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Influence of the formulation on the tolerance profile of nanoparticle-bound doxorubicin in healthy rats: Focus on cardio- and testicular toxicity

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Abstract

A toxicological study of doxorubicin bound to poly(butyl cyanoacrylate) or human serum albumin nanoparticles coated with polysorbate 80 was performed in healthy rats. The doxorubicin formulations were injected at a therapeutic dose regimen (3 × 1.5 mg/kg with a 72 h interval), and the animals were followed up for 15 or 30 days. The overall result of this study suggests that the surfactant-coated nanoparticle formulations of doxorubicin have favorable toxicological profiles. Specifically, these formulations display a considerably reduced cardio- and testicular toxicity, as compared to a free drug.

Keywords: Cardiotoxicity; Doxorubicin; Human serum albumin; Nanoparticles; Poly(butyl cyanoacrylate); Polysorbate 80; Rat; Testicular toxicity

1. Introduction

The anthracycline antibiotic doxorubicin is one of the most effective anticancer drugs, which is extensively used for the treatment of many neoplastic diseases. However, its clinical usefulness is often limited by severe adverse side effects, the most important of which are cardiomyopathy and congestive heart failure (Singal et al., 2000; Minotti et al., 2004). Over the past decades, substantial efforts have been directed towards better understanding of the mechanisms of activity and toxicity of doxorubicin and other anthracyclines and to the improvement of their therapeutic index.

Several strategies have been used, the search for a "better anthracycline" being first in the list. Indeed, nearly 2000 analogs were synthesized and evaluated; yet only few of them have reached the stage of clinical development and approval. Second generation analogs like mitoxantrone, epirubicin or idarubicin exhibit a decreased cardiotoxicity; however, these agents were not able to totally alleviate the risk of inducing cardiomyopathy (Anderlini et al., 1995; Ryberg et al., 1998; Gonsette, 2004).

Another approach attempts to counteract the destructive action of free radicals (reactive oxygen species) generated by the anthracycline molecule upon deglycosylation and intercalation of the aglycones into biologic membranes (Zucchi and Danesi, 2003; Minotti et al., 2004). Free radical generation also may occur via non-enzymatic pathways and is mediated by doxorubicin complexes with intracellular iron, which can reduce molecular oxygen, producing a burst of reactive oxygen species (Myers, 1998). Hence, this approach involves application of adjuvant cardioprotective therapy using agents that could defend cardiomyocytes against anthracycline-derived reactive oxygen species, such as antioxidants (i.e. thiol containing agents, piperidine nitroxides or vitamins A, E, and C) or iron chelators (i.e. dexrazoxane) (Hasinoff et al., 2003). However, the cardioprotective efficacy of these agents is often quite limited.

Further, it has been shown that the severity of doxorubicin-induced cardiotoxicity is directly correlated to the peak drug

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concentration in the heart and plasma rather than to plasma AUC (Danesi et al., 2002). Indeed, safety and efficacy of doxorubicin could be improved by replacing bolus administration with slow infusions, which correlated with the pharmacokinetic findings demonstrating that both plasma and left ventricular peak concentrations of doxorubicin and its active metabolite doxorubicinol were significantly lower after slow infusion than after bolus dosing (Cusack et al., 1993). Accordingly, a number of strategies are focused on the optimization of the drug biodistribution. One way to solve this problem is the development of the drug delivery systems that enhance distribution of anthracyclines within tumors, whereas exposure of healthy tissues to potentially toxic levels of the drug is decreased. Thus, liposomal formulations considerably improved the pharmacokinetics and therapeutic index of doxorubicin (Safra et al., 2000; Batist et al., 2001).

While liposomes are the most advanced example of this strategy, the nanoparticle-based delivery systems represent a promising alternative which enables broadening of the spectrum of drug activity. Thus, binding of doxorubicin to poly(alkyl cyanoacrylate) nanoparticles allowed overcoming of the multidrug resistance (Cuvier et al., 1992; Colin de Verdiere et al., 1997). Furthermore, recent studies suggest that the nanoparticle-based formulation of doxorubicin has a potential for the systemic chemotherapy of brain tumors, whereas the efficacy of free doxorubicin against this type of tumors is restricted due to the drug inability to cross the blood–brain barrier. Indeed, polysorbate 80-coated poly(butyl cyanoacrylate) (PBCA) nanoparticles enabled the brain delivery of doxorubicin after intravenous administration (Gulyaev et al., 1999) and yielded a high anti-tumoral effect against intracranial glioblastoma in rats (Steiniger et al., 2004).

The toxicological study of the above formulation revealed that binding of doxorubicin to the nanoparticles did not increase its acute toxicity (Gelperina et al., 2002). Moreover, the pharmacokinetic studies demonstrated that after intravenous injection of doxorubicin bound to polysorbate 80-coated PBCA nanoparticles the cardiac concentrations were significantly lower, as compared to the drug in solution (Gulyaev et al., 1999).

Another promising carrier system is based on human serum albumin (HSA) nanoparticles. Albumin is the most ubiquitous protein in the human body and is a natural carrier for many substances. HSA-nanoparticles represent a versatile colloidal drug carrier system with high drug loading capacity in combination with biodegradability and biocompatibility. The results of the recent studies of paclitaxel bound to albumin nanoparticles demonstrated a favorable safety profile of this formulation (Gradishar et al., 2005).

The objective of the present study was to gain further insight into the toxicological properties of the PBCA- and HSA-based nanoparticulate formulations of doxorubicin in a rat model, which appears to be most adequate among animal models for characterization of anthracycline-related toxicity (Mazue et al., 1995). The study was focused on the evaluation of cardiotoxicity and testicular toxicity, which is another important yet unabated adverse effect of doxorubicin.

2. Materials and methods

2.1. Chemicals

n-Butyl-2-cyanoacrylate (Sicomet® 6000) was obtained from Sichel–Werke (Hannover, Germany). Human serum albumin (HSA, fraction V, purity 96–99%, 65,000 Da), glutaraldehyde 8% solution, and dextran 70,000 were obtained from Sigma (Steinheim, Germany). Polysorbate 80 (Tween® 80) was supplied by ICI Chemicals (Essen, Germany); doxorubicin was from Sicor (Rho, Italy). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

2.2. Preparation and characterization of nanoparticles

2.2.1. Preparation of doxorubicin-loaded PBCA nanoparticles

Doxorubicin-loaded poly(butyl cyanoacrylate) nanoparticles (Dox-PBCA) were manufactured by anionic polymerization (Couvreur et al., 1979; Kreuter, 1983). One percentage of *n*-butyl-2-cyanoacrylate was added to a 1% dextran solution in 0.01N HCl under constant stirring. After 40 min, doxorubicin was added to the mixture to obtain a final doxorubicin concentration of 0.25%. Stirring was continued for 2.5 h, and then the polymerization was completed by neutralization with 0.1N NaOH. The mixture was filtered through a G1 sintered glass filter (Schott, Mainz, Germany) and lyophilized; 3% (w/v) mannitol was used as a cryoprotector.

