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# Paramagnetic species in the plasma of dogs with lymphoma prior to and after treatment with doxorubicin An ESR study

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**Abstract**

Doxorubicin is a potent cytostatic drug which is applied for the treatment of various kinds of malignant diseases. In spite of the routine use of this drug its major adverse effect, the dose-dependent cardiotoxicity, cannot be prevented yet. However, several clinical trials indicated that iron chelators are able to moderate the noxious effect more efficiently than radical scavenging antioxidants. This in turn supports the idea that doxorubicin–iron complexes are involved in triggering the cardiotoxicity of this drug by catalyzing the formation of oxygen radicals. However, both the mode of generation of doxorubicin–iron complexes and the consequences *in vivo* are not understood so far. In order to figure out whether or not doxorubicin can utilize iron from the transport protein transferrin for complex formation and prooxidative activities we studied the redox state of iron and its regulatory control by ceruloplasmin and ascorbate in the plasma of dogs suffering from malignant lymphoma by electron spin resonance spectroscopy. The respective electron spin resonance intensities prior to and after treatment with doxorubicin were compared with those from healthy controls. Our results revealed that dogs with lymphoma exhibit lower levels of paramagnetic copper in ceruloplasmin (−22%) and iron in transferrin (−33%) than healthy animals. Likewise the concentration of ascorbate radicals was lower in patients with lymphoma than in healthy subjects. The decreased cupric state of ceruloplasmin is equivalent to a diminished ferroxidase activity in plasma and therefore indicates indirectly an impaired antioxidant activity in these patients. Administration of doxorubicin *in vivo* further reduced the concentration of paramagnetic copper (−18%) and iron (−13%) while the concentration of ascorbate radicals remained unchanged. This decrease was also seen during the *in vitro* incubation of plasma with doxorubicin suggesting a direct interaction of the drug with the paramagnetic metal species. Model experiments revealed that the effect is based on a doxorubicin-induced release of iron from transferrin which is enhanced by ascorbate and the subsequent formation of doxorubicin–iron complexes. This mechanism was shown to trigger the formation of hydroxyl radicals from $H_2O_2$ and to cause an oxidation of the antioxidant ceruloplasmin. Our data demonstrate that cardiotoxic doxorubicin–iron complexes are not only formed in cardiomyocytes itself as generally assumed, but are also present in the circulation. Therefore, these findings provide an additional rationale for potential benefit of iron chelators during doxorubicin chemotherapy.


*Keywords:* Doxorubicin; Cancer; Electron spin resonance; Transferrin; Ceruloplasmin; Ascorbate free radicals

## 1. Introduction

Doxorubicin is clinically used for the treatment of a great variety of cancer diseases [1,2]. Besides typical adverse effects which are also common to other cytostatics doxorubicin causes a dose-dependent cardiotoxicity [1]. The mechanism of doxorubicin cardiotoxicity is still a matter of controversial debate [3]. We have recently shown that the selective cardiotoxic effect of doxorubicin is linked to the existence of a particular reductase in mitochondria of cardiomyocytes [4,5]. Other research groups reported that the development of cardiotoxicity is related to the redox

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*Abbreviations:* AFR, ascorbate free radicals; Apo-Tf, apo-transferrin; Asc, ascorbic acid/ascorbate mono anion (pH-dependent equilibrium); Cp, ceruloplasmin (protein); $Cu^{2+}$-Cp, copper(II) bound in ceruloplasmin; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; Doxo, doxorubicin; ESR, electron spin resonance; $Fe^{3+}$-Tf, iron(III) bound in transferrin (partially saturated with iron); ROS, reactive oxygen species; SOD, superoxide dismutase; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; Tf, transferrin (protein); TRIS, tris(hydroxymethyl)-aminomethan.

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activity of the various anthraquinone derivatives and the formation of oxygen radicals therefrom [3]. On the other hand doxorubicin–iron complexes were suggested to play a role as modulators of cardiotoxicity [6]. From *in vivo* studies it turned out that iron-chelating compounds were more efficient in protecting the heart from undesired side effects of anthraquinones than radical-scavenging antioxidants [7–9]. This observation suggests a key role of doxorubicin–iron complexes in toxic reactions of this drug [6]. However, the mechanism of the formation of doxorubicin–iron complexes and the involvement of these complexes in cardiotoxicity and general cytotoxicity is still a matter of speculation.

Ferrous iron ($Fe^{2+}$) after being resorbed from the intestine undergoes oxidation to the ferric form ($Fe^{3+}$), which allows its binding to transferrin [10]. Oxidation of $Fe^{2+}$ to $Fe^{3+}$ is mediated by ceruloplasmin-bound copper, which is reduced from the paramagnetic cupric ($Cu^{2+}$) into the diamagnetic cuprous ($Cu^{+}$) valence state. Therefore, the ferroxidase activity of ceruloplasmin in the blood determines the ratio between free iron and iron bound to transferrin. The electron shuttle between iron and ceruloplasmin can be made visible by means of electron spin resonance spectroscopy (ESR) due to the change from paramagnetic $Cu^{2+}$ to diamagnetic $Cu^{+}$. In contrast ascorbate may reduce free iron back into the ferrous state being itself oxidized to an ascorbate radical. The involvement of ascorbate as a reductant of free metal ions becomes visible from the detection of ascorbate free radicals by ESR.

