Adipose-derived stem cell sheet transplantation therapy in a porcine model of chronic heart failure

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Abstract

Adipose-derived stem cells (ASCs) are a promising resource for cell transplantation therapy for damaged heart tissue. Cell death in the graft early after transplantation represents the main cause of unsatisfactory therapeutic efficacy, but tissue-engineered cell sheets grown in temperature-responsive cell culture dishes may enable improved engraftment of transplanted cells. We investigated the therapeutic potential of this method in chronic myocardial ischemia in swine. We created a porcine model of chronic heart failure by implanting an ameroid constrictor around the main trunk of the left anterior descending artery, just distal to the circumflex branch. Simultaneously, ASCs were obtained from a piece of subcutaneous adipose tissue and expanded to form ASC sheets using temperature-responsive dishes. Four weeks after ameroid constrictor placement, triple-layered ASC sheets were transplanted onto the area of the ischemic myocardium (sheet group, $n = 7$). Controls ($n = 7$) received no sheet. Just before and 4 weeks after transplantation, left ventriculography (LVG) and coronary angiography (CAG) were performed. LVG revealed significant improvement in the left ventricular ejection fraction of the sheet group compared to controls (47.6 ± 2.9 vs. $41.4 \pm 2.8\%$, $p < 0.05$). Furthermore, development of collateral vessels was only detected in the sheet group with right CAG. Histological analysis demonstrated that engrafted ASC sheets grew to form a thickened layer that included newly formed vessels. ASC sheet transplantation therapy is an intriguing therapeutic method for ischemic heart failure.

Keywords: adipose-derived stem cells; cell sheet; heart failure

Running head: ASC sheet transplantation on heart failure model

Abbreviations

ASCs Adipose-derived stem cells

EPCs Endothelial progenitor cells

ECs Endothelial cells

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cTnT cardiac troponin-T

vWF von Willebrand Factor

Introduction

Heart failure continues to place significant burdens on healthcare systems worldwide[1]. Over the last decade, cell transplantation therapy for the treatment of heart failure has emerged as an alternative to cardiac transplantation, and a large body of experimental evidence has accumulated[2]. Adult stem cells such as skeletal myoblasts and endothelial progenitor cells have been proposed as promising sources for cardiac regeneration[3, 4]. However, recent clinical studies have shown unsatisfactory therapeutic results[5]. One possible reason for this may be cell death in the graft early after injection of cells due to hypoxia, inflammatory cytokines, and proapoptotic factors[6]. Alternative transplantation methods involving the use of tissue engineering techniques are therefore essential for achieving more effective cell delivery[7].

Our colleagues have developed an original method to create sheets of cells without using a scaffold[8]. Using a culture surface coated with the covalently linked temperature-responsive polymer poly-(*N*-isopropylacrylamide), cells can be harvested as intact sheets following a simple temperature change[9-11]. Furthermore, due to the presence of preserved adhesion proteins and because enzymatic digestion is not needed, thick, cell-dense tissues are constructed by layering these cell sheets[11-13]. Transplantation of cell sheets onto damaged heart tissue improves heart function in several animal models[14-18].

Adipose-derived stem cells (ASCs) are multipotent cells that are a promising source of cells for a range of therapeutic applications[19, 20]. We have previously reported that ASC sheets transplanted onto scarred myocardium in rats grow to form a thick stratum that includes newly formed vessels, undifferentiated cells, and small numbers of cardiomyocytes[18]. ASC sheets also act through paracrine pathways to trigger angiogenesis and improve cardiac function in rats with myocardial infarction[18]. The aim of our current study was to investigate the therapeutic potential of ASC sheet transplantation therapy on chronic myocardial ischemia in a pre-clinical setting.

Methods

Experimental Animals

The present study used male domestic pigs (weight, 15-20 kg; Kears, Osaka, Japan). All experimental procedures were approved by the Animal Care Committee at the National Cardiovascular Center. The study protocol included three stages: 1) induction of chronic heart failure; 2) ASC sheet transplantation; and 3) final evaluation.

