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Simplified qPCR method for detecting excessive mtDNA damage induced by exogenous factors

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Abstract

Damage to mitochondrial DNA (mtDNA) is a meaningful biomarker for evaluating genotoxicity of drugs and environmental toxins. Existing PCR methods utilize long mtDNA fragments (~8–10 kb), which complicates detecting exact sites of mtDNA damage. To identify the mtDNA regions most susceptible to damage, we have developed and validated a set of primers to amplify ~2 kb long fragments, while covering over 95% of mouse mtDNA. We have modified the detection method by greatly increasing the enrichment of mtDNA, which allows us solving the problem of non-specific primer annealing to nuclear DNA. To validate our approach, we have determined the most damage-susceptible mtDNA regions in mice treated *in vivo* and *in vitro* with rotenone and H₂O₂. The GTGR-sequence-enriched mtDNA segments located in the D-loop region were found to be especially susceptible to damage. Further, we demonstrate that H₂O₂-induced mtDNA damage facilitates the relaxation of mtDNA supercoiled conformation, making the sequences with minimal damage more accessible to DNA polymerase, which, in turn, results in a decrease in threshold cycle value. Overall, our modified PCR method is simpler and more selective to the specific sites of damage in mtDNA.

Keywords

Mitochondrial DNA; Oxidative damage; Hydrogen peroxide; Rotenone; RTGR-sequence

1. Introduction

Accumulation of lesions in mitochondrial DNA (mtDNA) due to oxidative and other types of damage is believed to be one of the causes of energy crisis in aging tissues (Harman, 2009). Lesion formation is promoted by the prokaryotic organization of mitochondrial genome, lack of histones, and less efficient than the nuclear one DNA repair system (Bogenhagen, 2012). Since mitochondrial reticulum contains multiple copies of mitochondrial genome, mtDNA lesions can be viewed not only as causes of metabolic

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Conflict of interest

None.

dysfunctions, but as biomarkers of the development of these dysfunctions and can be used to estimate the level of oxidative stress in mitochondria in various tissues.

The classical methods for evaluating the extent of DNA damage, such as Southern blot and high performance liquid chromatography, have a number of limitations. In particular, they require considerable amounts of DNA for analysis (10–50 µg) (Furda et al., 2012). Because of these limitations, many researchers have used long-range PCR (Van Houten et al., 2000; Ayala-Torres et al., 2000; Santos et al., 2006; Chan et al., 2011; Maslov et al., 2013; Czarny et al., 2013; Lehle et al., 2014) to access the levels of DNA damage, based on the assumption that DNA lesions (single-strand breaks, modified bases or their adducts) inhibit DNA polymerase and slow down accumulation of the PCR product. Therefore, the rate of PCR product accumulation would be inversely proportional to the number of damaged DNA molecules (Furda et al., 2012).

Many types of DNA lesions are induced by oxidative stress (Wallace, 2002). Mitochondria could be severely damaged not only by superoxide radical, singlet oxygen, and hydroxyl radical, but also by hydrogen peroxide. H₂O₂-induced DNA impairments are mediated by iron ions that catalyze formation of hydroxyl radicals (*OH) and cause single- and double-strand DNA breaks (Panayiotidis et al., 1999; Barbouti et al., 2001; Hegde et al., 2012). Another type of DNA lesions is thymine modification that leads to the formation of thymine dimers (TT) and thymine glycol (Basu et al., 1989). The eighth position of the purine imidazole ring is most susceptible to oxidative damage – its oxidation causes formation of 8-oxo-7,8-dihydro-2'-deoxyadenosine (8-oxoA) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG) (Wallace, 2002).

Most of these lesions inhibit DNA polymerase activity (Sikorsky et al., 2007) and therefore, can be detected by qPCR. The only exception is 8-oxoG, since amplification efficiencies and Cq values for templates containing a single 8-oxoG were not significantly perturbed, although the presence of two tandem 8-oxoGs substantially hindered amplification (Sikorsky et al., 2004). Numerous studies have shown that 8-oxoG is the most common type of DNA lesions in the nuclear genome (Cooke et al., 2003). A number of works demonstrated that aging is accompanied by accumulation of primarily 8-oxoG in DNA (Sohal et al., 1994; Rattan et al., 1995). In genomic DNA, these lesions are repaired mostly by DNA glycosylases that cleave oxidized purine bases. Thus, formamidopyrimidine DNA glycosylase (FPG), an enzyme specific to 8-oxoG, (Nelson et al., 2014) create breaks in DNA stands at the sites of 8-oxoG and prevents DNA synthesis by DNA polymerase (Maslov et al., 2013).

