Cardiac Analysis of Autologous Transplantation of Cocultured Skeletal Myoblasts and Mesenchymal Cells in a Rat Model Doxorubicin-Induced Cardiotoxicity: Histopathological and Functional Studies

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Abstract

Objective: Investigate the functional and histopathological effects of combined transplantation of mesenchymal stem cells (MSCs) and skeletal myoblasts (SM) in rats submitted to doxorubicin (DOX) induced cardiomyopathy.

Methods: Myocardiopathy was induced through intraperitoneal applications of 3.75 mg/kg/day DOX once a week for 4 weeks in all experimental animals. Echocardiography examination was conducted to evaluate heart function. Myocardial cell apoptosis was examined morphologically by optical microscopy. The expression of both, von Willebrand factor and MyoD proteins was analysed using immunohistochemical staining.

Results: Our results showed that all the experimental animals developed cardio-toxicity and exhibited decrease in heart function 4 weeks after the administration of DOX (P<0.05). The ventricular function significantly improved in rats that received sub-epicardial injection of co-culture of SM and MSC (CO group) when compared to rats that received only saline sub-epicardial injection (CG group) (P<0.05).

Conclusion: The results of this study provide evidences that the myocardial co-culture transplantation of SM and MSCs contributed to the treatment the DOX-induced cardiotoxicity.

Keywords: Doxorubicin; Cardiac failure; Cardiomyopathy; Cell therapy; Coculture

Introduction

Cancer affects more than 1.6 million of Americans and is believed to be the second most common cause of death (after accidents, injuries and intoxication) in children [1]. Unfortunately, cardiotoxicity is the most severe of chronic complications of antineoplastic therapy. Doxorubicin (DOX) is an anthracycline effective antitumor agent used in cancer treatment and the most widely used and successful chemotherapeutic for childhood cancers [2]. However, its clinical use is limited due to its severe and irreversible cardiotoxicity including the development of cardiomyopathy, and ultimately congestive heart failure [3,4].

The significant advances in therapeutic modalities, such as heart or ventricular transplantation, have emerged in the feld of regenerative medicine with the use of mesenchymal stem cells (MSCs) [5]. During the past decade, it has been demonstrated that MSCs possess outstanding potential for cardiac regeneration based on their multipotential differentiation ability, easy tissue accessibility and capacity for ex-vivo expansion [6].

Transplantation of satellite cell-derived myoblasts has therapeutic potential for repairing damaged heart and has been used both, experimentally and clinically, in an attempt to restore cardiac function [7]. Therefore, transplantation of cocultured cells remains an attractive approach for myocardial repair [8,9].

So, the current study was conducted to evaluate the functional and histopathological effect of combined transplantation of MSCs and SM in doxorubicin-induced cardiomyopathy.

Methods

All animal experimental procedures in this study were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and were approved by the Ethics Committee of the Pontifical Universidad Catholic of Paraná (protocol approval number 427).

Experimental animals

20 male Wistar rats at 5 months of age (young adult) were used in the study. DOX was administered intraperitoneally at a dose of 3.75 mg/kg/day DOX once a week for 4 weeks at a total cumulative dose of 15 mg/kg body weight, as previously described. The animals were
randomly divided into 2 groups (n=10/group): Non treated group (CG) received saline subepicardial injections, and cocultured group (CO) received combined MSCs and SM subepicardial injections. The heart of each experimental animal was exposed through a midline sternotomy under general anesthesia, as published [9]. 5 × 10^4 cocultured cells were subepicardial injected into independent sites in the heart region of CO animals 4 weeks after the administration of the last dose of DOX using a 50 μL Hamilton syringe. After the surgery, the sternum of the rats was closed with 3-0 silk sutures.

MSCs and SM isolation and coculture

MSCs were isolated from male Wistar rats by bone marrow aspiration of the posterosuperior iliac, following the protocol previously described [10]. SM was isolated after biopsy of the skeletal muscle of the lower limb, according to the technique of Delaporte [11]. In brief, the skeletal tissue was digested with 0.075% type II collagenase (Sigma, US) in a 5% CO2 incubator for 1 h at 37°C. This mixture was filtered through a 140 μm nylon mesh and centrifuged at 1,200 × g for 10 min at 4°C. MSCs were separated according to density using a Ficoll-Paque density gradient (1.077 g/ml) and 5% CO2 incubation in the dark for 1 h at room temperature with CD45-FITC or CD34-PE-Cy7 antibodies (BD Pharmingen, San Diego, CA, USA), respectively. The cells were washed with phosphate buffered solution (pH 7.4) and incubated in the dark for 1 h at room temperature with CD45-FITC or CD34-PE-Cy7 antibodies (BD Pharmingen, San Diego, CA, USA). The mixture was centrifuged at 1,200 × g for 10 min at 4°C. The assays were performed in 25 cm^2 flasks, and the cells were distributed in the proportion of 2 SM to 1 mesenchymal cell (2:1), approximately 5 × 10^4/ml. The resulting interphase was washed and cultured in Dulbecco's modified Eagle medium-low glucose (DMEM-LG; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (MDgenics, St. Louis, MO) and 5% CO2 incubator at 37°C for four to five passages for cell expansion before transplantation. The MSCs early passage (passage 3) were characterized by flow cytometry and analyzed for their multidirectional differentiation ability.

