EXPERIMENTAL WORKS

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CURCUMIN EXERTS PROTECTIVE EFFECTS AGAINST DOXORUBICIN-INDUCED CARDIOTOXICITY

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The mechanism of doxorubicin (Dox) cardiotoxicity involves different pathways, including oxidative stress and mitochondrial dysfunction. It's supposed that pharmacological effect on HIF gene expression may protect the heart against the detrimental effects of the doxorubicin-induced injury. We hypothesized that the cardioprotective effects of Curcumin (Curc) are exerted by regulating HIF and its target genes expression. To test this, an in vitro model of Dox-induced injury to primary myocardial cardiomyocytes was used. Isolated Wistar rat neonatal cardiomyocytes were incubated in the culture medium for 24 h in control, either with Dox (0.5 μmol/ml) or Curc (20 μmol/ml), or in their combination in the same doses. Mitochondria were isolated from rat cardiomyocytes culture. It was demonstrated that cardiomyocytes exposure to Dox led to an increase in the activity of oxidative stress markers in isolated mitochondria, a decrease in the efficiency of the respiratory chain and phosphorylation processes, decline of membrane potential and the rate of K^+ ions entry into mitochondria. Doxorubicin inhibited the expression of mRNA of both HIF-1α, 2α, 3α subunits and its important target genes PDK-1 and IGF-1 in mitochondria. A negative impact on the cardiomyocyte contractile activity was observed. The combined use of doxorubicin with curcumin led to an increase of cardiomyocytes viability and attenuation of oxidative stress in mitochondria, prevented the development of mitochondrial dysfunction and significantly improved the contractile activity of cardiomyocytes.

K e y w o r d s: cardiomyocyte, mitochondria, doxorubicin, oxidative stress, curcumin, expression of HIF subunits, membrane potential.

Antitumor doxorubicin treatment is closely linked to cardiovascular impairments, endothelial dysfunction, arterial stiffening, thrombosis, etc. [1] Doxorubicin-induced cardiotoxicity is related to genetic factors, mutations, telomere loss, oxidative stress, mitochondrial dysfunction, inflammation, immune disorders, and other homeostatic impairments.

It is known that mitochondria are responsible for ensuring a constant flow of energy to maintain the redox state of cells, pathogenetically causing an increase in the level of reactive oxygen species (ROS) [2, 3]. Excess ROS formation (predominantly in mitochondria) leads to the development of oxidative stress with contraventions of metabolic processes, structural and molecular components of cells, in particular, mitochondria itself and its genome, which is the cause or link of the severe disease pathogenesis. Thus, activation of free radical oxidation (FRO) and oxidative stress (OS) is one of the leading pathogenetic mechanisms of heart damage by doxorubicin [4].

In most antioxidant studies nowadays, compounds from natural sources are used. Curcumin, a yellow pigment derived from the rhizome part of the turmeric plant (Curcuma longa), in different *in vitro* and *in vivo* studies, showed varieties of pharmacological functions, including anti-inflammatory, antioxidative, antiproliferative, cholesterol-lowering and neuroprotective [5-8].

Regarding cardiovascular disorders, it is generally accepted that the overproduction of ROS plays a critical role in the genesis of heart tissue injury under pathological conditions, such as ischemia-reperfusion [9], hypoxia-reoxygenation [10], metabolic syndrome [11], as well as under cardiovascular diseases such as cardiomyopathy [12], coronary heart disease [13], vascular pathology [14], etc.

To the best of our knowledge, this is the first work showing the effects of curcumin on preventing OS development in heart tissue under doxorubicin treatment.

Materials and Methods

The experimental studies were performed on the primary culture of isolated cardiomyocytes from the heart ventricles of 50 newborn Wistar rats (5.0 ± 0.5 g). The experimental procedures were approved by the regional ethics committee according to Directive 2010/63/EU of the European Union.

Cultivation of neonatal cardiomyocytes. Isolation and cultivation of rat neonatal cardiomyocytes were performed according to the modified method [15]. Through cervical dislocation, the rats were immobilized and anesthetized. Thereafter, through the anterior longitudinal incision, the heart was removed. The ventricles were separated from the atrium and washed twice in a sterile buffered saline solution (pH 7.4). Enzymatic cleavage was carried out in a separation medium containing type II collagenase (95 U/ml) and pancreatin (0.6 mg/ml). The cells were precipitated by centrifugation at 400 g for 60 s and resuspended in a culture medium. The number of propidium-positive and propidium-negative cells was determined using staining cells with a 0.2% trypan blue solution. Cultivations were carried out in a nutrient medium of the aforementioned composition at 37°C in a gas medium containing 5% CO_2 and 95% atmospheric air.

Modeling of cardiotoxicity in the culture of neonatal cardiomyocytes with doxorubicin and its correction with curcumin. Incubation of neonatal cardiomyocytes with doxorubicin hydrochloride (Sigma Aldrich) at a concentration of 0.5 μmol/ml in DMEM + 199 culture medium was performed for 24 h. Curcumin (Sigma Aldrich) at a concentration of 20 μmol/ml was added to the culture medium for 24 h.

Estimation of the viability of cardiomyocytes in culture (staining of mitochondria with PI and Hoechst). To calculate the number of living and dead cells, methods of coloring bis-benzimide (Hoechst 33342) and propidium iodide at a concentration of 8.75 μmol/l and fluorescence microscopy (Nikon Eclipse E200), D/PI filter, wavelength excitation 330-380 and 510-560 nm for Hoechst and propidium iodide were used, respectively.

Estimation of the viability of cardiomyocytes in culture (MTT-test). To quantify the viability of cardiomyocytes and the mitochondria functional capacity during doxorubicin incubation, the MTT-test (MTT Protocol, Wallertand Provost Lab) was used. Cells were planted in 96-well plates and incubated with doxorubicin hydrochloride in DMEM + 199 for 24 h. Then, 20 μl of MTT stock solution (5 mg of MTT (Sigma Aldrich)) per ml of PBS was added to each well and incubated for 4 h. The result was evaluated by measuring CFL-45 ColorFlex (Hunter Lab Inc., USA) with a lysate optical density at a wavelength of 570 nm.

Investigation of the contractile activity of neonatal cardiomyocytes. To determine and evaluate the frequency of spontaneous contractions of isolated neonatal cardiomyocytes, the technique described in [16] was used. The contractile ability of cardiomyocytes was evaluated by determining the following indicators: peak reduction (PR, or amplitude reduction, in microns or %), time to achieve PR (ms), recovery time (ms), maximum contraction rate (MContr.R, μm/s, or -dl/dt) and relaxation (MRel.R, μm/s, or + dl/dt) [17-20]. Registration and analysis of the contractile function of the cells were performed using the IonOptix system, which scans the linearity of the edge of the cardiomyocyte at its reduction [21-24]. At all stages of the study, the cells were observed under a microscope (Olympus SKX 41) and on a computer monitor using the IonOptix MyoCam camera.

Isolation of mitochondria from the culture of rat neonatal cardiomyocytes. Mitochondria (Mx) from the myocardium were isolated by the generally accepted method of differential centrifugation, provided that the native state of the isolated organelles was preserved [25]. The animals were sacrificed, the heart was removed, and it was immediately cooled with a medium for isolating mitochondria (0.25 M sucrose solution and 1 mM EDTA solution, pH 7.4). One gram of tissue was taken and homogenized in a medium for isolating mitochondria at 2°C. The homogenate was centrifuged at 700 g. The supernatant containing mitochondria was separated and centrifuged at 9000 g for 15 min. The mitochondria

obtained in the precipitate were washed with a cold solution of 0.25 M sucrose and used for research.