It is known that mitochondrial antioxidant systems differ in different organs (*e.g.*, liver and brain), mostly in the activity of catalase and its involvement in the protection of mitochondria from H₂O₂ (Andreyev et al., 2015). For this reason, we used isolated brain and liver mitochondria to exclude the effects of cytosolic antioxidant systems, as well as to identify regions of mtDNA that are more vulnerable to damage induced by exogenous H₂O₂ and genotoxic agents, such as rotenone.

2. Materials and methods

2.1. Laboratory animals

Six months-old male C57BL6 mice were used in experiments. The animals were obtained from the Stolbovaya Nursery (Scientific Center for Biomedical Technology, Russia) and housed in plastic cages under standard conditions (25 °C; 12-h light/dark cycle; relative humidity, >40%) with *ad libitum* access to food (type ssniff Spezialdiäten GmbH, Germany) and water. Animal maintenance, injections and sacrifice were performed strictly in accordance with the rules set by Institutional Animal Care and Use Committee of Voronezh State University

2.2. mtDNA isolation from frozen tissue

mtDNA was isolated from frozen brain and liver tissues. Fifty mg of tissue was homogenized in 2 ml of PBS buffer (Invitrogen, USA). The homogenate was centrifuged at 13,000g for 1 min, and mtDNA was isolated with a Plasmid Miniprep Kit (Evrogen, Russia) as recommended by the manufacturer.

2.3. mtDNA isolation from isolated mitochondria

Three hundred mg of tissue was homogenized with a Dounce tissue grinder in 25 ml of the mitochondria isolation buffer containing 220 mM mannitol, 100 mM sucrose, 1 mM EGTA, 20 mM HEPES, 2 mg/ml BSA, pH 7.4. The homogenate was centrifuged at 900g for 5 min. The pellet was discarded, and the supernatant was centrifuged at 10,000g for 10 min. The resulting pellet was resuspended in the mitochondria isolation buffer without BSA and centrifuged at 10,000g for 10 min. The pellet was then resuspended in 1 ml of PBS buffer and divided between two 1.7-ml microtubes. H₂O₂ (500 µM) was added to one of the tubes, and the tubes were incubated for 30 min. mtDNA was then isolated from the treated and control mitochondria with a Plasmid Miniprep Kit.

mtDNA was additionally purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The beads were added to mtDNA solution at a 0.4x (v/v) ratio; the beads with the bound mtDNA were washed twice with 70% ethanol. mtDNA was eluted with 25 µl of 0.1X TE buffer.

The extent of mtDNA enrichment was determined by qPCR as described by Quispe-Tintaya et al. (2013) and calculated using the standard equation $RQ = 2^{-(Cq - Cq_{\text{control}})}$, where 1 was the

Cq value for total DNA isolated from tissue homogenate using Quick-gDNA MiniPrep kit (Zymo Research, USA).

2.4. Quantitative PCR

To validate qPCR as a method for assessing mtDNA damage, mtDNA fragments of different lengths (295, 1326, 2069, 4546, and 9158 bp) were amplified using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) (Table 1) (). The reaction conditions were: 5 min at 95 °C, followed by 35 cycles of 95 °C for 10 s, 61 °C for 30 s, 72 °C for 2–16 min (depending on the fragment length). The reaction mixture contained 0.4 µl of Encyclopolymerase, 2 µl of 10X Encyclo buffer, 0.2 mM of each dNTP (all reagents from Evrogen,

Russia), 1X SYBR Green Master Mix (BioDye, Russia), and 0.5 μ M forward and reverse primers in a total volume of 20 μ l. The linear character of PCR was confirmed using serial mtDNA dilutions (from 1 ng to 1 pg of template DNA); the reaction efficiency was calculated from the Eq. (1) (Yuan et al., 2006):

$$E = 10^{(-1/\text{slope})} \quad (1)$$

After qPCR optimization mentioned above, primers for the detection of lesions were designed. Primers for amplification of nine mtDNA fragments (Table 2) () were designed using the primer3 software (Untergasser et al., 2012). The extent of excessive mtDNA damage which was induced by H₂O₂ or rotenone was estimated using the Cq method:

Cq for the control and experimental (damaged) long fragments was compared to Cq for the control and experimental short fragments. Since the amplified fragments differed in length, the number of mtDNA lesions was calculated per 10 kb of mtDNA using the Eq. (2) (Rothfuss et al., 2010)

$$\text{Lesions per 10 kb} = \left(1 - 2^{-(\Delta \text{long} - \Delta \text{short})}\right) * 10000 \text{ bp} / \text{fragment length, bp} \quad (2)$$

To detect 8-oxoG, 50 ng of mtDNA was incubated in 50 μ l of reaction mixture containing 4 U of FPG (NEB, USA) at 37 °C for 1 h followed by 20 min incubation at 60 °C to inactivate the enzyme. mtDNA fragments were then amplified, and the number of lesions was calculated.