Although the doxorubicin–iron complex formation was proposed to occur from intracellular iron resources [8,11] upon intravenous application the drug already in the blood plasma gets in contact with iron-containing proteins. Therefore, the aim of the recent study was to test the hypothesis that doxorubicin can release iron from the plasma protein transferrin under formation of toxic complexes, which could be responsible for oxidative damage in the heart and other parts of the organism. In order to elucidate this question and possibly involved mechanisms we studied the concentration of $Fe^{3+}$-transferrin ($Fe^{3+}$-Tf), $Cu^{2+}$-ceruloplasmin ($Cu^{2+}$-Cp) and ascorbate free radicals (AFR) quantitatively by ESR in healthy dogs in comparison with dogs suffering from malignant lymphoma prior to and after the administration of doxorubicin.

## 2. Materials and methods

### 2.1. Animals

Male healthy beagle dogs were selected for control samples (N = 34, mean age 2.3 years). Male tumor-bearing dogs were recruited from the patients of the veterinary hospital and were included in our study after diagnosis of malignant lymphoma by histologic evaluation of biopsy material. The mean age of patients was 8.1 years (range 6–11 years, N = 12).

### 2.2. Doxorubicin treatment

After confirmation of the diagnosis dogs with lymphoma received five times 30 mg/m$^2$ Adriablastin (doxorubicin, Pharmacia & Upjohn) every 3 weeks and in addition patients were given three times 10,000 U/m$^2$ L-asparaginase weekly at the start of the therapeutic cycle.

### 2.3. Blood sampling

Blood samples (about 5 mL) were taken into heparinized tubes from both control animals and tumor patients by venipuncture of the jugular vein. All samples were taken from dogs that were fasted for 12 hr. For tumor patients samples were obtained during clinical examination prior to chemotherapy. For selected patients (N = 6) 30 min after chemotherapy blood samples were drawn again. Immediately after sampling the blood was centrifuged (2340 *g*, 7 min) at 4°. Afterwards the plasma was separated from cellular components and each 500 μL of plasma was aspirated into Braun OMINFIX–F 1 mL disposable syringes from which the luer lock connection was cut off. Filled syringes were frozen immediately in liquid nitrogen. The frozen plasma cylinders were pushed out of the syringes and stored at 77 K.

### 2.4. ESR spectroscopy of plasma samples

Plasma pellets were transferred into a quartz finger dewar at 77 K for ESR analysis. ESR spectra for $Fe^{3+}$-Tf and $Cu^{2+}$-Cp were recorded on a Bruker ESP 300 instrument and a $TE_{102}$-cavity using the following instrument settings: 9.44 GHz microwave frequency, 50 mW microwave power, 2500 G center field, 3000 G sweep, 10 G modulation amplitude, $5 \times 10^4$ receiver gain, 1072 G/min scan rate, 0.655 s time constant, 1 scan, and 77 K temperature. The intensities of $Fe^{3+}$-Tf and $Cu^{2+}$-Cp were measured in arbitrary units from the baseline-corrected peak-to-peak intensities. The ratio $Fe^{3+}$-Tf/$Cu^{2+}$-Cp was calculated for each individual ESR measurement prior to statistical analysis of the data. For measurements of the transferrin saturation plasma samples were thawed, supplemented with an excess of $Fe^{3+}$ (1 mM $FeCl_3$, Merck), and frozen again to 77 K. The saturation was calculated as ratio of the ESR intensities prior to and after the addition of iron.

In order to determine AFR concentrations plasma pellets were thawed under controlled conditions (10 min, 298 K) and filled into a quartz flat cell. ESR measurements were carried out in a TM-cavity using following instrument parameters: 9.80 GHz microwave frequency, 20 mW microwave power, 3486 G center field, 20 G sweep, 1 G modulation amplitude, $1 \times 10^6$ receiver gain, 3.58 G/min scan rate, 1.31 s time constant, 2 scans, and 298 K temperature. The concentration of AFR was calculated from the double integral of the ESR signal in comparison with a

500 nM 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO, Sigma) standard sample.

Experiments in the presence of desferrioxamine mesylate (Novartis), penicillamine (Sigma), superoxide dismutase (SOD, Sigma), catalase (Sigma) and ascorbic acid (Merck) were performed under the same conditions with freshly unfrozen plasma samples.

### 2.5. ESR spectroscopy of model systems

Ascorbic acid, $FeCl_3$, 5,5-dimethyl-1-pyrroline-*N*-oxide (Sigma), $H_2O_2$ (Merck), doxorubicin, purified ceruloplasmin (Sigma) and iron-depleted transferrin (Sigma) were used for these experiments. All reagent solutions were prepared from Chelex-treated water or buffer. Doxorubicin–iron complexes were prepared immediately prior to use by mixing doxorubicin and $FeCl_3$ in a molar ratio 3:1.

