

Effect of Bone Marrow Mononuclear Cells Transplantation on Acute Myocardial Infarction

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Abstract: **Objective** To investigate whether uninduced autologous bone marrow mononuclear cell (ABM MNC) could survive and differentiate into myocardial cells and endothelial cells in the infarcted heart. **Methods** 40 male big-ear Japanese rabbits were divided into two groups randomly: the transplanted group (n=20) and the control group (n=20). The model of acute myocardial infarction was made by left anterior descending artery ligation, which was confirmed by ECG. The cardiac function was evaluated by the echocardiography. 7 days later, BrdU labeled ABM MNCs were injected into infarcted and marginal area myocardium in the transplanted group, while the control rabbits were injected with saline. 6 weeks later, the hearts were harvested for histology and immunohistochemistry evaluation. **Results** In the transplanted group, viable cells labeled with BrdU could be identified in the infarcted area, and myocytes and endothelial cells labeled with BrdU can also be found in the border area, these cells demonstrate myogenic differentiation with the expression of α -Actin by immunostaining. Moreover, the vessel density of the transplanted group in the borders of the infarction was higher than the control group ($P < 0.05$), but there was no difference in the infarcted areas between two groups ($P > 0.05$). At the 6 weeks after experiment, the cardiac function was improved in both groups, but the transplanted group improved more than that in the control group ($P < 0.05$). **Conclusion** Autologous bone marrow mononuclear cells injected into the infarcted myocardium could survive in both the infarcted and the border areas, differentiated into endothelial cells and other cells which have obtained the characters of myocytes, and increase the vessel density in border area, improved the cardiac function.

Key words: bone marrow mononuclear cells; autologous; cell transplantation; myocardial infarction

自体骨髓单核细胞移植对急性心肌梗死的作用

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[摘要] 目的 探讨未经诱导的自体骨髓单核细胞可否在梗死心肌环境中存活并分化为心肌细胞及血管内皮细胞。方法 40只日本大耳雄兔随机分为两组:移植组及对照组,每组各20只。采用结扎冠状动脉左前降支的方法建立急性心梗模型,以心电图证实模型成功,由超声心动图评价心功能。模型建立后7天,将BrdU标记的自体骨髓单核细胞注射到移植组动物心肌梗死区及周边区,而对照组动物相同部位仅注射等量生理盐水。移植后6周,收集动物心脏进行组织学及免疫组化分析。结果 抗BrdU免疫组化发现移植组动物心肌梗死区及周边区内均存在染色阳性的移植细胞,且周边区内的移植细胞呈心肌细胞及血管内皮细胞的形态特点,同时这些细胞抗心肌特异性肌动蛋白抗体染色阳性,证实其肌源性分化。另外,移植组动物梗死周边区血管密度显著高于对照组($P < 0.05$),但两组动物在心肌梗死区内的血管密度没有统计学差异($P > 0.05$)。移植后6周,两组动物心功能均有改善,移植组明显优于对照组($P < 0.05$)。结论 自体骨髓单核细胞移植于梗死心肌后,可在梗死区及周边区存活,并在周边区分化为血管内皮细胞及具有心肌细胞形态特点,增加梗死周边区的血管密度,改善心功能。

[关键词] 骨髓单核细胞;自体;细胞移植;心肌梗死

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With the development of pharmacological treatments and mechanical interventions, the mortality of

myocardial infarction has been decreased remarkably. However, these methods could not rescue the infarcted myocardium, studies have been focused on the cellular cardiomyoplasty^[1] (CCM) recently. Since Wakitanis^[2] and his colleagues found that bone marrow-derived mesenchymal stem cells induced by 5-azacytidine could differentiate into myogenic cells in 1995, 5-azacytidine was applied in most vitro experiments about regeneration of myocardium. However,

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5-azacytidine belongs to demethylation agent that can cause an out-put-control upregulation of a wide variety of genes^[3], which limit its clinical application. Therefore, we hypothesized that autologous bone-marrow mononuclear cells (ABM-MNCs) without pretreatment could differentiate into the myocardial cells and endothelial cells, expecting to provide evidence for its clinical applications.

1 Subjects and Methods

1.1 Animals Male big-ear Japanese rabbits (1900 ~ 2100 g) were obtained from the Animal Experimental Center of the Second Hospital Of Harbin Medical University (HeiLongJiang province, China), which were divided into two groups randomly: the transplanted group and the control group, 20 animals in each group.

1.2 Animal Model Under 1% pentobarbital (30 mg/kg) anaesthesia by injection via auricular vein, the heart of the rabbit was exposed and the left anterior descending coronary artery was ligated. Before and after the ligation, the electrocardiography (ECG) was monitored to make sure the establishment of myocardium infarction.