The size of the PCR products was determined by electrophoresis in 2% agarose gel.

2.5. Induction of mtDNA breaks by restriction enzymes

mtDNA was treated with AhoI and BamHI restriction endonucleases (SibEnzyme, Russia) separately, and fragments 1–4 were then amplified by qPCR. Fragment 1 had an AhoI recognition site (ChrM 1206–1211); fragment 2 had a BamHI recognition site (ChrM 3222–3227); fragment 3 had an AhoI recognition site (ChrM 4965–4970) and two BamHI recognition sites (ChrM 3656–3570 and 4274–4279); fragment 4 lacked recognition sites for either AhoI or BamHI.

The number of mtDNA lesions was calculated from the Eq. (2) from the difference between amplification values for the digested and non-digested mtDNA.

2.6. In vivo induction of mtDNA lesions

Mice were injected intraperitoneally with a single dose of rotenone (3.0 mg/kg body weight; Sigma-Aldrich, USA) or vehicle (DMSO). Twenty-four hours after injection, the animals were sacrificed, and their midbrains and cortices were dissected. mtDNA was isolated from tissue homogenates without preliminary mitochondria isolation using Plasmid Miniprep Kit as described in Section 2.2. In the experiments with rotenone, mtDNA was isolated from tissue, not from isolated mitochondria. For this reason the extent of mtDNA damage was

assessed by qPCR amplification of fragments 1, 2, 3, 7, and 9 because these fragments do not have nuclear pseudogenes.

2.7. Statistical analysis

Statistical analysis was performed using statistical variance methods. The results are presented as mean $\pm$ standard error of the mean (SEM). The significance of differences between groups was estimated with the Student's *t*-test; only statistically significant differences ($p < 0.05$) are discussed in this paper. Correlation analysis was performed using the Spearman's rank correlation coefficient (r_s).

3. Results

3.1. mtDNA fold enrichment

The fold enrichment of mtDNA purified from liver and brain tissues was 6.8 ± 1.4 and 12.2 ± 4.2 , respectively (Fig. 2). The fold enrichment of mtDNA purified from liver and brain tissues mitochondria was 52 ± 11.4 and 57.2 ± 6.3 , respectively. We further purified mtDNA isolated from mitochondria with AMPure beads on magnetic stands. This procedure increases the fold enrichment values to 125.7 ± 20.3 for liver mtDNA and 235 ± 10.8 for brain mtDNA (data not presented).

3.2. Optimization of qPCR

Amplification of the 2069-bp mtDNA fragment was characterized by a high degree of linearity ($R^2 = 0.9919$) and over 90% efficiency. Amplification of longer DNA fragments (template DNA, 1 ng to 1 pg) was less linear: $R^2 = 0.9498$ for the 4546-bp fragment and $R^2 = 0.9186$ for the 9158-bp fragment. The amplification efficiency for these fragments was quite low: 64.1% and 33.6%, respectively (Table 1).

3.3. H₂O₂-induced damage of mtDNA in brain mitochondria

Incubation of brain mitochondria with 500 μ M H₂O₂ resulted in a significant damage to mtDNA: 1.44 ± 0.4 , 1.35 ± 0.8 , and 3.18 ± 0.8 lesions/10 kb in fragments 1, 2, and 9, respectively. The number of lesions in fragment 3 was insignificant – 0.33 ± 0.2 lesions/10 kb. In fragments 5, 6, and 7, the number of lesions was even less than in the control (-0.28 ± 0.4 ; -0.15 ± 0.3 , and -0.17 ± 0.8 lesions/10 kb, respectively), although these results were not statistically significant. Additional incubation of mtDNA with FPG increased the number of lesions; however, this increase was statistically significant only for fragment 1 (2.56 ± 0.6 vs. 1.44 ± 0.4 lesions/10 kb in the presence or absence of FPG, respectively). The difference between the average numbers of lesions for all the nine fragments with (0.86 ± 0.4 lesions/10 kb) or without (0.66 ± 0.4 lesions/10 kb) FPG treatment was not statistically significant (Fig. 3).

