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Original Article

Effect of human thymus adipose tissue-derived mesenchymal stem cells on myocardial infarction in rat model

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ABSTRACT

Background and objective: Stem cell (SC) therapy exhibits promising therapeutic efficiency against cardiovascular disease. The thymus adipose tissue (TAT) is familiar to cardiac surgeons with sternotomy; however, the application of TAT in SC therapy remains unknown. We assessed the effectiveness of TAT-derived mesenchymal SCs (TAT-MSCs) in the rat myocardial infarction (MI) model.

Methods: The human TATs were obtained from the patients who underwent coronary artery bypass graft surgery. In cell studies, we performed the cumulative population doubling level assessment, fluorescence-activated cell sorting analysis, and differentiation study. In animal studies, we segregated Sprague–Dawley rats (ischemia-reperfusion model) into three (sham, vehicle, and TAT-MSC) groups based on their corresponding treatment. Trans-thoracic echocardiogram (TTE) was obtained to assess the recovery of heart function in the 1st, 4th, 8th, and 12th week after surgical manipulations. After echocardiographic study, infarcted area of the heart was measured using triphenyl tetrazolium chloride (TTC) stain.

Results: The sham group exhibited significantly better systolic and diastolic function (SDF) than the other groups did. After one week of TAT-MSC or vehicle injection, the TAT-MSC group exhibited a significant improvement in the E/E′ value (25.75 ± 1.09 vs. 24.20 ± 0.91, $p < 0.001$) compared to the vehicle group. Although statistically insignificant, the trend of improvement in SDF was better in the TAT-MSC group than in the vehicle group. The infarcted area measured by TTC staining was 22.81 ± 6.41% and 29.95 ± 9.09% in the TAT-MSC and vehicle groups, respectively ($p = 0.04$).

Conclusion: Although TTE results exhibited insignificant variations in SDF, a trend with improvement in the SDF of the heart was observed in the TAT-MSC group compared to the vehicle group. The infarcted area of heart indicated significant reduction in the TAT-MSC group compared to the vehicle group as confirmed by histopathological study.

1. Introduction

Heart failure (HF) threatens the survival and lowers the quality of life in patients. Several causes trigger the onset of HF; however, coronary artery disease is one of the main causes and is highly prevalent prior to the development of HF [1]. The current treatment is merely reducing the progression rate of failing heart in addition to complete treatment using natural (donated) and artificial heart transplantations. However, a major limitation to HF treatment is the lack of transplant donors and application of artificial hearts in bridge therapy. Recently, stem cell (SC) therapies exhibited better efficiency in the treatment of cardiovascular diseases, especially HF. Multiple phase I and II clinical trials reported the promising effect of SCs on HF [2–19]. These studies reported the increase in left ventricle ejection fraction (EF) and exercise capacity, decrease in infarction size, and improved clinical outcomes. In another study, various sources of SCs including mononuclear cells, mesenchymal SCs (MSCs), skeletal muscle cells, and cardiac progenitor cells were used to treat HF.

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MSCs are multipotent stromal cells that differentiate into osteocytes, chondrocytes, adipocytes, and myocytes. Adipose tissue is an abundant source of MSCs and several studies utilized subcutaneous adipose tissue. The thymus is characterized by its immune function at a young age and is replaced by adipose tissue with age. The thymus adipose tissue (TAT) consists MSCs (TAT-MSCs); however, the application of TAT-MSCs in HF treatment remains unknown. Till date, most of the researches were performed using laboratory cell culture experiments [20–23]. Therefore, we investigated whether MSCs derived from the TAT are effective to treat myocardial infarction in rat model.

2. Materials and methods

2.1. Collection of tissue samples

The TATs were obtained from the patients with ischemic heart disease who underwent coronary artery bypass graft surgery at the Daegu Catholic University Medical Center. The TATs were collected immediately after opening the pericardium and all the procedures related to this protocol were approved by the Institutional Review Board of our medical center. In this study, particular patients were selected to harvest and collect TATs based on the following inclusion and exclusion criteria:

Inclusion criteria
1. Patients scheduled to undergo coronary artery bypass surgery.
2. Age of patient is ≥20 years.

Exclusion criteria
1. Patients with a history of malignant neoplasms.
2. Patients administered with immunosuppressive drugs.
3. Patients with a history of chemotherapy and radiation therapy.
4. Patients without the symptoms of infection.

