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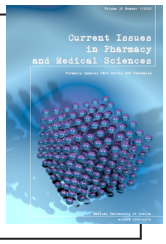

Can Dexrazoxane and Carvedilol prevent Doxorubicin-induced nephrotoxicity?

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ABSTRACT

Doxorubicin (*DOX*) is a cytostatic drug with a broad spectrum of anticancer activity that has been used in oncology for over 50 years. Among many adverse effects in humans, the most dangerous is late dilated cardiomyopathy, which appears even years after completion of therapy. However, in cats, the critical organ for the toxic effects of *DOX* is the kidney. Herein, nephrotoxicity is manifested as azotemia. The main aim of our study was to evaluate the protective effect of dexrazoxane (*DEX*) and carvedilol (*CVD*) against the nephrotoxic effects of *DOX*. Nephrotoxicity studies were performed in a rat model of repeated *DOX* administration. Analyzed blood morphological, biochemical and histopathological findings revealed that DEX has a dual effect: it positively impacts *DOX*-induced histological alterations and creatinine levels while negatively affecting urea concentration. Thus, the results do not support univocally recommend *DEX* to prevent nephrotoxicity caused by *DOX* in cats. However, further studies using initially lower doses of *DEX* are needed to assess the prevention of nephrotoxicity in cats clinically treated with *DOX*.

INTRODUCTION

Doxorubicin (*DOX*) is an effective cytostatic drug used to treat many human cancers. Side effects include myelotoxicity, gamete toxicity and organ toxicity. In humans, the most dangerous side effect is cardiotoxicity, manifested by dilated cardiomyopathy. This is an irreversible change leading to death [1]. The incidence rate of dilated cardiomyopathy depends primarily on the dose of *DOX*. At a cumulative dose of 650 mg/m^2, the incidence rate is as high as over 35%. Reducing the cumulative dose to 400 mg/m^2 minimizes the risk of cardiomyopathy to approximately 5%, but even lowering the cumulative dose to as low as 240-360 mg/m^2 does not eliminate the risk of cardiomyopathy [2] particularly with anthracyclines, is frequently associated with cardiotoxicity, an effect exacerbated by trastuzumab. Several compounds are in use clinically to attenuate the cardiac-damaging effects of chemotherapy drugs, including angiotensin-converting enzyme (ACE. In humans, *DOX* is not associated with nephrotoxicosis. However, risk factors for renal failure contraindicate administration. These factors include age and female sex, as both are associated with decreased muscle mass and total body water and, therefore, are likely an underrepresentation of pre-existing kidney dysfunction, as defined by serum creatinine concentration, before administration of chemotherapy [3].

DOX is also used in cancer diseases in pets such as cats, dogs, rabbits and guinea pigs. In cats, *DOX* is used in various cancers in mono- and multidrug therapy [4]. The most frequently employed protocols in the clinical setting are based on the CHOP (cyclophosphamide, *DOX* (hydroxydaunorubicin), vincristine (Oncovin), and prednisone/prednisolone) protocol. Anthracycline-dependent cardiotoxicity, including cardiomyopathy, has been demonstrated in dogs. In cats, cardiomyopathy occasionally appears as hypertrophic change, mild left atrial enlargement, mild right ventricular dilatation and left ventricular enlargement. Still, these changes had not been associated with clinical evidence of cardiac insufficiency. Hematological changes in cats are observed in approximately 25-50% of all cats treated with *DOX* (25 mg/m^2) as leukopenia. Several percent of cats developed

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persistent, nonregenerative anemia after administration of cumulative doses over 50 mg/m^2 [5].

The most dangerous toxic effect of *DOX* in cats is nephrotoxicity, which is manifested by overt azotemia [3]. When *DOX* alone was administered to cats with sarcoma after approximately eight months, 9% (5/55) became azotemic [4]. In another study, two of 17 (12%) cats with several different cancer types became azotemic after receiving over 100 mg/m2 cumulative *DOX* dose in combination with 2800 mg/m2 cyclophosphamide [5]. Indeed, experimental results show that 17% of all cats receiving *DOX* become azotemic. However, there was no difference concerning blood urea nitrogen (BUN) and serum creatinine concentrations between groups [3]. Thus, 9% and 17% of all cats receiving *DOX* become azotemic based on traditional evaluation of serum creatinine concentration outside of its reference interval without considering muscle loss. In addition to azotemia, other side effects have been observed in cats, such as anorexia, alopecia, myelosuppression, weight loss, vomiting, diarrhea and kidney injury [6] the use of this drug in cats has been associated with side effects such as renal injury, myelosuppression, anorexia, and weight loss. The goal of this study was to compare the toxicities associated with two dosing schemes for doxorubicin in tumor-bearing cats. Group A cats received 1 mg/kg of doxorubicin, while group B cats received 25 mg/m^2 of doxorubicin plus 22 ml lactated Ringer's solution per kilogram body weight subcutaneously. Toxicities were evaluated using laboratory data, physical examination, and history, and were graded using a standardized scale and compared between groups. Post-treatment neutrophil counts were significantly lower among cats in group B compared to cats in group A (P< or = 0.001.