3.4. H₂O₂-induced mtDNA damage in liver mitochondria

Incubation of liver mitochondria with 500 μ M H₂O₂ also led to mtDNA damage; but it was less pronounced than in the brain mitochondria. Fragments 1, 2, and 9 were most prone to damage (0.65 ± 0.1 , 0.54 ± 0.2 , and 1.74 ± 0.4 lesions/10 kb, respectively); however, the

extent of damage was considerably lower than for the same fragments in the brain mtDNA. The number of lesions for liver mtDNA fragments 4, 7, and 8 was statistically significant (0.12 ± 0.1 , 0.14 ± 0.1 , and 1.2 ± 0.1 lesions/10 kb, respectively) (Fig. 3). The average number of lesions for all the nine fragments was 0.38 ± 0.2 lesions/10 kb.

3.5. Damage of brain mtDNA by restriction endonucleases

The extent of damage revealed by qPCR amplification in the brain mtDNA fragment 3 (containing both BamHI and AhoI) after mtDNA treatment with either of the endonucleases was 5.99–6.3 lesions/10 kb. Amplification of mtDNA fragments with only one endonuclease recognition site (AhoI in fragment 1 and BamHI in fragment 2) or lacking both sites (fragment 4) produced negative values – from -0.3 to -0.6 lesions/10 kb (Fig. 4).

3.6. Rotenone-induced mtDNA damage

Single rotenone injection caused significant damage of the fragments 1, 2, and 9 of mtDNA isolated from the cortex 24 h after injection (1.74 ± 0.34 ; of 1.52 ± 0.24 , and 2.17 ± 0.32 lesions/10 kb, respectively) (Fig. 5). No significant difference in the number of mtDNA lesions from the control was observed for the fragment 3 (0.19 ± 0.41 lesions/10 kb); in fragment 7, the number of lesions was less than in the control (-0.31 ± 0.21 lesions/10 kb) (Fig. 5).

In the midbrain, rotenone damaged mtDNA to a similar extent in all mtDNA fragments studied: 1.70 ± 0.37 , 1.73 ± 0.40 , 1.53 ± 0.41 , 1.21 ± 0.39 , and 0.84 ± 0.28 lesions/10 kb in fragments 1, 2, 3, 7, and 9, respectively (Fig. 5).

4. Discussion

Many studies compare DNA damage in the nuclear and mitochondrial genomes. To ensure full coverage of the nuclear genome, the amplified fragments are usually over 10 kb in length for both nuclear and mitochondrial DNA (Van Houten et al., 2000; Ayala-Torres et al., 2000; Santos et al., 2006; Maslov et al., 2013; Lehle et al., 2014). However, when applied to evaluate the damage of mtDNA, this approach has several flaws. First, the use of only one or two amplicons does not allow to determine, which fragments of the mitochondrial genome are more susceptible to the damage. Second, it makes difficult to estimate the number of lesions, since the method will only register one lesion per 10-kb fragment of a single mtDNA copy, even when this fragment has multiple lesions.

Moreover, we showed that the increase in the amplified fragment length results in the decrease in PCR efficiency, which lowers the sensitivity and the reproducibility of the method. It is believed that the optimal PCR efficiency is between 90% and 110%, and the linearity of the standard curve (R^2) must be greater than 0.980 (Taylor et al., 2010). PCR amplification of the 2069-bp fragment with Encyclo-polymerase well conformed to these parameters, while for the 4546-bp fragment, both the reaction efficiency (64.1%) and linearity ($R^2 = 0.9498$) were considerably lower. Hence, Encyclo-polymerase-catalyzed amplification of mtDNA fragments much longer than 2 kb will be of low efficiency and produce no valid results. For this reason, to determine the extent of rotenone- and H_2O_2 -induced mtDNA damage, we used amplicons that were no longer than 2334 bp. The use of

short amplicons solved the above-mentioned problems and allowed us to considerably shorten the elongation step and to reduce the total reaction duration. On the other hand, using such short fragments (~1 kb) significantly reduced total coverage of the mitochondrial genome. In experiments of Rothfuss et al. (2010), the extent of mtDNA damage was determined by studying less than 25% of the mitochondrial genome. In this work, we used amplicons 1.3–2.3 kb long, which allowed us to cover over 95% of the mitochondrial genome (Fig. 1).

