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Increase in Mitochondrial DNA Copy Number in Response to Ochratoxin A and Methanol-Induced Mitochondrial DNA Damage in *Drosophila*

Ayse Gul Mutlu

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Abstract The current study examined the effects of ochratoxin A (OTA) and methanol on mitochondrial DNA (mtDNA). The results showed that methanol application caused approximately 29 % more damage than the control group, and that there were no significant differences among the other groups in terms of mtDNA damage. The application of 0.04 ng/mL OTA and 0.04 ng/mL OTA with methanol increased mtDNA copy number compared with the control and 0.005 ng/mL OTA with methanol groups. The copy number in the 0.04 ng/mL OTA group was approximately 16 % greater than in the control.

Keywords Methanol · Ochratoxin A · mtDNA copy number · mtDNA damage

Ochratoxin A (OTA) is a ubiquitous mycotoxin produced by fungi in improperly stored food products. The International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen. Currently, the mode of carcinogenic action by OTA is unknown, but it is genotoxic following oxidative metabolism. This activity is thought to play a central role in OTA-mediated carcinogenesis, and may be divided into direct (covalent DNA adduction) and indirect (oxidative DNA damage) mechanisms of action (Pfohl-Leszkowicz and Manderville 2006). Methanol occurs naturally in the human body as a product of metabolism and through intake of fruits, vegetables, and alcoholic beverages (US EPA 2009). Methanol can react with hydroxyl radicals to spontaneously yield formaldehyde (Harris et al. 2003), which is a known genotoxic substance (Li et al. 2004).

Mitochondrial DNA (mtDNA) damage following oxidative stress is more extensive and persistent than nuclear DNA (nDNA) damage in human cells (Yakes and Van Houten 1997). Some toxic materials may damage mtDNA (Mutlu and Fiskin 2009), triggering mitochondrial dysfunction (Lesnefsky et al. 2001). Damage to mtDNA could be more important than deletions in nDNA because the entire mitochondrial genome codes for expressed genes, while nDNA contains a large amount of non-transcribed sequences. Additionally, unlike nDNA, mtDNA is continuously replicated, even in terminally differentiated cells such as neurons and cardiomyocytes. Hence, somatic mtDNA damage may cause more adverse effects on cellular functions than somatic nDNA damage does (Liang and Godley 2003). There has been some investigation of DNA mutations generated by ochratoxins and methanol; however there is no information regarding the effects of these substances on mtDNA. Therefore, the aim of the current study was to investigate the effects of OTA and methanol on mtDNA and copy number in *Drosophila melanogaster*.

Materials and Methods

Two-day-old wild type (Oregon) *D. melanogaster* (fruit fly) were used in the experiments. Fruit flies are a useful model organism because of their small size and short generation time (Hedges 2002). Flies were fed corn meal medium containing water, corn flour, sugar, yeast, agar, and propionic acid as an antifungal. Flies were housed in glass bottles and incubated at 24 ± 1°C on a 12 h day–night cycle. Treatment groups were as follows: 1/100

A. G. Mutlu (✉)
Department of Biology, Mehmet Akif Ersoy University, Burdur, Turkey
e-mail: agmutlu@gmail.com; agmutlu@mehmetakif.edu.tr

methanol (v/v; methanol/corn meal); 0.005 ng OTA (dissolved in methanol)/mL corn meal; 0.02 ng OTA (dissolved in methanol)/mL corn meal; 0.04 ng OTA (dissolved in methanol)/mL corn meal; and 0.04 ng OTA/mL corn meal. At 48 h post-application, DNA was isolated from 12 flies from each group using GenElute Genomic DNA Extraction kits (Sigma Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions. Pico Green dsDNA quantitation dye (Invitrogen, Carlsbad, CA, USA) and a QUBIT 2.0 fluorometer (Invitrogen) were used for both template DNA quantification and the fluorometric analysis of PCR products. A crucial step of qPCR is the concentration of the DNA sample. The accuracy of the assay relies on initial template quantity because all of the samples must have exactly the same amount of DNA. The Pico Green dye has not only proved efficient in regard to template quantitation but also in PCR product analysis (Santos et al. 2002). DMSO (4 % of total volume) and 5 ng of template DNA were added to each PCR reaction.

