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Daunorubicin does not induce immunohistochemically detectable endothelial dysfunction in rabbit aorta and femoral artery

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Summary. Anthracyclines are one of the most effective anticancer drugs ever developed, but their clinical use has been hampered by the risk of severe cardiotoxicity. In this study, we investigated whether rabbits exposed to a different cumulative dose of anthracycline suffer from immunohistochemically detectable vascular toxicity and endothelial dysfunction.

Daunorubicin (3 mg/kg, i.e. 50 mg/m^2) was administered i.v. to rabbits once weekly for 1-10 weeks to reach different cumulative doses of the drug (50-500 mg/m^2), while control rabbits received saline. The rabbits were sacrificed either 24 hours or 7 days after reaching each particular cumulative dose, and aortas and right femoral arteries were collected for immunohistochemical analysis.

Immunohistochemical analysis showed ICAM-1 staining in many aortas from both saline and daunorubicin-treated rabbits without any relationship to the anthracycline treatment. On the contrary, unlike in the lipopolysaccharide-treated or hypercholesterolemic rabbits, no distinct immunoreactivity for other markers of inflammation, oxidative and nitrosative stress (VCAM-1, 4-HNE, iNOS and nitrotyrosine) were detected in aortas and femoral arteries from either control or daunorubicin-treated animals. No relationship to the cumulative dose of the drug or post-expose set up of harvesting was found.

In this study, we have demonstrated that daunorubicin does not induce gross histopathological changes in the studied arteries and it fails to induce immunohistochemically detectable endothelial dysfunction. Thus, we propose that endothelial cells are much less susceptible to anthracycline toxicity than cardiac myocytes. In addition, our data suggest that vascular toxicity of anthracyclines plays rather a minor role in the cardiovascular complications of anthracycline chemotherapy.

Key words: Anthracyclines, Endothelial dysfunction, Immunohistochemistry, Rabbit, Aorta

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Introduction

Anthracyclines (e.g., doxorubicin or daunorubicin) are one of the most effective anticancer drugs ever developed, but their clinical use has been hampered by the risk of severe cardiotoxicity. Although the pathogenesis of anthracycline cardiotoxicity remains elusive, oxidative stress-related hypothesis remains a leading opinion in the field (Ferreira et al., 2008). Several lines of evidence point to NO synthesis impairment and nitrosative stress as important pathogenetic mechanisms significantly contributing to the development of myocardial damage and left ventricular dysfunction (Fogli et al., 2004; Barry et al., 2007).

Recent experimental results have strongly suggested that besides the heart, vessels might also be an important target for anthracycline toxicity. A growing body of experimental evidence shows that the endothelium might be particularly sensitive to anthracycline treatment (Wakabayashi and Groschner, 2003). *In vitro* experiments have shown that anthracyclines induce oxidative stress, ATP depletion and apoptosis in cultured

endothelial cells (Thorburn and Frankel, 2006). Furthermore, they have been found to induce inflammation accompanied with markedly increased expression of cell adhesion molecules and facilitated adhesion of blood mononuclear cells (Abou El Hassan et al., 2003). Besides that, anthracyclines have been shown to induce a prothrombotic state by increasing thrombin synthesis and decreasing the endothelium-based protein C anticoagulant pathway (Woodley-Cook et al., 2006; Swystun et al., 2009).

Although there are several lines of evidence that anthracyclines induce endothelial damage *in vitro*, there is much less data from *in vivo* experiments. So far, doxorubicin has been described to induce moderate damage to endothelial ultrastructure of rat aorta (Yamac et al., 2006). In addition, anthracyclines have also been suggested to impair endothelium-dependent as well as independent vasodilatation of rabbit and rat aorta and to increase expression of both iNOS and eNOS (Murata et al., 2001). Furthermore, pilot clinical data has indirectly supported the idea that endothelial damage might contribute to overall cardiovascular toxicity burden induced by anthracyclines (Chow et al., 2006).

This study was designed to systematically assess whether exposure of rabbits to different cumulative doses of the anthracycline anticancer drug is associated with significant immunohistochemically detectable vascular toxicity.

Materials and methods

Animals

Adult Chinchilla male rabbits of an average initial weight 3.51±0.02 kg were housed under a 12-h light cycle, constant temperature and humidity. The animals had free access to water and a standard laboratory pellet diet. Drug administration and other mini invasive experimental procedures were performed under ketamine anesthesia (50 mg/kg, i.m.). Intravenous pentobarbital overdose was used for the sacrifice of the rabbits. The animals were used in this study according to Guide for the Care and Use of Laboratory Animals as approved and supervised by Ethical Committee of the Faculty of Medicine in Hradec Kralove.

Experimental design

Daunorubicin (3 mg/kg, i.e. 50 mg/m^2) was administered i.v. to rabbits (n=72) once weekly to reach different cumulative doses of the drug: 50, 150, 250, 400 and 500 mg/m^2 (n=12 in each group). The schedule of administration and cumulative doses was selected to cover the period sufficient for the development of severe chronic anthracycline cardiotoxicity (Gersl and Hrdina, 1994; Simunek et al., 2004). Rabbits were sacrificed either 24 hours or 7 days after reaching each particular cumulative dose (n=6 in each subgroup). Control rabbits received either a single bolus of saline and were sacrificed 24 hours thereafter (n=6) or were injected with saline once weekly for 10 weeks and sacrificed 7 days after the last dose (n=6). During the autopsy aortas and right femoral arteries were collected for immunohistochemical analysis.

Immunohistochemistry

For immunohistochemical analysis the aortic arch and right femoral artery were used. The aorta was dissected before the origin of brachiocephalic artery and approximately 1 cm segment of the vessel also containing a part of the adjacent descendent aorta was taken for analysis. The right femoral artery of approximately same length was dissected above the origin of saphena magna artery, and used for further analysis. Segments of vessels were immersed in OCT compound (Leica, Germany), snap frozen in liquid nitrogen, and stored at -80°C. Serial cross-sections (7 μm) were cut on a cryostat (Leica, Germany) and placed on gelatin-coated slides. Sections were air-dried and then slides were fixed for 20 minutes in acetone at -20°C. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in phosphate buffered saline (PBS, 15 minute). After blocking nonspecific binding sites with 10% normal goat serum (Sigma-Aldrich, Germany) in PBS solution (pH 7.4) for 30 min, slides were incubated with primary antibodies for 1 hour at room temperature. After a PBS rinse, the slides were developed with a secondary antibody - goat anti-mouse IgG conjugated to peroxidase-labelled polymer (DAKO EnVision™, USA). Bound antibodies were visualized with diaminobenzidine (DAB substrate-chromogen solution, DAKO, USA) and counterstained with hematoxylin. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Primary antibodies were used as follows: monoclonal mouse anti-rabbit VCAM-1 (Rb1/9, IgG1) and monoclonal mouse anti-rabbit ICAM-1 (Rb2/3, IgG1) diluted 1:50 and 1:20, respectively (generous gift from Dr. MI Cybulsky, University of Toronto, Toronto, Canada), monoclonal mouse anti-human CD31 (PECAM-1, JC/70A, IgG_1, DAKO, USA) ready-to-use, monoclonal mouse anti-nitrotyrosine (Chemicon, USA, clone 2A8.2) diluted 1:100, mouse anti-4-HNE (Oxis International Inc., USA) diluted 1:50 and monoclonal mouse anti-human iNOS diluted 1:200 (clone 54, IgG1, BD Transduction Laboratories, USA). This mouse antibody cross-reacts with rabbit activated endothelial cells (Hoshida et al., 1999).