Simultaneously, new solutions to minimize the risk of cardiomyopathy in *DOX*-treated patients are continuously being investigated. A few compounds have entered clinical trials for the registration of drugs with an anti-anthracycline-induced dilated cardiomyopathy activity, and *CVD* (NCT04023110) is one of them. It is a β-blocker with an antioxidant feature. *CVD* prevented anthracycline-induced dilated cardiomyopathy in two randomized clinical studies. The studies have demonstrated that *CVD* favors cardiac mitochondria *in vitro*, *ex vivo*, and *in vivo* models. *CVD*, in particular, is considered to act as an inhibitor of mitochondrial complex-I, which is recognized as a donor of NADH for the *DOX* redox cycle and, as a result, the cause of anthracycline-induced dilated cardiomyopathy. *CVD* was shown to be more effective than propranolol in preventing *DOX*-induced cardiomyopathy [7].

The second cardioprotective compound is dexrazoxane (*DEX*), which alleviates some of the side effects caused by *DOX*, and is the only pharmaceutical approved by the FDA to prevent the development of anthracycline cardiomyopathy [8]. *DEX* as an antidote for *DOX*-induced extravasations, was initially studied in rodents and used in humans. In 2012, a recommendation appeared for using *DEX* in extravasations caused by *DOX* in dogs. In dogs, DEX also attenuated haematologic, gastrointestinal and cardiovascular toxicities induced by *DOX*. To alleviate *DOX*-induced extravasation in cats, *DEX* was used for the first time in 2007 [9].

Several factors limit the use of *DEX*. Primarily, its protective impact may not be entirely successful, as it can diminish the pharmacological action of *DOX*. Furthermore, the drug itself can lead to severe side effects, such as myelotoxic effects, including the potential development of leukemia. Thus, exploring novel therapy approaches to attain the most effective protection strategies is reasonable. An effective approach could involve co-administering *DEX* with another molecule to synergistically enhance its protective impact, potentially leading to a lower dosage of *DEX* in the future. Over many years, research studies have consistently shown the efficacy of various substances with antioxidant properties. However, none has proven their efficacy in preventing *DOX*-induced cardiotoxicity.

To the best of our knowledge, the effects of *DEX* and *CVD* on *DOX*-induced nephrotoxicity and myelotoxicity in cats have not been studied so far. Therefore, this study has attempted to evaluate the effects of *DEX* and *CVD* administered in the model of rats receiving *DOX*, as well as the impact of *CVD* on the assessed nephro- and myelotoxicity parameters in rats receiving *DOX*+*DEX*. Additionally, correlations between markers of kidney damage and heart function parameters determined by ultrasound (ECHO) were assessed.

MATERIALS AND METHODS

Animals

The 100 male Wistar rats, all of whom were aged eight weeks, were acquired from the Experimental Medicine Center within the Medical University of Lublin, Poland. The rats in the experiment were housed under controlled environmental conditions, with a temperature varying from 22±3°C, relative humidity kept at 50±5%, and a continuous 12-hour light/dark cycle. The animals had unlimited access to drinking water, along with a standardized diet for rodents. The procedures were carried out from 9 a.m. to 3 p.m. The animal research procedure (123/2018) for Animal Experiments within the University of Life Sciences in Lublin, Poland was approved by the Local Ethical Committee on 03 December 2018. The experimental animal protocols adhered to the European Committee Directive for Care and Use of Laboratory Animals (2010/63/EU). The animals were continuously monitored by veterinarians, and every precaution was taken to reduce the risk of harm.

Experimental Design

The rats underwent a 7-day acclimation phase before the study, as previously described [10,11]. The animals were subsequently allocated to five research groups through random assignment. At first, there were 20 rats in each study group, with 50% of the animals being sacrificed in the 11th week, a week after the treatment period ended. The animals that remained were sacrificed in the 21st week, 10 weeks after the administration ended.

The experimental groups included the control group (CTR), *DOX* without prior treatment, *DEX* and *CVD* prior treatment 30 minutes before *DOX* administration, DEX prior treatment 30 minutes before *DOX* administration, and *CVD* pretreatment 30 minutes prior to *DOX* administration (Table 1). During the experiment, there was a shift in the amount

of rats in the two groups at the 21st week of the study (DOX group consisted of 5 animals, DOX + CVD group consisted of 6 animals) due to increased mortality as described in [11].

The group names are in regular font (e.g., DOX), and the substances are in italics (e.g., *DOX*) to distinguish the group symbol from the substance name in the manuscript.

DOX, *DEX*, and *CVD* were acquired from Merck in Darmstadt, Germany. The solutions were prepared at a concentration of 0.01 mL per gram of body weight shortly prior to administration, and administered by intraperitoneal injections (i.p.) one a week for 10 weeks, as described in Table 1. The rats were sacrificed using 3.5% isoflurane anesthesia, followed by decapitation. Their kidneys were subsequently collected for pathology, biochemical and molecular investigation. The blood was collected for further biochemical analysis.