2.2.2. Characterization of doxorubicin-loaded PBCA nanoparticles

The particle size and polydispersity of the size distribution were measured by photon correlation spectroscopy (PCS) using a Malvern zetasizer 3000HSA (Malvern Instruments Ltd., Malvern, UK). The samples were diluted with purified water and measured at a temperature of 25 °C at a scattering angle of 90°. An average particle diameter of 240 ± 40 nm was found. The average surface charge was -13.0 ± 2.1 mV.

The drug content was measured spectrophotometrically after dissolution of the freeze-dried formulation in DMSO. The percentage of the nanoparticle-bound drug was calculated as the difference between the drug content and amount of the free drug after its separation by ultrafiltration of the redispersed formulation, as follows: 400 μl of the suspension were centrifuged for 15 min at $16{,}100 \times g$ in centrifugal membrane filter devices (Microcon 30 kDa, Millipore, USA). The concentration of the free drug in the filtrate was assessed by spectrophotometry (480 nm). It was shown that $56 \pm 1.2\%$ of the drug in suspension was associated with the nanoparticles. The loading capacity was 137.5 ± 3.5 μg Dox per 1 mg PBCA.

Empty PBCA-NP were synthesized using the same technique and had an average diameter of 250 ± 30 nm. The average surface charge was -17.0 ± 2.5 mV.

Table 1
Physicochemical characteristics of doxorubicin-loaded nanoparticles

Formulation	Average particle diameter (nm)	Polydispersity	Zeta potential (mV)	Drug loading (%)	Capacity (Dox/NP) (μg/mg)
Empty PBCA NP	250 ± 30	0.221 ± 0.038	−17.0 ± 2.5	–	–
Dox-PBCA	240 ± 40	0.349 ± 0.035	−13.0 ± 2.1	56 ± 1.2	137.5 ± 3.5
Dox-HSA	404 ± 24	0.112 ± 0.033	−56.1 ± 1.4	84 ± 0.6	44.3 ± 0.3

Results are given as mean ± standard deviation, $n = 3$.

2.2.3. Preparation of doxorubicin-loaded HSA nanoparticles

HSA nanoparticles were prepared by a desolvation method described earlier (Weber et al., 2000; Langer et al., 2003). For this purpose, 1.0 mg of doxorubicin and 20 mg of HSA were dissolved in 1 ml of purified water and stirred for 2 h to achieve adsorption of the drug to the protein. Under constant stirring (650 rpm) NPs were formed by addition of 3 ml of ethanol (1 ml/min) using a tubing pump (Ismatec IPN, Glattbrugg, Switzerland).

Following the desolvation process, the particles were stabilised by the addition of an 8% aqueous glutaraldehyde solution (0.588 μl per mg HSA). The crosslinking process was performed under stirring of the suspension over 24 h. The resulting nanoparticles were purified by threefold centrifugation (16,100 × *g*, 12 min) followed by redispersion in purified water by ultrasonication. The supernatants were collected, and the drug content was measured by HPLC, as described below.

2.2.4. Characterization of doxorubicin-loaded HSA nanoparticles

The particle size of the doxorubicin-loaded HSA nanoparticles was measured by PCS, as described above. An average particle diameter of 404 ± 24 nm was found. The average surface charge was −56.1 ± 1.4 mV.

The nanoparticle content of the final dispersion was determined gravimetrically: a 50.0 μl aliquot of the nanoparticle suspension was transferred to aluminium boats (Lüdi, Flawil, Switzerland) and dried for 2 h at 80 °C; the mass of the particle residue was determined using a Supermicro balance (Sartorius, Göttingen, Germany).

The efficiency of doxorubicin loading in HSA nanoparticles was calculated as the difference between amount of doxorubicin added into the reaction medium and amount detected in the supernatants. The assay was performed by HPLC. A Hitachi D7000 HPLC system (Hitachi Ltd., Tokyo, Japan) with a reverse phase column (LiChroCART® 250-4 LiChrospher® 100 RP-18, Merck, Darmstadt, Germany) was used; the mobile phase was an isocratic mixture of water and acetonitrile (70%:30%) containing 0.1% trifluoroacetic acid. The flow rate was set to 0.8 ml/min. The quantification of doxorubicin in the samples was performed using UV (250 nm) and fluorescence (λ_{ex} 560 nm, λ_{em} 650 nm) detection. Under these conditions, the retention time for doxorubicin was about 12 min. The loading capacity was 44.3 ± 0.3 μg Dox per 1 mg HSA nanoparticles.

The physicochemical characteristics of both types of the nanoparticles are summarized in Table 1.

2.3. Toxicological study

The animal experiments were performed in compliance with the EU and Russian Guidelines for Animal Experiments and Welfare authorized by the Russian Ministry of Health (1045-73 and 52-F3).

2.3.1. Animals

Adult male Wistar rats weighing 120–150 g were obtained from animal production unit of the Russian Academy of Medical Sciences (Kryukovo, Moscow, Russia). Animals were acclimatized for 2 weeks and caged in groups of five. They were fed *ad libitum* with standard laboratory food and water.

2.3.2. Formulations

The following formulations were tested: (1) doxorubicin substance, solution in water (Dox); (2) doxorubicin substance, solution in 1% polysorbate 80 (Dox + PS); (3) empty PBCA nanoparticles coated with polysorbate 80 (PBCA + PS); (4) doxorubicin-loaded PBCA nanoparticles (Dox-PBCA); (5) doxorubicin-loaded PBCA nanoparticles coated with polysorbate 80 (Dox-PBCA + PS); (6) doxorubicin-loaded HSA nanoparticles (Dox-HSA); (7) doxorubicin-loaded HSA nanoparticles coated with polysorbate 80 (Dox-HSA + PS).

For injection, the formulations were prepared as follows: Dox and Dox-PBCA were dissolved or resuspended, respectively, in distilled water. For the Dox + PS, PBCA + PS, and Dox-PBCA + PS formulations, a 1% polysorbate solution in distilled water was used, and the nanoparticles were incubated in this solution for 30 min at 20 °C before administration. The polysorbate 80 concentration was chosen in accordance with the previous studies (Gulyaev et al., 1999; Steiniger et al., 2004). The final doxorubicin concentration in these preparations was 0.2%.

The Dox-HSA nanoparticles were used at a final doxorubicin concentration of 0.1% without and with PS coating. For coating, PS was added into the nanoparticle suspension to obtain a final concentration of 1% (m/v), and the suspension was incubated for 1 h at 20 °C.

The coating did not change the size of both types of the particles significantly, i.e. the size increases after coating were within the variability of the photon correlation measurements (data not shown).