In order to test the sensitivity of $Cu^{2+}$-Cp signals to different compounds ceruloplasmin was diluted in TRIS buffer and incubated with the respective reagents for 5 min at 37°. Afterwards each 500 μL of the solution were frozen and measured as described for plasma samples. $Fe^{3+}$-Tf complexes were obtained by mixing $FeCl_3$ with iron-depleted transferrin dissolved in buffer (40 mM TRIS, 10 mM $NaHCO_3$, pH 7.4) yielding a saturation of 50%. The iron release from $Fe^{3+}$-Tf was tested by incubation with doxorubicin and/or ascorbic acid for 30 min at 37°. After preparation of frozen pellets ESR measurements were performed as reported for plasma samples.

For spin trapping experiments each 500 μL $Fe^{3+}$-Tf solution (50% saturation) or 500 μL buffer were mixed with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO, Sigma), $H_2O_2$, doxorubicin, and ascorbic acid if required. Afterwards solutions were transferred into a quartz flat cell and ESR measurements were started using the following instrument settings: 9.80 GHz microwave frequency, 20 mW microwave power, 3487 G center field, 80 G sweep, 1 G modulation amplitude, $1 \times 10^6$ receiver gain, 28.6 G/min scan rate, 0.163 s time constant, 1 scan, and 298 K temperature.

### 2.6. Statistics

All data were analyzed using ORIGIN 4.1 (Microcal Software Inc.) or the statistical package SPSS 9.0 for Windows (SPSS Inc.). Bar graphs contain error bars which correspond to standard errors. Statistical significances were calculated by Student's *t*-test.

## 3. Results

### 3.1. Measurement of paramagnetic species in plasma

Plasma samples prepared from heparinized blood exhibited absorptions in the ESR spectra which arise from different paramagnetic species. At low temperature (77 K) ESR signals, which correspond to trivalent iron bound in transferrin ($Fe^{3+}$-Tf, $g = 4.15$), and to divalent copper complexed in ceruloplasmin ($Cu^{2+}$-Cp, $g = 2.04$) were observed (Fig. 1A). In contrast thawed samples at room temperature showed a doublet signal (Fig. 1C), which can be attributed to AFR, $g = 2.00$, $a_H = 1.81$ G. Specimen stored over a period of 1 month at 77 K did not exhibit significant changes in the ESR signal intensity of the paramagnetic markers. Pooled plasma samples unfrozen after different storage times exhibited AFR signal intensities which were not significantly different. Therefore, all plasma samples from patients were stored under these conditions up to 4 weeks prior to ESR measurements.

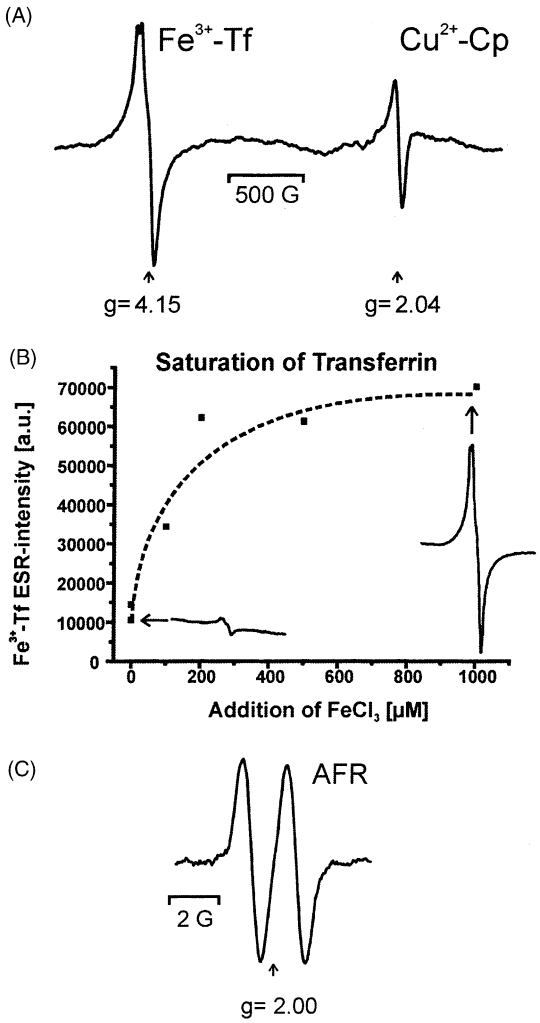


Fig. 1. ESR signals of paramagnetic species detected in the plasma of healthy dogs and dogs with malignant lymphoma. (A) Two independent ESR signals were detected at low temperature (77 K) in plasma pellets. According to their *g*-values the signals were attributed to iron bound in transferrin ($Fe^{3+}$-Tf) and to copper bound in ceruloplasmin ($Cu^{2+}$-Cp) [31,32]. (B) $Fe^{3+}$-Tf ESR signal intensity as a function of different amounts of $FeCl_3$ added to plasma samples. ESR signals of $Fe^{3+}$-Tf from the native plasma sample (left) and after addition of 1 mM $FeCl_3$ (right). (C) At room temperature a doublet ESR signal was detected, which can be assigned to AFR [33].

