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CELL-FREE MITOCHONDRIAL DNA IN DOXORUBICIN-TREATED KCL22 AND K562 CELL LINES

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Cell-free mitochondrial DNA (cf-mtDNA) is widely used as a biomarker of pathological processes, but its application for assessing the effects of environmental factors is limited. For the first time, cf-mtDNA was identified in the KCL22 cell culture treated with doxorubicin, which is known for its ability to disrupt mitochondrial functions. The results obtained indicate the effectiveness of using cf-mtDNA as a biomarker of genotoxic and cytotoxic effects.

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***Keywords*:** doxorubicin, cell-free mtDNA, PCR, gel electrophoresis, cytotoxicity, genotoxicity, KCL22 and K562 cells.

Introduction. Studies in human populations indicate that environmental toxicants, including polycyclic aromatic hydrocarbons, particulate air pollutants and black carbon, heavy metals, endocrine-disrupting chemicals, pesticides and nanomaterials as well as doxorubicin (DOX) can induce mitochondrial stress and impair mitochondrial function [1].

Upon mtDNA damage, multiple processes including mtDNA repair, clearance, and release occur to maintain mitochondrial quality and normal cell function [2]. If repair fails, direct degradation of damaged mtDNA by mitochondrial nucleases within mitochondria may occur. Dysfunctional mitochondria containing damaged mtDNA can be removed by mitophagy. Damaged mtDNA can be released into the cytosol, then cytosolic mtDNA can degraded or further released to extracellular space by extracellular vesicles or by cell membrane pores. Finally, the whole mitochondria carrying damaged mtDNA can be released to extracellular space by mitocytosis. In addition, upon severe damage, cell integrity is impaired and apoptosis or necrosis is induced, leading to the release of mtDNA into extracellular fluids [2]. Thus, mtDNA in the cytoplasm or extracellular space can occur due to mitochondrial damage.

Cell-free mitochondrial DNA (cf-mtDNA) in the humans was first reported in plasma and serum of type 2 diabetes mellitus patients [3]. A mitochondrial mutation in cf-mtDNA was also found in patients with diabetes. Currently, cfDNA is gaining

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attention as a potentially versatile biomarker for a wide range of diseases, aging, psychosocial and physical stress [4].

mtDNA is more susceptible to stress-induced damages than nuclear DNA, in part due to the close proximity to the ROS production and lack of the protection by histones. The mitochondria also have reduced DNA repair capacity in comparison to the nucleus [5].

DOX is one of the most studied compounds that damage mitochondria. In the inner mitochondrial membrane DOX specifically binds the phospholipid cardiolipin which leads to mitochondrial accumulation of the drug [6] at concentrations 100-fold higher than in plasma [7]. This would disrupt the electron transport chain and would lead to ROS production that can underlie DOX-induced toxicity and especially cardiotoxicity [6, 8]. DOX induces DNA damage through three main mechanisms: the formation of DNA adducts, single- and double-strand breaks, in which DNA strands remain bound to trapped topoisomerase enzymes through DNA–protein crosslinks and intercalation of DOX in the DNA molecule [9]. Circular and covalently closed nature of mtDNA allows easy access to intercalating agents [6]. DOX also inhibits topoisomerase, induces senescence, autophagy, apoptosis, pyroptosis, ferroptosis, or necrosis of cancer cells depending on the dose of the drug and the cellular context [9].

The use of circulating genomic DNA and mtDNA in human population and *in vivo* and *in vitro* studies will provide a better understanding of the impact of environmental toxicants. Research using cfDNA as a biomarker of environmental exposure is limited and needs to be developed. Thus, the aim of this study is the identification of cf-mtDNA in DOX-induced KCL22 and K562 cell lines using PCR with primers for whole human mitogenome.

Materials and Methods.

Cell Cultures. The K562 and KCL22 cell lines (both human chronic myeloid leukemia) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C.