2.3.3. Experimental design

The animals were randomized according to weight and divided into groups ($n = 12$), which received one of the above

Table 2
Body weight gain and weight indices of the rat internal organs on day 15 post-treatment using doxorubicin formulations

Groups	Increase of body weight (in % of the body weight at day 0)	Weight indices (organ weight relative to body weight on day 15) [%]						
		Heart	Lung	Kidney	Liver	Spleen	Testis	Thymus
Control	37.43 ± 1.40	0.33 ± 0.04	0.90 ± 0.05	0.34 ± 0.04	3.74 ± 0.08	0.32 ± 0.04	0.51 ± 0.05	0.24 ± 0.01
PBCA + PS	30.07 ± 1.07*	0.31 ± 0.03	0.92 ± 0.08	0.36 ± 0.03	3.75 ± 0.07	0.31 ± 0.02	0.52 ± 0.05	0.22 ± 0.01
Dox	28.35 ± 1.30*	0.30 ± 0.02	0.93 ± 0.06	0.33 ± 0.02	3.53 ± 0.09*	0.27 ± 0.03	0.45 ± 0.05	0.15 ± 0.02*
Dox + PS	11.71 ± 1.18**	0.32 ± 0.05	0.97 ± 0.07	0.38 ± 0.03	3.70 ± 0.08**	0.27 ± 0.02	0.47 ± 0.10	0.13 ± 0.02*
Dox-PBCA	22.37 ± 1.52**	0.31 ± 0.05	0.91 ± 0.08	0.34 ± 0.04	3.59 ± 0.09	0.26 ± 0.01*	0.46 ± 0.04	0.12 ± 0.03*
Dox-PBCA + PS	20.11 ± 1.90**	0.31 ± 0.03	0.96 ± 0.05	0.36 ± 0.02	3.54 ± 0.06*	0.25 ± 0.03	0.51 ± 0.04	0.19 ± 0.04
Dox-HSA + PS	34.00 ± 2.90**	0.34 ± 0.06	0.88 ± 0.08	0.37 ± 0.04	3.93 ± 0.08**	0.24 ± 0.03*	0.56 ± 0.07	0.19 ± 0.02*
Dox-HSA	29.00 ± 3.10*	0.34 ± 0.03	0.84 ± 0.03	0.38 ± 0.05	3.70 ± 0.09	0.23 ± 0.04*	0.54 ± 0.03	0.18 ± 0.03*

* Significantly different from control ($p \leq 0.05$).
** Significantly different from Dox ($p \leq 0.05$).

preparations. Untreated animals were used as a control. All preparations were injected i.v. into the tail caudal vein with the rate of 1 ml/min. The treatment regimen was 3 × 1.5 mg/kg (as doxorubicin) with a 72-h interval between injections. This regimen was considered to be relevant because it proved to be effective for chemotherapy of a brain tumor (Steiniger et al., 2004). The placebo nanoparticles (PBCA + PS) were administered in the regimen of 3 × 6 mg/kg (as PBCA) with a 72-h interval between injections.

Before treatment, clinical blood tests and electrocardiography were performed and the body weight was determined for all animals.

Six animals in each group were sacrificed 15 days post-treatment, and the remaining six animals 30 days post-treatment. In the course of the observation periods, the animals were followed up for any adverse effects. The body weight was evaluated on days 15 and 30. Clinical blood tests (hemoglobin concentration, leucocyte count, erythrocyte count, and differential blood count) were performed on days 3, 5, 7, 14, and 30 post-treatment course. The electrocardiographic examination (second standard lead) was performed on days 15 and 30 post-treatment (electrocardiograph AKSION EK1E-07, Russia).

2.3.4. Post-mortem examinations

After necropsy, the organs were inspected macroscopically. The weights of heart, lungs, liver, spleen, kidneys, thymus, and testes were measured, and relative organ weights (weight index, WI) were calculated on the basis of body weight measurement prior to necropsy.

The histological evaluation was performed for heart and testes. The organs were separated, fixed in 10% neutral formalin and cut. The cuts were stained with haematoxillin–eosin.

Statistical analysis was performed using Student's *t*-test. Mean values and standard deviations were calculated for body weights, hematological parameters, electrocardiographic parameters, and WI. The difference between the groups was considered significant at $p \leq 0.05$.

3. Results

3.1. Clinical observations

The administration of the doxorubicin formulations and the placebo (PBCA + PS) did not cause mortality in any of the treated groups. There were no signs of general clinical symptoms of toxicity or abnormal behavioral reactions.

3.2. Body weight

The dynamics of body weight was evaluated as the relative increase in body weight (% of the body weight on day 0) (Tables 2 and 3). In the 15-day groups, the gain in body weight was decreased in all experimental groups compared to control. This decrease was most pronounced in the group treated with

Table 3
Body weight gain and weight indices of the rat internal organs on day 30 post-treatment using doxorubicin formulations

Groups	Increase in body weight (in % of the body weight at day 0)	Weight indices (organ weight relative to body weight on day 30) [%]						
		Heart	Lung	Kidney	Liver	Spleen	Testis	Thymus
Control	50.82 ± 2.57	0.32 ± 0.02	0.74 ± 0.04	0.33 ± 0.02	3.49 ± 0.12	0.22 ± 0.03	0.49 ± 0.06	0.17 ± 0.04
PBCA + PS	51.68 ± 2.15	0.29 ± 0.03	0.57 ± 0.05*	0.28 ± 0.01*	3.26 ± 0.09*	0.23 ± 0.02	0.46 ± 0.06	0.18 ± 0.02
Dox	34.59 ± 1.79*	0.32 ± 0.03	0.73 ± 0.07	0.34 ± 0.015	3.55 ± 0.08	0.25 ± 0.012	0.31 ± 0.05*	0.20 ± 0.03
Dox + PS	41.63 ± 1.93*	0.31 ± 0.05	0.94 ± 0.03**	0.32 ± 0.03	3.53 ± 0.08	0.23 ± 0.01	0.30 ± 0.04*	0.22 ± 0.03
Dox-PBCA	30.23 ± 0.97*	0.31 ± 0.04	1.18 ± 0.06**	0.34 ± 0.03	3.48 ± 0.07	0.31 ± 0.03**	0.30 ± 0.03*	0.21 ± 0.02
Dox-PBCA + PS	20.82 ± 1.13*	0.32 ± 0.05	1.00 ± 0.04**	0.33 ± 0.02	3.58 ± 0.11	0.32 ± 0.02**	0.35 ± 0.04*	0.21 ± 0.05
Dox-HSA + PS	57.10 ± 4.10	0.32 ± 0.03	0.67 ± 0.04	0.33 ± 0.03	3.52 ± 0.09	0.26 ± 0.05	0.46 ± 0.04**	0.13 ± 0.04
Dox-HSA	85.50 ± 6.20**	0.32 ± 0.02	0.72 ± 0.02	0.36 ± 0.04	3.47 ± 0.07	0.25 ± 0.03	0.49 ± 0.05**	0.13 ± 0.05

* Significantly different from control ($p \leq 0.05$).
** Significantly different from Dox ($p \leq 0.05$).

