Exosomal doxorubicin reduces the cardiac toxicity of doxorubicin

Aim: To test the efficacy and toxicity of exosomal doxorubicin (exoDOX) compared with free doxorubicin. **Materials & methods:** The cytotoxic effects of exoDOX were tested *in vitro* and in nude mice by measuring the tumor volume. The toxic effects were evaluated by measuring the bodyweight and through histopathologic analyses. The biodistribution of DOX was assessed by MS. **Results:** *In vitro* and *in vivo* studies showed that exosomes did not decrease the efficacy of DOX. Surprisingly, exoDOX showed no cardiotoxicity as observed in DOX-treated mice and MS studies confirmed that the accumulation of exoDOX in the heart was reduced by approximately 40%. **Conclusion:** We demonstrated that exoDOX was less toxic than DOX through its altered biodistribution.

Keywords: breast cancer • drug delivery • exosomal doxorubicin • exosome • nanomedicine • toxicity

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A branch of nanomedicine focuses on the efficient and novel delivery of drugs to improve their therapeutic index. For the most part in oncology, drugs have a narrow window of effect and the accumulation of drug in the tumor tissue while decreasing exposure to healthy tissue is a key issue.

Organic nanoparticles have been successful due to low toxicity and high biocompatibility [1]. Among them, liposomes have been considered for this purpose and have attracted the attention of the pharmaceutical industry with the success of Doxil, a liposomal formulation of doxorubicin (DOX) [2]. This type of drug has a better circulation time and biodistribution, and utilizes passive targeting, a phenomena known as enhanced permeability and retention effects. What we learned from these pioneering 'nanodrugs' was that the alteration of parameters such as pharmacokinetics and biodistribution could shift the equilibrium from toxicity to efficacy [3].

In nature, there are many vehicle systems (e.g., virus) that are of great interest because of their ability to actively target and enter cells while avoiding the immune system [4]. Exosomes (Exo), vesicular bodies with sizes ranging from 30 to 200 nm, have intrinsic properties, which make them ideal candidates for drug delivery purposes [4]. A general idea is that exosomes are released in the microenvironment as a source of biological material to communicate among different cells. It has been demonstrated that they can deliver miRNA, mRNA, DNA and proteins [5–8]. Based on those studies, the term 'exocure' was created, a drug delivery system (DDS) that utilizes exosomes to deliver material of interest to cure pathology [9]. Interestingly, exosomes have a neutral lipid molar ratio similar to the optimal composition for liposome fusion and stability [10,11].

In this study, we tested the hypothesis that exosomes could increase the efficacy of doxorubicin by actively targeting tumor cells and decreasing toxicity by altering the biodistribution of the drug. We found that exosomes can deliver DOX with the same antitumor efficacy as free DOX in *in vitro* and *in vivo* models [12,13]. Surprisingly, in a mouse model, we demonstrated that exosomes reduce the cardiac toxicity of DOX

by altering the biodistribution of the drug, suggesting new therapeutic opportunities for exosomal drug delivery in cancer patients.

Materials & methods

Reagents

Cells were purchased from Sigma-Aldrich (Sigma-Aldrich, Switzerland; HCT-116) and Cell Biolabs (Cell Biolabs, CA, USA; MDA-MB-231) and grown as indicated.

Nude mice were purchased from Harlan Laboratories (IN, USA) and the procedures were approved by the Institutional Ethical Committee for Animal Experimentation, and were performed in accordance with the institutional guidelines. We utilized at least three female mice of 8 weeks of age per data point. Data are reported as mean and standard error.

Exosomes loading & characterization

Exosomes from MDA-MB-231 and HCT-116 cell lines were prepared from exosome-depleted medium, conditioned for 48–72 h and purified with exoquick-TC™ solutions according to the instructions (SBI Bioscience, CA, USA). 200 µg of exosomes were mixed with 200 µg of DOX in the electroporation buffer (1.15 mM potassium phosphate, 25 mM potassium chloride and 21% Optiprep) and electroporated at 150 V, 0.125 × 1000 µF under maximum capacitance in a 0.4 cm cuvette. Exosomes were collected by centrifugation and washed three-times with PBS. Quantification was done by the Bradford method. The loading efficiency was calculated as loaded DOX/total DOX (w/w) by measuring the absorbance at 490 nm. The size of exosomes was determined by nanoparticle tracking analysis with a NanoSight LM10 instrument (Malvern Instruments, UK) in a PBS buffer by diluting the samples at a concentration of about 10–20 $\mu g ml^{-1}$.

