ANALYSIS OF DOXORUBICIN-INDUCED MITOCHONDRIAL DNA DELETIONS IN HUMAN BLOOD LEUKOCYTES AND THP-1 CELL LINE BY PCR

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Doxorubicin (DOX) is an antineoplastic drug with potent cardiotoxic activity due to selective accumulation and disruption of mitochondrial functioning in cardiomyocytes. Accumulating evidence indicates that DOX can also induce genotoxic side effects in other cells. The mutagenic effects of DOX in human peripheral blood leukocytes and THP-1 leukemia cells were analyzed using PCR. It was revealed that DOX could induce deletions of different sizes and loci of mitogenomes of normal human leukocytes and THP-1 leukemic cells.

https://doi.org/10.46991/PYSU:B/2023.57.1.032

Keywords: doxorubicin, mtDNA, PCR, human blood, THP-1 cells.

Introduction. Mitochondria are double-membrane organelles that produce over 90% of ATP via oxidative phosphorylation (OXPHOS) in mammalian cells [1]. In somatic cells, each mitochondrion carries several copies of intronless circular mitochondrial DNA (mtDNA) containing 16,569 bp and 37 genes that encode for 13 protein subunits of the OXPHOS, two rRNAs (12S and 16S) and 22 tRNAs [2]. Due to a lack of histones and effective repair mechanisms of mtDNA, it is susceptible to various types of somatic mutations, which can be observed in different pathological conditions including cancer [3], Alzheimer’s disease [4], diabetes [5], cardiomyopathies [6], and Parkinson’s disease [7]. Therefore, the identification of the environmental factors that can induce mtDNA mutations is of high importance. In particular, drugs that cause mitochondrial damage and disruptions of mtDNA have gained much interest.

Doxorubicin (DOX) is a highly effective antineoplastic agent commonly used for the treatment of multiple cancer types [8] and has a wide range of mechanisms of toxicity. DOX is a topoisomerase II poison that results in the induction of DNA double-strand breaks and cell death in dividing cells. Therefore, DOX is more toxic to cancer cells with high proliferative activity. However, DOX induces dose-dependent acute and chronic toxic side effects such as cardiotoxicity and therapy-related malignancies [9].

Not only nuclear, but also mtDNA was shown to be the target of the action of DOX. Using the fluorescent DNA dye PicoGreen, it was shown that anthracycline

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drugs, including DOX, intercalate into nuclear DNA and mtDNA within living cells. Intercalation of mtDNA by anthracyclines causes nucleoid aggregation and mitochondrial interlinking and, thus, may contribute to the marked mitochondrial toxicity associated with these drugs. These effects were influenced by ATM/p53 activation, thereby showing mitochondrial nucleoids are linked to the genomic DNA damage response [10, 11].

It was shown that mitochondrial damage plays a pivotal role in DOX-induced cardiac failure and cell death [12]. The mitochondrial accumulation and toxicity of DOX could be explained by its high affinity to the inner mitochondrial membrane phospholipid cardiolipin. Cardiolipin directs the anchoring of cytochrome c on the outer surface of the inner mitochondrial membrane [13]. Thus, the binding of DOX to cardiolipin might impair the interaction of cytochrome c with the mitochondrial membrane [14]. Aryal and Rao [15] demonstrated that B lymphocytes from patients with Barth syndrome lacking cardiolipin are more resistant to DOX-induced oxidative stress and cytotoxicity in comparison to B lymphocytes from healthy individuals.

Accumulating evidence indicates that other mechanisms might also be involved in the side effects of DOX [16]. In mitochondria, DOX is reduced to a semiquinone leading to the production of superoxide and hydrogen peroxide [17]. It was suggested that mtDNA is highly susceptible to oxidative stress-induced damage due to its proximity to the electron transport chain which can impair mitochondrial functioning [18].

Lebrecht et al. [19] revealed a 324 bp common mtDNA deletion in the heart autopsies of patients exposed to DOX. Adachi et al. [20] revealed a 4 kb deletion in myocardial mtDNA of mice chronically exposed to DOX. It was suggested that the deleted fragment of mtDNA included the sequence between NADH dehydrogenase and ATP6 coding regions [20]. Similar results were obtained by Lebrecht et al. [21] in rat cardiomyocytes where a 459 bp common mtDNA deletion was observed due to chronic exposure to DOX. Thus, disruption of mtDNA integrity might be involved in the cardiotoxic effects of DOX in humans.