The next key factor is the quality and purity of the used mtDNA. It is known that many large fragments of mtDNA are represented in the nuclear genome as pseudogenes (Malik et al., 2011). Using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Pearson and Lipman, 1988), we have identified mouse mtDNA regions that are duplicated in the nuclear genome (Fig. 6). The highest degree of homology (99.94%) was found for the chromosome 1 fragment Chr1: 24611535–24616188 (NC_000067.6) homologous to the mtDNA region ChrM: 6390–11042 (NC_005089.1). Among other mtDNA fragments duplicated in the nuclear genome as pseudogenes, we should mention the following: ChrM: 4441–7679 homologous (97.36%) to Chr2: 22317430–22320676 (NC_000068.7); ChrM: 12488–15359 homologous (93.18%) to Chr4: 78551799–78554673 (NC_000070.6); and ChrM: 6216–6871 homologous (90.7%) to Chr11: 90538482 – 90539137 (NC_000077.6). In cases of all other analyzed mtDNA fragments, either the level of homology was below 90% or the size of the homologous region was less than 500 bp.

Despite the fact that most of the primers in our study corresponded to unique regions of mtDNA, some amplicons (*e. g.*, fragment 4 and 5) had sequences that were completely homologous to fragments of the nuclear genome. Therefore, we believe that estimation of the extent of mtDNA damage by qPCR of mtDNA fragments using total DNA as a template is incorrect. However, all previous studies have used total DNA to evaluate the mtDNA damage. Since mammalian mtDNA resembles in its properties bacterial plasmid DNA, we used Plasmid Miniprep kit to isolate mtDNA from liver and brain mitochondria, which allowed us to obtain mtDNA preparations with a high enrichment fold (52 ± 13.4 for liver mitochondrial mtDNA and 57.2 ± 6.3 for brain mitochondrial mtDNA). Although further mtDNA purification on AMPure beads significantly increased the fold enrichment; such positive selection might cause elimination of damaged molecules from mtDNA preparations. Therefore, mtDNA purified with AMPure beads can be used for detection of mutations by NGS methods, but not for estimating the extent of mtDNA damage by qPCR. We believe that a 50-fold mtDNA enrichment is sufficient to neglect the theoretical possibility of non-specific annealing of mitochondrial primers onto nuclear DNA.

To induce lesions in mtDNA, isolated brain and liver mitochondria were incubated with 500 μM H_2O_2 for 30 min. According to published data, H_2O_2 damages mtDNA in cell cultures at concentrations ranging from 20 μM to 1000 μM (Rothfuss et al., 2010; Maslov et al., 2013; Czarny et al., 2013; Wang et al., 2016). Mammals have complex enzymatic and non-enzymatic antioxidant protective systems (Andreyev et al., 2015). In particular, isolated brain mitochondria are able to utilize exogenous H_2O_2 at a rate of 8–12 nmol H_2O_2 /min/mg protein (Zoccarato et al., 2004; Drechsel and Patel, 2010; Starkov et al., 2014). The maximal

physiological H₂O₂ concentration of 100 μ M was recorded in the brains of rats subjected to ischemia with subsequent reperfusion (Hyslop et al., 1995).

Fig. 3 showed that fragment 9 that corresponds to the non-coding area of mtDNA, the so-called D-loop, was most susceptible to H₂O₂-induced damage in the brain mitochondria (3.2 ± 0.8 lesions/10 kb). This correlates well the data by Rothfuss et al. (2010) obtained by treatment of human neuroblastoma cells with H₂O₂ and with earlier data showing increased susceptibility for somatic mutations of the mtDNA regulatory region in cancer (Mambo et al., 2003). On the other hand, Rothfuss et al. (2010) demonstrated similar extent of damage in the other three regions of mtDNA, whereas our data show significant variations in the number of lesions in different regions of mtDNA (from 1.44 ± 0.4 lesions/10 kb in fragment 1 to -0.28 ± 0.4 lesions/10 kb in fragment 5). The fragment corresponding to the ND1 and ND2 genes showed much greater susceptibility to the damage in the experiments of Rothfuss et al. (2010) than in our experiments (~ 2 lesions/10 kb vs. 0.33 ± 0.2 lesions/10 kb, respectively) even when the same H₂O₂ concentration were used, therefore, the observed differences could not be gene-specific. And might be explained by the difference in the nucleotide sequences of mtDNA in mouse brain and human neuroblastoma.