Cell Viability. Cell viability was assessed using trypan blue exclusion test as previously described [10]. K562 and KCL22 cells were treated with 0.025–0.56 $\mu g/mL$ of DOX for 72 *h* at 37°C. 100 μL of cell suspension was mixed with 100 μL of trypan blue dye and analyzed using the Neubauer chamber.

DOX Treatment of K562 and KCL22 Cells. For the analysis of micronuclei induction and cf-mtDNA K562 and KCL22 cells were treated with 0.025, 0.035, and 0.05 $\mu g/mL$ of DOX for 72 *h* at 37°C. The implemented doses were selected based on cell viability analysis and our previous study of DOX-induced mtDNA insertion in nuclear genome [11, 12] and DOX-induced mtDNA deletions [13].

Cytokinesis-Block Micronucleus (CBMN) Assay. CBMN assay was performed for the analysis of levels of DOX-induced chromosome damage according to Fenech [14]. Cytochalasin B (3 $\mu g/mL$) was added after 44 *h* of incubation in order to block cytokinesis and obtain binucleated cells. In total, KCL22 and K562 cells were incubated for 72 *h* at 37°C. Hypotonic treatment was performed for 3 *min* in 0.075 *M* KCl at 4°C. Fixation was done twice using ice-cold (–20°C) ethanol/acetic acid (3:1 v/v). Slides were prepared by prewashing with fixative and cell suspension was added from a very low distance. After air-drying, slides were stained with 10%

Giemsa. To determine the MN frequency, at least 1000 binucleated cells were evaluated per each experimental point.

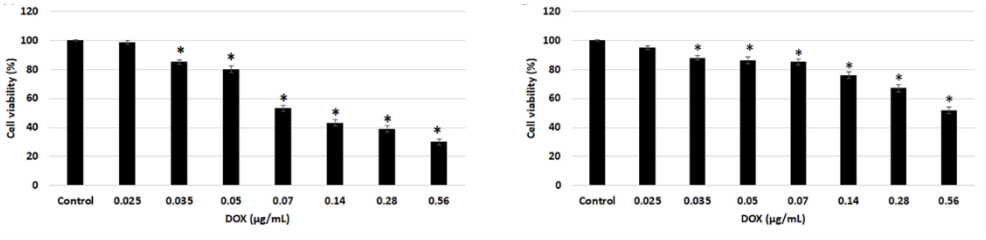
PCR Analysis of cf-mtDNA. After 72 *h* incubation of KCL22 and K562 cells with DOX, cell suspensions were centrifuged for 5 *min* at 2000 *rpm* and supernatants were filtered with a 0.45 μm filter. The extracellular DNA was isolated with a DNA isolation kit (Qiagen). PCR analysis was performed using primers designed for amplification of the whole mitogenome (see Table) [15]. 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, 4 μL of template DNA, and 12 μL of H_2O. 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*. PCR results were analyzed by gel electrophoresis.

Sequences and sizes of primer pairs used for the whole human mitogenome amplification [15]

Amplicon number and length, *bp*	Forward and reverse primer sequences (5'–3')
mt1 (1822)	for – tagccatgcactactcaccaga rev – ggatgaggcaggaatcaaagac
mt2 (1758)	for – ctgtatccgacatctggttcct rev – gtttagctcagagcggtcaagt
mt3 (2543)	for – acttaagggtcgaaggtggatt rev – tcgatgttgaagcctgagacta
mt4 (3005)	for – aagtcaccctagccatcattcta rev – gatatcatagctcagaccatacc
mt5 (2664)	for – ctgctggcatcactatactacta rev – gattggtgggtcattatgtgttg
mt6 (1738)	for – cttaccacaaggcacacctaca rev – ggcacaatattggctaagaggg
mt7 (1866)	for – gtctggcctatgagtgactaca rev – cagttcttgtgagctttctcgg
mt8 (1853)	for – ctccctctacatatttaccacaac rev – aagtcctaggaaagtgacagcga
mt9 (1872)	for – gcaggaatacctttcctcacag rev – gtgcaagaataggaggtggagt

Results and Discussion. It was shown that the concentration of cf-mtDNA in blood of cancer patients is higher than in blood of healthy subjects [16]. We hypothesized that DNA may be more likely to be released from tumor cells than normal cells and chose this model as presumably more sensitive for our study. DOX is known to cause mtDNA deletions [13, 17] and cytotoxic effects [9], both of which can lead to the release of DNA from cells. Accordingly, before studying extracellular mtDNA, the cytotoxicity and genotoxicity of DOX in K562 and KCL22 cell lines were analyzed.

