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The influence of a thyroxine supplemented diet on selected hepatic redox equilibrium markers, liver morphology and the serum lipid profile in rats treated with doxorubicin*

Wpływ diety suplementowanej tyroksyną na wybrane wykładniki równowagi redox, morfologię wątroby i profil lipidowy szczurów poddanych działaniu doksorubicyny

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Summary

Introduction: Cytotoxicity of doxorubicin (DOX) – an anticancer drug, mostly results from reactive oxygen species (ROS) generation. Some enzymes catalyzing this process and enzymes of antioxidant defense are regulated by iodothyronine hormones. Thus, disorders in iodothyronine hormone status may affect doxorubicin-induced redox imbalance and anabolic/catabolic disorders. The aim of this study was to evaluate the influence of doxorubicin and thyroxine (T4) associated treatment on liver morphology, markers of oxidative stress and plasma lipid parameters.

Materials and methods: Rats were intraperitoneally treated with doxorubicin (1.5 mg/kg) once a week for ten weeks. Thyroxine was simultaneously given in drinking water (0.2 or 2.0 mg/l) for 14 weeks.

Results: There were higher hepatic level of malonyldialdehyde (MDA) of all tested groups and at the same time in rats treated with DOX plus T4 lower concentrations of total glutathione compared to controls were observed. Morphology of liver did not show any features of necrosis or steatosis but a decrease of glycogen content in T4+DOX groups compared to DOX treatment was observed. The concomitant administration of a lower dose of thyroxine and doxorubicin decreased triglycerides (TG) and increased LDL level compared to the DOX group.

Discussion: Thyroxin supplementation caused redox equilibrium disorders and oxidative stress in liver of rats receiving DOX. The study revealed the normalizing influence of thyroxin on glycogen deposits that were observed after doxorubicin treatment. Apart from an adverse impact of thyroxine administration on LDL in rats treated with doxorubicin, a beneficial effect of lower dose of thyroxine on serum TG level was revealed.

Keywords: **doxorubicin • thyroid hormones • hepatotoxicity • oxidative stress • serum lipids**

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Streszczenie

Wprowadzenie: Cytotoksyczność doksorubicyny (DOX) – leku przeciwnowotworowego, jest w głównej mierze wynikiem generowania reaktywnych form tlenu (RFT). Niektóre z enzymów katalizujących tę reakcję, a także enzymów obrony antyoksydacyjnej są regulowane przez hormony jodotyroninowe. W związku z tym zmiany w statusie hormonów jodotyroninowych mogą mieć wpływ na zaburzenia równowagi redoks oraz równowagi procesów anabolicznych i katabolicznych wywołane działaniem doksorubicyny. Celem badań była ocena wpływu skojarzonego podawania DOX i tyroksyny (T4) na morfologię wątroby, markery stresu oksydacyjnego i gospodarki lipidowej we krwi.

Metody: Szczury otrzymywały doksorubicynę dootrzewnowo (1,5 mg/kg m.c.) przez dziesięć tygodni, jeden raz w tygodniu. Jednocześnie podawano tyroksynę (0,2 lub 2,0 mg/l) w wodzie do picia przez czternaście tygodni.

Wyniki: We wszystkich grupach badanych zwierząt stwierdzono wyższy poziom malonylodialdehydu (MDA). Jednocześnie u zwierząt, którym podawano DOX i T4 obserwowano mniejsze stężenie glutationu całkowitego w porównaniu do kontroli. Ocena morfologiczna wątroby nie wykazała oznak martwicy ani stłuszczenia. Stwierdzono natomiast zmniejszoną zawartość glikogenu w grupach DOX+T4 w porównaniu do grupy otrzymującej wyłącznie DOX. Równoczesne podawanie niższej dawki tyroksyny wraz z doksorubicyną wpłynęło na obniżenie stężenia triglicerydów (TG) oraz podwyższenie frakcji LDL cholesterolu.

