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Comparative Study of Seeding and Culture Methods to Vascular Smooth Muscle Cells on Biodegradable Scaffold

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Abstract How to improve the cell culture method on scaffolds is important in the tissue engineering fileld. In this study, we optimized seeding and culture methods to vascular smooth muscle cells (VSMCs) on biodegradable polymer scaffold. The primary culture of VSMCs obtained from canine external jugular vein was accomplished by applying the explant-derived method. The primary cultured VSMCs were seeded into scaffolds and then cultured by using various different methods; static or dynamic seeding, static or dynamic culture. The difference in proliferative response of VSMCs was analyzed with an alamar blue assay. Cell-polymer construct was examined by histochemical method and scanning electron microscopy. Mesh type scaffold (10×10×0.4 mm) was made of polyglycolic acid (PGA) suture thread. The PGA mesh type scaffold was 45% in porosity, and 0.03 g in weight. The primary cultured VSMCs were confirmed with immunohistochemical staining using monoclonal anti-α-smooth muscle actin. The density and distribution of proliferated VSMCs within the scaffold and cellular adherence on the surface of the scaffold showed better results in the static seeding condition than in the dynamic condition. Under the same condition of seeding method as the static condition, the dynamic culture condition showed enhanced proliferation rates of the VSMCs when compared to the static culture condition. In conclusion, to improve the VSMCs proliferation *in vitro*, static seeding is better than the dynamic condition. In the culture condition, however, culture under the dynamic status is better than the static condition. This was a pilot study to manufacture artificial vascular vessel by tissue engineering.

Key words: Vascular smooth muscle cells, scaffold, tissue engineering, seeding, culture

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Surgical mainstays of therapy for affected vessels less than 6 mm in diameter include bypass grafting with autologous veins or arteries [26]. However, adequate vessel for bypass conduits is lacking among many patients. Artificial materials, when used in bypass arteries that are less than 6 mm in diameter, have thrombosis rates greater than 40% after 6 months [21]. Although novel approaches for producing small-caliber arterial grafts have been developed, problems with mechanical property [24] or the utilization of neonatal cell [13] have heretofore prevented clinical implementation [18].

One of the more recent applications of absorbable polymeric biomaterials is in the growing field of tissue engineering research [2-5, 7, 11, 12, 23, 24, 28]. The shortage of donor organs and high cost as well as possible complications derived from transplant surgery have driven the need for an alternate mammalian tissue source. Absorbable materials are a potential option. They can be sculpted into a particular tissue shape and can be used as a delivery vehicle on which mammalian cells can be seeded *in vitro* before transplantation takes place. The material offers not only the guiding shape, but also the mechanical structure to help induce the development of the appropriate tissue structure. Once implanted, the polymer gradually absorbs as the new tissue develops [4].

Several types of scaffolds have been used to engineer smooth muscle (SM) tissues in past studies. In the majority of past work conducted in this area, type-I collagen gels have been used as scaffolds [9, 25, 27, 29] More recently, scaffolds have been fabricated from synthetic, biodegradable polymers [e.g., polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), and poly (L-lactic acid)(PLLA)] for engineering SM tissue. Use of these scaffolds has allowed for the study of cell seeding and culture methods [8, 10, 16].

Tissue-engineered vascular vessel has emerged as a new alternative to currently available prosthetic vascular vessel. The most common mode of engineering new vascular vessel is based on seeding isolated cells onto three-dimensional scaffolds by *in vitro* culture. The scaffolds serve as physical supports and templates for cell attachment and tissue development. The currently used materials for maintaining three-dimensional structure in tissue engineering are either polymers composed of chemical substances like polyglycolic acid, polylactic acid, or collagen out of extracellular matrix, etc. Unfortunately, these materials are still far from ideal. One of the drawbacks of these materials is their low mechanical strength. To improve the mechanical strength of scaffolds, we used surgical suture threads, which were woven into a spindle mesh type of scaffolds.

In this study, we optimized the method of distributing cells onto the matrix to maintain polymer integrity and to maximize cellular adhesion and proliferation.

