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HYDROGEN PEROXIDE-INDUCED MITOCHONDRIAL DNA DELETION IN *DANIO RERIO*

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Abstract

Oxidative stress has profound effect on mitochondrial DNA (mtDNA) and could cause mitochondrial dysfunction if remains unrepaired. Here, we showed that 20 μM H_2O_2 stress over 96 hours could induce mtDNA deletion in *Danio rerio* model. Although the fishes did well upon such a challenge with an LC_{50} value of 257.494 ± 19.434 μM, deletion in their egg mtDNA was observed but degradation was observed for the brain mtDNA. The water quality was not altered with the addition of 20 μM H_2O_2. We have successfully identified a deleted region of the *Danio rerio* mtDNA. The estimated size of the deletion was 7124 bp and was observed in both egg and brain mtDNA. We deduced that such deletion could have occurred from a recombination between an inverted repeat pair of *Danio rerio* mtDNA, and a fusion protein composed of cytochrome c oxidase II and cytochrome b could be generated upon such deletion. Such fusion protein could lead to mitochondrial dysfunction further.

Introduction

Mitochondrial activities are governed by both nuclear and mitochondrial genomes and a loss in the mitochondrial genome can affect the normal physiological roles of mitochondria(1). These include disruption in oxidative phosphorylation to produce energy and to regulate the apoptotic pathway(2). Mitochondria are one of the major cellular locations where reactive oxygen species are generated in the electron transport chain in normal physiological condition. Therefore, mitochondria are in constant challenge from oxidative stress and mitochondrial DNA (mtDNA) is prone to more oxidative damage as compared to the nuclear DNA(3). Most of these damages include the oxidation of bases, specifically

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the generation of 8-oxodG and these damaged bases are first excised by 8-oxodG DNA glycosylase 1 (OGG1) as a part of the base excision repair in DNA single-strand break repair pathway[4]. However, when the frequency of 8-oxodG increases, the process fails to cope up and multiple pathways engage to save the cell that could ultimately lead to mtDNA deletion or degradation[5]. Age-related and oxidation-related mtDNA deletion has been described previously and the size of such deletion could range from 5-12 kb[6,7]. Deletions in the mtDNA allow mtDNA rearrangements and could produce fusion genes. Previous reports have detected transcripts of these fusion genes, specifically the fusion product of cytochrome c oxidase II and cytochrome b[8]. However, such fusion transcript may fail to produce any detectable protein though stable fusion proteins were detected in cells containing around 60% or less mutated mtDNA as compared to the wildtype mtDNA[9].

Mitochondrial functions seemed unaffected in heteroplasmy, as the wildtype mtDNA sufficiently produces complexes associated with electron transport chain[10]. Thereby, nonsense-mediated mRNA decay for the fusion gene transcript could be promoted. Since the deletion reduces the size of an mtDNA, the deleted mtDNA is preferentially amplified increasing the copy number of deleted mtDNA inside a mitochondrion, and the propagation advantages of the deleted mtDNA over wildtype mtDNA follow a stable equilibrium state[9]. As the portion of deleted mtDNA increases, more fusion transcripts are generated and eventually reduce the counts of oxidative phosphorylation complexes. On top of this, stability of the protein products from the fusion transcript is crucial as some of these proteins are stable but others are not based on the size and location of the deletion and on the proportion of amino acids contributed by the fusion partners[8,9]. When the protein is stable, such protein could affect the whole respiratory function of an individual mitochondrion. Thus, the cells with mitochondria enriched with deleted mtDNA showed a drastic reduction in oxygen consumption and growth impairment[8]. Nonetheless, mitochondria containing deleted mtDNA would try to fuse with mitochondria with wildtype mtDNA to follow homologous recombination to correct the error. If the error is not correctable, the deleted mtDNA would accumulate further mutation and deletion resulting in mtDNA degradation[4,6]. Hence, it is likely that the mtDNA would first accumulate mtDNA deletion and failure to repair such damaged DNA would lead to mtDNA degradation.