Table 1. The experimental administration design

Symbol of Group	IP Administration, once a week for 10 weeks
CTR	mL 0.9% NaCl per g body weight
DOX	1.6 mg DOX per kg of body weight
DOX+DEX	25 mg DEX per kg of body weight 30 minutes prior DOX; 1.6 mg DOX per kg of body weight
DOX+DEX+CVD	1 mg CVD per kg of body weight 30 minutes prior DOX; 25 mg DEX per kg of body weight 30 minutes prior DOX; 1.6 mg DOX per kg of body weight
DOX+CVD	1 mg CVD per kg of body weight 30 minutes prior DOX. 1.6 mg DOX per kg of body weight

CTR – control, CVD – carvedilol, DEX – dexrazoxane, DOX – doxorubicin, IP – intraperitoneal

Biochemical Analysis

The assessment of morphological parameters was performed with an Abacus Junior Vet 5 (Diatron MI PLC, Budapest, Hungary) hematology analyzer. Creatinine blood serum levels were measured by Liquick Cor-Creatinine assay following the manufacturer's instructions (PZ Cormay, Lublin, Poland). Phosphorus concentrations were measured in blood serum by photometric measurement using Liquick Cor-Phosphorus assay following the manufacturer's instructions (PZ Cormay, Lublin, Poland). Urea blood serum levels were measured by Liquick Cor-Urea assay following the manufacturer's instructions (PZ Cormay, Lublin, Poland). Concentrations were calculated based on standard curves.

Histological Staining

Sections of the kidney were obtained from each rat and stored in buffered 10% formalin with a pH of 7.4. These specimens were then processed into paraffin blocks. Four-micrometer slides were prepared using a microtome and stained with hematoxylin and eosin. An experienced blind pathologist examined the slides with a light microscope. Each animal had slices from two kidneys assessed for staining, with 20 slides stained with hematoxylin and eosin per group. The histological alterations were categorized as follows: "–" for no changes, "+" for mild changes, "++" for moderate changes, and "+++" for large changes.

Statistical Analysis

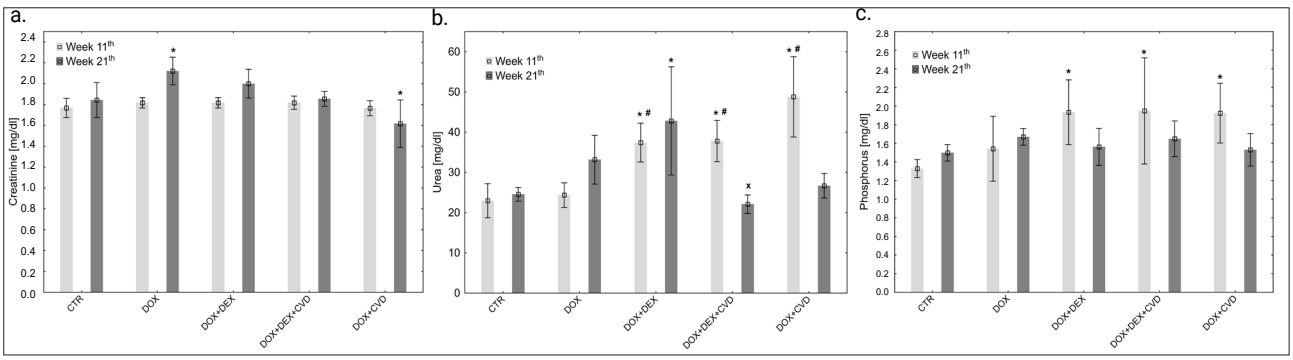
Statistical analysis was conducted using Statistica13 software (StatSoft, Krakow, Poland). The normal distribution of the variables was established by the Shapiro-Wilk test. Tukey's HSD post hoc test was used together with one-way ANOVA for statistical analysis. The body weight changes over 21 weeks was analyzed by applying one-way ANOVA for repeated measurements and Tukey's HSD test. Pearson correlation coefficient was employed to examine the relationships between echocardiographic parameters with creatinine, urea and phosphorus in the 11th and 21st weeks of the study. Histological abnormalities incidence was assessed via chi-squared testing. The data were computed as the mean ± the standard deviation. Statistically significant differences between the groups were considered when the *p*-value was less than 0.05.

RESULTS

Biochemical analysis

There were no alterations in creatinine levels among any of the research groups by the 11th week (Figure 1a). However, in week 21, higher creatinine levels were found after administration of *DOX* alone. In the remaining groups, no changes were observed in comparison to the control group.

At week 11, there were no changes in urea levels in rats receiving *DOX* alone (Figure 1b). In contrast, in the DOX+DEX, DOX+DEX+CVD and DEX+CVD groups, a significant increase in urea was observed compared to the control and the DOX groups. In turn, in the 21st week of the study, despite the lack of effect of *DOX* itself on the level of urea, its level increased when the rats received *DEX* in addition to *DOX* (DOX+DEX group). There was no increase in urea levels when rats received *CVD* in addition to *DOX* and *DEX*.



The values are presented as a mean ± SD. Statistical significance: *p≤0.05 *vs*. control group; #p≤0.05 *vs*. DOX; ˣ≤0.05 for comparison of DOX+DEX+CVD *vs* DOX+DEX. (one -way ANOVA with Turkey's post-hoc test). CTR, control; CVD, carvedilol; DOX, doxorubicin; DEX, dexrazoxane

Figure 1. Statistical differences between **a.** creatinine, **b.** urea, **c.** phosphorus levels in rat's blood serum sacrificed in the 11th or 21st week of study

Also, in the case of phosphorus (Figure 1c), no changes were observed in the 11th week after administration of *DOX* alone, but in the three remaining study groups, where *DOX* was administered together with protective agents, a significant increase in phosphorus levels was observed. There were no changes in phosphorus concentration in week 21 of the study.