Flow cytometric analysis

MSCs were washed with phosphate buffered solution (pH 7.4) and incubated in the dark for 1 h at room temperature with CD45-FITC or CD34-PE-Cy7 antibodies (BD Pharmingen, San Diego, CA, USA), following the protocol previously described [12]. The specific fluorescence of 10,000 cells was analyzed on FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using Cell Quest Pro software.

Echocardiography, histological and immunohistochemical (IHC) analysis

Echocardiographic studies were performed in all the experimental animals before DOX administration, 4 weeks after DOX administration, and 4 weeks after cell transplantation [7,8]. Echocardiography was performed using HP Sonos 5500 transducer (Philips medical system, Andover, MA) equipped with a 12 MHz sectorial probe. The parasternal longitudinal image was used for the planimetry of the left ventricle (LV) by the Simpson method to obtain left ventricular end systolic volume (LVES), end diastolic volume (LVEDV) and ejection fraction (LVEF), as previously described [13]. After echocardiography measurements, rats were euthanized by pentobarbital sodium, and their hearts were collected for further analyses [14].

Four weeks posterior to the cell transplantation, after the animals were euthanized by overdosing of pentobarbital sodium, the hearts of animals were harvested, fixed in 4% paraformaldehyde were paraffin embedded and 5 μm sections were stained with hematoxylin and eosin (HE). For IHC staining, slides were warmed up to 60°C for 1 h, deparaffinized in xylene and rehydrated. An endogenous peroxidase block was performed for 10 min in 3% H2O2/PBS and incubated 1 h at room temperature with antibodies specific for the detection of the following proteins: MyoD (1:50; Dako, Cambridge, UK) and von Willebrand factor (1:100; Dako, Cambridge, UK). Detection was performed with an Envision+System-HRP labelled polymer anti-rabbit (K4003, Dako, Cambridge, UK). Sections were imaged by light microscopy.

Statistical analysis

The Statistical analysis was performed using two-way analysis of variance followed by a Turkey multiple comparisons posttest. The P-values of at least<0.05 were considered significant. Data were analysed with the software Statistica v.12.0 (StatSoft Inc.).

Results

The coculture presented typical morphological characteristics of both cell lines 15 days after culture (Figure 1C). The transplanted MSCs were characterized by the expression of CD45 and CD34 surface antigen by flow cytometry (data not show).

The echocardiographic values obtained for each group (CG and CO) before and after the induction of cardiomyopathy were considered as healthy control (baseline), and positive control (DOX induced cardiotoxicity). Our results showed that in both groups, CG and CO, the animals exhibited decrease in heart function 4 weeks after DOX administration, as measured by the echocardiographic evaluation of left ventricular ejection fraction (LVEF%) (50.33 ± 4.52 vs 45.15 ± 2.60; p=0.007 in CG group, and 53.85 ± 4.45 vs 59.16 ± 6.71; p=0.03 in CO group; LVES: 0.23 ± 0.05 vs 0.26 ± 0.08; p=0.03 in CG group, and 0.22 ± 0.04 vs 0.29 ± 0.08; p=0.03 in CO group), 4 weeks after the cell transplantation (Figure 2).

The analysis of the left ventricle sections 30 days after DOX administration by HE light microscopy staining showed a significant tissue injury with cytoplasmatic vacuolization, picnotic nucleus,
apoptotic and necrotic areas in CG group demonstrating that the DOX induced cardiomyopathy was effective (Figures 1A and 1B). The morphological analysis of MSCs and SM by phase contrast microscopy showed that the both cells had mainly spindle and fibroblast-like shapes with two processes that extended in opposite directions from the cell body after 15 days of coculture (Figure 1C). The von Willebrand factor immunohistochemical staining (vWF) of the myocardium section of CO group showed positively stained cells with brown granules indicating the presence of vascular endothelial cell and microvessel 30 days after myocardial combined cells transplantation (Figure 1D).

Figure 2: Echocardiographic evaluation of MSCs.

HE light microscopy staining of myocardium section of CO group showed proliferation of cells with morphologic characteristics of skeletal muscle cells in the myocardial fibers (Figure 1E). This result demonstrates that there was connectivity of SM and cardiac tissue in CO group, which are suggestive of muscle formation. Additionally, the immunohistochemical staining for MyoD in cardiac tissue of CO group revealed the presence of skeletal muscle satellite cells, a kind of adult stem cell responsible for skeletal muscle hypertrophy and regeneration (Figure 1F).

