

Bone Marrow Mesenchymal Stem Cells to Improve Damaged Heart Function after Modified Myocardial Infarction Rat Model

Modifiye Edilmiş Miyokardiyal Enfarktüs Rat Modelinden Sonra Hasarlı Kalp Fonksiyonlarının Kemik İliği Mezenşimal Kök Hücreler İle İyileştirilmesi

Research Article

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ABSTRACT

Although nowadays the treatment methods were improved, acute myocardial infarction (MI) and heart failure are the most important cardiovascular mortality and morbidity causes. In this study, mesenchymal stem cells (MSCs) collected from the bone marrow was injected into myocardial regeneration areas of the Wistar albino rats with modified ischemic heart model and this areas were investigated by using electrocardiographic (ECG) data and histopathological analysis. In stem cell implementation group, an increase in the amount of vessels and cells were observed. In this group, the amplitudes of P waves, QRS complex and T waves had no changes, whereas there was a significant reduction on T wave duration and heart beat rate reduced significantly ($p \leq 0,001$). S-T depression increased to normal levels was observed in reference and control groups more than in stem cell implementation group.

Key Words

Ischemia, bone marrow mesenchymal stem cell, cauterization, electrocardiogram.

ÖZET

Akut miyokard infarktüsü ve kalp yetmezliği; tedavideki gelişmelere rağmen günümüzde kardiyovasküler mortalite ve morbiditenin en önemli nedenlerindedir. Bu çalışmada, modifiye edilmiş iskemik kalp modeli oluşturulan Wistar albino sıçanlarda, kemik iliğinden elde edilen mezenşimal kök hücreler ile rejenerasyon alanları, elektrokardiyografik (EKG) ve histopatolojik analizler yapılarak gözlenmiştir. Kök hücre uygulama grubunda damar ve hücre sayısında diğer gruplara göre zamana bağlı olarak artış gözlenmiştir. Ayrıca bu grupta EKG verilerinden P dalgası, QRS kompleksi, T dalgası amplitüdüleri diğer gruplara göre farklılık göstermezken, T dalgasının süresi ve kalp atım sayısında önemli derecede azalma gözlenmiştir ($p \leq 0,001$). Referans grubuna göre kontrol grubunda S-T çökmesinde artış gözlenirken, kök hücre uygulama grubunda azalma gözlenmiştir.

Anahtar Kelimeler

İskemi, kemik iliği mezenşimal kök hücreler, koterizasyon, elektrokardiyogram.

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INTRODUCTION

Ischemic heart disease is a life-threatening event that may cause sudden cardiac death and heart failure. Even though angioplasty and thrombolytic agents can relieve the symptom of the infarction, no medication or procedure used clinically has shown efficacy in replacing myocardial scar with functioning contractile tissue, thus cardiac dysfunction after myocardial infarction (MI) is still the major worldwide cardiovascular disorder [1]. In this case, there is a need for new therapeutics to regenerate cardiac tissue. Regenerative medicine is focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause. For a long time, the use of cells for transplantation has been restrained by the limited plasticity of somatic cells. Major advances have been permitted by the discovery and isolation of stem cells that harbour the capacity for self-renewal and the potential to differentiate into one or more types of specialized cells. In theory, the ideal cell type for cellular therapy is likely to be a less committed one that can undergo full cardiomyocyte differentiation, enhance angiogenesis, and trigger vasculogenesis. Under the circumstances, mesenchymal stem cells (MSCs) may have essential combination of plasticity and viability.

MSCs-based regenerative therapy is currently regarded as an alternative approach to salvage the MI hearts [1]. MSCs has been shown, *in vivo* and *in vitro*, to proliferate extensively and to differentiate not only into osteoblasts, neurons, and skeletal muscle cells but also into vascular endothelial cells and cardiomyocytes [2-7].

Therefore, this study was designed to investigate whether transplantation of MSCs induces myogenesis and angiogenesis, and thereby changes myocardial histomorphology and improves cardiac function in a rat model of myocardial infarction.

