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ORIGINAL ARTICLE

The Effect of Doxorubicin on Viability and Morphology of Human Embryonic Stem Cell-derived Cardiomyocytes

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KEYWORDS

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ABSTRACT: In spite of serious cardiotoxicity side-effects, doxorubicin is frequently used for treatment of several types of cancers. Isolated human adult cardiomyocytes could be the best model for assessing drug-induced cardiotoxicity, while harvesting mature cardiomyocytes is restricted by some limitations such as biopsy size, cell numbers, viability, proliferative capacity and their disability to be passaged as a cell line. In the present study, human embryonic stem cell (hESC)-derived cardiomyocytes applied as a model for evaluation of doxorubicin cardiotoxicity. In this process, cardiogenic differentiated hESCs spheroids were exposed to different concentrations of doxorubicin for 24, 48 and 72 hours. The viability of spheroids as well as their morphology was assessed as important criterion of cardiotoxicity. Findings of the study showed that the viability of spheroids was significantly reduced at doses of 3 and 30 µM ($P<0.05$). Moreover, cell morphology was changed in the presence of same doses. Overall hESC-derived cardiomyocytes (hESC-CMs) could be a useful in vitro model for evaluating drug-induced toxicity.

INTRODUCTION

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most effective anti-tumor agents used in the treatment of several adults and pediatric cancers, such as solid tumors, leukemia, lymphomas and breast cancer. However, their usefulness is limited by cumulative dose-dependent cardiotoxicity. Severe cardiotoxicity may occur months or even years after administration of DOX, including alteration in the ability of the heart to contract and/or relax, change in cardiac rhythm, alteration in blood pressure and ischaemia [1].To date, the exact molecular mechanism of DOX-induced cardiotoxicity still remains unclear. Among the proposed hypothesis, increase in cardiac oxidative stress, as evidenced by generation of reactive oxygen species (ROS) is one of the center mediators of direct and indirect cardiac adverse consequences[2].

Furthermore, heart repercussions are even more prevalent because of this organ's greater sensitivity to

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damage induced by free radicals, given the high oxidative metabolism of the heart and its lower level of antioxidant defenses[3].

This cardiotoxicity causes DNA damage, myofibrillar disarray, membrane damage due to lipid oxidation, mitochondrial dysfunction, altered calcium handling, cytoplasmic vacuolization, an increase in number of lysosomes, p53 accumulation, activation of apoptotic signaling cascades and inhibition of survival signaling pathways[4].

In the last few years many predictive models have been used for preclinical drug development including: in vivo and ex vivo animal models, non-human primary cell cultures and immortalized cell lines. However in spite of physiological similarities, there are a few problems due to species-specific pharmacotoxicological effects; so all of them fail to fully recapitulate relevant human biology[5].

Although isolated human adult cardiomyocytes could be one of the best models for assessing drug-induced cardiotoxicity, harvesting mature cardiomyocytes is confounded by limitations in biopsy size, cell numbers, viability, proliferative capacity and their disability to be passaged as a cell line. So cardiotoxic side-effects of drugs remain a major challenge in the cardiac research field[6].

The first derivation of human pluripotent stem cell (hPSCs) lines[7], either hESCs or human induced pluripotent stem cell (hiPSCs)[8], have received much attention because of their potential to differentiate into numerous types of functional cells. The ability to differentiate them into the cardiomyocytes[9] led to great excitement in drug discovery and development because hPSC-CM can be used as a considerable and useful in vitro model for evaluating drug-induced cardiotoxicity.

hPSC-CM display gene expression and microRNA expression profiles, ion channel functionality, ultra-structures, and pharmacological responses sharing similarities with an embryonic/fetal cardiac phenotype[10] .So far, several protocols have been utilized for hESCs differentiation into cardiomyocytes in vitro[11]. In the present study, it was tried to differentiate spheres generated from hESCs into cardiomyocytes in a defined medium using small molecules. The physiology of mentioned cells has been evaluated in response to doxorubicin an effective anti-cancer drug with cardiotoxicity side-effect.

