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Doxorubicin has *in vivo* toxicological effects on *ex vivo* cultured mesenchymal stem cells

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HIGHLIGHTS

- Mesenchymal stem cells (MSCs) were isolated from rats that received doxorubicin.
- Doxorubicin had toxic effects on MSCs.
- MSCs grew up in lower rates and had decreased alkaline phosphatase production.
- MSCs failed to express connexin 43 and troponin T.

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ABSTRACT

Doxorubicin (dox) is an effective chemotherapeutic agent that leads to cardiotoxicity. An alternative treatment for dox-cardiotoxicity is autologous mesenchymal stem cells (MSCs) transplantation. It remains unclear if dox has deleterious effects on MSCs from subjects under chemotherapy, therefore this study aimed to evaluate dox *in vivo* toxicological effects on *ex vivo* cultured MSCs, inferring whether autologous transplantation may be an alternative treatment in patients who are exposed to the drug. Wistar rats received either dox or saline. Following treatments, animals were sacrificed and bone marrow MSCs were isolated, characterized for cell surface markers and assessed according to their viability, alkaline phosphatase production, and proliferation kinetics. Moreover, MSCs were primed to cardiac differentiation and troponin T and connexin 43 expressions were evaluated. Compared to control, undifferentiated MSCs from dox group kept the pattern for surface marker and had similar viability results. In contrast, they showed lower alkaline phosphatase production, proliferation rate, and connexin 43 expression. Primed MSCs from dox group showed lower troponin T levels. It was demonstrated a toxic effect of dox in host MSCs. This result renders the possibility of autologous MSCs transplantation to treat dox-cardiotoxicity, which could be a non-suitable option for subjects receiving such antineoplastic agent.

1. Introduction

One of the most effective chemotherapeutic agents used for treatment of hematological and solid tumors is doxorubicin (dox) (Butany et al., 2009) However, it causes a dose-dependent cardiotoxicity that may lead to irreversible heart failure by different pathogenic mechanisms which have not been completely elucidated (Gianni et al., 2008). Production of reactive oxygen species, calcium imbalance and apoptosis are considered among the pathological mechanisms for dox-induced cardiotoxicity (Gianni et al., 2008; Tan et al., 2010; Zhang et al., 2011).

A progressive reduction of left ventricular function, detected by different echocardiography approaches, either in animal models (Oliveira et al., 2013), or in a significant proportion of pediatric (Lipshultz et al., 2012), adult (Tassan-Mangina et al., 2006) and elderly (Aapro et al., 2011) patients, is observed during the course of the anti-cancer therapy, leading to a life-threatening congestive heart failure. While cardiotoxicity can develop at no specific time once the treatment has begun, identifying cardioprotective strategies to minimize the long-term damage caused by anthracyclines is imperative. In this context, many alternatives have been investigated either in experimental models or in clinical trials in

Abbreviations: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; dox, doxorubicin; FBS, fetal bovine serum; MSCs, mesenchymal stem cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium salt; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TBS, tris buffered saline; TBST, tris buffered saline with Tween.

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order to prevent or diminish cardiac dysfunction, such as dexrazoxane (Lipshultz et al., 2010), carvedilol (Machado et al., 2008), plant extracts (Du and Lou, 2008) and stem cell therapy (Chen et al., 2010). Nevertheless, none of those are really established in routine medical practice to date. Between 1966 and 2010, Van-Dalen et al. (2011) investigated randomized controlled trials in which any cardioprotective agent was compared to no additional therapy or placebo in cancer patients (children and adults) receiving anthracyclines. Although dexrazoxane had showed the potential to prevent heart damage, the authors found no definitive conclusions about a real cardioprotective agent. A promising alternative for prevention or treatment of tissues and organs is the regenerative medicine using stem cells. Autologous mesenchymal stem cell (MSCs) transplantation is an obvious first option when thinking of a routine practice, given the ease of cell isolation and *in vitro* expansion required for therapy (Mayhall et al., 2004; Wang et al., 2012). However, the autologous MSCs transplantation for cardiac repair in patients under chemotherapy must be re-thought. It is paramount to firstly understand the effects of dox in such cell population before considering its use. For this reason, the aim of the present research was to study whether MSCs niches are affected by dox treatment in subjects under chemotherapy.

