Physiol. Res. 64 (Suppl. 5): S653-S660, 2015

Different Doxorubicin Formulations Affect Plasma 4-Hydroxy-2-Nonenal and Gene Expression of Aldehyde Dehydrogenase 3A1 and Thioredoxin Reductase 2 in Rat

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Received March 27, 2015
Accepted July 22, 2015
On-line December 15, 2015

Summary

Increased oxidative stress is indisputably an important mechanism of doxorubicin side effects, especially its cardiotoxicity. To prevent impairment of non-tumorous tissue and to improve the specificity in targeting the tumor tissue, new drug nanotransporters are developed. In many cases preclinical therapeutic advantage has been shown when compared with the administration of conventional drug solution. Three forms of doxorubicin – conventional (DOX), encapsulated in liposomes (lipoDOX) and in apoferritin (apoDOX) were applied to Wistar rats. After 24 h exposition, the plasma level of 4-hydroxy-2-nonenal (4-HNE) as a marker of lipoperoxidation and tissue gene expression of thioredoxin reductase 2 (*TXNRD2*) and aldehyde dehydrogenase 3A1 (*ALDH3A1*) as an important part of antioxidative system were determined. Only conventional DOX significantly increases the level of 4-HNE; encapsulated forms on the other hand show significant decrease in plasma levels of 4-HNE in comparison with DOX. They also cause significant decrease in gene expression of *ALDH3A1* and *TXNRD2* in liver as a main detoxification organ, and a mild influence on the expression of these enzymes in left heart ventricle as a potential target of toxicity. Thus, 4-HNE seems to be a good potential biomarker of oxidative stress induced by various forms of doxorubicin.

Key words

Doxorubicin • Drug nanotransporters • Oxidative stress • 4-hydroxy-2-nonenal • Thioredoxin reductase 2 • Aldehyde dehydrogenase 3A1

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Introduction

Anthracycline doxorubicin (DOX) is one of the most frequently used antitumor drugs, with high activity against an extensive variety of malignancies, both hematological and solid tumors – gynecological, urogenital, endocrine, stomach cancer, as well as Ewing and Kaposi's sarcomas (reviewed by Carvalho *et al.* 2009).

Numerous side effects which limit the wider application of doxorubicin were reported, including nausea, vomiting and fever (reviewed by Carvalho *et al.* 2014). Moreover, significant incidence of acute and chronic cardiovascular side effects that may even endanger the patient's life were also observed (Singal and Iliskovic 1998). In general, acute DOX side effects are reversible and well treatable. Nevertheless, when the lifetime cumulative DOX dose is nearing 500 mg/m^2 and above this value, iatrogenic life-threatening cardiomyopathy becomes more probable; it can even develop into dilated cardiomyopathy and eventually to congestive heart failure (Chatterjee *et al.* 2010, Shi *et al.* 2011).

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However, despite a significant research effort, the mechanism of DOX chronic cardiotoxicity is not fully clarified even after years of its clinical application. Suggested pathways responsible for its cardiotoxicity include inactivation of key enzymes, suppression of DNA, RNA and protein synthesis, abnormalities in Ca^{2+} handling, and above all oxygen free radical formation and iron oxidation (Sawyer *et al.* 2010). High level of oxidative stress generated by DOX treatment is related with its molecular structure, which contains quinone moiety able to undergo a redox cycling producing superoxide anion radical $O_2{\cdot}^-$ (Millic Torres and Dragojevic Simic 2012). In a presence of metal ions, especially iron, highly toxic hydroxyl radical (OH·) is generated from $O_2{\cdot}^-$. OH· is extremely toxic and can't be detoxified by any antioxidative enzyme. It reacts besides other substrates willingly with polyunsaturated fatty acids of lipid membranes and impairs them by lipid peroxidation. This chain of reactions leads to a high level of secondary peroxidation products, such as acrolein, malondialdehyde, 4-hydroxy-2-nonenal (4-HNE) and many other aldehydes. 4-HNE is considered to be highly reactive and cytotoxic with potential to damage all major types of macromolecules. There is a variety of defensive mechanisms for antioxidant protection in the cells, for example superoxide dismutase for converting $O_2{\cdot}^-$ to less harmful hydrogen peroxide. Among the other defense mechanisms, an important role is seen in the aldehyde dehydrogenase (ALDH), which metabolizes toxic aldehydes originating from lipid peroxidation. Last but not least there are also thiol-dependent systems. These are critical for cellular redox environment control by maintaining dithiol/disulfide balance of numerous proteins involved in essential cell functions (Lu and Holmgren 2014). Thioredoxin belongs to these systems and thioredoxin reductase (TXNRD) plays an important role in cellular defensive system.