We also measured a correlation between echocardiographic parameters (11) with creatinine, urea and phosphorus in the 11th and 21st weeks of the study (Table 2). Only in one case was a significant correlation observed. A positive correlation was found in the 11th week of the study between the urea level and the left atrial diameter, with $r = 0.401806$. Correlation studies between the level of markers of kidney function disorders: creatinine, urea and phosphorus and heart function parameters in the echocardiography study show that there are practically no mutual dependencies between these parameters in the 11th and 21st week of the study.

Table 2. Results of correlation of echocardiographic parameters with creatinine, urea and phosphorus in the 11th and 21st weeks of the study

Echocardiography parameter	Week of the study	Creatinine	Urea	Phosphorus
		Pearson correlation coefficient (r)		
Left ventricular ejection fraction	11th	-0.0486	-0.2653	-0.0453
Fractional shortening		-0.0192	-0.2789	-0.0555
Left ventricular end-diastolic diameter		-0.1024	0.0318	-0.0607
Left atrial diameter		-0.1176	0.4018	-0.0126
Ascendic aorta diameter		0.0721	-0.0496	-0.2319
Left ventricular ejection fraction	21st	0.0994	0.3463	-0.3840
Fractional shortening		0.1223	0.3194	-0.3929
Left ventricular end-diastolic diameter		-0.1049	0.0158	0.0429
Left atrial diameter		-0.1065	-0.1270	-0.0379
Ascendic aorta diameter		-0.4023	-0.1602	-0.1241

Data were measured by Pearson correlation coefficient (r) and strong correlation was marked by red color

Histopathological evaluation

Histopathological examinations of the kidney showed minor changes in the DOX+DEX group compared to the DOX group (Table 3, Figure 2 a-g). Adding *CVD* to rats receiving *DOX* and *DEX* led to a moderate increase in mononuclear cell infiltration and moderate changes in intratubular casts relative to the DOX+DEX group. The most significant unfavorable changes in the assessed parameters were found in the DOX+CVD group.

Cell morphology assessment

In week 11 of the study, *DOX* was responsible for a significant decrease in the number and percentage of monocytes (Table 4). Both *DEX* and *CVD* limited the decline in the number of monocytes and normalized their rate. The most effective reduction in monocyte number decline occurred when *DEX* was administered together with *CVD* (DOX+DEX+CVD *vs*. DOX group). However, in the DOX+DEX+CVD group, the percentage of lymphocytes decreased, which was not observed in the other study groups. In contrast, in all study groups except DOX, the number of lymphocytes decreased. There was also a decrease in WBCs

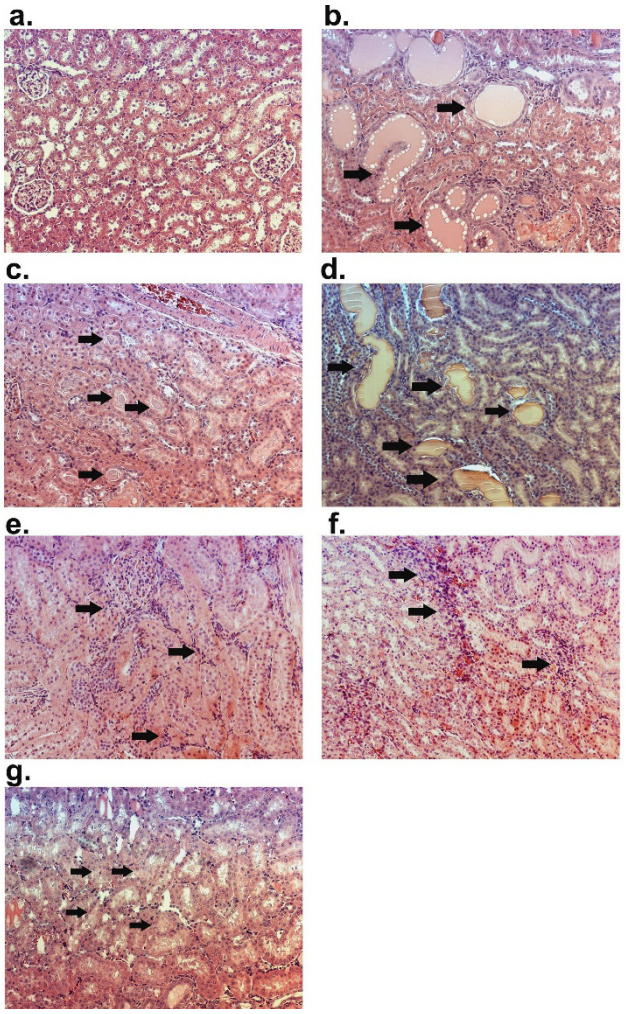


Figure 2. Hematoxylin and eosin staining of kidney, magnification 200×. (a) Control group; (b) Tubule dilatation (→) in DOX+DEX+CVD group, the 11th week of the study; (c) Intratubular casts (→) in DOX+DEX+CVD group, the 11th week of the study; (d) Intratubular casts (→) in DOX+CVD group, the 11th week of the study; (e) Intertstitial mononuclear cell infiltration (→) in DOX group, the 11th week of the study; (f) Intertstitial mononuclear cell infiltration (→) in DOX+DEX+CVD group, the 11th week of the study; (g) Focal necrosis (→) in DOX+CVD group, the 11th week of the study. CVD – carvedilol, DOX – doxorubicin, DEX – dexrazoxane