Investigation of oxidative phosphorylation, the level of the potential-sensitive fluorescence component in mitochondria, and the rate of potassium ion's entry to mitochondria. The processes of mitochondrial respiration and phosphorylation were investigated by [26] using a polarographic method using Clark's closed electrode and the Oxygraph System (Hansatech, England). Mitochondria isolation was carried out at 4°C. As an inhibitor of mitochondrial enzyme complex I served rotenone – 2 μmol/l. Breathing was stimulated by insertion into a polarographic cell of 200 μM ADP. Using the obtained chrono-amperographic curves, the parameters of mitochondrial respiration were calculated: active respiration with the addition of ADP (V3), controlled respiration (V4/ATP), respiratory control (V3/V4ATP), the coefficient of phosphorylation efficiency (ADP/O) by [27]. The protein concentration was determined by [28]. The evaluation of the ATP-dependent K^+ input rate in mitochondria (which characterizes the activity of the mitoK_{ATP} channels) was determined by the polarographic method mentioned above for the difference in the mitochondria respiration rate in the metabolic state 4 (V4ATP), which was recorded in the presence and absence of 300 μM ATP with the addition of 2 μM oligomycin. For the quantitative estimation of the ATP-dependent input of potassium ions in mitochondria, known stoichiometric coefficients between oxygen uptake and transport of monovalent cations (1:7 using succinate as a substrate of oxidation) were used [29].

For the indirect determination of the mitochondrial membrane potential value, Mitotracker DeepRedFM (Sigma Aldrich), a DMSO dilute, at a concentration of 100 nM was used as a lifetime fluorescence dye. The intensity of the coloration of living mitochondria was measured using a Nikon Eclipse E200 microscope at a wavelength of 665-644 nm [30]. The degree of fluorescence intensity indirectly indicated the magnitude of the mitochondrial membrane potential [31].

Methods of evaluation of mitochondrial pro- and antioxidant systems activity in neonatal cardiomyocyte culture. To evaluate the activity of pro- and antioxidant systems, mitochondria were taken from the cups with 0.2% EDTA, pH 8.1 containing 0.15% trypsin, incubating with the above solution for 15 min. Protein content was determined using the Bradford method [32]. The degree of oxidative stress was evaluated by the content of active products of 2-thiobarbituric acid (TBARS) and hydrogen peroxide in mitochondria. Antioxidant protection was assessed by the level of Mn-superoxide dismutase (Mn-SOD) and catalase (CAT) activity. The content of TBARS was determined spectrophotometrically by the method [33]. The content of hydrogen peroxide (H_2O_2) was determined by the method [34] in the system of lactoperoxidase/H_2O_2/potassium iodide. The activity of CAT was determined spectrophotometrically by the method [35]. The activity of Mn-SOD was determined spectrophotometrically [36].

Isolation of total RNA. Isolation of total RNA from the culture of neonatal cardiomyocytes was performed using a phenol-chloroform extraction method with Sigma reagents (USA). The isolated RNA was stored at -35°C or immediately used for work after measuring its concentration spectrophotometrically.

Semi-Quantitative Reverse Transcription and Real-time Polymerase-Chain Reaction (PCR). Semi-quantitative reverse transcription was performed using cytokine synthesis kits containing the reverse transcriptase "RevertAid H Minus M-MuLV RT" (Thermo Scientific, USA). PCR was performed in the Applied Biosystems 2700 (Perkin Elmer, USA) thermocycler according to individual programs for each gene. Real-time PCR was performed in the "7500 Fast Real-Time PCR System" thermocycler. Expression of genes was standardized according to the expression of GAPDH gene of the ribosomal subunit and the actin β-subunit as an endogenous control. PCR amplification of the gene was performed in 10 μl SYBR Green PCR Master Mix containing 30 pmol of each primer. The amplification program began with the previous activation of AmpliTaq Gold® DNA polymerase for 10 min at 95°C. For PDK-1, TERT, and IGF-1 genes, PCR amplification was performed in 10 μl of SYBR Green PCR Master Mix containing 30 pmol of each primer. The amplification program and specificity control did not differ from the ones outlined above.

Statistical analysis. Statistical processing of the results was performed using Microsoft® Excel 2003 software, as well as ImageJ, Origin, 7500 Fast Real-Time PCR System Software and SPSS Statistics (Version 17) software. The average values of the indicators (M) and the average error (m) were calculated. The normality of distributions was checked using the Kolmogorov-Smirnov test. The intergroup differences with the normal sample were evaluated

using Student's *t*-test or ANOVA. To calculate the error, a Bonferroni correction was used. For samples that did not satisfy the normality criterion, Mann-Whitney's nonparametric criterion was applied. To determine the correlation between the individual indicators, the Pearson correlation coefficient (*r*) and the methods of correlation-regression analysis were used [37]. To determine the validity of the differences between the expected splitting and the obtained method, $\chi 2$ was used. Results were considered statistically significant at $P < 0.05$.

Results

To determine the doses of doxorubicin and curcumin that are optimal for further studies, we conducted an MTT test. According to its results, the level of cytotoxicity of different concentrations of doxorubicin and the viability of cardiomyocytes can be estimated. When incubated with doxorubicin at concentrations of 0.1, 0.5, and 1.0 μM, the number of live cells relative to control decreased by 10.9 ± 9.99, 23.6 ± 8.76, and 31.5 ± 9.86%, respectively ($P < 0.05$ in all cases) (Fig. 1).

This confirmed the literature data concerning the cardiotoxicity of anthracyclines and made it possible to select an average dose of doxorubicin of 0.5 μM as the optimum for further studies. The correlation coefficient between the number of live cells and the concentration of doxorubicin added to the culture was -0.95.

As for the dose of curcumin, we were guided by the literature data. Thus, different authors showed that curcumin at 10–20 μM reduces the cardiac ischemia-reperfusion injury and high glucose-induced apoptosis of cardiomyocytes [38-40]. So, we decided to use 20 μM curcumin for our *in vitro* studies.

Estimation of cell death in the culture of neonatal cardiomyocytes. Using the method of dual staining cells with fluorescent dyes Hoechst 33342 and propidium iodide, it was shown that after incubation of the neonatal cardiomyocyte culture with doxorubicin (0.5 μM), the proportion of propidium iodide negative cells fell by 30.4 ± 0.015%, and the number of propidium iodide positive cells raised by 4.5 times compared with the control ($P < 0.05$) (Fig. 2). Incubation with doxorubicin and curcumin resulted in an increase in cell viability of 9.4 ± 0.01%, and the number of propidium iodide positive cells decreased by 19.5 ± 0.01% versus doxorubicin alone.

Investigation of the potential-sensitive fluorescent component level in mitochondria of rat neonatal cardiomyocyte culture. The mitochondrial membrane potential can be estimated by determining the level of a number of potential-sensitive fluorescent components *in vitro*. The Mitotracker dye fluorescence activity may indirectly indicate the magnitude of the mitochondrial membrane potential [41]. Using the fluorescence method (Fig. 3), we found a significant decrease in the intensity of mitochondria coloring under the influence of doxorubicin - by 42% compared to control ($P < 0.05$). The co-incubation of cells with curcumin and doxorubicin resulted in a significant increase in the fluorescence intensity of mitochondria (2-fold, compared to the use of doxorubicin alone) (Fig. 3).

Expression of mRNA HIF-1α, 2α, and 3α and their target genes (PDK-1, TERT, and IGF-1) in mitochondria isolated from neonatal cardiomyocyte culture. In the study of the HIF-1α gene and its target genes TERT, IGF-1, and PDK-1 expression levels in the culture of neonatal cardiomyocytes, it was found that the HIF-1α mRNA level was reduced by

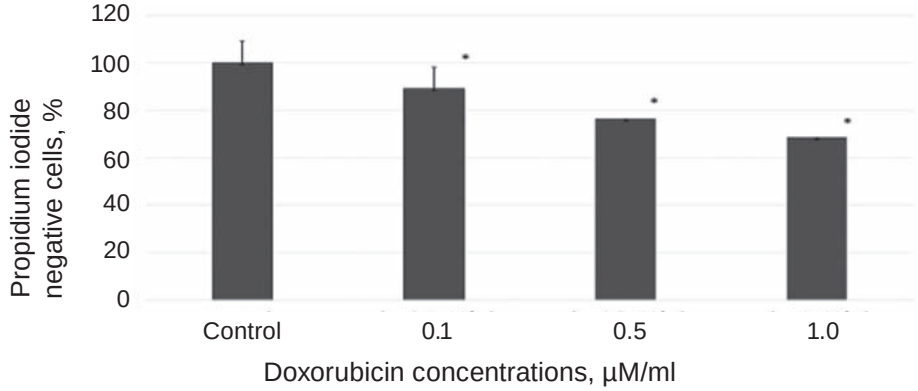

*Fig. 1. Percentage of live cardiomyocytes with 0.1, 0.5 and 1 μM doxorubicin according to MTT-test. *P < 0.05 compared to control*

