Asian Biomedicine Vol. 6 No. 1 February 2012; 9-18 DOI: 10.5372/1905-7415.0506.121

Original article

Application of cell-sheet seeding to improve seeding efficiency of monolayer cells on the surface of biomaterials

Zhenyu Wang[a], Ning Ma[a], Dehua Chang[b], Huimin Huang[a]
[a]Heart Center, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai 200127, China, [b]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo 1628666, Japan

Background: Cell seeding technique is a fundamental component of the tissue engineering construction. Effective cell seeding can not only shorten the construction time but also improve the final outcome of tissue engineered substitutes.
Objective: We improved the seeding efficiency of monolayer cells on the surface of biomaterials in tissue engineering. Cell sheet technology was applied to seed monolayer cell sheet instead of concentrated cell suspension, which was termed cell-sheet seeding.
Methods: Equal quantities of endothelial cells were seeded on the surface of equally sized acellular bovine pericardia by cell-sheet seeding and cell suspension seeding respectively. After culturing cell-seeded pericardia for 24 hours, the effects of these 2 cell seeding methods were evaluated and compared.
Results: As compared to cell suspension seeding, a larger quantity of loading cells, higher tissue-plasminogen activator (t-PA) and prostacyclin (PGI-2) content, and higher homogeneity of cells distribution on the surface of the pericardia was achieved with cell-sheet seeding. Furthermore, cell-sheet seeding did not affect metabolism or increase endothelial cells damage.
Conclusion: Cell-sheet seeding is an effective and time-saving method of seeding monolayer cells on the surface of biomaterials. This novel seeding method is particularly suitable for building tissue engineered substitutes with monolayered cells distribution and 2-dimensional structure.

Keywords: Cell adhesion, cell seeding, cell sheet, extracellular matrix, tissue engineering

Tissue engineering technology has been widely applied for repairing or replacing defective tissues and organs. A conventional strategy of constructing tissue engineered substitutes involves the cell seeding method, which largely in□uences the □nal outcome of engineered tissues. Traditional method is cell suspension seeding that concentrated cell suspension was dripped on scaffolds with the intention of cellular attachment and migration on the scaffolds. Although this seeding method involves only one seeding step, it carries the risk of cells loss and the seeding efficiency at a low cell concentration and is not satisfactory [1].

Correspondence to: Huimin Huang, PhD, Heart Center, Shanghai Children's Medical Center, School of Medicine, Shanghai Jiaotong University, Shanghai 200127, China. E-mail: 13391281688@qq.com

Recently, several new methods are designed that utilized external mediators such as centrifugal force or magnetic forces to increase the cell seeding efficiency [2, 3]. However, these seeding methods require special equipment, including a special rotor or magnetite nanoparticles. Far more important is that these methods are shown to be suitable for constructing engineered tissues with 3-dimensional structure. However, some tissues only have a monolayered rather than multilayered cells distribution, such as the vascular intima, bladder epithelium, and cornea, therefore these seeding methods are unsuitable for constructing engineered tissues with 2-dimensional structure.

In 1990, Okano developed cell sheet technology based on the temperature-responsive culture dish [4]. By covalently immobilizing the temperature-responsive

polymer poly-*N*-isopropylacrylamide (PIPAAm) onto the surface of an ordinary polystyrene culture dish, an intact cell sheet can be achieved by simple temperature changes. Since this technology does not involve proteolytic enzyme treatment, the cell sheet maintains its extracellular matrix (ECM), which makes the cell sheet easily adhere to any biomaterials or host tissues without using any mediators such as fibrin glue [5, 6]. This technology has been extensively applied in building tissue engineered substitutes such as skin [7], corneal epithelium [8], bladder urothelium [9], periodontal ligaments [10], cardiac muscle [11], and liver lobules [12]. However, cell sheet technology has not been tried to improve the efficiency of seeding cells on biomaterials.

In the present study, cell sheet technology was firstly applied to seed monolayer endothelial cell sheet on the surface of acellular bovine pericardia, which was termed cell-sheet seeding, to build tissue engineered cardiovascular patches with monolayered cells distribution and 2-dimensional structure. Equal quantities of endothelial cells were seeded on the surface of equally sized acellular bovine pericardia by cell-sheet seeding and traditional cell suspension seeding respectively. And then the short-term seeding effects of these 2 cell seeding methods were compared. Our goal was to identify whether the cell-sheet seeding method could improve the efficiency of seeding monolayer cells on the surface of biomaterials.

Materials and methods

Animal preparation

One-year-old cattle used in this study were obtained from a local dealer. All animals received care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health after approval from the Animal Care and Use Committee of Shanghai Jiaotong University School of Medicine.