Scanning electron microscopy

To perform scanning electron microscopy (SEM) analyses, exosomes were dehydrated in a graded 30–100% ethanol series, dried in a CO_2 apparatus at a critical point (Bal-Tec; EM Technology and Application, Liechtenstein), sputter coated with gold in an Edwards S150A apparatus (Edwards High Vacuum, UK), and examined with a Leica Stereoscan 430i scanning electron microscope (Leica Cambridge Ltd, UK).

Transmission electron microscopy

Exosomes were resuspended in PBS 2% glutaraldehyde (30 min) and deposited on formvar/carbon-coated electron microscopy (EM) grids. Two percent of uranyl acetate was added to the exosome-coated grids for 10 min and washed three-times with water. After drying in air, exosomes were imaged with a transmission electron microscope (EM 208, Philips, Eindhoven, The Netherlands). Micrographs were taken with a Quemesa Camera (Olympus Soft Imaging Solutions, Munster, Germany).

Cell viability assay

Cells (5×10^2) were plated in 96-well culture plates and the day after seeding, treated with DOX or exosomal doxorubicin (exoDOX) as indicated in the figure. Cell viability was measured after 96 h, according to the supplier (Promega, WI, USA; G7571), with a Tecan F200 instrument (Tecan, Switzerland). Averages and standard errors were obtained from three different experiments.

In vitro cellular internalization

MDA-MB-231 cells were seeded in 24-multiwell plates at a density of 1×10^5 cells/well. The following day, the cells were treated with DOX or exoDOX at a concentration of 100 ng/ml and incubated as indicated in the figure. The cells were imaged with a Leica fluorescence microscope at 20× magnification.

Xenograft

In xenograft tumor assays, 4×10^6 MDA-MB-231 cells were mixed with 30% of Matrigel (BD Bioscience, CA, USA) and implanted subcutaneously into the flanks of 8-week-old female nude mice. Once tumors reached measurable size (about 100 mm^3), mice were treated intravenously with indicated drugs two-times per week for seven treatments. Tumor volume was measured with a caliper instrument and calculated by using the formula: $(\text{length} \times \text{width}^2)/2$.

Histopathology

The hearts of mice were collected and fixed in phosphate-buffered 10% formalin, embedded in paraffin, sectioned at a thickness of 3 um and stained with H&E. The myocardial lesions induced by doxorubicin were assessed by light microscopic examination using different magnifications. Morphological details were studied at 40× objective.

Biodistribution

Mice were treated by intraperitoneal injection with 15 mg/kg^{-1} of DOX and sacrificed after 3 h. Tissues were washed with 10 ml of cold PBS/heparin before collection. Blood was collected from the left ventricle of the heart under anesthesia. The organs were diluted in 500 ul of PBS/BSA 4% and homogenized with Qiagen TissueRuptor for 20 sec at power 4 in ice. In the pharmacokinetic (PK) experiments, mice were treated with 3 mg/kg (ip.) and the blood was collected after

0.08, 0.25, 0.5, 1, 3, 18, 36 and 72 h. Samples were stored at -80°C.