Animal Model

All surgical procedures in this study were performed under general anesthesia and continuous monitoring of the heart rate, three-lead electrocardiography, and oxygen saturation. A porcine model of chronic heart failure was created[21]. After induction of anesthesia with intramuscular injection of ketamine (750 mg) and xylazine (150 mg), animals underwent endotracheal intubation and were connected to a mechanical ventilator, followed by continuous intravenous injection of propofol (5 mg/kg/h) during the procedure. A left thoracotomy was made through the third intercostal space, and the pericardium was opened. The left anterior descending artery (LAD) was ligated distal to the second diagonal branch, followed by implantation of an ameroid constrictor (2.5 mm diameter; Research Instruments SW, Escondido, CA) around the main trunk of the LAD, just distal to the circumflex branch. A lidocaine injection (1 mg/min, continuously intravenous) was used to avoid arrhythmia. After stabilization, the pericardium was approximated, and the thorax was closed.

Isolation and Expansion of ASCs

ASCs were obtained from a piece of subcutaneous adipose tissue (about 1-2 g) that was collected from the iliac area during ameroid constrictor placement. After mincing into small pieces, adipose tissue was digested with collagenase type I (25 mg/ml in phosphate-buffered saline) at 37 °C with constant shaking for 2 h. After filtration through 100-µm polyethylene

1 mesh and centrifugation, isolated cells were suspended in α -minimum essential medium
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4 supplemented with 10% fetal calf serum, plated onto a 100-mm dish, and incubated at 37 °C in
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7 5% CO₂. Plastic-adherent spindle-shaped cells became apparent 3-4 days later and were isolated
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9 with trypsinization after reaching 80-90% confluence[18, 20].
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11 12 13 ***Characterization of ASCs*** 14

15 Identity of the isolated ASCs was determined by assessing their surface markers using
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17 fluorescence-activated cell sorting (FACScan flow cytometer, Becton Dickinson, Mountain
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19 View, CA) using the following antibodies for porcine cell markers: CD29, CD90, CD31, and
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21 CD45 (BD Biosciences, San Jose, CA). Cells at passage 3 were used for flow cytometric
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23 analysis.
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26 The differentiation potential of ASCs was also demonstrated using previously reported
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28 methods[22]. Induction of differentiation into adipocytes and osteoblasts was examined with Oil
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30 Red O and Alizarin Red S staining, respectively.
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36 ***Formation and Transplantation of ASC Sheets*** 37

38 ASCs at passage 3 or 4 were used for ASC sheet formation. ASCs were cultured on 100-mm
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40 temperature-responsive dishes (CellSeed, Inc., Tokyo, Japan) at 1×10^7 cells/dish at 37 °C. After
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42 reaching confluence, ASCs were detached in a sheet at 20 °C as a monolayer. ASC sheets were
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44 allowed to gently pile up to form a triple-layered sheet on the dish and were used as
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46 transplantation grafts.
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49 Four weeks after placement of the ameroid constrictor, a midline sternotomy was
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51 performed in each chronic heart failure model pig, and the anterior aspect of the left ventricle
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53 wall was exposed. The sheet group (n = 7) received transplantation of triple-layered ASC sheets
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55 onto the border area of the ischemic and infarcted myocardium, whereas the control group (n =
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57 7) received a sham operation and no sheet. After confirmation of secure transplantation, a
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GoreTex pericardium membrane (expanded polytetrafluoroethylene, 0.1 mm; W.L. Gore & Associates, Flagstaff, AZ) was sutured onto the pericardial edge over the transplantation site to avoid direct adhesion in both the sheet and the control group pigs.

Cardiac Catheter Study

At 4 and 8 weeks after ameroid constrictor placement, cardiac catheter studies were conducted on each animal. After systemic heparin administration (2,000 IU intravenously) through a 5-Fr sheath placed in a femoral artery, a fluid-filled 5-Fr pig-tail catheter was passed into the left ventricle cavity, and left ventriculography (LVG) was performed using an OEC 9600 mobile C-arm (GE Yokogawa Medical Systems, Tokyo, Japan). Results were recorded on digital videotapes for off-line analysis. Left ventricular volumes were measured using the area-length method and left ventricular ejection fraction (LVEF) was evaluated.

