Research Article

The Redox Imbalance and the Reduction of Contractile Protein Content in Rat Hearts Administered with L-Thyroxine and Doxorubicin

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Oxidative stress and disorders in calcium balance play a crucial role in the doxorubicin-induced cardiotoxicity. Moreover, many cardiotoxic targets of doxorubicin are regulated by iodothyronine hormones. The aim of the study was to evaluate effects of tetraiodothyronine (0.2, 2 mg/L) on oxidative stress in the cardiac muscle as well as contractility and cardiomyocyte damage markers in rats receiving doxorubicin (1.5 mg/kg) once a week for ten weeks. Doxorubicin was administered alone (DOX) or together with a lower (0.2T₄ + DOX) and higher dose of tetraiodothyronine (2T₄ + DOX). Two groups received only tetraiodothyronine (0.2T₄, 2T₄). Coadministration of tetraiodothyronine and doxorubicin increased the level of lipid peroxidation products and reduced RyR2 level when compared to untreated control and group exposed exclusively to doxorubicin. Insignificant differences in SERCA2 and occasional histological changes were observed. In conclusion, an increase of tetraiodothyronine level may be an additional risk factor of redox imbalance and RyR2 reduction in anthracycline cardiotoxicity.

1. Introduction

Doxorubicin (DOX) is one of the widely used antitumor drugs, but its cumulative cardiac toxicity has been a major concern for oncologists [1–5]. The analysis of the main mechanisms responsible for DOX cardiotoxicity, oxidative stress [6, 7], and cardiac contractility disorders [8–10] indicates that some of them may be targeted by thyroid hormones (THs). Therefore, it seems that hyperthyreosis may be an additional risk factor in anthracycline cardiotoxicity.

There is an increasing body of evidence that the synthesis of many molecules involved in the heart systole and diastole is regulated during transcription by THs [11, 12]. Such an effect was proved for the expression of ryanodine receptor (RyR2) and cardiac sarcoplasmic reticulum Ca²⁺ ATP-are

(SERCA2), which are responsible for the heart systole and diastole, respectively [13, 14]. Moreover, THs generate reactive oxygen species (ROS) which are signaling molecules in the regulation of RyR2 and SERCA2 functions [15–18].

ROS oversynthesis [19, 20] is mediated by active metabolites of thyroxine (T₄): (3,3'-5) triiodothyronine (T₃) and diiodothyronine (3,5-T₂) in a short-term mechanism due to allosteric activation of cytochrome c oxidase [21, 22] and T₃-dependent long-term signalling inducing synthesis of enzymes involved in mitochondrial function, for example, respiratory chain proteins. This results in acceleration of the mitochondrial consumption of O₂ [23] and, consequently, an enhanced generation of superoxide and H₂O₂ [24]. T₃-dependent ROS oversynthesis [25, 26] may also increase due to the transcriptional activation of

FIGURE 1: A schedule of drug administration (DOX-doxorubicin, T₄-tetraiodothyronine).

NADPH-dependent enzymes: cytochrome P450 reductase (P450R) [19, 27, 28], oxidase [23], and nitric oxide synthase (NOS) [29]. Iodothyronine hormones also control the production of key enzymes responsible for the synthesis of NADPH, which is essential for the oxidative stress defense in the glutathione regeneration mechanism [30–32]. It should be stressed that NADPH is a source of ROS (as a cofactor of P450R, NOS and NADPH oxidase) and at the same time an indispensable factor in antioxidative stress defense.

DOX may elicit cardiotoxicity which becomes evident even many years after chemotherapy is completed and which may result in death [4, 5]. ROS generation in the presence of DOX is strictly connected with one electron reduction catalyzed by NADPH-dependent enzyme controlled by T₄ [33–36] and others [37–39]. On the other hand, the disturbance in the cardiac contractility produced by DOX might be at least partly related to changes in the expressions and activity of RyR2 and SERCA2 [9, 40].

The fact that the expression of some genes encoding proteins responsible for the cardiac muscle contractility and for the regulation of key enzymes involved in redox balance is affected by iodothyronine hormones suggests that the effect of anthracyclines on these processes may differ in hyperthyroid and euthyroid individuals. Therefore, it might be of clinical importance if a high level of iodothyronine hormones is or is not a risk factor for anthracycline cardiotoxicity. The aim of the present study was to evaluate the effect of diet supplemented with T₄ on RyR2 and SERCA2 protein level and on markers of cardiac redox imbalance and the incidence of necrosis of cardiomyocytes in rats receiving doxorubicin.

