Protective Effect of Ubiquinone and Precursors of Its Synthesis on Mitochondrial Respiratory Chain and Activity of Matrix Metalloproteinases in Animal Tissues under Effect of Doxorubicin

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Abstract: Mitochondrial dysfunction, oxidative stress, and their regulation are important fields of study in modern clinical research. Exogenous CoQ is an efficient therapeutic agent, yet its application has leads to continued suppression of endogenous CoQ synthesis, which limits CoQ applicability. Our aim was to study the state of mitochondrial electron transport chain components, CoQ content and redox state, superoxide anion radicals and NO production rates, and active MMP-2 and MMP-9 content in rat liver and heart under treatment with Doxorubicin, CoQ₁₀, and complex preparation of modulators and precursors of CoQ biosynthesis (EPM complex). The results demonstrate that treatment with EPM complex and CoQ₁₀ in addition to Doxorubicin administration exerts protective effect on liver and heart mitochondria, evidenced by restoration of electron transport in respiratory chain, which is expressed as decreased nitrile complexes formation with Fe-S-proteins and increased ubisemiquinone content. The protective effects of EPM complex on mitochondrial electron transport chain under Doxorubicin administration is on par with those of CoQ₁₀, and decreased MMP2 and MMP9 activities signify lessened extracellular matrix destruction. These results demonstrate the viability of approaches to correct adverse effects of Doxorubicin by treatment with CoQ₁₀ and e complex of precursors and modulators of its biosynthesis.

Key words: Ubiquinone, mitochondrion, matrix metalloproteinases, doxorubicin.

1. Introduction

The mechanisms underlying mitochondrial dysfunction and its role in etiology of certain pathologies, namely cardiovascular and oncological, are intensively studied lately. One of the causes

behind it is a disruption of regulation mechanisms for production and elimination of reactive oxygen species (ROS). The mechanisms of these effects remain unestablished. Thus, the study of mechanism of development of mitochondrial dysfunction, oxidative stress, and development of new strategies for regulation of such processes is urgent for modern medicine [1-3].

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The reaction of oxidation of ubisemiquinones by molecular oxygen in mitochondrial electron transport chain (ETC) is one of the main processes responsible for generation of superoxide anion-radicals [1, 3]. Ubisemiquinones function as electron and proton carriers between complexes I, II, and III of ETC. Changes in the pool of these radicals are typical under malfunction of mitochondrial ETC, and the assessment of its level allows to diagnose the dysfunctions of the latter [4, 5].

Massive disruptions of many physiological processes take place under mitochondrial dysfunction through redox-dependent mechanisms. These include heightende destruction of extracellular matrix through protease activation [6], that is an integral part of numerous pathologies, including oncological diseases [7]. Zincum-dependent endopeptidases, namely matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9, or gelatinases), play a key role in protolysis of proteins of extracellular matrix [8].

Doxorubicin is an anthracycline antibiotic widely used as antineoplastic agent. The antitumor effect of Doxorubicin correlates with dose-dependent liver toxicity, cardiac toxicity, etc., however [9, 10]. The main role in Doxorubicin toxicity may belong to increased ROS levels, that lead to oxidative damage to lipids, proteins, nuclear and mitochondrial DNA. It has been proposed that under antioxidant treatment (such as vitamin E and ubiquinone) the antineoplastic activity of Doxorubicin is improved via decreased levels of mitochondrial-produced ROS [9-11].

Ubiquinone (Coenzyme Q, CoQ) plays an important role in mechanisms of cellular functioning not only as an ETC component, but also as a regulator of gene expression, signal transduction, apoptosis, etc. [12]. The inhibition of regulation and level of CoQ biosynthesis its dietary intake cannot satisfy the physiological needs of mammal organism fully, especially so under progression or manifestation of pathological conditions associated with malfunction of bioenergetical metabolism [12-14]. Thus, an

additional supplementation of CoQ in the form of oral pharmaceuticals is required to satiate the organism's CoQ needs, and these are effectively used for therapy of a wide spectrum of diseases [12, 13]. Nevertheless, the approach based on substrate activation has certain drawbacks. Particularly it is the high cost of therapy course (that frequently requires dosage of 100 – 350 mg per day for 5 – 6 months), the continued suppression of endogenous CoQ synthesis (possibly through the substrate-enzyme inhibition mechanism) that limits CoQ applicability in medical practice [12].