In freshwater bodies, few oxidants (< 1 μM) like H_2O_2, superoxides and other free radicals are originated from the combined effect of solar irradiation and chromophoric organic matters[11]. Thereby, *Danio rerio* have antioxidant systems strong enough to encounter such oxidative stress as they have free-radical scavenging antioxidant systems[12]. Previous report showed that 100 μM H_2O_2 for a week altered the expression of apoptosis- and antioxidant-associated genes in *Danio rerio* embryos and these embryos suffered from developmental disorders. Nonetheless, H_2O_2 is considered as a deleterious molecule and the fish mtDNA is supposed to produce some mtDNA deletion prior to mtDNA degradation upon prolonged H_2O_2 treatment. However, mtDNA deletion from oxidative stress in the model organism *Danio rerio* has been described poorly. This is mostly due to the fact that fish mtDNA is prone to degradation upon oxidative stress[13]. Here, we showed the fate of *Danio rerio* mtDNA exposed to secondary oxidative stress inducer (H_2O_2) at 20 μM for 96 hours. Our study indicated that the

increased oxidative stress could affect the normal fish physiology as they accumulate mtDNA deletion and degradation. Deleted mtDNA could serve as an oxidative stress marker in fishes.

Materials and Methods

Collection and maintenance of fish: Five-months-old female *D. rerio* were purchased from local hatchery and were reared in a circulatory system at a maximum density of 6 fishes per litre. Fishes were kept for two weeks for acclimatization before using in the experiments and were maintained in clean tanks with continuous aeration. Fishes were fed twice per day with micro-pelleted feed collected from local market and the water was changed every day. Fishes were carefully handled to minimize any stress.

H_2O_2 treatment of D. rerio: Ten fishes were selected for each group of treatment or control. All fishes were kept starved before 12 hours of the experiments and during the experiments. They were either kept untreated (control) or treated with various concentrations of H_2O_2 ranging from 20-100 μM for 96 hours. Water with or without peroxide was changed every 6 hours during the experiments. The behaviour and survival of fishes were monitored and recorded during exposure to H_2O_2. LC_{50} was calculated by probit model[14]. The water quality (pH, temperature and DO) was monitored daily with standard protocol.

Enrichment of mitochondrial fraction: Ten female fishes were exposed with or without 20 μM H_2O_2 for 96 hours. Brains and eggs were collected after such exposure. Mitochondrial fraction was enriched with methods described previously, with some modifications[15,16]. Briefly, a tissue sample was minced coarsely using sterile scissors in 500 μl of cold homogenization buffer (0.32 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4). Then the lysate was prepared on ice by passing through a 21-gauge blunt needle with a syringe for 10 times followed by 10 more times with 26-gauge blunt needle. The lysate was collected using a cell strainer (40 μm) and centrifuged at 1,000 x g for 5 minutes at 4°C to remove nuclei. The supernatant was prepared centrifuged at 14,000 x g for 15 minutes at 4°C to obtain the crude mitochondrial pellet. The pellet was further washed with ice-cold homogenization buffer twice by the differential centrifugation process to remove any nuclei or genomic DNA.

Mitochondrial DNA extraction: Mitochondrial DNA was extracted from crude mitochondrial pellets with phenol-chloroform-isoamyl alcohol method[17]. Briefly, the mitochondrial pellet was suspended with 500 μl of lysis buffer (100 mM Tris HCl, 5 mM EDTA, 400 mM NaCl, 2% SDS, pH 8.5) and incubated at 55°C in a heat block for an hour. Then an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) was added to the sample and was mixed properly. The sample was then centrifuged at 13,000 rpm for 15 minutes at 4°C and the aqueous layer was collected. An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added to the collected aqueous layer and was centrifugation again at 13,000 rpm for 15 minutes at 4°C. Then the upper layer was collected, and DNA was precipitated with equal amount of isopropanol. The precipitated DNA was washed with 70% ethanol and dissolved in PCR-grade water. A 200 ng of DNA was resolved in a 1% agarose gel followed by visualization of mtDNA band with ethidium bromide under a UV-transilluminator.