In all rabbits, 40 slides were cut from both aorta and femoral artery for the immunohistochemical analysis. Five slides for each antibody were used for the staining. The results were compared to outcomes of immunohistochemical analyses of aorta obtained previously from hypercholesterolemic and lipopolysaccharide (LPS)-treated rabbits. Hypercholesterolemic rabbits consumed an atherogenic diet

(0.4% cholesterol, 3% fat, 19% proteins) for three months. The LPS-treated rabbits received 0.6 mg/kg of Lipopolysaccharides from *Escherichia coli* (055:B5, Sigma-Aldrich) and were sacrificed 20 hours thereafter. The hypercholesterolemic rabbits with atherosclerotic plaques and LPS-treated rabbits with induced systemic inflammation visible in all types of vessels are considered to be the positive control with respect to immunohistochemical staining for above-mentioned antibodies (Li et al., 1993; Nachtigal et al., 2004). Photo documentation and image digitizing from the microscope were performed with Olympus AX 70, with a digital firewire camera Pixelink PL-A642 (Vitana Corp. Canada) with image analysis software NIS (Laboratory Imaging, Czech Republic).

Stereological analysis

Stereological methods were used for the estimation of immunohistochemical staining of ICAM-1 endothelial expression in all groups of rabbits. The principle of estimation was similar as described previously (Nachtigal et al., 2004). Briefly, the systematic uniform random sampling and the principle of the point-counting method were used for the estimation. A total number of 40 consecutive serial cross sections were cut into 7 μm thick slices, which gave us 0.280 μm long pieces of the vessel called the reference volume. A systematic uniform random sampling in the reference volume was used. The first section for each immunohistochemical staining was randomly positioned in the reference volume and then each eight section was used, thus five sections for ICAM-1 staining were used for the stereological estimation. More than 100 test points hitting the brown staining in the endothelium per vessel was necessary for an appropriate estimation. The positivity and test points hitting was assessed by two independent observers.

Stereological analysis was performed with PointGrid module of the ELLIPSE software (ViDiTo, Slovakia).

Statistical analysis

All values in the graphs are presented as mean ± SEM of n=6 animals. Statistical significance in the differences between the groups was assessed by ANOVA followed by "Dunnett's Multiple Comparison Test" with the use of the GraphPad Prism (version 5.0). P values of 0.05 or less were considered statistically significant.

Results

Daunorubicin treatment induced premature death in two animals. The first of these animals received the complete cumulative dose of daunorubicin (500 mg/m^2) but died during the scheduled one week follow up, whereas second animal received cumulative dose of 450 mg/m^2. Both animals died 6 days after receiving last daunorubicin dose. Necropsy in both of these animals showed massive pleural and abdominal effusions with both heart ventricles dilated, as well as lung and liver hyperemia. In some of the animals receiving daunorubicin cumulative dose higher than 400 mg/m^2, minor pleural and/or abdominal effusions were also found. No such findings were observed in the control animals. Macroscopic inspection of aorta and a. femoralis and other arteries (e.g., a. carotis) showed no gross pathological changes in comparison to the saline-receiving controls.

Light microscopy examination of the arteries did not reveal any pathological changes in either group.

All antibodies used for the immunohistochemistry were first tested on positive reaction in aortas of LPS-treated and hypercholesterolemic rabbits. In LPS-treated rabbits, the strong endothelial positivity was detected for ICAM-1, VCAM-1 and weaker positivity was visible for iNOS (Figs. 3C, 4C, 7C). However, there was no positive reaction for 4-HNE and nitrotyrosine in these animals. Therefore, we used these antibodies on aortas collected from hypercholesterolemic rabbits with fibrous atherosclerotic lesions located in the aortic arch. Both 4-HNE and nitrotyrosine immunoreactivity were detected inside the atherosclerotic plaque (Figs. 5C, 6C), but not in the endothelium covering the plaque.

The presence of intact endothelial cells in aorta of all animals was detected by PECAM-1 staining (Nachtigal et al., 2005). The immunohistochemical staining of aorta and femoral artery with PECAM-1 antibody revealed strong positivity in luminal endothelial cells in both saline and daunorubicin-treated rabbits (Fig. 2). ICAM-1 staining was detected in many aortas from both saline and daunorubicin-receiving rabbits (Fig. 3). On the other hand, some aortas from other animals treated by either different cumulative doses of daunorubicin or saline showed no immunoreactivity. Moreover, stereological analysis of ICAM-1 endothelial expression showed no changes in ICAM-1 expression in daunorubicin treated rabbits when compared with control rabbits (Fig. 1). Hence, no direct association between ICAM-1 expression and daunorubicin treatment could be

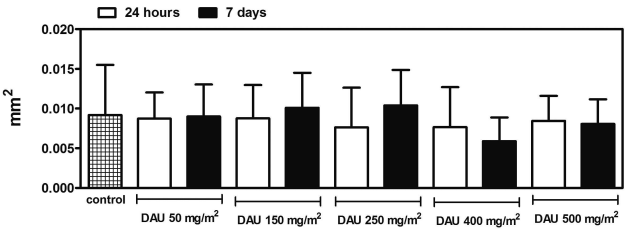

Fig. 1. Stereological analysis of ICAM-1 staining in endothelium in all groups of rabbits. The cumulative dose of daunorubicin in each group (n=6) and time from last dose to the animal sacrifice (24 hours and 7 days) are indicated. Stereological analysis showed no significant changes in ICAM-1 endothelial expression in daunorubicin treated rabbits when compared to control rabbits.

identified. Very weak or no ICAM-1 expression was detected in femoral arteries collected from the control group and the same results were found in arteries of daunorubicin-treated animals receiving either cumulative dose (data not shown). On the contrary, no VCAM-1 staining was detectable in any animal from control or daunorubicin-treated groups either in aorta or femoral artery (Fig. 4).

