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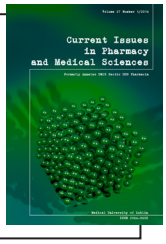

The effect of thyroxin on hepatic redox equilibrium and lipid metabolism in rats treated with doxorubicin

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ABSTRACT

The main side effects of the administration of doxorubicin, a widely used anticancer drug, is the generation of a reactive oxygen species (ROS) in normal cells. As a result, redox disorders and secondary oxidative stress are developed. Doxorubicin ROS generation is attributed to enzymes that are produced abundantly in hepatocytes. Oxidative stress has been a well-known risk factor of doxorubicin-related toxicity. However, in addition, according to the data collected in the last decade, changes in thyroxin status can propagate ROS generation, and, thus, initiate the doxorubicin hepatic effect. Moreover, both compounds have an impact on the cell metabolism. The aim of the study was to verify the thesis that thyroxin can modulate the effect of doxorubicin with regard to redox status and lipid metabolism disorders. In our work, we determined the ratio of $NADP^+$/NADPH and NAD^+/NADH in liver homogenates, blood ketone bodies and triglycerides in the liver and blood in rats treated with doxorubicin and thyroxin. Our results indicate that thyroxin has an insignificant effect on NAD^+/NADH, $NADP^+$/NADPH ratios and on hepatic and blood triglycerides. Moreover, thyroxin administration normalized the level of blood ketone bodies that was disturbed by doxorubicin.

INTRODUCTION

Doxorubicin is a very efficient and widely used cytostatic that belongs to the antracycline chemical group. Its toxic mechanism is different than that of its anticancer properties, and relies upon redox disorders and secondary oxidative stress. The doxorubicin chemical structure predisposes it to being part of a univalent redox cycle catalyzed by NADPH and NADH dependent enzymes [16]. However, thyroxin can modulate the effect of doxorubicin with regard to redox status and lipid metabolism disorders. NADPH and NADH dependent enzymes are especially abundant in hepatocytes [4]. A constantly repeated redox cycle may deplete NADH and NADPH quantities, resulting in redox balance disorders. Moreover, the changes in NAD+/NADH, NADP+/NADPH ratios may have an impact on lipid metabolism [10]. Yet, NADPH is also necessary in antioxidative defenses. The main enzymes that regenerate NADPH, e.g.: dehydrogenase glucose 6-phosphate (G6PDH), dehydrogenase 6-phosphoglukonate and malic enzyme are under the control of triiodothyronine [6,13]. Thus, in the current study, to bring about an understanding of the role played by doxorubicin and thyroxin administration, we determined the ratios of NADP+/NADPH and NAD+/NADH in liver homogenates, blood ketone bodies and triglycerides in the liver and blood of rats treated with both substances.

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METHODS

Animals and treatment

The study was approved by the Local Ethical Committee at the Medical University of Lublin. Male Wistar rats (180-220 g) were kept under conventional laboratory conditions (temperature 22°C, humidity 60-70%, 12h light/dark cycle) and fed with standard rodent granulated fodder LSM® (AGROPOL, Poland). Food and water were freely available. The animals were randomly placed into three experimental and control groups (six animals per group): control (saline *i.p.*, Polfa, Poland), DOX – doxorubicin (1.5 mg/kg), T4 – thyroxin (4 mg/l), T4 + DOX (thyroxin 4 mg/l + DOX 1.5 mg/kg b.w.). The rats received, once a week, a intraperitoneal injection of doxorubicin (EBEWE Arzneimittel Ges.m.b.H., Austria) at a dose of 1.5 mg/kg for 12 weeks. Thyroxin (tetraiodotyronin, T4; Sigma, USA) was taken with drinking water (4 mg/l). The administration of T4 was started a week before the first injection of doxorubicin and was stopped three weeks after doxorubicin cessation. Three weeks after the last dose of doxorubicin (cumulative dose 18 mg/kg b.w.), blood and liver samples were taken during pentobarbital anesthesia. The serum and liver was kept at -70°C until the determination time. The serum and hepatic concentration of triglycerides (Cormay, Polska), serum ß-hydroxybutyrate (Assay Kit, Cayman, USA) and NAD(P)$^+$/NADH(P) (BioChain, USA) ratios were determined using a spectrophotometric plate reader PowerWaveXS (Bio-Tek, USA), according to the manufacturer's procedures.