We designed the following primers for *Drosophila* mtDNA small fragment amplification (100 bp): 11426, 5′-TAAGAAAATTCCGAGGGATTCA-3′, and 11525, 5′-GGTCGAGCTCCAATTCAAGTTA-3′. The following primers were designed for large fragment amplification (10,629 bp): 1880, 5′-ATGGTGGAGCTTCAGTTGATTT-3′, and 12508, 5′-CAACCTTTTTGTGATGCGATTA-3′. For long fragment PCR amplification, DNA was denatured at 75°C for 1 min and then 95°C for 1 min, followed by 21 cycles of 94°C for 15 s, 52°C for 45 s, and 65°C for 11 min, and a final extension of 68°C for 10 min. For small fragment PCR amplification, DNA was denatured at 75°C for 2 min and then 95°C for 15 s, followed by 21 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s, with a final extension of 72°C for 10 min.

A qPCR method was used to measure mtDNA damage. Lesions in DNA block the progression of any thermostable polymerase on the template, resulting in decreased DNA amplification of damaged template. qPCR is a highly sensitive method for measuring DNA damage and repair. mtDNA damage was quantified by comparing the relative efficiency of amplification of long fragments of DNA, and then normalizing this to gene copy numbers by the amplification of smaller fragments, which have a statistically negligible likelihood of containing damaged bases (Mutlu 2012; Santos et al. 2002; Venkatraman et al. 2004; Yakes and Van Houten 1997). To calculate normalized amplification, the long template qPCR values were divided by the corresponding short qPCR results to account for potential copy number differences between samples (mtDNA/total DNA value may be different in 5 ng template total DNA of each PCR tube). The copy number results do not indicate the damage. Minitab Release 13.0 software was used for statistical analysis. The results were estimated using the Kruskal–Wallis test.

Results and Discussion

Mitochondrial DNA (mtDNA) damage and mtDNA copy number results are shown in Table 1. mtDNA damage in the methanol application group was significantly greater than the control group (Fig. 1; Table 1). There were no significant differences among the other treatment groups in terms of mtDNA damage. Methanol metabolism generates formaldehyde (Harris et al. 2003), which may be the ultimate carcinogen from methanol exposure (Bailey et al. 2012; US EPA 2009) and may induce DNA damage (Emri et al. 2004). Formaldehyde is a known genotoxic substance (Speit and Merk 2001). Numerous studies have shown that formaldehyde can induce DNA–DNA and DNA–protein crosslinks. Li et al. (2004) showed that low concentrations of formaldehyde induced DNA strand breaks, while formaldehyde at higher concentrations induced DNA–DNA and DNA–protein crosslinks. However, some studies do not support the theory that DNA damage is generated by methanol exposure (Mc Callum et al. 2010, 2011).

Mitochondrial DNA (mtDNA) damage in the OTA treatment groups slightly increased but was not statistically significant at the tested doses. OTA is weakly genotoxic to mammalian cells (Pfohl-Leszkowicz et al. 1991) and is a potent nephrotoxin and renal carcinogen in rodents. However, the mechanism of OTA-induced tumour formation is

Table 1 mtDNA damage (relative amplification) and mtDNA copy number results

Groups	mtDNA damage (relative amplification ± SE)	mtDNA copy number (small fragment amplification ± SE)
Control	1.026 ± 0.138	364.13 ± 11.10[b]
Methanol	0.727 ± 0.056[a]	340.78 ± 13.46[b]
0.005 ng/mL OTA with methanol	0.781 ± 0.069	372.33 ± 9.42[b]
0.02 ng/mL OTA with methanol	0.787 ± 0.052	374 ± 10.86
0.04 ng/mL OTA with methanol	0.79 ± 0.053	415.38 ± 17.86
0.04 ng/mL OTA	0.896 ± 0.078	421.55 ± 19.61

SE standard error of the mean

[a] Values statistically different from control group ($p < 0.05$)

[b] Values statistically different from 0.04 ng/mL OTA (with and without methanol) groups ($p < 0.05$)