Table 1
Influence of different compounds on the $Cu^{2+}$-related ESR signal of isolated ceruloplasmin (11 U/mL)

| Additions | ESR intensity of $Cu^{2+}$-Cp (%) |
|---|---|
| None | 100 ± 3 |
| Ascorbic acid (2 mM) | 0 |
| Ascorbic acid (100 μM) | 63 ± 1 |
| $Fe^{2+}$ (100 μM) | 0 |
| $Fe^{3+}$ (16.6 μM) | 101 ± 7 |
| Doxorubicin (50 μM) | 104 ± 2 |
| $Fe^{3+}$(doxorubicin)$_3$ (16.6 μM) | 67 ± 3 |

Experiments were performed in a Chelex-treated buffer (50 mM TRIS, pH 7.4) (N = 4, means ± standard errors).

Table 2
Changes of the AFR-related ESR signal intensity in pooled plasma samples (500 μL) by the addition of antioxidants and metal chelators

| Additions | ESR intensity of AFR (%) |
|---|---|
| None | 100 ± 4 |
| Ascorbic acid (1 mM) | 98 ± 1 |
| SOD (80 μg/mL) | 155 ± 1 |
| Catalase (80 μg/mL) | 106 ± 2 |
| Desferrioxamine (4 mM) | 78 ± 1 |
| Penicillamine (4 mM) | 4 ± 1 |

N = 3, means ± standard errors.

At first we tried to elucidate the origin of these ESR absorptions and the influence of other plasma components related to the iron metabolism on the signal intensities. Ceruloplasmin is synthesized in the liver and contains upon release into the circulation 6–7 copper ions. As shown in Table 1, the addition of ascorbic acid and free iron ($Fe^{2+}$) strongly diminished the ESR signal intensity of $Cu^{2+}$-Cp demonstrating the close relation of this signal to the redox state in plasma and the iron metabolism.

In contrast to ceruloplasmin transferrin, which circulates in the blood, is only partially saturated with metal ions. By incorporation of free $Fe^{3+}$ from the blood it prevents harmful redox-cycling of these metal ions which otherwise could trigger oxidative stress. This antioxidant function of transferrin depends on the degree of saturation with $Fe^{3+}$. The addition of $FeCl_3$ to plasma samples stepwise increased the $Fe^{3+}$-Tf signal up to a certain intensity (Fig. 1B), which is referred as the total iron-binding capacity. Excess iron did neither significantly disturb the ESR signal shape nor its intensity (Fig. 1B). Control samples with water instead of plasma, which were prepared in the heparinized tubes for blood sampling, demonstrated that in the iron-overloaded plasma sample the contribution of heparin–iron complexes to the peak-to-peak intensity of $Fe^{3+}$-Tf is less than 5%. After the addition of fluoride ions, which form paramagnetic $FeF_6^{3-}$ complexes ($g = 2$) with unbound $Fe^{3+}$, to iron overloaded samples the ESR spectra exhibited only very small additional signals at $g = 2$ indicating that most excess iron was reduced to $Fe^{2+}$ in these samples (data not shown). Therefore, the degree of saturation of transferrin in plasma samples was obtained from the titration of the $Fe^{3+}$-Tf ESR signal with 1 mM $FeCl_3$ and comparison with the ESR signal intensity of the native sample.

Ascorbate free radicals may arise from both prooxidant and antioxidant reactions in the plasma. The addition of ascorbic acid to plasma samples demonstrated that AFR signals were not dependent on ascorbic acid concentrations under these experimental conditions (Table 2). During the antioxidative activity of ascorbic acid AFR can result from the reaction of ascorbate with either hydroxyl or superoxide radicals. However, neither the addition of catalase, which removes $H_2O_2$ as precursor of hydroxyl radicals, nor

