Laboratory Research

Cardiac atrioventricular conduction improved by autologous transplantation of mesenchymal stem cells in canine atrioventricular block models

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Objective Atrioventricular block (AVB) is a common and serious arrhythmia. At present, there is no perfect method of treatment for this kind of arrhythmia. The purpose of this study was to regenerate cardiac atrioventricular conduction by autologous transplantation of bone marrow mesenchymal stem cells (MSCs), and explore new methods for therapy of atrioventricular block. Methods Eleven Mongrel canines were randomized to MSCs transplantation (n=6) or control (n=5) group. The models of permanent and complete AVB in 11 canines were established by ablating His bundle with radiofrequency technique. At 4 weeks after AVB, bone marrow was aspirated from the iliac crest. MSCs were isolated and culture-expanded by means of gradient centrifugal and adherence to growth technique, and differentiated by 5-azacytidine in vitro. Differentiated MSCs (1ml, 1.5×10^7 cells) labeled with BrdU were autotransplanted into His bundle area of canines by direct injection in the experimental group, and 1ml DMEM in the control group. At 1-12 weeks after operation, the effects of autologous MSCs transplantation on AVB models were evaluated by electrocardiogram, pathologic and immunohistochemical staining technique. Results Compared with the control group, there was a distinct improvement in atrioventricular conduction function in the experimental group. MSCs transplanted in His bundle were differentiated into analogous conduction system cells and endothelial cells in vivo, and established gap junction with host cardiomyocytes. Conclusions The committed-induced MSCs transplanted into His bundle area could differentiate into analogous conduction system cells and improve His conduction function in canine AVB models. (J Geriatr Cardiol 2007;4:238-243.)

Key Words mesenchymal stem cells; His bundle; cell transplantation; differentiation; 5-azacytidine

Introduction

Advanced atrioventricular block (AVB) is a severe arrhythmia which requires implantation of permanent pacemakers. While modern pacing technology has developed to a high level, pacemaker implantation for the treatment of AVB still has a lot of limitations. Recent studies have shown that adult stem cells can proliferate, and also cross-differentiate according to the external environment. Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells found in adult bone marrow, which show stable cell phenotype, easy separation and culture, self-reproduction, multilineage differentiation and high proliferation potential. MSCs can differentiate into muscle cells, nerve cells, marrow stroma support cells and other mesenchymal tissue cells. Because MSCs have many advantages, such as easy separation and culture, multilineage differentiation, massive proliferation, and no immune rejection, there is a great potential for clinical application in repairing myocardial injury. This study aims to use bone marrow MSCs for treating atrioventricular block, and to explore new ways of biological interventional treatment for bradycardia arrhythmia.

Methods

Design of experimental animals

Eleven Mongrel canines, male or female, weight 25-30kg, were randomly assigned to two groups. In the experimental group (n = 6) the dog’s His bundle was severed by radiofrequency ablation, creating a permanent third degree AVB model. Then from the bone marrow, MSCs were separated and induced to differentiate into cardiac myocytes. At four weeks after radiofrequency ablation, thoracotomy was performed, and differentiated cells (1 ml, 1.5 × 10^7 cells) labeled with BrdU (BrdU labeling and detection kit ¢à Roche) were injected into the His bundle ablation area at multi-point site. In the control group (n = 5) the dogs underwent radiofrequency ablation of the His bundle, causing permanent third degree AVB. After four weeks, thoracotomy was performed and 1 ml DMEM (Dulbecco’s Modified Eagle Medium) was multi-point injected into the His bundle ablation site. After 12 weeks, atrioventricular conduction...
function, cell proliferation and differentiation, and cell connectivity were evaluated in both groups.

**Preparation of third degree AV block model**

All experimental dogs were given ketamine anesthesia, artificial respirators-assisted breathing, and ECG monitoring and records (Multi-channel physiological recording device: NIHON KOHDEN P1100). The right femoral vein was isolated and opened with a small incision. A 7 F sheath was inserted into the femoral vein, and radiofrequency catheter was advanced to the right atrium under fluoroscopic guidance. His bundle was ablated by radiofrequency current (Radiofrequency ablation device: KYKY-RFG II, Beijing Inter Grafx Equipment Company, Beijing, China), power 20 W, discharge time 30-60 s. Then an ideal animal model with third degree atrioventricular block model was created and ready for testing (Figure 1).