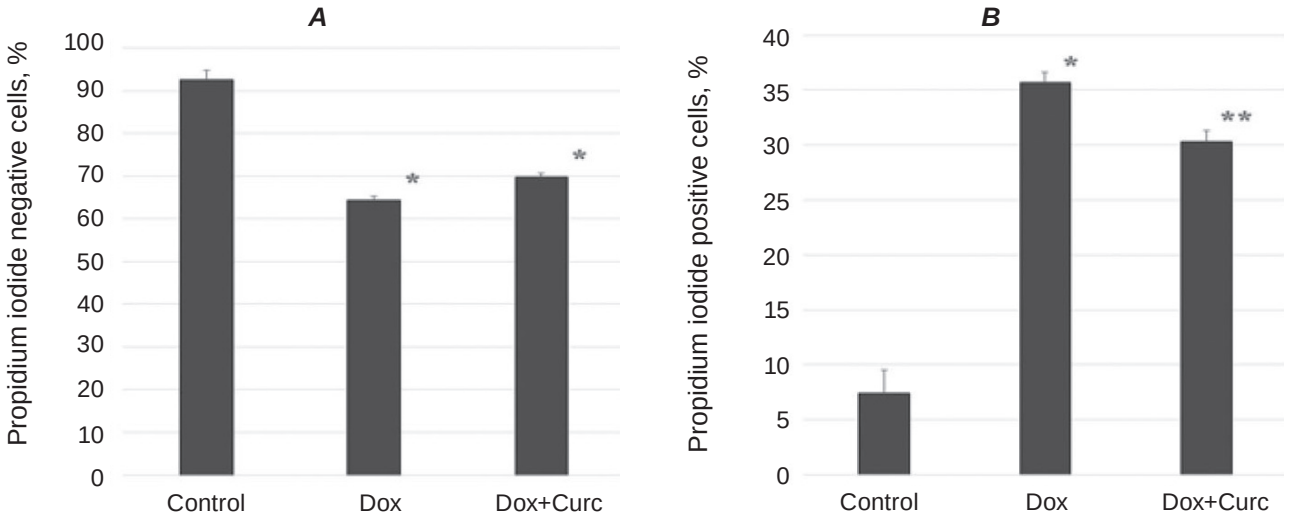

*Fig. 2. Percentage of live (**A**) and propidium iodide positive (**B**) cells with 0.5 μM doxorubicin (Dox) alone and 20 μM curcumin (Curc) according to fluorescence microscopy. *P < 0.05 compared to control; **P < 0.05 compared to doxorubicin administration*

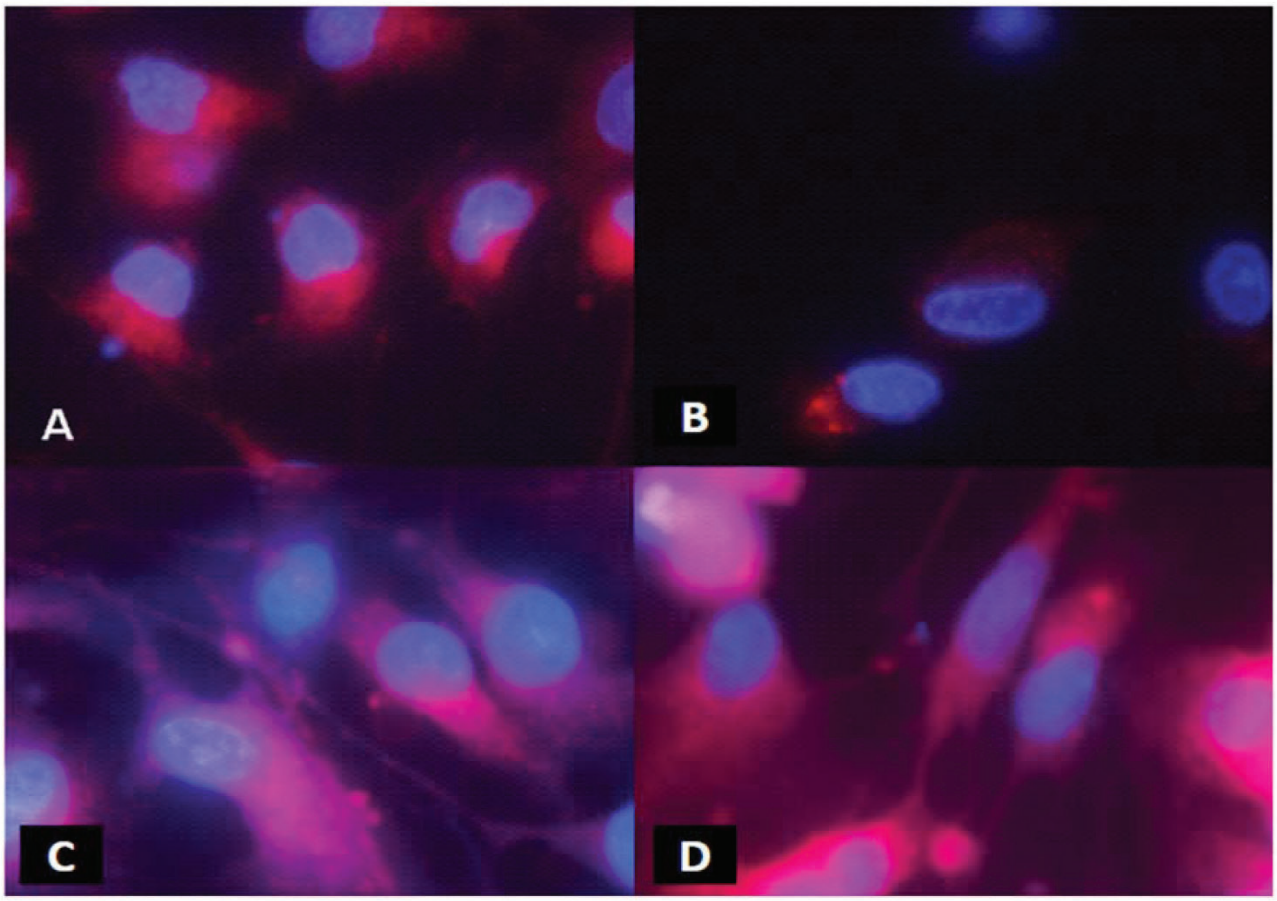

*Fig. 3. Changes in the fluorescence intensity of mitochondria in the culture of neonatal cardiomyocytes (staining with dyes Mitotracker DeepRed FM and Hoeshst 33342). **A** – control; **B** – after incubation with doxorubicin (0.5 μmol/ml); **C** – with curcumin (20 μmol/ml), **D** – when using them together in the same doses. Fluorescence microscope, × 40000*

50.3% when doxorubicin was administered and by 63.3% under curcumin administration, compared to control. With the combined administration of doxorubicin and curcumin, the expression of the HIF-1α gene decreased by 55.7% compared to doxorubicin alone (Fig. 4, *A*). The HIF-2α mRNA level fell dramatically (39 times) when doxorubicin was administered and 1.5 times under curcumin administration compared to the control. With the combined effect of doxorubicin and curcumin, the expression of the HIF-2α gene increased 17 times compared with the addition of doxorubicin alone (Fig. 4, *B*). The HIF-3α mRNA level did not almost express when doxorubicin was administered; its level decreased 3.8 times under curcumin administration and 48 times under the combined effect of doxorubicin with curcumin (Fig. 4, *C*).

Furthermore, there was a rise in the expression level of the TERT target gene under the action of doxorubicin and curcumin alone, by 51.3 and 24%, respectively, compared to the control. The use of doxorubicin with curcumin caused a decrease in the expression of the TERT gene (57.2%) compared to doxorubicin alone (Fig. 4, *D*).

The expression of the target gene IGF-1 was significantly reduced by doxorubicin and curcumin use alone and their combined use by 51.3, 68.1, and 59.3%, respectively, compared to control (Fig. 4, *E*).

The level of the target gene PDK-1 exposure to doxorubicin decreased by 37.7% compared to the control. In the case of curcumin administration and their combined use, expression of this gene was 43.1 and 44.8% lower than control, respectively (Fig. 4, *F*).

