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Research Article

Tirapazamine-Doxorubicin Interaction Referring to Heart Oxidative Stress and Ca^{2+} Balance Protein Levels

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Doxorubicin (DOX) causes long-term cardiomyopathy that is dependent on oxidative stress and contractility disorders. Tirapazamine (TP), an experimental adjuvant drug, passes the same red-ox transformation as DOX. The aim of the study was to evaluate an effect of tirapazamine on oxidative stress, contractile protein level, and cardiomyocyte necrosis in rats administered doxorubicin. Rats were intraperitoneally injected six times once a week with tirapazamine in two doses, 5 (5TP) and 10 mg/kg (10TP), while doxorubicin was administered in dose 1.8 mg/kg (DOX). Subsequent two groups received both drugs simultaneously (5TP+DOX and 10TP+DOX). Tirapazamine reduced heart lipid peroxidation and normalised RyR2 protein level altered by doxorubicin. There were no significant changes in GSH/GSSG ratio, total glutathione, cTnI, AST, and SERCA2 level between DOX and TP+DOX groups. Cardiomyocyte necrosis was observed in groups 10TP and 10TP+DOX.

1. Introduction

Doxorubicin (DOX) is a very efficient antitumor drug, but its use is limited by a dose-dependent, irreversible, and progressive cardiomyopathy, which may become evident years after completion of therapy [1–5]. The main pathomechanism of cardiotoxicity is connected with oxidative stress and disorders in function of contractile proteins, which are at least partly related to oxidative stress [6–10], and thus we assumed that any agent causing changes in red-ox balance may have an influence on DOX-related cardiotoxic effects.

Tirapazamine (TP), designed especially for eradicating hypoxic tumor cells, which are commonly resistant to classical radio- and chemotherapy [11, 12], passes the same red-ox metabolic changes as DOX. The drug has a moderate antitumor activity [13, 14] but, as it has been proved in many experimental studies, in combination with classical chemotherapeutics and radiotherapy it can increase the anticancer effectiveness compared to the treatment with classical chemotherapeutics alone [15–17] and thus giving hope for it to be used as an adjuvant.

Moreover both drugs TP and DOX pass similar red-ox-related metabolic transformations (Figure 1). These metabolic pathways of TP in normoxic conditions and DOX are related to one-electron reduction with the formation of radicals, TP* or DOX* respectively [18, 19]. Many NADPH and NADH-dependent enzymes catalyzing that reaction are common for DOX and TP, for example, NADPH cytochrome P450 reductase [16, 20], NOS [21–24], NADPH oxidase [25–27], and catalase [28, 29]. In the next step, TP* and DOX* are reoxygenated to a nonradical parent compound and at the same time a superoxide radical is formed ($O_2^{\bullet -}$). This

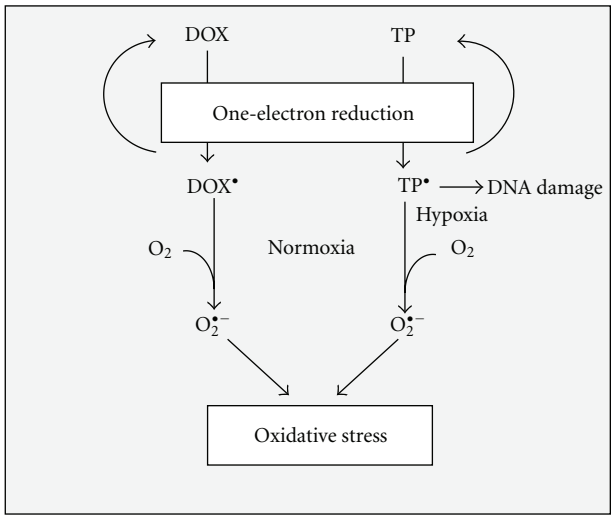


Figure 1: Schematic presentation of one-electron reduction of DOX and TP in hypoxic and normoxic conditions. Independent of hypoxia or normoxia, both DOX and TP go through one-electron reduction catalyzed by NADH dehydrogenase, xanthine oxidase, NOS and NADPH cytochrome P-450 reductase. In normoxic conditions DOX and TP radicals transfer one electron to oxygen and transform back to their parent compounds. However, in hypoxia, TP radical preferentially destroys DNA.

cycle of reactions may repeat many times leading to an overproduction of $O_2^{\bullet -}$, which may be a source of hydrogen peroxide and the much more toxic hydroxyl radical [10]. These reactive oxygen species (ROS) are responsible for the oxidative stress.