Acellular bovine pericardia

Specific procedures for the preparation of acellular bovine pericardia were according to the method previously described [13]. Briefly, the pericardium (7×7 cm) was dissected after thoracotomy of a bovine under anesthesia. After removing excess adipose tissue, the pericardium was incubated with 0.25% trypsin at 37°C for 24 hours, followed by continuous shaking with 0.5% SDS detergent to extract the digested cells. After treatment, the decellularized pericardium was frozen at -80°C for three to four hours and then quickly re-warmed. After three to four cycles of freezing-dissolving processing, it was dehydrated in a vacuum freeze dryer at temperature lower than -20°C and then sterilized by epoxy ethane. The histological sections of these acellular bovine pericardia were observed under microscopy to confirm that no cells remained in the matrix.

Cell source

The endothelial cells were isolated from a bovine carotid artery according to the method previously described [13]. Endothelial cells were cultured in endothelial cell growth medium-2 (EGM - 2, Lonza, USA) at 37°C in a humidi□ed atmosphere of 5% CO_2. Cells were monitored daily using phase contrast microscopy and subcultured when they were confluent. The cultured endothelial cells were identified by FITC-labeled CD31 and vWF antibodies using immuno-fluorescence techniques.

Preparation of endothelial cell sheets

The endothelial cell sheets were made according to Asakawa's method [14]. The vitality of endothelial cells was con□rmed by trypan blue staining before making cell sheets. Cells at passage four were seeded onto the surface of a PIPAAm grafted dish (temperature-responsive culture dish, Φ3.5cm, UpCell®, CellSeed, Tokyo, Japan) at a cell density of 1×10^6 cells per dish and cultured in EGM-2 at 37°C in a humidi□ed atmosphere of 5% CO_2. Cells were incubated for two days to reach confluence and the temperature-responsive culture dish was then transferred to an incubator controlled at 20°C and 5% CO_2 for harvesting an intact monolayer endothelial cell sheet with its diameter no bigger than 1.5 cm.

Cell seeding

Before cell seeding, one piece of acellular bovine pericardium was rinsed in PBS three times, was cut into small pieces (1.51×.5 cm), and was soaked in EGM-2 overnight at 37°C. Endothelial cells were seeded onto the surface of a 1.5×1.5 cm acellular bovine pericardium by two different cell seeding methods. There were five samples in each group. In an effort to minimize the discrepancy between the samples, the same passage 4 cells were used. The first method used was cell suspension seeding. Trypan blue staining was performed to confirm cell viability, and a 0.1 mL

concentrated cell suspension (1×10^6 cells) was seeded uniformly on the surface of a wet 1.5×1.5 cm acellular bovine pericardium. The cell-seeded pericardium was incubated for two hours at 37°C before transfer to a new 24-well cell culture plate. An additional 1 mL EGM-2 was added to the culture well. The second method used was cell-sheet seeding. A previously prepared endothelial cell sheet (1×10^6 cells) was gently transferred onto the surface of a wet 1.5×1.5 cm acellular bovine pericardium. The cell-seeded pericardium was treated the same way as the cell suspension seeding method described above. Finally, all cell-seeded pericardia were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 hours. The seeding effects of these two different methods were compared, including the loading cells quantity, metabolism, damage, secreting function, and distribution. The experiment was performed in triplicate.

Effects evaluation

Cells quantity

Loading cells quantity was determined using a Cell Counting Kit-8 assay kit (CCK-8, Dojindo, Kumamoto, Japan), with a highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] according to the manufacturer's instructions. Each cell-seeded pericardium (n=5/group) was rinsed with PBS 3 times and was placed in a new well of a 24-well plate containing 1 mL of fresh EGM-2, to which 100 μL CCK-8 was added. The pericardia were incubated in the dark at 37°C in a humidified atmosphere of 5% CO_2 for 2 hours. Afterwards, 100 μL culture medium of each sample was removed and transferred to a 96-well plate and the absorbance was read at 450 nm using a microplate reader (Well Scan MK2, Labsystems, Dragon, Finland). Unseeded acellular bovine pericardia (n=2) processed in the same way served as controls. The absorbance accurately reflected the quantity of loading viable cells.

Cells metabolism and damage

Cells metabolism and damage were assessed by measuring the concentrations of glucose (Glu), lactate (Lac), and lactate dehydrogenase (LDH) in the culture medium using a commercial kit (Johnson & Johnson, Vitros-250, USA). Culture medium (200 μL) was collected (n=5/group) and was centrifuged at 4°C/1000 RPM for five minutes to deposit the nonadherent cells. The supernatant was immediately analyzed for concentrations of Glu, Lac, and LDH. Unseeded acellular bovine pericardia (n=2) served as controls.

