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Treatment of dilated cardiomyopathy in rabbits with mesenchymal stem cell transplantation and platelet-rich plasma

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ABSTRACT

Dilated cardiomyopathy (DCM) is a major cause of cardiovascular mortality and morbidity, and there is evidence to suggest that stem cell transplantation may be a viable treatment option for this condition. Therefore, the goal of the present study was to assess myocardial regeneration in rabbits with doxorubicin-induced DCM treated with adipose mesenchymal stem cells (MSC) alone or in combination with platelet-rich plasma (PRP). Twenty New Zealand rabbits received doxorubicin for the induction of DCM and were divided into four groups according to treatment: saline, MSC, PRP and MSC + RP. Treatment agents were injected directly into the left ventricular myocardium following a thoracoscopy. Rabbits were assessed through echocardiographic and electrocardiographic examinations, as well as serum cardiac troponin I measurements at baseline, after the induction of DCM and 15 days after treatment. Animals were euthanased following the last assessment, and hearts were collected for histopathological analyses.

The MSC group showed improvements in all parameters assessed, while the PRP group showed significantly impaired heart function. Histopathology of the heart revealed that the MSC group displayed the lowest number of lesions, while rabbits in the MSC + PRP, saline and PRP groups had steadily advancing lesions. These results suggest that MSC transplantation can improve heart function in rabbits with DCM, and underscore the need for further studies of the effects of PRP on the myocardium.

Introduction

In spite of significant advances in medical and surgical care, congestive heart failure is still one of the main causes of cardiovascular morbidity and mortality. Dilated cardiomyopathy (DCM) is a primary myocardial disease of unknown aetiology, characterised by a loss of cardiomyocytes and an increase in fibroblasts, as well as a common cause of heart failure. Although both myocyte mitosis and cardiac precursor cells have been found in adult hearts, the death of a large number of cardiomyocytes can still result in heart failure. As such, restoring cardiomyocyte levels may be an adequate treatment strategy for DCM (Nagaya et al., 2005).

Mesenchymal stem cells (MSC) help repair damaged myocardial tissues through several mechanisms, the main one being the production of repair factors, which increase the local expression of growth factors and cytokines. MSC may also suppress local inflammation, repair damaged cells and contribute to the creation of a favourable environment for endogenous tissue repair. These findings have established MSC as a promising new treatment approach for several cardiovascular conditions (Souza et al., 2010).

MSC are generally applied to an aqueous medium, from which they can be easily isolated, especially when they come into direct contact with the circulatory system. Stem cells are combined with scaffolds to optimise the patency of the implanted grafts (Huang et al., 2005). Platelet-rich plasma (PRP) is an autologous blood product which contains a high concentration of growth factors. PRP has been widely used in the healing of skeletal muscle, for which it has proven to be a safe and effective treatment. In spite of growing evidence of the safety and efficacy of PRP, few studies have analysed its effects on cardiovascular tissues (Mischra et al., 2010).

Therefore, the goal of the present study was to assess myocardial regeneration in rabbits with doxorubicin-induced DCM treated with adipose MSC, either with or without the use of PRP as a scaffold.

Materials and methods

Animals

Twenty-one New Zealand rabbits (*Oryctolagus* cuniculus), comprising a male donor and 20 females, aged between 3 and 4 months, weighing 2–3.5 kg, were used in this study. The animals received doxorubicin to induce heart failure and were divided into four groups containing five rabbits each, which were labelled according to the treatment received: MSC resuspended in PRP (MSC + PRP group), PRP (PRP group),

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MSC suspended in a culture medium (MSC group) and 0.9% sodium chloride solution (NaCl group). The number of MSC used was in the order of 10^6 cells/animal. PRP-treated animals received a 1 mL of the substance.