Dyskusja: Suplementacja tyroksyny spowodowała zaburzenia równowagi redoks oraz stres oksydacyjny w wątrobie szczurów otrzymujących DOX. Badania wykazały normalizujący wpływ tyroksyny na obecność depozytów glikogenu, obserwowanych po podawaniu doksorubicyny. Poza niekorzystnym wpływem tyroksyny na poziom LDL u szczurów otrzymujących doksorubicynę, wykazano korzystne oddziaływanie mniejszej dawki tyroksyny na stężenie TG w surowicy.

Słowa kluczowe: **doksorubicyna • hormony tarczycy • hepatotoksyczność • stres oksydacyjny • lipidy surowicy krwi**

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Introduction

Doxorubicin is one of the most widely used cytostatics belonging to the anthracycline group, which is characterized by high antitumor efficacy. The main limitation of the drug therapy is its toxicity. In normal tissues, its mechanism is associated with the occurrence of oxidative stress [1,2,22]. The liver is an organ in which extensive metabolism of the drug takes place, leading to the formation of not only more toxic metabolites, but also reactive oxygen species [3]. The associated redox changes can lead to anabolic/catabolic imbalances. On the other hand, iodothyronine hormones synthesized by the thyroid gland are very important for maintaining such balance, particularly in relation to lipids. There has been evidence that activation of the synthesis of free radicals is mediated by changes in the concentrations of thyroxine [7,17]. This suggests that these hormones may modify the organism's response in the field of the oxidative stress mediated by doxorubicin, which implies changes in the lipid balance. Confirmation of these assumptions may have potential importance in clinical practice for hepatic changes induced by anthracyclines in individuals with disturbance of iodothyronine hormone balance. The aim of the study was to evaluate the impact of co-administration of doxorubicin and thyroxine on selected parameters of oxidative stress, liver morphology and serum lipid concentration.

Materials and Methods

Experimental model

The study was conducted on sexually mature albino rats of Wistar CRL: (WI)WUBR strain, obtained from a commercial breeder (Warsaw-Rembertow, Poland). The experimental protocol was approved by the Local Bioethical Committee of the Medical University of Lublin. 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 given standardized granulated fodder LSM® (AGROPOL, Poland). The rats were intraperitoneally (i.p.) dosed with doxorubicin (DOX; Ebewe, Austria) while thyroxine (T4; Sigma-Aldrich, USA) was administered in drinking water. The animals were randomly divided into six groups (n=8): DOX – doxorubicin 1.5 mg/kg; 2T4+DOX – thyroxine 2.0 mg/l and doxorubicin 1.5 mg/kg; 0.2T4+DOX – thyroxine 0.2 mg/l and doxorubicin 1.5 mg/kg; 2T4 – thyroxine 2.0 mg/l; 0.2T4 – thyroxine 0.2 mg/l; and the untreated control group. Rats in groups DOX, 2T4+DOX and 0.2T4+DOX received doxorubicin (1.5 mg/kg) for 10 weeks (once a week). In addition, the animals from 2T4+DOX and 0.2T4+DOX groups received thyroxine in drinking water at a concentration of 2.0 and 0.2 mg/l, respectively. The thyroxine administration started one week before the first dose of doxorubicin, and was completed three weeks after the last dose to exclude the presence of cytostatic at the time of obtaining tissue. The rats of groups 2T4 and 0.2T4 were i.p. treated with saline and orally with thyroxine at the same concentrations (2.0 and 0.2 mg/l, respectively).

The animals were anesthetized with pentobarbital. Blood for biochemical studies was collected from the left ventricle to Vacuette tubes with clot activator. Two separate sections of the right liver lobe for histological and biochemical studies were collected to buffered formalin or sterile tubes, which were then frozen in liquid nitrogen and stored at -75°C. After thawing, the tissue sections were homogenized in 20 mM phosphate buffer at pH 7.4 at a ratio of 0.5 g of tissue in 2 cm^3 of the buffer. A homogenizer with a Teflon piston (Glas-col, USA) was used for homogenization (5 min at 4000 rpm). The obtained homogenates were centrifuged for 20 minutes at 14 000 rpm at 4°C.

Biochemical determinations

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).