Materials and Methods

Polymer Scaffold

The scaffold was made from Polyglycolic Acid (PGA) fibers (approximately 10-15 µm in diameter) that was obtained from Samyang (Taechon, Korea). Mesh scaffold is composed of 11 vertical lines and 200 cross lines. It is square in shape, measuring 1 cm wide, and made by a weaving method. There is an interval of 1 mm between the vertical lines. It is 0.03-0.035 g in weight, 0.5 mm in thickness, and approximately 45% in porosity. The mesh scaffold was treated with methylenchloride for 10 min to improve cell attachment. The scaffold was sterilized with ethylene oxide gas. Before the seeding, the mesh scaffold was prewet in tubes containing 15 ml of 10% fetal bovine serum (FBS), antibiotic-antimycotic in the M199 medium. The medium and PGA scaffolds were mixed at 100 rpm for 24 h by using a refrigerated incubator shaker (New Brunswick Scientific Co, Inc., Edison, NJ, U.S.A.), prior to cell seeding to allow the adsorption of attachment proteins contained in the serum onto the PGA fibers.

SMC Isolation and Culture

Under general anesthesia, the external jugular veins of Mongrel dogs were harvested and placed into ice-cold serum-free medium. The vascular smooth muscle cells (VSMCs) were isolated from the harvested vein and cultured by the modified techniques described previously [19]. Briefly, the harvested vein was rinsed in a sterile Petri dish with M199 medium containg 10% heat-inactivated FBS to remove blood. It was opened longitudinally to expose the inner surface of the vessel. Using forceps, the vessel was stripped of its intima, then it was flipped over to remove the adventitia by using a scraping action with a scalpel. The cleaned media layer of the harvested vein was cut into small pieces and placed onto the scratched surface of a 35-mm Petri dish. The sections were spread flat on the plate. The dishes were allowed to sit in the hood for 30-60 min to allow the explants to attach, then a few drops of the M199 medium+10%FBS were added directly onto the tissue pieces to moisten them. They were then transferred to a humidified 5% CO_2 incubator at 37°C. On the next and the following days, a small amount of medium was gently added each day so a total of 3-4 ml/per dish by the end of the first week was made. Once the explants had firmly been attached, the media were changed twice a week. Two weeks after the primary culture, the tissue pieces of the media layer of harvested vein were removed and the attached VSMCs were retrieved with 0.25% trypsin/EDTA. To subcultivate the explanted cells, they were passaged at a 1:3 split using 0.25% trypsin/EDTA. The medium for growth and subcultivation was M199+10% heat-inactive FBS.

The Optimum Number of Seeding Cell

A certain minimal cell seeding density was necessary to maintain a construct structural integrity. Scaffolds seeded with less than 0.1×10^7 cells lost their integrity after the culture, whereas scaffolds seeded with more than 0.1×10^7 cells maintained their structural integrity. Three weeks after seeding the VSMCs onto the scaffold, an alamar blue assay was performed to identify the optimal cell seeding

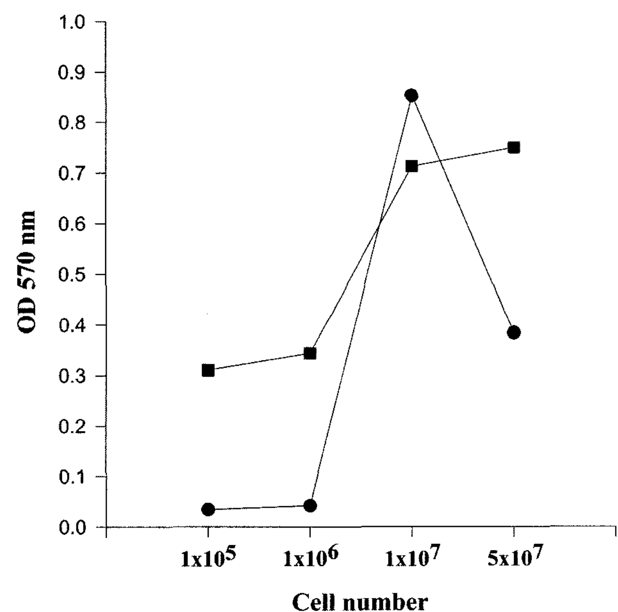


Fig. 1. A comparison of the cell number of static (●) and dynamic (■) seeding conditions.
The number of cells per scaffold was determined by an alamar blue assay. Optimizing cell number was determined as 0.5×10^7- 1×10^7 cells/ scaffold.