MATERIAL AND METHODS

Isolation, Culturing and Characterization of Mesenchymal Stem Cells

Male Wistar albino rats (150 and 350 g) were used with the approval of the Animal Care and

Use Committee, Hacettepe University. 150 g rats were used for cell isolation, characterization, and transplantation. Bone marrow stromal cells were collected from tibias and femurs of rats. After the ether inhalation, both tibias and femurs were removed and their proximal ends were cut. Bone marrow were collected in a centrifuge tube within cell culture medium (Dulbecco's Modified Eagle's Medium-F12, DMEM/F12; Sigma Chemical, USA) containing antibiotics (0,5% penicillin-streptomycin). Bone marrow were homogenized by pipetting and centrifuged at 800 rpm for 10 minutes. The marrow pellet were resuspended in cell culture medium (Dulbecco's Modified Eagle's Medium-F12, DMEM-F12) containing 10% fetal bovine serum (FBS) and antibiotics (0,2% penicillin-streptomycin) incubated in a humidified atmosphere of 5% CO₂ at 37°C. The medium was replaced every three days and the non-adherent cells were discarded. The attached cells were incubated in same medium under standard culture conditions. When the cells become confluent, they were trypsinized. The culture medium were removed, and the cells were harvested using a mixture of 0.25% trypsin-10 mM EDTA (Biochrom AG, Germany) and the mesenchymal stem cells were passaged three times. All experiments were performed using cells from the third passage.

Cells were confirmed to be MSCs by cell surface markers, CD13-FITC (fluorescein isothiocyanate), CD13-TR (Texas red) (data not shown), CD29-FITC and CD29-TR (Santa Cruz Biotechn., USA) which are specific for MSCs. After cell culture medium was removed, cells were washed with phosphate buffer saline (PBS, Santa Cruz Biotechn., USA). Cells surface were coated with -10°C methanol for 5 minutes for fixation. After washing with PBS for a few times, cells were incubated with primary antibodies (CD13 and CD29) for 60 minutes. After this duration, cells were washed with PBS several times again and after that they were incubated with secondary antibodies (goat anti-mouse IgG-FITC and goat anti-mouse IgG-TR) for 45 minutes. At the end of this process, cell surfaces were covered with UltraCruz™ mounting medium (SantaCruz Biotechn., USA) and they were examined under inverted microscope (Olympus IX70 Inverted Microscope, Japan) (Figure 1(a), (b), (c), (d), (e), (f),(g) and (h)).