Selective coronary angiographies (CAGs) were subsequently performed on each animal to confirm stenosis of the LAD and to examine the formation of collateral vessels. Collateral flow to the LAD area was graded angiographically using the Rentrop score[23].

Pathological and Histological Assessment

At the end of the study, after humane euthanasia, hearts were harvested and cut into 5-6 slices of 8-mm thickness along the longitudinal axis. Slices were stained using tetrazolium red, and each basal side was photographed. Slices were fixed with 10% neutral formaldehyde for 24 h, embedded in paraffin, and cut into 4- μ m slices. A few sections were prepared and stained using hematoxylin and eosin or Masson's trichrome for histological examinations.

To examine cell differentiation, immunohistochemical staining for endothelial cells and cardiomyocytes was performed using rabbit anti-human von Willebrand Factor (vWF) antibody (1:50; DAKO, Glostrup, Denmark) and mouse anti-rabbit cardiac troponin-T (cTnT)

antibody (1:100; Neomarkers, Fremont, CA), respectively. Visualization was performed with diaminobenzidine staining (EnVision+ System/HRP Rabbit, DAKO).

Neovascularization in the ischemic myocardium was evaluated by counting the number of capillaries that were identified with vWF-positive endothelial cells. Five different slices from the border zone between viable and infarcted myocardium were selected. Pictures were taken under light microscopy at 40× magnification in five random fields. Vessels with a diameter smaller than 50 µm were counted. The capillary density was expressed as the number of capillaries per square millimeter.

Statistical Analysis

GraphPad Prism (GraphPad Software, San Diego, CA) was used for all statistical analysis. All values are expressed as the mean ± standard error of the mean. The Mann-Whitney U-test was used to compare differences between groups. The Wilcoxon signed rank test was used to determine within-group differences. Comparisons of LVEF at different time points were made with two-way analysis of variance followed by the Bonferroni post-test. P values <0.05 were considered statistically significant.

Results

Porcine Model of Chronic Heart Failure

We created a porcine model of chronic heart failure by placing an ameroid constrictor around the main trunk of the LAD and ligating the distal LAD (Fig. 1A). This created a highly stenosed (99% with delay) proximal LAD and total occlusion of the distal LAD, as confirmed with CAG in each case (Fig. 1B). Furthermore, the area of LAD perfusion in this model comprised viable myocardium in the proximal area and infarcted myocardium in the distal area (Fig. 1C).

Characteristics of ASCs

Most cultured ASCs expressed CD29 and CD90 (Fig. 2). In contrast, the majority of ASCs were negative for CD31 and CD45 (Fig. 2), which is in agreement with previous reports[24, 25]. The multipotency of ASCs was confirmed by their ability to differentiate into adipocytes, which contained oil droplets stained with Oil Red O, and osteoblasts, which produced calcium stained with Alizarin Red S (data not shown).

Formation and Transplantation of ASC Sheets

ASCs were grown on temperature-responsive dishes and harvested as ASC sheets after reaching confluence by reducing the temperature from 37 °C to 20 °C. ASC sheets shrank by two-thirds in size following detachment from the dish surface. ASC sheets were then manually piled onto each other to form triple-layered sheets, which were used as transplantation grafts (Fig. 3A-C).

We transplanted three triple-layered ASC sheets onto the ischemic area in the anterior wall of each heart in the sheet group 4 weeks after ameroid constrictor placement (Fig. 3D).

Growth and Differentiation of ASC Sheets after Transplantation

We examined the hearts of both groups 8 weeks after ameroid constrictor placement. ASC sheets transplanted onto the ischemic myocardium in the sheet group grew to form thickened layers of cells. ASC layers were 10-15 mm thick and stained blue with Masson's trichrome (Fig. 3E, F). Conversely, no layer was observed on the surface of control group hearts.