Dox + PS: the gain in body weight was only 11% versus 37% in the control group. As shown in Table 2, the gain in body weight in the groups treated with the PBCA-based formulations of doxorubicin was significantly lower, as compared to those treated with the drug solution in water (Dox). In the group treated with Dox-HSA, the gain in body weight did not differ from that of the group treated with doxorubicin. The weight of the animals treated with Dox-HSA + PS was increasing faster, in comparison to the group treated with doxorubicin and slower in comparison to control.

The parameters of body weight for 30-day groups are presented in Table 3. By day 30, the body weight gain in the groups treated with Dox-PBCA and Dox-PBCA + PS was significantly lower than that in the control group. The group treated with placebo (PBCA + PS) did not differ from control. In the group treated with Dox + PS, the animals not only compensated for the lag in body weight gain observed during 15-day period but exceeded the weight gain in the groups treated with Dox, Dox-PBCA, and Dox-PBCA + PS. In the group treated with Dox-PBCA + PS, the decline of gain weight was ∼20%, which is significantly lower, as compared to all other experimental groups. In the group treated with Dox-HSA + PS, the gain of weight did not differ from control, whereas Dox-HSA caused a significant increase in this parameter, as compared to both, Dox-treated and control groups.

3.3. Hematology

The influence of doxorubicin formulations on the hematological parameters was assessed by clinical blood tests on days 3, 5, 7, 14, and 30 post-treatment. In general, throughout the period of observation there were no clinically important hematological effects, and all fluctuations of the hematological parameters remained within the physiological range. The following fluctuations were observed:

On day 3 post-treatment, the total leukocyte count was increased in the group treated with PBCA + PS. The analysis of the blood differential count demonstrated that this increase could be attributed to the increased level of segmental neutrophils. Doxorubicin did not influence the total leukocyte count on day 3, but on day 5 the count in this group was decreased. On the contrary, Dox + PS, Dox-PBCA, Dox-PBCA + PS, and Dox-HSA + PS induced a temporary but significant decrease of this parameter, which was back to normal by day 5 post-treatment. On day 7, the count was again decreased in the group treated with Dox-PBCA. Further tests did not reveal any changes in the leukocyte count in any of the experimental groups. It has to be mentioned that true leucopenia (leukocyte count $<6 \times 10^3/mm^3$) was not observed in any of the experimental groups throughout the period of observation. The results of the total leukocyte count obtained on days 0, 3, 7, and 30 are presented in Fig. 1.

In general, all doxorubicin formulations induced a decrease of lymphocyte count and an increase of the number of segmental cells, which was not associated with a left shift of the differential leukocyte count (a number of stab neutrophils was not increased, and immature leucocytes were absent). These fluctuations remained within the physiological range during the period of the observation.

The erythrocyte count was moderately influenced by Dox + PS, Dox-PBCA, and Dox-PBCA + PS (Fig. 2). In these groups, the erythrocyte count was decreasing from days 3 to 7. In the group treated with Dox, this effect was somewhat delayed, starting only from day 5. This decrease was found to be in parallel with the decreased level of hemoglobin (not shown). True erythrocytopenia was not observed, and these parameters also remained within the physiological range. After day 7, the counts were gradually increasing and by day 30 were similar to the pretreatment values in all groups. In the groups treated with HSA-based formulations, the erythrocyte count was similar to control throughout the entire period of observation.

3.4. Heart functions

The results of the electrocardiographic examination performed in a second standard lead on days 15 and 30 post-treatment are presented in Table 4.

On day 15, a slight but statistically significant increase of the QT-interval was observed only in the group treated with Dox, as compared to control. By day 30, this increase was

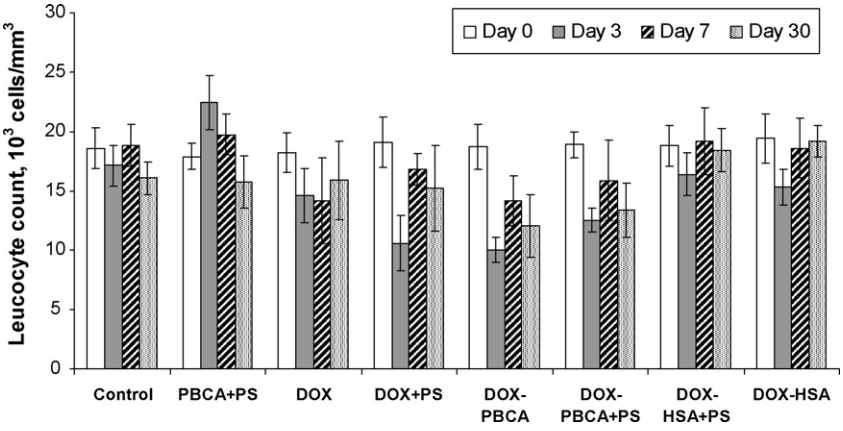

Fig. 1. Influence of doxorubicin formulations on the rat total leukocyte count.

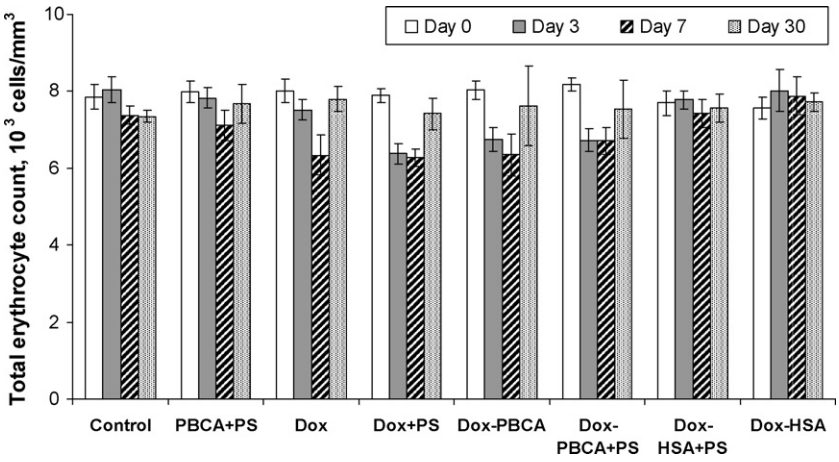

Fig. 2. Influence of doxorubicin formulations on the rat total erythrocytes count.

found to be more pronounced and was associated was tachycardia. At this time, the signs of the cardiac decompensation were also observed in the groups treated with Dox + PS and Dox-PBCA. Importantly, administration of Dox-PBCA + PS, Dox-HSA + PS, and Dox-HSA did not induce the increase of the QT-interval or tachycardia throughout the period of observation.

3.5. Internal organs

The influence of the drug treatment on the relative weight of the internal organs (weight indices, WI – i.e organ weight relative to body weight) is presented in Tables 2 and 3.

The lung WI on day 15 in all groups did not differ from control. By day 30, the lung WI increased in the groups treated with Dox + PS, Dox-PBCA, and Dox-PBCA + PS, as compared to doxorubicin and control; whereas in the group that received PBCA + PS this WI appeared to be significantly lower than that of the control group.

The kidney WI on day 15 was similar to control in most groups. On day 30, PBCA + PS caused significant decrease of the kidney WI compared to all other groups.