Therefore, the aim of this project was to study the state of mitochondrial ETC components, CoQ content and redox state, superoxide anion radicals and NO production rates, and active MMP-2 and MMP-9 content in rat liver and heart tissues under treatment with Doxorubicin, CoQ₁₀ medical, and complex preparation of modulators and precursors of CoQ biosynthesis.

2. Materials and Methods

Male white rats of 180-220 g body mass were assigned into 4 groups: (1) Control (intact) animals; (2) Animals subjected to Doxorubicin treatment; (3) Animals subjected to combined Doxorubicin and α -tocopherol acetate, 4-hydroxybenzoic acid, and methionine treatment ("EPM" complex); (4) Animals subjected to combined Doxorubicin and CoQ₁₀ treatment ("Qudesan", Akvion Co, the Russian Federation).

Doxorubicin (Kyivmedpreparat JSC, Ukraine) was administered intraperitoneally in doses of 2.2 mg/kg of body mass daily for 8 days [15, 16]. The rats of the Control group were administered the corresponding volume of normal saline. The animals were sacrificed by decapitation in accordance with international regulations on treatment of laboratory animals.

CoQ was purified from tissue samples by TLC. 1.5 mL of 40% KOH solution, 8.0 mL of methanol and 90 mg of ascorbic acid were added to 3 mL of tissue homogenate. The samples were incubated at 75 °C

water bath for 30 min to stimulate alkali hydrolysis, with mixing every 5 min. and rapid cooling afterwards. The lipids that remained unhydrolyzed were extracted with four elutions of 8 ml of petroleum ether (40-70 °C fraction). The extracts were settled, then decanted and washed with distilled H₂O till pH reached neutral values (4 times of equal H₂O volume was enough in most cases). The water was decanted, and additional step of drying with a couple of drops of methanol was performed. The extracts were afterwards evaporated in bulbs under vacuum at 40-44 °C.

The dried extracts were dissolved in petroleum ether and applied on stationary phase (silica gel L5/40 μ + 13% CaSO₄) of thin layer chromatography. The chromatography was performed in mobile phase that consisted of hexane : diethyl ether 70 : 30. The tocopherols and ubiquinones blots were identified at marker (α -tocopherol acetate) level under ultraviolet illumination (λ = 254 nm) and eluted with diethyl ether. Another thin-layer chromatography was performed afterwards in order to separate CoQ from tocopherols (stationary phase, standard Silufol plates; mobile phase, acetone:H₂O = 5:95). The CoQ blots were identified at marker (CoQ₁₀) level, eluted with diethyl ether and evaporated in glass bulbs under vacuum.

The eluted samples were then dissolved in ethanol. Quantification of CoQ content was performed spectrophotometrically according to differences in spectra of oxidized and reduced forms of CoQ. The spectra of samples were first recorded, then the samples were mixed with KBH₄ (reducing agent) and left for 10 min. The spectra samples were then recorded again, and CoQ content was calculated from the differences between extinctions of oxidized and reduced forms of CoQ.

Active MMP-2 and MMP-9 content was determined with zymography on PAA gel [17, 18]. The tissue samples were snap frozen in liquid nitrogen immediately after excision and stored at -70 °C.

Tissue samples were homogenized on ice in SDS buffer solution (pH 6.8), then centrifugated 10 min at 2500 g, supernatant was used for further measurements. Concentration of MMP-2 and MMP-9 latent and active forms were determined in tissue protein fraction by enzymography in 12% polyacrylamide gel with SDS and gelatin as substrate. Electrophoresis was carried out at +4 °C, 150 V for 4 h. After protein separation, gel was washed in triton X-100 solution, then incubation in buffer with CaCl₂ (pH 7.5) for 18 h at 37 °C, fixed and stained with 0.25% Cumassie brilliant blue. Estimation of proteolytic activity was done by measurement of the clear area of lysis zone, using standards for MMP-2 and MMP-9 (Sigma, USA) and standard program TotalLab 1.01.