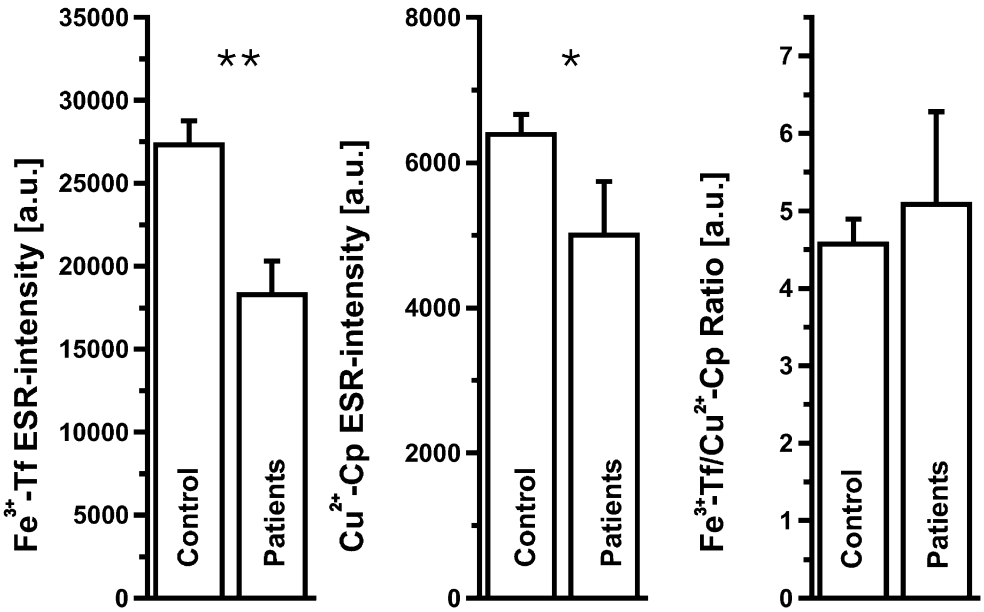


Fig. 2. Changes of ESR intensities of paramagnetic metal ions $Fe^{3+}$-Tf, $Cu^{2+}$-Cp and the ratio $Fe^{3+}$-Tf/$Cu^{2+}$-Cp in dogs with malignant lymphoma prior to treatment with doxorubicin (N = 12) in comparison with healthy dogs (N = 34). (**) $P < 0.01$; (*) $P < 0.05$.

the addition of superoxide dismutase, which eliminates $O_2^{\bullet -}$, diminished the ESR signal in plasma samples. In contrast the addition of SOD even increased AFR concentrations in the respective plasma samples. Metal chelators, like desferrioxamine (rather specific for Fe) and penicillamine (rather specific for Cu), were found to inhibit the AFR signals partially or even completely (Table 2). The data suggest that AFR in plasma samples from untreated animals preferably result from the reaction of ascorbate with redox-active copper ions.

### 3.2. Changes of paramagnetic markers under pathophysiological conditions

All selected paramagnetic markers are closely related to the iron and copper metabolism in the organism. Since it is commonly known that plasma levels of iron and copper are modulated under different pathophysiological conditions we compared the concentrations of paramagnetic metal ions in lymphoma patients with healthy animals. Our experiments demonstrated that the malignant disease caused a significant alteration of these parameters as well (Fig. 2). In male dogs with malignant lymphoma a significant decline of $Fe^{3+}$-Tf and $Cu^{2+}$-Cp was observed in comparison with healthy animals while the ratio $Fe^{3+}$-Tf/$Cu^{2+}$-Cp remained unchanged. The lower $Fe^{3+}$-Tf values were caused by a significantly decreased iron saturation of transferrin (from $62 \pm 3\%$ in controls ($N = 14$) to $43 \pm 7\%$ in patients ($N = 8$), $P < 0.01$) while the total apo-transferrin concentration was unchanged. Furthermore, a decreased concentration of AFR ($115 \pm 16$ nM, $N = 12$) in patients with malignant lymphoma was observed in comparison with healthy dogs ($153 \pm 10$ nM, $N = 34$) which was, however, not statistically significant at the level of $P = 0.05$.

### 3.3. Influence of doxorubicin on paramagnetic species in vivo and in vitro

In order to elucidate the influence of doxorubicin treatment on paramagnetic species in plasma we studied the effect of this drug *in vivo* and *in vitro*. Preparatory experiments revealed that doxorubicin–iron complexes in low concentrations did not interfere with the measurement of $Fe^{3+}$-Tf by ESR.

Our data from *in vivo* experiments (Fig. 3A) which were obtained from plasma samples of dogs with malignant lymphoma prior to and after treatment with doxorubicin (30 min) demonstrated that doxorubicin caused a decrease of both $Fe^{3+}$-Tf and $Cu^{2+}$-Cp ESR signals, but did not change AFR levels.

Repeating these experiments *in vitro* the incubation of pooled plasma samples with doxorubicin decreased the concentration of both $Fe^{3+}$ bound to transferrin and $Cu^{2+}$ in ceruloplasmin (Fig. 3B). This suggests that doxorubicin is able to release iron from transferrin although the latter is