The liver WI in the groups treated with Dox and Dox-PBCA + PS on day 15 were significantly lower in comparison to control. In the groups treated with Dox + PS and Dox, the WI values did not significantly differ from control. On day 30, the liver WI were similar to control in all groups, with the exception of the group treated with PBCA + PS, where this WI was decreased.

The spleen WI on day 15 were decreased in the groups treated with Dox-PBCA, Dox-HSA, and Dox-HSA + PS. On day 30, in the groups treated with Dox-PBCA and Dox-PBCA + PS this WI was significantly higher, as compared to all experimental groups and control.

The testis WI on day 15 was similar to control in all groups. On day 30, this WI was significantly decreased in the groups treated with Dox-PBCA and Dox-PBCA + PS, as compared to the control group and the group treated with PBCA + PS. In the groups treated with HSA-based formulations, the testis WI was similar to control.

The thymus WI on day 15 was significantly decreased in the groups treated with Dox, Dox + PS, Dox-PBCA, Dox-HSA, and Dox-HSA + PS, as compared to control and Dox-PBCA + PS treated group. On day 30, this WI was similar to control in all groups.

The heart WI was not influenced by any of the formulations within both periods of observation.

Table 4
Electrocardiographic parameters post-treatment using doxorubicin formulations

ECG parameters	Control	PBCA + PS	Dox	Dox + PS	Dox-PBCA	Dox-PBCA + PS	Dox-HSA + PS	Dox-HSA
15 days post-treatment								
R-R (ms)	163 ± 11	161 ± 15	152 ± 14	149 ± 13	146 ± 7	153 ± 13	147 ± 26	151 ± 9
QT (ms)	71 ± 4	67 ± 7	67 ± 1	67 ± 8	65 ± 6	67 ± 5	63 ± 7	65 ± 7
QT (%)	43.8 ± 0.9	44.3 ± 0.75	46.2 ± 1.2*	45.1 ± 0.8	44.2 ± 1.3	43.9 ± 0.7	42.8 ± 0.5**	43.2 ± 0.6**
Pulse rate (bpm)	368 ± 28.7	372 ± 49	396 ± 41	403 ± 26	411 ± 39	393 ± 61	407 ± 31	396 ± 24
30 days post-treatment								
R-R (ms)	153 ± 14	146 ± 12	117 ± 8*	126 ± 18	120 ± 5*	139 ± 6	142 ± 27	144 ± 9
QT (ms)	65 ± 9	64 ± 8	62 ± 7	62 ± 8	61 ± 6	72 ± 8	65 ± 8	66 ± 6
QT (%)	42.5 ± 3.8	44.2 ± 1.7	52.8 ± 0.6*	49.8 ± 1.4*	50.3 ± 2.7*	51.7 ± 5.4	46.1 ± 1.3**	45.9 ± 1.4**
Pulse rate (bpm)	395 ± 35.5	412 ± 43.2	512 ± 57.6*	476 ± 23.7*	497 ± 44.6*	431 ± 27	423 ± 17.9**	418 ± 32.1**

* Significantly different from control ($p \leq 0.05$).
** Significantly different from Dox ($p \leq 0.05$).

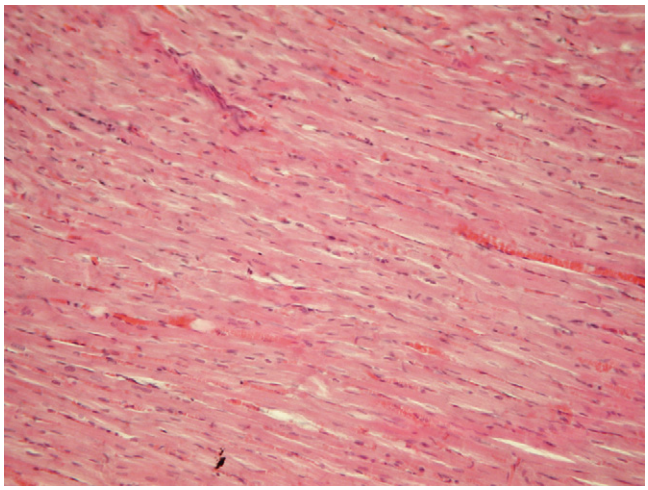

Fig. 3. Rat myocardium: control; 20×.

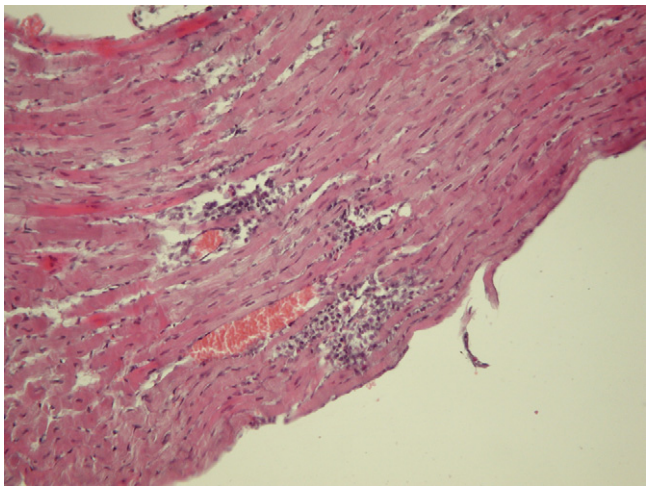

Fig. 5. Rat myocardium: Dox-PBCA, 30 days post-treatment. Vacuolization and atrophy of myofibrils co-localized with vessels, moderate lymphoid infiltration; 20×.

3.6. Histological evaluation

3.6.1. Myocardium

The results of the histological evaluation of the rat myocardium are shown in Figs. 3–6. No pathological changes were observed in the groups treated with Dox-HSA + PS and Dox-HSA throughout the period of observation, and the myocardium of these animals was similar to control (Fig. 3). The myocardium of the rats treated with PBCA + PS also displayed no signs of toxicity.

On day 15, administration of Dox, Dox + PS, Dox-PBCA, and Dox-PBCA + PS produced similar moderate changes of the myocardium. These changes included single minor lymphoid infiltrations and rare lesions of toxicity-related cardiomyopathy associated with single edematous myofibers.

On day 30, the response of the animals to treatment in these groups was clearly different depending on the formulation used. Thus, in the groups treated with Dox and Dox + PS, by day 30 the myocardium displayed edematous lesions and focal vacuolar degeneration of myofibers associated with the lymphoid infiltrations (Fig. 4). These findings are typical for doxorubicin and correlate with the observations of other authors (Klugmann et al., 1984; Gebbia et al., 1985).

In the group treated with Dox-PBCA, the myocardium of 4/6 animals was similar to that of Dox-treated animals. However, in 2/6 animals the pathological changes were less pronounced. These animals displayed fewer and less prominent edematous foci; the lesions of the vacuolar degeneration of the myocytes were observed mainly in the vicinity of the blood vessels and were associated with only moderate lymphoid infiltrations (Fig. 5).