Studies of Henle et al. (1999) showed that different DNA segments have different susceptibility to the H₂O₂-induced damage. Specific interactions of Fe²⁺ ions with thymine in the purine–T–G–purine context make the RTGR sequence (where R is A or G) more prone to the Fenton reaction (Henle et al., 1999), as confirmed by the nuclear magnetic resonance studies (Rai et al., 2001). However, recent studies by Lee et al. (2016) showed the correlation between ROS-induced DNA damage and the frequency of occurrence of GTGR (*i.e.*, GTGG and GTGA) sequences, but not of the RTGR sequence.

In the study of Rothfuss et al. (2010), all four fragments had approximately equal contents of the RTGR sequences (0.6%; 0.8%; 0.5% and 0.8%). In our experiments, the RTGR sequences were mostly represented in fragments 1 and 2 (1.2% and 1.3%, respectively), and these fragments displayed the highest numbers of lesions in mtDNA from the H₂O₂-treated mitochondria. However, correlation analysis showed that the number of lesions correlated well the content of the GTGR sequences ($r_s = 0.78$; $p = 0.01$), whereas for the RTGR sequences, the correlation was statistically insignificant ($r_s = 0.53$; $p = 0.07$). Similar correlation between the percentage content of GTGR sequences and mtDNA damage was observed for liver mitochondria ($r_s = 0.9$; $p = 0.002$). Our data are more consistent with the results obtained by Lee et al. (2016), than with the earlier data of Henle et al. (1999). However, the mechanism of the damage of specific nucleotide sequences described by Henle et al. (1999) well explains our findings.

The amplified fragment of the D-loop had the highest content of GTGR sequences (0.4%); however, the high extent of mtDNA damage could not be explained by this fact only. In the experiments of Rothfuss et al. (2010), the content of GTGR sequences in the D-loop fragment was 0.2% (*i.e.*, similar to that in other fragments), but the extent of DNA damage was much higher. This could be related to the fact that the D-loop has a very complex structure and includes the initiation and termination sites for transcription and translation. This noncoding region also contains the light strand promoter (L-strand promoter, LSP), the

heavy strand promoter (H-strand promoter, HSP), the origin of replication (OR), and conserved sequences involved in the translation and transcription termination (termination-associated sequence, TAS) (Holt et al., 2007). Formation of the D-loop between OR and TAS is accompanied by hybridization of the 7S DNA (Pohjoismäki and Goffart, 2011). The resulting triple-strand structure is presumably more susceptible to various types of damage. These data are consistent with the studies of point mutations in brain mtDNA. Brain mtDNA often displays heteroplasmy in the non-coding region of the D-loop, which might be related to a high frequency of somatic mutations (Cantuti-Castelvetri et al., 2005), and somatic mutations can be caused by mtDNA damage. Despite the fact that most of these mutations are lethal and suppress mtDNA replication, some lesions (such as 8-oxoG and 8-oxoA) are potentially mutagenic and can lead to the accumulation of somatic mutations (Wallace, 2002). In our experiments, 8-oxoG constituted less than 25% of all mtDNA lesions induced by treatment of brain mitochondria with 500 μ M H₂O₂.

The negative number of lesions in mtDNA fragments 5, 6, and 7 was surprising. An insignificant extent of mtDNA damage might be explained by the low content of GTGR sequences in these fragments (0.05%, 0.1%, and 0.1%, respectively). However, low GTGR content does not explain the obtained negative values. It is possible that the reasons for the “reduced” damage are changes in the conformation of the supercoiled mtDNA induced by its exposure to deleterious factors. In cells, mtDNA exists in various topological configurations: a supercoiled configuration with an average of 100 negatively superhelical turns, relaxed circular configuration, and linear configuration (Chan and Chen, 2009). It is known that oxidative stress, including one induced by H₂O₂, not only damages individual bases, but can also initiate the relaxation of the supercoiled mtDNA (Chen et al., 2007). We assume that H₂O₂ causes significant mtDNA damage in the GTGR-enriched regions that leads to the formation of single-strand breaks. The presence of single-strand breaks results in the inhibition of DNA polymerase, raises PCR threshold and, therefore, increases the calculated number of lesions per 10 kb. However, single-strand breaks also cause relaxation of the supercoiled mtDNA, making it more accessible to DNA polymerase in the regions, where the damage is minimal. This decreases the PCR threshold, which results in the appearance of negative calculated values for the number of lesions per 10 kb, as compared to the control (Fig. 3).