MATERIALS AND METHODS

Suspension cell culture and differentiation protocol

Royan H5 hESC line[12] was used in this study. Suspension culture of hESCs was performed according to a recently published protocol[13]. Briefly, cells were treated with 10 mM ROCK inhibitor Y-27632 (Sigma-Aldrich, Y0503) for 1 h prior to dissociation from Matrigel. Cells then were washed by Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS; Gibco, 21600-051) and incubated with 0.05% trypsin at 37°C for 4–5 min. Dissociated cells were transferred into non-adhesive bacterial plates (60 mm; Griner, 628102) at density of 15×10^4 viable cells/ ml in hESC medium which had been conditioned on mouse embryonic fibroblasts (MEFs), containing 10 mM ROCK inhibitor. After 2 days, half of the medium was replaced by the hESC medium conditioned on MEFs. The medium was changed every other day. Differentiation of the cells into cardiomyocytes in suspension was performed according to the Gonzalez et al. protocol[14], with some modifications. Briefly, 6-day old spheres were treated with small molecules CHIR99021, purmorphamine, SB431542 and IWP in RPMI medium (Gibco, 51800-035) supplemented with 2% B27 without vitamin A. At day 4, the spheres were plated on gelatin-coated plates in RPMI/B27 medium without cytokines. Beating clusters were observed about 8 days post-plating.

Toxicity studies using hESC-derived cardiomyocytes

In order to evaluate DOX (Ebedoxo, EBEWE Pharma, Austria) cytotoxicity, beating spheres derived from hESC-CM were trypsinized, seeded at 25,000 cells/100 µl in each well of a 96-well tissue culture plate and subsequently treated with 0.03, 0.3, 3, 30 µM concentrations of DOX for 24, 48 and 72h. The percentage of viable cells was assessed by MTS assay, according to this way, at the end of DOX treatment, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide (MTS/PMS, G5421, Promega) was added to the cells in 96-well plates (10 µl per 100 µl medium in each well). After 4 h incubation in 37°C, the resulted Formazan crystals were dissolved in isopropanol. Absorbance at 490 nm was read by an ELISA reader system (Elx800, BioTek, USA). Cellular abnormalities in morphologic level could also be detected using phase contrast microscopy. Human foreskin fibroblasts (HFF) were used as control cells to distinguish cardiac specific toxicity from general cytotoxicity.

STATISTICAL ANALYSIS

The data were expressed as mean ± standard error (SE). One-way ANOVA followed by the Tukey‘s post hoc test was used for data analysis. All experiments were repeated in three independent replicates. Differences between groups were considered as statistically significant at $P < 0.05$. All data was analyzed using SPSS software version 18.

RESULTS

Royan H5 colonies (Figure 1, A) were treated with trypsin and dissociated cells were transferred into non-adhesive bacterial plates (Figure 2, B).After 6 days obtained spheres (Figure 1, C) were treated with small molecules and beating spheres plated on gelatin-coated plates about 4 days later (Figure 1, D). In order to evaluate DOX cytotoxicity, the trypsinized beating spheres derived from hESCs were treated with 0.03, 0.3, 3 and 30 µM DOX for 24, 48 and 72h. According to the results of the MTS assay and comparison of data with the control group, doses of 3 and 30 µM of doxorubicin, have significantly reduced the viability of spheres in a dose- and time-dependent manner (Figure 2). Human Foreskin fibroblasts (HFF) were used as control cells to distinguish cardiac specific toxicity from general cytotoxicity. In contrast, the effect of doxorubicin on HFF was much lower, showing a toxic effect only at the two highest tested concentrations tested (data not shown). In addition, cell morphology was changed (Figure 3).