1.3 ABM-MNCs Preparation And Labeling 6 d after the myocardial infarction, BM-MNCs were derived from bone marrow aspirates of themselves. Then ABM-MNCs were isolated by density gradient centrifugation (2000 r/min, 20 min) on Lymphocyte Separation Medium (1.077 g/L, the Blood Research Institute of Chinese Academy of Medical Science), followed by twice rinse (1500 r/min, 5 min). For the cell-labeling procedures, ABM-MNCs were cultured with BrdU (10 μ mol/L, Sigma, USA) in 1640 culture medium (Hyclone, Utah, USA) containing 20% fetal bovine serum (H & Y Bio.Co.Ltd, Tianjin, China) at 37 °C in a humidified atmosphere 5% carbon dioxide for 24 h.

1.4 Implantation Of ABM-MNCs 7 d after the ligation, ABM-MNCs labeled with BrdU were harvested, and were finally resuspended in 0.7 ml saline, a small fraction of which were used for cell counting and viability measuring with Trypan Blue. The average viability was 98.87%. After that, anesthesia was induced and the heart was exposed again through the former thoracotomy incision. Then, the ABM-MNCs suspension were injected into the infar-

tion and the marginal area, 16 injections (mean of 3.41×10^7 cells/rabbit) were delivered. While the control rabbits only accepted the injection of equal volume saline solution.

1.5 Evaluation Of The Cardiac Function Color-Doppler Echocardiography was used to evaluate the cardiac function before and 1 d after the myocardial infarction and 6 weeks after the implantation. The data included ejection fraction (EF), fractional shortening (FS), left ventricular wall extent (LVWE), end-systolic thickness of left ventricular wall (LVWTs), end-diastolic thickness of left ventricular wall (LVWTD), thickening fraction (ΔT) and heart rate (HR).

1.6 Histopathology And Immunohistochemistry 6 weeks after the implantation, all rabbits were killed by overdosing with pentobarbital (100 mg/kg), the hearts were harvested, fixed with 10% buffered formaldehyde and embedded in paraffin. Some sections (4 μ m in thickness) were stained with hematoxylin and eosin for morphology, cytoplasm and so on. Other sections were mounted on a set of poly-L-lysine-coated (Sigma, USA) glass slides to ensure immunohistochemistry procedure. Sections were incubated with mouse monoclonal anti-BrdU (Sigma, USA) and rabbit polyclonal anti-actin (Boster Biotechnology Co.Ltd, Wuhan, China) antibody, TRITC-conjugated affinipure goat anti-mouse IgG and FITC-conjugated affinipure goat anti-rabbit IgG were used for the second antibody, respectively.

1.7 The Count Of Vessel Numbers Randomly selected 8 high powered microscopic fields (HPF) were analyzed in every H-E section, and the average vessel numbers per HPF were counted.

1.8 Statistical Analysis The data were presented as ($\bar{x} \pm s$). Student *t* test were used for the statistical analysis, $\alpha = 0.05$.

2 Results

2.1 Evidence Of Myocardial Infarction ECG showed continuously R wave reduced voltage, ST segment elevated gradually, and Q wave developed in the relative leads at the end of ligation, the myocardial infarction was confirmed. In addition, the echocardiography showed that EF, FS, LVWE, LVWTs, LVWTD, ΔT decreased ($P < 0.05$) and HR increased ($P < 0.05$) obviously 24 h after the myocardi-

al infarction compared to pre-ligation .

2.2 The Evaluation Of The Cardiac Function The cardiac function decreased ($P<0.05$) remarkably after the ligation and improved 6 weeks after the im-

plantation in both groups , but the cardiac functions of the transplanted group was better than that of the control group ($P<0.05$) evidently (Table 1) .

Table 1 . Comparison of Cardiac Function between Two Groups Six Weeks after Implantation

Groups	EF(%)	FS(%)	LVWE(mm)	LVWTs(mm)	LVWTD(mm)	Δ T(%)	HR(/ min)
Control	51 .67 ±3 .87	23 .53 ±2 .41	1 .86 ±0 .31	2 .88 ±0 .33	1 .84 ±0 .23	33 .41 ±4 .28	269 .75 ±5 .21
Transplanted	61 .61 ±4 .90 ^a	30 .02 ±2 .90 ^a	2 .32 ±0 .33 ^a	3 .38 ±0 .18 ^a	2 .08 ±0 .15 ^a	38 .02 ±5 .19 ^b	249 .08 ±28 .52 ^b

NOTES : a . $P<0.005$; b . $P<0.05$.