The evaluation of the lipid peroxidation product was based on malondialdehyde (MDA) concentration in hepatic homogenates. A 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. The concentration of total glutathione (GSHT) was determined colorimetrically in the supernatants obtained from liver homogenates using the commercial set of reagents (Oxis-Research, USA).

Concentrations of total cholesterol (CHT), low density lipoprotein (LDL) and triglycerides (TG) were determined colorimetrically using kits of reagents according to the manufacturer's recommendations (Cormay, Poland).

Preparing for the assessment of histological preparations

4 μm histological slides obtained from paraffin blocks were routinely processed and stained with hematoxylin and eosin (H&E), as well as using periodic acid-Schiff method (paS) and paS after treatment with diastase (d-paS). Frozen tissue sections were stained with Sudan III. Histological specimens were evaluated by means of light microscopy.

Statistical Analysis

Materials and Methods

The obtained data were expressed as mean ± SD and analyzed using STATISTICA 5.0 software. 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 Student's t-test or Mann-Whitney U test and group-to-group comparisons were assessed by one-way ANOVA and post-hoc Tukey's HSD test. The value of $p < 0.05$ was considered statistically significant.

Results

The serum concentration level of free thyroxine (FT4) was significantly higher in animals treated with 2.0 mg of thyroxine than in the untreated control group (Table 1).

Table 1. Concentration (ng/l) of free thyroxine in rats' serum

	FT4
Control	22.38±3.18
2T4	29.77±5.65[a]
0.2T4	21.39±4.42

Data presented as mean ±SD; [a] $p<0.05$ *vs* control

A significantly lower concentration of total glutathione in liver homogenates was found in rats receiving both doses of thyroxine concomitantly with doxorubicin. Interestingly, after applying only doxorubicin or a higher dose of thyroxine alone, insignificant differences were observed while a lower concentration of thyroxine caused a significant increase in the total glutathione level. There were insignificant changes in GSHT level in groups of T4+DOX vs DOX. A significantly higher concentration of hepatic lipid peroxidation products (Table 2) was revealed in all groups of animals compared to the controls. However, insignificant changes of MDA concentration were found between T4+DOX and DOX groups.

Table 2. The concentration of GSH_T [nmol/g sample] and MDA [nmol/g sample] in the liver

	GSH T	MDA
Control	245.33 ± 36.07	2.85 ± 0.43
DOX	206.44 ± 76.97	3.77 ± 0.52[a]
2T4+DOX	186.89 ± 33.99[a]	3.88 ± 0.33[a]
0.2T4+DOX	167.50 ± 22.67[a]	4.59 ± 0.86[a]
2T4	252.89 ± 50.04	4.61 ± 0.57[a]
0.2T4	310.00 ± 31.10[a]	4.52 ± 0.59[a]

Data presented as mean±SD; [a] p<0.05 *vs* control

After doxorubicin administration, the total cholesterol concentration in serum was significantly higher when compared to the control value (Table 3). These differences in mean values compared to the control are intensified even more in animals that received doxorubicin in addition to thyroxine in both doses while differences in the concentrations of cholesterol among groups of T4+DOX versus DOX were not significant. In groups 0.2T4 and 2T4, the concentration of cholesterol was similar to the control. Doxorubicin administration did not significantly change LDL level when compared with the control group (Table 3). However, when doxorubicin was administered with T4 in both doses, the level of LDL was several fold higher compared to the control and DOX group.

A higher concentration of triglycerides (TG) in serum (Table 3) was found in all groups receiving doxorubicin (DOX, 2T4+DOX and 0.2T4+DOX). In rats administered with doxorubicin with a higher dose of thyroxine the mean value of TG concentration was decreased compared to the group of DOX but the difference was not statistically significant. However, it was found that the lower dose of T4 significantly reduced serum TG level in rats receiving doxorubicin (0.2T4+DOX) compared to the group of DOX.