2. Materials and methods

2.1. Dox cytotoxicity testing

Neonatal ventricular cardiomyocytes, adipose tissue derived MSCs and breast cancer MDA-MB 231 (ATCC® HTB-26™) cell cultures were evaluated for dox cytotoxicity. Cardiomyocytes (Ott et al., 2008) and MSCs (Oliveira et al., 2013) were obtained as previously described. The cell cultures were seeded on 24-well plates at 5×10^4 cells/cm^2. Thereafter, they were incubated in a humidified atmosphere with 5% CO_2 at 37 °C using *Dulbecco's modified Eagle's* medium (DMEM) high glucose (Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS) (Cripion Biotecnologia LTDA), 5 mM sodium bicarbonate (Cinética Química Ltda), penicillin (100 U/mL), streptomycin (0.1 mg/mL), amphotericin B (0.25 mg/mL) (Sigma–Aldrich), and gentamicin (60 mg/L; Schering-Plough). After 24 h, media was changed and was added 5 μmol/l of dox [dox hydrochloride (Adriblastine®, Pfizer)] on the plates. Dox concentration was used as previously described (Spallarossa et al., 2004). The length of incubation was determined by evaluating the time where 50% of cardiomyocytes died. Then, this incubation time was performed for all cell cultures. Afterward, to determine the cytotoxic effect of dox on the different cell types, cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Cat. M-6494; Invitrogen) as previously described by Paula et al. (2013). Briefly, 24 h after plating the cells, medium was changed to 170 μl of the MTT solution (5 mg/ml) and 210 μl of basal media (DMEM/10% FBS). Cells were, then, incubated in a humid 5% CO_2 atmosphere at 37 °C. 2-h later, the precipitated formazan crystals were solubilized by adding 210 μl of sodium dodecyl sulfate (SDS)-10% HCl. After 18 h, 100 μl of solution was transferred to a 96-well plate, and the optical density was measured at 595 nm using a microplate reader. Data is from three independent experiments.

2.2. Animals and experimental groups

Sixteen male 6–8 weeks old Wistar rats weighing 300–350 g were studied. Animals were kept in climate-controlled environment under a 12-h light/dark cycle with free access to standard rodent chow and water. All experimental protocols were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee in place at the University (protocol number 176/2010).

Animals were allocated into two groups containing eight rats each: control group [saline intraperitoneal injection (i.p.)] and dox-treated group [dox hydrochloride (Adriblastine®, Pfizer) 5 mg/kg; i.p.]. Animals were weighted weekly and the dox dosage was properly calculated to each animal. The injections were given once a week for four weeks. After 48 h of the last dox/saline administration, animals were anaesthetized and sacrificed by overdose of isoflurane anesthesia. The cumulative dose of dox used was previously demonstrated to be effective and to promote cardiotoxicity and heart failure (Oliveira et al., 2013). In order to confirm the occurrence of cardiac dysfunction, animals from both groups were evaluated by echocardiography examination before (baseline) and after dox treatment.

2.3. Bone marrow derived MSCs isolation and culture

Following the euthanasia, bone marrow MSCs were isolated from rats of both control and dox groups, as previously described (Assis et al., 2010) with minor modifications. Briefly, bone marrow was flushed out from tibias and femurs of both control and dox-treated rats, using DMEM high glucose supplemented with sodium bicarbonate, penicillin, streptomycin, amphotericin B, and gentamicin. This content was centrifuged, and the pellet containing MSCs was put into suspension in the aforementioned medium added with 10% FBS and, then, plated in 75 cm^2 flasks. Cell cultures were kept in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h before the first medium change. The medium was then changed every 2–3 days. Cell adherence to cell culture flask guaranteed mesenchymal cell population enrichment. The cells were allowed to grow near confluence, and were split in a 1:3 ratio using 0.25% trypsin–EDTA. Fourth passage bone marrow derived MSCs were used in all experiments.

2.4. Cellular characterization by indirect immunofluorescence

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized in 0.1% Triton X-100 (Sigma) for 1 h at room temperature. Samples were blocked with 1% bovine serum albumin (BSA) and 5% goat serum in phosphate buffered saline (PBS) solution and incubated for 1 h at room temperature followed by two washes with PBS. Primary antibodies [CD45 (cat.# 610266), CD54 (cat.# 554967), CD73 (cat.# 551123) and CD90 (cat.# 554895), all from BD Biosciences, USA] were added in 1% BSA in PBS solution and incubated overnight at 4 °C at 1:100 dilution. Samples were washed with PBS twice. Secondary antibodies [Alexa Fluor dye conjugated (Invitrogen, USA)] were diluted (1:500) in the same solution as the primary antibodies and incubated at room temperature for 1 h in dark. Samples were washed with PBS twice. Coverslips were sealed with hydramount aqueous media (Cat. HS-106; National diagnostics) and analyzed with a Zeiss LSM 510—Meta confocal microscope. Nine random fields were collected from each experimental group. Data is from three independent experiments.

2.5. Cell viability assay

Cell viability was assessed by the MTT assay as aforementioned. In these studies, cultures of 5×10^4 cells/cm^2 were seeded on 24-well plates. Data is from three independent experiments.

2.6. Alkaline phosphatase (AP) activity

AP activity was evaluated with the 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium salt (NBT) Kit assay as