2. Material and Methods

2.1. Animals and Treatment. The experimental protocol was approved by the Local Bioethical Council of the Medical University in Lublin. The study was conducted on sexually mature albino rats of Wistar CRL: (WI) WUBR strain, obtained from a commercial breeder (Warsaw-Rembertow, Poland). The 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 were given standardized granulated fodder LSM (AGROPOL, Poland).

All the animals were randomly divided into six groups ($n = 8$): DOX-doxorubicin 1.5 mg/kg; 2T₄+ DOX-thyroxin 2.0 mg/L and doxorubicin 1.5 mg/kg; 0.2T₄+ DOX-thyroxin 0.2 mg/L and doxorubicin 1.5 mg/kg; 2T₄-thyroxin 2.0 mg/L; 0.2T₄-thyroxin 0.2 mg/L; the untreated control (Figure 1).

Thyroxin (Sigma, USA) was administered in drinking water in two doses: 0.2 (0.2T₄, 0.2T₄+ DOX) and 2 mg/L (2T₄, 2T₄+ DOX) throughout the 14 weeks of the experiment. Since the end of the first week, doxorubicin (Ebewe, Austria) was intraperitoneally injected (1.5 mg/kg) once a week for ten weeks in groups DOX, 2T₄+ DOX, and 0.2T₄+ DOX (Figure 1). The doses for both compounds were chosen according to the previous data [14, 41, 42]. Two days after T₄ treatment was terminated (experimental day 100) and animals were sacrificed. The blood and heart were collected during autopsy.

The heart was washed with 20 mL of saline then sectioned along the interventricular and coronal groove. The left ventricular wall was placed in liquid nitrogen and stored at –75°C until the biochemical and molecular analyses were conducted. The right ventricular wall samples were fixed in buffered 10% formalin and routinely processed histologically.

2.2. Evaluation of Serum Biochemical Parameters. Serum-free tetraiodothyronine (FT₄) concentration was determined using a competitive ELISA test (Novatec, Germany) according to the manufacturer's manual, based on the immune complex formed by the enzyme-labeled antigen and final absorption reading at 450 nm with a microwell plate reader (BIO-TEK XS PowerWave, USA).

Heart cTnI, H-FABP, and BNP concentrations were assessed in rat serum with ELISA commercial kits as follows: Life Diagnostic (USA) for cTnI and H-FABP and Assay Pro (USA) for BNP, where two types of antibodies were used in the evaluation: antibodies covering microtiter plate and the secondary antibodies bound to horseradish peroxidase. The product of the catalytic reaction was spectrophotometrically detected at 450 nm.

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

2.3. Evaluation of Tissue Markers for Redox Imbalance. The evaluation of lipid peroxidation product was based on the malondialdehyde (MDA) concentration in cardiac homogenates. The commercial kit TBARS (Cayman, USA) was used for the assessment. The concept of the method is based on the reaction between MDA and thiobarbituric acid at 100°C and low pH.

NADPH concentration was determined by the spectrophotometric method described in the commercial kit (Bio Vision, USA). The obtained and frozen cardiac samples (~20 mg) were homogenized in an extraction buffer provided by the manufacturer and then incubated at 60°C in order to decompose NADP particles. The final readings were made using Power Wave Microplate Spectrophotometer (Bio-Tek) at 450 nm.

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

DNA oxidative damage in the cardiac muscle was evaluated by measuring the amount of abasic sites (so-called AP) with a commercial kit (Dojindo, Japan). The 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 was used for the colorimetric detection at 650 nm.

2.5. Evaluation of Expression of SERCA2 and RyR2 Proteins. Cardiac samples were homogenized in 20 mM phosphate buffer (pH 7.4; proportions: 0.5 g of tissue and 2 cm³ 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. Six 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 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 the nitrocellulose membrane were placed between two layers of the filter paper and put between two electrodes of XCell II Blot Module electrotransfer apparatus (Invitrogen, USA). The transfer was conducted for 60 minutes at the constant voltage of 30 V. 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 the 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

TABLE 1: Concentration (ng/L) of free thyroxin in rats' serum.