It has been evidenced that some ROS-dependent cardiotoxic effects are also observed after DOX administration [30–32]. Among them are disorders in Ca^{2+} intracellular balance [33], mitochondrial dysfunction [34], cardiomyocytes death [35], and heart remodeling [36], ultimately leading to disturbance in contractility and heart failure [1, 2, 33]. ROS-related disorders in Ca^{2+} intracellular balance resulting in cardiac contractility disturbance are partly related to post-transcriptional regulation of RyR2 and SERCA2 [7, 9, 37–39]. Transcriptional regulation of both proteins, mediated by DOX, has also been found [40, 41].

Summarizing, DOX and TP are involved in the same type of one-electron reduction resulting in ROS overproduction. In the light of ROS-dependent mechanisms responsible for cardiotoxicity, it is possible that TP will change the response of cardiomyocytes to DOX. Therefore, if there is an interaction between the two drugs, it might be of clinical importance. The aim of the study was to evaluate the effect of TP on oxidative stress, contractile protein level, and cardiomyocytes necrosis in rats administered DOX.

2. Materials and Methods

2.1. Animals and Treatment. The experimental protocol was approved by the Local Bioethical Committee of the Medical University in Lublin. The study was conducted on sexually mature male albino rats of Wistar CRL: (WI)WUBR strain, obtained from a commercial breeder (Warsaw-Rembertow, Poland). Animals with the initial body weight of 160–195 g were maintained in stable conditions at 22°C with a 12 h light/dark cycle and given standardized granulated fodder LSM (AGROPOL, Poland). The rats were *intraperitoneally* (*i.p.*) exposed to doxorubicin (DOX; Ebewe, Austria) and/or tirapazamine (TP; International Laboratory, USA).

The animals were randomly divided into six groups ($n = 7$): DOX: doxorubicin 1.8 mg/kg; 5TP: tirapazamine 5 mg/kg; 10TP: tirapazamine 10 mg/kg; 5TP+DOX: 1.8 mg/kg doxorubicin and 5 mg/kg tirapazamine; 10TP+DOX: 1.8 mg/kg doxorubicin and 10 mg/kg tirapazamine; control was given *i.p.* 0.9% NaCl solution. Doxorubicin (1.8 mg/kg) and tirapazamine in both doses were intraperitoneally injected once a week for six weeks in all study groups. A week after administration of both compounds the study was terminated. The animals were sacrificed and the blood and heart samples were collected during autopsy.

The heart was washed with 20 mL of saline then sectioned along the interventricular and coronal grove. The wall of the left ventricle was placed in liquid nitrogen and stored at −75°C until the time of biochemical and molecular analysis. The right ventricular wall sample was fixed in buffered 10% formalin and histologically processed.

2.2. Determination of Serum Biochemical Parameters. Heart cTnI (commercial kits Life Diagnostic, USA) concentration in rat serum was assessed with ELISA where two types of antibodies were used in the determination: antibodies covering microtiter plate and secondary antibodies bound to horseradish peroxidase. The product of enzymatic reaction was spectrophotometrically detected at 450 nm.

Aspartate aminotransferase (AST) activity was determined by the kinetic method with commercial diagnostic kits (Cormay, Poland) where the transfer of amino group between L-aspartate and 2-oxoglutarate was catalyzed by AST and the decrease in NADH absorbance was measured at 340 nm.

2.3. Determination of Tissue Markers for Red-Ox Imbalance. All measurements were conducted on homogenates obtained from ∼20 mg of frozen cardiac samples using the extraction buffer provided by the manufacturer of each commercial kit.