Table 1. Characteristics of experimental groups.

	Seeding	Culture
	24 hours	2 weeks
Group I	Static	Static
Group II	Static	Dynamic
Group III	Dynamic	Static
Group IV	Dynamic	Dynamic

density (Fig. 1). The optimum density of VSMCs for seeding was 0.5×10^7- 1×10^7 cells/scaffold.

Condition of Seeding and Culture

VSMCs were seeded onto the scaffold in one of two manners (Tables 1 and 2). The static seeding method was performed by pipetting 0.1 ml of the cell suspension containing 0.1×10^7 cells onto the surface of the scaffold, 5 times every 2 h. As a result, the total number of seeded cells onto the scaffold was 0.5×10^7 cells/scaffold. After the final pipetting for seeding, the medium was carefully filled. For the dynamic seeding method, the scaffold and cells (0.5×10^7 cells/scaffold) in 50 ml of medium were added to a spinner flask (250 ml). The dynamic seeding condition was obtained by stirring for 24 h at approximately 50 rpm under the incubation period. The following day, the scaffolds were removed from the spinner flask and they were washed with phosphate-buffered saline (PBS).

The scaffolds seeded with VSMCs were cultured in one of two manners (Tables 1 and 2). In the static culture, mesh scaffold seeded with the static or dynamic methods were cultured in 6-well plates (cm^2) for 2 weeks. The medium (5 ml/scaffold) in the static condition was changed every other day. The dynamic culture condition was performed using a spinner flask stirred at 50 rpm for 2 weeks in a humidified 5% CO_2 incubator.

Metabolic Activity: Alamar Blue Assay

The stock solution of alarmar blue was aliquoted and kept in the dark at 4°C [1]. To measure the proliferation rates of VSMCs quantitatively, 300 μl of alarmar blue (10% of the incubation volume) was added to the culture dish containing the scaffold. After incubating for 3- 4 h, metabolic activity was determined by measuring the fluorescence with a wavelength of 560 nm or absorbance at 570 nm.

Table 2. Characteristics of experimental groups.

	Seeding	Culture		
		1st week	2nd week	3rd week
Group V	Static	Static	Static	Static
Group VI	Static	Static	Dynamic	Dynamic
Group VII	Static	Dynamic	Dynamic	Dynamic

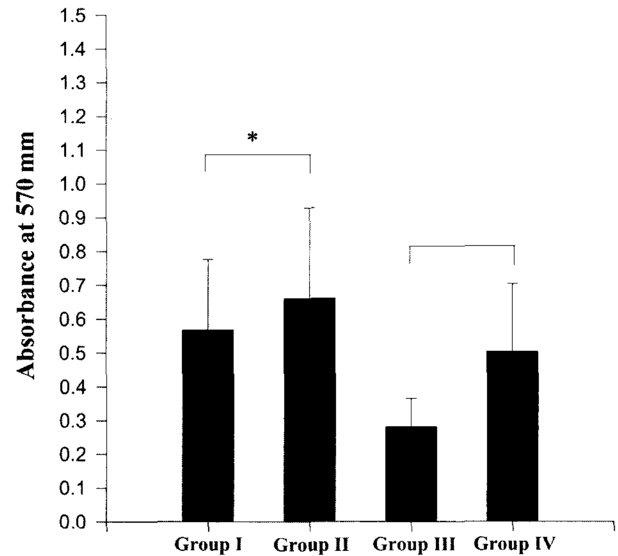


Fig. 2. The alamar blue assay was read on absorbance at 570 nm. Quantification of cell density demonstrated that the static seeding groups (Groups I & II) had a much higher cell proliferation than the dynamic seeding groups (Groups III, IV).