Study of pro- and antioxidant system indices in mitochondria isolated from neonatal cardiomyocytes. Our studies have shown that doxorubicin intoxication leads to a significant rise in secondary lipid TBARS by 3.6 times and hydrogen peroxide by 64% over the control group ($P < 0.05$) (Fig. 5), indicating an intensification of FRO in cardiomyocytes. Prolonged administration of doxorubicin reduced the enzymatic activity of Mn-SOD by 32%, while catalase activity was increased by 72% compared to control ($P < 0.05$) (Fig. 5). Adding curcumin at a dose of 20 μM to the cardiomyocyte cell culture increased the activity of Mn-SOD by 14% and catalase by 23% compared to control ($P < 0.05$). Furthermore, 20 μM of curcumin added to the neonatal cardiomyocyte culture for 24 h raised the TBARS and H_2O_2 levels by 1.4 times ($P < 0.05$) and by 20%, respectively, compared to control ($P < 0.05$). Incubation of the cardiomyocyte culture with doxorubicin and curcumin resulted in a significant fall in the level of free radical processes, in contrast to doxorubicin intoxication. Reduction in the content of TBARS and H_2O_2 (by 56.7 and 18.4%, respectively, $P < 0.05$), with a decrease in hyperactivation of catalase (by 19%, $P < 0.05$) and an increase in activity of Mn-SOD (by 35%, $P < 0.05$) (Fig. 5), indicates signs of restoring the prooxidant-antioxidant balance in cardiomyocytes.

We also found a link between the viability of neonatal cardiomyocytes and the level of FRO in these cells under the doxorubicin influence. Thus, a correlation-regression analysis of the relationship between the level of living cardiomyocytes and the content of TBARS, H_2O_2, and catalase activity allowed us to establish a negative relationship between the indicated parameters. In particular, the r correlation coefficient for TBARS was -0.97, for H_2O_2 -0.63, and for catalase -0.62. Thereby, the higher the level of OS, the lower the viability of cardiomyocytes.

Estimation of changes in parameters of respiration and phosphorylation of mitochondria isolated from neonatal cardiomyocyte culture. We have found that in mitochondria after doxorubicin administration, the active respiration rate (V3) decreased by 43.8%, the respiratory control ratio (V3/V4) – by 47%, and the ADP/O ratio – by 31.7% compared to control (Table 1). The ADP/O ratio (ADP converted to ATP per unit oxygen consumed) provides an index of the efficiency of ATP production. The respiratory control ratio (RCR or ratio of state 3 to state 4 respiration) also provides an index of the efficiency of ATP formation.

We investigated the role of mitochondrial KATP channels in regulating mitochondrial energy metabolism. A study of the transport rate of potassium ions showed that under the action of doxorubicin, the entry of K^+ in the mitochondria declined by 27.7% compared to control (Table 1). Thus, it can be assumed that doxorubicin suppresses the entry of K^+ through KATP channels.

To test this hypothesis, we investigated the effect of the classical inhibitor of ATP-dependent mitochondrial potassium channels 5-hydroxidecanoate and obtained similar results: the input of K^+ in mitochondria showed a significant fall (by 21% compared to control), and the index of oxidative phosphorylation efficiency also changed similarly.

At the same time, after the doxorubicin and curcumin combined administration, mitochondrial

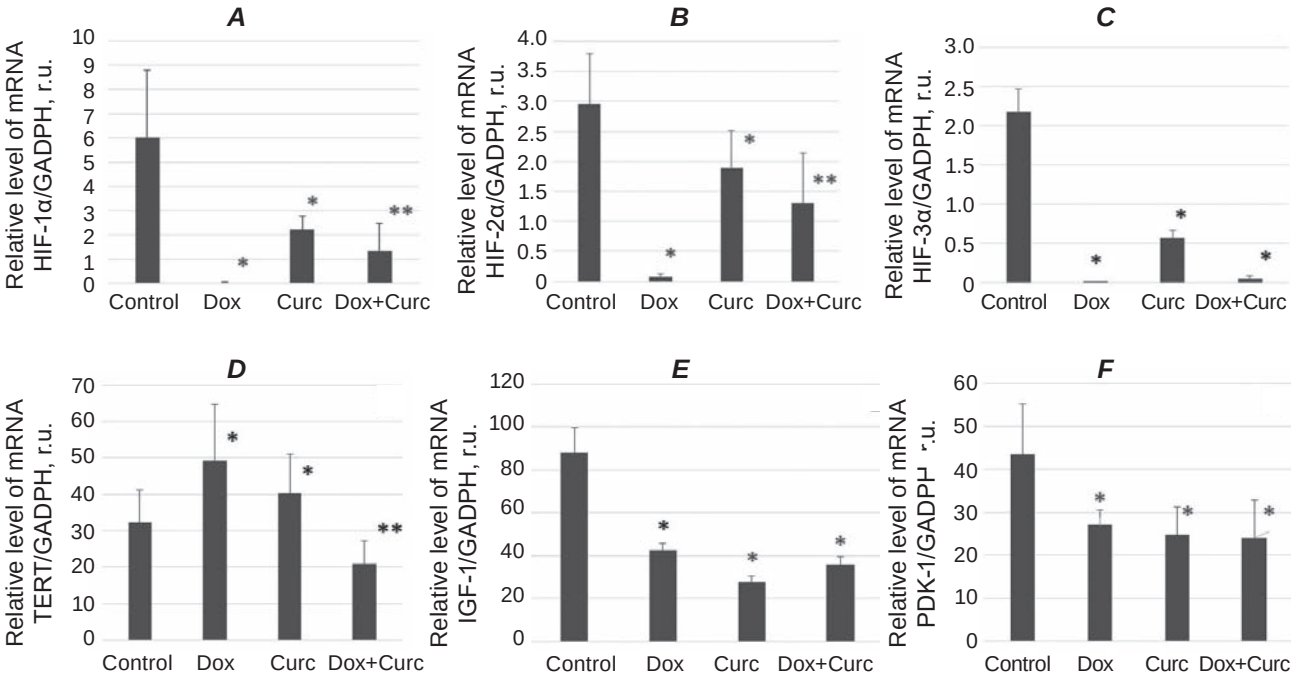

*Fig. 4. Expression of mRNA HIF-1α (**A**), HIF-2α (**B**), and HIF-3α (**C**) and target genes TERT (**D**), IGF-1 (**E**), and PDK-1 (**F**) in the culture of neonatal cardiomyocytes of rats in real-time PCR: *P < 0.05 compared to control; **P < 0.05 compared to doxorubicin administration*