Cancer chemotherapy is generally limited by a lack of specificity in the targeting to tumor tissue. Accordingly, reduction of the side effects on non-tumorous tissue is one of the major challenges in oncology. One of the approaches to overcome such limitations is encapsulation of conventional drugs. Encapsulation of DOX into the nanomaterial carrier decreases the toxic effect caused by this cytostatic drug and protects healthy tissues. It also improves tumor targeting *via* the enhanced permeation and retention effect which has been demonstrated for a range of nanosized drug delivery systems. Besides liposomal nanocarriers, which were recently introduced into clinical practice, transporters based on protein (apo)ferritin (Todd *et al.* 2013) and/or carriers utilizing carbon nanomaterials, such as fullerenes or carbon nanotubes (Wong *et al.* 2013) are intensively studied.

The aim of the study was to verify whether 4-HNE is good potential biomarker of oxidative stress and apply it for comparison of the effects of conventional doxorubicin and its two encapsulated forms (liposomal DOX anSd DOX in apoferritin transporter) on the level of oxidative stress in rat, using 4-HNE as a marker of lipoperoxidation and two selected lipoperoxidation-related genes – aldehyde dehydrogenase 3A1 (*ALDH3A1*) and thioredoxin reductase 2 (*TXNRD2*).

Materials and Methods

Materials

All chemicals used in biochemical analyses were of the p.a. or HPLC grade, purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard of 4-HNE was purchased from Cayman Chemical (Ann Arbor, MI, USA). Kits used for RNA isolation and PCR reaction were manufactured by Roche (Basil, Switzerland).

All doxorubicin compounds were kindly provided by Laboratory of Metallomics and Nanotechnologies, Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University, Brno, Czech Republic. The applied forms of doxorubicin were as follows: conventional doxorubicin – DOX (Teva 2 mg/ml powder for infusion solution), doxorubicin encapsulated in apoferritin transporter – apoDOX (characterized in Gumulec *et al.* 2014), and liposomal doxorubicin – lipoDOX (DOX encapsulated in liposome 10, characterized in Kominkova *et al.* 2014).

Experimental animals

All experiments were carried out according to the recommendations of the European Community Guide for the Care and Use of Laboratory Animals and according to the experimental protocol approved by the local Committee on the Protection of Animals, Masaryk University and by the Committee of Ministry of Agriculture of the Czech Republic. Animals were housed in the Animal Breeding and Experimental Facility, Faculty of Medicine, Masaryk University, Brno, Czech Republic in temperature, pressure and humidity controlled environment, with light cycle 12/12 light/dark.

In the study, 30 male Wistar rats (body mass

302.2±9.3 g) were used. Animals were divided into four groups: group A, n=8, 306.4±17.2 g; group B, n=7, 302.9±26.1 g; group C, n=8, 293.4±18.4 g; and group D, n=7, 306.7±15.0 g. Three days before drug exposure, animals were housed individually in glass metabolic cages (Simax, Czech Republic) for adaptation. After adaptation period, DOX (5 mg/kg), apoDOX (equal to 5 mg/kg of DOX), and lipoDOX (equal to 5 mg/kg of DOX), were applied by single intraperitoneal injection in light inhalation anesthesia (group B, C, and D, respectively). To group A, which served as a negative control, vehiculum – aqua pro injectione (2.78 ml/kg) was applied.

Twenty four hours after application, animals were deeply anesthetized (Ketamine 100 mg/kg i.p. and Xylazine 10 mg/kg i.p.), thoracotomy was performed, blood samples were collected into the syringe with EDTA by direct intracardial puncture and the heart was excised. Samples from heart, liver, kidney and testes were promptly obtained, immediately frozen in liquid nitrogen and stored at −80 °C for further analyses.