The immunohistochemical staining with 4-HNE antibody showed negative results in both aorta and femoral artery in all saline and in most daunorubicin-treated animals (Fig. 5). The only clearly positive finding was witnessed in the aorta of one rabbit which received the highest cumulative dose of daunorubicin - 500 mg/m^2 (data not shown). In addition, no distinct expression of iNOS and nitrotyrosine was detected in any aorta or femoral artery from either control or daunorubicin-treated groups (Figs. 6, 7). Thus, no association between 4-HNE, nitrotyrosine and iNOS expression and daunorubicin treatment could be recognized.

Discussion

Several lines of evidence have pointed to the possibility that overall cardiovascular toxicity of anthracycline anticancer drugs might be at least partially co-determined by the toxic damage to the vessels and to

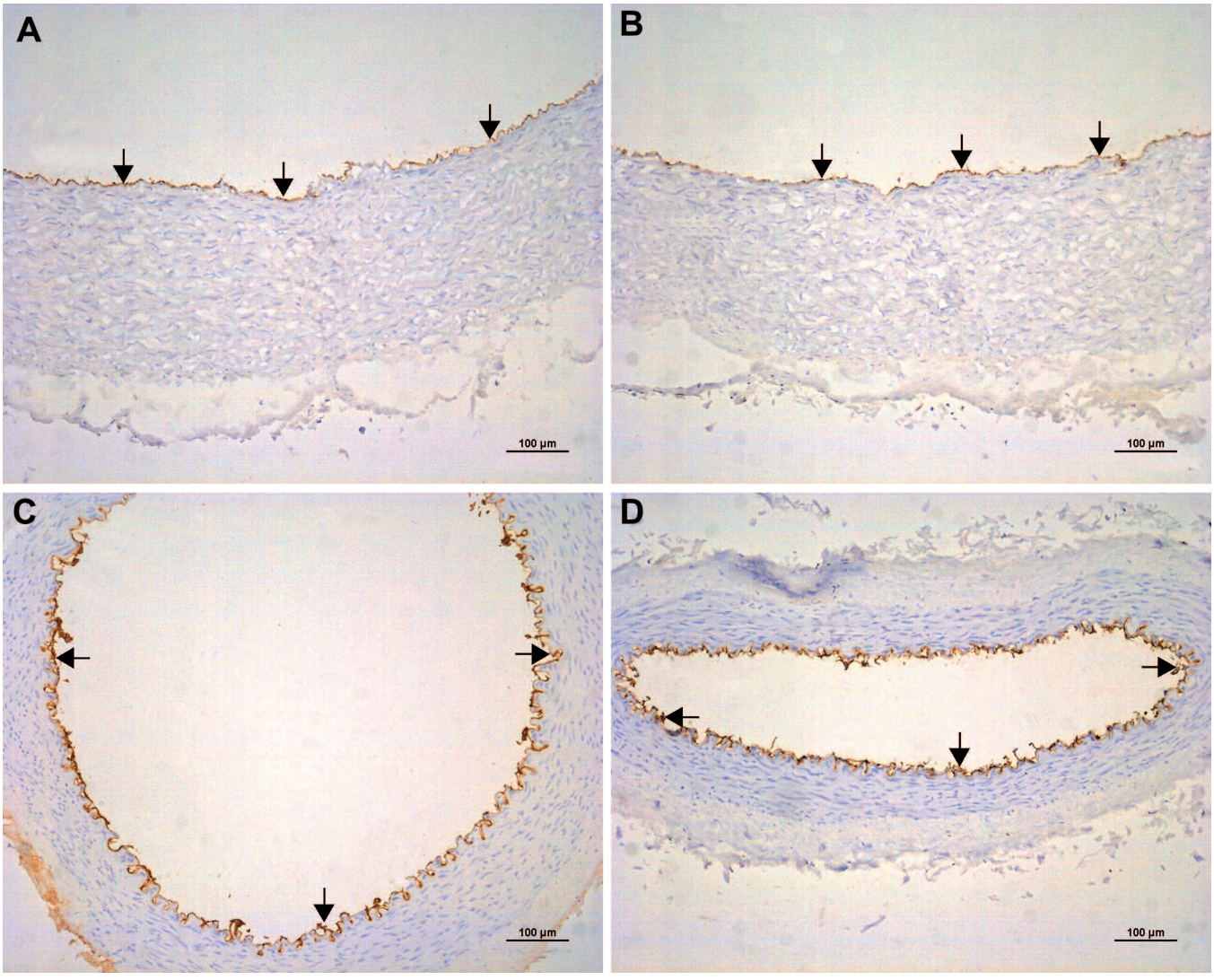

Fig. 2. Representative sections of PECAM-1 staining in aorta and femoral artery from both control **(A, C)** and daunorubicin (cumulative dose 500 mg/m^2) treated rabbits **(B, D)**. PECAM-1 staining was detected in endothelial cells in all rabbits, both in the aorta and femoral artery (arrows) regardless of daunorubicin treatment, suggesting the presence of intact endothelium in these vessels. The slides were counterstained with hematoxyline. Scale bar: 100 μm.

the endothelium in particular. The endothelium is well recognized to be a key player responsible for the maintenance of the very delicate balance of vessel homeostasis. It mediates several physiologically essential effects in the vasculatures, such as vasodilatatory, antiproliferative, and antithrombotic effects (Mitchell et al., 2008). Therefore, it is no surprise that impaired endothelial function and/or full blown endothelial dysfunction are early features of considerably important vascular diseases such as atherosclerosis (Ross, 1999).

Although endothelial dysfunction should be understood as a complex process, there are several points that create a cornerstone of this phenomenon - impaired NO availability (decreased NO production and/or NO inactivation), production of proinflammatory cell adhesion molecules (Cybulsky et al., 2001), and increased oxidative (Higashi et al., 2009) and nitrosative stress (Souza et al., 2008). Interestingly, the toxicological profile of anthracyclines creates a very good rationale for suspecting that these drugs are able to induce endothelial dysfunction. First of all, anthracyclines are administered intravenously - thus endothelial cells are among the first cells within the body to be exposed to these drugs. In addition, before drug distribution takes place, the endothelium has to face very high concentrations of anthracyclines. Furthermore, anthracyclines have been well described to be able to