The evaluation of lipid peroxidation in cardiac homogenates was based on malondialdehyde and 4-hydroxyalkenals concentration (MDA+4HAE). The commercial kit Biotech LPO-586 for MDA+4HAE (*Oxis*Research, USA) was used for the assessment. The concept of the method is based on the reaction between MDA and 4HAE with N-methyl-2-phenylindol. After mixing N-methyl-2-phenylindole and methanol with the supernatant acquired from the homogenization, methanesulfonic acid was added and all reagents were placed in temperature 45°C for 60 minutes. Next, the solution was centrifuged and the supernatant containing the product was transferred to the plastic plate used in the spectrophotometric reader PowerWave XS (BioTek USA) at 586 nm. Subsequently, the procedure was conducted according to

the manufacture description and the concentration of MDA+4HNE in tested samples was calculated from formula of calibration curve $y = 0.0896x - 0.008$. The obtained date was calculated taking into account recommendations described in the procedure. Obtained results were expressed in nmol/g cardiac sample.

NADPH and NADH concentrations were determined by the comparable spectrophotometric method described in the commercial kit (Bio Vision, USA). The cardiac homogenate was incubated at 60°C in order to decompose NADP particles. Final readings were made using PowerWave XS Microplate Spectrophotometer (Bio-Tek, USA) at 450 nm.

Glutathione determination was conducted using commercial kit GSH/GSSG-412 (OxisResearch, USA). The frozen cardiac samples (~20 mg) were homogenized in extraction buffer provided by the manufacturer. Total glutathione (GSH_t; GSH+GSSG) was determined in the enzymatic reaction, where Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid) reacts with GSH forming color product with the maximum of absorbance at 412 nm. The concentrations of GSH and GSSG and GSH/GSSG ratio were assessed after measuring the speed of the reaction and establishing the calibrations curves. The concentrations of GSH and GSSG were determined based on calibration curve described by the formula $y = 0.1447x + 0.0004$ and $y = 0.1476x$. On the basis of the obtained date the GSH/GSSG ratio was calculated.

2.4. Determination of DNA Oxidative Damage. Commercial kit (Fermentas, Lithuania) was used for the isolation of cardiac DNA according to the manufacturer manual. Frozen tissue was pulverized in liquid nitrogen and suspended in TRIS-EDTA (TE) buffer. Then, the sample was incubated in lysis buffer at 65°C. Released DNA was extracted with the use of chloroform and then precipitated with precipitation factor.

DNA oxidative damage in cardiac muscle was evaluated by measuring the amount of basic sites (so-called AP) with commercial kit (Dojindo, Japan). Isolated DNA was labeled with ARP reagent, which can recognize aldehyde group of the open ring in AP sites and can combine with biotin. Then, biotin-avidin-specific connection and horseradish peroxidase were used for the colorimetric detection at 650 nm.

2.5. Determination of SERCA2 and RyR2 Proteins Level. Tissue samples were homogenized in 20 mM phosphate buffer (pH 7.4; proportions: 0.5 g of tissue and 2 cm^3 of buffer) with a protease inhibitor cocktail (Sigma-Aldrich, USA) in a homogenizer with a Teflon piston (5 minutes at 4000 rpm). Then, homogenates were centrifuged at 14000 rpm at 4°C for 20 minutes and used to quantify SERCA2 and RyR2 proteins by immunoblotting. Five randomly selected samples were taken from different randomly selected animals in each study group. 20 μg of protein was loaded in a polyacrylamide gel, and a 50-minute electrophoretic separation was performed under reducing conditions in XCellSureLock apparatus (Invitrogen, USA) at constant voltage of 200 V. After separation, the gel and nitrocellulose membrane were placed between two layers of filter paper and put between two electrodes of XCell II Blot Module electrotransfer apparatus (Invitrogen, USA). The transfer was conducted for 60 minutes at a constant voltage of 30 V. The blots were developed using Western Breeze Chromogenic Detection Kit (Invitrogen, USA). Nonspecific antibody-binding sites were blocked, and the membrane was incubated in the solution of a primary antibody: monoclonal mouse antibody against rat SERCA2 protein (clone 2A7-A1; Affinity BioReagents, USA) or against rat RyR2 protein (clone 34C; Affinity BioReagents, USA). After washing, the membrane was incubated with the secondary antibody combined with alkaline phosphatase. The last step was to mark the sites of reaction with antigen by the reaction of alkaline phosphatase and chromogen. The relative intensities of the bands were quantified using the 1D Image Analysis Software program (Kodak, USA), and all the values were normalized to the intensities of the respective beta-actin signal that was used as a loading control (Abcam, USA).