Rabbits are particularly susceptible to the cardiotoxicity of doxorubicin and this experimental model pioneered studies on the pathophysiology of heart failure. This experimental model develops lesions similar to those described in humans, including cytoplasmic vacuolisation, interstitial edema and myofibrillar rupture. All animals were housed and handled according to Brazilian Animal Experimentation Code and Animal Research Ethics Committee guidelines, based on the law 11.794, of October 8, 2008. This study was approved by the Research Ethics Committee of the Porto Alegre Clinical Hospital (HCPA) (protocol number 11-0279).

Adipose tissue collection

A rabbit was premedicated with tramadol chlorhydrate 5 mg/kg (União Química), midazolam 1 mg/kg (Dormonid, Roche) and ketamine 20 mg/kg (Cetamin, Syntec), administered intramuscularly (IM), followed by isoflurane (Isoforine, Cristália) to facilitate orotracheal intubation, and maintained with isoflurane and 100% oxygen. The animal was placed in a sternal decubitus position and, after antisepsis, adipose tissue was collected from the interscapular region. The skin was then sutured.

Adipose MSC isolation

The adipose tissue was placed in type I collagenase solution (1 mg/mL) in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 9 mM HEPES, for 1 h at 37 °C to facilitate tissue digestion. After digestion, the collagenase was inactivated by dilution with DMEM containing 10% foetal bovine serum (FBS, Invitrogen). Once isolated, cells were cultivated in a low-glucose DMEM medium supplemented with 9 mM HEPES, 20% FBS and an antibiotic solution containing 100 U/mL penicillin and 100 mg/mL streptomycin. The culture was kept at a constant temperature of 37 °C, in an atmosphere of 5% CO_2 and 100% humidity. After 24 h, the culture medium was aspirated and replaced by fresh medium. When the culture reached 80% confluence, the adherent cells were removed from the dishes by the addition of 0.5% trypsin/EDTA (Gibco). The material was then placed in DMEM supplemented with 10% FBS (complete medium).

In the second passage, MSCs were added to FBS supplemented with 5% dimethylsulfoxide (DMSO) and stored in a freezer at –80 °C. Approximately 1 week before the transplant, the cells were thawed and expanded. The transplants were performed using cells between the third and fourth passages. MSCs were classified using in vitro morphology and differentiation into chondrogenic, osteogenic and adipogenic lineages.

Doxorubicin-induced dilated cardiomyopathy

DCM was induced using doxorubicin chlorhydrate (Glenmark), which was applied once a week at a dose of 2 mg/kg in the first 2 weeks and a dose of 3 mg/kg in the last 2 weeks, reaching a cumulative dose of 10 mg/kg. Rabbits were first sedated with midazolam (2 mg/kg) and ketamine (10 mg/kg) IM, after which doxorubicin was injected intravenously (IV). The animals were assessed before and after the induction of DCM as well as 15 days after treatment using echocardiography (Mylab 30 Vet bi-dimensional echocardiography equipment), electrocardiograms (TEB PC Vet electrocardiograph) and serum cardiac troponin I concentration (Boehring Diagnostics Opus Plus Kit). Electrocardiography (heart rate, QRS morphology and duration) and echocardiography (systolic left ventricular diameter, shortening and ejection fractions) results were recorded as the mean of three measurements made by an examiner blind to group assignment.

PRP preparation

Prior to the surgical procedure, 10 mL blood was collected from the central ear artery of each animal, placed in a plastic tube containing sodium citrate and centrifuged at 300 g for 10 min. A total of 500 μL was then collected from the top layer of the plasma and placed in a separate sterile 15 mL tube labelled with the letter A, containing 150 μL calcium gluconate. The remaining plasma in the upper and intermediate layers was placed in another sterile plastic tube, labelled with the letter B. Both tubes were centrifuged at 640 *g* for 10 min.

After centrifugation, half of the material in tube B was discarded, and the remaining solution was homogenised. Two mL of the contents of tube B and 1 mL of the material drawn from tube A were then placed in a 2:1 ratio (2 mL PRP:1 mL thrombin) in another sterile plastic tube labelled with the letter C. One mL of the material in tube C was then placed in a microtube, at which point the PRP, with or without the MSC, was prepared for implantation into the myocardium.