TATs were collected from 5 patients, ages 59, 61, 64, 66, and 81, respectively. The weight of each tissue was about 5–10 g.

2.2. Preparation of primary cell cultures

The TATs obtained from patients were sent to the Daegu Gyeongbuk Medical Innovation Foundation (DGMIF). These TATs was subjected to tissue trimming and TAT-MSCs were extracted using collagenase type I. These TAT-MSCs were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, plasmocin, platelet-derived growth factor, and fibroblast growth factor at 37 °C in a 5% CO_2 incubator. These TAT-MSC cultures were subjected to 5–10 passages to be utilized in the preparation of injection (Fig. 1). Cells were obtained at approximately 2×10^6 at passage 0.

After five primary cultures from different individuals, three different lots of cells were secured. A random lot was chosen and an experiment was performed using this lot. The selected lot comprised cells from a 61-year-old male who had a history of diabetes, hypertension, and underwent coronary artery bypass surgery.

All three lots of cells had the same phenotype: the cells were adherent, and fibroblast-like spindle shaped. All cells were extracted using the same method described above in the materials and methods.

2.3. Animal model

Sprague–Dawley (SD) rats were segregated into the sham, vehicle, and TAT-MSC groups based on the treatments and further experiments were performed. A left anterior descending artery (LAD) was ligated and reperfusion was performed to develop a myocardial infarction model. After 3 days of operation, the animals were re-opened the chest and the normal saline (vehicle group) or MSCs (stem cell group) were administered. The sham group was similar to the other groups except that the left anterior coronary artery ligation was not performed and normal saline was administered after 3 days of reopening the surgical wound. Each group included 10–12 rats based on the results of previous publications and our preliminary experiments [24]. This experiment was approved by the Institutional Animal Care and Use Committee of the Experimental Animal Center of the DGMIF based on the Animal Protection Act.

2.3.1. Breeding and management of SD rats

Rats ($n = 2$ or 3 per cage) were housed in well-ventilated cages (395 width × 346 depth × 213 height mm). At the end of purification period, rats were moved to the operating room to perform surgeries. Rats that underwent surgery were kept in isolation and general symptoms were observed daily. The incubation temperature was 22 ± 3 °C, relative humidity was 50 ± 20%, the ventilation frequency was 10–20 times/h, and the dark and light cycle was 12 h/day (07:00–19:00). During the refinement period, the cage was exchanged twice per week, the breeding cage was cleaned using an automatic washer, and sterilized in a high pressure steam sterilizer.

2.3.2. Development of SD rat model with ischemic reperfusion

Male SD rats (age: 8–9 weeks) were anesthetized using 50 mg alfaxan/kg body weight and 5 mg xylazine/kg body weight. The body temperature and electrocardiogram were regularly monitored and controlled breathing of rat was maintained using an animal ventilator (Harvard Apparatus Instora ASV model 55-7058). We performed left thoracotomy to remove the pericardium and expose the heart. The LAD was snared using a polyethylene (PE) tube for 30 min and PE tube was taken out of the thoracic cavity. Later, PE tube was removed, thoracic cavity was closed, and a negative pressure and sutures were applied. Reperfusion was performed for 2 h after the ischemic induction for 30 min. The procedure to develop the sham group was similar to that of the other groups except that the left anterior coronary artery ligation was not performed.

2.3.3. Re-thoracotomy to perform intra-myocardial injection

The rats were anesthetized using 50 mg alfaxan/kg body weight and 5 mg xylazine/kg body weight to perform the intra-myocardial injection of either TAT-MSCs or vehicle after 3 days subsequent to the induction of ischemic reperfusion injury. Their body temperature and electrocardiogram were monitored and controlled breathing was maintained using an animal ventilator. The left thoracotomy was re-performed to expose the heart and TAT-MSCs (2×10^5 cells) or vehicle (normal saline) was administered to all the three sites (total volume: 30 μl; 10 μl/site) in the heart and myocardial ischemic border zone. The concentration of TAT-MSCs to prepare the injection was referred from the related literature [24].