Cytotoxicity of DOX in KCL22 and K562 Cells. To select the range of genotoxic concentrations of DOX that are expected to induce cf-mtDNA, a preliminary analysis of cytotoxicity of different DOX concentrations was carried out using trypan blue exclusion test of cell viability. KCL22 and K562 cells were treated with 0.025 to 0.56 $\mu g/mL$ of DOX for 72 *h*. The Inhibitory Concentrations 50 (IC_{50}) determined in our experiments for KCL22 and K562 cells were 0.07 $\mu g/mL$ (Fig. 1, a) and 0.56 $\mu g/mL$, respectively (Fig. 1, b).

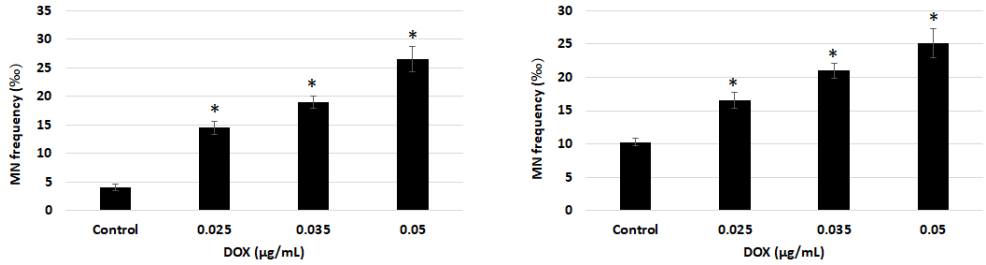


a b

Fig. 1. Levels of viability of KCL22 (a) and K562 (b) cells exposed to DOX. * $p < 0.05$ – significant difference compared to control.

Genotoxicity of DOX in KCL22 and K562 Cells. The genotoxicity of DOX at doses 0.025, 0.035 and 0.05 *μg/mL*, selected on the basis of cytotoxicity data, was analyzed by micronucleus test. The obtained data showed significant increase in the frequency of micronuclei (MNi) in both cell lines. In KCL22 cells, treated with DOX at concentrations 0.025, 0.035 and 0.05 *μg/mL* the levels of MNi were 14.5±1.2‰, 19.0±1.1‰ and 26.1±2.2‰, respectively versus 4.1±0.6‰ in control (Fig. 2, a). The spontaneous level of MNi in K562 cells was 10.3±0.8‰; after treatment with DOX at concentrations 0.025, 0.035 and 0.05 *μg/mL*, the frequency of MNi increased to 16.5±1.4‰, 21.0±1.8‰ and 25.1±2.9‰, respectively (Fig. 2, b).

The induction of MNi by DOX confirmed its ability to damage DNA at selected concentrations and conditions. The results obtained served as the basis for the use of these concentrations for studying cf-mtDNA induction.



a b

Fig. 2. DOX-induced MNi levels in KCL22 (a) and K562 (b) cells. * $p < 0.05$ – significant difference compared to control.

Extracellular mtDNA in DOX-Treated KCL22 and K562 Cells Cultures. Cell-free DNA was isolated from control and DOX-treated KCL22 and K562 cell cultures and amplified using primers for the amplification of whole mitogenome. Gel electrophoresis images of obtained PCR products of mtDNA in control and DOX-treated KCL22 cells are shown in Fig. 3.