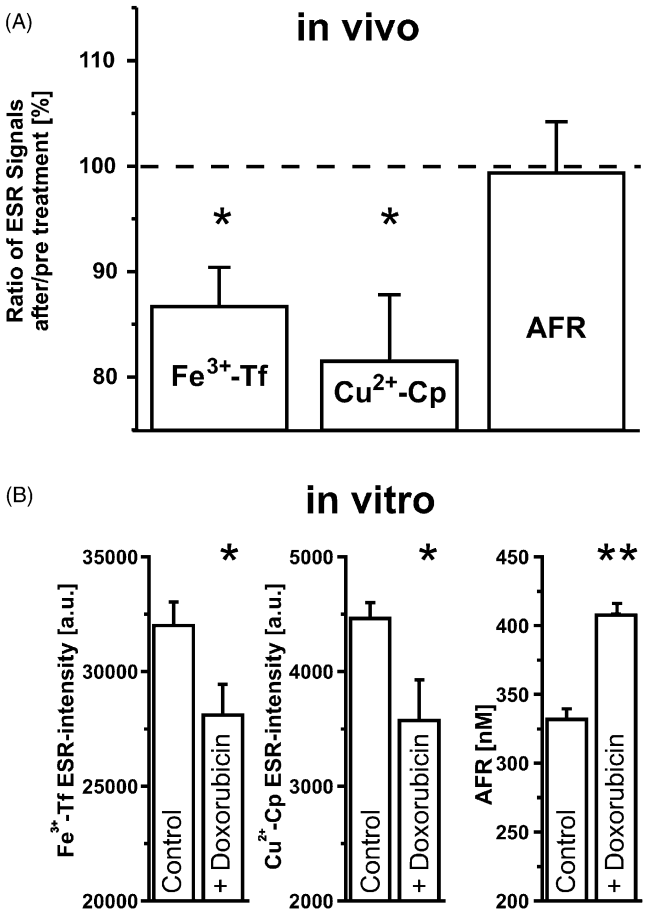


Fig. 3. Influence of doxorubicin on the concentration of paramagnetic species in the plasma. (A) Plasma samples were drawn from dogs with malignant lymphoma prior to doxorubicin treatment and 30 min after parental application of the drug ($N = 6$). Afterwards the paramagnetic species were measured and the intensity after doxorubicin treatment was expressed as percentage of the signal intensity prior to the treatment for each patient. (B) Freshly obtained plasma samples of healthy dogs were pooled and supplemented with doxorubicin (50 μM), incubated for 30 min at 37°, and analyzed by ESR ($N = 3$). (**) $P < 0.01$; (*) $P < 0.05$.

an extremely strong complexing agent for trivalent iron. The lower intensity of $Cu^{2+}$-Cp is indicative of reductive processes which can occur if free $Fe^{2+}$ or doxorubicin–iron complexes exist in the plasma (Table 1). Likewise the increased AFR formation in the presence of doxorubicin (Fig. 3B) supports the idea that this agent can recruit iron from the redox-inactive $Fe^{3+}$-Tf complex thereby allowing iron to undergo harmful redox-cycling reactions between the ferric and the ferrous state.

### 3.4. Iron release from transferrin in the presence of doxorubicin and reactive oxygen species (ROS) formation by doxorubicin–iron complexes

The liberation of redox-inactive iron bound in transport or storage proteins is likely to be a trigger mechanism for oxidative stress during cytostatic therapy with doxorubicin. In order to elucidate the mechanism of this iron release we

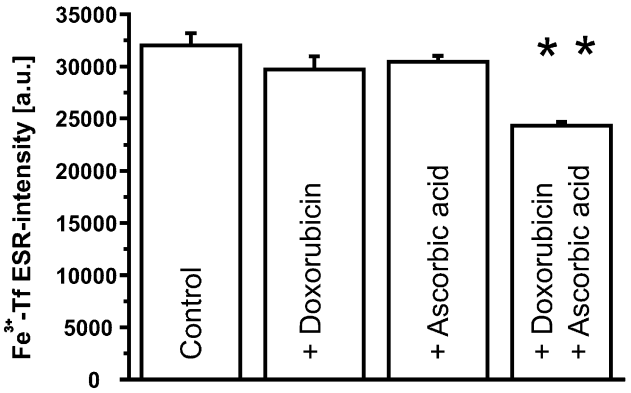


Fig. 4. Decrease of the $Fe^{3+}$-Tf ESR signal intensity induced by doxorubicin and ascorbic acid in buffer (40 mM TRIS, 10 mM $NaHCO_3$, pH 7.4). Purified transferrin (5 mg/mL ≈ 65 μM) was saturated with iron to 50% and supplemented with either doxorubicin (50 μM) or ascorbic acid (1 mM) alone or a combination of both compounds. Afterwards the samples were frozen and the $Fe^{3+}$-Tf intensities were measured by ESR. N = 4, (**) $P < 0.01$.

incubated purified transferrin, which was saturated with iron to about 50%, with different reactants. As shown in Fig. 4 neither ascorbate nor doxorubicin alone induced a detectable release of iron from transferrin. However, the combination of both compounds decreased the $Fe^{3+}$-Tf signal up to 25%. Since these three compounds coexist in the plasma during chemotherapy this effect can occur also *in vivo* (Fig. 3A).

In order to demonstrate the prospective consequences of this iron release in the presence of $H_2O_2$, which can be liberated into the plasma from different cells, we performed ESR spin trapping experiments using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap compound. Neither $Fe^{3+}$-Tf (Fig. 5A) nor doxorubicin (Fig. 5B) alone triggered the formation of detectable DMPO spin adducts in the presence of $H_2O_2$. However, in the presence of both $Fe^{3+}$-Tf and doxorubicin in this system a quartet ESR signal was observed (Fig. 5C). The splitting constants of $a_N = 14.9$ G and $a_H = 14.8$ G suggest the formation of a hydroxyl radical DMPO adduct (DMPO/$^{\bullet}$OH). After the addition of ascorbic acid to the incubation mixture the intensity of the DMPO/$^{\bullet}$OH adduct was strongly increased (Fig. 5D) and in addition a doublet signal corresponding to AFR (Fig. 5D) was superimposed ($a_H = 1.8$ G).