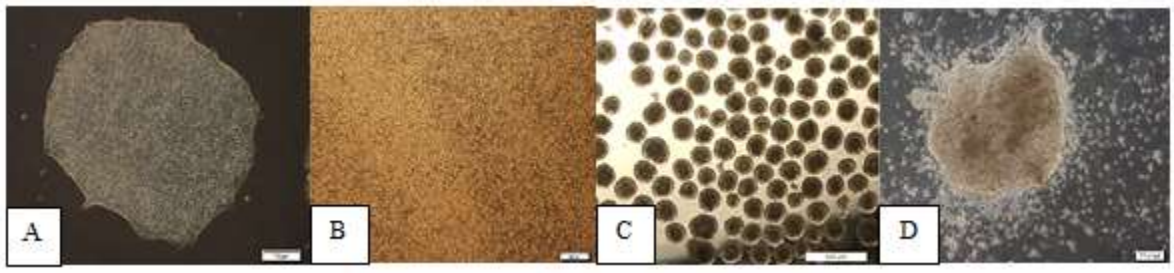

Figure 1. Cell morphologies during our cardiogenic differentiation protocol. A. Royan H5 colony on matrigel , B. Royan H5 single cells in bacterial dishes, C. spheres generated from hESCs, D. beating cardiomyocytes cluster differentiated from hESC.

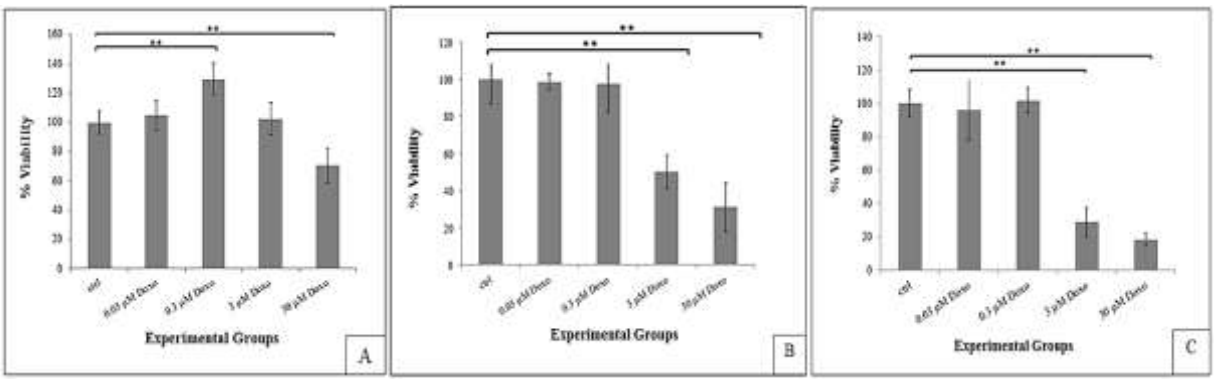

Figure 2. Percentage of viable cells after Doxorubicin treatment evaluated by MTS assay at 24h (A), 48h (B) and 72h (C). Data is presented as mean percentage of cell viability ± SE.