Table 3. The presence and intensity of morphological changes in rats' kidneys after the study treatment

Morphological feature	Week of the study	Study group				
		CTR	DOX	DOX +DEX	DOX +DEX +CVD	DOX +CVD
Intertstitial mononuclear cell infiltration	11	-	++ (10/10)	-	+ (9/10)	+ (8/10)
	21	+ (6/10)	+ (5/5)	+	++ (9/10)	+ (6/6)
Tubule dilatation	11	-	+ (9/10)	-	-	++ (9/10)
	21	-	+ (5/5)	-	-	+ (6/6)
Intratubular casts	11	-	-	-	+ (8/10)	+ (8/10)
	21	-	-	-	+ (7/10)	-
Necrosis	11	-	+ (8/10)	-	-	++ (8/10)
	21	-	-	-	-	-

"-" – no changes, "+" – changes of minor intensity. "++" – moderate changes, "+++" – changes of major intensity, incidence in the group

in the DOX+DEX and DOX+CVD groups. In week 21 of the study, no statistically significant changes were found in group comparisons.

Table 4. Statistical differences between white blood cell count and percent in rats sacrificed in the 11th or 21st week of study

Biochemical parameter	Week of the study	Study group				
		CTR	DOX	DOX +DEX	DOX +DEX +CVD	DOX +CVD
WBC [10^3/μL]	11	6.55 ±2.20	3.88 ±2.55	3.25 ±0.98*	3.86 ±1.44	3.96 ±0.92*
	21	6.08 ±2.67	4.67 ±2.43	6.76 ±2.34	6.78 ±3.02	7.49 ±2.41
LYM [10^3/μL]	11	4.31 ±1.15	2.85 ±1.36	2.10 ±0.60*	1.87 ±0.81*	2.29 ±0.64*
	21	3.85 ±2.15	2.39 ±1.35	4.34 ±1.78	4.35 ±1.65	4.50 ±2.68
MON [10^3/μL]	11	0.69 ±0.16	0.08 ±0.07*	0.20 ±0.17*	0.33 ±0.11*#	0.26 ±0.26*
	21	0.51 ±0.31	0.43 ±0.21	0.49 ±0.25	0.46 ±0.53	0.57 ±0.65
NEU [10^3/μL]	11	1.55 ±0.90	1.41 ±1.40	0.94 ±0.30	1.65 ±0.47	1.41 ±0.35
	21	1.61 ±0.60	1.85 ±0.96	1.93 ±0.62	1.97 ±0.94	2.42 ±1.76
LYM [%]	11	66.73 ±4.12	62.50 ±12.24	65.06 ±4.13	47.43 ±9.67*x	57.83 ±8.74
	21	63.24 ±11.38	49.45 ±7.56	62.76 ±6.77	66.00 ±5.83	59.53 ±13.71
MON [%]	11	10.80 ±1.06	3.47 ±3.34*	6.06 ±3.84	8.90 ±2.48	5.58 ±5.00
	21	8.60 ±3.52	9.60 ±1.86	7.36 ±3.93	5.23 ±4.85	7.53 ±5.48
NEU [%]	11	22.50 ±5.23	34.00 ±11.83	28.90 ±4.12	28.77 ±7.61	32.88 ±8.95
	21	28.17 ±11.89	40.96 ±8.13	29.90 ±7.42	28.78 ±4.71	32.88 ±9.15

The values are presented as a mean ± SD. Statistical significance: *p≤0.05 *vs.* control group; #p≤0.05 *vs.* DOX; ^{x} ≤0.05 for comparison of DOX+DEX+CVD *vs* DOX+DEX. (one-way ANOVA with Turkey's post-hoc test)

CTR – control, CVD – carvedilol, DOX – doxorubicin, DEX – dexrazoxane, LYM – Lymphocytes, MON – Monocytes, NEU – Neutrophils, WBC – White blood cells

Of all the red blood cell panel parameters tested, only the RDWc values changed in the DOX group (Table 5). A similar increase in RDWc versus control was observed in all study groups. When, in addition to *DOX*, *DEX* was administered (DOX+DEX group), an increase in other parameters *vs*. DOX (RBC, HGB, HCT, MCHC, and RDWc) and/or *vs*. control (MCH, HCHC, RDWc) was observed. In the DOX+CVD and DOX+DEX+CVD groups, a similar increase in almost all determined parameters was noted. When rats were additionally administered *CVD* in addition to *DOX* and *DEX* (comparison of DOX+DEX+CVD *vs*. DOX+DEX), normalization of RBC and HGB to the control level was demonstrated. Despite the described statistically significant differences in individual parameters of the red blood cell system, the maximum percentage deviations from the average of the appropriate control for different study times were small.

Among the platelet parameters, *DOX* showed an influence only on the MPV value (Table 6). A significant reduction in this parameter was indicated in the DOX group. In the DOX+DEX group, there were no differences in any of the tested parameters of this panel compared to the control. Reductions in PCT and PDWc values were observed in the DOX+DEX+CVD and DOX+CVD groups, respectively.