**Bone marrow aspiration, culture and differentiation of marrow mesenchymal stem cells**

Two weeks after, the ECG showed a stable third degree atrioventricular block. Under general anesthesia, 10ml of canine autologous bone marrow fluid were collected into an anti-coagulation tube. The fluid was diluted and bluntered with contour DMEM (10ml), and centrifuged at 600 g × 30 min with Percoll solution (density 1.063 g/ml). The liquid interface flocculi were extracted, centrifuged and washed. The sediments with rich mononuclear cells were suspended in 5 ml DMEM with 10% fetal bovine serum(FBS). The isolated cells were transferred into a 75 ml flask, and cultured in incubator at 37°C, 5% CO2. After 24 h incubation, the medium was replaced for removal of red blood cells, hematopoietic stem cells and other non adherent bone marrow stem cells. The culture medium was replaced every three days. After bone marrow MSCs were cultured for 24 h, myocytes-induced agent 5-azacytidine (20 mmol / L)(Sigma Corporation) was added to the cell medium and incubated cells for 48 h. Then the medium with 5-azacytidine was discarded, and the MSCs continued to be cultured for 2 weeks in ordinary medium (added 5% fetal bovine serum). Then, MSCs were sub-cultured for 3-4 weeks in 24-well plates, with low-density (5000cells/well) inoculation and low concentrations of serum (2% FBS) medium. The formation of muscular tube was observed under optical microscopy. The cells immunochemical staining for vWF (factor related antigen) and α-actinin (primary antibody from Beijing Zhongshan Biotechnology Ltd.), myosin (Sigma Corporation), α-actin (Sigma Corporation), troponin I (Sigma Corporation) and connexin 43 (Santa Cruz Biotechnology, Inc.) were carried out. The ultrastructure of differentiated stem cells was observed under electron microscope (transmission electron microscope: JEOL JEM-1010).

**BrdU labeling of transplanted bone marrow stem cells**

Bone marrow cells were induced to proliferate for 2 weeks, and cell volume reached 1.5 × 10^7. Then the transplanted cells were labeled with BrdU, which can be incorporated into thymidine site of DNA, and detected rapidly by BrdU antibody with fluorescent dyes. The specific method was that BrdU (10 mol /L) was added to cellular medium, incubated with cells for 12 h, and incorporated in nucleus during cellular proliferative phase. BrdU labeling rate was detected by BrdU antibody staining.

**Cell preparation before transplantation**

Phosphate-buffered saline (PBS) was used for removing BrdU without incorporation. The stem cells in flasks were digested and isolated with 0.125% trypsin, and centrifuged 3 times for rinsing BrdU. The harvested stem cells (about 1.5 × 10^7 cells) were added to 1 ml DMEM and stored in the sterile tube for preparation.

**Autologous bone marrow stem cell transplantation**

The experimental dog was anesthetized with ketamine; after the left pleura was opened, the heart was exposed with the superior vena cava, inferior vena cava and azygos vein blocked. The right atrium of the dog was opened and blood in right atrium was removed with aspirator for fully exposing the right atrium wall. The mesenchymal stem cells (1 ml, 1.5 × 10^7 cells) were multi-point injected into the His bundle branch node and surrounding area with 27G needle. In the control animal, 1ml DMEM medium was injected instead. After the procedure, antibiotics were given to each animal.

**Detection of AV conduction function and treatment of experimental animals**

One to 12 weeks after autologous stem cells or placebo transplantation, regular ECG examination was done in each animal to determine their AV conduction function and recovery.
Myocardial histological and immunohistochemical studies 4, 6, 7

Twelve weeks after autologous cell transplantation, the animals were sacrificed to obtain tissue samples from the transplanted areas. The tissue was fixed with 10% Formalin, then was embedded for staining: routine hematoxylin and eosin staining for general observation of radiofrequency ablation zone and cell transplantation zone; BrdU immunohistochemistry staining (BrdU labeling and detection kit Æ Roche) for observation of survival and proliferation of transplanted stem cells in His bundle zone; BrdU and vWF double-staining for observing new vessels and vascular cell differentiation of transplantation stem cells; BrdU and troponin I double-staining for observation of stem cells differentiation state; BrdU and connexin 43 double-staining to observe gap junction between the transplanted cells and the host cells.

Results

Preparation of 3rd degree atrioventricular block models

Stable third degree atrioventricular block was successfully created in all 11 experimental dogs. At four weeks after model preparation, third degree AV-B is still being evidenced by electrocardiogram.

Culture and differentiation of mesenchymal stem cells in vitro

The bone marrow MSCs were separated by Percoll gradient centrifugation. The MSCs were of spindle shape, tightly adherent and producing proliferation (Figure 2). The hematopoietic stem cells which were round and not adherent, were removed. At 7 days, almost all the cultured cells were spindle-shaped MSCs (Figure 3). After adding 5-azacytidine for 14 days, bone marrow MSCs showed no obvious morphological changes. After continuous training for 21 to 28 days, stem cells had fusion phenomenon, evolving from the spindle into polygonal, circular, and connecting with each other to form myotubes. After application of specific muscle cell antibody for immunocytochemical staining, about 70-80% cells showed A-actin, A-actinin, myosin and troponin I positive cells (Figure 4). Under electron microscope, the cells showed spindle, nuclei oval in the center, and had

![Figure 2](image2.png) A single mesenchymal stem cell isolated from canine bone marrow liquid (× 200).