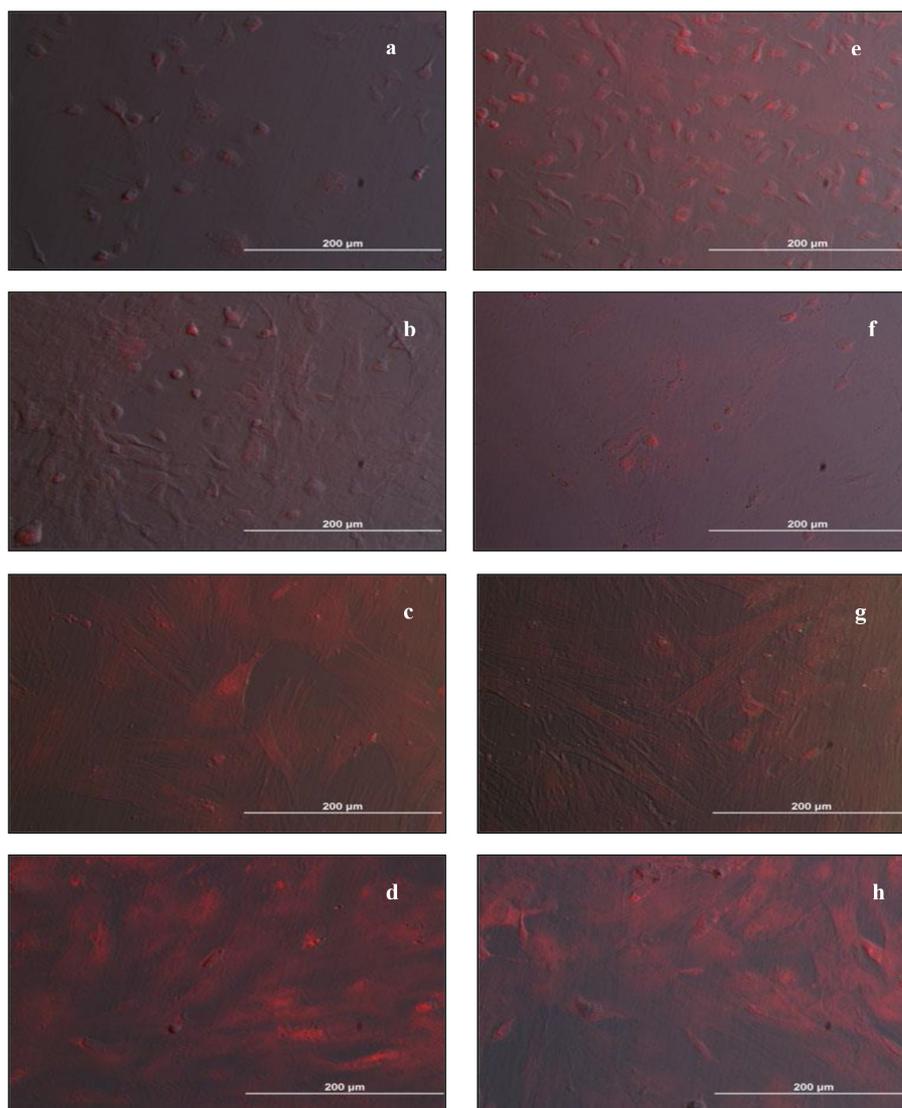


Figure 1. Immunocytochemistry studies for CD29 expression in (a) cells at passage 0, (b) cells at passage 1, (c) cells at passage 2 and (d) cells at passage 3 and CD13 expression in (e) cells at passage 0, (f) cells at passage 1, (g) cells at passage 2 and (h) cells at passage 3 [Protein expression was confirmed by Texas Red (TR) stain - Red color] [Adobe Photoshop CS2 light microscopic and TR fluorescent staining superimpose filter images (545-580 nm)].

Myocardial Infarction Model and MSCs Transplantation

Experimental MI was induced by cauterization of the left anterior descending coronary artery that a novel modified model described before [8]. 350 g animals were used for the *in vivo* study. The rats were divided randomly into three groups: sham-operated group, control group and stem cell implementation group. Briefly, 34 male Wistar albino rats were anesthetized with ketamin (40 mg/kg) and xylazine (1 mg/kg) by intramuscular administration. After the absence of the pedal

reflex, the rats were placed in the supine position. Under sterile conditions, the rats were subjected to left lateral thoracotomy between fourth and fifth ribs. The costas were gently retracted to maintain surgical exposure. The pericardium was incised to expose the anterior surface of the heart and the left anterior descending artery was cauterized at the midpoint through the starting point and the cardiac apex. Correct position of the occlusion was confirmed by S-T segment elevation on the ECG and regional necrosis of the myocardial surface. The control-operated group