The results obtained show the absence of extracellular mtDNA in the non-exposed KCL22 cell cultures (Fig. 3, a). An approximately 300 *bp* amplicon (mt9 primers) was detected in KCL22 cells treated with DOX at a concentration of 0.025 *μg/mL* (Fig. 3, b). Amplification of fragments of approximately 200 *bp* and 1870 *bp* in length (mt9 primers) was observed in KCL22 cells treated with DOX at concentration 0.035 *μg/mL* (Fig. 3, c). When KCL22 cells were treated with the highest concentration of DOX (0.05 *μg/mL*), amplicons of approximately 1500 *bp* (mt7 primers), 1870 *bp* and 500 *bp* (mt9 primers) were found (Fig. 3, d).

No DOX-induced extracellular mtDNA fragments were detected in the K562 cell line (data not shown).

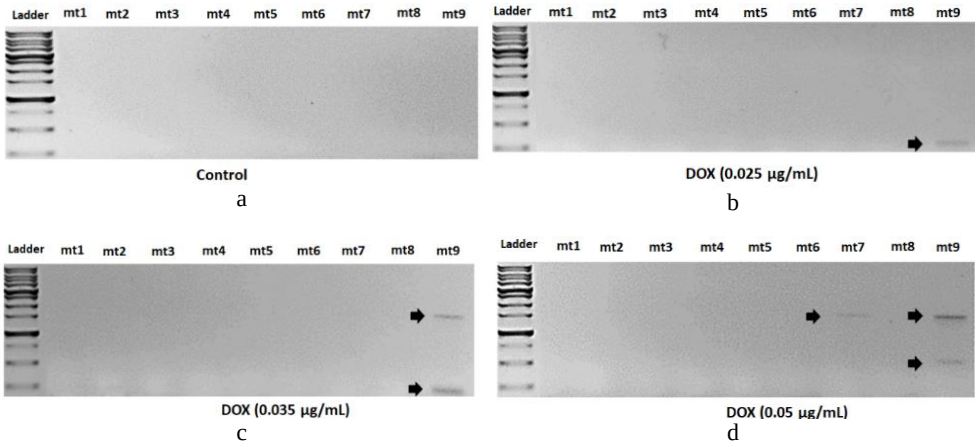


Fig. 3. Agarose gel electrophoresis showing PCR products of cf-mtDNA amplification in KCL22 cell culture: a) No cf-mtDNA fragments were detected in the control probe; b) 300 *bp* cf-mtDNA in cells treated with 0.025 $\mu g/mL$ DOX; c) 200 *bp* and 1870 *bp* cf-mtDNA in cells treated with 0.035 $\mu g/mL$ DOX; d) 1500 *bp*, 1870 *bp*, and 500 *bp* cf-mtDNA in cells treated with 0.05 $\mu g/mL$ DOX. Amplified fragments are indicated with black arrows.

In this study, we reported, for the first time, the elevation of cf-mtDNA in KCL22 cell culture treated with DOX. Recent studies have demonstrated cf-mtDNA as a molecular marker for various diseases [4] and, therefore, it appears as a result of endogenous damage under pathological conditions. Our study demonstrates that cf-mtDNA can also be detected under the influence of exogenous genotoxic factors.

Earlier DOX-induced deletion of mitochondrial DNA was shown *in vivo* in cardiomyocytes of mouse with chronic DOX-induced cardiotoxicity [17] and *in vitro* in human blood leukocytes [13]. Thus, DOX-induced cf-mtDNA in KCL22 cells could be formed as a result of DNA deletion and subsequent release into the cytoplasm and out of the cell as well as a result of DOX-induced various forms of cell death, including apoptosis, pyroptosis, ferroptosis, or necrosis [9].

The absence of cfDNA in K562 cell culture can be explained by the limited scope of research and the unequal response of the two cell types to DOX. cfDNA is usually released from cells in small quantities and associated detection problems may be responsible for the negative result in K562 cells. It is worth to note that the IC_{50} concentration of DOX in K562 cells is higher (0.56 $\mu g/mL$) compared to KCL22 cells (0.07 $\mu g/mL$). Thus, the resistance of K562 cells to DOX could also be in play for the absence of cf-mtDNA in our experiments. In addition, the formation of cfDNA in KCL22 cells under the present experimental conditions may be explained by their higher proliferative activity. The doubling time of K562 and KCL22 cells are 30-40 *h* and 24 *h*, respectively [18]. Thus, during the cultivation period it is expected that the KCL22 cells will go through more division cycles and the DNA of dividing cells is expected to be more prone to accumulation of damage.