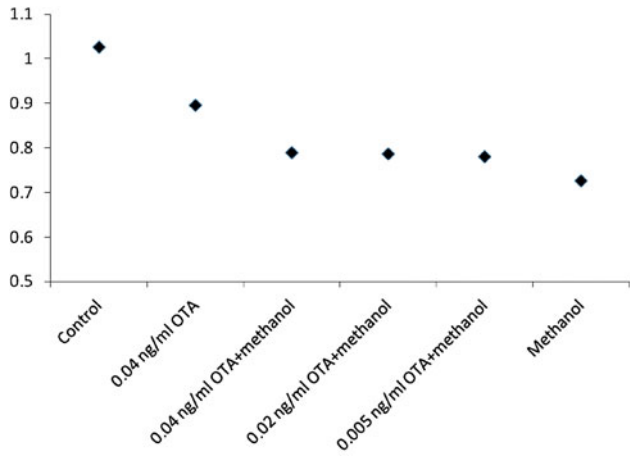

Fig. 1 mtDNA damage in application groups according to qPCR relative amplification results. Decrease of relative amplification indicate the increased DNA damage

unknown, and conflicting results regarding the potential of OTA to react with DNA have been obtained (Mally and Dekant 2005). Ochratoxin has been suggested by various researchers to mediate its toxic effects by inducing apoptosis, disrupting mitochondrial respiration and/or the cytoskeleton, or generating DNA adducts. Thus, it remains unclear if the predominant mechanism is genotoxic or epigenetic (O'Brien and Dietrich 2005).

As shown by mtDNA copy number experiments, groups treated with 0.04 ng/mL OTA (0.04 ng/mL OTA or 0.04 ng/mL OTA with methanol) had significantly increased mtDNA copy numbers compared to the control, methanol, and 0.005 ng/mL OTA with methanol groups (Fig. 2; Table 1). There are few investigations into the relationship between methanol and mtDNA copy number. However, in one study, Spikings et al. (2012) demonstrated a decrease in mtDNA content following methanol treatment in ovarian follicules of zebrafish. Similarly, in the

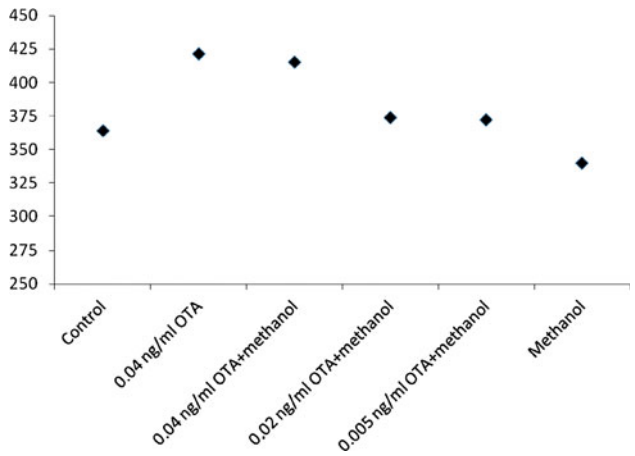

Fig. 2 mtDNA copy number in application groups according to qPCR small fragment amplification results

current study, mtDNA copy number slightly decreased in the methanol application group. However, according to our mtDNA copy number results, the 0.04 ng/mL OTA application groups (both with and without methanol) had increased mtDNA copy numbers. To the best of our knowledge, there is no published study regarding the relationship between OTA and mtDNA copy number. However, some other toxic materials have been investigated. Carugno et al. (2012) showed that blood mtDNA copy number was increased in persons exposed to low benzene levels. The authors commented that this could potentially reflect mtDNA damage and dysfunction. OTA triggers the production of reactive oxygen species in the organisms (Arbillaga et al. 2007; Meki and Hussein 2001; Petrik et al. 2003; Schaaf et al. 2002). Lee et al. (2000) suggested that oxidative stress can increase the mtDNA copy number. Similarly, in the current study, we hypothesize that mtDNA copy number may be increased by the oxidative stress that originated from OTA.

This study revealed that exposure to OTA, with or without methanol, increases the mtDNA copy number. Additionally, OTA was not found to induce mtDNA damage; however, mtDNA damage in the methanol application group increased significantly. Slight increases in mtDNA damage were observed in the groups treated with lower doses of OTA (not statistically significant). An increase in mtDNA copy number in parallel with OTA dose may compensate for the potential damage. Oxidative stress induced by OTA may trigger the increase in mtDNA copy number. However, the observed increased in mtDNA damage in the methanol group may be generated by an unknown formaldehyde-mediated mechanism.

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