Quantitative changes of infarcted myocardium according to different time of treatment for human mesenchymal stem cell transplantation in rat with myocardial infarction

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감사의 글

먼저 본 논문이 완성되기까지 세심한 지도와 관심 어린 격려로 이끌어 주신 이승환 교수님께 깊은 감사를 드립니다. 또한 연구라는 것이 어려운 것이 아니라 새로운 것을 발견하고 찾아가는 즐거운 과정이라는 것을 깨닫게 해주신 유병수 선생님, 항상 용기를 북돋아 주시고 긍정적으로 생각하고 고민할 수 있도록 도와주신 김현수 교수님께도 감사를 드립니다.

실험 진행에 많은 어려움을 겪으면서 데이터를 만들어 준 윤영진 선생님께 진심으로 감사 드립니다.

마지막으로, 말없이 뒤에서 조용히 지켜봐 주시는 부모님, 아들처럼 아껴주시는 장모님, 곁에서 남편을 위해 물심양면으로 성원해주는 아내 윤정이와 사랑하는 딸 다연이에게 감사를 전합니다.

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저자 씀

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Abstract

Quantitative changes of infarcted myocardium according to different time of treatment for human mesenchymal stem cell transplantation in rat with acute myocardial infarction

Background and Objectives: Mesenchymal stem cell (MSC) therapy has shown potential benefits for repairing myocardial damage. But, the therapeutic effect is modest and the optimal time for treatment is not established. The aim of this study was to assess an optimal time of treatment for acute myocardial infarction (AMI) in rat model.

Methods: AMI was induced by ligation of the left anterior descending coronary artery in male Sprague-Dawley (SD) rats. Human bone marrow MSCs were directly injected into the infarct boarder zone at 1 hour and 1 week after AMI. Control groups were injected equal volume of phosphate buffered saline (PBS). The hearts were excised, and the left ventricle was sectioned into 4 slices. Quantitative analysis of infarct size was done by using the SigmaScan planimetry measurement.

Results: The infarct area of 1 hour group (N=5) was reduced compared with 1 week group (N=5) and control group (N=3) (1 hour group vs. 1 week group vs. control group:

12.86 ± 6.55 vs. 17.52 ± 6.64 vs. 17.93 ± 4.10). But there was no statistical significance according to different time of treatment (p = 0.295 for 1 hour group vs. 1 week group; p = 0.279 for 1 hour group vs. control group; p = 0.926 for 1 week group vs. control group).

Conclusion: Our study failed to demonstrate the optimal time for treatment and the effect of MSC in rats with AMI. But, numerical improvement of infarct area in 1 hour group seems to be confirmed in further large study..

Key words: Mesenchymal stem cells; Myocardial infarction

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1. Introduction

Although advanced therapeutic strategies for treating acute myocardial infarction (AMI) reduced early mortality and morbidity, the cardiovascular disease is one of the main burden of death worldwide.[1-3] Experimental studies showed that stem cell therapy could improve myocardial function after AMI.[4-8] However, pooled analysis of clinical trials showed only modest effect.[9] Data using mesenchymal stem cell (MSC) in human clinical

trials were limited. MSC is considered to be an attractive candidate because of many advantages as follows: high capacity for replication, paracrine effect, preserved potency and no adverse reaction to allogenic versus autologous transplants.[10-11]

The optimal timing of stem cell transplantation has been slightly overlooked. There are limited data for the optimal timing of stem cell transplantation in humans. Experimental studies suggest the existence of a temporal window of opportunity bound by the acute inflammatory response on one hand and by scar formation on the other.[12] Therefore, we decided to evaluate the optimal time for MSC transplantation in rats with AMI.

2. Materials and methods

2. 1 Animal model and procedure

Male Sprague-Dawley (SD) rats, weighing 200-300g were anesthetized with ether and placed in a supine position, followed by endotracheal intubation with a 14-gauge catheter. Tracheal ventilation was performed at 70 cycles/min with 2.5-3.0 mL tidal volume, room air supplemented with oxygen (Harvard Rodent Ventilator, Model 683, Harvard Apparatus Co, Inc). A left intercostal thoracotomy was performed under aseptic technique.

The fourth-intercostal space was opened carefully to avoid accidentally cutting any vessels including the intermal mammary artery. The fourth and fifth ribs were separated with a small retractor to explore heart. The pericardium was removed and the left anterior descending artery (LAD) and its branch was observed. The LAD was ligated proximally with 8-0 silk suture. Rats were randomized into three group: 1 hour group, 1 week group and control group.