2.6. Preparation of Slides for Histological Evaluation. 4 μm histological slides obtained from paraffin blocks were routinely processed and stained with hematoxylin and eosin (H&E). Selye's method was also used to visualize cardiomyocyte necrosis.

2.7. Statistical Analysis. The obtained data was expressed as mean ± SD and statistically analyzed by STATISTICA 5.0 software. Continuous data were compared among experimental groups using the Kolmogorov-Smirnov test. The statistical significance of differences between control and study groups was evaluated by Student's t-test or U Mann-Whitney test. Group-to-group comparisons were made by one-way ANOVA. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Markers of Oxidative Stress. Levels of MDA+4HNE were significantly higher in all study groups compared to control (Table 1). However they were significantly lower in group 10TP+DOX compared to DOX. Since the change in marker of lipid peroxidation was mostly pronounced in the groups receiving higher dose of TP with DOX, markers of DNA oxidative damage were measured in these groups: levels of oxidative DNA damage were higher in groups receiving DOX and 10TP+DOX compared to control but there was no significant difference between these two groups (Figure 5). Level of NADPH (Table 1) was significantly higher in group 10TP+DOX compared to both control and DOX group, while level of total glutathione was significantly elevated in the group treated only with doxorubicin when compared to control. The GSH/GSSG ratio was elevated in all study groups compared to control. However, there were no significant differences when compared to DOX. NADH level was significantly decreased in group 5TP compared to control but increased in 10TP+DOX compared to the DOX group. Together, it was stated that DOX and TP increased levels of markers involved in oxidative stress. However,

Table 1: Markers of oxidative stress (heart homogenates).

	n	MDA+4HAE (nmol/g)	NADPH (ng/g)	NADH (ng/g)	GSH_t (nmol/g)	GSH/GSSG
Control	7	37.45 ± 6.75	17.04 ± 3.94	28.08 ± 8.66	742.71 ± 148.62	16.96 ± 5.46
DOX	7	164.07 ± 103.78[a]	18.67 ± 2.98	21.66 ± 6.01	962.45 ± 134.73[a]	38.70 ± 16.85[a]
5TP+DOX	7	114.66 ± 68.42[a]	27.87 ± 15.39	27.21 ± 4.85	824.52 ± 122.94	37.67 ± 6.01[a]
10TP+DOX	7	59.90 ± 16.05[a,b]	30.42 ± 12.74[a,b]	42.5 ± 15.5[b]	891.62 ± 178.11	38.96 ± 8.13[a]
5TP	7	68.70 ± 13.60[a]	15.23 ± 2.35	15.76 ± 4.57[a]	857.00 ± 128.46	38.12 ± 9.08[a]
10TP	7	62.88 ± 13.73[a]	15.83 ± 2.16	21.24 ± 3.55	891.62 ± 178.11	38.11 ± 3.13[a]

[a] $P < 0.05$ versus control, [b] $P < 0.05$ versus DOX.

among all these tested parameters, the statistical significance between DOX versus 10TP+DOX was the only observed referring to lipid peroxidation marker.

3.2. Light Microscopy Evaluation of the Myocardium. The morphological evaluation of hearts in the DOX group did not reveal signs of necrosis, neither in H&E nor in Selye's staining, specific for necrosis. In contrast, signs of cardiomyocyte necrosis were found in 10TP and 10TP+DOX and confirmed in both aforementioned stainings (Figures 2(a) and 2(b)). Low-intensity inflammatory infiltration was observed near necrotic foci.

3.3. Plasma Concentrations of cTnI and AST Plasma Activity. Levels of cTnI were significantly higher in groups 10TP and 10TP+DOX compared to control, which is accompanied by morphological changes of rat myocardium (Table 2). Insignificant change in AST plasma activity was found in all study groups.