The concentrations of DOX in serum and tissues were measured by LC-MS/MS. The proteins were precipitated with two volumes of cold acetonitrile containing 20 ng/ml daunorubicin as an internal standard. The cleared supernatant was diluted with two volumes of 0.2% formic acid and 10 ul were injected on LC-MS/MS system. The chromatographic separation was performed on Accucore-150 30 × 2.1 mm 2.6 um C18 column (Thermo Scientific, MA, USA) equilibrated with a 0.7 ml/min of 0.2% formic/acetonitrile (95:5) and maintained at 50°C. An elution gradient B from 5 to 80% of acetonitrile over 5 min was applied and 3 min of equilibration A 4000 QTRAP MS/MS system equipped with Turbo ESI source (AB Sciex, MA, USA) was applied in positive-ion mode. The transitions of DOX and daunorubicin were monitored in multireaction monitoring mode at m/z 544.1→397.2 and 528.2→321.1, respectively. The spray voltage was set at 5000 V with the source temperature at 400°C. The curtain gas, nebulizer gas (gas1) and auxiliary gas (gas 2) were set at 20, 50 and 50 arbitrary units, respectively. The declustering potential and collision energy voltages for both DOX and daunorubicin were set at 45 and 16 V, respectively.

Statistical analysis

The statistical significance was determined using a t-test. A p-value less than 0.05 was considered significant for all comparisons. All data were expressed as mean ± standard error.

Results & discussion

In vitro characterization of exosomal doxorubicin

Exosomes were isolated from cancer cells since it has been hypothesized that they have an intrinsic property to target the tumor microenvironment [14–19]. To enrich the fraction of exosomes from the supernatant culture of MDA-MB-231 and HCT-116 cell lines, exoquick-TC™ solution was utilized. The hydrodynamic dimension of vesicles was characterized by nanoparticle tracking analysis [20]. The mean and standard deviation values were 155 ± 55 and 156 ± 55 in MDA-MB-231 and HCT-116 cell lines, respectively