Biochemical analysis

The level of 4-HNE was measured by modified method according to Kinter (1996). Plasma samples were obtained from blood with EDTA and stored at −80 °C until assayed. Preparation of sample included the addition of butylated hydroxytoluene to avoid artificial oxidation and deproteination by perchloric acid with subsequent centrifugation. Then 2,4-dinitrophenylhydrazine as a derivatization reagent was mixed with aliquot of supernatant and incubated for 1 h in dark place and then the product was extracted to hexane. Sample was evaporated to dryness under nitrogen and redissolved in 60 % acetonitrile. The amount of the product was measured by HPLC with UV detection. Conditions applied for measurement were as follows: column Kinetex C-18 (100 mm × 4.6 mm, 5 μm), acetonitrile mobile phase (55:45, v/v), isocratic conditions with flow rate 0.5 ml/min, UV detector (LCD 2084.2, ECOM, CZ) set at 355 nm and software for analysis Clarity^{TM} (DataApex, CZ). The plasma 4-HNE concentration was calculated according to the standard curve.

RNA isolation and reverse transcription

High pure total RNA isolation kit was used for isolation. The medium was removed and the samples were twice washed with 5 ml of ice-cold PBS. The cells were scraped off, transferred to clean tubes and centrifuged at 20,800 × g for 5 min at 4 °C. After this step, a lysis buffer was added and RNA isolation was carried out according to manufacturer's instructions. The isolated RNA was used for cDNA synthesis. RNA (600 ng) was transcribed using transcriptor first strand cDNA synthesis kit, which was applied according to manufacturer's instructions. The cDNA (20 μl) prepared from the total RNA was diluted with RNase free water to 100 μl and the amount of 5 μl was directly analyzed by using the Lightcycler 480 II RT-PCR system (Roche, Basel, Switzerland).

Quantitative real-time polymerase chain reaction

q-PCR was performed in triplicate using the TaqMan gene expression assay system with the Lightcycler 480 II RT-PCR system; the amplified DNA was analyzed by the comparative Ct method using β-actin (*ACTB*) as an endogenous control for *ALDH3A1* and *TXNRD2* gene expression quantification. The primer and probe sets for ACTB (assay ID: Rn00667869_m1), *ALDH3A1* (Rn00694669_m1), and *TXNRD2* (Rn00574868_m1), were selected from TaqMan gene expression assays (Life Technologies, Waltham, MA, USA). q-PCR was performed under the following amplification conditions: total volume of 20 μl, initial incubation at 50 °C/2 min, followed by denaturation at 95 °C/10 min, then 45 cycles at 95 °C/15 s and at 60 °C/1 min.

Statistical analysis

One-way ANOVA followed by planned comparisons (contrast analysis) was used to reveal dependencies between variables. Data were tested for normality and log-normal data were transformed accordingly. Unless noted otherwise, P level <0.05 was considered significant. Software Statistica 12 (StatSoft, Tulsa, OK, USA) was used for analysis.

Results

Effect of DOX forms on plasma level of 4-HNE

First, the effect of doxorubicin modification on the plasma level of 4-HNE was analyzed using univariate analysis. There was a significant effect on the plasma level of this metabolite, $F(3,26)=34.85$, $p<0.001$. To reveal differences between individual forms of doxorubicin modifications and negative control, planned comparisons (contrast analysis) was performed. The plasma concentration of 4-HNE was significantly

(4.35-fold) higher when treated with conventional DOX as compared to negative control ($p<0.001$). In the next step, novel doxorubicin modifications (lipoDOX and apoDOX) were compared to conventional DOX. The plasma 4-HNE level was significantly (2.41-fold) lower after exposure to those novel forms as compared to conventional DOX, $p<0.001$. Additionally, although the plasma 4-HNE level was 1.46-fold lower after apoDOX treatment as compared to lipoDOX, no significance was observed ($p=0.06$) (Fig. 1).