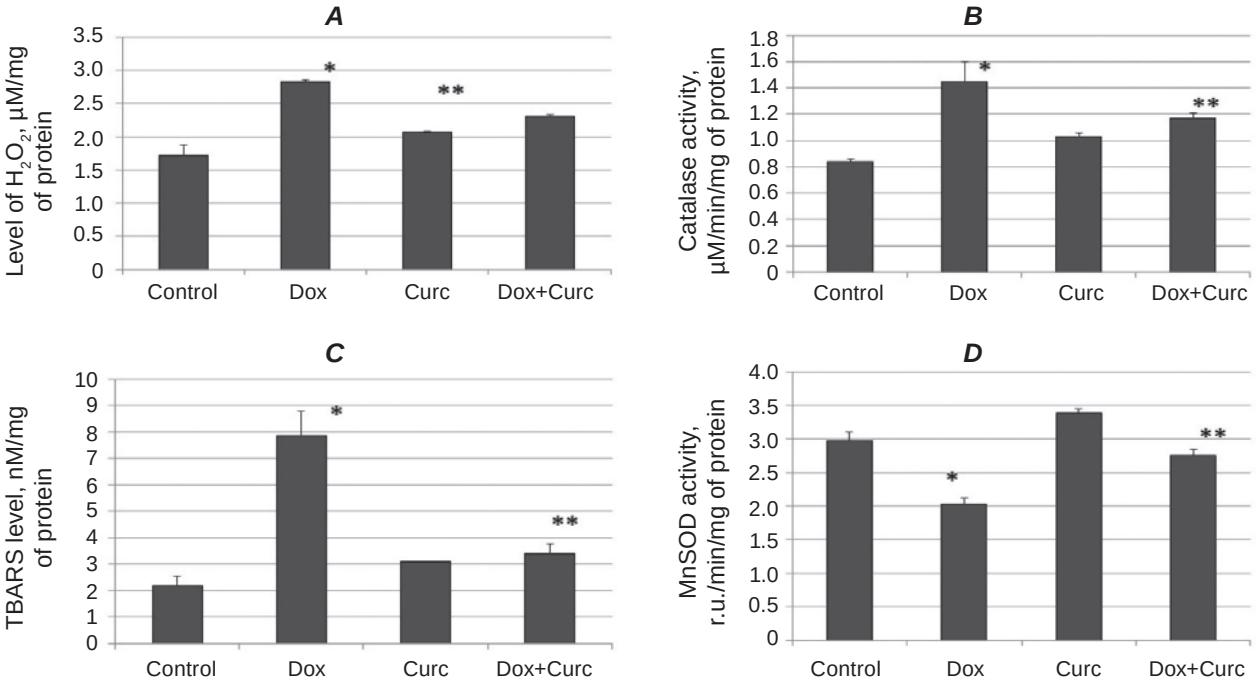

*Fig. 5. The levels of H_2O_2 (**A**), catalase activity (**B**), TBARS (**C**) and activity of Mn-SOD (D) in the culture of neonatal cardiomyocytes under incubation with doxorubicin (0.5 μM), curcumin (20 μM) and their combined administration. *Compared to control (P < 0.05), **compared with the action of doxorubicin (P < 0.05)*

Table 1. Indices of oxidative phosphorylation and the rate of potassium ions entry in rat myocardial mitochondria

Parametrs	Control	Doxorubicin	Doxorubicin + curcumin
V3, nM·min^{-1}/mg of protein	63.70 ± 3.44	35.80 ± 2.77*	44.70 ± 2.13*$^{\#}$
V4ATP, nM·min^{-1}/mg of protein	22.10 ± 2.23	23.50 ± 2.11	24.90 ± 2.18
V3/V4	2.88 ± 0.19	1.52 ± 0.09*	1.80 ± 0.11*$^{\#}$
ADP/O	1.67 ± 0.12	1.14 ± 0.05*	1.28 ± 0.04*$^{\#}$
The rate of the K ions entry, μM·min^{-1}/mg	52.20 ± 2.57	37.6 ± 2.2*	46.07 ± 2.05*$^{\#}$

Note. Oxidation substrate: 5 mM succinate Na. *$P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with the introduction of doxorubicin; $n = 12$

respiration and phosphorylation rates improved compared to those with the use of doxorubicin alone. Thus, all the parameters were raised: V3 – by 25%, the V3/V4 ratio – by 18%, and the index of phosphorylation efficiency ADP/O – by 12%. The rate of K^+ entry into mitochondria also increased by 22.5% compared to the administration of doxorubicin alone (Table 1).

Investigation of the contractile activity of neonatal cardiomyocytes. It has been established that doxorubicin causes significant changes in all parameters of their contractile activity compared to control. Thus, there was a rise in the frequency of spontaneous contractions by 2 times, a violation of their rhythmicity (fluctuations from 0.98 ± 0.25 to 3.32 ± 0.80 s), a decrease in amplitude (0.98 ± 0.10 μm), a percentage of shortening (reduction to 4.07 ± 0.47%), and an increase in the maximum speed of contraction and relaxation (by 20.5 and 19.5%, respectively) without significant changes in the duration of these processes (Fig. 6).

Incubation with doxorubicin and curcumin resulted in a significant reduction in the frequency of spontaneous contractions (3 times), the restoration of their rhythmicity, a rise in amplitude and percentage of shortening by 2 times, and an increase in the maximum reduction speed and in the relaxation speed by 1.8 and 16%, respectively. There were no significant changes in the duration of these processes compared with doxorubicin monoconduction (Fig. 6).

Discussion

It has been established that the activation of free radical oxidation is one of the leading pathogenetic mechanisms of cardiomyocyte damage by anthracyclines antibiotics, which are known to be particularly cardiotoxic and have the ability to potentiate and cumulate [4, 42]. Doxorubicin is capable of directly damaging the cytoplasmic membrane [43]. Besides the typical cytostatic side effects, doxorubicin has a significantly more pronounced toxic effect on the heart [44]. It causes destructive-dystrophic changes in the myocardium of animals: swelling of separate muscle fibers, degeneration of myofibrils, homogenization, vacuolization, resorption of sarcoplasm, and violation of the nuclear structure [42, 45], which in turn adversely affects the contractile ability of cardiomyocytes. The above changes lead to myocardial dysfunction and the development of heart failure.

The HIF-system activation is crucial to protecting the body against oxidative stress conditions. HIF-1 also activates transcription of genes encoding glucose transporter GLUT1 and hexokinases HK1 and HK2, which are required for the high level of glucose uptake and phosphorylation that is observed in metastatic cancer cells, and pyruvate dehydrogenase kinase 1 (PDK1), which shunts pyruvate away from the mitochondria, thereby increasing lactate production [46, 47]. However, daily administration of doxorubicin potently inhibited the transcription of a HIF-1-dependent reporter gene as well as endogenous HIF-1 target genes [48].

Currently, various substances, both chemical and vegetable, are used as protection agents against oxidative stress. We decided to use the plant antioxidant curcumin as a possible cardioprotective agent against oxidative stress. This substance has a protective effect on myocardial damage and helps maintain heart functions [49] while simultaneously affecting the activity of cytokines, enzymes, and transcriptional factors associated with the development of oxidative stress [50] and hypoxia, also reducing the toxic effect of doxorubicin on the heart due to its powerful antioxidant properties [51].