Although cardiomyocyte is the most studied cellular target of DOX accumulating evidence indicates that other cells might be targets of DOX too [22]. Lipshultz et al. [23] demonstrated the elevation of mtDNA copy number/cell in peripheral blood mononuclear cells in childhood survivors of high-risk acute lymphoblastic leukemia treated with DOX. This compensatory increase in mtDNA copies might be due to maintaining overall OXPHOS and ATP production in cells [23]. Mei et al [24] suggested that the elevation of mtDNA copy number in HEp-2, HNE2, and A549 tumor cell lines treated with DOX could be a self-protective mechanism to prevent DOX-induced apoptosis.

Previously our group demonstrated that DOX can induce the translocation of mtDNA fragments into the nuclear genome in healthy human blood leukocytes in vitro [25, 26]. However, it is not clear which fragments of mitogenome were deleted and inserted in chromosomes. Recently Wei et al. [27] demonstrated that nuclear mtDNA fragments (NUMTs) in the human genome preferentially involve non-coding fragments of mitogenome. However, it is not clear if there is a relationship between mtDNA deletions and NUMTs. Thus, the aim of this study is the identification of DOX-induced mtDNA deletions and their location in mitogenome in human blood leukocytes and the THP-1 human leukemia monocytic cell line using PCR.
Materials and Methods.

Cell Cultures. Blood samples were collected by venipuncture from two healthy nonsmoking donors (one female and one male) aged 27–29 years without a history of exposure to DOX or other anthracyclines. This study was approved by the Ethic Committee of the National Center of Bioethics (Faculty of Biology, YSU), and informed consent was obtained from all study donors. The venous blood (2 mL from each donor) was collected into vacutainers with heparin. Blood samples and THP-1 cells were cultivated in RPMI-1640 medium, containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C for 48 h. For the induction of lymphocyte divisions in the blood 10 μg/mL phytohemagglutinin-L was used.

DOX Treatment of Human Blood and THP-1 Cells. For the analysis of mtDNA deletions whole blood and THP-1 cells were treated with 0.025, 0.035, and 0.05 μg/mL of DOX for 24 h at 37°C. The implemented doses were selected based on our previous study where DOX-induced mtDNA transfer into the chromosomes was shown [25, 26].

PCR Analysis of mtDNA Deletions. MtDNA deletions were analyzed using PCR followed by gel electrophoresis. Total DNA was isolated using a DNeasy kit (Qiagen). PCR analysis was performed using 9 pairs of primers designed for amplification of the whole mitochondrial genome (see Table) [28]. Each reaction mixture contained 2 μL of FIREPol Master Mix Ready to Load (Solis Biodyne), 1 μL of forward primer, 1 μL of reverse primer, 2 μL of template DNA, and 14 μL of H2O. PCR conditions were the following: initial denaturing at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s, 72°C for 1 min, followed by 72°C for 10 min.

Primer pair | Product length, bp | Sequences of primer pairs (5’–3’)
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The graphical visualization of the mitogenome was realized using the OGDRAW toolkit [29]. Localization of the deleted fragments in mitogenome was analyzed by alignment of primer pairs of deletion fragments against mtDNA.
Results and Discussion. In the current manuscript, the results of the analysis of mtDNA deletions induced by DOX at concentrations 0.025, 0.035 and 0.05 \( \mu \text{g/mL} \) in human peripheral blood (Fig. 1) and THP-1 leukemia cell line (Fig. 2) are presented. MtDNA deletions were not identified in control samples of the blood (Fig. 1, A) and THP-1 cells (Fig. 2, A).

In peripheral blood leukocytes of male donor PCR analysis revealed mtDNA deletion induced by DOX at 0.05 \( \mu \text{g/mL} \) in samples amplified with mt2 primer pair (Fig. 1, B). To analyze what part of the mitogenome was deleted forward and reverse primers of the mt2 primer pair were aligned against the mtDNA reference sequence (NCBI RefSeq: NC_012920.1) using the NCBI BLAST tool. BLAST analysis revealed that the mt2 primer pair amplifies the 1758 \( \text{bp} \) fragment from the 16488–1677 \( \text{bp} \) region of the mitogenome. The deleted fragment (\( \Delta1758 \text{bp} \)) resulted in the removal of 659 \( \text{bp} \) of D-loop region, TRNF (tRNA-Phe), RNR1 (12S rRNA), TRNV (tRNA-Val) genes, and 8 \( \text{bp} \) of RNR2 (16S rRNA) gene from mtDNA (Fig. 1, C).

In peripheral blood leukocytes of female donor DOX-induced deletions were not identified.