The components of ETC, rates of superoxide radicals and NO generation and ubisemiquinones content was evaluated by EPR on digitalized PE-1307 spectrometer with cylinder MW resonator with the mode H₀₁₁ with frequency 9.15 GHz. The microwave power radiation was 40 mW and the magnetic field modulation frequency 100 kHz. Tissue samples were frozen *ex tempore* in liquid nitrogen (T = 77K) as cylinders with the diameter of 4.5 mm and length of 40 mm. 1-hydroxy-2,2,6,6-tetramethyl-4-oxopiperidine was used as spin trap to determine superoxide radical generation rate at room temperature [19, 20]. Magnetic field modulation amplitude was 0.5 G. Sodium diethyldithiocarbamate was used as spin trap to determine NO generation rates by NO-synthases in liquid N₂ coolant (77 K). Magnetic field modulation amplitude was 5 G in this case.

NADH-CoQ-oxidoreductase (NQR) (EC1.6.5.3) activity and succinate-CoQ-oxidoreductase (SQR) (EC 1.3.5.1) activity were assayed after [21] and [22], correspondingly. The rate of CoQ reduction (which results in production of free CoQH₂) was measured for SQR enzyme system by the rate of reduction of 2,6-dichlorophenolindophenol by CoQH₂ at λ = 600

nm, and for NQR enzyme system by the rate of oxidation of NADH at $\lambda = 340$ nm.

The buffer solution for SQR activity assay contained Tris-HCl, potassium succinate, EDTA, Triton-X100, CoQ₁, and phenazine metasulfate. 2,6-dichlorophenolindophenol sodium salt was applied to initiate reaction. H₂O was added to control solutions instead of 2,6-dichlorophenolindophenol. The decrease in optical density of buffer solution (due to 2,6-dichlorophenolindophenol reduction) was registered for 5 min. The data were used to calculate the rate of activity of the SQR complex.

The buffer solution for NQR activity assay contained Tris-HCl, sucrose, deoxycholic acid, EDTA, and CoQ₁. NADH was applied to initiate reaction. The procedure was generally analogous to that of SQR assay.

Cytochrome c oxidase activity (EC 1.9.3.1) was assayed spectrophotometrically after intensity of oxidation of reduced cytochrome c by the enzyme [23]. Reduced cytochrome c was added to mitochondria sample, and changes in optical density were measured for 3 min at $\lambda = 550$ nm.

Protein content was determined with Lowry method [24].

The results were processed with variational statistics methods. The numerical data are presented as median with standard deviation ($M \pm m$). Reliability of inter-median differences was assayed after Student's t-test.

3. Results and Discussion

EPR spectra of liver and heart tissues of animals from control group, Doxorubicin group, Doxorubicin + EPM group, and Doxorubicin + CoQ₁₀ group are presented at Figs. 1 and 2, accordingly.

According to [3, 25, 26] Fe-S proteins make the biggest contribution to EPR spectra of liver tissue of control animals. This form, intensity, and signal correlation in EPR spectrum characterizes the functional state of mitochondria from liver tissues of

control animals. EPR spectrum of liver and heart tissues of animals from group that was administered Doxorubicin has differences that may be explained as an oxidative shift in mitochondrial redox-state, characterized by decreased activity of certain centers, intensification of others, and increased production rates of superoxide anion-radicals and NO.

In liver and heart tissues of animals that received Doxorubicin appears to be a propagation of triplet EPR signal with $g = 2.007$, decrease in EPR signal intensity with $g = 1.94$ (N-2 center of NADH-CoQ-oxidoreductase) (Fig. 3a), and increase in EPR signal intensity with $g = 2.03$, that is characteristic of formation of complexes between NO

Fig. 1 EPR spectra of liver tissues. (a) liver tissue of control animals; (b) liver tissue of animals administered Doxorubicin; (c) liver tissue of animals administered Doxorubicin and EPM; (d) liver tissue of animals administered Doxorubicin and CoQ₁₀.

Fig. 2 EPR spectra of heart tissues. (a) heart tissue of control animals; (b) heart tissue of animals administered Doxorubicin; (c) heart tissue of animals administered Doxorubicin and EPM; (d) heart tissue of animals administered Doxorubicin and CoQ₁₀.

and non-heme proteins (Fig. 3b), as well as a decreased mitochondrial ubiquinone content ($g = 2.003$) (Fig. 6). The changes as observed may lead to loss of capacity for ATP synthesis by mitochondria and to switch to superoxide anion-radicals generation.