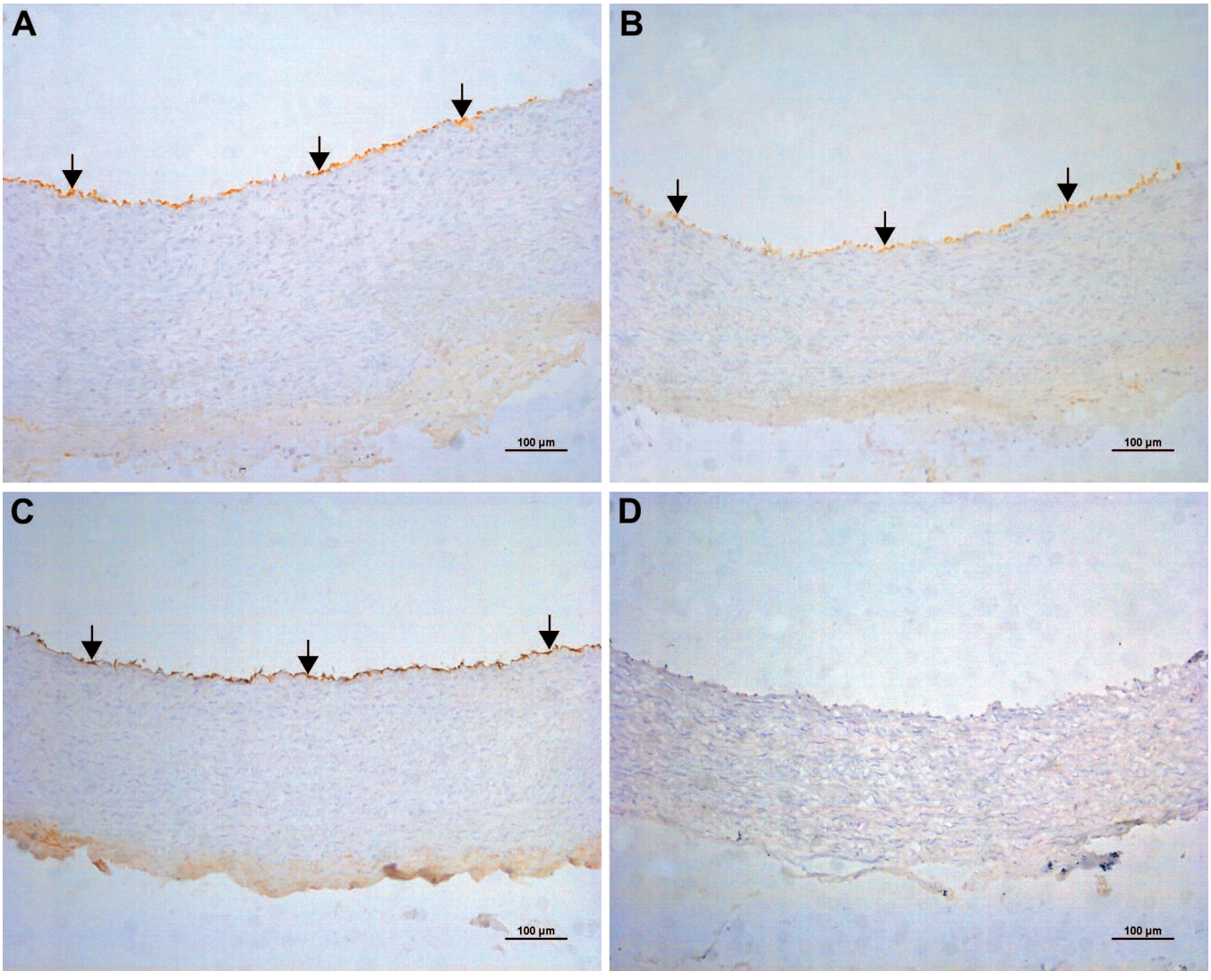

Fig. 3. Representative sections of ICAM-1 endothelial staining in rabbit aorta. ICAM-1 staining (arrows) was detected in some animals from the control group **(A)** and the same was true for animals receiving different cumulative doses of DAU **(B)** and from LPS-treated rabbits **(C)**. In general, ICAM-1 staining intensity was not affected by daunorubicin treatment. The slides were counterstained with hematoxyline. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins **(D)**. Scale bar: 100 μm.

induce significant oxidative and nitrosative stress to different kinds of cells (Chen et al., 2007). Moreover, they have been shown to affect NO synthesis and availability and trigger an inflammatory response (Wakabayashi and Groschner, 2003).

Bearing all this information in mind, the importance of careful assessment of the potential toxic effects on endothelium seems more than justified. For this purpose, we have utilized our well-accepted model of chronic daunorubicin-induced cardiotoxicity in rabbits. In this model, we have previously described all the functional, biochemical as well as morphological features of heart damage (Gersl et al., 1995; Simunek et al., 2004). Indeed, using this particular schedule of daunorubicin administration, we have reported a progressive decrease of the left ventricular contractility (commencing the cumulative dose at 350 mg/m^2 and culminating at 500 mg/m^2) (Sterba et al., 2006; Popelova et al., 2009). Furthermore, elevations of plasma biomarkers of myocardial injury strongly suggest that significant toxicity already develops from the cumulative dose of 250 mg/m^2 (Adamcova et al., 2007; Sterba et al., 2007). Examination of myocardium from rabbits receiving a complete cumulative dose (500 mg/m^2) has traditionally shown increased lipoperoxidation along with very dramatic histopathological changes (Popelova et al., 2008, 2009). Hence, daunorubicin administration in this schedule to rabbits induces a very significant myocardial