![Figure 3](image3.png) The mesenchymal stem cells presented fusion, a spindle-shaped, spiral growth after single bone marrow stem cell was cultured for seven days (× 200).

![Figure 4](image4.png) 5-Aza induced bone marrow mesenchymal stem cells into myocardial cells, and its specific antigen troponin I was positive (× 200).

![Figure 5](image5.png) 5 - Aza induced bone marrow mesenchymal stem cells in vitro to differentiate into cardiac cells, electron microscope showed myofilament (× 20 000).
myofilament and atrial particles in cytoplasm (Figure 5). BrdU labeling rate was 80±12% in vitro.

**Recovery of atrioventricular conduction function**

The atrioventricular function in two of six dogs with autologous bone marrow cell transplantation improved from third degree to second degree atrioventricular block, or 3:1 or 4:1 conduction. In the control group, the atrioventricular function did not improve, and the ECG still showed third degree AVB in all five dogs at 12 weeks.

**Histological features at stem cells transplantation area**

Histological features at stem cells transplantation area. There was coagulation necrosis with fibrous tissue hyperplasia in the radiofrequency ablation zone of the His bundle. In the control group, it showed fibrosis with low vascular density and no BrdU labeling cells. The island newborn myocardial cells and BrdU-positive cells can be seen in the experimental group (Figure 7). The newborn BrdU-positive cells had troponin I positive staining (Figure 8), which showed that bone marrow mesenchymal stem cells had been differentiated into cardiac myocytes. There were a lot of new vessels with BrdU-positive endothelial cells in stem cells transplantation zone (Figure 9); the endothelial
cells were vWF-positive staining. These images proved that vascular endothelial cells were derived from bone marrow cells. There were positive connexin 43 staining between transplantation bone marrow cells (BrdU-positive) and the host myocardial cells (Figure 10), indicating that gap junction had been set up between myocardial cells and newly implanted MSC. There were no infiltrating lymphocytes and immune exclusion phenomenon in the cells transplantation area. The cartilage, bone cells, fat cells and tumor-like cells had not been seen in the same transplantation area.

The relationship between the immune histological changes and atrioventricular improvement

BrdU labeling positive newborn island myocardial cells and vascular endothelial cells were found at His bundle area in all experimental dogs, but not found in the control group. Atrioventricular function was improved in 2 of 6 experimental dogs, but not improved in all control groups, which still had third degree atrioventricular block in ECG.

Discussion

Atrioventricular block is a severe brady-arrhythmia. Although the pacemaker can solve certain problems for this condition, there are a lot of limitations. Bone marrow MSCs transplantation has many advantages in repair of myocardial injury. Therefore, the application of bone marrow MSCs transplantation for the treatment of atrioventricular block could be a very attractive method of treatment. The results of our study showed that MSCs in vitro could be indeed induced and differentiated to cardiomyocytes with typical cardiac myocyte structure, pacing and cardiac conduction function. Such differentiation cells showed surface receptor function, and could be regulated by neurohormones. In theory, differentiation and transplantation of bone marrow MSCs in the His bundle zone could substitute injured conduction cells and play its conduction function, because therapy of brady-arrhythmias by bone marrow MSCs has its safety and feasibility. MSCs could differentiate to myocardial cells with mature structure and function in vivo and in vitro. The differentiation cells have sinus-like and myocyte-like potential in patch-clamp detection. The differentiated cells can automatically beat and automatically depolarize. These cells have connexin 43 expression, form gap connection with surrounding host myocardial cells and electricity conduction. MSCs transplantation can improve or restore nerve conduction function in animal models of spinal cord injury.

In our study, we transplanted bone marrow MSCs to His bundle area of experimental animal models with the third degree atrioventricular block, and expected that bone marrow MSCs could differentiate to mature cardiomyocytes with conduction function for substituting injured His bundle by means of role of microenvironment in vivo and in vitro. The results of this study showed that MSCs transplanted in injured His bundle zone could differentiate to His-like cells with myocyte structure and conduction function in the dual role of the internal and external environment. Transplanted stem cells not only differentiated to special myocardial cells with typical phenotype and vascular endothelial cells, but constructed gap connection with host myocardial cells, and played a conduction role substituting His bundle. Although these results were still preliminary, they were very promising and indicated that further study was necessary to firmly establish a new treatment method of complete atrioventricular block. Because bone MSC can be easily obtained, a large number of amplification in vitro, its use without immunosuppression, and no social ethics problems, they could have significant strengths and potential in clinical application of brady-arrhythmias treatment.

Conclusion

The committed-induced MSCs transplanted into His bundle area could differentiate into analogous conduction system cells and improve His conduction function in canine AVB models. Although the results were very promising, the preliminary results indicated that further study was necessary for establishing a new treatment of complete atrioventricular block.

Acknowledgement

This study was supported by National Natural Science Foundation (approved No. 30370567; No 30170374).

References

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