We tested this hypothesis in a set of control experiments. The same mtDNA was treated with restriction endonucleases AhoI and BamHI, which significantly inhibited polymerase activity when the amplified fragments contained recognition sites for these enzymes (~6 lesions/10 kb). Fragments that lacked the enzyme recognition sites exhibited a decreased number of lesions (−0.3 to −0.6 lesions/10 kb) (Fig. 4). Both cleavage and H₂O₂-induced damage of supercoiled mtDNA cause its relaxation and increase the accessibility of undamaged mtDNA regions to polymerases. For this reason, some researchers used restriction endonucleases to increase the efficiency of mtDNA amplification by PCR (Maslov et al., 2013).

Treatment with FPG showed no significant elevation in the number of lesions induced by H₂O₂, with the only exception of fragment 1, for which the number of lesions increased by 78% ($p < 0.05$). There was a trend toward the increase in the extent of damage in fragment 7,

but the data were statistically insignificant ($p = 0.12$). These results confirm that peroxidation causes a broad spectrum of lesions in mtDNA.

mtDNA from liver mitochondria was less susceptible to the damaging effect of H_2O_2 . However, statistical analysis of the average number of lesions in brain and liver mtDNA (fragments 1–9), revealed no statistically significant difference between the tissues (0.66 ± 0.4 lesions/10 kb in the brain and 0.38 ± 0.2 lesions/10 kb in the liver; $p = 0.1$), due to a large variability in the number of lesions in brain mtDNA fragments 6, 7, and 8 caused by the relaxation of the supercoiled mtDNA (see above). The difference between the extent of damage in fragments 1, 2, and 9, which are most susceptible to the damage, was statistically significant (1.99 ± 0.6 lesions/10 kb in the brain vs. 0.96 ± 0.4 lesions/10 kb in the liver, $p < 0.05$). The lesser number of lesions induced by exogenous H_2O_2 in liver mtDNA was probably due to the properties of the antioxidant system in liver mitochondria. Liver mitochondria are characterized by the highest known catalase activity (825 ± 15 U/mg protein) (Salvi et al., 2007), while the catalase activity in brain mitochondria is considerably lower (72 ng/mg, or 1.8 U/mg protein) (Andreyev et al., 2015).

In addition to studying mtDNA in isolated mitochondria, we examined the *in vivo* effect of genotoxic agents on mtDNA. Previous studies by Sanders et al. (2014) showed the rotenone induced accumulation of mtDNA structure lesions in midbrain dopaminergic neurons, but not in cortical neurons, where in the number of lesions decreases. Here, we demonstrated that rotenone-induced mtDNA lesions in the cortex are unevenly distributed along the mtDNA sequence and concentrate in GTGR-rich regions and in the D-loop. The other mtDNA regions display the same, or even lower numbers of lesions than the controls (Fig. 5). Our results are consistent to a certain extent with the data of Sanders et al. (2014), who observed negative values for the number of lesions, likely because they estimated the extent of mtDNA damage using a single amplicon that contained no D-loop, a region that is especially susceptible to mtDNA damage. This, in its turn, could lead to the disruption of the supercoiled mtDNA and to false decrease in the number of lesions by the mechanism described above. Similarly to Sanders et al. (2014), we also observed significant mtDNA damage in the midbrain caused by a single injection of rotenone, the extent of damage being similar in all analyzed mtDNA fragments.

5. Conclusions

Assessment of mtDNA lesions by qPCR might become a useful tool for studying oxidative stress and development of pathologies associated with disturbances in mitochondrial metabolism. Optimization of qPCR for its application in mice, which serve as main model subjects in numerous pharmacological studies, in combination with new generation sequencing methods, will help to assess the damage of particular regions of mtDNA in trials aimed to evaluate possible mutagenic effects and safety of newly developed drugs.

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Abbreviations

mtDNA	mitochondrial DNA
8-oxoG	8-oxo-7,8-dihydro-2'-deoxyguanosine
FPG	formamidopyrimidine DNA glycosylase
HSP	H-strand promoter
OR	origin of replication
TAS	termination-associated sequence

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Fig. 1.

Schematic view of covering of mtDNA genes by 9 PCR fragments. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1) ChrM: 298–2036; 2) ChrM: 2078–3403; 3) ChrM: 3318–4992; 4) ChrM: 5784–7517; 5) ChrM: 7858–9763; 6) ChrM: 9895–11856; 7) ChrM: 11775–13717; 8) ChrM: 13650–15381; 9) ChrM: 15361–369. Black lines indicate PCR products corresponding fragments. Blue line corresponds to non-coding D-loop region. Orange lines correspond to ribosomal RNA genes. Red lines correspond to subunits I complex genes. Green line corresponds to subunit III complex gene. Yellow lines correspond to subunits IV complex genes. Dark blue lines correspond to ATPase genes. Grey lines correspond to 22 tRNA.