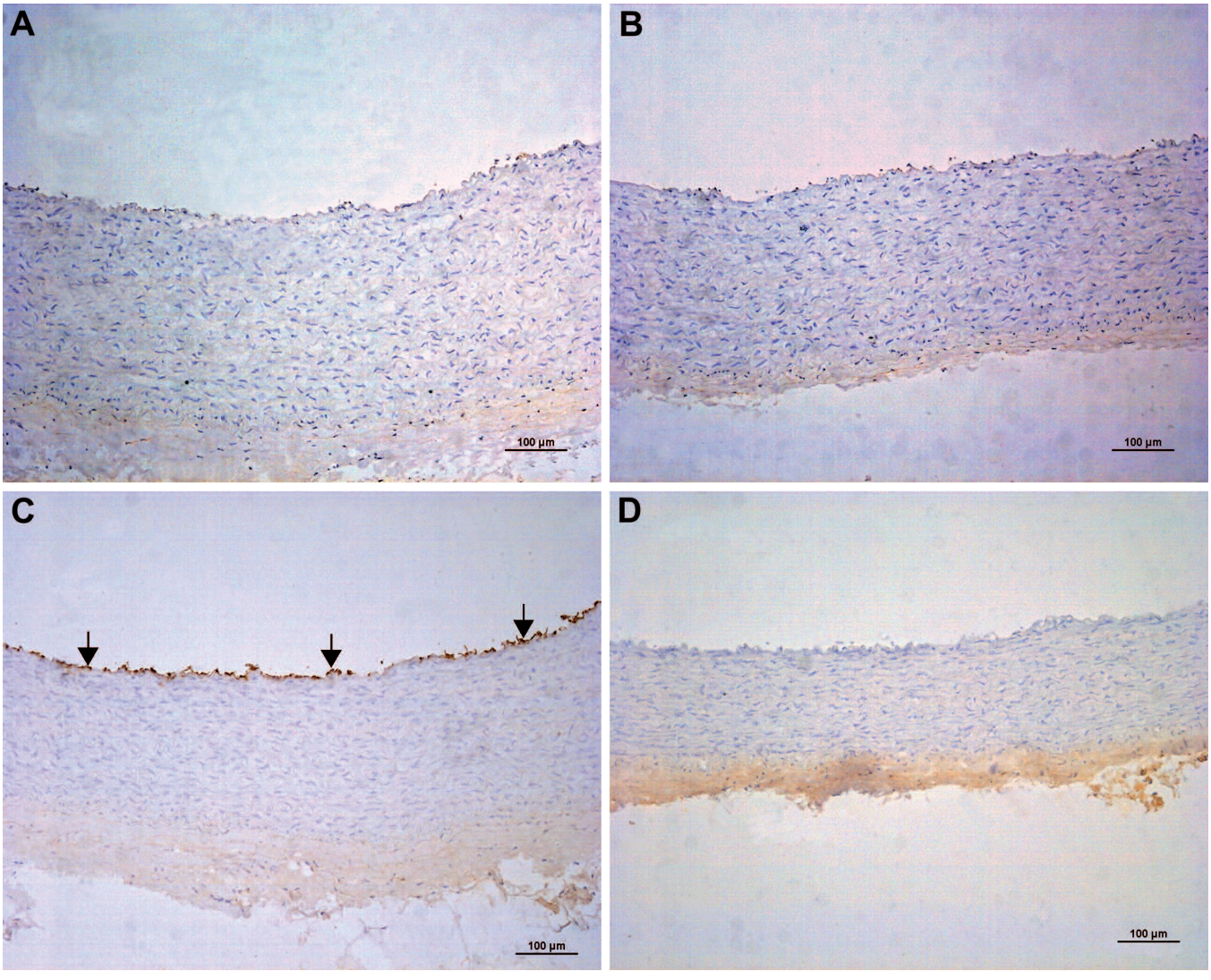

Fig. 4. Representative sections of VCAM-1 staining in rabbits. VCAM-1 expression was not detected in any aorta from either control **(A)** or daunorubicin-treated **(B)** rabbits. On the other hand, strong immunoreactivity for VCAM-1 (arrows) in aortic endothelium was detected in LPS treated rabbits **(C)**. The slides were counterstained with hematoxyline. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins **(D)**. Scale bar: 100 μm.

perturbation, the severity of which is determined by the cumulative dose of the drug. The autopsy and necropsy data from the present study seem to be well in line with these findings. However, in contrast to myocardium, the daunorubicin-induced toxic damage to vessels and arteries in particular, remained unclear.

In this study, we have harvested aortas and femoral arteries from rabbits injected with daunorubicin once weekly to reach a different cumulative dose. We focused on the aortic arch and adjacent part of the descendent aorta, where the most pronounced atherosclerotic changes have been previously described when compared to other vessels (Nachtigal et al., 2002).Thus, we believed that this is the most susceptible place to detect early changes in endothelium. In addition, we have collected femoral arteries, as an example of histologically different muscular arteries. Thus, this study covered the period of subclinical (50-400 mg/m^2) as well as clinically manifest cardiotoxicity (400-500 mg/m^2) (Simunek et al., 2004; Popelova et al., 2008). Moreover, the present study has been designed to recognize both possibilities – the development of potential changes in the arteries, which might have a cumulative character, or potentially decreased response on repeated exposure, which might be due to some adaptation changes. We have also deliberately selected two different time intervals after the last drug administration (24 hours and 7 days) for collection of the