Protective application of CoQ₁₀ and EPM complex leads to renewal of electron transport through mitochondrial ETC, which is characterized by restoration of EPR spectra structural properties and decrease in rates of mitochondrial superoxide radicals production. Triplet EPR signal ($g = 2.007$) is no longer registered in EPR spectra of animal tissues, EPR signal level of N-2 Fe-S center of NADH-CoQ-oxidoreductase increases (fig. 3a) and EPR signal level of complexes between NO and non-heme proteins decreases (fig. 3b). This can be

interpreted as the evidence of the ability of EPM complex and CoQ₁₀ preparation to protect the components of mitochondrial electron transport chain from oxidation, which helps to maintain its function and, therefore, cellular energy flow.

Triplet structures at $g = 2.007$ in EPR spectra reflect the level of damage to mitochondrial ETC complexes (Figs. 1 and 2) [3]. The prominence of EPR signal change correlates with levels of NO production. Kinetics of formation of EPR signal with triplet structures at $g = 2.007$ has been used as a molecular marker for malignant transformation [3]. It is worth mentioning that in our studies such changes in mitochondrial ETC are observed in animals with no tumors.

Our studies demonstrate the decrease of NQR activity in liver mitochondria under Doxorubicin administration (Table 1). No changes in the activity of this complex in heart mitochondria were observed. Administration of EPM Complex leads to increase in NQR activity to levels of control animals.

SQR activity in liver mitochondria was observed to decrease reliably under administration of Doxorubicin. The activity of this enzyme complex in liver and heart mitochondria reliably increased under EPM administration (Table 1).

We showed decreased cytochrome c oxidase activity in heart and liver mitochondria under Doxorubicin administration. As cytochrome c oxidase is the terminal oxidase of mitochondrial electron transport chain, such negative changes in its function may prevent electron transport to ATP synthase complex and thus disrupt mitochondrial respiration. If EPM complex or CoQ₁₀ preparation had been administered concurrently to Doxorubicin administration, the cytochrome c oxidase activity of heart and liver mitochondria increased (Table 1).

Mitochondrial ETC components damage under Doxorubicin is tied to activation of NO synthesis by mitochondrial NO-synthase and by generation of NO-FeS complexes in iron-sulfur proteins of complex

Fig. 3 EPR signal intensity of liver and heart tissues. (a) N2 cluster of NADH-CoQ-oxidoreductase; (b) complexes of non-heme proteins with NO.

Table 1 NQR (complex I), SQR (complex II), and Cytochrome c oxidase (complex IV) activities of heart and lever mitochondria under Doxorubicin administration and effect of EPM complex and CoQ₁₀ preparation ($M \pm m$, $n = 6$).

	Control	D	D+EPM	D+CoQ ₁₀
Liver mitochondria				
Complex I, mmol NADH $\times$ (min $\times$ mg of protein) ⁻¹	12.26 $\pm$ 1.41	7.45 $\pm$ 1.01*	15.93 $\pm$ 2.42#	12.62 $\pm$ 1.99#
Complex II, mmol of succinate $\times$ (min $\times$ mg of protein) ⁻¹	16.38 $\pm$ 0.83	12.66 $\pm$ 1.29*	19.71 $\pm$ 2.96#	17.18 $\pm$ 1.28#
Complex IV, mmol of cytochrome c $\times$ (hr $\times$ mg of protein) ⁻¹	1.69 $\pm$ 0.26	1.18 $\pm$ 0.13*	1.43 $\pm$ 0.04#	1.34 $\pm$ 0.09
Heart mitochondria				
Complex I, mmol NADH $\times$ (min $\times$ mg of protein) ⁻¹	4.86 $\pm$ 0.11	5.18 $\pm$ 0.41	5.47 $\pm$ 0.42	5.02 $\pm$ 0.31
Complex II, mmol of succinate $\times$ (min $\times$ mg of protein) ⁻¹	14.90 $\pm$ 0.24	7.76 $\pm$ 0.87*	26.32 $\pm$ 1.43*#	17.45 $\pm$ 0.96#
Complex IV, mmol of cytochrome c $\times$ (hr $\times$ mg of protein) ⁻¹	4.97 $\pm$ 0.07	3.12 $\pm$ 0.16*	4.01 $\pm$ 0.69	4.64 $\pm$ 0.21#

* – the difference with Control group is statistically significant;

– the difference with Doxorubicin group is statistically significant; $P < 0.05$.