Fig. 2. Fold enrichment of brain and liver mtDNA over nuclear DNA using two different approaches. Dark columns, extraction of mtDNA from frozen tissue; Light columns, extraction of mtDNA from isolated mitochondria.

Fig. 3. Number of lesions per 10 kb mtDNA induced by 500 μ M H_2O_2 for each of the nine fragments. Dark grey column shows the number of lesions for brain mtDNA. Light gray column shows the number of lesions for brain mtDNA incubated with FPG, which induced additional strand-break in 8-oxoG. White column shows the number of lesions for brain mtDNA.

Fig. 4.

Number of lesions per 10 kb mtDNA induced by AhlI and BamHI restriction endonucleases. Fragment 1 had an AhlI recognition site; fragment 2 had a BamHI recognition site; fragment 3 had recognition site for both restriction endonucleases; fragment 4 lacked recognition sites for either AhlI or BamHI.

Fig. 5.
Number of lesions per 10 kb mtDNA induced by single intraperitoneal injection of rotenone.
Dark column shows the number of lesions for cortex, light column for midbrain.

Fig. 6. Duplication of the mitochondrial genome in the nuclear genome. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The query representing the mouse mitochondrial genome. The red lines correspond to genomic pseudogenes. Yellow lines correspond to 9 fragments that were assessed in this paper.

Table 1

Optimized mtDNA qPCR primer set.

		PCR product	Length fragment	PCR efficiency, %	Linearity, R ²
ChrM: For.	ACGAGGGTCCAACTGTCCTTA				
ChrM: Rev. 1	TAGGGTAACTTGGTCCTCGTTGAT	2078–2372	295 bp	99.7	0.9956
ChrM: Rev. 2	CCGGCTGCGTATTCTACGTT	2078–3403	1326 bp	92.2	0.9974
ChrM: Rev. 3	TAGTTGAGTACGATGCCAGGA	2078–4146	2069 bp	90.1	0.9919
ChrM: Rev. 4	GCCCAGGAAATGTTGAGGGA	2078–6623	4546 bp	64.1	0.9498
ChrM: Rev. 5	TGGCTATAAGTGGGAAGACCAIT	2078–11235	9158 bp	33.6	0.9186

Table 2

qPCR primer set.

	Sequence	Sequence	PCR product	Length fragment
ChrM: For. 1	TAAATTTCTGTCCAGCCACC	ChrM: Rev. 1 (short)	298–369	72 bp
		ChrM: Rev. 1 (long)	298–2036	1739 bp
ChrM: For. 2	ACGAGGGTCCAACTGCTCTTA	ChrM: Rev. 2 (short)	2078–2174	97 bp
		ChrM: Rev. 2 (long)	2078–3403	1326 bp
ChrM: For. 3	CTAGCAGAAACAAACCGGGC	ChrM: Rev. 3 (short)	3318–3403	86 bp
		ChrM: Rev. 3 (long)	3318–4992	1675 bp
ChrM: For. 4	GGCGGTAGAAGTCTTAGTAGAGAT	ChrM: Rev. 4 (short)	5184–5319	136 bp
		ChrM: Rev. 4 (long)	5184–7517	2334 bp
ChrM: For. 5	AACATTCCCACTGGCACCTT	ChrM: Rev. 5 (short)	7858–7965	108 bp
		ChrM: Rev. 5 (long)	7858–9763	1925 bp
ChrM: For. 6	ACCTCACCATAGCCTTCTCAC	ChrM: Rev. 6 (short)	9895–9982	87 bp
		ChrM: Rev. 6 (long)	9895–11856	1960 bp
ChrM: For. 7	TCATTCTTCTACTATCCCCAATCC	ChrM: Rev. 7 (short)	11775–11856	81 bp
		ChrM: Rev. 7 (long)	11775–13717	1942 bp
ChrM: For. 8	CCCCAATCCCTCCTTCCAAC	ChrM: Rev. 8 (short)	13650–13717	68 bp
		ChrM: Rev. 8 (long)	13650–15381	1732 bp
ChrM: For. 9	AAGAAAGGAGCTACTCCCAAC	ChrM: Rev. 9 (short)	15361–15499	139 bp
		ChrM: Rev. 9 (long)	15361–369	1308 bp