Histological Analysis

Scaffold taken for histological analysis was rinsed in PBS, then fixed overnight in 10% formalin. They were embedded in paraffin and cross-sectioned 4-μm-thick [14]. Sections were stained with hematoxylin and eosin (H&E) [6]. For immunohistochememistry (IHC) assessment, the deparaffinized sections were incubated with a mouse anti-

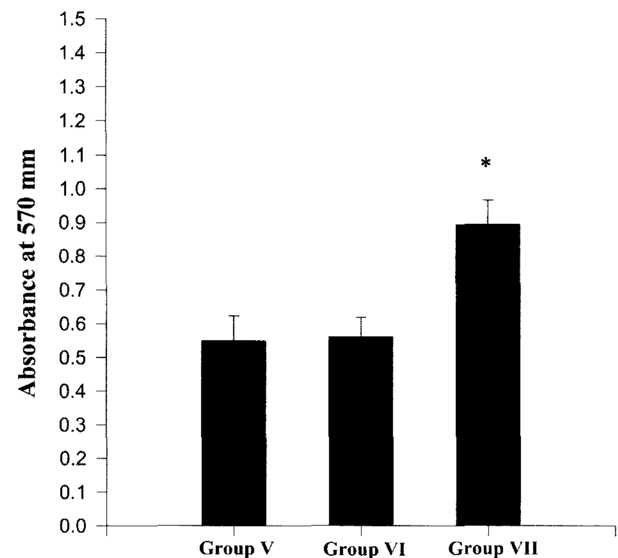


Fig. 3. The alamar blue assay was read on absorbance at 570 nm.
Quantification of cell density demonstrated that the dynamic culture group (Group VII) had a much higher cell proliferation than the static culture group (Group V).

human a-actin for 25 min, rinsed and incubated with streptavidin peroxidase. For this procedure, we used a Large Volume DAKO LSAB® kit (DAKO). For the scanning electron microscopy (SEM), the scaffold was fixed in 2.5% glutaraldehyde solution for 12 h and then rinsed in Millonig buffer for 2 h, followed by post-fixing in 1% osmium tetroxide for 1 h. It was then dehydrated in a graded ethanol series (70- 100%), after which it was dried in a critical point drying system (Hitachi, HCP-1, Tokyo, Japan). Finally, it was mounted on holders and sputter coated with platinum in an ion-coater system (Eiko, IB 5) and then observed and photographed under SEM (Hitachi, S-800, Tokyo, Japan).

Statistical methods

Statistical analysis was completed by one-way ANOVA. For making a comparison between the two groups, the unpaired Student's t-test was used. A significant difference was taken at P<.05. Data are given as mean±SD.

RESULTS

Alamar Blue Assay

The number of adherent VSMCs were subsequently quantified using the alamar blue assay (Fig. 2). The number of adherent cells per scaffold increased according to the concentration of seeded cells, but more cells adhered with the static seeding groups (Groups I & II) than the dynamic seeding groups (Groups III & IV).

Under the same condition of seeding method as the static condition, the experimental groups were divided according to the culture condition. Quantification of cell density in these constructs confirmed that the dynamic culture cell