Table 3. Total cholesterol (CHT), low density lipoproteins (LDL) and triglycerides (TG) [mmol/l] in serum

	CH_T	LDL	TG
Control	2.51 ± 0.15	0.37 ± 0.12	1.30 ± 0.27
DOX	4.75 ± 1.88[a]	0.79 ± 0.70	6.21 ± 4.18[a]
2T4+DOX	6.24 ± 0.88[a]	3.01 ± 0.53[a,b]	4.63 ± 3.21[a]
0.2T4+DOX	5.33 ± 2.77[a]	2.54 ± 0.54[a,b]	1.73 ± 0.66[b]
2T4	2.57 ± 0.26	1.81 ± 0.10[a]	1.40 ± 0.47
0.2T4	2.47 ± 0.13	1.84 ± 0.04[a]	1.22 ± 0.19

Data presented as mean±SD ; a P <0.05 vs Control, b p <0.05 vs. DOX

The liver of animals treated with doxorubicin presents hepatocytes with clear, granular cytoplasm. There were also hepatocytes with blurred boundaries focally seen, merging with each other (Fig. 1). A few clusters of mononuclear cells between the hepatocytes were also present, with no clear tendency to locate in certain areas of the lobules. On the slides stained with d-paS an increase in hepatic glycogen content in comparison with the control group was observed (Fig. 2). The hepatocytes in rats treated with doxorubicin simultaneously with thyroxine revealed granular, eosinophilic cytoplasm with the appearance of parenchymatous degeneration. Lack of changes in glycogen content was found when compared with the control. Hydropic degeneration was occasionally seen in the same hepatocytes of the periportal zone (Fig. 3). A few hepatocytes were observed with a more eosinophilic, homogeneous cytoplasm and pycnotic nuclei. In none of the studied groups were the features of steatosis or necrosis noted.

Discussion

The mechanism of the cytotoxic effect of doxorubicin in cancer cells is different from that in normal cells, in which the main mechanism – as outlined in the introduction – is associated with the generation of free radicals and consequent formation of oxidative stress [1,2,22]. The physiological dynamics of ROS formation changes in the imbalance of thyroxine levels [7,17]. It can be assumed that the effects caused by ROS generated in the presence of doxorubicin will be different in individuals with iodothyronine hormone impairment. The common objective of the impact of doxorubicin and iodothyronine hormone on the synthesis of ROS is the mitochondria. The drug inhibits the first electron transport mitochondrial complex [22], and thyroxine,

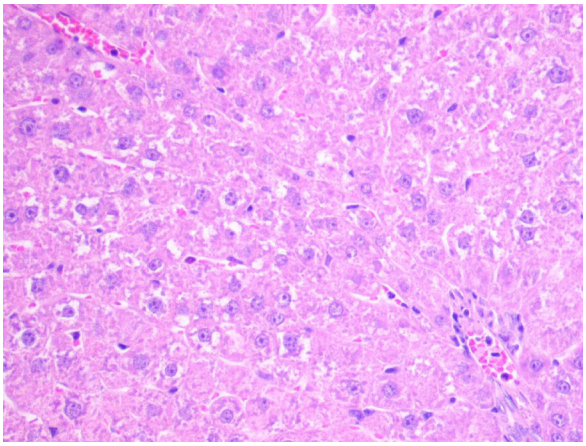

Fig. 1. Hepatocytes with clear cytoplasm. Focal blurring of boundaries between hepatocytes. DOX group (H & E, mag. 200x; DOX group)

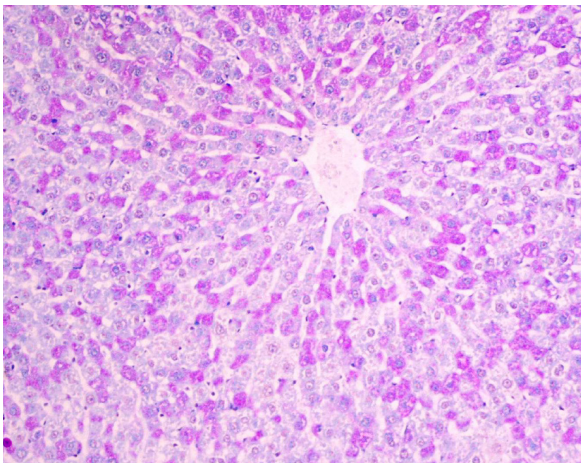

Fig. 2. Deposits of glycogen in hepatocytes (paS , mag. 100x; DOX group)