2.2 MSC preparation

MSCs were obtained under local anesthesia from the posterior iliac crest of healthy person. MSCs were provided by FCB-Pharmicell Company Limited (Seongnam, South Korea) and all manufacturing and product testing procedures for the generation of clinical-grade autologous MSCs were carried out under good manufacturing practice. Mononuclear cells were separated from the bone marrow by density gradient centrifugation (HISTOPAQUE-1077; Sigma–Aldrich, St. Louis, MO, USA) and washed with phosphate-buffered saline (PBS). Cells were resuspended in Dulbecco's modified Eagle's medium-low glucose (DMEM; Gibco, Grand Island, NY, USA) containing 10% fatal bovine serum (Gibco), 100 U/mL penicillin/100 μg/mL and streptomycin (Gibco).

They were plated at 2–3×10^5 cells/cm^2 into 75 cm^2 flasks. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 5–7 days, non-adherent cells were removed by replacing the medium; adherent cells were cultured for another 2–3 days. When the cultures were near confluence (70–80%), adherent cells were detached by using trypsin containing ethylene diamine tetra-acetic acid (EDTA; Gibco) and replated at 4–5×10^3 cells/cm^2 in 175 cm^2 flasks. Cells were serially subcultured up to passage 4 or passage 5 for infusion. MSCs were harvested using trypsin and EDTA, washed twice with PBS and once with saline solution, and resuspended to a final concentration of 1×10^6 cells per mL in saline solution. The criteria for the release of MSCs for experimental use included viability >80%, absence of microbial contamination (bacteria, fungus, virus, mycoplasma) if undertaken 3–4 days before administration, and expression of CD73 and CD105 by >90% of cells and absence of CD14, CD34, and CD45 by <3% of cells as assessed by flow cytometry. Also, the *in-vitro* osteogenic and cardiomyogenic differentiation potential of MSCs was tested before release as a potency test.

2.3 MSC injection

Cell transplantation was performed via intramuscular (IM) injection to the border zone

of infarct area 1 hour and 1 week after AMI. Each rat was injected twice for a total of 1×10^6 cells. Control group were injected with the same volume of PBS.

2.4 Histologic analysis

The rats were sacrificed 4 weeks after AMI. The left ventricles were cut into 2-mm-thick transverse slices, which were incubated for 15 minutes at 37ºC in 1% triphenyl-tetrazolium chloride (TTC) solution to visualize the infarct myocardium. Myocardial fibrosis was analyzed by slides stained with Masson's trichrome. The slides were photographed and the area of infarction was quantified by using planimetry software (Sigma Scan Pro version 5).

2.5 Statistical analysis

A single investigator blinded to the treatment group performed all histological measurements. All values are expressed as means ± SD. The unpaired Student's t-test with the use of a two-tailed distribution was used to calculate the statistical significance between the means of the two groups. A P value of <0.05 was considered to be significant. The statistical analysis was performed with the SPSS version 15 software (SPSS, Inc., Chicago, Illinois).

3. Results

3.1 Mortality

We experienced high mortality related to surgical procedure and MSC injection. A total of 30 rats were used and 15 rats (50%) were died in the present study. 3 of 8 rats were died in control group (only LAD ligation group) and 6 of 11 rats were died in 1 hr group during procedure. Among 11 rats in 1 week group, 5 were died during MSC injection and one was died immediately due to procedure-related complication. Deaths were presumed to be from acute respiratory failure or injection-related malignant arrhythmia.

3.2 Histological finding

Extensive left ventricular myocardial infarction was grossly identified and then confirmed histopathologically using Masson's trichrome stain. (Figure 1). No infarct area in 2 rats of control group were observed and these two rats were excluded from analysis.