Polymerase chain reaction: Extracted mtDNA was amplified by PCR using forward primer 5′-CTATACATTATCATCGCAATAG-3′ and reverse primer 5′-TAGTTTAATTTAGAATCCTGGCTTTG-3′ that amplifies 8345 bp covering a mutation hotspot[18]. Briefly, 20 μl of PCR reaction was set up in a PCR tube consisting of 50 ng of template, PCR master mix, PCR-grade water and 1 μl of each forward and reverse primers at 25 μM final concentrations. Then the tubes were placed in a thermocycler and amplified at 95°C for 5 minutes followed by 40 cycles of 95°C for 30 seconds, 45°C for 30 seconds and 72°C for 5 minutes, followed by a final extension at 72°C for 10 minutes. The PCR product was resolved in a 1.5 % agarose gel followed by visualization under UV-transillumintaor with ethidium bromide staining.

Bioinformatics analysis: Repeats in *D. rerio* mtDNA were searched using RepFinder (http://skl.scau.edu.cn/repfinder) using the sequence NC_002333 retrieved from the NCBI. Both direct and inverted repeats were considered and the minimum length for each repeat was 10 bp. The minimum gap between the repetitive sequences was considered as 6 kb. Structures of all proteins were deduced with Phyre2[19].

Results and Discussion

Overburden of 8-oxodG from excessive oxidative stress results in disproportionate base excision repair that ultimately causes mtDNA degradation[13]. Also, oxidant-induced oxidative stress causes rapid deletion of mtDNA, sometimes such deletion can cover up to 8 kb[7]. H_2O_2 is one of stable environmental reactive oxygen species that is generated in fresh water from chromophoric dissolved organic matter through solar irradiation[11]. Thereby, we hypothesized that oxidant, like H_2O_2, could induce mtDNA deletion. We induced oxidative stress in *D. rerio* with different concentrations of H_2O_2 for 96 hours and examined the mtDNA deletions in these fishes. Eggs and neurons are two cell types that have a higher mitochondrial content and mtDNA copy number compared to other cell types[10]. The average weight of selected fishes was 0.886 ± 0.13 g and the length was 4.1 ± 0.3 cm.

The concentration of H_2O_2 has been reported to reach up to 0.5 μM in a shallow freshwater body and such concentration can affect the normal physiology of fish, despite algae and bacteria present in the freshwater can deplete the generated H_2O_2[11]. The concentration of H_2O_2 can be 100 times higher in the cellular environment or when combined with the endogenous H_2O_2[11,20]. Thereby, we tested the effect of H_2O_2 at different concentrations on fish. Since water quality is an important aspect for the fitness and wellness of fish[21], the physico-chemical properties such as temperature, DO and pH of water was monitored for all concentrations of H_2O_2 during the 96 hours of experimental period (Table 1).We did not observe any drastic changes in these parameters during our experiments. Fish behaviors of exposed and non-exposed fish were monitored during the experiment. No unusual behavior of fish was observed during this experiment and no fish death was observed at 0, 20, 50 and 100 μM concentration during 96 hours exposure. Nevertheless, during the 96-hour treatment with H_2O_2, a total of 1, 2, and 8 fish out of 10 died at concentrations of 200 μM, 250 μM, and 275 μM, respectively. After probit analysis, the 96 hours LC_{50} value

was estimated as 257.494 ± 19.434 μM (95% confidence interval). This data indicated that *D. rerio* might have the capacity to tolerate H_2O_2 originated from environmental or external sources[22]. H_2O_2 at higher concentrations could have a rapid effect leading to apoptosis eventually leading to fish death[12]. Next, we sought to investigate the physiological or molecular effect of H_2O_2 at lower concentrations on *D. rerio*.