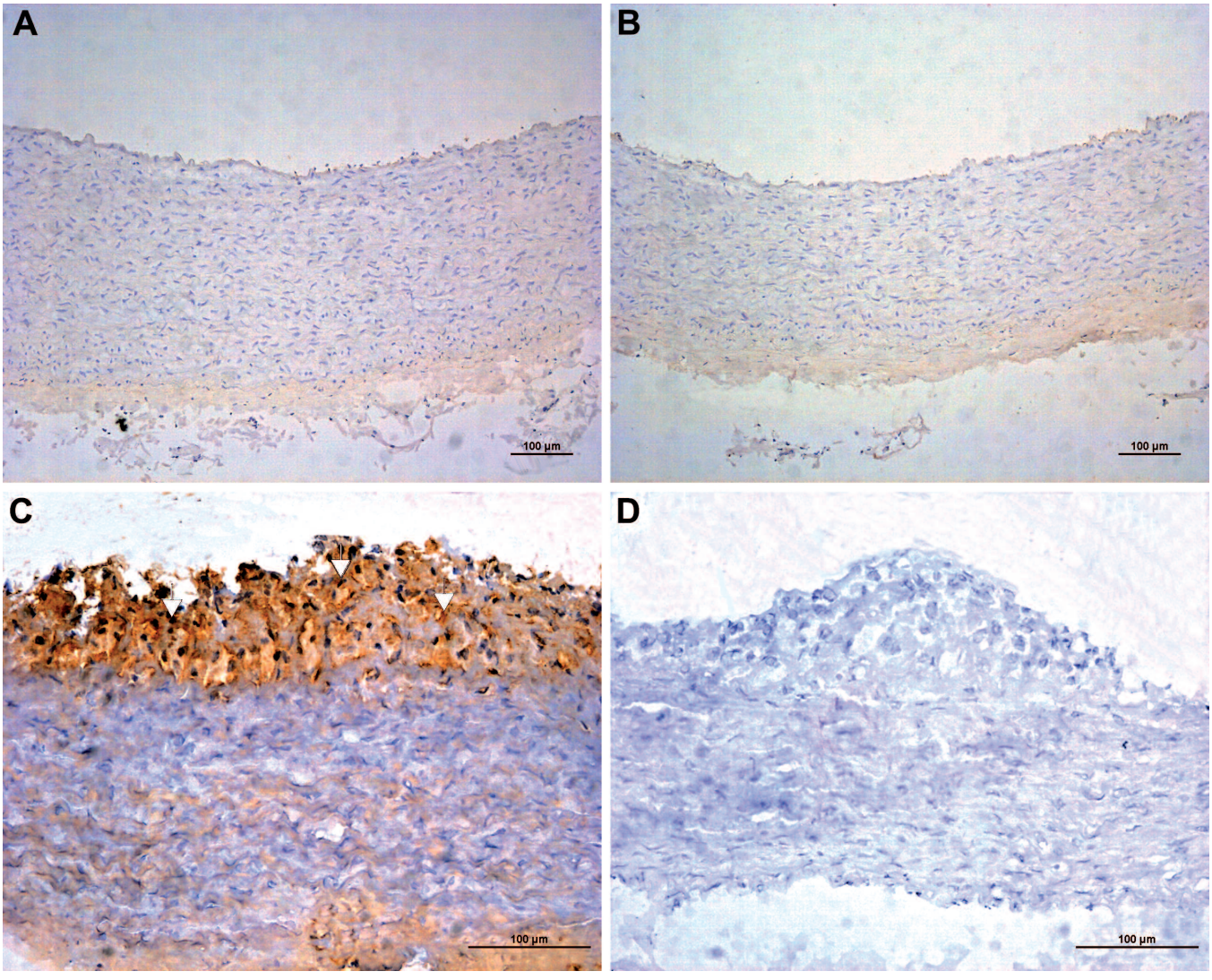

Fig. 5. Representative sections of 4-HNE immunostaining in rabbit aortas. 4-HNE immunoreactivity was not detected in any aorta from either control group **(A)** or daunorubicin-treated groups **(B)**. On the other hand, strong immunoreactivity for 4-HNE was detected inside atherosclerotic lesion (arrows) in hypercholesterolemic rabbits **(C)**. The slides were counterstained with hematoxyline. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins **(D)**. Scale bar: 100 μm.

arteries to detect possible acute changes, which might be normalized within few days post-dose, as well as those potential changes showing a cumulative character.

Taking all the data from the present experiment together we strongly suggest that daunorubicin does not induce significant oxidative and nitrosative stress within the arteries. Furthermore, the total cumulative dose of the drug or timing of arteries collection post-dose showed no impact on the data obtained. This finding might be considered surprising as anthracyclines are known to be able to produce reactive species formation using at least two independent pathways: quinone-semiquinone cycling of an anthracycline aglycone and redox cycling of anthracycline-iron complexes (Keizer et al., 1990; Simunek et al., 2009). Hydroxyl and superoxide radicals produced may interact with lipids and cause lipoperoxidation. Therefore, in the present study we have evaluated 4-hydroxy-2-nonenal (4-HNE) as a major end-product of peroxidation of membrane n-6-polyunsaturated fatty acids and also one of the most frequent markers of oxidative stress (Poli et al., 2008). However, unlike in the case of aorta obtained from hypercholesterolemic rabbits, daunorubicin treatment showed no impact on this marker. Moreover, using both immunohistochemistry and selective HPLC technique we have previously clearly shown significantly increased lipoperoxidation in the myocardium of rabbits undergoing the same treatment. Hence, it is evident that both arteries analysed in this study showed a different response to drug administration than rabbit myocardium

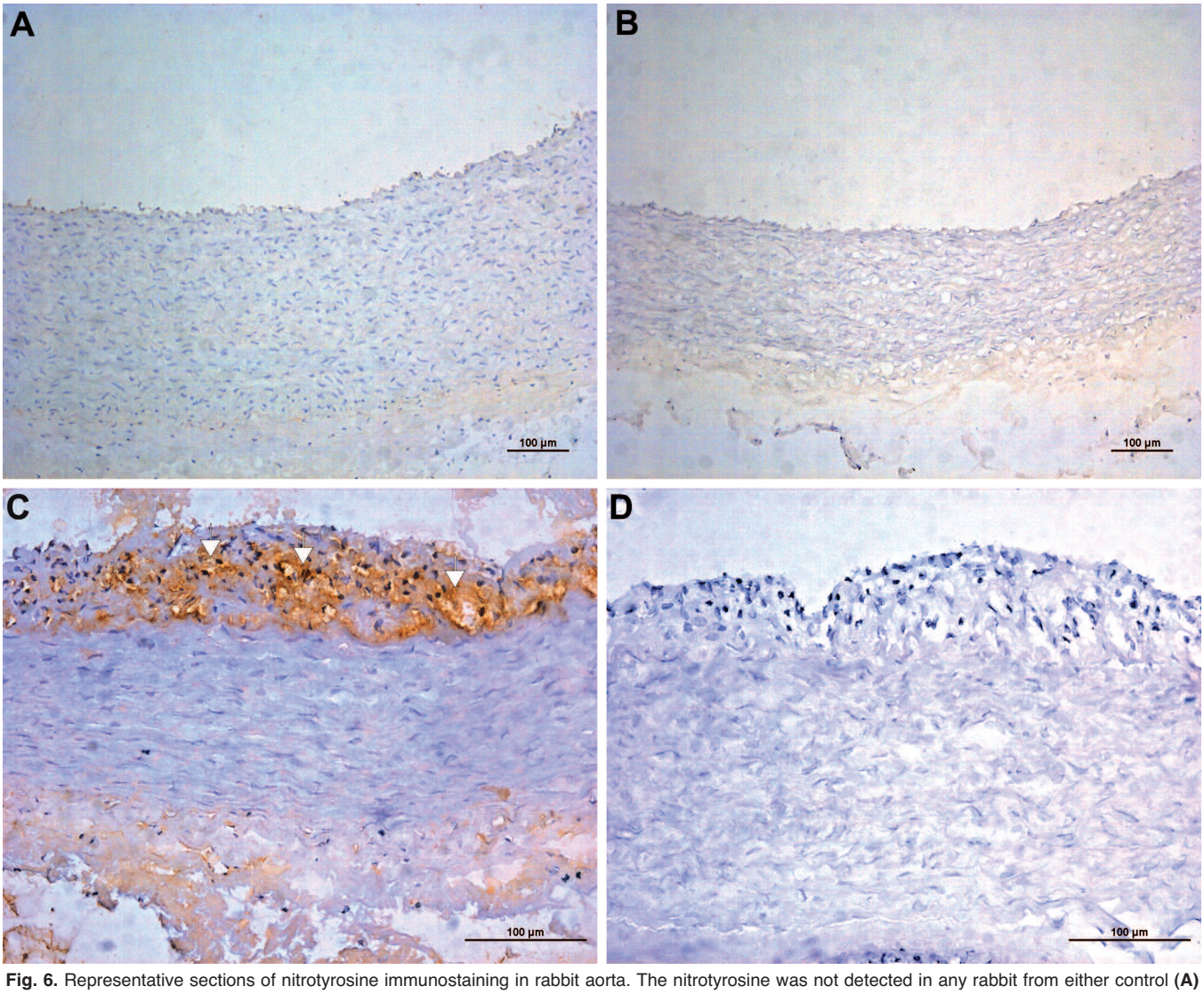

Fig. 6. Representative sections of nitrotyrosine immunostaining in rabbit aorta. The nitrotyrosine was not detected in any rabbit from either control **(A)** or daunorubicin-treated **(B)** groups. On the other hand, strong immunoreactivity for nitrotyrosine was detected inside atherosclerotic lesion (arrows) in hypercholesterolemic rabbits **(C)**. The slides were counterstained with hematoxyline. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins **(D)**. Scale bar: 100 μm.