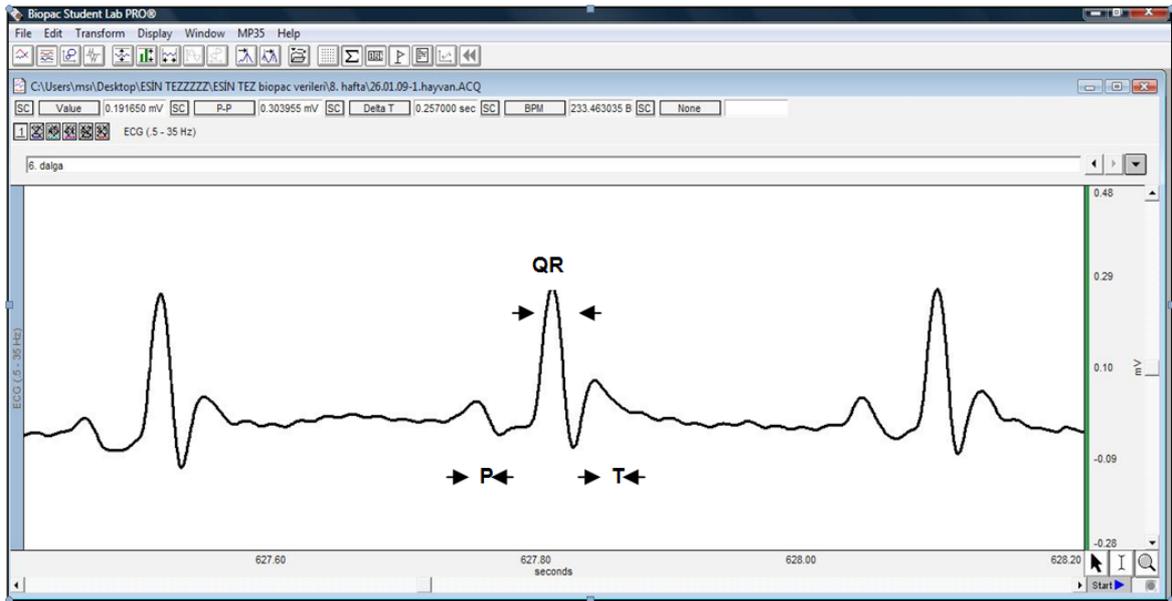


Figure 2. Electrocardiogram sample of rats taken by MP35 data acquisition system (BIOPAC System, Inc.).

received the same procedure of thoracotomy. Five minutes after the cauterization, to the stem cell implementation group a total of 2×10^4 MSCs suspended in 100 μ l of PBS were separately injected with a 30-G tuberculin syringe into three different sites for each infarcted area. Control-operated rats received the same volume of PBS injection. The sham group underwent the identical surgery with neither cauterization of the coronary artery nor MSC transplantation.

MP35 Monitorization ve Electrocardiography

ECG records were taken by MP35 data acquisition system (BIOPAC System, Inc.). For this purpose BSL PRO 3.7 sample files were used. Records were taken over the Channel-1 for ECG 0.5-35 Hz. Records were performed with 200 sample per second, 66.50 Hz sampling rate and x1000 gain. All records were adjusted for two hours.

Electrocardiographic records were taken from all rats before and during operation and also before sacrifice. Standard limb lead electrocardiograms (ECGs) at derivation-II (DII) were monitored throughout the surgical procedure. Body surface ECGs were made with rats in the supine position. ECGs were recorded using three subcutaneously placed needle electrodes: two on each hind limb and one on

right front limb. Electrode on the right hind limb functioned as a reference electrode. The three orthogonal ECG signals were recorded with a 66.50-Hz sampling rate. Registrations were performed with a maximum sample size of 200 sample/second to allow for beat selection and subsequent averaging, further improving the signal-to-noise ratio (Figure 2).

Histopathology

All rats were sacrificed 1, 2, 4 and 8 weeks after cell transplantation. The heart was exposed by median sternotomy and quickly excised and perfused with normal saline. The hearts were cut longitudinally into two equal slice samples. Samples were fixed in 10% formalin and embedded in paraffin and stained with H&E. Serial sections of 5 μ m thickness were cut transversely. Ten randomly selected fields per section were analyzed for cell number and capillary density.

The number of microvessels and cells in the infarcted area were counted in paraffin-embedded sections with H&E staining under inverted microscope (Olympus IX70 Inverted Microscope, Japan) (magnification x200). Microvessels and cells were counted at the same magnification by Analysis LS Research program (Soft Imaging System, An Olympus Company, Germany). The