Table 1. Physico-chemical properties of water during the experiment

Physico-chemical properties	Mean ± SD
Temperature (°C)	28.50 ± 0.21
DO (mg/L)	5.70 ± 1.30
pH	7.56 ± 0.23
LC_{50} (μM)	257.494 ± 19.434

To justify the molecular effect of H_2O_2 on fishes at lower concentrations, we collected eggs and brain samples of fishes and isolated mitochondria from these samples. A previous report detected a rapid 3.8 kb deletion in mtDNA of rat liver exposed to oxidant at milder concentration (20 μM)[7]. Other reports suggested rapid degradation of mtDNA upon treatment with oxidants like H_2O_2[13]. We detected multiple deletions in mtDNA of eggs and complete degradation of mtDNA of neurons when *D. rerio* fishes were treated with 20 μM H_2O_2 for 96 hours (Fig. 1). Sizes of egg mtDNA deletions ranged from 5-12 kb, indicating massive mtDNA damage. Oxidative stress in neurons is usually higher as compared to eggs and thus such endogenous stress could combine with the exogenous stress, resulting in sufficient oxidative insult to degrade the mtDNA due to excessive base excision repair[23,24]. Nonetheless, unrepaired single-strand breaks in mtDNA could result in apoptosis and DNA degradation is promoted during apoptosis[25]. Therefore, our data suggests that the oxidative stress from 20 μM H_2O_2 over 96 hours could cause mtDNA damage sufficient enough to induce persistent single-strand DNA break that could lead to mtDNA deletion or degradation. Such mtDNA deletion or degradation could have profound physiological effects on fish as mtDNA deletion was found to be associated with aging.

Next, we wanted to deduce any specific mtDNA deletion in fish exposed to 20 μM H_2O_2 for 96 hours. For this, we designed a primer set flanking a mutational hotspot of fish mtDNA (8244-16589 bp) and the PCR would amplify an 8345 bp region of the wildtype mtDNA. To enhance the amplification from deleted mtDNA, we reduced the amplification time to 5 minutes which would prevent the amplification of full-length amplicon of the mtDNA and would preferentially amplify the deleted mtDNA. Thereby, if any mtDNA deletion resides within the primer flanking region, this would generate a smaller amplicon which would be detected on agarose gel after PCR. In fact, we detected a band close to 1200 bp after the PCR in both egg and brain samples (Fig. 2). The intensity of the PCR band from egg mtDNA was brighter whereas the intensity of the PCR band from brain mtDNA was fainter though equal amount (10 μl) was loaded in both cases; indicating a small portion of brain mtDNA was intact or partly degraded but harboured the deletion region. The

deletion harbouring brain mtDNA might experience severe oxidative damage leading to complete mtDNA degradation. Nonetheless, the mitochondria could repair the damaged DNA or replicate the remaining wildtype mtDNA to recover the loss if the oxidative stress is reduced close to normal conditions[3].

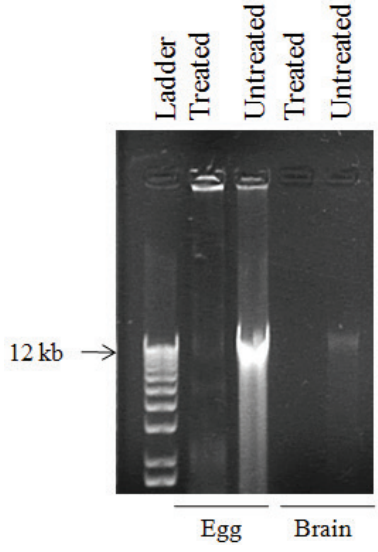

Fig. 1. Detection of mtDNA deletion in *D. rerio* egg and brain. After exposure to 20 μM H_2O_2 for 96 hours, DNA from mitochondria enriched fractions was visualized by agarose gel electrophoresis.