	FT ₄
Control	22.38 ± 3.18
2T ₄	29.77 ± 5.65 ^a
0.2T ₄	21.39 ± 4.42

Data presented as mean ± SD; ^a*P* < 0.05 versus control.

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 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) as well as using van Gieson's method.

2.7. Statistical Analysis. The obtained data was statistically analyzed using STATISTICA 5.0 software. Results are expressed as mean ± standard deviation (SD). Continuous data were compared among the experimental groups using the Kolmogorov-Smirnov test. The statistical significance of differences between control and the other groups was evaluated either by *t*-Student test or *U* Mann-Whitney test, and group to group comparisons were made by one-way ANOVA. The value of *P* ≤ 0.05 was considered as statistically significant.

3. Results

The level of free thyroxin (FT₄) was significantly higher in animals treated with 2 mg/L of T₄ than in the untreated control group (Table 1). However, an insignificant change in T₄ blood level was observed when the animals were treated with a lower hormone dose (0.2 mg/L).

A significantly lower concentration of NADPH in the cardiac muscle was observed only in 0.2T₄+ DOX group compared to the control and to the group exposed exclusively to DOX (Table 2). Lipid peroxidation products, MDA, were also significantly higher in 2T₄+ DOX group and in 0.2T₄+ DOX group when compared to the control group. A similar effect was observed in animals receiving a higher dose of thyroxin (Table 2). Insignificant differences in the oxidative DNA damage in the cardiac muscle were revealed in all examined groups (Table 2). It is worth mentioning that a lowest number of abasic sites was observed in the group treated only with DOX, while the highest was found in the group exposed to DOX and a higher dose of T₄ (2T₄+ DOX).

There were insignificant changes in SERCA2 protein level in any tested group comparing to the control and between DOX and T₄+ DOX groups. When compared to the untreated control, a 25 and 16% decrease of SERCA2 protein level was found in 0.2T₄+ DOX and 2T₄+ DOX

TABLE 2: Concentration of NADPH (nmol/g tissue), MDA (nmol/g tissue), and the number of abasic sites in genomic DNA (AP/100 kbp) in the cardiac muscle.

	NADPH	MDA	AP in gDNA
Control	19.95 ± 5.2	2.37 ± 0.39	7.34 ± 2.84
DOX	24.53 ± 6.79	2.30 ± 0.66	6.56 ± 1.99
2T ₄ + DOX	16.12 ± 5.30	3.75 ± 0.66 ^{a,b}	9.65 ± 3.79
0.2T ₄ + DOX	9.27 ± 1.72 ^{a,b}	3.13 ± 0.65 ^{a,b}	6.84 ± 3.29
2T ₄	22.13 ± 5.25	3.84 ± 1.45 ^a	8.46 ± 5.48
0.2T ₄	18.10 ± 5.73	3.03 ± 0.94	6.29 ± 1.51

Data presented as mean ± SD; ^a*P* < 0.05 versus control; ^b*P* < 0.05 versus DOX.

FIGURE 2: (a) Representative Western blot analysis for SERCA2 protein in cardiac muscle homogenates. Beta-actin is shown as a loading control. (b) Densitometric analysis (mean ± SD) of total SERCA2 content expressed as percent changes with respect to the control group, which was established at 100%.

groups, respectively (Figure 2). Less intensive changes were noted in the group exposed exclusively to DOX. However, in animals receiving only the lower dose of T₄, a 10% increase of SERCA2 protein concentration was revealed.

The level of RyR2 protein was the lowest in 2T₄ + DOX group (13% of the control), and it was significantly different from the control and from the DOX group (Figure 3). A significant decrease of the parameter was also detected in

FIGURE 3: (a) Representative Western blot analysis for RyR2 protein in cardiac muscle homogenates. Beta-actin is shown as a loading control. (b) 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.

groups: DOX (41%) and 0.2T₄ + DOX (71%) as compared to the control.

A significant decrease of BNP level was found in all drug-exposed groups when compared with the untreated control (Table 3). The most significant differences (about 50%) were observed in 2T₄ + DOX and 2T₄ groups.

TABLE 3: Concentration (ng/mL) of BNP, H-FABP and cTnI, and activity (IU/L) of AST in rats' serum.