In the group treated with Dox-PBCA + PS, by day 30 all animals displayed fewer and less prominent foci of edema, vacuolation and degeneration of the myocytes, as compared with the group treated with the free drug in solution. The lymphoid infiltrations were not observed (Fig. 6).

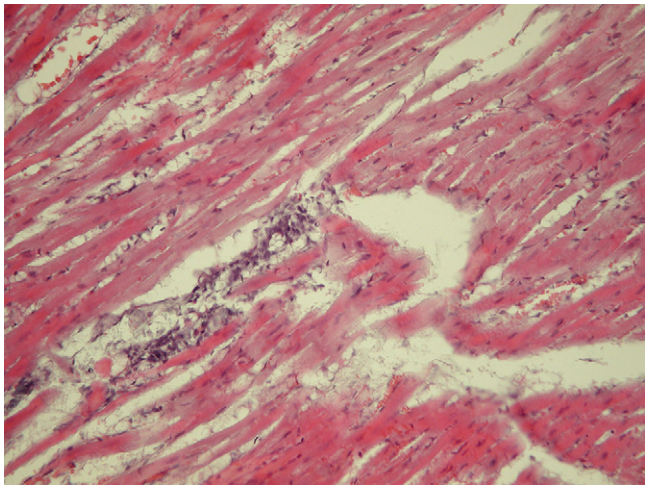

Fig. 4. Rat myocardium: Dox, 30 days post-treatment. Focal destruction of cardiomyocytes, pronounced edema, lymphoid infiltrates associated with foci of myofibrils atrophy; 20×.

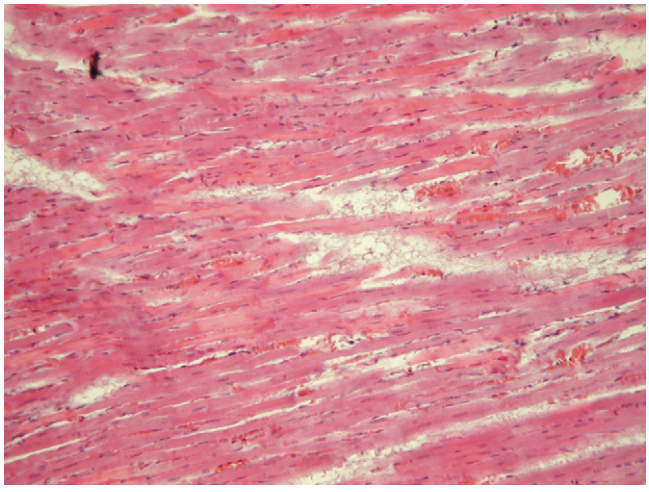

Fig. 6. Rat myocardium: Dox-PBCA + PS, 30 days post-treatment. Lesions of vacuolization, edema, and atrophy can be seen but the number of these lesions and their size are decreased. Lymphoid infiltrates are absent; 20×.

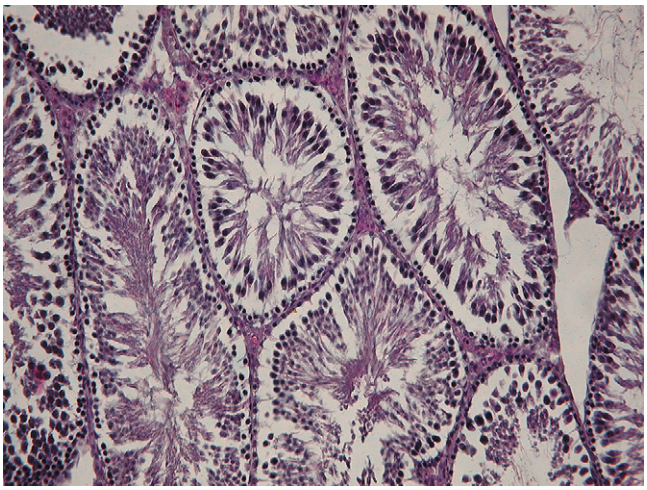

Fig. 7. Rat seminiferous tubules: Control. Normal spermatogenesis characterized by the presence of all typical cell generations; 20×.

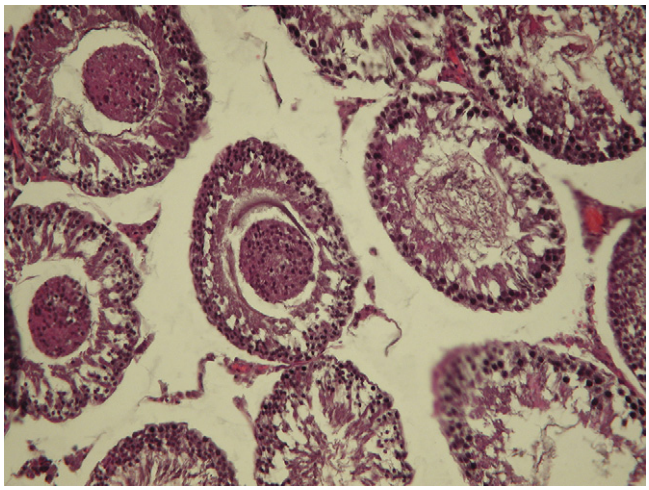

Fig. 9. Rat seminiferous tubules: Dox, 15 days post-treatment. Dyscomplexation and exfoliation of spermatogenic epithelium, formation of luminal clumps; 20×.

3.6.2. Testes

The seminiferous tubules of the control animals displayed all types of cell generations typical for normal spermatogenesis (Fig. 7).

In the group treated with PBCA + PS, on day 15 two of six animals displayed single immature cells of spermatogenic epithelium in some tubules. Similar observations were made on day 30. Testes of other animals in this group were normal throughout the period of observation (Fig. 8).

On day 15, some of the seminiferous tubules of the animals treated with Dox, Dox + PS, Dox-PBCA, and Dox-PBCA + PS displayed similar well-pronounced abnormalities such as disorganization of spermatogenic epithelia and its desquamation with luminal clumps formation. In some of the tubules, decomposition of nuclear chromatin was observed in form of dark type A spermatogonia and type B spermatogonia. The lumina of some tubules were filled with stainable amorphous material, possibly proteinacious by nature. The testis of a rat treated with Dox is shown in Fig. 9 and is typical for all animals of the above mentioned groups at this time point.

However, on day 30, the influence of the dosage form on the testicular toxicity became visible. The testes of the rats treated with Dox and Dox + PS underwent significant degenerative changes. Most tubules displayed deep atrophy of the spermatogenic epithelia. Some of the tubules were necrotic, whereas in other tubules type A spermatogonia were still preserved (Fig. 10).

In the group treated with Dox-PBCA, by day 30 the spermatogenic epithelia in most tubules was degenerated; however, some of the tubules displayed all types of cell generations typical for normal spermatogenesis.

In the group treated with Dox-PBCA + PS, the spermatogenic epithelia were degenerated in 2/3 of the tubules. These tubules displayed only Sertoli cells or few dark or light type A spermatogonia and single type B spermatogonia. At the same time, 1/3 tubules were fully or partially functional (Fig. 11).