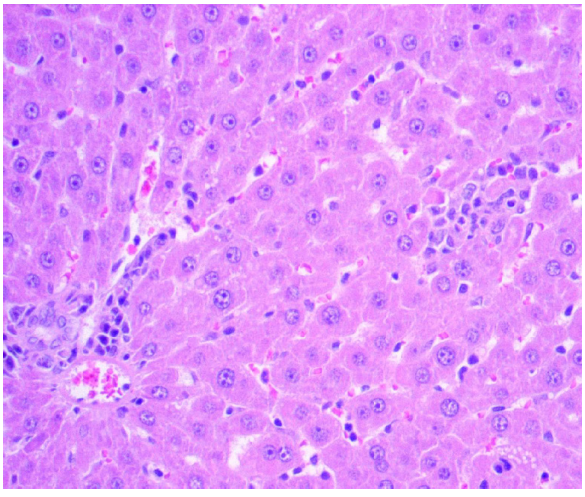

Fig. 3. Hepatocytes with granular, eosinophilic cytoplasm; mononuclear cell infiltration and sparse hepatocytes around triad with hydropic change (H & E, mag. 200x; 2T4+DOX group)

through its T2 metabolite, regulates the formation of ATP [4,15]. Any change in the rate of electron transport in the mitochondrial respiratory chain up-regulates ROS production [11,20]. However, while doxorubicin exerts only a peroxidative effect through the synthesis of ROS and the consumption of NADPH to their own bioactivation [22], iodothyronine hormones, apart from the peroxidative effect through the regulation of mitochondrial function, also regulate the synthesis of the antioxidant NADPH, necessary for regeneration of glutathione. Thyroxine activates G6PDH and malic enzyme, which are the main source of cellular NADPH [6,23].

The study showed a decrease of total glutathione levels in the liver of rats treated with thyroxine (0.2 and 2.0 mg/l) with doxorubicin compared to controls, with no such changes in rats treated with doxorubicin alone. Based on the MDA level, oxidative stress was detected with similar intensity in all groups. The level of antioxidant enzyme was not tested because it does not give direct information about the severity of oxidative damage of molecules. The highest concentrations of cholesterol and LDL were demonstrated in the groups receiving doxorubicin with thyroxine. Thyroxine greatly elevated LDL levels in rats administered doxorubicin. The mean concentrations of TG in group 0.2T4+DOX were lower than in the group receiving DOX alone. The study showed no morphological features of necrosis or steatosis in any of the studied groups of animals, but showed a lower content of glycogen in the liver of rats treated with T4+DOX (0.2 and 2.0 mg/l) compared to rats treated with doxorubicin alone.

In the present study, the most severe changes associated with redox imbalance were observed in animals that received doxorubicin in addition to thyroxine. It is only in these groups that there were simultaneously reduced glutathione concentrations and an increase in the total lipid peroxidation, suggesting that the oxidative stress is a consequence of disturbances in the antioxidant system. The effect of lipid peroxidation may impair the integrity and permeability of the cell membrane, in extreme cases leading to necrosis. However, the lack of histological features of liver necrosis indicates that the observed increase in lipid peroxidation was not extremely high.