In the THP-1 leukemia cell line PCR analysis revealed mtDNA deletion induced by DOX at 0.025 \( \mu \text{g/mL} \) in samples amplified with mt4 primer pair (Fig. 2, B). BLAST analysis revealed that the mt4 primer pair amplifies the 3006 \( \text{bp} \) fragment from the 3734–6739 \( \text{bp} \) region of the mitogenome. The deleted fragment (\( \Delta3006 \text{bp} \)) resulted in the removal of 528 \( \text{bp} \) from ND1, TRNI (tRNA-Ile), TRNQ (tRNA-Gln), TRNM (tRNA-Met), ND2, TRNW (tRNA-Trp), TRNA (tRNA-Ala),
TRNN (tRNA-Asn), TRNC (tRNA-Cys), and TRNY (tRNA-Tyr) genes and 706 bp of COX1 gene from mtDNA (Fig. 2, C).

Fig. 2. PCR analysis of DOX-induced mtDNA deletions in THP-1 leukemia cell line using 9 pairs of primers. All 9 mtDNA fragments (mt1–9) are amplified in the control sample (A). A deletion of the 3006 bp fragment (Δ3006 bp) can be detected in the sample treated with DOX at 0.025 µg/mL and amplified with mt4 primer pair (B). The DOX-induced deletion of the 3006 bp fragment of mtDNA is shown on the mitogenome map (the schematic depiction of the deletion fragment is a graphical approximation) (C).

Previously it was shown that NUMT sequences of tRNAs, 12S rRNA, 16S rRNA, COX1, COX3, CYTB and short fragments of ND1, ND5, and ND6 genes in the nuclear reference genome are overrepresented [30]. Therefore, alignments of the Δ1758 bp and Δ3006 bp deleted fragments against the human reference genome were performed. The highest score of alignment of Δ1758 bp fragment was identified against 1155 bp fragment in 11p15.4 loci (score: 1058; 95.6% identity) containing MTRNR2L8 pseudogene. The highest score of alignment of Δ3006 bp fragment was identified against 2911 bp fragment in 17p11.2 loci (score: 1945; 84.3% identity) containing MTND1P15, MTND2P13, and MTCO1P13 pseudogenes.

Although the main mechanism of DOX-induced cell death is due to its ability to intercalate into DNA and interfere with topoisomerase II-DNA cleavage complex in chromosomes, it can also result in toxic effects via damaging mtDNA. Nevertheless, mutagen-induced deletions of mtDNA are poorly studied. Damaged mtDNA molecules usually only represent a small fraction of mtDNAs in a human cell, and their limiting accumulation may involve the clearance of the damaged mitochondrial pool by mitophagy. In addition, damaged mtDNA molecules may sometimes be degraded rather than repaired and healthy molecules will replicate to restore mtDNA copy number, but a complete understanding of what regulates the balance of degradation versus repair remains unclear [31, 32]. Moreover, several studies have shown that mitochondria are resistant to mutagenesis after exposure to known mutagens. For example, Valente et al. [33] demonstrated that benzo[a]pyrene or N-ethyl-N-nitrosourea produced no significant increases in mtDNA point mutations or deletions while inducing mutations in nDNA in Muta™Mouse mice. These findings contradict models of mtDNA mutagenesis and suggest that mitochondrial damage responses can repress mutagen-induced mutations. The authors suggest that
mitochondria appear to have mechanisms to avoid or eliminate mutagenic DNA lesions, and pathways such as mtDNA repair, degradation, and mitochondrial dynamics could contribute individually or in combination. Thus, further research should be implemented to clarify the mechanisms of the formation and elimination of mutagen-induced mutations in mtDNA.

In this article DOX-induced deletions of different sizes and regions of mitogenomes in human peripheral blood leukocytes and THP-1 leukemia cell line are demonstrated. Previous studies have shown that DOX exposure can result in deletions of different regions of mitogenome in human [19] and animal [20, 21] cardiomyocytes. Thus, the obtained results are in line with the limited data of the literature.

In peripheral blood leukocytes the $\Delta 1758$ bp was induced by DOX at 0.05 $\mu$g/mL while in THP-1 leukemia cells the $\Delta 3006$ bp was induced at 0.025 $\mu$g/mL. There is limited data on differences in sensitivity between normal human peripheral blood leukocytes and leukemia cells to DOX. Jedrzejczyk et al. [34] compared the cytotoxic effects of DOX in human peripheral blood mononuclear cells (PBMCs) and the K562 leukemia cell line by analyzing the mitochondrial activity of respiratory chain oxidoreductases. It was shown that the IC$_{50}$ concentration of DOX in the K562 leukemia cell line is significantly lower compared to PBMCs [34]. However, there is no data demonstrating differences between mtDNA deletions in blood leukocytes and leukemia cells.