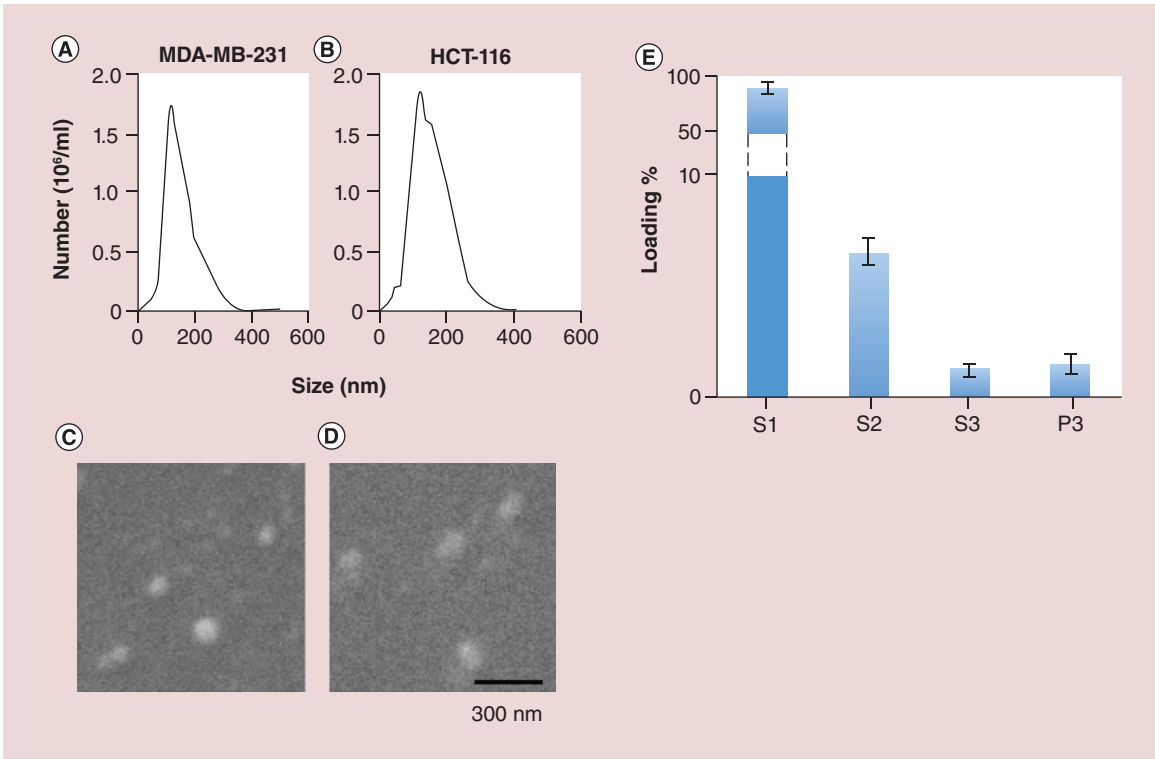

Figure 1. Characterization and loading efficiency of exosomes. **(A & C)** MDA-MB-231 and **(B & D)** HCT-116 nanoparticle tracking analysis (upper panel) and scanning electron microscope images (lower panel) demonstrate the shape and dimension of exosomes (preloading). **(E)** Loading efficiency of exosomal doxorubicin. After loading, the exosomes were washed three-times and the quantity of drug released in the supernatant or entrapped in the pellet was measured.
P: Pellet; P3: Pellet (exosomes) after three washes; S: Supernatant; S1, S2 and S3: Supernatant after one, two and three washes, respectively.
For color figures, please see online at www.futuremedicine.com/doi/full/10.2217/NNM.15.118

(Figure 1A & B). Exosomes were also characterized by surface (SEM) and transmission electron microscope. The Figure 1C & D & Supplementary Figure 1 depict a regular size with a spherical shape [21]. The vesicles were loaded through electroporation [22,23] and the loading of DOX was confirmed by measuring dimension of the exosomes, which increased to 176 ± 53 and 209 ± 54 nm in MDA-MB-231 and HCT-116 cell lines, respectively. Compared with free diffusion, electroporation increases the efficiency of loading of about three-times with a yield of 1.5 ± 0.5% (Figure 1E).

After exosome isolation and characterization, cells were treated with three different concentrations of DOX (100 ng/ml, 50 ng/ml and 25 ng/ml) and exoDOX from MDA-MB-231 and HCT-116 cell lines. The exoDOX were crossed on both cell lines in order to test if there was cell lineage specificity. The cell viability was measured at different time points as reported in Figure 2A & B. Similar to data reported in other published papers [12,13], we observed that exoDOX killed cancer cells at the same extent as free DOX. The same effect was also observed in two others colon cancer cell lines; LoVo and DLD-1 (Supplementary Figure 2).

The interaction and internalization of exosomes with cells has been the subject of many research papers. Exosomes can deliver different biological messengers such as RNA, miRNA, proteins, etc., utilizing 'native' mechanisms, which can also be utilized to deliver exogenous materials such as drugs. Different mechanisms of interaction with cells have been postulated: receptor- or lipid raft-mediated endocytosis, macropinocytosis or direct fusion with cell membranes. It appears from those studies that the content of the exosomes is efficiently released in the cytoplasm [24–28]. Time course analysis of DOX and exoDOX internalization in MDA-MB-231 cells showed