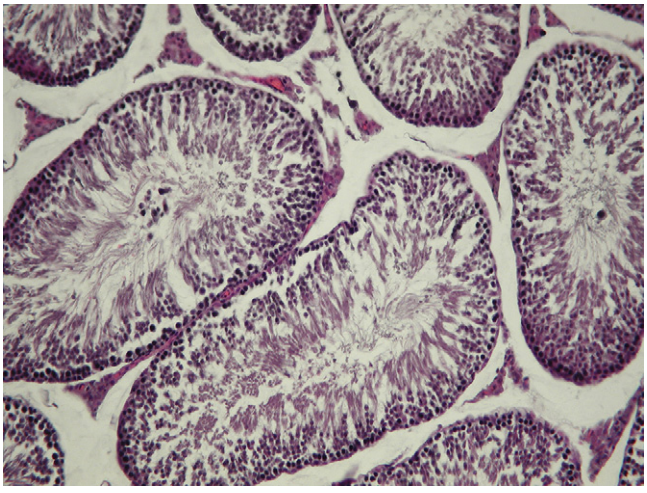

Fig. 8. Rat seminiferous tubules: PBCA + PS, 30 days post-treatment. Single immature cells of spermatogenic epithelium can be seen within some tubules; 20×.

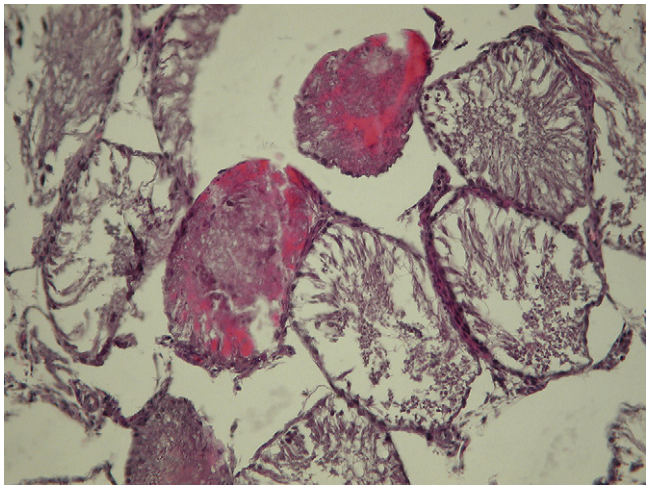

Fig. 10. Rat seminiferous tubules: Dox, 30 days post-treatment. Severe atrophy of spermatogenic epithelium, necrosis, edema of the interstitium; 20×.

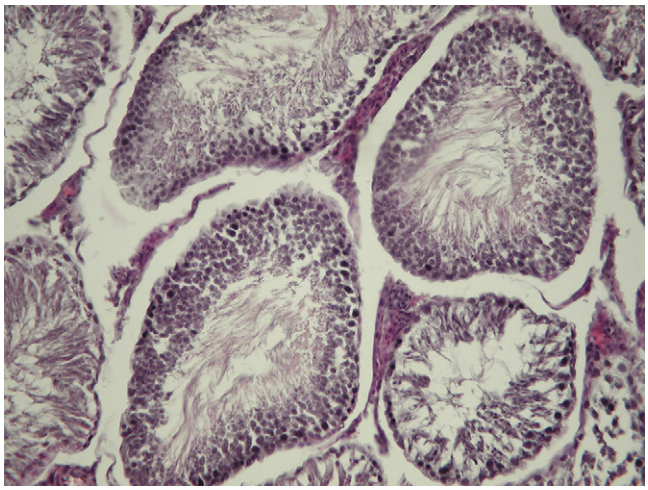

Fig. 11. Rat seminiferous tubules: Dox-PBCA + PS, 30 days post-treatment. Degeneration of the spermatogenic epithelia in 2/3 of the tubules; 1/3 of the tubules are fully or partially functional; 20×.

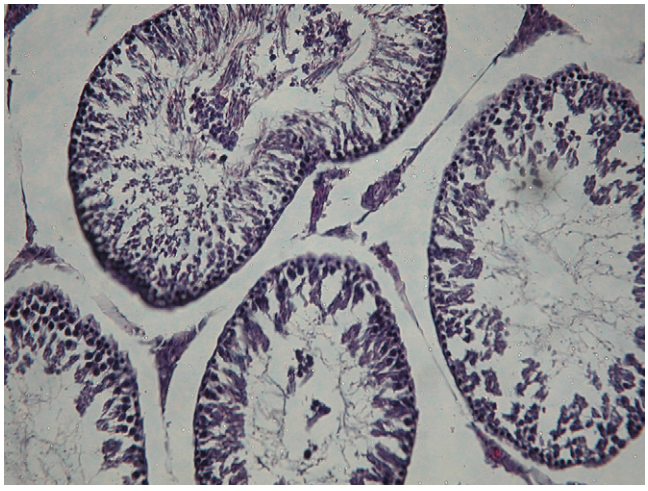

Fig. 12. Rat seminiferous tubules: Dox-HSA + PS, 30 days post-treatment. Moderate atrophy of the spermatogenic epithelia (dyscomplexation or few immature cells in the lumina).

The effect of HSA-based formulations on rat testes was less detrimental. On day 15, the testes of the animals treated with Dox-HSA + PS or Dox-HSA were similar to control. On day 30, a moderate atrophy of the spermatogenic epithelia (disorganization or few immature cells in the lumina) was visible in some of the tubules (Fig. 12).

4. Discussion

Especially PBCA but also HSA nanoparticles have been extensively studied as drug carriers (Kreuter, 2002; Vauthier et al., 2003; Gradishar et al., 2005; Michaelis et al., 2006). However, the toxicological parameters of the formulations based on these particles have only been marginally addressed so far. In the present study, comparative evaluation of the toxicological profiles of the nanoparticle-based formulations of doxorubicin versus free drug was performed at the therapeutic regimen that previously proved to be effective for the chemotherapy of the intracranial glioblastoma in rats (Steiniger et al., 2004).

In general, the clinical and morphological signs of doxorubicin toxicity observed in this study correlate with the findings of other authors (Mazue et al., 1995; Singal et al., 2000; Tsunenari et al., 2000). Thus, the treatment induced moderate changes of the gain in body weight. The hematological toxicity of all formulations was minor, and although slight fluctuations of the hematological parameters were observed, they remained within the physiological range throughout the study. This moderate toxicity is most probably explained by the dose-sparing treatment regimen. At the same time, the design of the study allowed to reveal the influence and impact of the formulation parameters on the toxicological profile of doxorubicin.