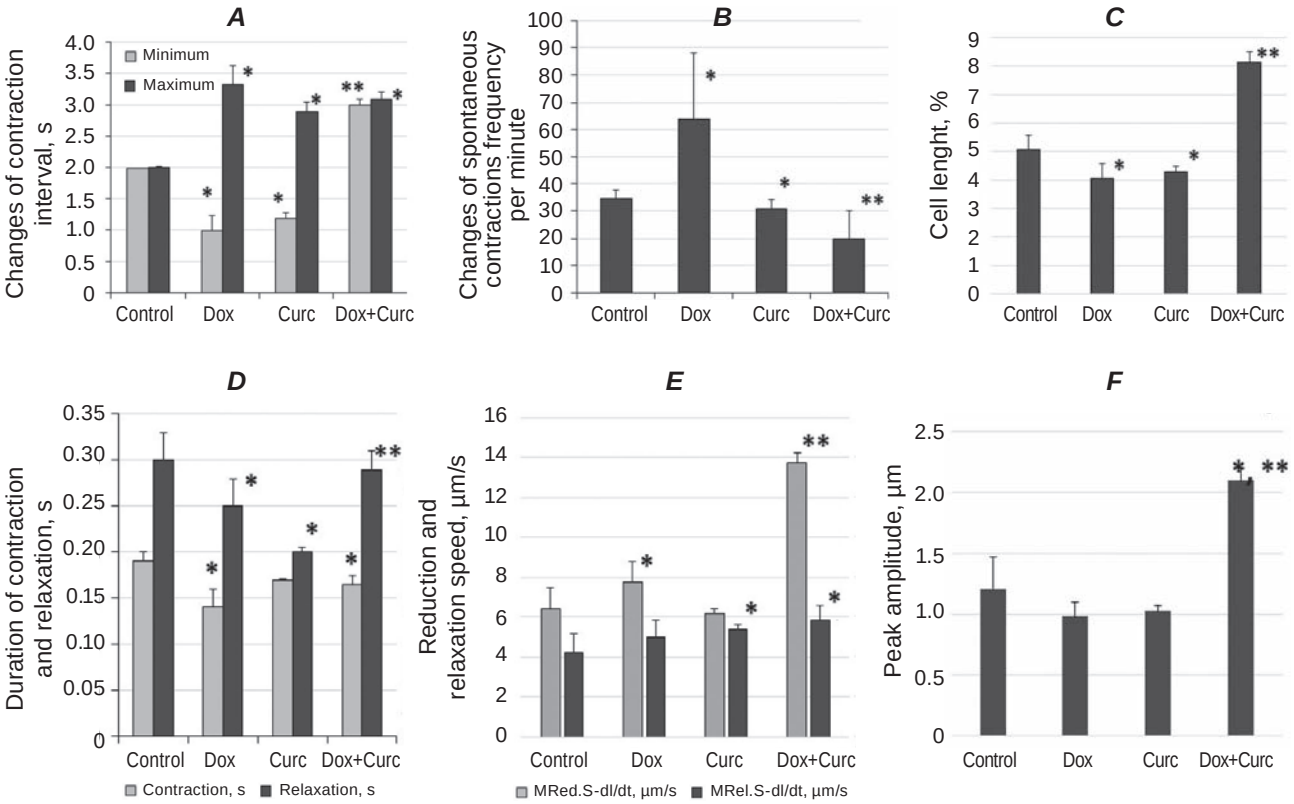

*Fig. 6. Abbreviations rhythmicity (**A**), frequency of spontaneous contractions (**B**), cell length changes (**C**), duration of contraction and relaxation (**D**), maximum reduction and relaxation speed (**E**), and change of peak amplitude (**F**) of neonatal cardiomyocytes after incubation with doxorubicin (0.5 µmol/ml), with doxorubicin (0.5 µmol/ml) and curcumin (20 µmol/ml). *P < 0.05 compared with control; **P < 0.05 compared with the influence of doxorubicin*

This led us to use the doxorubicin model for the reproduction of oxidative stress *in vitro* and made it possible to study the deep genetic-molecular mechanisms of the anthracyclines antibiotic influence on the mitochondrial apparatus of the heart cells.

There is a large variety of doxorubicin doses in the literature, which are used to simulate doxorubicin-induced oxidative stress in the culture of cardiomyocytes [52, 53]. The same situation exists with curcumin, whose dose depends on many factors, such as administration methods, fast or delayed effect we expect, etc. Therefore, foremost, we needed to determine the concentration of doxorubicin, which could cause changes that are characteristic for oxidative damage but did not lead to significant death of cardiomyocytes. It is known that the MTT test characterises the intensity of oxidative-reducing processes. According to its results, it is possible to draw conclusions about the level of cytotoxicity of different doses of doxorubicin and the viability of cardiomyocytes. When incubated with doxorubicin in doses of 0.1, 0.5, and 1.0 µmol, the number of live cells relative to control decreased in a dose-dependent manner. This confirmed the literature data on the cardiotoxicity of anthracyclines and made it possible to select an average dose of doxorubicin of 0.5 µmol as optimal for our studies [54].

As for the dose of curcumin, we were guided by the literature data. Thus, Kim et al. (2012) showed that the curcumin (10 µM) pretreatment reduced the cardiotoxicity caused by TNF-alpha, peptidoglycan, or H/R in rat cardiomyocytes [38]. Curcumin also attenuated the I/R-induced toxicity through the SIRT1 pathway [39]. Treatment of primary cultures of neonatal rat cardiomyocytes with curcumin (10-20 µM) reduced the glucose-induced toxicity by decreasing the apoptotic rate and ROS production. Curcumin reduced the toxicity through the NADPH-mediated oxidative stress and PI3K/Akt pathway [40]. So, we decided to use 20 µM curcumin for our *in vitro* studies.

Relying on facts that doxorubicin-induced oxidative stress causes cardiomyocyte death through the different pathways (apoptosis, autophagy, necrosis), and the use of curcumin alone, in turn, is both antioxidant and apoptosis-provoking, we investigated the level of neonatal cardiomyocyte cell death. Using the method of cell dual staining with Hoechst and propidium iodide fluorescent dyes, it was shown that after the incubation of the neonatal cardiomyocyte culture with doxorubicin at a dose of 0.5 μm, the proportion of living cells significantly decreased and the number of propidium positive cells increased by 4.5 times compared to control ($P < 0.05$). Cell death was mainly due to necrosis, which resulted in the loss of the integrity of the cell's plasma membrane and its ruptures [55]. After incubation with curcumin at a dose of 20 μmol, the number of live cells decreased slightly, and the rate of propidium iodide positive cells increased by 3.25 times ($P < 0.05$) versus control, which may be due to the proapoptotic effect of curcumin on the neonatal cardiomyocyte culture in its direct application. In this case, the combined use of doxorubicin and curcumin increased the viability of cells, and the number of dead cells decreased accordingly. Our data about doxorubicin-induced necrosis of cardiomyocytes coincide entirely with the studies of doxorubicin-induced necrosis in kidney cells [56]. The authors found that this form of necrosis is mediated by the action of PARP1 (poly-(ADP-ribose)-polymerase 1), a nuclear protein that induces inhibition of ATP synthesis, ROS generation, DNA damage, and cell cycle stopping [57].

In addition, the results of the MTT test allowed us to conclude such changes in the level of cytotoxic effects of doxorubicin alone and in combination with curcumin on neonatal cardiomyocytes as follows: incubation with doxorubicin at a dose of 0.5 μmol resulted in a significant death of living cells; incubation of cells with doxorubicin and curcumin (20 μmol) together contributed to a decrease in the percentage of propidium iodide positive cells compared with doxorubicin alone [58]. This confirmed the literature data concerning the cardioprotective properties of curcumin.

The study of changes in the expression of HIF and its target genes under the double and sequential exposure of doxorubicin and curcumin to the non-tumor cells has not yet been performed. At the same time, it was recently shown that HIF 3-α plays a specific regulatory role in oxidative stress that accompanies load hypoxia [59]. So, we investigated changes in the level of hypoxia-induced factors HIF-1,-2,-3 α and their target genes TERT, IGF-1 and PDK-1 expression in the neonatal cardiomyocyte culture by real-time polymerase chain reaction.

In the neonatal cardiomyocyte culture, the following data were obtained: the level of mRNA HIF-1α was significantly reduced when monotherapy was administered with doxorubicin or curcumin (versus control). With their combined use, such expression decreased by half compared with the addition of doxorubicin alone. These results reaffirm data on the ability of doxorubicin and curcumin to inhibit HIF-1α expression and confirm the enormous antioxidant potential of curcumin [60, 61]. The same results were obtained in our study of the mRNA HIF-2α and HIF-3α levels. Thus, the mRNA HIF-2α level fell significantly, and the mRNA HIF-3α level did not almost express when doxorubicin was administered alone. With the combined administration of doxorubicin and curcumin, the expression of the HIF-2α gene increased, but the HIF-3α gene level decreased dramatically 3.8 times under curcumin administration and 48 times under the combined effect of doxorubicin with curcumin.

Another HIF target gene, telomerase (TERT), increases the viability of both tumor and stem cells by reducing ROS production and acting as a transcription cofactor in the Wnt-β-catenin pathway [62]. We found that there was a rise in the expression level of the TERT target gene under the separate action of doxorubicin and curcumin in cardiomyocyte culture. The combined use of doxorubicin with curcumin caused a decrease in the expression of this gene compared to the action of doxorubicin alone.

Our data suggest that the deterioration in the viability of neonatal cardiomyocytes under doxorubicin administration is associated with a decrease in the expression of the telomerase and the PDK-1 genes. This assumption is based on several experimental data sets that confirm the cytoprotective properties of telomerase and PDK-1. In turn, another study has shown that telomerase has a neuroprotective effect under oxygen and glucose deprivation, the mechanisms of which are currently unknown [63]. However, it is believed that this effect is due to a decrease in the Bcl-2/Bax ratio, enhancement of ROS production, and a decrease in the mitochondrial potential (Ψm). Thus, our data to some extent explain the mechanisms by which the cell death level in the neonatal cardiomyocyte culture increases while incubated with doxorubicin.

It is known that insulin-like growth factor 1 (IGF-1) protects cardiomyocytes from apoptosis [64]. Overexpression of this gene reduces the heart cell death rate in mice during a heart attack, counteracting dilatation of the ventricles and heart hypertrophy [65]. Researchers have shown the cardioprotective effect of this factor in cardiomyocyte apoptosis caused by doxorubicin-induced oxidative stress [66, 67], which is completely confirmed by our experiments. Furthermore, by influencing the regulation of the expression of IGF-1R and IGFBP-3, doxorubicin inhibits H9c2 cell responses to these key factors for survival in apoptosis [67]. Anyway, there remains a need to study the effects of curcumin on the HIF expression level and its target genes to confirm the effectiveness of this method of myocardium protection at the molecular genetic level under the anthracyclines use. We showed that the expression of the target gene IGF-1 in cardiomyocyte culture was significantly reduced by doxorubicin and curcumin alone use and their combined use.