3.4. Proteins SERCA2 and RyR2. SERCA2 protein content after administration of lower dose of TP with DOX was significantly higher with regard to control and DOX group (Figure 3). DOX reduces and 5TP increases RYR2 protein level comparing to the control (Figure 4). In 5TP+DOX group the level of RYR2 was significantly higher versus DOX group, but there was no difference compared to the control. Together, the comparison between groups of DOX and TP+DOX has shown that both doses of TP increased RyR2 and lower dose of TP increased SERCA2 concentration in rats receiving DOX.

4. Discussion

As it was stressed above, the metabolism of TP in normoxic conditions runs via the same pathways as DOX cardiotoxic-related mechanisms [16, 19, 20, 25, 26, 28, 29]. It was assumed that coadministration of both drugs leads to interaction referring to red-ox status and cardiomyocytes cytotoxicity. For these reasons the objective of the study was to assess oxidative stress and cardiomyocyte necrosis in rats administered TP and DOX simultaneously. Moreover, due to DOX having disadvantageous effect on RyR2 and SERCA2 [7, 9, 37–39] and the key contractile molecules, the content of these proteins has been also assessed.

Table 2: Plasma concentrations of cTnI and AST activity.

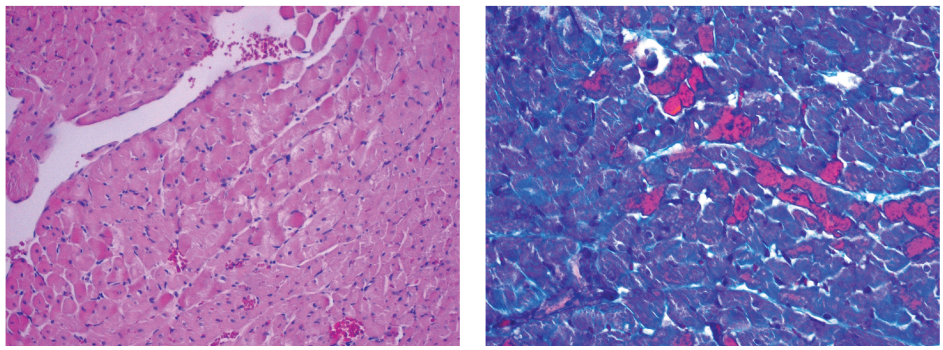
	n	cTnI (ng/mL)	AST (IU/I)
Control	7	1.14 ± 0.85	112.00 ± 24.58
DOX	7	1.98 ± 2.35	158.60 ± 151.36
5TP+DOX	7	2.40 ± 2.14	153.40 ± 52.76
10TP+DOX	7	2.90 ± 1.06[a]	149.25 ± 49.26
5TP	7	2.40 ± 2,23	107.00 ± 13.19
10TP	7	3.11 ± 0.96[a]	133.0 ± 33.83

[a] $P < 0.05$ versus control.

The main results of the current study indicate protective effect of TP on oxidative stress and RyR2 protein level disturbed by DOX. However, an additive effect referring to SERCA2 protein level was found when lower dose of TP was given with DOX. There was no additive effect on cardiomyocytes necrosis when rats were treated with both drugs at the same time.

As it was highlighted in Section 1, NADPH is used in one-electron reduction of both studied drugs causing ROS generation [10, 25–27]. Paradoxically, on the other hand, NADPH is an indispensable factor in quenching of ROS by glutathione regeneration mechanism. In the light of this fact a decrease in NADPH level may be expected. However, there were no changes in NADPH concentrations in any groups receiving DOX or TP alone. Moreover, a significant increase was found in 10TP+DOX comparing to the control and DOX. This observed effect is presumably a result of adaptation mechanism enabling overproduction of NADPH, for example, by G6PDH and malic enzymes, the main cell sources of that factor, in response to oxidative stress. NADPH-dependent GSH/GSSG ratio is higher not only in 10TP+DOX but over twofold increase versus control was found in every tested group. Normal level of NADPH and at the same time higher GSH/GSSG ratio in the rest of groups indicate that the physiological capacity of NADPH-dependent regeneration of GSH is very efficient. The question arises why GSH/GSSG ratio is elevated. It was revealed that MDA+4HNE concentration is also higher in every tested group, and, thus, GSH/GSSG ratio even in higher level is not enough to quench lipid peroxidation and oxidative stress is still present even one week after the final dose.