Table 5. Statistical differences between red blood cell parameters in rats sacrificed in the 11th or 21st week of study

Biochemical parameter	Week of the study	Study group				
		CTR	DOX	DOX +DEX	DOX +DEX +CVD	DOX +CVD
RBC [10^6/μL]	11	9.43 ±0.49	9.06 ±0.77	9.10 ±0.38	9.08 ±0.48	9.74 ±0.41
	21	9.13 ±0.64	8.09 ±0.66	9.67 ±0.32#	9.10 ±0.25#x	9.33 ±0.77
HGB [g/dL]	11	16.67 ±0.70	15.75 ±0.79	18.24 ±0.90#	17.88 ±0.17*#	18.35 ±0.43#
	21	17.00 ±0.95	16.22 ±1.52	17.96 ±0.45	16.52 ±0.56x	17.02 ±0.91
HCT [%]	11	50.63 ±2.48	48.15 ±3.94	50.89 ±1.10	50.44 ±2.54	51.98 ±1.80
	21	49.00 ±2.42	45.85 ±3.66	52.16 ±2.29#	49.01 ±2.61	50.79 ±3.42
MCV [fL]	11	54.00 ±1.00	53.25 ±0.50	56.20 ±1.92#	55.67 ±1.21#	53.50 ±1.64
	21	53.43 ±1.60	56.67 ±1.86	54.00 ±1.87	53.83 ±1.72	54.50 ±1.38
MCH [pg]	11	17.70 ±0.17	17.42 ±0.69	20.04 ±1.15*#	19.75 ±0.88*#	18.87 ±0.61*#
	21	18.64 ±0.49	20.07 ±1.20	18.58 ±0.29	18.12 ±0.39	18.30 ±0.94
MCHC [g/dL]	11	32.93 ±0.58	32.82 ±1.23	35.82 ±1.52*	35.53 ±1.57*	35.32 ±0.71*
	21	34.66 ±0.74	35.35 ±1.60	34.48 ±1.13	33.67 ±1.04	33.57 ±0.89
RDWc [%]	11	16.67 ±0.29	17.70 ±0.24*	17.80 ±0.90*	18.05 ±0.68*	17.95 ±0.65*
	21	17.56 ±1.67	18.18 ±0.93	16.52 ±0.40#	16.37 ±0.35#	17.98 ±0.66

The values are presented as a mean ± SD. Statistical significance: *p≤0.05 *vs.* control group; #p≤0.05 *vs.* DOX; ^{x}≤0.05 for comparison of DOX+DEX+CVD *vs.* DOX+DEX (one-way ANOVA with Turkey's post-hoc test)

CTR – control, CVD – carvedilol, DOX – doxorubicin, DEX – dexrazoxane, HCT – hematocrit, HGB – hemoglobin, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, MCV – mean corpuscular volume, RBC – red blood cell, RDW – red cell distribution width

Table 6. Statistical differences between platelet cell parameters in rats sacrificed in the 11th or 21st week of study

Biochemical parameter	Week of the study	Study group				
		CTR	DOX	DOX +DEX	DOX +DEX +CVD	DOX +CVD
PLT [10^3/μL]	11	605.67 ±161.26	621.33 ±235.87	540.60 ±158.92	424.67 ±167.63	625.00 ±124.87
	21	689.00 ±127.32	665.17 ±78.02	718.40 ±91.91	629.17 ±139.41	579.17 ±354.08
PCT [%]	11	0.47 ±0.02	0.45 ±0.17	0.41 ±0.12	0.32 ±0.12*	0.46 ±0.09
	21	0.51 ±0.08	0.50 ±0.05	0.55 ±0.06	0.49 ±0.09	0.43 ±0.26
MPV [fL]	11	7.87 ±0.30	7.33 ±0.21*	7.54 ±0.29	7.50 ±0.21	7.47 ±0.41
	21	7.43 ±0.47	7.62 ±0.75	7.72 ±0.19	7.82 ±0.25	7.48 ±0.43
PDWc [%]	11	35.27 ±0.50	34.23 ±1.11	34.90 ±1.93	34.17 ±1.45	33.72 ±0.98*
	21	33.73 ±1.16	34.00 ±1.32	34.54 ±1.06	34.78 ±0.63	34.57 ±3.13

The values are presented as a mean ± SD. Statistical significance: *p≤0.05 *vs*. control group; #p≤0.05 *vs*. DOX (one-way ANOVA with Turkey's post-hoc test)

CTR – control, CVD – carvedilol, DOX – doxorubicin, DEX – dexrazoxane, MPV – mean platelet volume, PCT – platelet count and plateletcrit, PDWc – platelet distribution width, PLT – platelet count

DISCUSSION

The general findings of our studies are that *DEX* alleviates the effects of histological changes in the kidney and normalizes the creatinine level affected by *DOX*. *DEX* was found to adversely affect the level of urea and phosphorus in the blood serum of rats receiving *DOX* one week after the end of the administration of the compounds. However, ten weeks after the end of administration of the compounds, the changes normalize. Among the tested blood count parameters, *DOX* altered several parameters, but it was transient and probably not clinically significant. *DEX* and *CVD* were administered separately, leading to changes in many

parameters of the white and red blood cell panels and the platelet panel. Still, although statistically significant, these changes do not appear clinically significant. The assessment of the correlation between the level of serum markers of renal function disorders: creatinine, urea and phosphorus, and heart function parameters in the echocardiography examination does not show any mutual dependencies between these parameters.