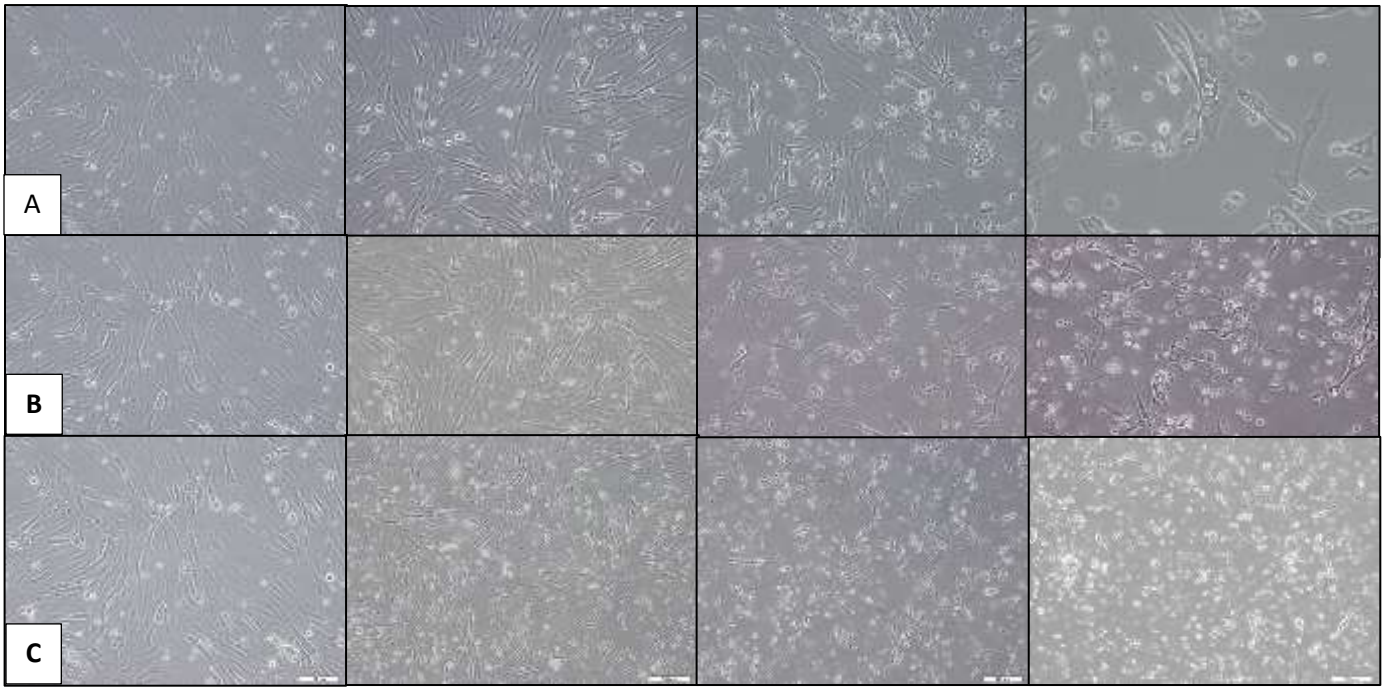

Figure 3. The cardiomyocytes morphology treated with different concentration of doxorubicin at 24h (A), 48h (B) and 72h (C), each line from left to right: control, 0.3, 3, 30μM. As it is obvious, the morphology has been changed. Debris and apoptotic cell have increased during treatment.

DISCUSSION

In this study, the viability of spheroids was significantly reduced at doses of 3 and 30 μM. Likewise; cell morphology was changed in the presence of same doses. Considering drug safety, cardiovascular system has been attracted scientists' critical focus as any heart damage can be reflected immediately and directly threaten human's life. Cardiotoxicity is a general term that describes cardiomyocytes damage which causes decrease in cardiac function. In recent years, high rates of drug attrition and withdrawal from market have been occurred because of unexpected cardiotoxicity which imposed multi-billion dollar scathe for pharmaceutical companies[15]; so an effective predictive model is required to evaluate potential toxicities of cardiac and non-cardiac drugs. Previous works of cardiotoxicity testing were based on animal models such as the mouse,

guinea pig, rabbit and dog for in vivo testing or animal-derived primary cardiomyocytes for in vitro assays[16]; but it is obvious that the application of animals is very expensive, time-consuming and involves lots of ethical concerns. Although using ESC-CM for drug cardiotoxicity testing can overcome some of these problems, these produced cells are often heterogeneous and fail to fully recapitulate relevant human physiology. Nowadays highly efficient production of cardiomyocytes from hPSCs (hESCs and hiPSCs) is one of the most important subjects in stem cell research especially for drug screening. So far, several protocols have been utilized for efficient and high yield differentiation of hPSC into cardiomyocytes in vitro[17]. As it is reported using small molecules instead of growth factors, cytokines and unknown factors in serum enhanced cardiomyocytes differentiation efficiency[18]. So in the present study, it was tried to differentiate spheres generated from hESCs into cardiomyocytes in a defined medium just by using small molecules. Then we have evaluated cells' response to doxorubicin an effective anti-cancer drug that targets DNA but produces off-target cardiotoxicity side-effect independent of their DNA-binding ability, potentially through increased reactive oxygen species. Results of the study showed that cell response to doxorubicin is dose- and time- dependent. At doses of 3 and 30 µM of doxorubicin, the viability was significantly reduced and cell morphology was changed. In addition some vacuoles were seen in cardiomyocytes cytoplasm by increasing doxorubicin concentration.

CONCLUSION

HESC-CMs showed expected specific toxicity; so they could be a useful model for in vitro evaluation of drug-induced cardiotoxicity and could be used as one of the most similar models to human's heart cells.

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