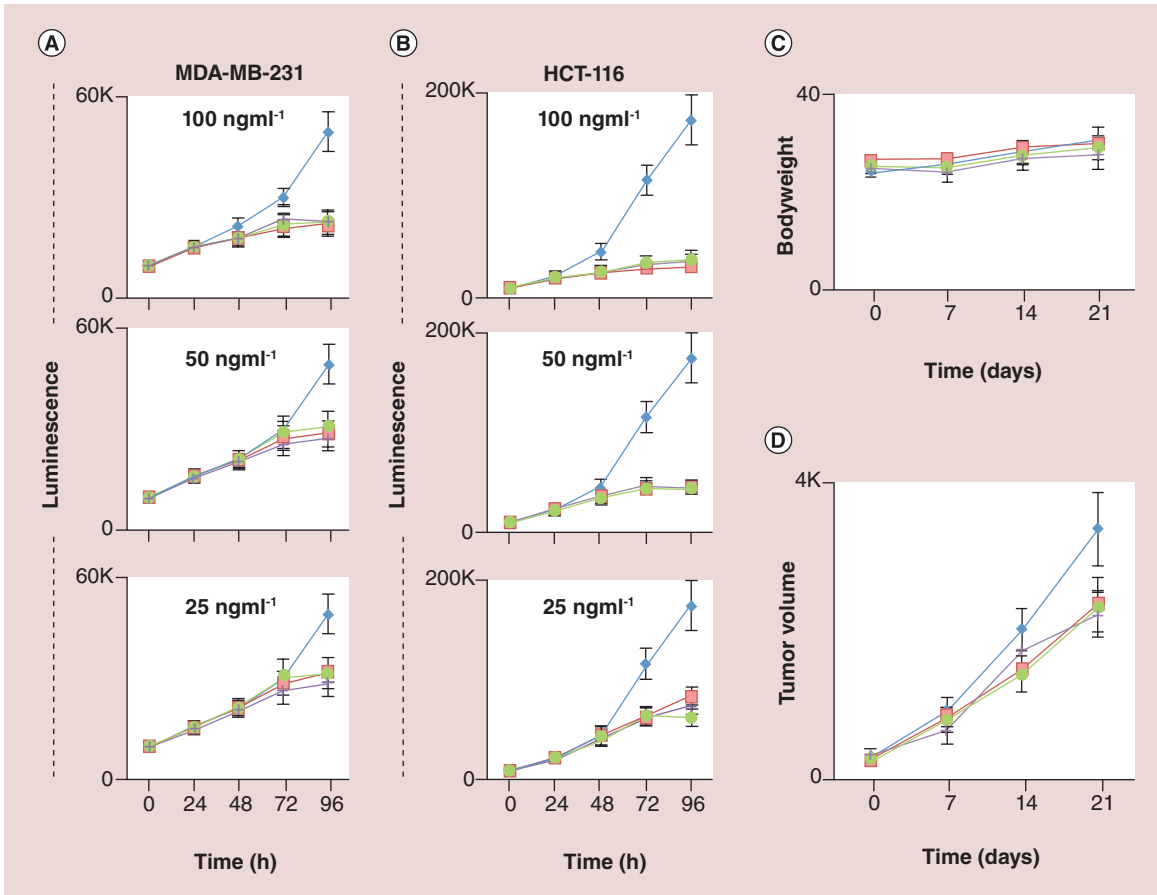

Figure 2. Exosomal doxorubicin has the same effect of doxorubicin *in vitro* and *in vivo*. Cell viability assays showed no difference among treated cells. **(A)** MDA-MB-231 and **(B)** HCT-116 cells were treated with different concentrations of DOX (■), exo231DOX (•), exoHCTDOX (□) and control (♦). Y-axis: luminescence refers to count per second. **(C & D)** MDA-MB-231 cells were implanted in the flank of nude mice. The animals were treated by intravenous injection (1.5 mg/kg^{-1}) with DOX (■), exo231DOX (•), exoHCTDOX (□) and control (♦). The tumor volume was monitored for 21 days every week. No difference was observed between treatments. The bodyweight of treated mice was normal suggesting no evidence of toxicity with treatments. (K: 1000).
exoDOX: Exosomal doxorubicin; exoHCTDOX: Exosomes derived from HCT-116 cell line.

a similar pattern of drug accumulation in the nucleus (Supplementary Figure 3). These data suggest that exosomes could efficiently release the DOX to exert its cytotoxic effect. *In vitro* analyses in static conditions have several limitations and it is more appropriate to use an *in vivo* model to mimic physiologic conditions. In addition, pegylated liposomal doxorubicin was two-times less effective compared with free DOX in *in vitro* cell viability assay but more effective *in vivo* [29]. In this setting, the MDA-MB-231 cancer cell line was injected as a subcutaneous xenograft and treated with free DOX or exoDOX prepared from MDA-MB-231 or HCT-116 cell lines. At a dose in which DOX did not produce any significant toxic side effect in mice (1.5 mg/kg, Figure 2C) [30], the efficacy of exoDOX was comparable to that of free DOX. The volume of the tumors was inhibited by about 30% compared with untreated mice (Figure 2D).