Discussion

Previous studies have shown that chemotherapy by DOX induces cardiovascular alterations by free radical generation and mitochondrial dysfunction, leading to ischemic or idiopathic dilated cardiomyopathy [15,16]. The exact causative mechanisms of DOX-induced cardiotoxicity remain incompletely understood. In other idiopathic dilated cardiomyopathy, as Chagas disease, the main cause of tissue injury is associated with inflammation of the myocardium generating cardiomyocyte loss and ventricular arrhythmias [17].

The present study demonstrated that coculture showed typical morphological and phenotypical characteristics of both cell lines 15 days after culture. These cells were considered to be multipotent and so they are interesting for cell therapy [18-21].

Flow cytometric analyses were employed to mesenchymal stem cell characterization by CD73, CD90, and CD105 positive cell surface marker expression, while being negative for CD34, CD45, and HLA-DR surface markers [12,22].

Echocardiographic findings demonstrated that there was a functional improvement of the LVEF, and LVES after cell therapy, when CG and CO groups are compared (Figure 1). Our results provide evidence that the transplantation of cocultured SM and MSCs contributed to the treatment of DOX-induced cardiotoxicity (CO group). In our previous study, it has been shown that the co-transplant of stem cells and skeletal myoblasts was functionally effective in the Chagas disease ventricular dysfunction, characterized by diffuse fibrosis and impairment of microcirculation [21].

Successful recovery of cardiac function must include restoration of the vascular network and formation of new muscle fibers. Angiogenesis may be induced by MSCs and myoblasts, which secrete proangiogenic growth factors. Satellite cells are muscle precursor cells that are activated by muscle injury and become the primary effectors of muscle repair [23]. In our study, the histopathological analysis presented an increase in myocardial angiogenesis, demonstrated by our immunohistochemical vWF (Figure 1D).

In addition, our data suggested that SM cells enhance engraftment and promote neovascularization in myocardium area, improving cardiac function (Figure 1F). These results are in accordance with recent studies that had reported that transplantation of SM exerts a positive effect on cardiac function [24]. It was also proposed that the survivals of transplanted cells and the myocardial perfusion are increased by induced angiogenesis, and the reciprocal interaction between satellite and endothelial cells within SM may be required for appropriate in vivo cellular function [25].

The results of this study showed that myocardium histological examination exhibited a significant tissue injury with cytoplasmatic vacuolization, picnotic nucleus, apoptotic and necrotic areas. These results supported the findings of previous studies by Carvalho KA et al. showed that DOX treatment increased myocardial cytoplasmatic vacuoles in rat heart [26].

Stem cell transplantation may be considered as an option to prevent or repair cardiac injuries due to promising data from experimental studies involving several heart failure models [27]. Various undifferentiated stem cell sources have been proposed for regenerative medicine, each having their advantages and disadvantages. Autologous SM is acceptable candidates due to their easy isolation, great proliferative potential, and absence of tumorigenicity [28].

Animal studies have demonstrated that the transplantation of autologous SM ameliorated the mechanical function and electrical activity of the animal heart after experimentally induced myocardium infarction [29,30]. In many instances, however, transplanting SM to injured heart area is not enough for repair the cardiac lesion, since angiogenesis also plays an important role in myocardial regeneration [31]. Furthermore, other main disadvantage of SM application is an increased risk of arrhythmias [32].

MSCs are advantageous because they can be isolated from a variety of different tissues, including bone marrow, adipose tissue, and cord blood and can differentiate in different cells lineages, including cardiomyocytes, skeletal myoblasts, chondrocytes, and adipose tissue [33].

However, MSCs cocultured with neonatal cardiomyocytes displayed limited cardiomyogenic plasticity and no significant cardiomyocyte differentiation after transplantation, in a mouse model of myocardial infarction [19]. Thus, MSCs potential clinical benefits for myocardial repair shows to be quite limited when used as unique cell source for cell-based therapy. One of the most interesting MSCs therapy aspects concerns about their angiogenic potential. MSCs transplantation can also contribute to therapeutic revascularization, increasing the blood supply [34]. Although MSCs support this therapeutic process, the relevance of their differentiation into endothelial lineages is still questioned [35].
In this way, the coculture of SM and MSCs seems to be a feasible alternative to restores cardiac function through the replacement of damaged cells by healthy ones and the increase of myocardial revascularization and blood supply [20]. In addition, other advantage of the coculture model is diminish the operational costs of the cultures and expansion [36].

In conclusion, the efficacy and safety of the transplantation of coculture into a rat model doxorubicin-induced cardiotoxicity was demonstrated. The beneficial effects of coculture may be mediated at least in part by their differentiation into muscle and vascular cells, attributed to the abundance of various growth factors such as anti-apoptotic and vascular. However, new researches and further investigations are also require to promote a better understanding about cells coculture treatment protocols and regenerative medicine.

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References