Moreover, it has been indicated that lipid peroxidation in hearts of rats administered with DOX only was four



(a) (b)

Figure 2: (a) Increased eosinophilia of scattered cardiomyocytes (10TP+DOX group; H&E, objective magnification 10x). (b) Positive color reaction detecting necrosis (group 10TP+DOX; Selye's method, objective magnification 20x).

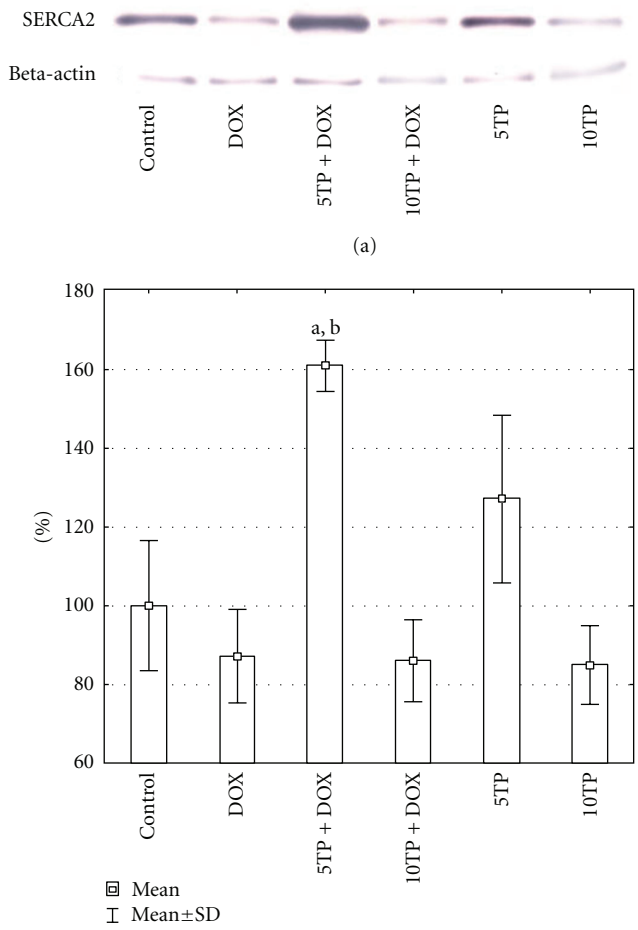


Figure 3: Representative Western blot analysis for SERCA2 protein in cardiac muscle homogenates (beta-actin is shown as a loading control) and densitometric analysis (mean ± SD) of total SERCA2 content expressed as percent changes with respect to the control group, which was established at 100%. [a]$P < 0.05$ versus control, [b]$P < 0.05$ versus DOX.