Cells secretion function

The loading cells secretion function was assessed by quantitating the cumulative content of secreted tissue-plasminogen activator (t-PA) and prostacyclin (PGI-2) in the culture medium using ELISA kits (Hushang Biotechnology Co. Ltd., China). Culture medium (100 μL) was collected (n=5/group) and centrifuged at 4°C/1000 RPM for five minutes to deposit the nonadherent cells. The supernatant was stored at -80°C until further analysis for t-PA and PGI-2. Unseeded acellular bovine pericardia (n=2) served as controls.

Light microscopy

The cell-seeded pericardia were rinsed with PBS three times and fixed in 10% neutral buffered formalin solution. The fixed samples were conventionally dehydrated in sequentially increasing ethanol solutions to 100% ethanol, immersed in xylene, and embedded in paraffin. The samples were sectioned (thickness= 4 μm) and stained with hematoxylin and eosin (H&E) to visualize the content and distribution of the loading cells and ECM. The sample sections were observed by light microscopy (DM-LB2, Leica, Germany).

Scanning electron microscopy

The cell-seeded pericardia were rinsed with PBS three times and fixed in 2.5% glutaraldehyde solution. The samples were rinsed with sodium cacodylate buffer, dehydrated in a graded series of ethanol, and dried with tetramethylsilane. Critical point drying was performed with an apparatus (HCP-2, Hitachi, Japan). The samples were sputter coated with gold (IB-2, Hitachi, Japan) and the pericardium samples were photographed using a scanning electron microscope (S-520, Hitachi, Japan) at an acceleration voltage of 20 kV. The density and distribution of loading cells as well as cell-cell and cell-pericardium relationships were observed.

Statistical analysis

All experimental data are presented as mean□standard deviation (SD). Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS 16.0) by one-way ANOVA analysis

for multiple comparisons and *t*-test for comparisons between two groups, and confidence levels of 95% ($P<0.05$) were considered statistically significant.

Results

Cells quantity

The CCK-8 assay was used to accurately evaluate the quantity of cells adhering to the acellular bovine pericardium. **Figure 1** showed that the optical density (OD) values of the three groups (unseeded= 0.01±0.00, cell suspension seeding=0.79±0.04, cell-sheet seeding=1.12±0.08). Cell-sheet seeding group was significantly higher than those of the cell suspension seeding group and the control group.

Cells metabolism activity

The concentrations of Glu (mmol/L) and Lac (mmol/L) in the culture medium 24 hours after cell seeding are shown in **Figure 2** (Glu: unseeded= 5.57±0.05, cell suspension seeding=3.69□0.26, cell-sheet seeding=2.64±0.33; Lac: unseeded=0.07±0.05, cell suspension seeding=1.12□0.20, cell-sheet seeding=1.24±0.24). The Glu concentration of the cell-sheet seeding group was significantly lower than those of the cell suspension seeding group and the control group. In contrast, the Lac concentration of the cell-sheet seeding group was significantly higher than that of the control group. However, the Lac concentration of the cell-sheet seeding group was similar to that of the cell suspension seeding group and no significant difference was observed between the two groups.

Cells damage

The cumulative concentration of LDH (U/L) leakage in the culture medium 24 hours after cell seeding is shown in **Figure 3** (unseeded=89.17±3.76, cell suspension seeding=145.40±9.16, cell-sheet seeding=151.87±11.19). Both of the two cell seeding groups showed signi□cantly higher LDH than the control group. However, the LDH leakage of the cell-sheet seeding group was only slightly increased compared to the cell suspension seeding group and no significant difference was observed between the two groups.

Cells secretion function

The cumulative concentrations of t-PA (□g/L) and PGI-2 (ng/L) in the culture medium of the three groups 24 hours after cell seeding (t-PA (**Figure 4A**): unseeded=0.78±0.01, cell suspension seeding= 16.84±1.01, cell-sheet seeding=22.29□0.96; PGI-2 (**Figure 4B**): unseeded=11.89□0.20, cell suspension seeding=231.81±31.45, cell-sheet seeding=329.29± 23.44). Both the t-PA and PGI-2 concentrations of the two cell seeding groups were signi□cantly higher than those of the control group. Furthermore, the concentrations of t-PA and PGI-2 in the cell-sheet seeding group were signi□cantly higher than those of the cell suspension seeding group.