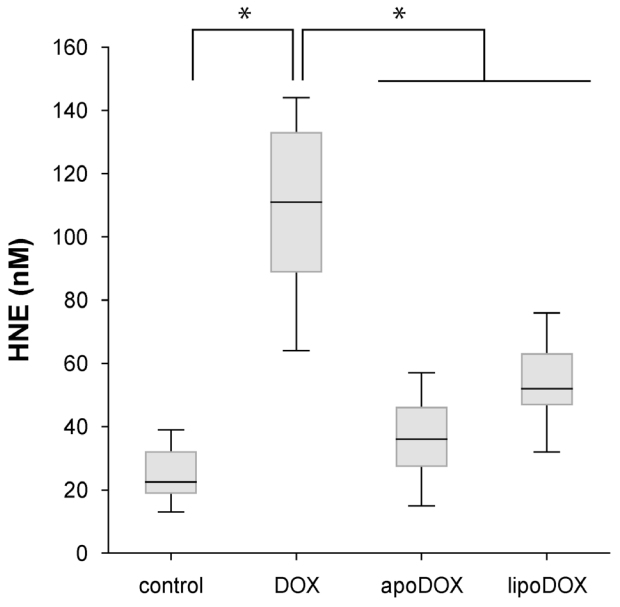

Fig. 1. Plasma level of 4-hydroxy-2-nonenal after 24 h exposure to various doxorubicin modifications. Asterisks indicate a significant difference at $p<0.05$. Displayed as median, box plots indicate 25-75 %, whiskers indicate non-outlier range of variables.

Effect on gene expression

Next, the mRNA expression level of *TXNRD2* and *ALDH3A1* was analyzed in two tissues – liver and left ventricle. Multivariate testing revealed that the effect of DOX treatment on the expression of these genes was significant in both liver tissue $F(9,58)=7.53$, $p<0.001$ and left ventricular tissue, $F(9,48)=4.02$, $p<0.001$. As apparent from Figure 2, the trend of these genes after the treatment with negative control, conventional doxorubicin and novel forms is similar as the trend of 4-HNE (compare Fig. 1 and Fig. 2). With regard to the liver tissue, highest expression of *ALDH3A1* and *TXNRD2* was observed after conventional DOX treatment, as compared to novel forms of DOX modifications, $p=0.03$ and 0.01 for *ALDH3A1* and *TXNRD2*, respectively. Although the gene expression of these genes after DOX treatment was higher as compared to negative control, the trend was below the significance level. Additionally, no significant difference in gene expression after application of apoDOX and lipoDOX was observed in liver tissue.

With regard to the left ventricle, similar gene expression pattern was observed in *ALDH3A1* gene. Nevertheless, there were no significant differences among the groups.

Discussion

Doxorubicin-induced cardiotoxicity is a threatening side effect of oncological treatment. Acute toxicity occurs in approximately 11 % of patients and comprises pericarditis-myocarditis and arrhythmias, but is quite well treatable and preventable by various drug administrations. Chronic cardiomyopathy, on the other hand, is the most serious drawback of clinical use of anthracyclines. It can arise in 20 % of patients and usually has a clinical picture of dilated cardiomyopathy and congestive heart failure. The mortality of these patients reaches 50 %, increasing significantly with increasing cumulative DOX doses (Chatterjee *et al.* 2010). The induction of generation of free radicals and initiation of oxidative stress is a known mechanism through which DOX causes cellular injury (Injac and Strukelj 2008) that can participate in the development of cardiotoxicity.

In this study, we focused on applicability of 4-HNE in plasma as a marker of oxidative stress after administration of various forms of DOX because 4-HNE is highly reactive secondary product of lipid peroxidation. It derives from ω-6 polyunsaturated fatty acids, which are, apart from others, important component of cardiolipin – relatively cardiospecific phospholipid of the inner mitochondrial membrane. Besides its role as a modulator of cell function (Leonarduzzi *et al.* 2000), the involvement of 4-HNE in pathogenesis of cardiac disorders such as atherosclerosis, hypertrophy or arrhythmias was reported (Mali and Palaniyandi 2014). In our animal model, significantly elevated plasma 4-HNE level was found after DOX treatment (4.35 fold higher when compared to negative control) that reflects an increase of oxidative stress. Elevated plasma level of 4-HNE associated with various pathologies was also found in clinical studies (Mali and Palaniyandi 2014, Riahi *et al.* 2010). In order to counteract the adverse effects of 4-HNE and another lipid peroxidation-derived