## 4. Discussion

Doxorubicin was successfully used in cancer chemotherapy during the last decades [2,12]. However, its major adverse effect, the dose-related cardiotoxicity, cannot be avoided yet [2,13]. Clinical trials in the past revealed that certain iron chelators (e.g. dexrazoxane) were more effective than radical-scavenging antioxidants (e.g. Vitamin E) in the prevention of cardiotoxic side effects [7,14]. Various research groups suggested that doxorubicin–iron complexes are involved both in the cardiotoxicity and in general cytotoxicity of this drug [6,15]. However, the mode of formation of these complexes *in vivo* allowing an evaluation of their pathogenetic significance is not clear so far.

The storage and redox state of iron in the blood are controlled by transferrin (Tf), the iron-reducing agent ascorbic acid (Asc) and the iron-oxidizing enzyme ceruloplasmin (Cp). While iron in Tf ($Fe^{3+}$-Tf) is exclusively in the paramagnetic trivalent state (Eq. (1)), the redox state of copper in ceruloplasmin is variable involving the paramagnetic cupric state ($Cu^{2+}$-Cp) and the diamagnetic cuprous state ($Cu^{+}$-Cp) (Eq. (3)). This redox equilibrium is strongly influenced by the presence of $Fe^{2+}$ and antioxidants (ascorbic acid and thiols) in the plasma. Asc in the

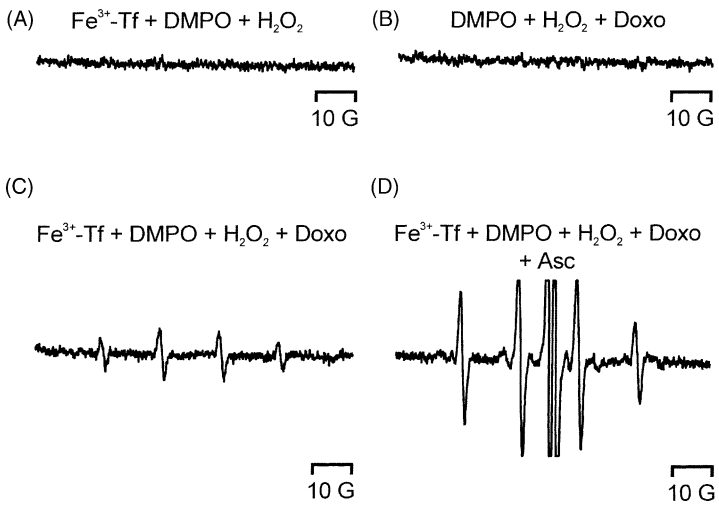


Fig. 5. ESR spectra of spin trapping experiments demonstrating the consequences of iron release from $Fe^{3+}$-Tf by doxorubicin (Doxo) in the presence of $H_2O_2$. The incubation mixture contained 10 mM DMPO as spin trap compound and 1 mM $H_2O_2$ in Chelex-treated buffer (40 mM TRIS, 10 mM $NaHCO_3$, pH 7.4). The spectra were recorded after 10 min incubation with (A) 65 μM $Fe^{3+}$-Tf (50% Fe-saturation), (B) 50 μM doxorubicin, (C) 65 μM $Fe^{3+}$-Tf (50% Fe-saturation) + 50 μM doxorubicin. Spectrum (D) was obtained after incubation of sample (C) with 0.5 μM ascorbic acid (Asc) for 1 min.

plasma is in equilibrium with its half oxidized state, the AFR, and the fully oxidized species dehydroascorbate (Eqs. (2) and (4)).

$$\text{apo-Tf} + Fe^{3+} \rightleftharpoons Fe^{3+}\text{-Tf} \quad (1)$$

$$Fe^{3+} + \text{Asc} \rightarrow Fe^{2+} + \text{AFR} \quad (2)$$

$$Cu^{2+}\text{-Cp} + Fe^{2+} \rightarrow Cu^{+}\text{-Cp} + Fe^{3+} \quad (3)$$

$$Cu^{2+} + \text{Asc} \rightarrow Cu^{+} + \text{AFR} \quad (4)$$

Reactions (1) and (3) proceed in the plasma of patients and healthy subjects.

Unexpectedly the AFR formation in plasma is only poorly inhibited (see Table 2) by a strong iron chelator, like desferrioxamine ($\log K = 31$ for $Fe^{3+}$) as it would be expected from Eq. (2). The observed sensitivity of AFR signals towards penicillamine, which exhibits a higher binding constant for $Cu^{2+}$ ($\log K = 16.5$) than for $Fe^{2+}$ ($\log K = 7.6$) [16], suggests that free Cu ions are mainly responsible for AFR signals in plasma samples in the absence of doxorubicin (Eq. (4)). However, as shown in Fig. 3B in the presence of doxorubicin also doxorubicin–iron complexes may contribute to increased AFR levels. The conclusion that AFR result from the reduction of transition metals according to Eqs. (2) and (4) is also supported by the inability of ROS-detoxifying enzymes (SOD and catalase) to inhibit AFR formation in plasma samples.