In the transplanted ASC layer, vWF-positive cells were observed in apparent vascular structures (Fig. 3H). This implied that ASCs had differentiated into endothelial cells and formed blood vessels to support further growth. Unfortunately, no cTnT-positive cells were observed (Fig. 3I).

Recovery of Cardiac Function after ASC Sheet Transplantation

To evaluate left ventricular function before and after ASC sheet transplantation, we performed LVG on animals in the control and sheet groups at both 4 and 8 weeks after ameroid constrictor placement. LVG revealed significantly improved LVEF in the sheet group (from $41.4 \pm 2.8\%$ to $47.6 \pm 2.9\%$) compared to the control group ($38.9 \pm 4.8\%$ to $34.6 \pm 1.9\%$, $p < 0.05$) after ASC sheet transplantation (Fig. 4).

Furthermore, right CAG was performed to assess collateral formation to the LAD-responsive area from the right coronary artery. Development of collateral vessels was only detected in the sheet group with right CAG (Fig. 5A), although no statistically significant differences in the Rentrop score were observed between groups (Fig. 5B).

The capillary density in the ischemic myocardium in the LAD responsive area was significantly greater in the sheet group than in the control group (905.5 ± 89.3 vs. 330.0 ± 83.4 capillaries per mm^3 , $p < 0.05$) (Fig. 6).

Discussion

In the present study, we demonstrated that ASC sheet transplantation significantly improved LVEF in a porcine chronic heart failure model. Furthermore, ASC sheets increased in thickness up to 15 mm, and vascular formation was observed after transplantation onto the ischemic myocardium.

ASCs are defined as an isolated, plastic-adherent, multipotent cell population[20] and currently appear to represent one of the most suitable sources of material for cell therapy for heart failure[26]. These cells show differentiation potential for endothelial cells and cardiomyocytes and show strong secretion of vascular growth factors[18, 20, 27]. Furthermore, ASCs are easily expandable[26] and can be harvested with a minimally invasive procedure. These features will be advantageous for clinical application of ASC transplantation therapy[28]. Several animal studies have demonstrated the therapeutic benefits of ASCs for ischemic heart

1 failure[18, 24, 25, 29-31]. The heterogeneity of ASCs had sometimes been discussed.