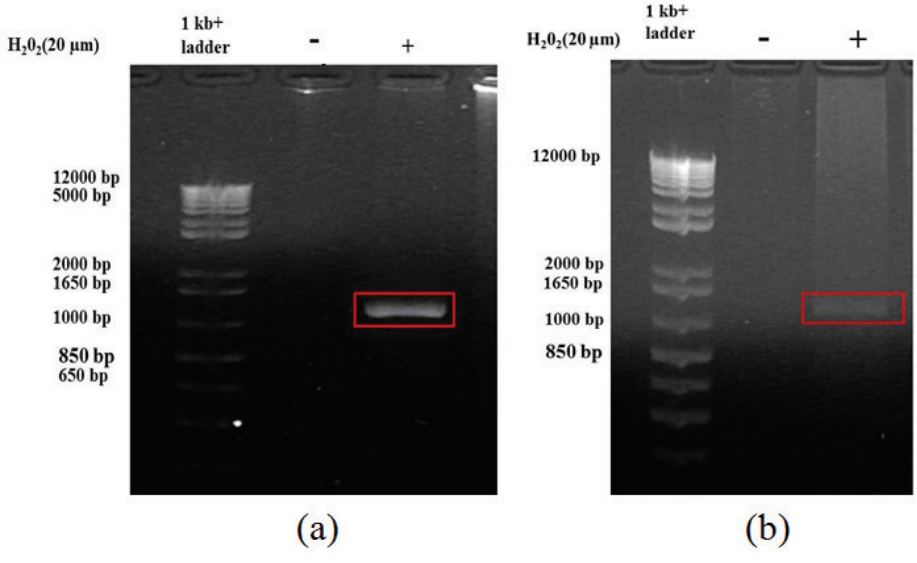

Fig. 2. Deletion in mtDNA of *D. rerio* (a) egg and (b) brain. Fishes were treated with or without 20 μM H_2O_2 for 96 hours and PCR was done with extracted mtDNA. Red boxes indicate the band originated after mtDNA deletion due to a shortened amplicon.

Next, we purified the PCR band for sequencing; however, the sequencing reaction failed even though we repeated the process several times. This finding indicates that the PCR band might contain multiple amplicons and required topoisomerase-based cloning of each fragment into a cloning vector followed by sequencing. Due to funding limitations, we could not follow this process, but we did look for repeats within the amplicon region to justify our results (Table 2). During excessive oxidative damage, a desperate DNA repair process like homologous recombination or micro-homology mediated end joining could result in deletion of DNA flanked by direct or inverted repeats[6]. The size of the repeats could vary from 6-20 bp, and we found at least 67 direct repeats and 51 inverted repeats of sizes ≥10 bp within the region of our amplicon. Therefore, recombination between these repeats could cause massive deletion of mtDNA, specifically within the region we amplified. One of these repeats includes an inverted repeat located at 8454-8463 and 15596-15587 that could produce a deletion of 7124 bp from *D. rerio* mtDNA (Table 2). Since the size of our amplicon was 8345 bp, such deletion would produce an amplicon of 1221 bp which is close to the size of our PCR product. Based on this analysis, we predicted that a deletion of 7124 bp took place in *D. rerio* mtDNA during 20 μM H_2O_2 stress for 96 hours.

Table 2. Locations of repeats within mtDNA region 8244-16589 bp

Repeat type	Locations	Sequence (5′→3′)	Length(bp)	Deletion size (bp)
Direct repeats	9385-9397; 15734-15746	GCCACAGTAATTA	13	6349
	8644-8656; 14728-14740	GACCATCGAATAG	13	6084
	9027-9036, 15079-15088	ACAAAAATGA	10	6052
	10439-10448; 16442-16451	TGAAGCTGCC	10	6003
Inverted repeats	9898-9908; 16360-16350	TCCAATGATGA	11	6442
	8581-8591; 15111-15101	GAATTTTAGTT	11	6510
	8454-8463; 15596-15587	GAATATACAG	10	7124

Next, we looked for the gene coverage of the predicted deletion and found that such deletion could completely knockout 15 genes. Depending on the location of deletion it could impact genes like tRNA-Lys, ATP synthase 8, ATP synthase 6, cytochrome c oxidase III, tRNA-Gly, NADH dehydrogenase 3, tRNA-Arg, NADH dehydrogenase 4L, NADH dehydrogenase 4, tRNA-His, tRNA-Ser 2, tRNA-Leu 2, NADH dehydrogenase 5, NADH dehydrogenase 6 and tRNA-Glu. The deletion could also cause a partial deletion of from the C-terminal region of cytochrome c oxidase II (118 amino acids) and N-terminal region of cytochrome b (97 amino acids). Thus, the deletion and subsequent recombination could produce a fusion protein composed of 387 amino acids, of which the first 112 amino acids would be identical to the N-terminal 112 amino acids of cytochrome c oxidase II and the rest would be identical to the C-terminal 275 amino acids of cytochrome b (Fig. 3a). Predicted

structure showed that the N-terminal portion of the fusion protein is similar to that of cytochrome c oxidase II and the C-terminal portion of the fusion protein is similar to that of cytochrome b (Fig. 3b). Thereby, we suggest that the fusion protein could structurally mimic parts of both partner proteins. Given that the cytochrome b is part of respiratory complex III and the cytochrome c oxidase II is part of complex IV, such fusion protein could destabilize the structure of each of these complexes and could form a different complex. Formation of such complex could potentially result in a short circuit across the electron transport chain of mitochondria and affect fish normal physiology[26-28].