(Popelova et al., 2009).

In addition, when excessive superoxide radicals are present in the cell with a high NO content, both molecules may rapidly interact yielding a powerful oxidant and nitrating agent - peroxynitrite ($ONOO^-$). Peroxynitrite-derived $\cdot OH$ and $\cdot NO_2$ promote the nitration of tyrosine residues (Halliwell, 1997). The other proximal nitrating agents involve hemoperoxidases in the presence of hydrogen peroxide (H_2O_2) and nitrite (NO_2^-) (Souza et al., 2008). Nitration of free and protein-associated tyrosine produces 3-nitrotyrosine (3-NT), which is considered to be a marker of nitrosative stress. However, unlike in the LPS-treated aortas, we have detected no increase in 3-NT immunoreactivity in either arteries after the daunorubicin treatment. These outcomes are different to the effect of the single high dose of anthracycline on mice myocardium as has been reported by others (Zanon et al., 1980). This latter discrepancy might be explained by much higher complexity and clinical relevance (repeated administration of clinically relevant dose of daunorubicin) of the present *in vivo* experiment.

The data from the literature have suggested that anthracyclines might cause a significant induction of the inflammation process associated with expression of cell adhesion molecules. Anthracyclines have been shown previously to induce VCAM-1 and P-selectin expression *in vitro* on human vascular endothelial cells. Furthermore, they have been suggested to induce neutrophil adhesion of vascular endothelial cells, and

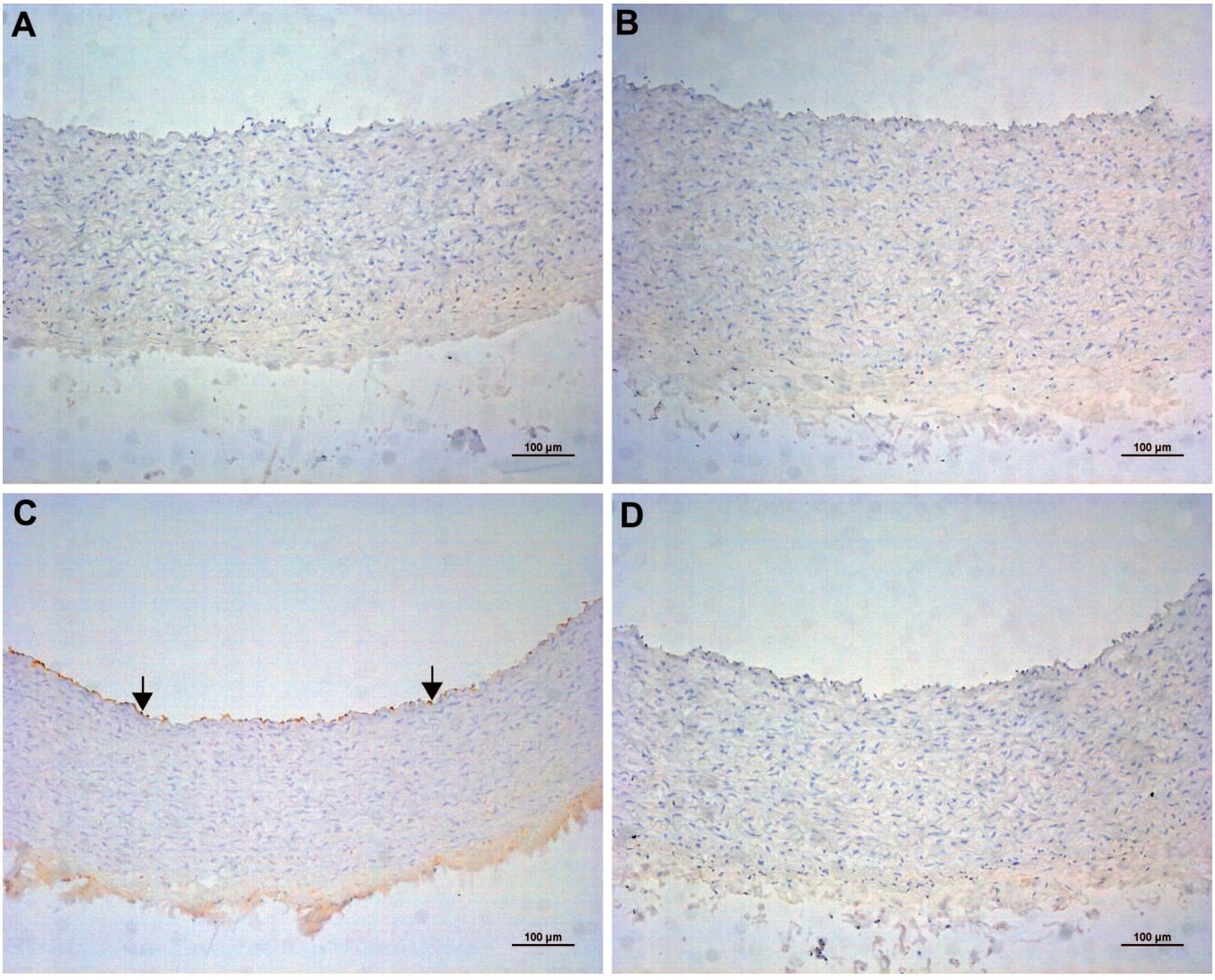

Fig. 7. Representative sections of iNOS staining in rabbit aorta. iNOS expression was not detected in any aorta from either control **(A)** or any daunorubicin-treated **(B)** rabbits. However, weak positivity (arrows) was detected in LPS treated rabbit **(C)**. The slides were counterstained with hematoxyline. The specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins **(D)**. Scale bar: 100 *µ*m.