The results of the current study indicate that DOX-induced deletions occur at different loci of mitogenomes of blood leukocytes and THP-1 leukemia cells. In blood leukocytes, DOX-induced $\Delta 1758$ bp fragment includes sequences encoding mitochondrial 12S and 16S rRNAs, TRNV and TRNF, and D-loop region. While in THP-1 leukemia cells DOX-induced $\Delta 3006$ bp fragment includes ND1, ND2, COX1, and eight tRNAs genes of the mitogenome.

Previous studies have shown that deletions of mitochondrial genes are associated with a wide range of human disorders including ophthalmoplegia [35], Kearns-Sayre syndrome [36], Pearson syndrome [37], and cancers [3]. In the majority of these studies, the deletion fragment of mtDNA includes a common mtDNA deletion ($\Delta 4977$ bp) between 8470-13447 bp region of mitogenome. However, the DOX-induced deletions in blood leukocytes and THP-1 leukemia cells do not overlap with the $\Delta 4977$ bp fragment.

It was suggested that the lack of methods for the manipulation of mtDNA significantly renders the functional analysis of mitochondrial genes [38]. In this study, it was shown that $\Delta 1758$ bp and $\Delta 3006$ bp fragments include sequences of genes involved in the mitochondrial translation apparatus and subunits of proteins of the OXPHOS system. Thus, the obtained results show that mutagen-induced deletions can be considered an effective tool for studies of the functional effects of different genes of mitogenome.

This work was supported by the Science Committee of the MESCS RA, in the frames of the research project No. 21AG-1F068.
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ԴՈՔՍՈՐՈՒԲԻՑԻՆՈՎ ՄԱԿԱԾՎԱԾ ՄՏԴՆԹ -Ի ԴԵԼԵՑԻԱՆԵՐԻ ՎԵՐԼՈՒԾՈՒԹՅՈՒՆԸ ՄԱՐԴՈՒ ԱՐՅԱՆ ԼԵՅԿՈՑԻՏՆԵՐՈՒՄ ԵՎ THP-1 ԲՋՋԱՅԻՆ ԳԾՈՒՄ ՊՇՐ ՄԵԹՈԴՈՎ:

Դոքսորուբիցին (DOX) հակաքաղցկեղային դեղամիջոց է, որը նույն ժամանակ կարդիոսպիրալ ակտիվություններ է ունի մարդու արյան կարդիոմիոցիտների միտոքոնդրիումների տեսակետից։ Դոքսորուբիցինը կարող է ստիպել իր միտոքոնդրիումների ֆունկցիայի խաթարմամբ և դրանց ֆիզիոլոգիայի ինհարացման։ Չնայած պրոցեսը գուրանց է, որ DOX-ը կարող է մայրաքառորդ միտոքոնդրիումների կենսաչափերի ստեղծման մակարդակ բարձրացնել, Դոքսորուբիցինը DOX-ի միտոքոնդրիումի վերականգնման դեղամիջոցների կատարելիությանը և THP-1 լեյկոցիտների ինհարացման համար միաժամանակ։ Ուտոր, որ DOX-ը կարող է մայրաքառորդ մայրաքառորդ միտոքոնդրիումների ինհարացման համար հավանական միտոքոնդրիումների հաղթականությանը և THP-1 միտոքոնդրիումների ինհարացման համար միաժամանակ:

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ԱНАЛИЗ ДЕЛЕЦИЙ МИТОХОНДРИАЛЬНЫХ ДНК, ИНДУЦИРОВАННЫХ ДОКСОРУБИЦИНОМ В ЛЕЙКОЦИТАХ КРОВИ ЧЕЛОВЕКА И КЛЕТОЧНОЙ ЛИНИИ THP-1 МЕТОДОМ ПЦР

Доксорубицин (DOX) – противоопухолевой препарат с мощной кардиотоксической активностью за счет избирательного накопления и нарушения функционирования митохондрий в кардиомиоцитах. Многочисленные данные показывают, что DOX может индуцировать генотоксические побочные эффекты и в других клетках. В данной работе с использованием ПЦР были оценены мутагенные эффекты DOX в лейкоцитах периферической крови человека и клетках лейкемии THP-1. Было выявлено, что DOX может индуцировать деления различного размера и локусов митогеномов нормальных лейкоцитов человека и лейкемических клеток THP-1.