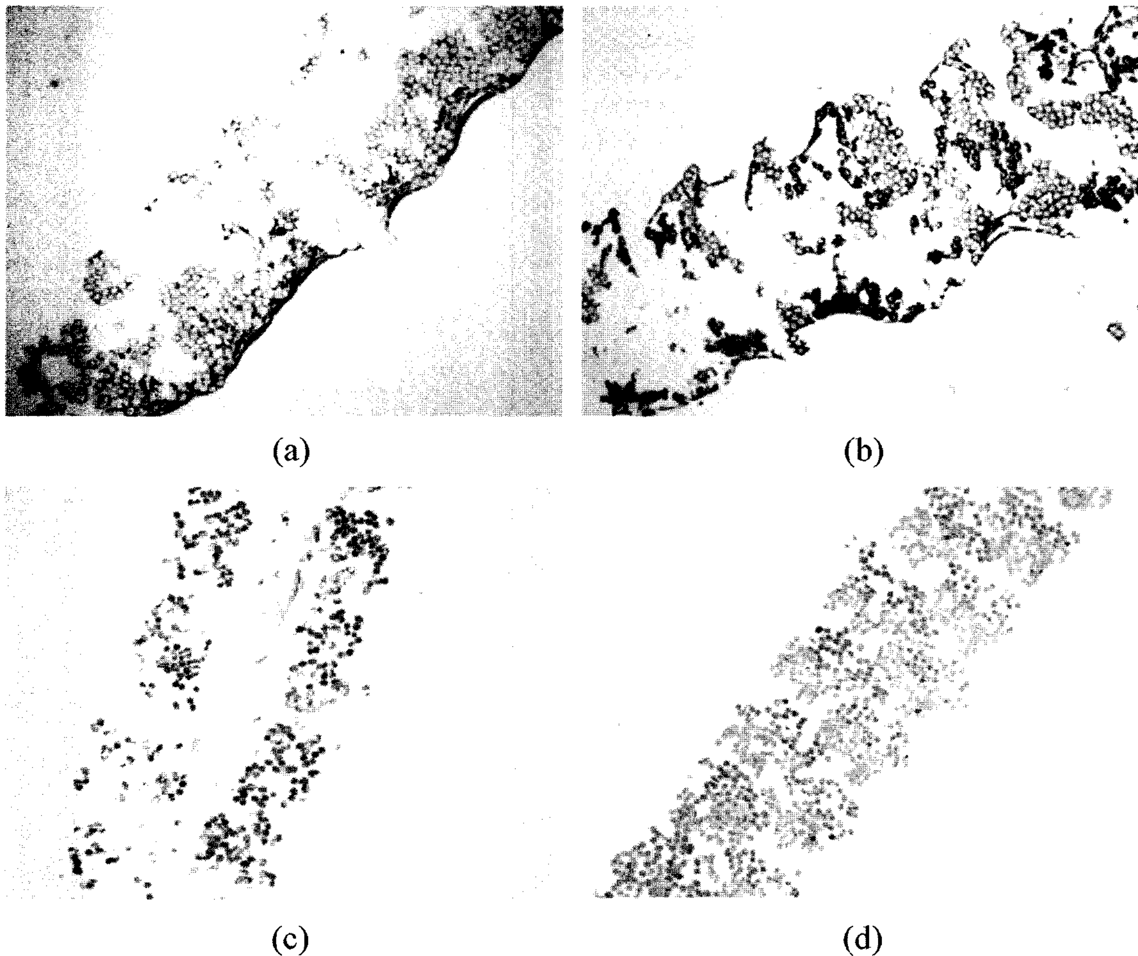
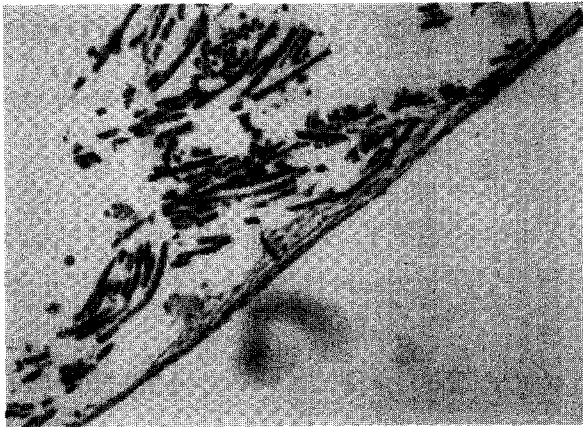
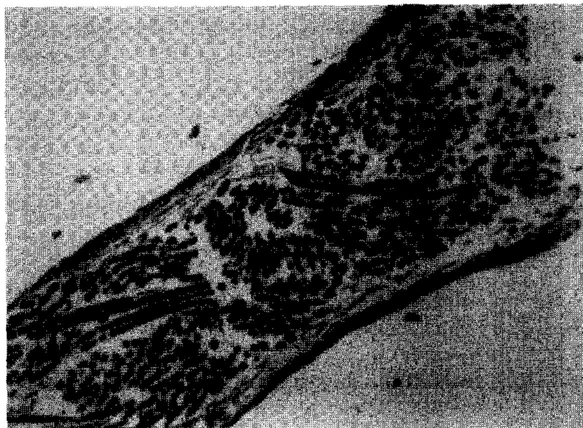


Fig. 4. Histological cross-section of seeding cellular scaffold appearance of cells within the scaffold.
Sections were stained with hematoxylin and eosin stain (original magnification×200). The scaffold seeded with the static condition (Groups I & II) contained more vascular smooth muscle cells (VSMCs) than those seeded with the dynamic condition (Groups III & IV). Furthermore, VSMCs in cell-polymer scaffold seeded with the static condition were distributed more uniformly throughout the constructs compared to those seeded with the dynamic condition. (a) Group I, (b) Group II, (c) Group III, (d) Group IV.