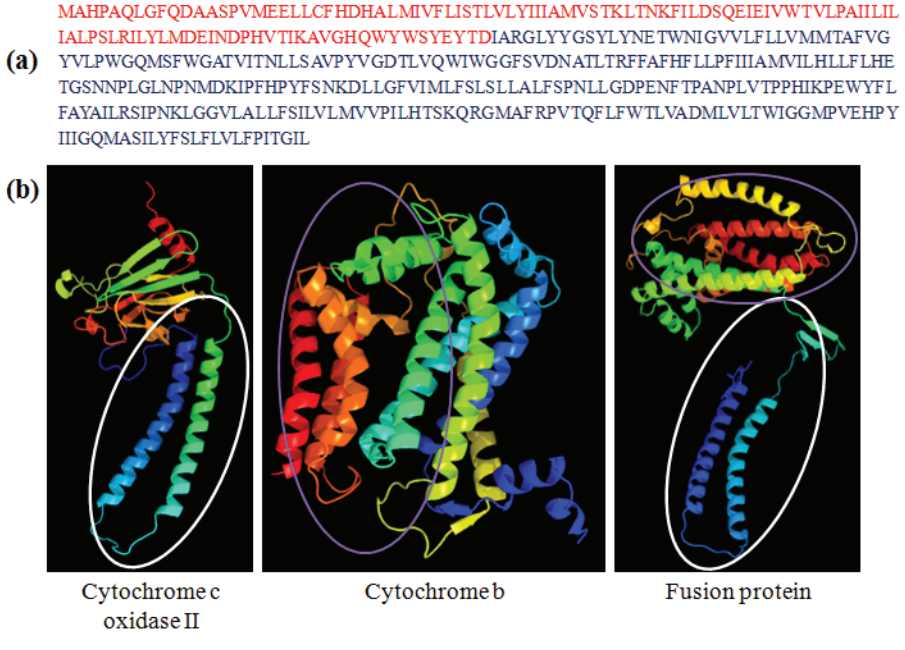

Fig. 3. Sequence and structure of the cytochrome c oxidase II and cytochrome b fusion protein. (a) A full sequence of the fusion protein, red indicates part of cytochrome c oxidase II and dark blue indicates cytochrome b; (b) structures of the predicted proteins as indicated. Note that the β-sheets of cytochrome c oxidase II and three α-helix of cytochrome b are absent in the fusion protein.

Conclusion

In this study we reported that *Danio rerio* can tolerate 20 μM H_2O_2 stress for 96 hours but such stress could cause loss of mtDNA. We found that an approximately 7124 bp deletion occurred in *Danio rerio* mtDNA and such deletion could produce a fusion protein of cytochrome c oxidase II and cytochrome b. Such fusion protein could impair the normal function of the mitochondrial electron transport chain and could ultimately affect normal fish physiology[28]. A follow-on study would deduce the exact breakpoint for the mtDNA deletion and the effect of such deletion in mitochondrial physiology in future. PCR-based detection of mtDNA-specific region deletions could serve as an effective molecular toxicological marker in fish.

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Author contributions

MMKC conceptualized the research, wrote and managed the UGC grant. MMKC, MSA and MSK designed all the experiments. MKC, EN, SC and JH performed all the experiments. EN, JH and MGR maintained *Danio rerio*. MKC, EN, SC and MSA analyzed the data. EN, SC, MSA and MSK did the literature review and handled fishes. MMKC, MSA and MSK wrote the article. All authors read, critically reviewed and agreed to publish this research.

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