Milder changes in the redox system, however, may modulate the dynamics of anabolic/catabolic processes, which appear as disorders of lipid and carbohydrate metabolism. A model example of such changes is alcoholic hyperlipidemia followed by hepatic steatosis, as a result of the increase in the NADH/NAD ratio and caused by alcohol metabolism. In this study, higher concentrations of cholesterol were observed in the T4+DOX groups and TG in the group 2T4+DOX. It should be assumed that these changes result from the activities of the administered chemotherapeutic agent. This is supported by the higher levels of both parameters in rats treated with doxorubicin only and with no differences in the groups of rats treated with thyroxine alone. In rats receiving both doses of thyroxine with doxorubicin, on the other hand, the observed

interactions in relation to LDL should be mainly assigned to thyroxine, since in animals treated with doxorubicin alone insignificantly higher levels of LDL were observed. In groups receiving the same doses of thyroxine the increase compared to control was significant but lower than in the T4+DOX groups. The results related to the effects induced by doxorubicin are confirmed in studies by other authors who revealed that receiving the drug frequently leads to the development of hyperlipidemia manifested as an increase in triglycerides, total cholesterol, LDL and phospholipid levels in serum [5,14,18,19]. The effect of thyroxine on lipid metabolism is multidirectional. Examination of 4000 genes in the liver of mice with short- and long-term hypo- and hyperthyroidism revealed that triiodothyronine (the more active metabolite of thyroxine) controls the expression of many genes whose products are enzymes involved in lipogenesis, fat mobilization and activation of mitochondrial free fatty acid (FFA) oxidation [8]. The effect of thyroxine on lipid metabolism is observed in clinical practice. Hypothyroidism is accompanied by an increase in blood lipids, whereas the administration of thyroxine in patients with hypothyroidism leads to reduced lipid concentrations. Such activity was also observed in the current study since a significant decrease of TG level was found in the 0.2T4+DOX group in comparison with the DOX group.

Taking into account the characteristics of doxorubicin, it may inhibit the activity of the respiratory chain in mitochondria. Moreover, some previous studies demonstrated inhibition of key mitochondrial enzymes by DOX, including complexes I and II, cytochrome oxidase and Fo/F1 ATP synthase [9,10,16]. The consequence of the complexes I and III inhibition chain is the increase of the synthesis of mitochondrial reactive oxygen species: $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ [16]. As mentioned in the present study, it was observed that the most serious changes associated with oxidative stress occurred in animals that received DOX with T4, and only in those groups was there a simultaneous increase of lipid peroxidation and a decrease of the total glutathione level, which may indicate a disturbance in the antioxidant system. The respiratory chain inhibition by DOX should lead to a reduction of NADH oxidation. Furthermore, superoxide anion formed in the presence of DOX is a potent inhibitor of aconitase, and lipid peroxidation products – especially 4-hydroxy-2-nonenal (HNE) – inhibit the activity of α-ketoglutarate dehydrogenase [13,21]. It is also important that both enzymes take part in the cycle as well, and inhibition of its course leads to weakening of the synthesis of NADH [13,21]. The increase in the NADH/NAD ratio results in the weakening of the β-oxidation of fatty acids which are toxic to cells and must be neutralized. The increased synthesis of triglycerides can therefore occur in hepatocytes, which are then transported into the bloodstream in the form of very low density lipoprotein (VLDL). With significant inhibition of β-oxidation, fatty acids are transformed to TG. As a result, TG may accumulate in cells. The assessment of liver histological features did not show steatosis. It seems that TG are effectively removed to the blood. This thesis may explain the cause of a higher blood TG concentration observed in rats treated with DOX.

It is possible that the redox imbalance in the liver of rats treated with doxorubicin will influence the balance of carbohydrates. This supposition was confirmed based on paS and d-paS staining of sections obtained from the livers of animals treated exclusively with doxorubicin, where there was an increase in glycogen content. Interestingly, following co-treatment with DOX and thyroxine, no changes in glycogen amount were found. One reason for these differences may be an increase in glucose-6-phosphate dehydrogenase – a pentose phosphate pathway enzyme, whose synthesis is up-regulated by thyroxine [8,12].

Thyroxine supplementation caused redox equilibrium disorders and oxidative stress in the liver of animals receiving doxorubicin, but these changes do not cause steatosis or necrosis. The study revealed the normalizing influence of thyroxine on glycogen deposits that were observed after doxorubicin treatment. A lower dose of thyroxine had a positive impact on the serum triglyceride imbalance caused by doxorubicin. However, it has an adverse effect on the LDL cholesterol fraction in rats treated with doxorubicin.

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