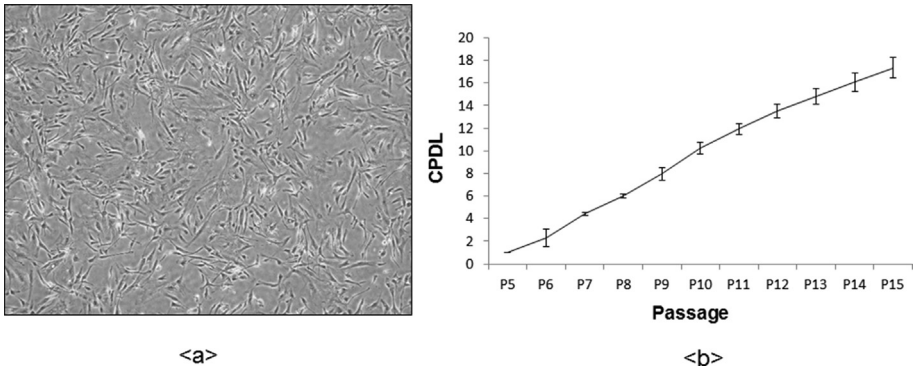


Fig. 1. Confirmation of the presence of TAT-MSCs. a. Image of TAT-MSCs at passage 6 under a bright field microscope. b. Cell population doubling level of TAT-MSCs, showing that cell growth progresses well from passage 5 to 15. TAT-MSC: thymus adipose tissue-derived mesenchymal stem cell; CPDL: cell population doubling level.

2.4. Mesenchymal stem cell culture and characterization

Cells were cultured in DMEM with 10% FBS, and MSC characterization was performed in passage 6. Reverse transcription polymerase chain reaction (RT-PCR) was used to identify stem cell markers. To confirm that the criteria of the International Standard of MSC were met, we examined the expression of specific markers in cultured cells using fluorescence activated cell sorting (FACS) [25]. The cultured cells were stained according to the protocol provided by the manufacturer (Beckman Coulter, USA). Briefly, the cultured cells were detached and washed several times with PBS. The detached cells were divided into groups (approximately 1×10^6 cells) and stained with specific antibodies. The antibodies used for FACS analysis were for cell surface markers, including CD105, CD90, CD73, CD45, CD34 and CD14 (BioLegend, CA, USA, anti-human). The antibodies were conjugated to the fluorescein dye, with IgG1 PE or Fluorescein isothiocyanate (FITC) used as control dyes. The analysis was conducted with a FACS cytometer (Gallios Flow Cytometer; Beckman Coulter, USA) and software (Kaluza for Gallios; Beckman Coulter, USA). Specific media and RT-PCR were used to identify osteogenesis, adipogenesis, and chondrogenesis.

2.5. Evaluation of the efficacy of ischemic reperfusion model

2.5.1. Echocardiogram

At the end of the 1st, 4th, 8th, and 12th weeks after performing the myocardial ischemic reperfusion insult, rats were anesthetized using alfaxan (50 mg/kg body weight) and xylazine (5 mg/kg body weight) to perform echocardiography. Rats were placed in the supine position on an ultrasonic workstation that monitors the electrocardiogram, respiratory rate, and pulse. Fractional shortening (FS) and EF were measured to determine the cardiac contractility and E′ and E/E′ values were measured to estimate the diastolic function. The estimation of cardiac contractility by measuring the short axis of heart in B-mode was performed using Visual Sonics Vevo 2100 imaging system and the cardiac relaxation was measured using pulse-wave Doppler mode and Tissue Doppler imaging.

2.5.2. Histopathological examination

After performing cardiac autopsy, the heart was placed on a Matrix (Acrylic rat heart matrix 2 mm, 72-5015, Harvard Apparatus) at 2 mm intervals to identify the ischemic site. The heart tissue was placed into 1% triphenyl tetrazolium chloride (TTC) dissolved in 1 × phosphate buffered saline in a 50-ml conical flask. After 10 min at 37 °C, the heart sections were imaged. The ischemic site (%) was measured by calculating the ischemic area relative to the left ventricular area (excluding ventricle lumen) using the Image J program for each section. Each of the four sections from the apex part of each individual were obtained and the percentage of each individuals ischemic area was calculated. The mean size of the hearts in each group was not different, and the calculated results reflected the degree of ischemia regardless of the size of the heart, reflecting the ischemic area relative to the left ventricle of each individual.