2.3 Histopathology And Immunohistochemistry

Immunochemistry with anti-BrdU antibody showed that the positive cells were found in the infarcted and the border areas 6 weeks after acceptance the ABM-MNCs transplantation . But the positive cells in infarcted area didn't show the morphology of the cardiomyocytes , but the fibroblasts (Fig.4.1 on the third cover) ; in the border area , some of the positive cells showed the modality of cardiomyocytes , others showed the appearance of the endothelial cells (Fig. 4.2 on the third cover) . The shape of the positive cardiomyocytes were similar with the host cardiomyocytes , there were no edema and cardiomyocytes necrosis , which existed in some host cardiomyocytes in the border area . There were no difference of morphology between the positive endotheliocytes and host ones . While , we didn't find the positive cells in control group . Immunohistofluorescence results : the positive cells which nuclei were stained with red fluorescence of anti-BrdU showed negative result when their cytoplasm stained with green fluorescence of anti-Actin (Fig.4.3 on the third cover) in the infarcted area ; in contrast , in the border area some BrdU positive cells showed both nuclear and cytoplasmic staining (Fig.4.4 on the third cover) , but they had a large nucleus-to-cytoplasm ratio .

2.4 The Count Of Vessel Numbers The capillary numbers were counted under the same magnification in H-E sections , the results showed that the vessels of the infarction center , the border zone and the distant myocardium in transplanted group were all higher than those in the control group , but the difference in the infarction area between two groups had no significance(Table 2) .

3 Discussion

Currently several donor cell sources^[4-5] have been used for cellular cardiomyoplasty (CCM) . The

haematopoietic cells and the mesenchymal stem cells are also contribute to the repair of the damaged organ . The hemangioblasts could participate in the neovascularization , the mesodermal progenitor cells could differentiate into the endothelial cells , the endothelial progenitors could differentiate into the cardiomyocytes^[6] , BM-MNCs are the most promising donor cells for CCM and have been used widely . Hence , we have performed the primary study on the model of rabbits' acute myocardial infarction .

Table 2 . The Vessel Count in Different Location o Both Groups Six Weeks After The Implantation

Groups	Infarction Area	Border Area	Distant Area
Control	10 .01 ±9 .89	35 .55 ±16 .22	14 .33 ±8 .50
Transplantation	15 .42 ±14 .12	80 .61 ±45 .38 ^a	27 .33 ±10 .02 ^a

NOTES : a . $P<0.05$.

The best time of stem cell transplantation has not been clear , however , we selected the 7 d after the myocardial infarction in order to avoid the inflammatory response , and could provide ABM-MNCs an better circumstance for their differentiation , because the plasma levels of vascular endothelial growth factor (VEGF) are significantly elevated , peaking on 7th day after acute myocardial infarction (AMI)^[7] . Although the exact source isn't clear , it is possible that ischemia cardiac tissues secrete VEGF because the promoter sequence of the VEGF gene contains hypoxia-responsive elements^[8] . Another study showed that the myocardial VEGF expression was enhanced in patients with AMI^[9] . In addition , the granulation tissue have developed enough capillaries at the same time , which can provide blood supply for the grafted ABM-MNCs . Therefore , we thought that the transplantation on the 7th day is benefit for the survival and differentiation of the grafted ABM-MNCs , especially the differentiation into the vessels .

We have observed that the survival grafted cellular cytoplasm in the infarction area did not express the

Actin through the fluorescence microscopy compared with normal cardiomyocytes, and the shapes were different to the host cardiomyocytes, but similar to the fibrous tissue nearby. While in the border area, the morphology and the Actin expression level of the grafted cells were similar to the normal cardiomyocytes. In addition, we could find few the endothelial cells derived from ABM MNCs through the immunohistochemistry of anti-BrdU antibody in the infarction area, which confirmed the result of vessel count: the difference of the infarction area between two groups was not significant ($P > 0.05$), but in the border area, there were more vessels in the transplantation group than the control group ($P < 0.05$). These results all supported the opinion that the certain local circumstances could induce the differentiation of the grafted cells^[10].

Another important point was that although the data of cardiac function in the transplantation group were better than the control group, the cardiac function of the control group also improved with statistical significance 6 weeks after only saline transplantation. We consider that this may be related to the delivery approach. In a relative study^[11], Yang et al adopt the same method, the ischemic area in the group of transmyocardial needle and laser puncture were decreased and angiogenesis were increased significantly compared with nontreatment myocardial ischemia group ($P < 0.05$). It is possible that epicardial transplant may stimulate cardiac tissue for angiogenesis^[12].

It is a pity that we didn't observe the ABM MNCs of untreated with 5-aza differentiated into the mature cardiomyocytes in the scar tissue and consequently replaced the scar, but the final results of the echocardiography showed the better heart function in the transplanted group. Therefore, these proved that the transplantation of simple ABM MNCs were effective. Of course, the exact mechanism of this methods still remains unclear, and there are many steps needed to be improved, but we believe deeply that the transplantation of ABM MNCs will be a promising method

for the myocardial infarction treatment.

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