	BNP	H-FABP	cTnI	AST
Control	0.309 ± 0.02	24.07 ± 2.49	2.68 ± 0.69	138.14 ± 38.40
DOX	0.193 ± 0.08 ^a	5.90 ± 1.34 ^a	0.78 ± 0.63 ^a	114.40 ± 56.79
2T ₄ + DOX	0.153 ± 0.06 ^a	4.50 ± 0.43 ^a	0.57 ± 0.66 ^a	80.83 ± 30.79 ^a
0.2T ₄ + DOX	0.254 ± 0.04 ^a	19.29 ± 4.36	2.64 ± 1.54	113.67 ± 23.37
2T ₄	0.184 ± 0.04 ^a	5.81 ± 1.13 ^a	0.83 ± 0.73 ^a	150.83 ± 14.62
0.2T ₄	0.236 ± 0.01 ^a	21.85 ± 3.87	3.12 ± 1.09	145.33 ± 17.71

Data presented as mean ± SD; ^a*P* < 0.05 versus control.

TABLE 4: Histological features of the cardiac muscle in experimental and control groups.

	Interstitial		Perivascular fibrosis		Cardiomyocytes		Inflammation
	fibrosis	edema		vacuolisation	irregular cytoplasm staining		
Control	2	—	1	—	—	—	1
DOX	2	—	—	2	1	—	3
2T ₄ + DOX	4	—	1	—	3	—	5
0.2T ₄ + DOX	4	—	—	3	2	—	3
2T ₄	3	3	—	—	1	—	1
0.2T ₄	2	8	—	—	—	—	3

Samples from all rats in all experimental and control groups were evaluated (*n* = 8/group). A single animal may be represented more than once in the listing of individual histological changes.

There was an insignificant increase of H-FABP level in all tested animal groups compared to the control. In DOX, 2T₄+ DOX, and 2T₄ group, significantly lower concentrations were observed (Table 3).

The level of the concentration of cTnI in three groups of DOX, 2T₄+ DOX, and 2T₄ was significantly lower than in the control and it was similar in all those groups.

AST activity was significantly decreased only in 2T₄+ DOX group when compared with the untreated control.

Histological cardiac changes were focal and accidentally observed (Table 4). Necrosis was not revealed in any of the examined slides, but other local changes with minimal or low intensity were revealed in all groups. In some cases, a small area with a subendomyocardial and perivascular fibrosis was found (Figure 4(a)). Interstitial connective tissue edema was observed in all rats treated with a lower dose of T₄ and in three rats from 2T₄ group (Figure 4(b)). Single animals from the groups of DOX and 0.2T₄+ DOX revealed focal vacuolization of sparse cardiomyocytes (Figure 4(c)). Several samples from different groups show focal irregular sarcoplasm staining. Small local accumulations of mononuclear cells were seen in all groups, while a large inflammatory infiltration without evidence of the cardiomyocyte damage was found only in single cases in DOX (*n* = 2), 2T₄+ DOX (*n* = 1), and 0.2T₄+ DOX groups (*n* = 1) (Figure 4(d)).

4. Discussion

As it was pointed out in the introduction, there are two principal mechanisms responsible for anthracycline cardiotoxicity: contractility dysfunction [8–10] and oxidative

stress [6, 7], which might be influenced by an abnormally high level of T₄ blood.

To find out if T₄ concentrations in the supplemented diet were proper for our studies, we assessed free tetraiodothyronine FT₄ level in the blood. A lower dose of FT₄ had no effect on FT₄ blood concentration, which is presumably caused by the adaptive mechanism based on the restriction of thyroxine synthesis by the thyroid. However, a higher dose of T₄ caused a significant increase in FT₄ blood concentration; therefore, the tested doses were chosen properly.

The studies revealed that T₄ in a higher dose causes an adverse interaction with DOX referring to the contractile protein RyR2 and lipid peroxidation. There was an insignificant adverse effect of T₄ on the necrosis of cardiomyocytes and cardiac morphology in rats receiving DOX.

The effect of DOX on RyR2 is consistent with the results obtained by Arai et al. [40], where rabbits were treated with 2.5 mg/kg of DOX every week for eight weeks and a lower expression of RyR2 was accompanied by a lower protein amount 8 weeks after the final injection. Those authors had a similar observation referring to SERCA2 mRNA and protein. However, in our studies, in the group where 1.5 mg/kg of DOX was administered to rats for ten weeks, a lower but insignificant concentration of SERCA2 protein was noted in all DOX-exposed groups. It is especially important since the protein level was increased three weeks after DOX administration was completed. Such data indicate that the level of RyR2 protein was constantly lower several weeks after the final dose of DOX and the question is if these changes are irreversible.