Video-assisted thoracoscopy

Rabbits were premedicated with pethidine chlorhydrate 3 mg/kg (União Química), midazolam 0.7 mg/kg and ketamine 14 mg/kg, administered IM. Anaesthesia was induced using an isoflurane mask and maintained by orotracheal intubation with vaporised isoflurane in 100% oxygen. After antisepsis, the chest cavity was accessed through an incision made above the sixth left intercostal space, 5 cm ventral to the costovertebral region, and a 5 mm trocar was used to perforate the pleura for the insertion of a rigid endoscope. In the fourth intercostal space, between the costochondral and sternal regions, a 13 × 4 mm hypodermic needle attached to a 1 mL syringe was used to inject the treatment agents into the least vascularised region of the left ventricular wall. The incision was closed and negative intrathoracic pressure was then reestablished. In the postoperative period, animals were treated with tramadol chlorhydrate 3 mg/kg subcutaneously (SC) and enrofloxacin 10 mg/kg (Zelotril, Agener) IM for 72 h.

After the 15-day assessment period, all rabbits were euthanased and their hearts were collected for examination. These were fixed in 10% buffered formalin for 24 h, after which they were embedded in paraffin wax and stained with haematoxylin and eosin (H&E). The hearts were investigated histopathologically for sarcoplasmic vacuolisation, myofiber necrosis and fibrosis.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using the SPSS software (v.18.0), and $P < 0.05$ was considered statistically significant. Electrocardiography and echocardiography data were subjected to a three-factor repeated measures analysis of variance (ANOVA). Troponin concentrations were assessed through a two-way repeated measures ANOVA assuming a symmetrical component correlation matrix between assessments. Statistically significant analyses were followed by Tukey post-hoc tests. Histopathological data were assessed through non-parametric Kruskal–Wallis tests, followed by Bonferroni post-hoc tests.

Results

The troponin I, echocardiography, electrocardiography and histology data indicated that DCM was successfully induced in all rabbits. The same exams were used to assess the results of the treatments administered.

Rabbits showed an increase in serum troponin I over time (Fig. 1). The most pronounced increase in troponin I at euthanasia was observed in the PRP group, followed by the saline group, the MSC + PRP group and, lastly, the MSC group. The presence of heart lesions was determined by the presence of troponin I concentrations >0.05 ng/mL (Alvarez et al., 2012).

The electrocardiography results showed no significant between-group differences in QRS configuration, although its duration increased throughout the experiment in the saline and MSC + PRP groups, while the remaining groups displayed an increase in this value followed by a decrease. Nonetheless, all QRS values were within the expected physiological range for the species (Fig. 2a).

Fig. 2b illustrates the changes in systolic left ventricular (LV) diameter observed in each treatment group throughout the experiment. At the pre-euthanasia assessment, LV diameter was found to have increased in the saline group, but decreased in the other treatment groups. The ejection fraction decreased over time in the MSC + PRP group, and was found to first decrease, then increase in the other three treatment groups, with this pattern being more pronounced in the MSC group (Fig. 2c). Fig. 2d shows a decrease in the shortening fraction following the induction of cardiomyopathy by doxorubicin, and an increase in this value following surgery in the MSC, MSC + PRP and PRP groups. The saline group displayed a decrease in the SF over the course of the study.

Histological analysis showed extensive sarcoplasmic vacuolisation with isolated myofiber necrosis and diffuse myocardial fibrosis (Fig. 3). Furthermore, in several animals in all groups, locally extensive myocardial fibrosis, attributed to the impact of the intracardiac injection, was also observed. The PRP group had statistically more lesions than the saline group. The MSC group had the best histological results out of all analysed groups. Data regarding between-group differences in histological findings ($P = 0.010$) can be found in Table 1. Significantly more histological alterations were found in the myocardium of PRP-treated rabbits than in MSC ($P = 0.007$) and MSC + PRP-treated ($P = 0.007$) animals (Table 2).