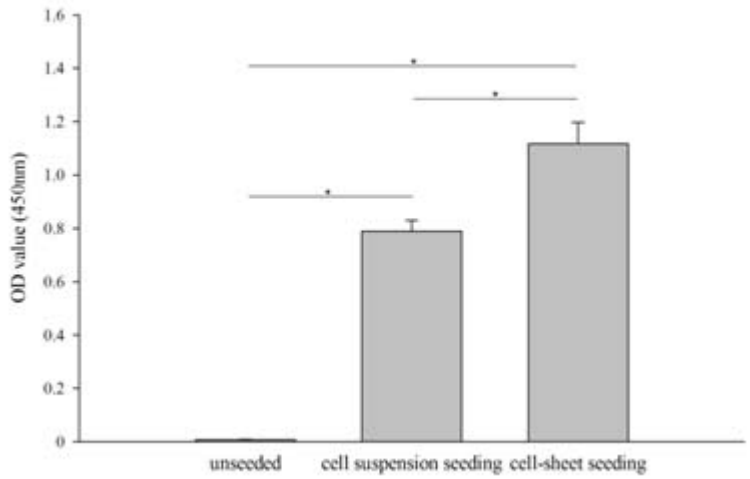

Figure 1. OD value of the three groups; unseeded=0.01±0.00, cell suspension seeding=0.79±0.04, and cell-sheet seeding=1.12±0.08. Each line represents a comparison between two groups. *$P<0.05$, significant difference in the OD value between the two groups.

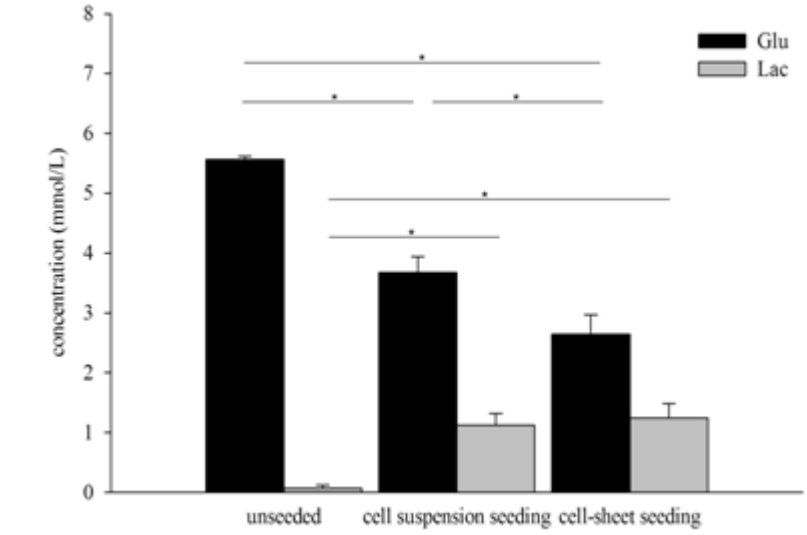

Figure 2. Concentrations of Glu and Lac in three groups: black bars represent concentration of Glu: unseeded=5.57±0.05, cell suspension seeding=3.69±0.26, and cell-sheet seeding=2.64±0.33; grey bars represent concentration of Lac: unseeded=0.07±0.05, cell suspension seeding=1.12±0.20, and cell-sheet seeding=1.24±0.24. Each line represents a comparison between two groups.*P<0.05 significant difference in the concentration between the two groups.

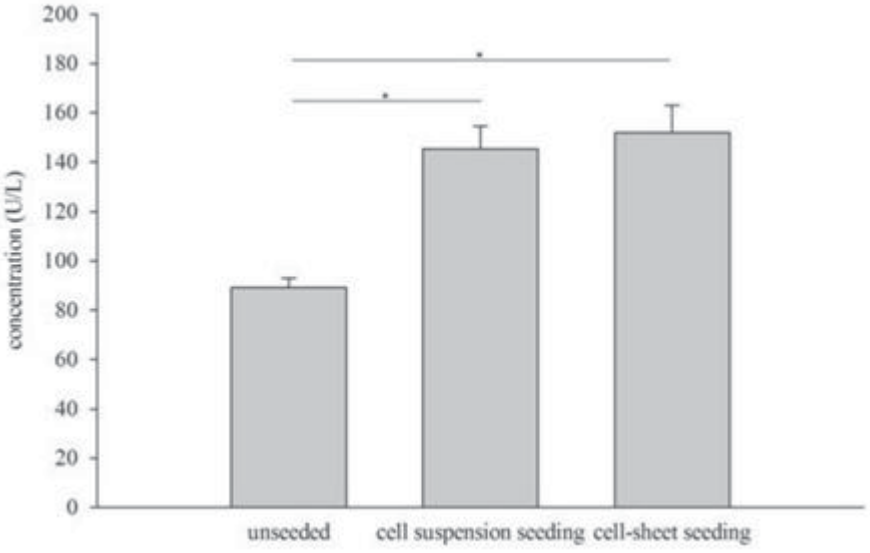

Figure 3. Concentration of LDH in the three groups: unseeded=89.17±3.76, cell suspension seeding=145.40±9.16, and cell-sheet seeding=151.87±11.19. Each line represents a comparison between two groups. *P<0.05 significant difference in the concentration between the two groups.