aldehydes, many specific cellular defense mechanisms employing aldehyde dehydrogenases have been developed. They differ in cellular location and specificity to various aldehydes. Therefore, this study pointed on ALDH3A1 which efficiently metabolizes 4-HNE (Black *et al.* 2012, Singh *et al.* 2013). Oxidative stress also causes collapse of the mitochondrial membrane potential leading to impairment of oxidative phosphorylation of ADP (Gunter *et al.* 1994). It also induces nonspecific permeabilization of inner mitochondrial membrane (Kowaltowski *et al.* 1998). With this regard, inner mitochondrial membrane is suspected of releasing of the mitochondrial constituents, including cytochrome c, into cytosol, and therefore inducing apoptosis (Green and Reed 1998). Accordingly, apoptosis is predominant form of cardiomyocyte cell death triggered by cytostatic drugs, and both the extrinsic and intrinsic apoptotic pathways are involved (Octavia *et al.* 2012).

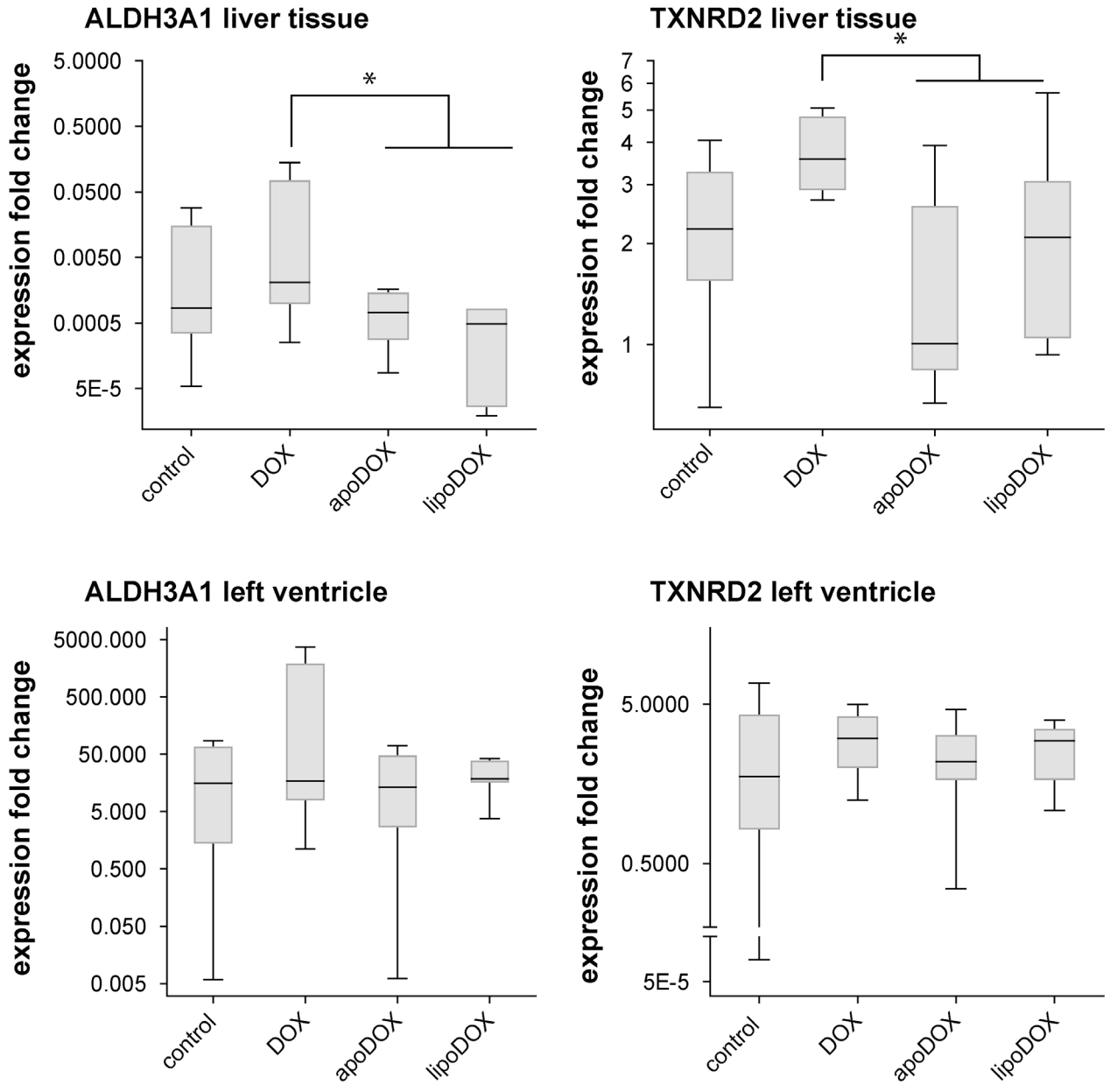

Fig. 2. Gene expression levels of *TXNRD2* and *ALDH3A1* after 24 h exposure to various doxorubicin modifications in liver and left ventricle. Asterisks indicate a significant difference at $p<0.05$. Displayed as median, box plots indicate 25-75 %, whiskers indicate non-outlier range of variables.

The line of cell antioxidant protection provided by thioredoxin reductase 2 (TXNRD2) as a part of thioredoxin antioxidant system, has apparently essential role. Therefore, apart from 4-HNE detection, an expression of these two antioxidant defense-related genes – *ALDH3A1* and *TXNRD2* – in two relevant tissues (liver and left ventricle) was analyzed in this study. Our results in multivariate testing show significant effect of DOX-treatment on the expression of both enzymes in liver and left ventricle and also a similar trend as compared to 4-HNE.

The importance of new drug formulations and their role in suppression of side effect of cancer treatment is high. Studies pursuing by new drug delivery systems have provided some evidence of the benefits resulting from their physical-chemical properties (Chau *et al.* 2006, van Vlerken *et al.* 2008). Only a few advanced delivery systems of DOX have been transferred into the clinical use – PEGylated and non-PEGylated liposomal nanocarrier of doxorubicin (Doxil® and Myocet®). Nowadays the research continues to find effective transporters, both liposomal and others, with beneficial functions and some of them are already in clinical studies (Perche and Torchilin 2013). We followed this trend by testing new nanotransporters – liposomal form without cholesterol, which shows better releasing of doxorubicin from liposomal particle (Kominkova *et al.