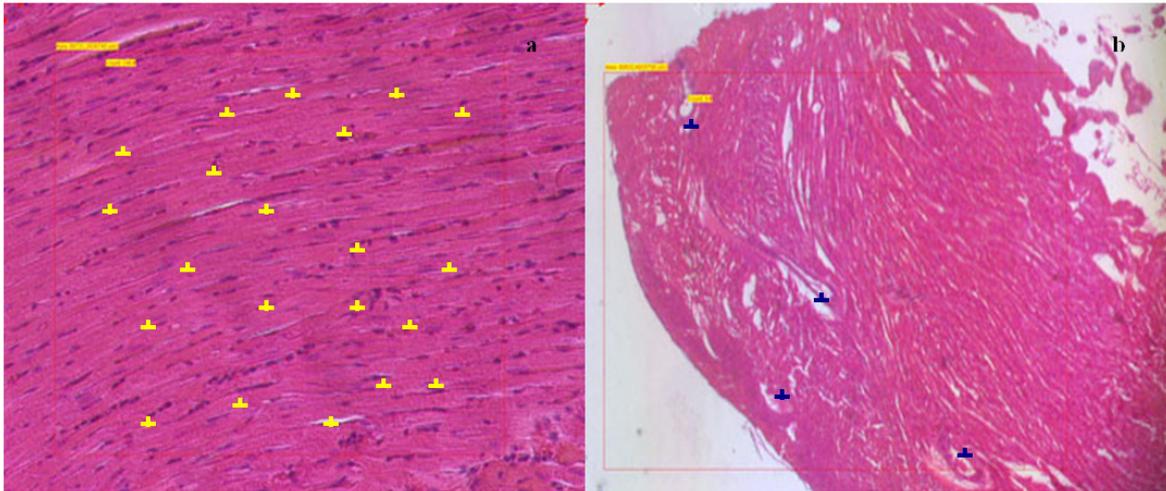


Figure 3. H&E staining of heart section for quantification of heart muscle cells (nucleus) and small vessels in ischemic area at week 1, 2, 4 and 8. CLI induction (n = 9). (a) Cell number of stem cell implementation groups belonging to the fixed field cell counts (+) X200. (b) Capillary number of stem cell implementation groups belonging to the fixed field cell counts (+) X40.

number of samples in each were averaged, vascular and cell densities were expressed as the number of microvessels and cells per unit area ($89572,6611 \mu\text{m}^2$) (Figure 3).

Statistical Analyses

Data were presented as mean \pm Standard deviation (SD). Statistical analysis were performed using a one-way ANOVA, and the difference between groups was determined by further analysis with Tukey and Hochberg's GT2 post test (SPSS for window, version 11.5). $P \leq 0.05$ was considered statistically significant.

RESULT AND DISCUSSIONS

Isolation, Cultivation and Characterization of MSCs

Three days after the MSCs were seeded on culture plates, inverted microscope demonstrated small colonies of adherent cells with typical fibroblast-shaped morphology. These primary cells reached monolayer confluence, after being plated for 7-10 days, when they were passaged for the first time. The cells were noted to have great potential for proliferation after subculture. The fibroblast-like morphology was also maintained after passages and throughout the culture period.

The third passage of MSCs was analyzed by cell surface markers for the expression. Isolated MSCs were characterized by staining CD13 and CD29 antibodies specific for them. The cells were found to be positive for CD13 and CD29.

Sample Size and Mortality

A total of 34 male Wistar rats were included in this part of the study. A mortality rate of 40% (14 rats) was observed with a total of 20 rats surviving to the experimental end point at 8 weeks. All of the mortalities occurred during the first 24 h after coronary occlusion. No late deaths were observed in surviving rats.

Histological Examination

H&E sections of heart showed that, in the infarcted area, there was a focal cell infiltration and sarcoplasm condensation (Figure 4). Also in a few area there was a congestion and especially cell border changes were observed in stem cell implementation group under a microscope (Figure 5). Compared to control group the cell borders were thicker in the stem cell implementation group. Sham group tissues exhibited no noticeable pathological change.

In stem cell implementation group, the increased amount of vessels and cells were observed. The results showed that MSC