Although mtDNA is more susceptible to stress-induced damages than nuclear DNA due to its proximity to the sites of oxidative phosphorylation and lack of the protection by histones [5] research on its application as a marker of genotoxic exposure is very limited. An increase or reduction in the mitochondrial DNA copy number has been documented after environmental and occupational exposure to benzene [19–21] and polycyclic aromatic hydrocarbons [22, 23], highlighting the role of mtDNA in exposure associated with intoxication. The only study of cf-mtDNA as a biomarker of environmental chemical exposure was realized by Budnik et al. [24] in individuals with fumigant (halogenated hydrocarbon pesticides) intoxication. Two fragments of circulated mtDNA were identified in blood of exposed persons, 79 *bp* and 230 *bp* in size using qPCR. The concentrations of total DNA, mtDNA-79 and mtDNA-230 was shown to be increased, and the mtDNA integrity is decreased in exposed patients compared with those in healthy individuals. Authors suggested circulating mtDNA as biomarker reflecting mitochondrial destabilization after environmental exposure to toxic chemicals.

Conclusion. mtDNA can be released from cells as a result, in particular, of mtDNA damage or cell death. Therefore, we suggest that the cf-mtDNA in the DOX-treated culture of KCL22 cells could be a consequence of genotoxic or cytotoxic effect of DOX or both effects together. Thus, further studies using substances of different mechanisms of action and other cellular models are needed to clarify the effectiveness of cf-mtDNA application as a marker of environmental exposures.

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ԱՐՏԱԲՋՋԱՅԻՆ ՄԻՏՈՔՈՆԴՐԻՈՒՄԱՅԻՆ ԴՆԹ-Ն ԴՈՔՍՈՐՈՒԲԻՑԻՆՈՎ ՄՇԱԿՎԱԾ KCL22 ԵՎ K562 ԲՋՋԱՅԻՆ ԳԾԵՐՈՒՄ

Չնայած կորիզային և միտոքոնդրիումային ծագման արտաբջջային ԴՆԹ-ի լայն կիրառմանը պաթոլոգիական գործընթացները բնութագրելու համար, այս կենսամարկերի օգտագործումը շրջակա միջավայրի գործոնների ազդեցության գնահատման համար սահմանափակ է: Ներկայացված աշխատանքում, օգտագործելով ՊՇՌ պրայմերներ ամբողջ միտոգենոմի համար, արտաբջջային մտԴՆԹ-ն գնահատվել է դոքսորուբիցինով մշակված K562 և KCL22 բջիջների կուլտուրաներում: KCL22 բջիջների կուլտուրայում հայտնաբերվել են արտաբջջային մտԴՆԹ-ի տարբեր երկարության հատվածներ, ինչը ցույց է տալիս արտաբջջային մտԴՆԹ-ի կիրառման արդյունավետությունը՝ որպես գենաթունային և բջջաթունային ազդեցությունների կենսամարկեր:

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ВНЕКЛЕТОЧНАЯ МИТОХОНДРИАЛЬНАЯ ДНК В ОБРАБОТАННЫХ ДОКСОРУБИЦИНОМ КЛЕТОЧНЫХ ЛИНИЯХ KCL22 И K562

Несмотря на широкое использование внеклеточной ДНК ядерного и митохондриального происхождения для характеристики патологических процессов, применение этого маркера для оценки эффектов средовых факторов ограничено. В представленном исследовании с использованием ПЦР-праймеров для всего митогенома была оценена вк-мтДНК в культурах клеток K562 и KCL22 при действии доксорубицина. Фрагменты вк-мтДНК различной длины были обнаружены в культуре клеток KCL22, что свидетельствует о эффективности применения вк-мтДНК как биомаркера генотоксических и цитотоксических эффектов.