Assessment of the nephrotoxic effect

Effective anticancer therapy with anthracyclines is limited because of their toxicity to various organs, including the heart and kidneys. In people with normal renal function, *DOX* does not pose a serious risk of life-threatening nephrotoxicity. However, renal failure is a contraindication to the use of *DOX* in patients [12]. In non-nephropathic cats suffering from cancer, *DOX*-induced nephrotoxicity may occur in approximately 30% of all subjects [13].

In our studies, after administration of *DOX* alone, there were no changes in the levels of urea and phosphorus, neither in the 11th nor the 21st week of the study. However, a significantly higher creatinine level was evident at week 21. Most studies on models of nephrotoxic effects of *DOX* involve single administrations of *DOX* in very high doses – 10-20 mg/kg i.p. to induce acute nephrotoxicity (14). In these models, a significant increase in urea and creatinine is observed. Also, in models where *DOX* is administered several times but in relatively high doses (3-6 mg/kg b.w.; cumulative dose 18 mg/kg b.w.) [15] there is an increase in creatinine and urea. It is worth emphasizing, however, that in these studies, the parameters were determined 24 hours or seven days after the last drug administration. What is more, there are studies on rat models where high doses of *DOX* were used, 40 mg/kg daily for 16 days, and 2.5 mg DOX/kg without serum creatinine and urea determination [16,17].

The novelty of our research is the use of a rat model to study nephrotoxicity with a relatively low single dose (1.6 mg *DOX*/kg b.w.) administered once a week for ten weeks (cumulative dose 16 mg *DOX*/kg b.w.) and the tests were performed one week or ten weeks after the last administration. This is consistent with Reiman *et al.* [6]the use of this drug in cats has been associated with side effects such as renal injury, myelosuppression, anorexia, and weight loss. The goal of this study was to compare the toxicities associated with two dosing schemes for doxorubicin in tumor-bearing cats. Group A cats received 1 mg/kg of doxorubicin, while group B cats received 25 mg/m^2 of doxorubicin plus 22ml lactated Ringer's solution per kilogram body weight subcutaneously. Toxicities were evaluated using laboratory data, physical examination, and history, and were graded using a standardized scale and compared between groups. Post-treatment neutrophil counts were significantly lower among cats in group B compared to cats in group A (P< or = 0.001. As a standard, a dose of 20-40 mg/m^2 of body surface (equivalent to a dose of 1-2 mg/kg b.w.) is used every 21 days up to a maximum cumulative dose of 300-320 mg/m^2 of body surface (equivalent to a dose of 15-16 mg/kg b.w.) in cats treated for cancer. Our rat model is, therefore, a close equivalent of a single and cumulative dose in cats in terms of dose sizes. Moreover, this model is a good reflection of dosing in humans, because a week after the previous administration, no signs of heart failure were revealed in the echocardiography examination. In contrast, 11 weeks after the last administration, left ventricular ejection fraction disturbances occurred, indicating heart failure [11]. This model, therefore, reflects quite well the development of cardiomyopathy in humans many months and usually years after the end of *DOX* chemotherapy in humans. The increase in creatinine concentration in the DOX group in the 21st week of the study (10 weeks from the last administration) indicates a permanent change in kidney function. This thesis is confirmed by histopathological examinations of the kidneys of DOX group rats (Figure 2).

DOX-induced nephrotoxicity has been reported in many studies on rodents. The alterations in rats' kidneys include increased glomerular capillary permeability, tubular atrophy, and podocyte injury [18]. However, comparing the obtained results with the cited studies is not very reliable due to the diverse dosing methods, dose sizes and the different times in which the determinations were made since the last administration of *DOX*.

Both *DEX* and *CVD* were administered separately and together normalized the creatinine level increased by *DOX* in the 21st week of the study. Although there was no effect of *DOX* alone on urea and phosphorus levels, surprisingly, there was an adverse effect of *DEX* and *CVD* administered alone or in combination to rats receiving *DOX* on both urea and phosphorus levels at week 11. In turn, in the 21st week of the study, an unfavorable effect of *DEX* on the urea level was found in rats receiving *DOX* (DOX+DEX group), and a beneficial impact of *CVD*, which was manifested by a significant reduction in the urea level in the DOX+DEX group (comparison of DOX+DEX+CVD *vs*. DOX+ DEX). During this time (week 21), phosphorus levels normalized in all study groups.

Although the exact mechanism of *DOX*-induced nephrotoxicity remains unknown, it is believed that the toxicity may be mediated, as in the case of late cardiotoxicity, through free radical formation, iron-dependent oxidative damage of biological macromolecules, lipid peroxidation membrane and protein oxidation [19]. In the case of the heart muscle, in recent years, in addition to the free radical theory, there has been increasing evidence for another mechanism that is related to the inhibition of topoisomerase 2β (TOP2β) [8]. According to the second, *DOX* interrupts TOP2β's normal catalytic cycle, resulting in DNA double-strand breaks, which can lead to cardiomyocyte death. We do not know whether a similar mechanism also occurs in *DOX* nephrotoxicity in cats.