I, which initiates the generation of superoxide anion radicals, formulates the propagation of cellular hypoxia, and activates the matrix metalloproteinases.

True to this, we have shown that Doxorubicin administration to experimental leads to increased superoxide radicals generation in heart and liver tissues (Fig. 4).

At the same time we have observed statistically significant increase in NO levels of mentioned organs in comparison to control (Fig. 5).

We have observed statistically significant decrease in rates of superoxide radicals generation in lever and heart tissues of animals that received EPM complex and CoQ10 in addition to Doxorubicin administration (Fig. 4). The same changes were observed in NO levels—the concentrations of this mediator were approaching the levels in control animals (Fig. 5).

Doxorubicin administration correlates with twofold decrease in CoQ levels of liver tissue if compared to

control (Table 2). Concurrently to this, CoQ content in liver mitochondria increases in nearly 1.6 times, and in heart mitochondria in 2 times, if compared to control. The levels of CoQ in tissues and mitochondria of liver and heart are normalized under treatment with EPM complex and CoQ10 in addition to Doxorubicin administration.

The established consensus is that CoQ interacts with ETC enzyme complexes not directly, but through its semi-reduced free-radical form – ubisemiquinone. As the half-life period of ubisemiquinone is fairly short and it is transformed into ubiquinone or ubiquinol, the excessive amounts of CoQ are necessary to sustain the optimum concentrations of ubisemiquinone [14].

The results of studies of CoQ redox state in animals under Doxorubicin administration are presented in fig. 6. The ubisemiquinone content in liver and heart tissue under Doxorubicin administration was observed

Fig. 4 Rates of superoxide radicals generation in animal tissues under Doxorubicin administration and treatment with EPM Complex ($M \pm m$, $n=5$).

Fig. 5 NO levels in tissues of animals under Doxorubicin administration and effect of EPM complex ($M \pm m$, $n = 5$).

Table 2 CoQ content in tissue and mitochondria of liver and heart of animals under Doxorubicin administration and treatment with CoQ10 and EPM complex ($M \pm m$, $n = 6$).

Experimental groups	Liver tissue, $\mu\text{g/g}$ of protein	Liver mitochondria, $\mu\text{g/g}$ of protein	Heart mitochondria, $\mu\text{g/g}$ of protein
Control	190.70 ± 44.24	69.70 ± 9.41	110.07 ± 14.38
D	$99.01 \pm 18.46^*$	$114.74 \pm 4.67^*$	$286.60 \pm 37.46^*$
D + EPM	$207.15 \pm 37.99^\#$	$83.85 \pm 13.52^\#$	$101.26 \pm 13.23^\#$
D + CoQ ₁₀	$176.37 \pm 29.91^\#$	$63.02 \pm 2.74^\#$	$115.44 \pm 13.21^\#$

* – the difference with Control group is statistically significant;

– the difference with Doxorubicin group is statistically significant; $P < 0.05$.

to decrease in 2.1 and 2.3 times, accordingly, if compared to control. The increased CoQ content in liver and heart mitochondria (Table 2) that coincides with decreased ubiquinone content prompts us to suppose the transition of the available CoQ in these tissues into fully reduced or fully oxidized state. Treatment of experimental animals with EPM and CoQ in addition to Doxorubicin administration leads to increase in ubiquinone levels in liver and heart tissues in comparison to Doxorubicin-only group (Fig. 6), despite the fact that its content in heart tissue remains at lower levels than that of the control group animals.

The high liver levels of ubiquinones are characteristic for normal functioning of CoQ cycle in mitochondria [12]. The decrease in such levels may be tied to disruptions in electron transport, that may be the consequence of either increase in oxidative stress, or development of defensive mechanisms against increasing quantities of free radicals [5]. The drop in ubiquinone levels has been shown under ischemia-reperfusion, and cardiomyopathic pathologies [4, 5, 27].