(a)



(b)

(c)

Fig. 5. Histological cross-section of seeding cellular scaffold appearance of cells within scaffolds.
Sections were stained with hematoxylin and eosin stain (original magnification×200). Scaffold cultured with static condition (Group V) exhibited an uneven cell distribution and a low cellularity in the center of the scaffold after 3 weeks of culture. In contrast, scaffold cultured with dynamic condition (Group VII) showed a significantly higher cellularity and more uniform cell distribution through the constructs. (a) Group V, (b) Group VI, (c) Group VII.

constructs had a much higher cell density than the static cultured cell constructs (Fig. 3).

Histological Analysis

H&E staining indicated that 2-week cell-polymer scaffold seeded with the static condition (Groups I & II) contained more VSMCs than those seeded with the dynamic condition (Groups III & IV) (Fig. 4). Furthermore, VSMCs in cell-polymer scaffold seeded with the static condition were distributed more uniformly throughout the constructs compared to those seeded with the dynamic condition.

To investigate whether enhanced cell adherence in the culture system would improve tissue development, scaffolds seeded with the static seeding condition were cultured in a culture dish (static culture) or in spinner flasks (dynamic culture). Scaffolds cultured with the static condition (Group V) exhibited an uneven cell distribution and a low cellularity in the center of the scaffold after 3 weeks of culture (Fig. 5a). In contrast, scaffolds cultured with the dynamic condition (Group VII) showed a significantly higher cellularity and more uniform cell distribution through the constructs (Fig. 5c).

Scanning Electron Microscopy

After 24 h of seeding and 2 weeks' culture, the surface of the scaffold was examined by SEM (Fig. 6). The dynamic seeding groups (Groups III & IV) yielded a small number of cells adherent to the scaffolds. On the contrary, the static seeding groups (Groups I & II) yielded a high number of cells adherent to the scaffolds. Under the same condition of seeding method as the static condition, the experimental groups were divided according to the culture condition (Fig. 7). Static culture group (Group V) yielded a small number of cells adherent to the scaffolds (Fig. 7a). On the other hand, the dynamic culture group (Group VII) yielded a high number of cells adherent to the scaffolds (Fig. 7c).

Overall Assessment

Tables 3 & 4 summarize the qualitative assessment and score of each assay for seeding and culture conditions. In conclusion, to improve the VSMCs proliferation *in vitro*, static seeding was better than the dynamic condition. However, in the culture condition, culture under the dynamic status is better than the static condition.

DISCUSSION

Several assays have been reported to quantify cellular proliferation. These are incorporation of radioactive nucleotides, [^{3}H]thymidine or [^{125}I]iododeoxyuridine, cleavage of tetrazolium salts, MTT(3-(4,5-dimethyethialzol-2-yl)-2,5-diphenyltetrazolium bromide) [17] and XTT(sodium 3'-[1-