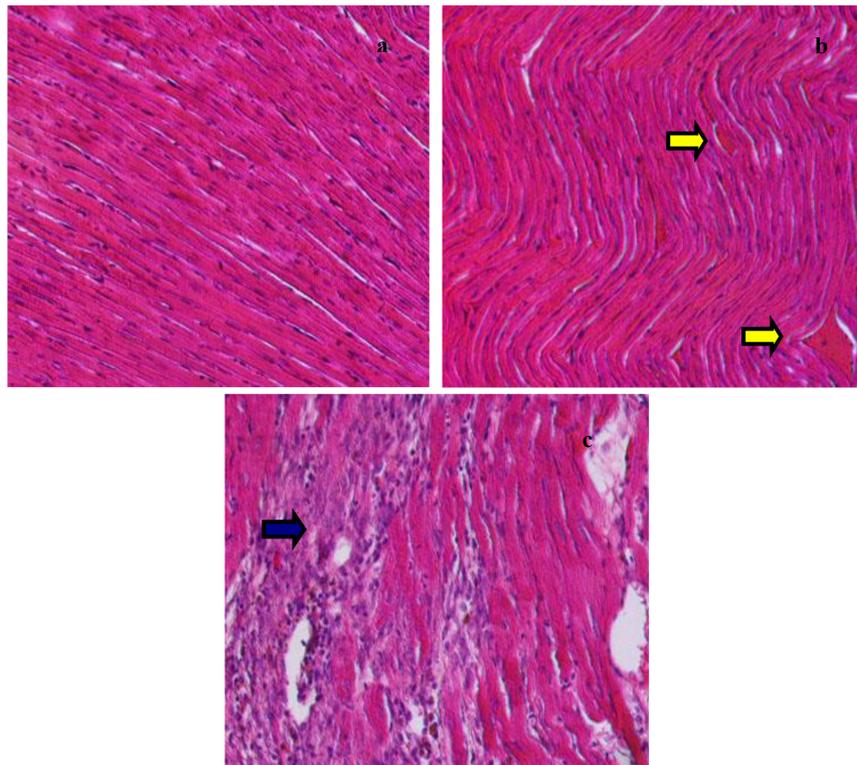


Figure 4. Histopathological staining of heart section (200x) at reference and stem cell implementation groups at day 7. Representative photomicrographs from sections stained with H&E 1 weeks following coronary cauterization of reference (a) and stem cell implementation groups (b-c) demonstrating a focal cell infiltration (blue arrow) and sarcoplasm condensation (yellow arrow). HPF = highpower field (200x).

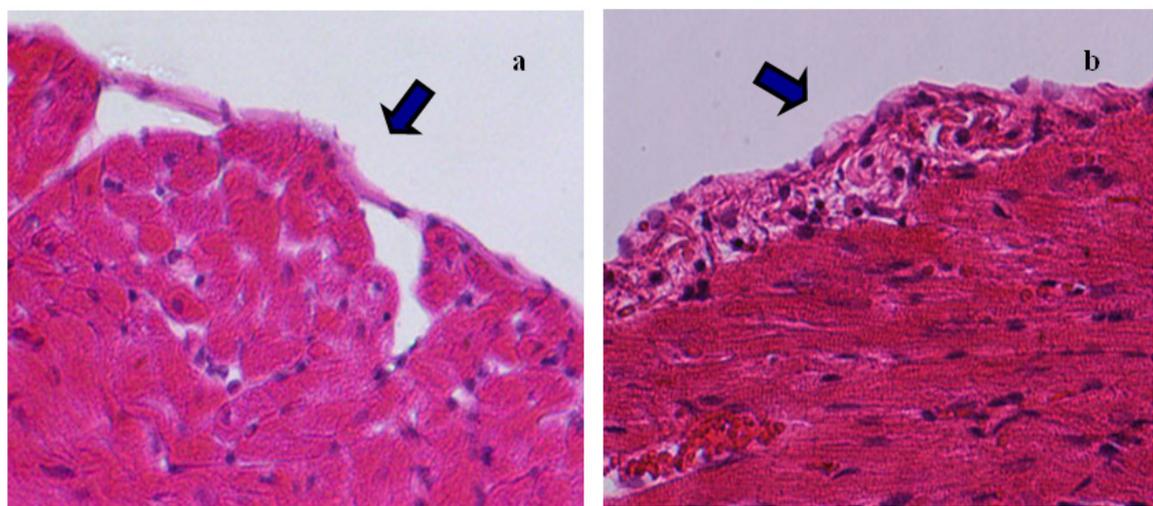


Figure 5. H&E staining of heart section (400x) of reference and stem cell implementation groups at week 8. Representative photomicrographs from sections stained with H&E, 8 weeks following coronary cauterization of reference (a) and stem cell implementation groups (b) demonstrating cell border changes. HPF = highpower field (400x).

Table 1. Cell number of the reference, control and stem cell implementation groups.

Cell number	Left side			
	1st week	2nd week	4th week	8th week
Reference	197.15±7.73	197.15±7.73	197.15±7.73	197.15±7.73
Control	250.00±15.51	243.25±17.20	266.25±8.37	198.00±4.89
Stem Cell Implementation group	241.40±6.75	204.93±7.34	193.33±7.73	231.80±6.56

Table 2. Capillary number of the reference, control and stem cell implementation groups.