Regarding curcumin, there is evidence that it has a significant anti-tumor effect by suppressing the expression of IGF-1 [68], and, as we have found, appropriate inhibition has also been observed with doxorubicin-induced oxidative stress in non-cancerous cells.

As we have already seen, PDK-1 is one of the HIF target genes, which leads to a decrease in the flow of pyruvate in mitochondria, thereby counteracting the reduction in electron transport efficiency under hypoxic conditions that could otherwise increase ROS levels [69, 70]. We investigated that *in vitro*, the level of PDK-1 expression under exposure to doxorubicin slightly decreased. In the case of curcumin and doxorubicin administration, its expression stayed lower than that in the control.

Thus, the analysis of molecular genetic changes (at the level of the HIF system) in rat cardiomyocytes under the administration of doxorubicin and curcumin suggests the following: it has been shown for the first time that doxorubicin may inhibit expression not only of the HIF-1α gene but also of its target genes (PDK-1, TERT, IGF-1). This suggests that the deterioration of cardiomyocyte viability with doxorubicin is partly due to a decrease in the ability of cardiomyocytes to withstand the development of oxidative stress by reducing the cytoprotective properties of these genes. Thus, a decrease in the expression of the PDK-1 gene may stimulate ROS production in mitochondria [70]. Reducing the expression of TERT, in addition to this effect, diminishes the size of the mitochondrial potential [64]. Concerning the reduction of the IGF-1 gene expression after the application of doxorubicin, it can be argued that it inhibits the severity of antiapoptotic defense [66]. We also showed that mechanisms for increasing the loss of cardiomyocytes in different ways under the action of doxorubicin are related to HIF-dependent processes and established the molecular-genetic ways of correction of doxorubicin-induced mitochondrial dysfunction in cardiomyocytes with the help of curcumin.

As we have already mentioned, the disorder of the cell's oxidative-reducing state causes an elevated level of active forms of ROS generation, the excessive formation of which leads to the development of oxidative stress with its potent damaging effect on metabolic processes and the cell's structural components, in particular mitochondria and their genome [71, 72]. According to our research [58], the effect of doxorubicin results in a significant increase in the content of active products of TBARS and hydrogen peroxide in cardiomyocytes. This indicates the intensification of free radical oxidation, which may lead to changes in the structural organization of myocardial cells. The degree of cardiomyocyte destructive-metabolic disturbances in these conditions depends on the state of enzyme and non-enzyme antioxidant systems, the coordinated action of which holds under the control of both the formation and inactivation of oxygen-active forms. Importance in these systems belongs to SOD and catalase enzymes [73, 74], which act in the interconnection. The initial stages of the process of free radical oxidation are controlled by SOD, which deactivates the superoxide radical and, respectively, reduces the overall toxic effect of ROS. Hydrogen peroxide, which is formed by the superoxide anion dismutation, decomposes with catalase. It is believed that Mn-SOD plays the most significant role in anti-radical protection by maintaining a superoxide anion-safe level and activating the expression of SOD protects cells from the oxidative stress of different genesis [75]. In our work, the continued incubation of cardiomyocytes with doxorubicin reduced the enzymatic activity of Mn-SOD, with catalase activity increasing. This can be explained by the compensatory increase in the activity of this enzyme in response to an increase in H_2O_2 production, which is known to act as an antiperoxide enzyme substrate [73].

Adding curcumin to the cell culture of cardiomyocytes contributed to increased activity of

Mn-SOD and catalase, which confirms data from other studies on the properties of this drug as an antioxidant [14]. It is known that the use of curcumin improves the antioxidant status and, thus, prevents heart damage, mainly due to its moderate antioxidant effect [12]. Antioxidant mechanisms of curcumin may include one or more of the following interactions: the neutralization of free radicals [76], inhibition by cytochrome P450 [77], the reduction of the availability of oxygen to the oxidative reaction, the interaction with the oxidation cascade and the prevention of its consequences [78], and the reduction of the oxidative properties of ions of such metals as iron [79, 80]. Thus, curcumin effectively prevents tissue damage by reducing oxidative stress and restoring antioxidant status. However, in our experiments, when added to the culture of neonatal cardiomyocytes curcumin at 20 μmol/ml for 24 h, these processes took place against the background of some increase in the TBARS and H_2O_2 content. The co-administration of doxorubicin and curcumin into the culture of cardiomyocytes led to a significant reduction in free radical processes, in contrast to the effect of doxorubicin alone. Reducing the TBARS and H_2O_2 content by eliminating hyperactivation of catalase and increasing Mn-SOD activity indicates a restoration of the cardiomyocyte pro- and antioxidant balance. Thus, the addition of curcumin to the cardiomyocyte culture with the use of doxorubicin had a positive corrective effect on free radical processes and antioxidant protection. The issue of a slight increase in lipid peroxidation due to the effects of curcumin is currently unclear. In the literature, there is only evidence of an increase in lipid peroxidation against the background of co-administration of curcumin and various damaging agents (including doxorubicin). As for the slight increase of H_2O_2 due to the action of curcumin, this can be explained by the fact that Mn-SOD protects against free radical damage by converting the O_2-radical into H_2O_2 and preventing the formation of OH radicals through the Fenton reaction, after which H_2O_2 can be removed by catalase [51].

We also found a link between the viability of neonatal cardiomyocytes and the level of radical oxidation in these cells under the doxorubicin influence. For the first time, a correlation-regression analysis of the relationship between the level of living cardiomyocytes and the content of TBARS, H_2O_2, and catalase activity allowed us to establish a negative inverse relationship between the indicated parameters. In particular, the correlation coefficient *r* for TBARS was -0.97, for H_2O_2 -0.63, and for catalase -0.62. Thus, the higher the level of oxidative oxidation, the lower the viability of cardiomyocytes.

It is known that neonatal cardiomyocytes can contract spontaneously. According to the literature, doxorubicin reduces SERCA activity and Ca^{2+} concentration in the sarcoplasmic reticulum [19], which may contribute to the activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and increase the leakage of Ca^{2+} with SPR, reducing cardiomyocyte calcium control [20].

Under the condition of oxidative stress, in particular, as a result of exposure to doxorubicin, the synthesis of ATP in mitochondria is suppressed, and a rapid decrease in the level of creatine phosphate and, subsequently, ATP occurs [81]. In this case, it was found that metabolic activity and sarcolemmal Ca^{2+}-ATPase (SERCA) are suppressed too [82]. All these disorders can lead to diseases of the cardiovascular system, and therefore, the search for mechanisms of possible cardioprotection is extremely relevant.

As a result of studies on the culture of neonatal cardiomyocytes, we found that doxorubicin causes significant changes in all of their contractile activity parameters compared to control [83, 84]. Namely: the increase in the spontaneous contraction frequency by two times, the violation of their rhythm, the amplitude, and the percentage of shortening decrease; the maximum rate of reduction and relaxation increase without significant changes in the duration of these processes. The decrease in the contraction amplitude of the neonatal cardiomyocytes and their loss of rhythmicity under the doxorubicin influence may be attributed to a violation of the Ca^{2+} redistribution between the depot and the cell myoplasm. The cardiomyocyte contractile function violation can also be explained by the fact that 1 h after the doxorubicin action (1 μmol/l), the calpain activity, which stimulates degradation of titin (an effect similar to calcium ionophore ionomycin), is tripled in neonatal cardiomyocytes [21].

Incubation with curcumin caused a significant decrease in the frequency of spontaneous contractions, a reduction in the maximum rate, a decrease in amplitude, and a percentage of shortening, a violation of their rhythmicity. As can be seen from the results, the incubation of cells with curcumin alone also worsened the functional state of neonatal cardiomyocytes compared to control, which may be explained by the curcumin apoptotic properties [78].

