA. Primer pairs were designed, and the following oligonucleotides were used. i) To quantify the deletions, the deletion-specific PCR product was amplified using the primer set P3 and P4. ii) For total mtDNA, the upstream primer P5 (nucleotide positions 2813-2834) was 5'-GGC TAC ATA CAA TTA CGC AAA G-3' and the downstream primer P6 (nucleotide positions 3058-3079) was 5'-TAG AAT TGC GGC GTA CGA AAG G-3'. The amplified fragment length was 267 bp. The primer pair P5 and P6 was directed to the ND1 region of...
the mitochondrial gene, which is a highly conserved sequence. As a result, the PCR product reflects the amount of total mtDNA. iii) For $\beta$-actin, the upstream primer P7 (nucleotide positions 2747-2771) was 5'-ATC CGT AAA GAC CTC TAT GCC AAC A-3', and the downstream primer P8 (nucleotide positions 2900-2923) was 5'-GGC TAC AAC TAC AGG GCT GAC CAC-3'. The amplified fragment length was 177 bp. Each PCR was performed using the SYBR Premix Ex Taq™ reagent (Takara Bio), according to the manufacturer’s instructions (Table I). The PCR mixture (20 µl) consisted of 10 µl of SYBR Premix Ex Taq™ reagent (Takara Bio), according to the manufacturer’s instructions (Table I). The PCR mixture (20 µl) consisted of 10 µl of SYBR Premix Ex Taq, 2 µl of mtDNA as the target (100 ng), 0.4 µl of each primer (final concentrations were 0.2 µM) and 7.2 µl of H$_2$O.

Standard curves for mtDNA (ND1) and $\beta$-actin were generated with normal control rats. Another standard curve for deletion-specific mtDNA was generated from rats 14 days after bile duct obstruction, when liver function was the most severely impaired. Templates in each reaction were diluted 1:100,000, 1:10,000, 1:1,000, 1:100, 1:10 and 1:1. Substrate concentrations were calculated according to the standard curves. The ratio of deletion-specific mtDNA to total mtDNA was determined as the relative amount of deletion-specific mtDNA in hepatocytes. The ratio of total mtDNA to $\beta$-actin was considered to be the relative amount of mtDNA copies in liver cells.

**Statistical analysis.** Variances within the data were determined with the F-test for variance. Based on these results, the Student’s t-test for equal or unequal variance was used to determine the statistical significance between different groups. All tests were two-sided and P<0.05 was considered to be statistically significant. Statistical software SPSS12.0 was used.

**Results**

**Analysis of mitochondrial function.** The mitochondrial function of the BDL group was badly compromised as is observed by the lower RCR, P/O ratio and ATP content in hepatic tissue, compared to the Sham group (P<0.01) at each time-point. This damage became more pronounced as the ligation time increased (Table II).

**Changes in hepatic function.** In the Sham group, there were no marked changes in indices of hepatic function, including ALT, AST, Alb and TBIL. However, in the BDL group, the hepatic function was clearly impaired, as observed by increases in ALT, AST and TBIL values, and the decreases in Alb levels, compared to the Sham (P<0.05) at each time-point. With prolonged ligation time, this damage became gradually more pronounced (Table III).

**Location of mtDNA deletions in hepatocytes of rats with OJ.** In the Sham group, products of ~15.5 kb in length were found, compared to the BDL group, where a small proportion of shorter products were found.
which are considered to be full-length mtDNA. However, in the BDL group, besides the full-length product, additional lower molecular weight products of ~4.4 kb in length were also identified. This appears to be due to an mtDNA deletion that, to the best of our knowledge, has not been reported previously (Fig. 1). Restriction enzyme digestion with SacI resulted in 2.6- and 12.9-kb fragments in the Sham group. However, besides these fragments, an additional fragment of 1.8 kb was detected in the BDL group (Fig. 2A). This result was consistent with restriction enzyme mapping of SacI, meaning these PCR products were full-length or deleted mtDNA. All rats in the BDL group had this mutation at each time-point.

To determine the location of the mtDNA deletion, PCR products were digested with restriction endonucleases ApaI and SauI. Digestion with ApaI resulted in 3.1- and 12.4-kb fragments in the Sham group, whereas in the BDL group, besides these two fragments, a 1.3-kb fragment was found (Fig. 2B). SauI digestion showed that in the Sham group, 1.9-, 3.7- and 9.9-kb fragments were generated. However, in the BDL group, an additional 4.4-kb fragment was identified (Fig. 2C). Comparing the results of SacI and SauI restriction enzyme digestion, the 4.4-kb fragment was not digested by SauI, indicating the absence of the restriction site at nucleotide positions 4390 and the end site is located between nucleotide positions 14954 and 15490.