2.6. Statistical analysis

In this study, all the data were expressed as mean ± SD and $p < 0.05$ was considered as significant value. The comparison among infarcted areas measured by TTC stain analysis in various groups was performed using Student's *t*-test and other data were analyzed using the IBM SPSS Statistics 25 program as a repeated measures analysis of variance.

3. Results

3.1. In vitro (laboratory) experiments

3.1.1. Cell proliferation and stem cell markers

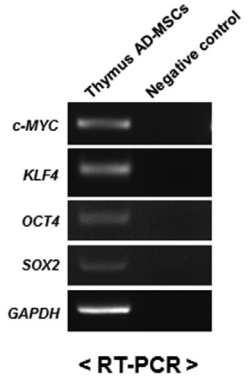
We confirmed the presence of TAT-MSCs by microscopy and confirmed that the cells proliferated well through passage 15 (Fig. 1). To determine if TAT-MSCs retained a stem cell marker phenotype after passage, we observed the expression levels of the markers OCT4, KLF4, c-Myc, and SOX2 using RT-PCR (Fig. 2). The TAT-MSCs expressed all markers tested, suggesting that these cells do retain a stem cell phenotype after *in vitro* passage.

3.1.2. Cell surface markers

To confirm that the criteria of the International Standard of MSC were met, we examined the expression of specific markers in cultured cells using FACS [25]. TAT-MSCs were positive for the markers CD105, CD90, and CD73 and negative for the markers CD45, CD34, and CD14 (Fig. 3). Based on these data, we concluded that our TAT-MSCs meet International Standard.

3.1.3. Differentiation study

To confirm that TAT-MSCs could differentiate into osteogenic, adipogenic, or chondrogenic lineages, cells were stained with the specific characterization medium, and RT-PCR was performed to detect particular genes that correspond to each lineage (Fig. 4). Alizarin Red S Staining and Von Kossa Staining, along with RT-PCR using specific osteogenic genes, confirmed that the cells underwent osteogenic differentiation. The production of lipid droplets in the



Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Cycle
GAPDH	CGCTGAGTACGTCGTGGAGT	GGAGGCATTGCTGATGATCT	21
c-Myc	GCGACTCTGAGGAGGAACAA	TGCGTAGTTGTGCTGATGTG	26
KLF4	AGGCACTACCGTAAACACACG	CGGGAAGACAGTGTGAAAAGT	31
OCT4	CTTCAGGAGATATGCAAAGCA	ACACTCGGACCACATCCTTC	31
SOX2	ACCAGCGCATGGACAGTTAC	CCGTTCATGTAGGTCTGCGA	35

Fig. 2. Validation of stem cell markers in TAT-MSCs by performing RT-PCR. Primer sequences and PCR cycles are shown below. TAT-MSC: thymus adipose tissue-derived mesenchymal stem cell; RT-PCR: reverse transcription polymerase chain reaction.