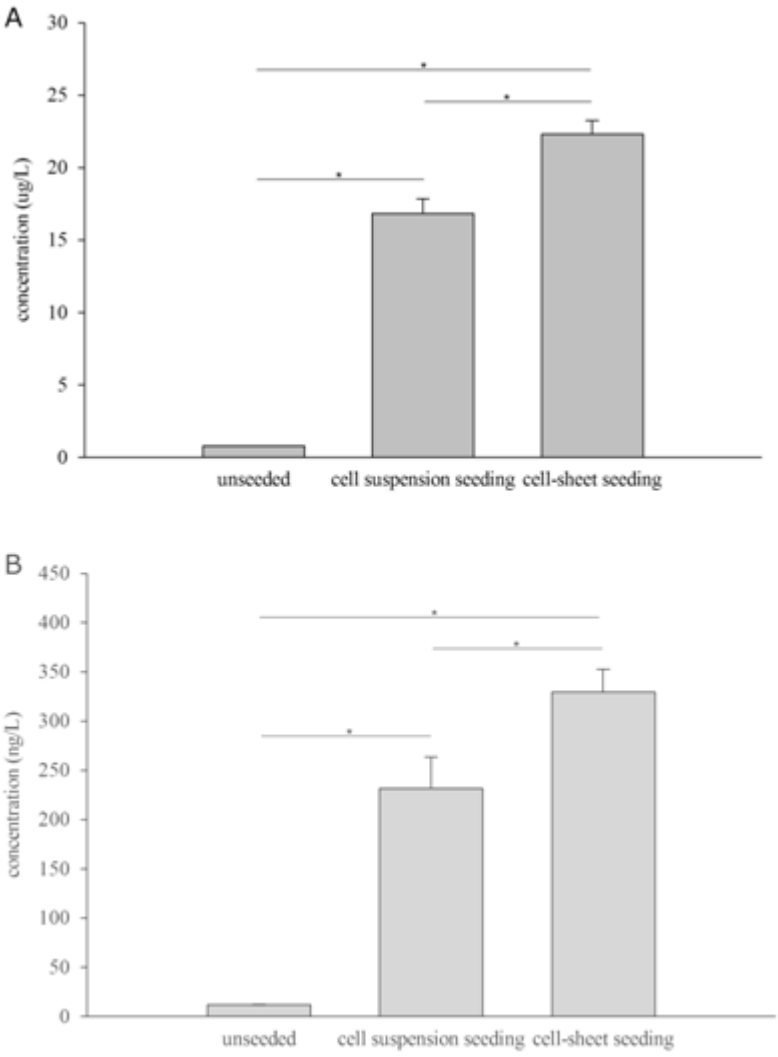

Figure 4. Concentrations of t-PA and PGI-2 in the three groups; (**A**) concentration of t-PA: unseeded=0.78□0.01, cell suspension seeding=16.84±1.01, and cell-sheet seeding=22.29±0.96; (**B**) concentration of PGI-2: unseeded=11.89±0.20, cell suspension seeding=231.81±31.45, and cell-sheet seeding=329.29±23.44. Each line represents a comparison between two groups. *P<0.05 significant difference in the concentration between the two groups.

Light microscopy

Figure 5 shows histological sections of cells attached to the acellular bovine pericardium 24 hours after cell seeding. No cells were observed on the surface of the acellular bovine pericardium (**Figure 5A**). Less cells and ECM adherence to acellular bovine pericardium were observed by using the cell suspension seeding method (**Figure 5B**) compared to the cell-sheet seeding method (**Figure 5C**). Furthermore, loading cells distributed more homogeneously and continuously in the cell-sheet seeding group.

Scanning electron microscopy

Scanning electron micrographs of the pericardia in three groups are shown in **Figure 6**. No cells were observed on the surface of the acellular bovine pericardium (**Figure 6A**). More loading cells and ECM were also observed in the cell-sheet seeding group (**Figure 6C**) compared to the cell suspension seeding group (**Figure 6B**). In addition, loading cells had already achieved confluence and the cells were arranged more closely in the cell-sheet seeding group.