* 2014) and apoferritin, the advantage of which is its potential to release its content in low pH environment, which is typical for tumor tissue (Gumulec *et al.* 2014). Our results support the idea of new transporters' benefits by showing significantly decreased level of 4-HNE in plasma and gene expression of *ALDH3A1* and *TXNRD2* in liver tissue as a main metabolizing organ after application of encapsulated forms of DOX in comparison with conventional DOX. Although this significant effect was not proved in left ventricle, the mild analogous trend was observed for *ALDH3A1*. It could be due to the fact that heart has low levels of some oxidative enzymes when compared with liver. Moreover, cardiomyocytes have a unique structure, in which 40 % of the organelles are mitochondria. Both these facts may explain why DOX is so toxic to heart (Ascensão *et al.* 2005). Taken together, our results show that plasma 4-HNE level sensitively reflects the changes of oxidative stress involved in the formation of cardiotoxicity after administration of different DOX formulations.

In summary, an association between doxorubicin form and the severity of oxidative stress was proven. Encapsulated forms (lipoDOX and apoDOX) show significantly lower level of lipoperoxidation marker 4-HNE in plasma. Gene expression of antioxidative enzymes thioredoxin reductase 2 and aldehyde dehydrogenase 3A1 was lower in liver tissue as a main detoxification organ after encapsulated form's administration, too. On the other hand, there was very mild difference in gene expression of *ALDH3A1* in the left ventricle as a main site of toxicity. It may be concluded that 4-HNE seems to be a good potential biomarker of doxorubicin-induced oxidative stress.

Although this study brought certain interesting findings, some other issues remain unclear. Thus, further research is planned, investigating wider range of parameters describing oxidative stress in the same model. Moreover, comparison of rat and human models of oxidative stress induced by DOX treatment might be interesting.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This publication was written at Masaryk University as part of the project "Příspěvek chemických a biochemických metodik ke studiu molekulární podstaty vybraných patologických stavů a onemocnění" number MUNI/A/1195/2014 and "Kardiovaskulární systém od buňky k lůžku pacienta" number MUNI/A/1326/2014 with the support of the Specific University Research Grant, as provided by the Ministry of Education, Youth and Sports of the Czech Republic in the year 2015.

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