The only FDA-approved drug for *DOX* cardioprotection in humans is *DEX.* Interestingly, there is evidence for the protective effect of *DEX* in a free radical and TOP2β-related mechanism. Herein, chelation of iron ions and the inhibition of ROS production were linked to the preventive effects of *DEX* reported in the clinic [20]. *DEX* was shown to bind TOP2β, preventing *DOX* binding to DNA [8]. We do not know whether the dual mechanism of *DEX* protection against *DOX*-induced cardiotoxicity will be analogous to *DOX*-induced nephrotoxicity in cats. So far, *DEX* has been used in humans and dogs for *DOX*-induced cardiomyopathy

and in cats – in extravasation after *DOX* administration [21] the efficacy of disopyramide in cats has not been reported. We treated a cat with HOCM with carvedilol and disopyramide cotherapy and monitored the changes in LVOT flow velocity and N-terminal pro B-type natriuretic peptide (NT-proBNP. However, no attempts have been made to mitigate cat nephrotoxic and myelotoxic effects.

CVD, in turn, is a drug from the group of β-blockers with an antioxidant component [7] and has not been used in anthracycline cardiomyopathy in cats. In one case study, disopyramide with *CVD* was administered to a cat with hypertrophic obstructive cardiomyopathy. The research results encouraged continuation. In another study of 21 cats with hypertrophic cardiomyopathy, a positive response was observed in 10 cats [22].

Biochemical studies in this project showed the beneficial effect of *DEX* a*nd CVD* on the creatinine level increased by *DOX. A* positive impact of *DEX* on changes in the histopathological picture induced by *DOX* was also found. However, *DEX* had a negative effect on the urea level in rats receiving *DOX*. In turn, *CVD* not only prevented the increase in creatinine induced by *DOX* but also acted against the increase in urea in the DOX+DEX group (comparison DOX+DEX+CVD *vs*. DOX+DEX). Still, unlike in the case of *DEX*, *CVD*, and *DEX* with *CVD* had a negative impact on the histopathological image of the kidney compared to changes induced by *DOX* alone. This indicates a differential effect of *DEX* and *CVD* compounds at the biochemical and tissue levels.

Assessment of the myelotoxic effect

Of all the white blood cell parameters tested, *DOX* only affected the number and percentage of monocytes. In week 11 of the study, *DOX* alone was responsible for a significant decrease in the number and rate of monocytes, which may indicate an unfavorable effect of *DOX*. Both *DEX* and *CVD* limited the decline in the number of monocytes and completely normalized their percentage. The most effective reduction in monocyte decline occurred when *DEX* was administered together with *CVD*. There was a reduction in other parameters of the white blood cell system (WBC and %LYM) when, in addition to *DOX*, *DEX* and/or *CVD* were administered, which was not observed in rats receiving *DOX* alone. These changes are not only statistically significant but may also be clinically significant because WBV and %LYM values were below the reference range, <4.4K/mm^3 for WBC and <61% for LYM, respectively [23]. Therefore, *DEX* and *CVD*, on the one hand, normalize the monocyte level reduced by *DOX*, but on the other hand, they may have an adverse effect on WBC and %LYM in rats receiving *DOX*. However, all unfavorable changes demonstrated in the white blood cell system in week 11 of the study were normalized in week 21, which indicates that there were no permanent changes.

Of all the tested parameters of the red blood cell system, *DOX* affected only RDWc (increase the value). Significant differences were found between the study groups *vs*. control, DOX and DOX+DEX. However, all statistically significant differences in individual parameters of the red blood cell system are clinically insignificant because the maximum percentage deviations from the mean of the corresponding control for different study times were not more significant than 10% concerning the control. Therefore, in this case, it is difficult to talk about the harmful effects of *DEX* and *CVD* in rats taking *DOX*. As in the case of red blood cell parameters, the observed changes in the panel of platelet parameters, although statistically significant, do not have clinical significance. It is worth emphasizing that in the 21st week, all parameters normalized, which can be confirmed by the lack of differences in the study groups compared to the control.

CONCLUSIONS

The analysis of the advantages and disadvantages of using *DEX* and/or *CVD* as a protective factor in potential *DOX* therapy in cats carried out in this study in a rat model indicates that *DEX* alone has the most beneficial effect in preventing *DOX*-induced kidney histological changes compared to *CVD* alone or *DEX* with *CVD*. *DEX* and *CVD* administered separately or together have a beneficial impact on the creatinine level increased by *DOX*. Still, although *DOX* alone did not increase urea concentration, simultaneous administration of *DOX* with *DEX*, with *CVD*, or *DEX* with *CVD* led to an increase in urea and phosphorus. This is the most significant limitation of the recommendation to use *DEX* with *DOX* because the main side effect of *DOX* in cats is azotemia [4,13]. Changes in blood count do not constitute a severe contraindication to *DOX* and *CVD* in preventing *DOX* disorders because, despite numerous changes in blood parameters, these changes were statistically significant but of little clinical importance. The resultant of these positive and negative phenomena may indicate the need to verify the positive effect of *DEX,* obtained in our studies, in preventing nephrotoxicity in cats clinically treated with *DOX*, starting with a minimal dose of *DEX*.

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INSTITUTIONAL REVIEW BOARD STATEMENT

This study was conducted in accordance with the Declaration of Helsinki, and approved by the Local Ethical Committee for Animal Experiments based at the University of Life Sciences in Lublin, Poland (protocol code 123/2018 and date of approval: 3 December 2018).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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