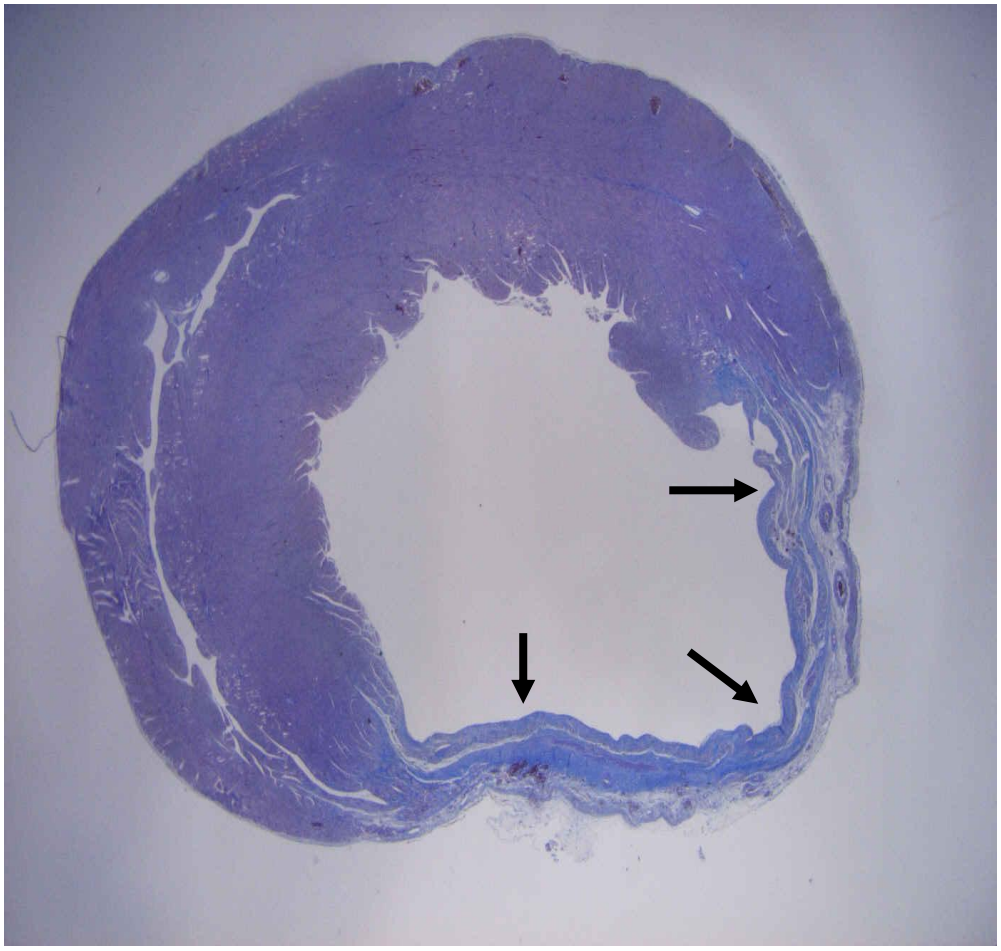

Figure 1. Histological finding of Masson's trichrome stain identified the collagen fibers (black arrow indicating infarcted myocardium with fibrosis)

3.3 Quantitative analysis of infarct area

Quantitative measurements of infarct area of each group were shown in Table 1. Mean infarct area in 1 hr group was reduced compared with 1 week group and control group. But the statistical differences were not significant among each groups (Table 2).

Table 1. Values of infarct area (%)

Number	1 hr group (N=5)	1 week group (N=5)	Control group (N=3)
1	17.5	20.8	17.2
2	12.1	21.6	14.3
3	10.1	19.2	0*
4	3.9	20.2	0*
5	20.7	5.8	22.3
Mean ± SD	12.86 ± 6.55	17.52 ± 6.64	17.93 ± 4.1

* was excluded from analysis.

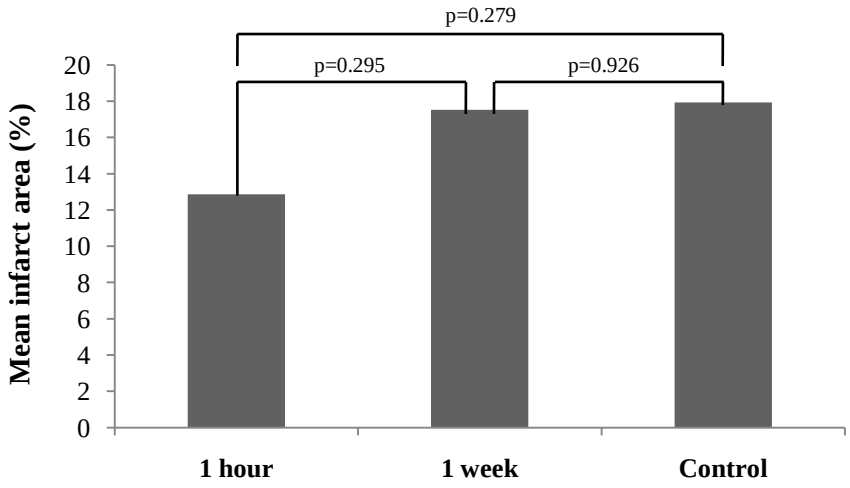

Figure 2. Comparison of mean infarct area (%)

4. Discussion

Our study did not showed the reduction of infarct area according to different time (1 hour and 1 week) of treatment of intramuscular MSC in rats with AMI. And we could not

find the beneficial effect of MSC on infarct size in rats with AMI.