Both SERCA2 and RyR2 are controlled by thyroid hormones [13, 14]. Our study revealed a higher RyR2 protein

FIGURE 4: Selected cardiac abnormalities found among the treated animals. (a) Perivascular fibrosis in a rat treated with doxorubicin 1.5 mg/kg (group DOX, van Gieson; objective mag. $\times 10$). (b) Interstitial connective tissue edema in a rat treated with thyroxine dose of 0.2 mg/L (group 0.2T₄; H&E objective mag. $\times 10$). (c) Focal vacuolization of sparse cardiomyocytes in a rat treated with 0.2 mg/L of thyroxine and 1.5 mg/kg of doxorubicin (group 0.2T₄ + DOX; H&E, objective mag. $\times 20$). (d) Sparse and small accumulations of mononuclear cells in myocardium in a rat treated with 0.2 mg/L of thyroxine and 1.5 mg/kg of doxorubicin (group 0.2T₄ + DOX; H&E, objective mag. $\times 20$).

level in rats after thyroxine treatment, with SERCA2 level also being higher but statistically insignificant in rats treated with a lower dose of T₄. These results are similar to Arai et al. [14] study, where rats were administered a daily dose of 200 μ g/kg of T₄ for 4 and 8 weeks, which is comparable to the dose used in the present study. An over 100% increase in SERCA2 mRNA concentration was detected in the study by Ojamaa et al. [43], where rats were treated with T₄ only for 72 hours. It seems that both DOX and thyroxine modulate the synthesis of RyR2 and SERCA2 by other mechanisms of regulation [9, 10, 13, 14].

Moreover, the obtained results confirmed that DOX leads to decrease, while 2T₄ to increase in the level of RyR2 when compared to the control group. On the basis of these effects, it might be expected that an abnormally high level of T₄ should normalize RyR2 concentration disturbed by DOX. Such an action was observed when DOX was coadministered with a low dose of T₄. However, a higher dose of T₄ in rats receiving DOX caused a much higher decrease in RyR2 level than it was in rats administered with DOX alone. Such data may indicate an adverse clinical implication in hyperthyroid individuals treated with DOX.

Furthermore, iodothyronine hormones and DOX not only affect RyR2 and SERCA2 level but may also influence their function via the redox dependent mechanism. There is increasing evidence that the Ca²⁺ transport activity of RyR2 and SERCA2 is redox dependent [15–18]. Both

iodothyronine hormones and DOX affect NADPH cell balance, which is seemingly paradoxically involved in oxidative stress generation and at the same time the antioxidative defense [23, 28, 29, 36].

The hypothesis that oxidative stress is the most important mechanism in long-term cardiotoxicity of anthracyclines leading to heart failure is widely accepted [7, 8]. Because lipid and DNA oxidative damage markers reflect changes in oxidants-antioxidants balance, we assessed the influence of the studied drugs on both marker levels. However, in DOX-exposed group of the present study, where the organ samples were examined three weeks after the last dose of DOX, a lack of oxidative stress—tested on the basis of lipids and DNA oxidation products—was found. According to the literature, oxidative stress markers are elevated a short time after drug administration (hours or even days) because DOX is still present in the tested tissue [35, 44, 45]. In these cases, oxidative stress in the heart is the direct effect of DOX participating in the transfer of electrons from NADPH to O₂, which results in superoxide overproduction. However, it does not explain the ROS-dependent mechanism of heart failure when clinical symptoms are observed several years after DOX-administration was completed. According to recent suggestions, ROS produced during DOX redox metabolism initiates early oxidative changes in the mitochondrial DNA (mtDNA) [46]. In that period, the symptoms of oxidative stress may be reduced parallel to DOX excretion from the