Capillary number	Left side			
	1st week	2nd week	4th week	8th week
Reference	3.95±0.29	3.95±0.29	3.95±0.29	3.95±0.29
Control	5.75±0.85	5.00±0.00	5.00±0.00	4.00±0.40
Stem Cell Implementation group	3.73±0.39	5.73±0.67 ^a	7.00±0.60 ^a	7.13±0.44 ^{a,b}

^a: Significantly different from Reference group ($p \leq 0.001$).

^b: Significantly different from control group ($p \leq 0.001$).

transplantation increased vessel density and this increase was significantly greater in 8 week stem cell implementation group compared to control and sham groups ($p \leq 0,001$). These results are also same for cell density measurement (Tables 1 and 2).

Electrocardiographic Assessment of Cardiac Function

Electrocardiographic assessments *in vivo* were obtained in MI and control groups before operation, during operation and after operation at 1st, 2nd, 4th and 8th weeks. The ECG waves showed significant differences among the MI rats and sham-operated animals. In stem cell implementation group, the amplitudes of P waves, QRS complex and T waves had no changes, whereas there was a significant reduction on T wave duration and heart beat rate reduced also significantly ($p \leq 0,001$) (Table 3). S-T depression increased to normal levels was observed in reference and control groups more than in stem cell implementation group (Figure 6).

DISCUSSION

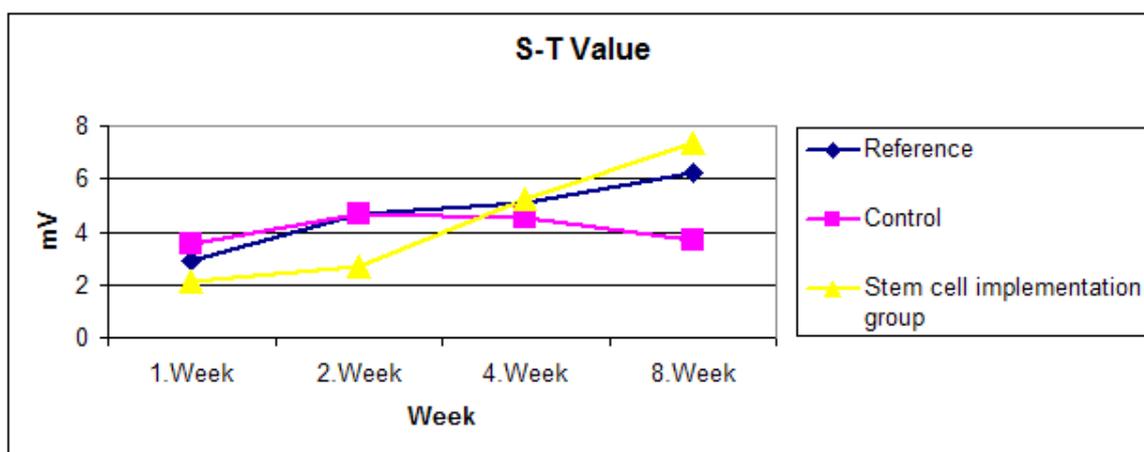
Bone marrow is a rich reservoir of stem cells and progenitor cells. This reservoir includes a population of MSCs, which have been shown to proliferate extensively *in vivo* and *in vitro* and, when cued by the appropriate microenvironment, to differentiate along multiple lineages giving rise to muscle, brain, liver, cartilage, bone, fat, and blood vessels [2,9-13]. Toma et al reported that the relatively purified MSCs engrafted in the myocardium appeared to differentiate into cardiomyocyte [14]. Thus, MSCs are commonly used in transplantation to repair the ripped myocardium with all these features.

Cell necrosis after an injury such as MI may cause the release of signals into the circulatory system, and these could induce the homing of stem cells to the myocardium [15-17]. The intense inflammatory reaction after MI may cause a local accumulation of mast cells, which might also contribute to the homing of MSCs [6,18-20]. The injured tissue may express receptors or ligands that facilitate the trafficking and adhesion of stem cells to the site of injury, where differentiation is

Table 3. ECG parameters of stem cell implementation and reference groups.