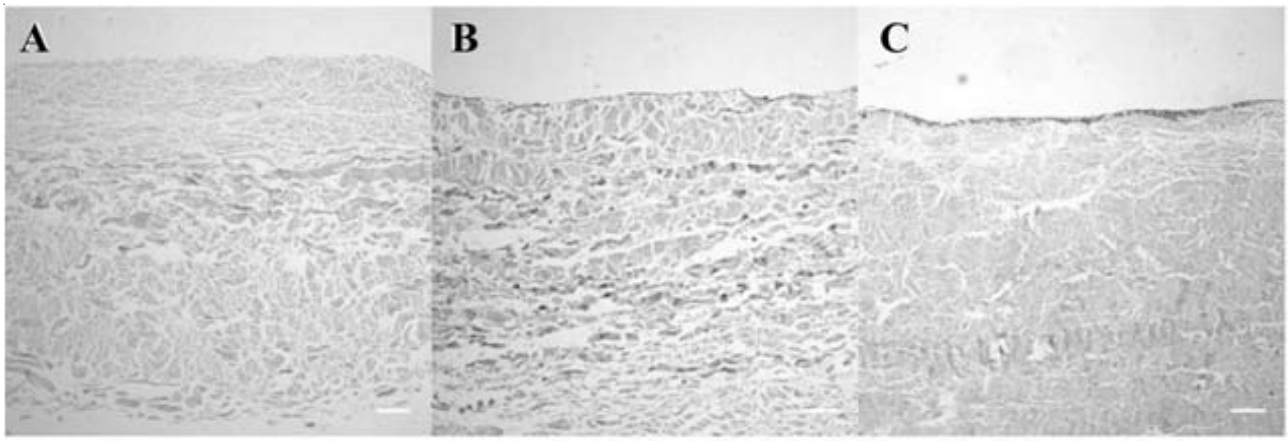

Figure 5. Light microscopy images of H&E stained (**A**) acellular bovine pericardium: no cells on the pericardium, (**B**) cell suspension seeding: less cells and ECM adhering to the pericardium, and (**C**) cell-sheet seeding: more cells and ECM adhering to the pericardium, cells distributed more homogeneously and continuously, bar=100 μmA

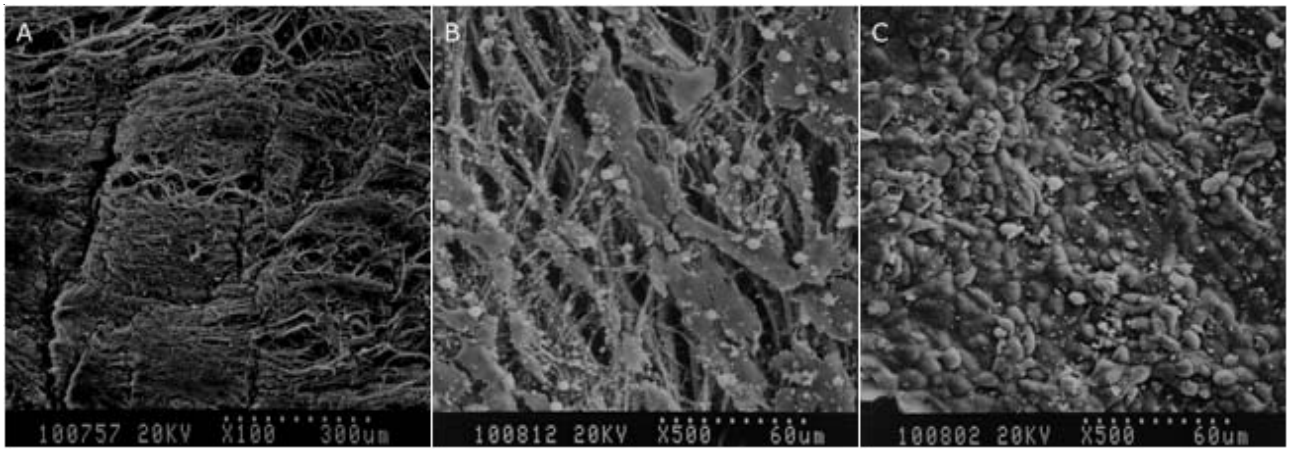

Figure 6. Scanning electron micrographs of (**A**) acellular bovine pericardium: no cells on the pericardium, (**B**) cell suspension seeding: less cells and ECM adhering to the pericardium, loading cells arranged separately, and (**C**) cell-sheet seeding: more cells and ECM adhering to the pericardium, loading cells achieved confluence and arranged closely.

Discussion

Organ dysfunction or damage can occur due to congenital disease, cancer, trauma or other pathophysiological conditions. Autologous tissues are the preferred choice for repairing or replacing the original tissues or organs, but sources of autologous tissues are limited. Therefore, engineered biological substitutes have huge potential for reconstructing defective tissues or organs. The ideal tissue engineered substitute requires normal tissue architecture, speci□c cell types, and adequate cellular quantity that are comparable to the native tissue. Cell seeding technique as a fundamental component of the construction strategy contributes significantly to cell adhesion [15 - 17]. Only upon adhering to the substrate can cells migrate, proliferate, and exert physiological function [18]. Therefore, effective cell seeding can not only shorten the construction time but also improve the final outcome of tissue engineered substitutes. Although many novel cell seeding methods have been devised to increase the seeding efficiency, most of them are focused on how to facilitate infiltration of cells into porous scaffolds. Unfortunately, how to improve the efficiency of seeding monolayer cells on the surface of biomaterials has not been reported. Our work is aimed to fill in the gap in this field.