MMP2 and MMP9 activity in heart and liver tissue increases significantly under Doxorubicin administration if compared to control (Figs. 7 and 8).

Fig. 6 Ubisemiquinone levels of liver and heart tissues of animals under Doxorubicin administration and treatment with CoQ₁₀ and EPM complex ($M \pm m$, $n = 5$). * – the difference with Control group is statistically significant, # – the difference with Doxorubicin group is statistically significant; $P < 0.05$.

Fig. 7 MMP2 activity (concentration of active forms of the enzyme) in liver and heart tissues of animals under Doxorubicin administration and treatment with EPM complex and CoQ₁₀ ($M \pm m$, $n = 5$); * – the difference with Control group is statistically significant, # – the difference with Doxorubicin group is statistically significant; $P < 0.05$.

Treatment of experimental animals with CoQ₁₀ in addition to Doxorubicin leads to decrease of many

times in activity of both gelatinases in mentioned organs. Treatment with EPM complexin addition to

Fig. 8 MMP9 activity (concentration of active forms of the enzyme) in lever and heart tissues of animals under Doxorubicin administration and treatment with EPM complex and CoQ₁₀ ($M \pm m$, $n = 5$); * – the difference with Control group is statistically significant, # – the difference with Doxorubicin group is statistically significant; $P < 0.05$.

Doxorubicin administration was also observed to significantly decrease the enzymes' activity (with the exception of liver tissue concentration of active forms of MMP9, which increased nearly tenfold).

It has been established that MMP9 is a vascular-specific gelatinase that plays a key part in angiogenesis. The EPM complex, as administered with Doxorubicin, may possibly promote angiogenesis through MMP9 activation as part of organism's adaptation to chronic effect of toxic agent.

We established the inverse correlation between ubiquinone content and gelatinases activity in heart and liver tissues (with the exception of liver MMP9) (with correlation coefficients of 0.33 to 0.64; $P < 0.05$). Thus Doxorubicin administration leads to increased gelatinases activity (which are known to be regulated by ROS) associated with decrease in ubiquinone content caused by disruption of electron transport. Treatment of experimental animals with EPM complex and CoQ₁₀ in addition to Doxorubicin administration leads to decrease in MMP2 and MMP9 activities accompanied by

increased ubiquinone content in the tissues. Consequently, the inverse correlation between these events reflects the magnitude and trend in oxidative stress development.

Therefore, Doxorubicin may deprive cells of one of the most important preconditions of their normal functioning – namely, the ability to maintain the redox state homeostasis and the pool of oxidative-reductive components. Mitochondrial dysfunction and, primarily, mitochondrial I and III complexes dysfunction, forms the basis of oxidative stress progression and is the molecular mechanism underlying the disorders of energy exchange that result in all known forms of hypoxia and proteinase activation [2, 5, 20].

We demonstrated that Doxorubicin administration damages mitochondrial ETC, evidenced by triplet structures formation at $g = 2.007$ in EPR spectra, and this can be used as a marker of Doxorubicin toxicity and as a target for therapeutic efforts. Moreover, decrease in ubiquinone level was also shown.

Increased MMP2 and MMP9 activities under

Doxorubicin administration may lead to damage to extracellular matrix integrity that, given the presence of tumor tissue, can increase the risk of metastasizing.

Treatment of experimental animals with EPM complex and CoQ10 in addition to Doxorubicin administration exerts a protective effect on liver and heart cells' mitochondria, evidenced by restoration of electron transport in respiratory chain, which is expressed as decreased nitrile complexes formation with Fe-S-proteins and increased ubisemiquinone content. It should be stressed that the protective effects of EPM complex on mitochondrial ETC under Doxorubicin administration is on par with those of CoQ10. Concurrently, MMP2 and MMP9 activities are decreased, which gives evidence of lessened extracellular matrix destruction. CoQ10 appeared to be more efficient at this if compared to EPM complex.

The experimental data obtained may become the basis of development of approaches to correction of adverse effects of Doxorubicin by treatment with CoQ10 and the complex of precursors and modulators of its biosynthesis. These data may be used to substantiate the application of these biologically active substances within frameworks of complex treatment of oncological pathologies.

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