Statistical analysis

The obtained data were analyzed using STATISTICA 5.0. (Statsoft Inc., USA). Statistical significance was evaluated by the U Mann-Witney test (vs. saline control) and by one-way analysis of variance (Kruskal-Wallis ANOVA). The post-hoc test (Newman-Keuls) was used to verify the null hypothesis, according to which the lowered thyroid hormones status influence the evaluated parameters. All data are expressed as mean ± standard deviation. The value of $p < 0.05$ was considered statistically significant.

RESULTS

There were no significant differences in hepatic NAD+/NADH and NADP+/NADPH ratios in all tested group, compared to the control and between the DOX+T4 versus DOX group (Tables 1 and 2).

In all tested groups, a significant decrease in hepatic triglycerides level was observed in comparison to the control, but the effect in group DOX+T4 was not summed up (Table. 3). There were no significant differences between the DOX+T4 versus DOX group.

Serum triglycerides level were several time higher in group DOX or T4 and this change is exacerbated in group DOX+T4, where the level was 6-fold higher than in the control (Table 4). There were, however, no significant changes between the DOX+T4 versus DOX group. Probably this effect was related to the individual differences in response – as was evidenced by high value of SD.

The blood β-hydroxybutyrate concentration was highly reduced in the rats that were treated with either doxorubicin or thyroxin (Table 5). However, in rats receiving both drugs together, its level was similar to the control and significantly higher versus the DOX group.

Table 1. Hepatic NADP+/NADPH ratios

	M	Me	Min	Max	SD	p
Control	1.43	1.15	0.93	2.865	0.814	
DOX	2.76	2.24	1.87	5.396	1.484	0.075
4T	1.17	1.10	0.92	1.445	0.234	0.916
DOX+4T	1.60	1.64	0.98	2.049	0.417	0.250

Table 2. Hepatic NAD+/NADH ratios

	M	Me	Min	Max	SD	P
Control	1.86	1.44	1.28	3.61	0.986	
DOX	2.83	2.59	2.32	4.10	0.729	0.075
4T	1.09	0.96	0.69	1.88	0.472	0.075
DOX+4T	1.74	1.71	1.39	2.09	0.260	0.347

Table 3. Hepatic homogenate triglycerides concentration [mg/l]

	M	Me	Min	Max	SD	p
Control	2426.18	2626.40	976.40	3788.20	1074.15	
DOX	1108.24	1025.84	871.35	1452.25	240.41	0.044
4T	693.37	698.31	401.69	852.81	179.39	0.009
DOX+4T	785.86	698.31	568.54	1161.80	247.05	0.017

Table 4. Serum triglycerides concentration [mg/l]

	M	Me	Min	Max	SD	p
Control	55.31	52.22	26.57	81.57	20.20	
DOX	144.19	140.28	96.40	202.70	39.96	0,001
4T	104.64	104.44	61.80	155.73	32.58	0.010
DOX+4T	330.62	310.84	122.36	739.10	225.13	0.004

Table 5. Serum β-hydroxybutyrate concentration (mM)

	M	Me	Min	Max	SD	p
Control	1.52	1.39	1.00	2.27	0.467	
DOX	0.18	0.16	0.10	0.34	0.086	0.004
4T	0.29	0.20	0.16	0.67	0.198	0.004
DOX+4T	1.64*	1.63	1.16	2.27	0.406	0.662

* $p \leq 0.05$ vs DOX

DISCUSSION

The best known side effect of doxorubicin is its cardiotoxicity. According to the current data, the drug programs changes in mitochondrial DNA (mtDNA), hence, synthesizes defected mitochondrial protein. This results in ROS production and repeated damage to mtDNA [2, 20]. Initially clinically silent, changes with time manifests as mitochondrial insufficiency and fatal delayed cardiomyopathy [5]. Because the enzymes that produce free radicals in the presence of doxorubicin have several times higher activity in the liver than in other organs [4] the mechanism may by similar. For this reason, in the current study, the model with cumulative

dose was used, and biochemical evaluation was performed three weeks after the last doxorubicin injection. It must be underlined, that the liver is the main organ responsible for the lipid and hydrocarbon metabolism that is under control, among others, of thyroid hormones [6,13].

The most important results of the study indicate that thyroxin did not change hepatic NAD^+/NADH and $NADP^+$/NADPH ratios, nor did it do so to hepatic and blood triglycerides levels. However, thyroxin normalize the level of ketone bodies in the blood that was disturbed by doxorubicin.