From clinical reference data it is known that the total iron and copper plasma contents are modulated by different pathophysiological mechanisms [10,17,18]. Our results demonstrate that this holds true for paramagnetic metal species in plasma as well. Studies in the past reporting on the existence of paramagnetic species in the blood of cancer patients were inconsistent [19–24]. Concentrations of paramagnetic species exhibiting ESR signals at $g = 2.04$ were found to be sex specific, to vary with malignant diseases and to be under the influence of estrogen intake [23,25]. Accordingly our data from healthy dogs exhibited significant sex differences (data not shown). Therefore, in our study only healthy male animals were compared with male dogs suffering from malignant lymphoma. In contrast to the study of Horn *et al.* [23], in which increasing $Cu^{2+}$-Cp levels but no changes of $Fe^{3+}$-Tf concentrations in humans with malignancies were found, we detected a significant decrease of $Fe^{3+}$-Tf and $Cu^{2+}$-Cp in tumor patients in comparison with control animals (Fig. 2). The reason for this discrepancy may be the use of serum by the former group, in which a strong increase of $Cu^{2+}$-Cp ESR signals during sample preparation was observed [23]. In contrast the $Cu^{2+}$-Cp signal in heparinized plasma samples remained unchanged even after thawing and freezing of the sample. Furthermore, our study included only male dogs with malignant lymphoma while Horn *et al.* [23] averaged data from a great variety of cancer diseases. Therefore, from the methodological point of view the data, which we obtained using plasma and selecting patients with only one type of cancer, may be more reliable. The decrease of $Fe^{3+}$-Tf in lymphoma patients (based on a decreased iron saturation) demonstrates that tumor patients have even a higher portion of unsaturated transferrin, which can bind ferric iron, than healthy animals. However, the controlled oxidation of ferrous iron to ferric iron requires the ferroxidase activity of ceruloplasmin. Therefore, a decreased level of $Cu^{2+}$-Cp in animals with malignancies suggests an increased accumulation of toxic $Fe^{2+}$ in plasma. Since ceruloplasmin was shown in other studies [26] directly to prevent the oxidative damage induced by doxorubicin [27], a decline of its activity is also equivalent to a reduced antioxidative protection in tumor patients.

In such already effected patients the application of doxorubicin additionally threatens the antioxidative defense. Our data suggest that doxorubicin can remove iron partially from transferrin both *in vivo* and *in vitro* (Fig. 3). If one considers the stability constant for $Fe^{3+}$-Tf ($\log K = 22.7$ and 22.1 for $2Fe^{3+}$ [28]) and $Fe^{3+}$ in doxorubicin complexes ($\log K = 18$, 1:1 complex [29]) a partial removal of iron from transferrin by doxorubicin seems to be thermodynamically possible. Since the binding constants for the two iron ions bound in transferrin are not extremely different the transferrin saturation may not significantly influence the iron mobilization from this transport protein. The release of iron from $Fe^{3+}$-Tf under formation of doxorubicin–iron complexes may explain the observed additional decline of $Cu^{2+}$-Cp after doxorubicin application *in vitro* and *in vivo* (Fig. 3A and B). This conclusion is supported by our finding that doxorubicin–iron complexes serve as a substrate for $Cu^{2+}$-Cp (Table 1). Although, the iron release from $Fe^{3+}$-Tf *in vitro* is accompanied by elevated AFR plasma levels no significant changes were observed *in vivo* implying additional recycling mechanisms for AFR in the whole blood [30].

Studies with purified $Fe^{3+}$-Tf revealed that the doxorubicin-catalyzed iron release from this transport protein became significant if ascorbic acid was present. This observation can be explained by the increased formation of $Fe^{2+}$–doxorubicin complexes in the presence of ascorbate [6] and the fact that $Fe^{2+}$ cannot be reinserted into apo-transferrin. The phenomenon that doxorubicin alone is able to release traces of iron from $Fe^{3+}$-Tf was indirectly demonstrated by the ESR spin trapping experiments. Iron released from $Fe^{3+}$-Tf in the presence of doxorubicin caused a cleavage of $H_2O_2$ to hydroxyl radicals (see Fig. 5C). This prooxidative reaction was enhanced in the presence of small amounts of ascorbic acid which in turn stimulates redox-cycling of doxorubicin–iron complexes.

From the results of this study one has to conclude that: (i) heparinized plasma samples are suitable for the quantitative detection of paramagnetic species in the blood of patients. (ii) Both $Fe^{3+}$-Tf and $Cu^{2+}$-Cp levels are

decreased in dogs with malignant lymphoma vs. control animals demonstrating a decreased ferroxidase activity and antioxidative defense. (iii) The treatment of tumor patients with doxorubicin further compromises the antioxidative defense by extraction of iron from $Fe^{3+}$-Tf under formation of highly redox active doxorubicin–iron complexes in the blood. (iv) The reaction of doxorubicin–iron complexes with $H_2O_2$ proved to be a potential source of hydroxyl radicals, thereby contributing to the cardio- and general cytotoxicity of doxorubicin.

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