The quantity of loading cells on biomaterials is extremely important for building tissue-engineered tissues or organs. Our results showed that in the early stage after cell seeding, a larger quantity of loading cells was achieved by cell-sheet seeding compared to cell suspension seeding. It might be attributed to the reason that the cell sheet maintained more ECM, which makes the cells stick to the acellular bovine pericardium more easily and tightly with minimal cell loss. Therefore, this approach might be suitable for endothelial cell seeding in cardiovascular tissue engineering, because it results in tight adherence of endothelial cells to biomaterials against the shear stress of blood flow.

The levels of Glu and Lac in culture medium reflect cellular metabolism. We observed an obviously low Glu concentration in the cell-sheet seeding group, consistent with the fact that a greater number of loading cells on the pericardium would consume more Glu. Thereby, the lower Glu level was explained by the fact that there was a large quantity of loading cells in the cell-sheet seeding group, resulting in vigorous cell metabolism and greater Glu consumption. Lac is a product of cellular anaerobic metabolism that is produced when the cellular aerobic metabolism cannot keep up with the energy demand. Our results showed that the Lac concentration of the two seeding groups was higher than that of the control group, which could be associated with the high concentration of seeding cells. A sharp increase in the cellular energy demand triggered anaerobic metabolism and produced a certain amount of Lac shortly after cell seeding. However, Lac concentrations were approximately equivalent between the two seeding groups, indicating that although cell-sheet seeding increased the total number of loading cells, it did not have an excessively adverse effect on aerobic metabolism compared to cell suspension seeding.

LDH leakage is a marker of cell damage or death. Our results showed a higher LDH concentration in the two seeding groups compared to the control group, which was possibly associated with the increased Lac concentration. Because an excessive amount of Lac could disturb the acid-base equilibrium of the culture medium, leading to cell damage. However, there was no significant difference in LDH concentration in the two seeding groups. The previous CCK-8 assay showed that there were more loading cells in the cell-sheet seeding group, suggesting that there was a lower incidence of cell damage in the cell-sheet seeding group than in the cell suspension seeding group.

The physiological functions of endothelial cells include synthesizing and secreting cytokines such as t-PA and PGI-2. Our study demonstrated that cell-sheet seeding resulted in higher t-PA and PGI-2 production comparing to cell suspension seeding. We propose that this result might be related to the quantity as well as the secreting ability of the loading cells. Several studies had proved that the ECM is an important nonpharmacologic cellular regulating factor that could influence many cellular physiological and pathological processes [19-22]. In addition, the cell sheet is harvested without using proteolytic enzymes, which might impact cellular function [23, 24]. Therefore, we speculate that cell-sheet seeding, maintaining more ECM, might enhance cellular secretion function compared to cell suspension seeding.

Cells distribution on the biomaterials is another important aspect affecting the final outcome of the tissue engineered substitute after implantation. Emphasis should be placed on the 2-dimensional rather than 3-dimensional structure when constructing some tissues with monolayer cells distribution. All above histological and morphological results in this study showed that cell-sheet seeding resulted in a more homogeneous and continuous distribution of the loading cells on the surface of the acellular bovine pericardium. At the same time, loading cells had already achieved confluence in the cell-sheet seeding group. Some studies had shown that although endothelial cell-seeded polymer material grafts showed enhanced potency in animal models, the consequences were not satisfying clinically and these disappointing outcomes were ascribed to the insufficient initial loading cells density, poor adhesion under blood flow, and failure to achieve cell confluence [16]. In addition, the spatially nonhomogeneous distribution of seeded cells, low cells density, and poor cellularity could also result in inferior preparation of tissue engineered constructions [25]. Our study showed that cell-sheet seeding method could compensate for these deficiencies to a large extent.

In conclusion, our study proved that cell-sheet seeding method could improve the efficiency of seeding monolayer endothelial cells on the surface of acellular bovine pericardium in different aspects. This novel seeding technique is particularly suitable for constructing tissue engineered substitutes with monolayered cell distribution and 2-dimensional structure.

Acknowledgements

We thank Assistant Prof. Tadashi Sasagawa and Assistant Prof. Yuji Haraguchi from Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, TWIns, for their expertise in making the cell sheet. This present work was supported by the grant from the National Natural Science Foundation of China (No. 81170151) and the grant for R&D Project of Combination Medicine and Engineering at Shanghai Jiaotong University (No. YG2010MS63). The authors have no conflict of interest to report.