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4 Fibroblasts, as well as endothelial cells, smooth muscle cells, and pericytes, could be included
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6 in the early stage of isolation process of ASCs, however, these are thought to be eliminated by a
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8 washing procedure in the cell culture process[28].
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11 The development of tissue engineering offers several methods for delivering cells to
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13 damaged cardiac regions[7]. The tissue engineering approach using substrates such as polylactic
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15 acid, polyglycerol acid, collagen, and alginates has enabled several transplantation approaches
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17 such as scaffolds and patches[7, 32]. However, improved methods are needed that will correct
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19 the unfavorable degradation properties that lead to acidification and inflammation of
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21 surrounding tissues[32].
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25 Cell sheets are generated from confluent cells on temperature-responsive culture
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27 dishes simply by lowering the temperature, preserving cell-cell and cell-extracellular matrix
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29 adhesions in the cell sheet[8]. The cell sheets are composed only of cells and attach well to
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31 native tissues due to the adhesion molecules on the surface[9, 11]. With these features, the cell
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33 sheet technique enables the delivery of a large number of cells without loss of the transplanted
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35 cells or damage to the host tissue. In addition, the superiority of the cell sheet technique over
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37 conventional needle injection has been appropriately demonstrated[14, 30].
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41 Following a study in a rat model of myocardial infarction[18], this is the first study to
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43 evaluate the efficacy and feasibility of ASC sheet transplantation therapy for ischemic heart
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45 failure in a large animal model. Because swine closely resemble humans in terms of anatomy
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47 and physiology, the results of this study have several implications for the clinical application of
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52 In this study, the transplanted ASC sheet showed increased thickness after
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54 transplantation onto the ischemic myocardium. Interestingly, newly formed vessels were
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56 included in the thickened layer of ASC sheets. These findings imply that some engrafted ASCs
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58 may have differentiated into endothelial cells and formed vascular networks to perfuse
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2 themselves and sustain further growth. Some advantageous properties of ASCs such as a higher
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4 proliferative potential and endothelial differentiation capacity[20, 26] may account for the
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6 efficient engraftment and proliferation after ASC sheet transplantation[18].
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9 In the sheet group, significant improvement in LVEF 4 weeks after transplantation was
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11 often accompanied by collateral vessel growth into the ischemic area. In addition, the capillary
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13 density of peri-infarct ischemic myocardium was significantly increased. These results suggest
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15 that the improvement in left ventricle function after ASC sheet transplantation was induced by
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17 the increased perfusion of hibernating myocardium through neovascularization[33]. A possible
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19 mechanism for the neovascularization is due to the secretion of paracrine factors from ASCs, as
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21 demonstrated in several studies[18, 30, 34]. We speculate that transplanted ASCs steadily
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23 increased in number and then strengthened their potential as reservoirs of angiogenic growth
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25 factors through paracrine effects, leading to recovery of the left ventricle contractility in
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27 hibernating myocardium, which is dependent on increased perfusion.
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31 A limitation of this study is the short period of observation, and a further study with
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33 longer observation is warranted to exclude the possibilities of tumorigenesis and
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35 arrhythmogenesis to provide evidence for the safety of ASC sheet transplantation therapy.
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38 In conclusion, ASC sheet transplantation therapy is an intriguing therapeutic method
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40 that may be used to treat ischemic heart failure in the future in humans.
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Figure legends

Figure 1. A porcine model of chronic heart failure. **A)** An ameroid constrictor (white arrow) was placed around the proximal LAD, and the distal LAD was ligated (yellow arrow). **B)** CAG of the left coronary artery showed stenosis in the proximal LAD and occlusion of the distal LAD. **C)** Transverse sections of the resected heart stained with tetrazolium red. Viable myocardium was stained red, and infarcted regions appeared white in color.

Figure 2. Flow cytometric analysis of the ASCs. Most ASCs expressed CD29 and CD90, whereas they were negative for CD31 and CD45.

Figure 3. A-C) Formation of ASC sheets. ASCs were incubated on a 10-cm temperature-responsive dish (**A**). After reaching confluence (**B**), ASCs were detached as sheets and piled up to form triple-layered sheets for use as grafts (**C**). **D-F)** Transplantation of ASC sheets. **D)** ASC sheets were transplanted onto the anterior aspect of the ischemic myocardium. **E)** A slice of the resected heart 4 weeks after ASC sheet transplantation. **F)** ASC layers were 10- to 15-mm thick and stained blue with Masson's trichrome staining. **G-I)** Differentiation of ASC sheets. Microscopic image of the transplanted ASC layers. **G)** Hematoxylin and eosin staining. **H)** vWF immunohistochemistry staining. **I)** cTnT immunohistochemistry staining. White bar applied to the Figure 3 I) represents 50 μ m.

Figure 4. Improvement in the ejection fraction after ASC sheet transplantation. LVG revealed significant improvement in the ejection fraction in the sheet group compared to controls 4 weeks after transplantation (from $41.4 \pm 2.8\%$ to $47.6 \pm 2.9\%$ in the sheet group vs. from $38.9 \pm 4.8\%$ to $34.6 \pm 1.9\%$ in the control group, $p < 0.05$). * $p < 0.05$.

Figure 5. Collateral formation after sheet transplantation. CAG (A) and the Rentrop score (B) before and 4 weeks after ASC sheet transplantation (n = 6).

Figure 6. Neovascularization after sheet transplantation. The sheet group showed significant improvement in the capillary density in the ischemic myocardium compared to the control group. White bar applied to the Figure 6 A) represents 50 μm . *p < 0.05.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

A

control

sheet

B