Both NAD^+/NADH and $NADP^+$/NADPH ratios play pivotal roles in the cell redox equilibrium. The NAD^+/NADH ratio is a main parameter of metabolic state because a lot of anabolic and catabolic processes are regulated by changes in the NAD^+/NADH ratio. Inhibition of some mitochondrial electron transport chain enzymes by doxorubicin [12] may cause the elevation of NADH, and, consequently, bring about an inhibition of fatty acid ß-oxidation. As a result, triglycerides increase in the liver, and, subsequently, should appear in the blood. However, our study showed that hepatic triglycerides level decreased to 50% of that of the control. This effect may be understood after a detailed explanation of the inhibition mechanism of the I mitochondrial complex through treatment with doxorubicin. The one enzyme of the I mitochondrial enzymatic complex, despite receiving a transfer electron from NADH to complex III, reduces doxorubicin, and this obtained electron from doxorubicin is transferred to the O_2, generating an oxidative stress reaction [15]. Because this process runs cyclically to the end of the availability of the substrates, the inhibition of the I complex may results in a diminishment of NADH as a result of conversion to the NAD^+. Immediately, the NAD^+ is transform, especially by way of the Krebs cycle and through fatty acids ß-oxidation. Thus, this effect may explain the lack of changes that we saw in the hepatic NAD^+/NADH ratio and the triglycerides level in rats treated with doxorubicin. What is more, thyroxin has no effect on these parameters in rats receiving doxorubicin, although basic metabolism transformation is observed during hypothyreosis [1]. Of note, the increase in serum triglycerides level in the group DOX and DOX+T4 seems to be dependent on metabolism effects within some other tissue. Our results, however, are consistent with those of Singal and Iliskovic's study [19].

NADPH is a second cofactor used by enzymes to reduce doxorubicin, but it is also the main agent regenerating reduced glutathione – the main enzymatic antioxidant counter in the cell. A proper $NADP^+$/NADPH ratio is crucial in keeping the cell's redox potential [8,14]. Moreover, according to the study by Ogasavara *et al.* [18], the $NADP^+$/NADPH ratio is a better oxidative stress marker than is glutathione, because the induced changes persists longer than do changes in GSH.

Based on the currently obtained data, doxorubicin did not have a significant effect on NADP+/NADPH ratio. Such a result is unexpected, since NADPH cytochrome P450 reductase and NADPH nitric oxide synthase are very efficient in the univalent reduction of doxorubicin [7,17]. Moreover, NADPH defends against the oxidative stress induced by univalent doxorubicin reduction. Thus, it seems that the produced NADP+ in both these reactions is regenerated in an efficient manner by glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malate enzyme. The genes encoding these enzymes are controlled by iodothyronine hormones [6,13]. However, thyroxin has an insignificant effect on the NADP+/NADPH ratio. This situation is interesting, especially since iodothyronine hormones led to accelerated mitochondrial electron transport, and, secondarily, to the increasing of ROS synthesis [21].

β-Hydroxybutyrate makes up an average of 75% of all ketone bodies in an organism, thus, it is a good marker for ketoacidosis. It is synthesized mainly in the liver during the oxidation of fatty acids. In our work, in rats treated with doxorubicin (cumulative dose 18mg/kg), the concentration of β-hydroxybutyrate was 8-fold lower than in the control. This effect is consistent with the diminishment of hepatic triglycerides that we also observed in this study. The study carried out by Carvalho *et al.* [3] and Hong *et al.* [9] shows that in other organs there is no increase in consumption of ketone bodies. A similar decrease in β-hydroxybutyrate level was observed in rats taking thyroxin with drinking water. What we saw was in opposition to our expectations because thyroxin exacerbates the β-oxidation of fatty acids [11]. Moreover, despite these decreasing in T4 and DOX groups, in rats treated with thyroxin together with doxorubicin, the level of β-hydroxybutyrate was similar to that of the control.

In summary, the obtained results reveal a delayed (3-week after cessation of drugs), significant inhibition of ketone bodies synthesis by doxorubicin and thyroxin, and an unpredictable effect of interaction between both drugs that leads to normalization of β-hydroxybutyrate levels. Yet, some of the obtained results are difficult to interpretation. However, an adaptive mechanism seems to play a major role and should be taken under consideration. To fully understand this effect, a study of a broader spectrum of biochemical parameters in several other organs (including, in particular, the muscles), should be undertaken.

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