Binding of doxorubicin to HSA-NP significantly reduced all manifestations of the drug toxicity, PS-coated formulation being more favorable in terms of its influence on the hematological parameters and body weight. PBCA nanoparticles also altered the toxicological profile of doxorubicin considerably. The gain in body weight in the group treated with Dox-PBCA was significantly lower, as compared to both, control and Dox-treated animals, which could be indicative of the increased gastrointestinal toxicity. The influence on the hematological parameters also was more pronounced, as demonstrated by the decrease of the leukocyte and erythrocyte counts by day 3 post-treatment, as well as by the decreased spleen WI by day 15. Coating of Dox-loaded PBCA nanoparticles with PS considerably reduced the hematological effects, as compared to both, Dox and Dox-PBCA.

Moderate toxicity was observed also for placebo (PBCA + PS), which produced a slightly decreased gain in body weight by day 15, slight leukocytosis by day 3, and decreased WIs of kidney, liver, and lungs observed by day 30.

The difference between the formulations was most visible in the manifestations of the cardiac and the testicular toxicity. As expected, binding of doxorubicin to nanoparticles significantly decreased cardiotoxicity. This phenomenon, observed previously for liposomal and nanoparticle formulations of doxorubicin (Couvreur et al., 1982; Drummond et al., 1999), obviously appears to be related to the altered biodistribution pattern of the carrier-bound drug.

Thus, whereas free doxorubicin induced pronounced cardiotoxicity that was most clearly demonstrated by morphological changes in myocardium such as the cytoplasm vacuolation and atrophy of myocytes, pycnosis of nuclei, loss of myofibrils, and lymphoid infiltrations, doxorubicin bound to HSA nanoparticles (Dox-HSA and Dox-HSA + PS) did not cause any morphological changes of the myocardium during the period of observation. The histopathological findings were supported by the results of electrocardiographic study that revealed no signs of cardiac decompensation for these formulations.

PBCA-based formulations also yielded a lower cardiotoxicity compared to the free drug. Although these formulations induced morphological changes of the myocardium similar to those of the free drug, these changes were considerably less pronounced: the foci of myocardial atrophy were smaller and less frequent; the lymphoid infiltrations were absent. As mentioned above, this

phenomenon is explained by the alteration of the drug pharmacokinetics, in particular, by the reduced doxorubicin uptake in the heart enabled by nanoparticles (Couvreur et al., 1982, 1986; Gulyaev et al., 1999). Indeed, as shown by the pharmacokinetic study performed in rats, after administration of Dox-PBCA and Dox-PBCA + PS, the heart concentrations of doxorubicin were below the detection limit (10 ng/g), whereas the peak concentration of the free drug reached a considerable level of ~8 μg/g (Gulyaev et al., 1999).

It is noteworthy, that in this study the cardiotoxicity of Dox-PBCA + PS was lower, as compared to Dox-PBCA, both by functional and histopathological criteria. Again, the most probable explanation of this lower cardiotoxicity is the favorable alteration of the drug pharmacokinetics produced by PS coating. Indeed, although in the study of Gulyaev et al. (1999) it was not feasible to compare the heart concentrations of these formulations (both were below the detection level, i.e. >10 ng/g), it was demonstrated that coating of the nanoparticles with PS increased the AUC as well as decreased the volume of distribution and the clearance of doxorubicin, which are beneficial factors known to be associated with the reduction of cardiotoxicity (Zucchi and Danesi, 2003).

Another important finding in the present study is the reduced testicular toxicity of the nanoparticle-bound doxorubicin, which confirms an earlier report by Couvreur et al. (1986). Although, generally, the drug delivery to the testes is impeded by the blood–testes barrier, doxorubicin induces considerable testicular toxicity in men and animals (Shamberger et al., 1981; Russell and Russell, 1991; Tsunenari et al., 2000). Primary targets of this adverse effect are rapidly dividing spermatogonia and spermatocytes, which appear to be most susceptible to the cytotoxic effects of doxorubicin such as inhibition of DNA replication and DNA-dependent RNA synthesis.

In this study, signs of testicular toxicity were observed for all doxorubicin formulations, indicating that the doxorubicin solutions as well as all other formulations were able to transport the drug across the blood–testes barrier. However, the severity of damage of rat spermatogenic epithelium was considerably more pronounced in the animals treated with the free drug. In contrast, after administration of Dox-PBCA + PS the epithelia in most of the seminiferous tubules were not affected, and those that were damaged displayed single spermatogonia suggesting their potential for recovery. Again, the non-coated Dox-PBCA formulation was more toxic than Dox-PBCA + PS and less toxic compared to free drug.

It is generally believed that the potential for recovery of the seminiferous epithelium depends on the extent of spermatogonia damage. However, the results of Russell and Russell (1991) suggest that the death of Sertoli cells also may considerably contribute to the damage of the seminiferous epithelium and even lead to the failure of recover. It is possible that the lower toxicity of Dox-PBCA + PS is explained by a less efficient uptake of the surfactant-coated and consequently less opsonized particles by the Sertoli cells that have a phagocytotic function. Accordingly, the uncoated particles being more prone to phagocytosis are more hazardous for the Sertoli cells. Inside the cell, these particles degrade and release the drug that then exerts its cytotoxic effect, thus causing more pronounced damage of the epithelium.

As mentioned above, binding of doxorubicin to HSA nanoparticles significantly reduced all manifestations of the drug toxicity. This difference between the two nanoparticle types can be due to their different physicochemical properties which has an impact on the drug release. In particular, the PBCA-based formulations contained both, free and adsorbed drug in equilibrium. Moreover, these particles quickly release the drug upon administration (burst effect). As shown by Soma et al. (2000), in the case of similar, poly(isobutyl cyanoacrylate) nanoparticles, ~70% of bound doxorubicin might be released within 15 min. The burst effect additionally can be enhanced by the presence of a surfactant (unpublished data). Nevertheless, probably due to the above-mentioned alteration of the biodistribution and a difference in the interaction with the different body cells, the doxorubicin toxicity in the Dox-PBCA + PS formulation was reduced. It is known that polysorbate 80 might inhibit the Pgp efflux system (Woodcock et al., 1992; Nerurkar et al., 1996). However, when this surfactant was injected together with the doxorubicin solution no major influence on the drug in-vivo performance was observable in the present study or in the study of Gulyaev et al. (1999). Moreover, as already observed by Calvo et al. (2001), the impact of the polysorbate on the biodistribution is considerably reduced in the presence of nanoparticles.

In contrast to the PBCA nanoparticles, in the HSA-based formulations all of the drug is totally retained within the particle matrix and is released in a slower manner (unpublished data), thus producing lower peak plasma concentrations, which in turn appears to be responsible for the even lower cardiotoxicity of the latter nanoparticles.

5. Conclusions

The results of the present study demonstrate that the hematological, cardiac, and testicular toxicity of doxorubicin can be reduced by binding the drug to poly(butyl cyanoacrylate) nanoparticles and even more considerably by binding to human serum albumin nanoparticles. Coating of poly(butyl cyanoacrylate) nanoparticles with polysorbate 80 contributed to the reduction of toxicity. The lower toxicity of the nanoparticulate formulations of doxorubicin is most probably explained by the altered biodistribution of the drug mediated by the nanoparticles.

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