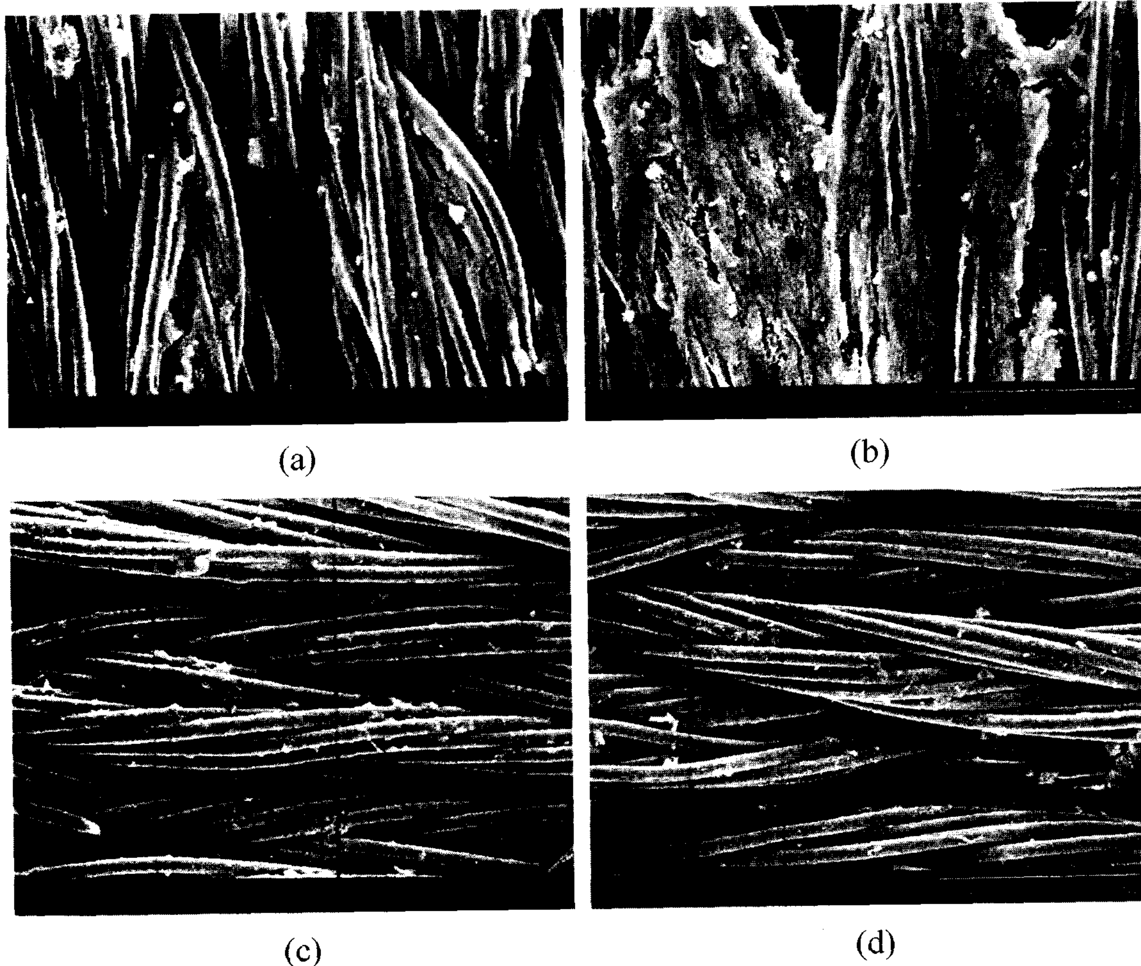


Fig. 6. Scanning electron micrographs of VSMCs adherent to PGA scaffold.
Static seeding groups (Groups I & II) yielded a high number of cells adherent to the scaffolds. On the contrary, dynamic seeding groups (Groups III & IV) yielded a small number of cells adherent to the scaffold. (a) Group I, (b) Group II, (c) Group III, (d) Group IV.

phenylamino]-carbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate [20, 22]. The [^{3}H]thymidine incorporation assay is the most commonly employed assay because of its sensitivity and relative reliability. However, there are many disadvantages. These include: (1) radiation hazard to personnel, (2) labor intensive as several steps are involved, (3) time consuming, (4) high toxicity level due to xylene-based scintillation fluids, (5) concerns related to safe disposal of radioactive waste, and (6) relatively high expense [15]. Thus the alamar blue assay, a one-step non-radioactive assay, has been accepted widely by many investigators. This assay is reliable, simple, and compares well with the [^{3}H]thymidine incorporation assay. The alamar blue assay is a good non-radioactive alternative to the [^{3}H]thymidine incorporation assay [1].

How to improve the rates of the cell proliferation on the biodegradable scaffold has been a popular issue in the

Table 3. Score of index of cellular proliferation.

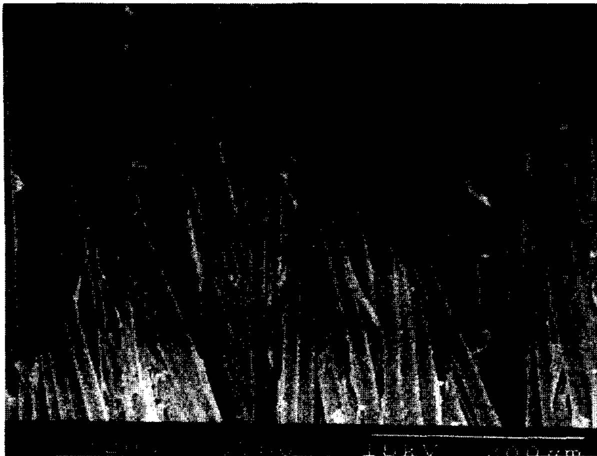
	Surface cellular distribution and attachment	Cellular distribution and attachment within matrix	Metabolic activity
Group I	3	3	3
Group II	4	4	4
Group III	2	2	1
Group IV	1	2	2

1: low; 2–3: intermediate; 4: high.