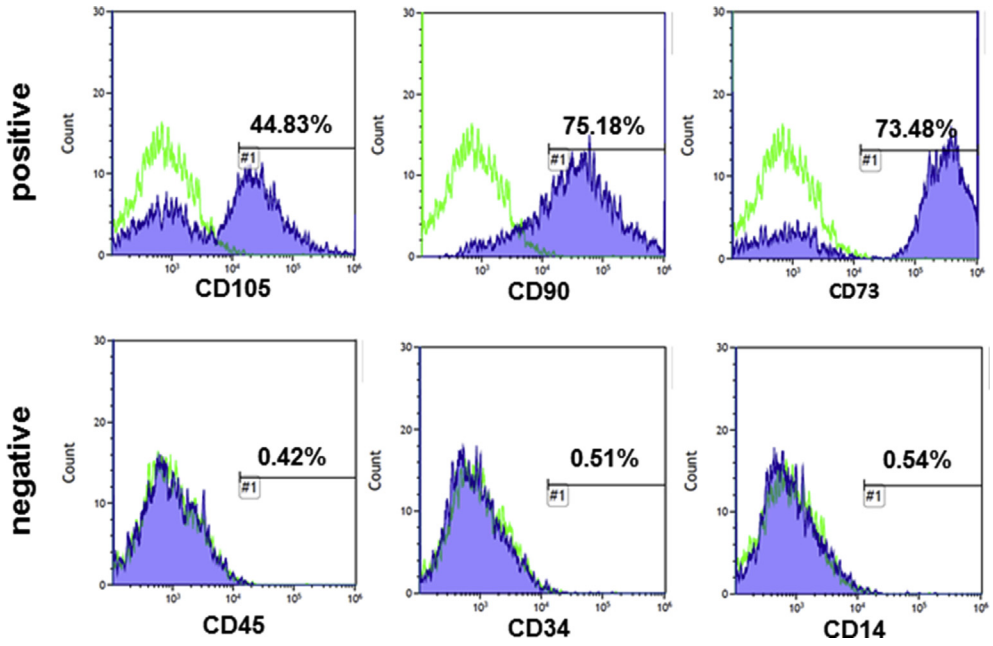


Fig. 3. Determination of cell surface markers in TAT-MSCs using FACS. TAT-MSCs were positive for CD105, CD90, and CD73 markers and negative for CD45, CD34, and CD14 markers. Of the two peaks observed in CD73 and CD105, the peak marked green is the control, and the peaks contained within it are considered a non-specific response in FACS analysis. TAT-MSC: thymus adipose tissue-derived mesenchymal stem cell; FACS: fluorescence activated cell sorting.

cells was confirmed by Oil Red-O Staining, while adipogenic differentiation was confirmed by RT-PCR. Finally, chondrocyte differentiation was confirmed by Toluidine Blue Staining and RT-PCR. Thus, this study revealed that TAT-MSCs have characteristics of mesenchymal cells.

3.2. *In vivo (animal) experiments*

3.2.1. *Echocardiogram*

To determine the efficacy of the ischemic reperfusion model, echocardiograms were obtained at the end of the 1st, 4th, 8th, and

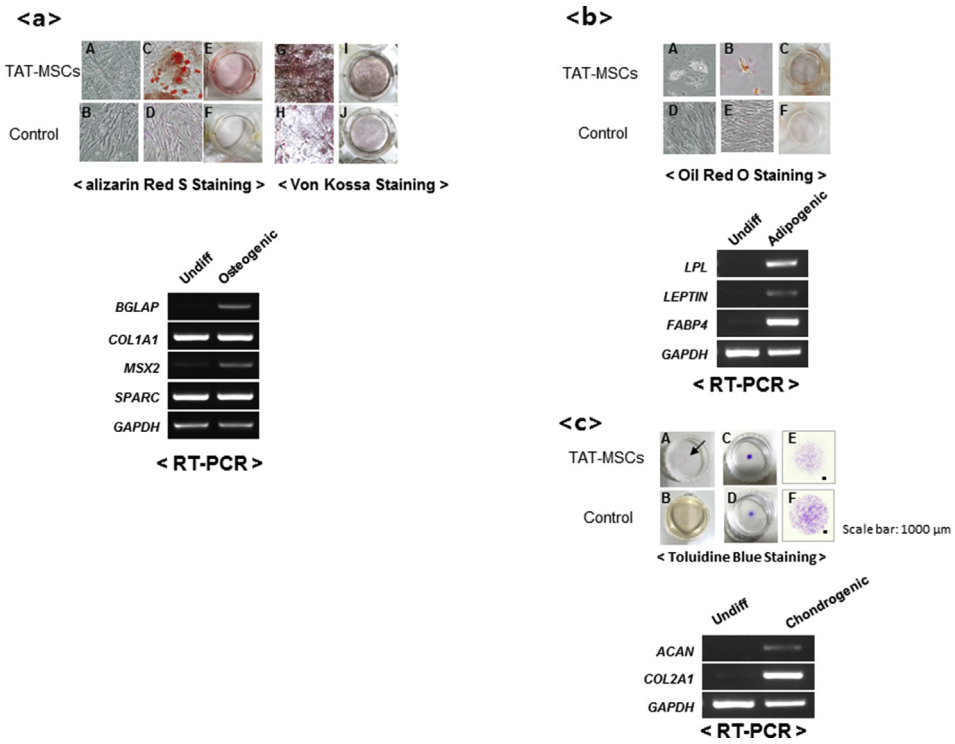


Fig. 4. Results of the differentiation study (a – osteogenesis, b – adipogenesis, and c – chondrogenesis). Osteogenesis, adipogenesis, and chondrogenesis were confirmed using the characterization medium and RT-PCR was performed to detect particular genes corresponding to each lineage. a. The TAT-MSCs group (A, C, E, G, I) showed positive results for alizarin Red S Staining and Von Kossa Staining while the negative control group (B, D, F, H, J) did not. b. Lipid droplets were confirmed by Oil Red O Staining in the TAT-MSCs group (A, B, C). c. Chondrogenic differentiation of TAT-MSCs was confirmed by Toluidine Blue Staining (A, C, E). TAT-MSC: thymus adipose tissue-derived mesenchymal stem cell.