	1st week	2nd week	4th week	8th week	Reference
P wave amplitude (mV)	0.037±0.001	0.040±0.001	0.039±0.001	0.049±0.014	0.043±0.001
QRS complex amplitude (mV)	0.249±0.007	0.271±0.007	0.280±0.006	0.294±0.012	0.264±0.005
QRS complex time (sec)	0.031±0.000	0.034±0.001	0.031±0.000	0.033±0.000	0.032±0.000
T wave amplitude (mV)	0.122±0.007	0.082±0.008	0.117±0.010	0.102±0.007	0.087±0.003
T wave time (sec)	0.035±0.001 ^a	0.025±0.000	0.043±0.003	0.033±0.001	1.162±1.132
Beat per Minute (BPM)	221.1±3.496 ^a	223.4±4.050 ^a	243.2±13.906 ^a	241.4±8.967 ^a	280.0±4.833

^a: Statistically significant ($p \leq 0.05$).

**Figure 6.** Changes of S-T derivation of reference, control and stem cell implementation groups during the different weeks.

initiated, resulting in the generation of cells of the appropriate lineage [21]. However, it is still unclear what environmental cues initiate the homing of MSCs to the ischemic heart.

This is consistent with previous studies, which demonstrated the capacity of MSCs to give rise to new myocytes, smooth muscle cells, and endothelial cells in ischemic myocardium [22,23]. The optimal amount of MSCs for transplantation to improve heart function and attenuate ventricular remodeling in the greatest extent is still not determined. Hou et al reported that 2×10^6 cell/50 μ l PBS affect the improvement of vessels density [24]. It was shown that 1×10^7 cell/500 μ l PBS not affected the restoration of the number of cells but affected the progression of the vessel

density [3,4,25]. In the light of these, this study demonstrated that 2×10^4 cell/100 μ l PBS can increase vessel density, attenuate cardiomyocyte apoptosis and improve heart function.

In studies on timing for MSCs injection and scheduling the analysis after therapy, Hou et al proposed that 8 weeks is ideal for seen the differences between groups in terms of angiogenesis [24]. Also our data indicated that 8 weeks provided the optimal timing for MSCs therapy that shown by capillary density in implementation groups.

Although ischemia induces VEGF expression and angiogenesis, blood supply is far from enough for tissue repair. As a result, ischemia

and anoxia may cause myocardial infarction in the end. MSCs secrete a variety of cytokines including VEGF. Thus MSCs can be transplanted into the infarcted myocardium. The VEGF secreted by MSCs will induce angiogenesis, which will increase regional blood flow to ischemia tissue and reduce infarct area [5,12,13,25-29]. In this study, results demonstrated that two months after MSCs transplantation, the vessel density of the stem cell implementation group varied greatly from that of the control and sham groups.

In this study, stem cell implementation were increased capillary density in the myocardium. Quantitative analysis demonstrated that capillary density was significantly higher in the stem cell implementation group at eight weeks compared with those in control and sham groups. Improvement in myocardial vascular supply has been shown to decrease necrosis and inflammation in MI. The previous study done by Nagaya et al reported that increased capillary density was associating with improved cardiac function and decreased infarct size following MSC transplantation in a rat model of MI [11]. These results propose that MSC-induced neovascularization may have contributed to the improvement of heart histopathology in this rat model of MI.

The present study evaluated that the functional improvement seen after MSC transplantation after two months by electrocardiographic analysis. Stem cell implementation group showed the expected return to baseline in S-T deviation. These results suggested that MSC transplantation might support myocardial regeneration and provide partial recovery of ischemic area.

An understanding of the limitations of the *in vivo* study is important to better understand the significance of the results. We did not attempt some quantitative analysis like left ventricular ejection fraction values and wall thickness in our study, which was a limitation of this research.

In conclusion, MSC transplantation was beneficial for the myocardial regeneration in experimental rats. The present study only

assessed the short-term effects of transplant MSC. Long-term studies task for the future to find the most practical and specific ways of improving the targeting including labeling the cells and tracking and thereafter detect the effectiveness of stem cell therapy. Also further experiments are required to optimize the location of transplantation of the cells after delivery to heart.

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