In vivo toxicity analyses of DOX & exoDOX

The clinical benefit of liposomal DOX is due to the reduced cardiac toxicity compared with free DOX [3]. In order to test the toxic side effects of exoDOX, the mice were treated at the acute toxic dose of 15 mg/kg of DOX [31,32]. After a period of 9 days, controls and exo-DOX-treated mice exhibited normal behavior and clinical appearance; however, DOX-treated mice showed lack of grooming, limited movement (hunched posture, ruffled hair coat, naso-ocular discharge) and were carefully sacrificed. As objective scale, we measured the bodyweight of mice, which is recognized a universal parameter to follow the health of the mice during the experiments. The animals treated with DOX lost more than 25% of their total bodyweight (Figure 3A & B). The bodyweight of exoDOX-treated mice decreased by an average of 10%, indicating less toxicity. The same mice were also analyzed by histopathology (see below).

Histopathology & biodistribution analyses of DOX & exoDOX

We hypothesized that the different observed toxicity was due to a distinctive biodistribution of DOX in the heart. The mice were injected with 15 mg/kg of DOX or exoDOX and after 3 h, the biodistribution of the drug was evaluated in the serum and heart. It was found that the concentration of DOX in the serum was comparable to exoDOX. This result suggested that the exoDOX preparations could diffuse in the body similar to free drug (Figure 3C & D). Differently from DOX, the accumulation of exoDOX in the heart was reduced by about 40% ($p < 0.05$). To better understand if the different biodistribution was due to an altered PK profile, DOX and exoDOX were evaluated in the serum at different time points.

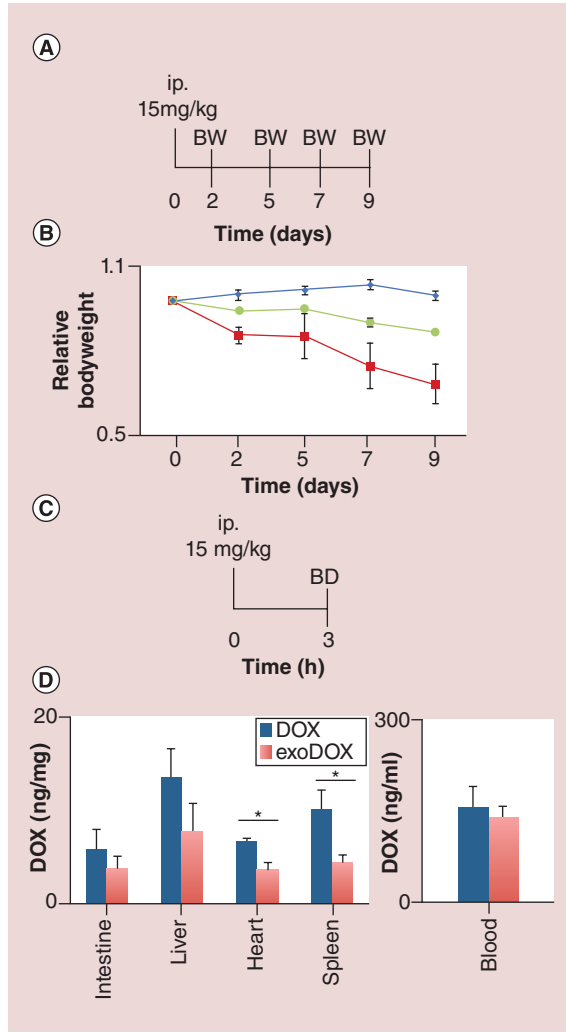

Figure 3. Doxorubicin is more toxic than exosomal doxorubicin in mice. **(A)** Schematic representation of mice treated by intraperitoneal injection with high levels (15 mg/kg^{-1}) of **(B)** DOX (■), exoHCTDOX (•) and controls (♦). Bodyweight was monitored at the indicated time points for 9 days. **(C)** Schematic representation of mice **(D)** treated by ip. injection with high levels (15 mg/kg^{-1}) of DOX and exoHCTDOX. The tissues were collected after 3 h. Although the level of DOX in the blood was equal, the heart tissue accumulated less exoDOX.
* $p < 0.05$.
BD: Biodistribution; BW: Bodyweight; DOX: Doxorubicin; exoDOX: Exosomal doxorubicin; exoHCTDOX: Exosomes derived from HCT-116 cell line; ip.: Intraperitoneal.