References

1. Oberpenning F, Meng J, Yoo JJ, Atala A. De novo reconstitution of a functional mammalian urinary bladder by tissue engineering. Nat Biotechnol. 1999; 17:149-55.
2. Godbey WT, Hindy SB, Sherman ME, Atala A. A novel use of centrifugal force for cell seeding into porous scaffolds. Biomaterials. 2004; 25:2799-805.
3. Shimizu K, Ito A, Honda H. Mag-seeding of rat bone marrow stromal cells into porous hydroxyapatite scaffolds for bone tissue engineering. J Biosci Bioeng. 2007; 104:171-7.
4. Yamada N, Okano T, Sakai H, Karikusa F, Sawasaki Y, Sakurai Y. Thermo-responsive polymeric surfaces; control of attachment and detachment of cultured cells. Makromol. Chem Rapid Commun. 1990; 11:571-6.
5. Yang J, Yamato M, Nishida K, Ohki T, Kanzaki M, Sekine H, Shimizu T, Okano T. Cell delivery in regenerative medicine: The cell sheet engineering approach. J Control Release. 2006; 116:193-203.
6. Yang J, Yamato M, Shimizu T, Sekine H, Ohashi K, Kanzaki M, Ohki T, Nishida K, Okano T. Reconstruction of functional tissues with cell sheet engineering. Biomaterials. 2007; 28:5033-43.
7. Yamato M, Utsumi M, Kushida A, Konno C, Kikuchi A, Okano T. Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. Tissue Eng. 2001; 7:473-80.
8. Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, Watanabe H, Yamamoto K, Nagai S, Kikuchi A, Tano Y, Okano T. Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. Transplantation. 2004; 77:379-85.
9. Shiroyanagi Y, Yamato M, Yamazaki Y, Toma H, Okano T. Urothelium regeneration using viable cultured urothelial cell sheets grafted on demucosalized gastric flaps. BJU Int. 2004; 93:1069-75.
10. Akizuki T, Oda S, Komaki M, Tsuchioka H, Kawakatsu N, Kikuchi A, Yamato M, Okano T, Ishikawa I. Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs. J Periodontal Res. 2005; 40:245-51.
11. Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res. 2002; 90:e40-8.
12. Harimoto M, Yamato M, Hirose M, Takahashi C, Isoi Y, Kikuchi A, Okano T. Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. J Biomed Mater Res. 2002; 62:464-70.
13. Huang HM, Wu SF, Ren H. Tissue-engineered graft constructed by self-derived cells and heterogeneous acellular matrix. J Zhejiang Univ Sci B. 2006; 7:351-6.
14. Asakawa N, Shimizu T, Tsuda Y, Sekiya S, Sasagawa T, Yamato M, Fukai F, Okano T. Pre-vascularization of in vitro three-dimensional tissues created by cell sheet. Biomaterials. 2010; 31:3903-9.
15. Doag□ IO, Savopol T, Neagu M, Neagu A, Kov□cs E. The kinetics of cell adhesion to solid scaffolds: an experimental and theoretical approach. J Biol Phys. 2008; 34:495-509.
16. Ravi S, Qu Z, Chaikof EL. Polymeric materials for tissue engineering of arterial substitutes. Vascular. 2009; 17: S45-54.
17. Takahashi Y. and Tabata Y. Homogeneous seeding of mesenchymal stem cells into nonwoven fabric for tissue engineering. Tissue Eng. 2003; 9:931-8.
18. Dewez JL, Lhoest JB, Detrait E, Berger V, Dupont-Gillain CC, Vincent LM, Schneider YJ, Bertrand P, Rouxhet PG. Adhesion of mammalian cells to polymer surfaces: From physical chemistry of surfaces to selective adhesion on defined patterns. Biomaterials. 1998; 19:1441-5.
19. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix Biol. 2001; 19:816-27.
20. Mongiat M, Fu J, Oldershaw R, Greenhalgh R, Gown AM, Iozzo RV. Perlecan protein core interacts with extracellular matrix protein 1 (ECM1), a glycoprotein involved in bone formation and angiogenesis. J Biol Chem. 2003; 278:17491-9.
21. Chan I. The role of extracellular matrix protein 1 in human

skin. Clin. Exp Dermatol. 2004; 29:52-6.
22. Prasad Chennazhy K, Krishnan LK. Effect of passage number and matrix characteristics on differentiation of endothelial cells cultured for tissue engineering. Biomaterials. 2005; 26:5658-67.
23. Kushida A, Yamato M, Isoi Y, Kikuchi A, Okano T. A noninvasive transfer system for polarized renal tubule epithelial cell sheets using temperature-responsive culture dishes. Eur Cell Mater. 2005; 10:23-30.
24. Kushida A, Yamato M, Kikuchi A, Okano T. Two-dimensional manipulation of differentiated Madin-Darby canine kidney (MDCK) cell sheets: the noninvasive harvest from temperature-responsive culture dishes and transfer to other surfaces. J Biomed Mater Res. 2001; 54:37-46.
25. Holy CE, Shoichet MS, Davies JE. Engineering three-dimensional bone tissue in vitro using biodegradable scaffolds: investigating initial cell-seeding density and culture period. J Biomed Mater Res. 2000; 51:376-82.