12th week after the development of the myocardial ischemic reperfusion rat model. FS and EF are cardiac contractility indicators, E′ reflects the left ventricular relaxation function at early diastolic velocity, E/E′ is an index of cardiac relaxation ability that can estimate the left ventricular filling capacity. The FS and EF values of the vehicle and TAT-MSC groups were significantly decreased compared to those of the sham group. Whereas the FS and EF values of the TAT-MSC group were not significantly different than those of the vehicle group, they were slightly increased (Fig. 5; $p = 0.882$ and $p = 0.914$, respectively). The E′ and E/E′ values of the vehicle group and TAT-MSC group were significantly decreased compared to those of the sham group. The E′ and E/E′ values of the TAT-MSC group were not significantly different from those of the vehicle group; however, they exhibited a decreasing trend (Fig. 5; $p = 0.937$ and $p = 0.263$, respectively). Overall, these data suggest an improving trend regarding the SDF of heart in the TAT-MSC group.

3.2.2. Histopathological study

To confirm the presence of a myocardial ischemic site, the cardiac tissue was resected at 2 mm intervals and stained using 1% TTC stain at 37 °C for 10 min. In the TAT-MSC group, the ischemic area was significantly smaller (22.81 ± 6.41%), compared to the vehicle group (29.95 ± 9.09%, Table 1, $p = 0.04$), suggesting a positive effect of TAT-MSCs on the infarcted heart.

4. Discussion

Till date, multiple studies were conducted on the SC therapy in heart diseases; however, data for the application of TAT in SC therapy is unavailable. As the SC potential of TAT is solely being studied by performing cell studies in several researches, to the best of our knowledge this study is the first to perform in animal experiments using TAT to treat heart diseases [20–23]. Previous studies reported that the surface markers of TAT-MSCs differ from those of MSCs derived from the other tissues [20–22]. Olivia-Olivera et al. have conducted the most intensive studies on TAT-MCSs [22]. Their studies have shown that TAT-MSCs from elderly people have higher levels of adipogenic and osteogenic capacities than those taken in middle age. More interesting are the cell surface markers. In general, CD14 and CD34, which were negative in MSCs, were reported to be positive in TAT-MSCs in their study. Russo et al. and Musina et al. reported that the TAT-MSCs were positive for CD34 compared with various fat-derived cells of the human body [20,21]. Contradictorily, the results of our experiments were very distinct with previous study, so we repeatedly estimated the CD14 and CD34 expression level and found that it was not expressed in TAT-MSCs acquired in our study. However, other study exists that supported our observation [23]. It is presumed that the passage number of TAT-MSCs used to determine the expression levels of surface markers among various studies is varied. In the previous studies in which CD14 and CD34 expression of TAT-MSCs was reported to be positive, cell studies were conducted between passage 1 and 3, whereas in our case, the experiments were conducted at passage 6.

Previous animal studies or clinical trials that used the TAT are unavailable except the characterization studies that reported cell experiments. The most commonly used source of adipose-derived SCs is abdominal adipose tissue that is relatively easy to acquire and large quantities of adipose cells are obtained through liposuction. The TAT is not extensively studied as it requires surgical treatment during its extraction compared to the other adipose or bone marrow tissues. However, the TAT is a familiar tissue that is always encountered by cardiac surgeons during the heart surgery