Supplementary Figure 4 showed that DOX and exo-DOX have the same PK profile. A paper recently published showed that exosomes accumulated less in muscle and heart tissues [33]. In the heart, the myocardium is supplied by vessels with tight junctions and the well-developed lymphatic system reduced the accumulation of membrane-based nanovectors such as liposomes [34]. Since a major problem of DOX is

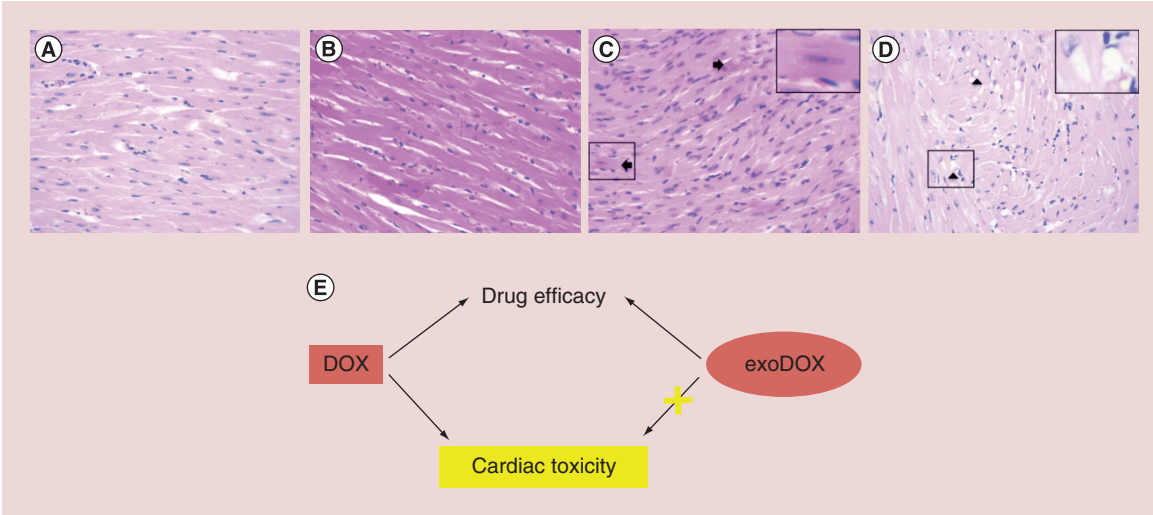

Figure 4. Exosomal doxorubicin-treated mice have normal cardiac tissue. Mice were treated with 15 mg/kg (intraperitoneal) of drug and analyzed after 9 days of treatment. DOX toxicity is well documented and that four mice were analyzed. In untreated **(A)** and exoDOX **(B)** treated mice, myocardial fibers showed the regular organized pattern of cytoplasmic striations with central located nuclei. The mice treated with DOX **(C & D)** exhibited scattered cytoplasmic paranuclear vacuoles (arrowheads) and focal intense eosinophilic (pink) cytoplasm, devoid of their transversal striations (arrows). The images in the boxes were enlarged (3×) in the top right corner. H&E staining, original magnification 40×. **(E)** Schematic summary of the beneficial effects of exoDOX compared with DOX. ExoDOX reduces the cardiac toxicity of doxorubicin.
DOX: Doxorubicin; exoDOX: Exosomal doxorubicin.

the cardiac toxicity, our results support exoDOX as an innovative and safe system to deliver DOX in cancer patients [35]. To support our observations, we analyzed the heart tissue by histopathology. The heart of mice treated with exoDOX or untreated appeared normal (Figure 4A & B). In accordance with the literature [36], mice treated with DOX showed vacuolization and hypereosinophilia of the cytoplasm compared with untreated mice (Figure 4C & D). Moderate disarrangement of cardiomyocytes was also evident in some sections. Other tissues such as intestine, liver, kidney, spleen, lung and brain did not reveal evident or significant toxicity (Supplementary Figure 5).