Co-incubation with doxorubicin and curcumin caused a significant decrease in the frequency of spontaneous contractions (3 times), the restoration of their rhythm, amplitude, and percentage of shortening increased, and the maximum rate of reduction and the rate of relaxation rose, without significant changes in the duration of these processes in comparison with monoincubation with doxorubicin. The co-administration of drugs greatly improved the contractile activity of the cells, which may be due to a decrease in the oxidative stress manifestations in cardiomyocytes. Namely, it reduced the frequency of spontaneous contractions (approached control values) and restored their rhythm, peak amplitude, and percentage of shortening. These phenomena can be explained by the fact that curcumin has a protective effect on myocardial damage and helps to preserve heart function [49], influences the cytokine activity, enzymes, and transcription factors associated with the development of oxidative stress [50] as well as significantly reduces doxorubicin toxic effects on the heart due to its antioxidant properties [51].

In further studies, we evaluated the degree of oxidative stress effects on the cardiomyocyte viability and the functional activity of their mitochondria (mitochondria respiration and oxidative phosphorylation, the magnitude of mitochondrial membrane potential, and the rate of potassium ion transport) [85].

The following changes were found in the assessment of cardiomyocyte mitochondria respiration parameters in rats, receiving doxorubicin injection, the active respiration rate (V3) almost doubled, the index of the coupling of respiration with phosphorylation (V3/V4), and the phosphorylation efficiency coefficient were also reduced in comparison with the control. These data indicate a decrease in the respiratory chain efficiency and the use of oxygen, a violation of the mitochondrial respiration energy regulation level, reduced respiration, and phosphorylation, which are indicators of doxorubicin-induced violation of the mitochondrial oxidative phosphorylation processes. At the same time, after co-administration of doxorubicin and curcumin, mitochondrial respiration (V3, V3/V4, phosphorylation efficiency) significantly improved compared to those with doxorubicin administration alone.

Using the fluorescence method, we found a significant decrease in the intensity of mitochondrial coloring under the doxorubicin influence – by 42% compared with control ($P < 0.05$), which completely confirms the data on the damaging effect on the mitochondrial device. The co-incubation of cells with curcumin and doxorubicin resulted in a significant increase in the mitochondria fluorescence intensity (2-fold, compared with the use of doxorubicin alone). The obtained data testify to the ability of curcumin to enhance neonatal cardiomyocyte culture viability and to increase or maintain the initial value of the mitochondrial membrane potential under the oxidative damage condition. It can be concluded that curcumin is a potential cardioprotector due to its antioxidant properties and the ability to maintain mitochondrial membrane potential, increasing the synthesis of ATP in mitochondria [83].

The sustainability of the living cells can be explained by the fact that curcumin activates p53 apoptotic modulator (PUMA) and NOXA, which in turn activates the proapoptotic multidomain of BCL-2 members of the Bax, Bim, Bak family and suppresses Bcl-2 and Bcl-xl. The loss of equilibrium between pro- and anti-apoptotic Bcl-2 proteins causes calcium flow in mitochondria and decreases the permeability of the external mitochondrial membrane (MOMP), which allows cytochrome C to penetrate the cytoplasm, resulting in caspase cascade activation, apoptosome formation, and apoptosis [50, 86].

In the literature, there is data on the ability of doxorubicin to lead to dilated cardiomyopathy with long-term use of this antibiotic [84, 87]. Moreover, this ability depends on the dose and duration of administration of the drug. In our case, an average dose of doxorubicin was administered for a week, which was sufficient to cause oxidative stress and lead to pathological remodeling and the onset of dilated cardiomyopathy signs [85].

Conclusion. In this work, we demonstrated the positive effect of curcumin on preventing the development of OS in heart tissue during doxorubicin treatment. In particular:

1. It was shown that the number of dead cells after incubation of the culture of neonatal cardiomyocytes with doxorubicin increased by 4.5 times compared to the control ($P < 0.05$). The combined use of doxorubicin with curcumin led to an increase in the viability of cardiomyocytes by $9.40 \pm 0.01\%$, and the number of dead cells decreased by $19.50 \pm 0.01\%$ compared to the use of doxorubicin alone ($P < 0.05$).

2. It was demonstrated that exposure to doxorubicin leads to the induction of oxidative stress and an increase in the activity of its marker indicators in mitochondria. The combined use of doxorubicin with

curcumin led to a significant decrease in markers of free-radical processes and restoration of the pro- and antioxidant balance in the mitochondria of cardiomyocytes. A negative correlation was established between the number of live cardiomyocytes and the content of TBARS and H_2O_2, as well as catalase activity (r = -0.97, -0.63, and -0.62, respectively).

3. It was found that the effect of doxorubicin on the mitochondria of cardiomyocytes led to a decrease in the efficiency of the respiratory chain and the use of O_2 in phosphorylation processes, a violation of the level of energy regulation of mitochondrial respiration, a decrease in the coupling of respiration and phosphorylation, and a decrease in the rate of entry of K ions into isolated mitochondria. The combined use of doxorubicin with curcumin significantly improved these indicators and prevented the development of mitochondrial dysfunction: V3 increased by 25%, V3/V4 –by 18%, the phosphorylation efficiency coefficient – by 12%, and the rate of entry of K ions into mitochondria increased by 22.5% compared to that under the action of doxorubicin alone ($P < 0.05$).

4. The level of the potential-sensitive fluorescent component in the mitochondria (which characterizes changes in the mitochondrial membrane potential) of neonatal cardiomyocytes significantly decreased by 42% under the influence of doxorubicin. Incubation of cells with curcumin and doxorubicin increased the viability of the culture of neonatal cardiomyocytes (2-fold compared to the use of doxorubicin alone), contributing to the increase or maintenance of the initial value of the mitochondrial membrane potential.

5. Doxorubicin significantly ($P < 0.05$) reduces the expression of not only the HIF-1α gene but also its important target genes (PDK-1, TERT, IGF-1), simultaneously increasing the expression of the HIF-3α gene in rat cardiomyocytes, while curcumin via different molecular-genetic ways corrected the doxorubicin-induced mitochondrial dysfunction in cardiomyocytes.

6. It was established that doxorubicin impairs the contractility of neonatal cardiomyocytes. The combined use of doxorubicin with curcumin significantly ($P < 0.05$) increased the contractile activity of cells (reduced the frequency of spontaneous contractions, restored their rhythmicity, peak amplitude, and percentage of shortening), which may be associated with a decrease in the manifestations of oxidative stress in cardiomyocytes.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ПРОТЕКТОРНИЙ ЕФЕКТ КУРКУМІНУ ЗА ДОКСОРУБІЦИН-ІНДУКОВАНІЙ КАРДІОТОКСИЧНОСТІ

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Механізм кардіотоксичності доксорубіцину (Dox) включає різні шляхи, зокрема окислювальний стрес і мітохондріальну дисфункцію. Передбачається, що фармакологічний вплив на експресію генів HIF, може захистити серце від шкідливих наслідків пошкодження, спричиненого доксорубіцином. Ми припустили, що кардіопротекторний ефект куркуміну (Curc) здійснюється шляхом регулювання HIF та експресії його цільових генів. Щоб перевірити це, була використана *in vitro* модель Dox-індукованого пошкодження первинних кардіоміоцитів міокарда щурів. Ізольовані неонатальні кардіоміоцити щурів Вістар інкубували в культуральному середовищі протягом 24 годин, з додаванням Dox (0,5 мкмоль/мл), або Curc (20 мкмоль/мл), чи в їх комбінації в тих самих дозах. Мітохондрії було виділено з культури кардіоміоцитів. Показано, що вплив Dox на кардіоміоцити призводить до підвищення активності маркерів окислювального стресу в ізольованих мітохондріях, зниження

ефективності дихального ланцюга та процесів фосфорилювання, зниження мембранного потенціалу та швидкості надходження іонів K у мітохондрії. Доксорубіцин інгібував експресію мРНК як субодиниць HIF-1α, 2α, 3α, так і його важливих цільових генів PDK-1 та IGF-1 у мітохондріях. Спостерігався негативний вплив на скорочувальну активність кардіоміоцитів. Комбіноване застосування доксорубіцину з куркуміном призводило до підвищення життєздатності кардіоміоцитів і послаблення окислювального стресу в мітохондріях, попереджало розвиток мітохондріальної дисфункції та значно покращувало скорочувальну активність кардіоміоцитів.

Ключові слова: кардіоміоцити, мітохондрії, доксорубіцин, оксидативний стрес, куркумін, експресія субодиниць HIF.

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