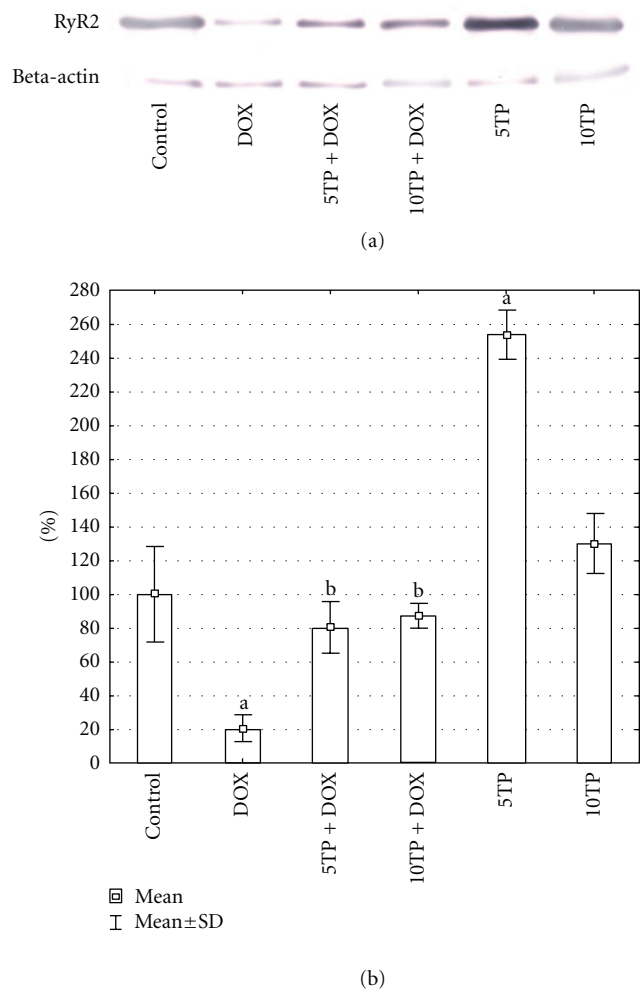


Figure 4: Representative Western blot analysis for RyR2 protein in cardiac muscle homogenates (beta-actin is shown as a loading control) and densitometric analysis (mean ± SD) of total RyR2 content expressed as percent changes with respect to the control group, which was established at 100%. $^{a}P < 0.05$ versus control, $^{b}P < 0.05$ versus DOX.

times higher compared to control. Administration with TP in animals treated with DOX decreased the level of lipid peroxidation, which suggests a protective influence of TP. The majority of prior researches indicated oxidative stress symptoms within short time after single-dose administration or last dose application of DOX. Knowing the red-ox metabolism of DOX, it is possible to assume that while the drug is still present in the tissue, there will be production of ROS and oxidative stress, which was evidenced in multiple studies [23, 42, 43]. Presence of oxidative stress one week after the last dose of DOX, therefore after the drug has been removed from the organism, is consistent with another study [44] and hypothesis explaining late cardiotoxicity of DOX based on oxidative stress [45, 46]. According to this hypothesis oxidative damage, formed in the presence of DOX, is "recorded" and after many years may lead to overt heart failure. ROS, formed in the presence of DOX, leads to oxidative mitochondrial DNA (mtDNA) damage [47–49]. These changes can then lead to disturbances in synthesis of proteins necessary to mitochondrial function, particularly complexes of electron transport chain metabolism pathways [50]. This, as a result, leads to an overproduction of ROS compared to physiological conditions. Increase in mitochondrial ROS level causes mitochondrial DNA damage and in this way the cycle of events may repeat itself until the productivity of mitochondria is finally limited [51], with possible subsequent serious implications in tissue with high metabolic rate. Finally, one can observe contractility disturbances and congestive heart failure. It is worth emphasizing that mitochondria are particularly vulnerable to an influence of oxidative stress as previously described [51].

There is abundant evidence proving toxic damage to mitochondria caused by DOX activity on morphological [52], biochemical [10, 53, 54], and molecular levels [45, 55, 56]. Additionally, it has also been stated that TP causes mitochondrial dysfunction [18]. In relation with the above, the level of NADH, main substrate for the electron transport chain, has been assessed. There was no significant difference in NADH level in any of the groups administered DOX compared to control (DOX, 5TP+DOX, 10TP+DOX); however, a significant increase in NADH level was observed in 10TP+DOX group compared to DOX. Therefore, the group

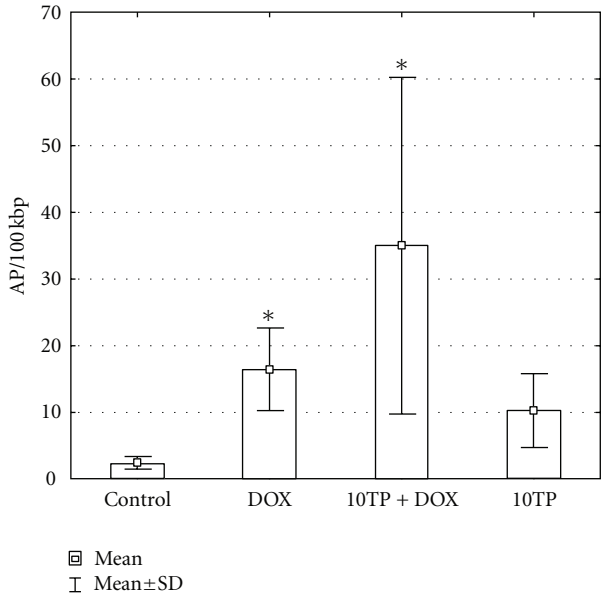


Figure 5: DNA oxidative damage (AP/100 kbp) in heart homogenates. $^*P < 0.05$ versus control.