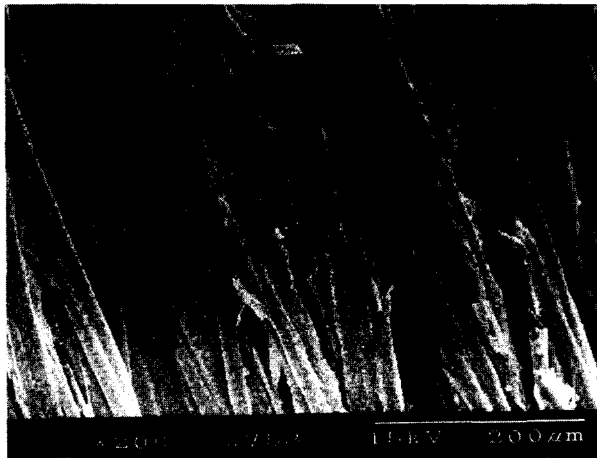
Table 4. Assessment of culture condition.

	Surface cellular distribution and attachment	Cellular distribution and attachment within matrix	Metabolic activity
Group V	1	1	1
Group VI	2	1	2
Group VII	3	3	3

1: low; 2: intermediate; 3: high.



(a)



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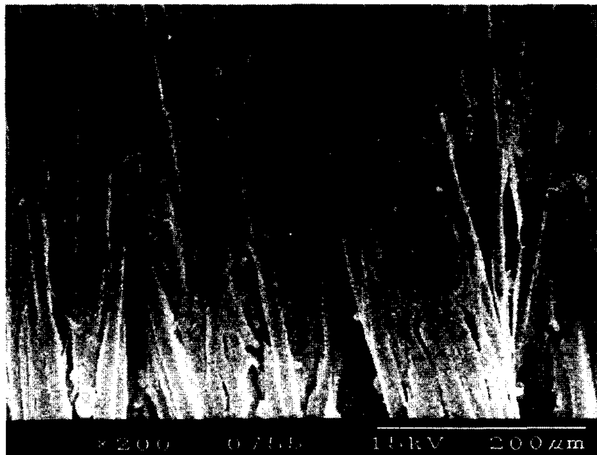

(c)

Fig. 7. Scanning electron micrographs of VSMCs adherent to PGA scaffold.
Static culture group (Group V) yielded a small number of cells adherent to the scaffolds. On the contrary, the dynamic culture group (Group VII) yielded high numbers of cell adherent to the scaffolds. (a) Group V, (b) Group VI, (c) Group VII.

tissue engineering filed. Dynamic cell seeding and culture conditions enhance the formation of cartilaginous tissues from seeded chondrocytes [8] and vascular smooth muscle tissues from seeded VSMCs [10]. However, our results showed that the static cell seeding condition enhanced more of the VSMCs proliferation than the dynamic cell seeding condition. These different results might be due to the difference of scaffold characteristics. The scaffolds used in previous studies [4, 10] were PGA sponge type scaffolds, having more than 96% in porosity. Seeded cells can fully infiltrate into the scaffolds. However, the woven mesh scaffolds out of PGA threads used in our study contained approximately 45% in porosity. Most VSMCs seeded onto these scaffolds with the dynamic seeding condition did not stay on the scaffolds. In contrast, the static seeding condition yielded a high number of cells adherent to the scaffolds. This might be due to quiescent VSMCs maintained on the surface of the scaffolds. Most of the cells probably stayed on the surface of the scaffold and did not come into contact with the inter-PGA fiber space in the low porous scaffolds (approximately 45%).

The mesh scaffolds woven out of PGA threads used in this study do not appear to offer suitable porosity and environment for cell attachment. To improve this problem, we will devise experiments to coat the surface of the mesh scaffold with hydrophilic molecules or loosely weave the mesh for future study.

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