reduce the viability of resting endothelial cells (Abou El Hassan et al., 2003). Members of the immunoglobulin superfamily of endothelial adhesion molecules like vascular cell adhesion molecule (VCAM-1) and intercellular cell adhesion molecule (ICAM-1), strongly participate in leukocyte adhesion to the endothelium during the inflammatory process. These inflammatory molecules are considered as markers of endothelial dysfunction and the beginning of the atherosclerotic process (Nachtigal et al., 2006). They are markedly expressed by aortic endothelium in regions predisposed to atherosclerosis (Cybulsky et al., 2001; Ley and Huo, 2001). However, in this study, immunohistochemical analysis showed no expression of VCAM-1 in any rabbit from the experiment. On the contrary, ICAM-1 expression was detectable in some animals irrespective of the treatment. Stereological analysis, however showed no significant differences in ICAM-1 expression due to the treatment. These results are in line with the fact that VCAM-1 was suggested to be a more specific marker of endothelial dysfunction because it is not expressed by aortic endothelium in healthy rabbits (Li et al., 1993) when compared with ICAM-1 (Iiyama et al., 1999). These data strongly suggest that administration of daunorubicin does not induce inflammation in atherosclerosis prone aortic and femoral artery endothelium. This is in line with the absence of inflammatory infiltration on hematoxylin-stained light microscopic preparations, though others have described an inflammatory reaction in veins (Hecker, 1990). Moreover, our data are not in line with results of the above-noted *in vitro* experiments (Abou El Hassan et al., 2003). Again, at this point we should note that our experiments employed appropriate doses and routes of administration of the anthracycline and therefore these conditions are certainly clinically relevant. However, this is not certain in the cell culture experiments, where many reactions are known to be strongly dependent on concentration, time and conditions of incubation and cell-type. Furthermore, expression of cell adhesion molecules might be differentially regulated under artificial *in vitro* conditions, where endothelial cells are not in contact with other vessel wall cells, blood cell and other factors, in the vessel wall or blood.

Many physiological functions of the healthy vessel are dependent on NO availability. The endothelium provides a constitutive supply of NO which is catalyzed by constitutively expressed eNOS. Under certain pathological conditions excessive amounts of NO can be produced by the catalytic activity of the inducible isoform of nitric oxide synthase (iNOS). High levels of NO produced from iNOS in endothelial cells and macrophages can cause endothelial injury (Esaki et al., 1997; Boyle, 2005). In addition, when superoxide radicals are present, toxic peroxynitrite ($ONOO^-$) might be formed. Surprisingly, epirubicin, another anthracycline derivative, has been shown to inhibit the induction of iNOS *in vitro* and *in vivo* in rats (Sakai et al., 1996). On the contrary, very recent data described increased iNOS expression in myocardium (Mukhopadhyay et al., 2009) after anthracycline treatment. Furthermore, *in vivo* data from rat experiments have suggested that both iNOS and eNOS expression in aorta might be induced due to anthracycline treatment (Olukman et al., 2009). However, in the present study we have failed to detect any induction of iNOS expression in arteries of daunorubicin-treated animals irrespective of the cumulative dose of the drug and time of harvesting. Interestingly, even the arteries of rabbits dying prematurely due to end-stage cardiotoxicity with marked dilation of both heart ventricles accompanied with massive pleural effusion and ascites were free of markedly increased iNOS expression. One may consider interspecies differences, but more relevant appears the factor of dose. In this study, a single, high dose of doxorubicin (20 mg/kg) was employed (Olukman et al., 2009). The clinical relevance of acute intoxication with single supratherapeutic dose is unsure as acute anthracycline cardiotoxicity is only very rarely a clinical issue and indeed, the pathogenetic mechanisms may differ considerable to those taking place in the chronic anthracycline cardiotoxicity (Yi et al., 2006). Others (Luo and Vincent, 1994) have suggested that rather than induction of endothelial NO synthase (eNOS), a reduction of its endothelial content and activity might be associated with anthracycline treatment. However, in line with our data other authors have also reported no effect of anthracyclines on eNOS levels in rat aortic strips or aorta (den Hartog et al., 2003).

Hence, all our data strongly suggest that at clinically relevant conditions daunorubicin treatment is not associated with immunohistochemically detectable endothelial dysfunction. These outcomes do not support the conclusion of the pilot clinical study (Chow et al., 2006). In this study, anthracycline treatment was shown to induce significant changes on the brachial artery reactivity test as detected using echocardiography. Although there was a significant variability in the data obtained, the difference in brachial reactivity was statistically significant. First, for practical reasons this study was dealing with the brachial artery and besides functional testing no other data was available. While there is no known reason why the brachial artery should react in a completely different manner than e.g., similar femoral artery, the relationship between the morphological approach and the above-discussed functional test is clearly much more complex. Interestingly, anthracycline containing chemotherapy has been previously shown to impair endothelial-dependent vasodilatatory response to acetylcholine or adenosine in a rabbit model of doxorubicin-induced cardiomyopathy (Kaye et al., 1994) and *in vitro* on organ culture system, in rabbit mesenteric arteries (Murata et al., 2001).

It should be noted that most of the studies, which were focused on the possibility of toxicity of anthracyclines to endothelial cells and vessels have used another anthracycline derivative - doxorubicin. Our model utilizes daunorubicin as this model anthracycline shows less extracardiac toxicity, which mainly concerns

reduced hematotoxicity and nephrotoxicity, and this makes the model more reproducible and reliable (Klimtova et al., 2002). Although we cannot rule out a different reaction of endothelium to different anthracycline derivatives administered, it should be noted that at least ROS production and cardiotoxicity are now well recognized as a class effects common for all anthracyclines introduced into clinical practice so far (Minotti et al., 2004). In addition, the data in the literature was also obtained with anthracyclines other than doxorubicin. Even the clinical study discussed above included 29% of patients treated with daunorubicin, but no marked differences between the anthracycline derivatives have been reported so far.

Thus, the present complex study shows that daunorubicin administration does not induce immunohistochemically detectable vascular toxicity and endothelial dysfunction at least in rabbits. This suggests that endothelium is much more resistant to anthracycline toxicity than cardiac myocytes. Unlike endothelial cells, cardiomyocytes have been repeatedly shown to be markedly affected by anthracycline-induced oxidative and nitrosative stress and undergo profound degeneration and both apoptotic and non-apoptotic cell death (Billingham et al., 1978; Popelova et al., 2009). In this context it is interesting that even early histopathological reports have indicated that unlike cardiomyocytes, myocardial endothelial cells are poorly affected by anthracycline toxicity (Billingham et al., 1978). This might be associated with a completely different phenotype of endothelial cells and highly specialized post-mitotic cardiomyocytes. Indeed, one always has to consider the biological difference between animals and human beings and therefore such data should be interpreted carefully. On the other hand, larger prospective clinical studies with long-term follow up including other than those indirect functional tests deserve to be performed to completely clarify this point. Moreover, necropsy data from patients who have undergone anthracycline treatment might further contribute to our understanding of this issue.

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