To determine the accurate location of the mtDNA deletion, primers were designed according to the primary location of the specific mtDNA deletion, and the PCR was performed with a 10-min extension time, which did not result in the generation of the full-length mtDNA. Following PCR amplification, a 600-700-bp fragment was generated in the BDL group, whereas no fragment was generated in the Sham group (Fig. 2D). The amplified PCR product was sent to Biotech Company (Shanghai, China) for sequencing by GeneRay. The result showed that the sequence of the fragment was exactly the same as that of mtDNA at nucleotide positions 3878-4100 and nucleotide positions 15295-15690. These results suggested the presence of a large (11,194 bp) mtDNA deletion in the BDL group, which was located at nucleotide positions 4101-15294.

Quantification of the mtDNA deletion in hepatocytes. In all rats in the BDL group the copy number of the total mtDNA was significantly lower (P<0.01), compared to the Sham group.
at the same time-point (Table IV). With prolonged ligation time, the decrease became more pronounced, and the ratio of deletion-specific mtDNA to total mtDNA in the BDL group increased. The difference was also statistically significant (P<0.01). In the Sham group, no mtDNA deletion was found.

Discussion

mtDNA is a double-stranded DNA ring that is packed into a nucleo-protein complex, known as a nucleoid (24,25). mtDNA lacks introns and encodes 13 proteins necessary for assembly of the respiratory chain, 22 tRNAs and two ribosomal RNAs (26). Due to inefficiencies in the DNA repair system and the intrinsic characteristics of mtDNA, it represents a critical cellular target for damage and is more susceptible to damage, compared to genomic DNA. The main reasons for this observation are: i) due to the lack of protective histones, mtDNA is almost ‘naked’ and is easily affected by external factors. ii) The ratio of lipid to mtDNA is high, leading to preferential accumulation of lipophilic carcinogens on mtDNA. iii) mtDNA is always replicating during the cell cycle, contributing to its poor stability. iv) The stability of mtDNA is constantly challenged by the endogenous production of mitochondrial reactive oxygen species (mtROS), which are generated during normal electron flux through mitochondrial electron transport. Unlike nuclear DNA, mtDNA is located in proximity to mtROS generation sites, which are located within complexes I and III of the electron transport chain (27). In fact, the steady-state levels of oxidatively induced lesions observed in mtDNA may be several-fold higher than those in nuclear DNA (28). Due to the mutagenic nature of many of the ROS-induced lesions, mitochondrial-free radicals are thought to be an important source of mtDNA mutations and DNA instability (5). The proofreading activity of DNA polymerase γ and the formation of a hairpin structure in the region of the tRNA gene cause high rates of mispairing during mtDNA replication (29).

Due to the characteristics mentioned above and the significance of mtDNA in regulating mitochondrial function, various studies (30) have shown that alterations in mtDNA, such as point mutations, insertion mutations and deletions, are associated with aging and many diseases in post-mitotic tissues, including skeletal muscle, heart and brain, all of which are heavily dependent on intact functional mitochondria. However, few studies have been conducted to provide insight into the effect of OJ on mtDNA, particularly on the structure of mtDNA.

We hypothesized that following BDL, the combination of increased ROS generation and impaired antioxidant capacity resulted in mtDNA damage. mtDNA damage may involve various pathways, leading to the generation of a large deletion. Typically, a single cell contains hundreds of mitochondria with multiple copies (2-10) of mtDNA, which are required to maintain normal respiratory function in mitochondria (31). mtDNA mutation-induced pathological consequences in a particular organ or tissue often require a minimum critical amount of damaged mtDNA (32-34). The amount of damaged mtDNA was also measured by real-time quantitative PCR. The results showed, not only a mtDNA deletion, but also a decrease in mtDNA copy number in the BDL group. With increasing ligation time, the damage to mtDNA became increasingly clear as the ratio of damaged mtDNA to total mtDNA in the BDL group increased. This mtDNA damage correlated with worsening hepatic function.

In conclusion, our results have confirmed the existence of a specific 11,194 bp mtDNA deletion located at nucleotide positions 4101-15,294 in hepatocytes of rats with OJ and a decrease of mtDNA copies. Our study gives insight into the correlation between mtDNA deletion and OJ and provides a mechanistic understanding of hepatic function affected by OJ.
This study provides information that may be useful during the perioperative period in protecting and improving impaired hepatic function caused by OJ.

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References