Some preclinical studies were directly focused on the timing of stem cell transplantation. Hu et al.[13] demonstrated that all groups treated with MSCs 1 hour, 1 week and 2 week after AMI showed significantly increased heart function and decreased infarct size compared to sham-operated group and control group. Moreover, they found the greater benefit in the 1 week group. Jiang et al.[14] reported similar results that MSC transplantation was beneficial for the recovery of cardiac function in experimental rats. MSC transplantation at 1 week post-MI exerted the best effects on cardiac function, anti-apoptosis and angiogenesis, suggesting that 1 week post-MI may be the optimal choice for MSC transplantation in rats.

There may exist the optimal period of effective stem cell therapy in several clinical trials and subgroup analysis. The evidence based on these studies suggests that bone marrow derived stem cell therapy should be conducted within the first month, but not within 24 h after AMI.[12] Very early treatment, i.e. within 6 hour after reperfusion has not been tried and might provide promising improvement of cardiac function over current treatment.[9]

There are several limitations in this study. First, a small number of study could not draw a clear conclusion. Second, there were high mortalities in all study groups. Most common causes of death is considered due to an unskillful surgical procedure or injected method. Surgical complications such as pneumothorax and minor bleeding could be the leading cause of death. Direct damage related to intramuscular injection might be the cause of malignant arrhythmia. Third, no myocardial injury was observed in 2 rats in control group. This suggests incomplete ligation of LAD. This also implicates the possibility of inconsistent surgical procedure for inducing myocardial infarction in other studied animals.

5. Conclusion

Our study failed to demonstrate the optimal time for treatment and the effect of MSC in rats with AMI. But, numerical improvement of infarct area in 1 hour group seems to be confirmed in further large study.

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국문 요약

흰쥐의 급성 심근경색 모델에서 사람의 중간엽 줄기 세포를 이용하여 다른 시기에 치료를 했을 때 경색 심근의 양적 변화에 대한 연구

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배경: 중간엽 줄기세포를 이용한 치료는 심근의 손상을 회복시키는데 있어서 가능성 있는 효과를 보여주었다. 하지만 치료의 효과는 그다지 크지 않으며 치료의 적절한 시기도 아직 정립되어 있지 않다. 본 연구의 목적은 급성 심근

경색을 유발한 흰쥐 모델에서 적절한 치료의 시기를 평가하기 위한 것이다. **방법:** 수컷 Sprague-Dawley (SD) 쥐의 좌전하행지 관동맥을 결찰하여 급성 심근경색을 유발하였다. 사람에서 채취한 골수 유래 중간엽 줄기세포를 이용하여 급성 심근경색을 유발한 1시간 뒤와 1주일 뒤에, 경색 주변 부위에 직접 심근내 주입을 하였다. 대조군은 동일한 양의 phosphate buffered saline을 경색 주변 부위에 투여하였다. 심근경색 유발 4주 후, 심장을 절개하고 좌심실을 4개의 절편으로 잘라내었다. Sigma Scan 프로그램을 이용하여 경색 범위의 면적을 정량적으로 측정하였다. **결과:** 경색 유발 1시간 후 투여군 (N=5)은 경색 유발 1주 후 투여군 (N=5)과 대조군 (N=3)과 비교하였을 때 경색 범위가 감소하였다 (1시간 후 투여군 vs. 1주일 후 투여군 vs. 대조군: 12.86 ± 6.55 vs. 17.52 ± 6.64 vs. 17.93 ± 4.10). 하지만 각 군간의 통계적으로 유의한 차이는 관찰할 수 없었다 (1시간 후 투여군 vs. 1주일 후 투여군, p=0.295; 1시간 후 투여군 vs. 대조군, p=0.279; 1주일 후 투여군 vs. 대조군; p=0.926). **결론:** 본 연구는 급성 심근경색을 유발한 쥐에서 다른 시간별로 중간엽 줄기세포를 투여하였을 때의

효과를 밝혀내지 못했다. 또한 중간엽 줄기세포를 투여받은 군이 대조군보다 유의한 효과가 있음을 보여주지 못했다. 하지만 1시간 후 투여군에서의 수치적인 심근경색 범위의 감소 소견은 추후 좀 더 대규모 연구를 통한 확인이 필요할 것으로 생각된다.

핵심단어: 중간엽 줄기세포; 심근 경색