Conclusion

The success of newly formulated drugs such as Doxil is primarily due to the reduced toxicity more than an increase in its efficacy. Specifically for DOX, the cardiac toxicity is a limiting factor during therapy. A different biodistribution of liposomal DOX limits the cardiac toxicity compared with DOX [3]. In this work, we tested the hypothesis that DOX encapsulated in exosomes has a different therapeutic activity compared with free DOX. Although tumor exosomes loaded with DOX could decrease the growth of cancer cells *in vitro* and *in vivo*, the efficacy was similar to free DOX. This result is supported by data from other publications in which exosomes were modified to perform active targeting [23]. In addition, we tested if exoDOX is less toxic than free DOX. The results were evident by visual inspection of the mice tissues. After 9 days of treatment, the DOX-treated mice lacked grooming, had hunched posture and lost significant body weight. The exoDOX-treated mice appeared healthy and the histopathologic analysis confirmed that the cardiac tissue in exoDOX-treated mice was normal. Biodistribution analyses showed a similar quantity of DOX and exoDOX in the serum but a reduced accumulation in the heart of exoDOX-treated mice compared with DOX.

Taken together, the results of this study suggest that exosomes could be used to reduce the toxicity of DOX by altering the biodistribution (Figure 4E). It could be of interest, to test other chemotherapeutic drugs to understand if exosomes are a valid drug delivery system for broad applications.

Future perspective

Drug delivery systems are an emerging nanotechnology for personalized therapy in cancer patients. The role of exosomes as messenger of functional cargo has thrust them into the spotlight as ideal DDS [24]. The concept is supported by the structure of exosomes; the lipid composition renders the exosomes stable in biological fluids and promotes their fusion. Proteins such as integrins facilitate adherence and internalization [10]. Recently, it was also suggested that exosomes derived from tumor cells showed some specificity toward cancer cells with an enhanced association compared with

liposomes of a similar size [11]. Although liposomes were successful in increasing stability, solubility and PK properties of free drugs, the application was modest due to the limited reproducibility during preparation and lack of knowledge of *in vivo* interactions and fate. Self-produced exosomes have natural properties that could overcome most of the limitations of liposomes. Different cells produce different exosomes, widening their application prospects. Similar to liposomes, exosomes can be purified and analytically validated for clinical applications [37]. From our PK data, we anticipate that exosomes could avoid side effects of liposomal DOX by limiting the accumulation in off-target sites such as the skin [38].

In the next decade, the functional study of exosomes could reveal a way to assemble superior DDS to reach the perfect match between high specificity and low toxicity. Considering the high quantity of drug necessary to obtain a therapeutic effect, we would increase the loading capacity by applying a similar remote loading system utilized for liposomes. In the long term, exosomes produced from a single patient could be manipulated to obtain a personalized exosomal therapy.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/NNM.15.118

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved

Executive summary

The exosomal doxorubicin has cytotoxic effects on MDA-MB-231 & HCT-116 cell lines *in vitro* & on MDA-MB-231 xenograft mouse model.

- Exosomes could be loaded with doxorubicin without decreasing the efficacy.
- The loading efficiency of exosomes increases with electroporation but still at low percentage.

The exosomal doxorubicin reduces the toxicity of doxorubicin

- Physical examination and bodyweight analyses of mice reveal a better safety profile of exosomal doxorubicin (exoDOX) than DOX.
- The heart tissue of mice treated with DOX exhibits scattered cytoplasmic paranuclear vacuoles and focal intense eosinophilic cytoplasm, devoid of their transversal striations with moderate disarrangement of cardiomyocytes. Conversely, the cardiac tissue of exoDOX-treated mice appears normal.

Exosomal doxorubicin biodistribution is different from doxorubicin

- The quantity of exoDOX in the blood after 3 h of intraperitoneal injection is similar to DOX, suggesting that exoDOX could easily redistribute in the body.
- The heart of mice shows a 40% reduction of exoDOX, explaining the reduced toxicity.

Conclusion

- ExoDOX has similar antitumor effect as free DOX but is less toxic through an altered biodistribution.
- ExoDOX could increase the therapeutic index of DOX by reducing toxic side effects.

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