indicating protective influence of TP (10TP+DOX) at the same time has also higher NADH level. It is a matter of discussion if increased level of NADH is a result of mitochondrial failure or escalated synthesis of the nucleotide itself. It seems that the first interpretation is more probable because NADH synthesis is based on beta-oxidation of fatty acids and Krebs cycle. Both of these metabolic pathways are hampered by DOX: beta-oxidation as a result of impediment of long-chain fatty acids transport [57] and Krebs cycle through blocking aconitase by superoxide formed in the presence of DOX [10]. Therefore, it seems that the higher concentration of NADH observed in group 10TP+DOX may be a result of cumulative unfavorable actions of both factors towards the electron transport chain. Considering the alleviating influence of higher dose of TP on lipid peroxidation in hearts of rats treated with DOX, DNA oxidative damage (sum of mitochondrial and nuclear) has also been assessed in these groups. The results confirmed the presence of oxidative stress in all evaluated groups; however, there was no interaction between the studied compounds.

It should also be stressed that the pathomechanism of contractility disturbances of myocardium is multidirectional. Oxidative stress plays an important role in the process and in the present study it was assessed based on MDA and oxDNA levels and was elicited by both compounds. Oxidative stress through cumulative and mitochondrial dysfunction (escalating with time) causes depletion in ATP [54, 58] necessary in the process of myocardial fiber contractions. It may also be a basis for pathological changes in calcium regulation through proteins RyR2 and SERCA2. Finally, oxidative stress can lead to myocardial necrosis, remodeling, and contractility disturbances. In the present study it has been evidenced that DOX majorly decreases RyR2 protein level. In contrast, after administering 5TP RyR2 level was over 2.5 times higher than in control. After increasing the dose of TP (10TP) RyR2 level was approximately 30% higher than control but the difference was not statistically significant. TP causes oxidative stress and simultaneously increases level of RyR2, which indicates that oxidative stress is not a factor limiting RyR2 synthesis in spite of disrupting the function of this protein. Therefore, the decrease in RyR2 level observed in DOX group does probably not result from oxidative stress. It may be expected that administration of two compounds with opposite actions will not elicit changes compared to control. This thesis is concordant with our observations in both groups 5TP+DOX and 10TP+DOX. Moreover, RyR2 level in groups administered DOX and TP was significantly higher when compared to group given DOX only, which suggests protective influence of TP on regulation of RyR2 level in hearts of animals treated with DOX. A RyR2 concentration decrease was also observed in other studies [40, 41] including our previous study [59] in which the cumulative dose of DOX was 15 mg/kg (1.5×10 weeks) and the samples were obtained three weeks after termination of drug administration. There was interaction between 5TP and DOX pertaining to SERCA2 level as SERCA2 level was significantly higher in group 5TP+DOX compared to DOX. It seems this change may have a negative influence on cardiomyocytes. These assumptions are supported by Burke et al. [60], which indicated significantly lower survival factor in mice with overexpression of SERCA2 gene treated with DOX as well as greater degree of histological damages compared to an isogenic control. There was no interaction between TP and DOX pertaining to cardiomyocyte necrosis. Observed morphological features of necrosis in group 10TP+DOX should be understood as TP action because features of necrosis were similar in groups 10TP and 10TP+DOX. Analogically, one can interpret cTnI concentration, which confirms the presence of cardiomyocyte necrosis in group 10TP+DOX.

In summary, the study revealed dose-dependent interaction between TP and DOX pertaining to myocardial oxidative stress and concentration of proteins taking part in heart muscle contraction. The open question remains if observed alterations are persistent or if the changes reverse after longer period of time. For this reason, further studies with different range of time after final dose of both drugs are needed to assess the effect of that interaction on contractility function of the heart.

Conflict of Interests

The authors declare that there is no conflict of interests.

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