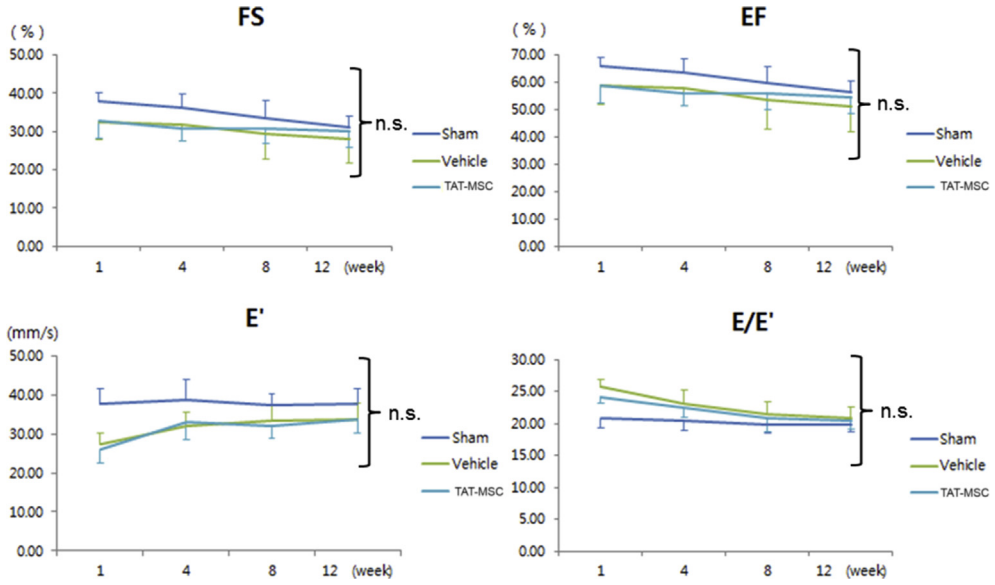
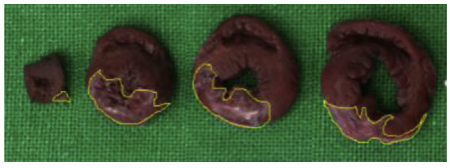


Fig. 5. Results of echocardiography in each group. After 12 weeks of follow-up, the absolute values of the contractile and relaxation capacities were improved in the TAT-MSC group, however this variation was not statistically significant. TAT-MSC: thymus adipose tissue-derived mesenchymal stem cell; n.s.: no significant differences.

Table 1
Representative images of the heart intercept. The area where the myocardium turned white is the infarct area (in yellow line). In the TAT-MSC group, the infarct area was significantly reduced compared to that of the vehicle group ($^{*}p < 0.05$ other groups vs. vehicle group). TAT-MSC: thymus adipose tissue-derived mesenchymal stem cell.

Group	Infarcted area (%)	Gross photography
Sham	–	
Vehicle	29.95 ± 9.09	
Cell	22.81 ± 6.41*	

and it is seldom removed to secure an operative field of view. If a method to extract TAT-MSCs might be expeditiously developed, a novel treatment for ischemic heart disease might be applied as SCs are collected from the TAT and administered back at the termination of cardiac surgery.

In animal experiments, from the researcher's point of view, it is important to understand whether the incidence of disease is consistently maintained after a disease model is developed. In this study, animal model was established through several preliminary experiments to induce a relatively constant degree of myocardial infarction in rats. The coronary artery adjacent to the first diagonal branch was ligated for 30 min and reperfusion was performed to allow the formation of 40% infarction. In future experiments, we plan to perform imaging assisted cell tracing using an agent that targets nucleus to estimate the infarct area prior to the euthanasia of animal model.

In our study, the concentration of TAT-MSCs administered to rats (2×10^5 cells/rat) was determined by referring to a previous study [24]. Further experiments are required to optimize the TAT-MSC concentration to be injected into rats.

In this study, although the results of several preliminary experiments were consistent, the question whether these effects are specifically caused by myocardial infarction, the appropriate dose or frequency of TAT-MSC administration, and the availability of any

other indicators that might be used to assess the efficacy of SC therapy remain as limitations. Further experiments that complement the aforementioned points are essential to establish TAT-MSC therapy for heart diseases.

5. Conclusions

In our study, although the TTE results were not statistically significant, the variations in FS, EF, E′, and E/E′ values exhibit an improving trend regarding the SDF of heart in the TAT-MSC group compared to that in the vehicle group. The infarcted area in the ischemic-reperfusion model of rat heart was significantly reduced in the TAT-MSC group compared to that in the vehicle group as confirmed by histopathological study. Further researches that consider the cell type, dose, infusion route, and time of injection are needed to confirm the positive effect of TAT-MSCs on the infarcted heart.

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Disclosure

The authors declare that there no conflict of interests.

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