body. However, an increase of mtDNA damages may result in a mutation leading to the synthesis of mitochondrial proteins with defects causing disorders in the mitochondrial physiology [47]. As a final result, ultrastructural changes through the mitochondrial electron chain of cardiomyocytes lead to mtDNA disorders [48–50] and to an increase of ROS production [46, 51]. Such a cycle is repeated constantly and no clinical symptoms are observed in the beginning but when mitochondria fail, a clinically detected heart failure may occur. Therefore, the explanation that markers indicating oxidative stress are seen at the early stage when DOX is present in the tissues and in the late period when disorders in electron transfer are serious seems reasonable. Referring to a recent study, no signs of oxidative stress in rats treated with DOX alone may be explained by the fact that within 3 weeks after the last dose of DOX the drug was removed from the body. However, the time of the experiment was too short to reveal the cumulative, oxidative effects of the mitochondrial DNA damage. The studies published by Robinson et al. [52], where rats were treated with 2 mg/kg of DOX for 13 weeks, confirmed that oxidative stress is not observed even 14 and 19 weeks after the final dose of the drug.

However, T_4 -supplemented diet in rats treated with DOX leads to an increase of lipid peroxidation products comparing to the group administered with DOX alone. It is the evidence that T_4 is responsible for oxidative stress in the heart of rats treated with DOX. It should be noticed that in the groups of T_4 + DOX, ROS affects lipids but not DNA. Similar to our results, there was no DNA damage after THs administration although lipid peroxidative changes were observed [25, 26]. The absence of DNA damage might be explained by the fact that ROS are neutralized by intracellular antioxidants before the nucleus is reached. Higher susceptibility of lipids to oxidative stress may form a protective barrier against DNA oxidative damages. Genomic DNA might be protected better than lipids due to histone binding, which makes it less susceptible to damage or abnormalities, with DNA being repaired faster than other molecules.

NADPH level was significantly lower in the heart of rats from $0.2T_4$ + DOX (as opposed to $2T_4$ + DOX) group compared to the control and DOX groups. This finding indicates that the observed changes are dose related. In the presence of DOX, NADPH is a source of ROS generation and at the same time it is a key factor in the antioxidative defence caused by ROS [8, 53, 54], whereas T_4 intensifies NADPH consumption, which is due to stimulated metabolism. Moreover, because of transcriptional activation of same genes responsible for the synthesis NADPH-dependent enzymes (e.g., P450R, NOS, and NADPH oxidase [23, 28, 29, 36]), T_4 may intensify NADPH consumption. It should be stressed that at the same time T_4 activated genes responsible for NADPH synthesis, that is, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malate enzyme [30–32], but this mechanism is insufficient to keep NADPH on the normal level in $0.2T_4$ + DOX group. However, when rats were administered a higher dose of thyroxine ($2T_4$ + DOX group) no differences in NADPH level comparing to the control and DOX groups were found. This may suggest that the synthesis of NADPH controlled by T_4 is

sufficient to compensate the stress stimulated by the action of DOX.

On the other hand, the oxidative damage of lipid membranes may result in the death of cardiomyocytes and remodeling of the heart muscle [55]. Therefore, cardiac morphology and various biochemical markers were evaluated. There was no effect of T_4 on cardiac histology in rats treated with DOX. Biochemical observations based on cTnI, H-FABP, and BNP, serum markers, or a detailed analysis of histological slides did not indicate necrosis of cardiomyocytes in any of the tested group. The obtained data suggest that oxidative stress estimated on the basis peroxidation of lipids products seen in both groups of T_4 + DOX is insufficient to elicit necrosis.

The level of H-FABP, cTnI, and BNP was even lower in the serum of rats treated with a higher dose of T_4 and DOX separately or together as compared to the control. Similar results referring to H-FABP after DOX treatment were presented by Sayed-Ahmed [56], who administered the drug in a cumulative dose of 18 mg/kg. There were a lot of studies confirming that BNP is affected by DOX [57–60] and T_4 [61, 62]. BNP is considered to be a marker diastolic dysfunction of heart ventricles [56, 59]. Presumably, a DOX-related low level of RyR2, cTnI, H-FABP, and BNP—key proteins in contractile activity—may depend partly on the unspecific manner of proteasome degradation, which was confirmed in recent studies by An et al. [63].

In conclusion, the present studies revealed that an abnormally high level of T_4 was responsible for the heart redox imbalance, oxidative stress, and RyR2 protein reduction in rats treated with DOX. Further studies on the heart function should be continued to assess if T_4 might be considered as an additional risk factor in anthracycline cardiotoxicity.

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