Comparison of three different methods for effective introduction of platelet-rich plasma on PLGA woven mesh

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Abstract
For successful tissue regeneration, effective cell delivery to defect site is very important. Various types of polymer biomaterials have been developed and applied for effective cell delivery. PLGA (poly lactic-co-glycolic acid), a synthetic polymer, is a commercially available and FDA approved material. Platelet-rich plasma (PRP) is an autologous growth factor cocktail containing various growth factors including PDGF, TGFβ-1 and BMPs, and has shown positive effects on cell behaviors. We hypothesized that PRP pretreatment on PLGA mesh using different methods would cause different patterns of platelet adhesion and stages which would modulate cell adhesion and proliferation on the PLGA mesh. In this study, we pretreated PRP on PLGA using three different methods including simple dripping (SD), dynamic oscillation (DO) and centrifugation (CE), then observed the amount of adhered platelets and their activation stage distribution. The highest amount of platelets was observed on CE mesh and calcium treated CE mesh. Moreover, calcium addition after PRP coating triggered dramatic activation of platelets which showed large and flat morphologies of platelets with rich fibrin networks. Human chondrocytes (hCs) and human bone marrow stromal cells (hBMSCs) were next cultured on PRP-pretreated PLGA meshes using different preparation methods. CE mesh showed a significant increase in the initial cell adhesion of hCs and proliferation of hBMSCs compared with SD and DO meshes. The results demonstrated that the centrifugation method can be considered as a promising coating method to introduce PRP on PLGA polymeric material which could improve cell-material interaction using a simple method.

1. Introduction

Tissue engineering and regenerative medicine are emerging fields to facilitate the healing process in injured sites by using various combinations of cells, bioactive molecules and scaffolds. Most of the cells used in tissue engineering have anchorage dependent property. Therefore, biomaterials for tissue regeneration should provide suitable physical support for initial cell adhesion and should have bioactive motives to modulate the behaviors of cells including migration, proliferation and differentiation (Hench and Polak 2002, Shin et al 2003, Langer and Tirrell 2004). Although synthetic poly(α-hydroxy ester), including poly(lactic-co-glycolic acid) (PLGA), is widely used as scaffolds for in vitro cell culture or in vivo tissue regeneration due to its biocompatibility and biodegradability, it has shown limited cell-material interaction due to its hydrophobic property and lack of biological cues (Shin et al 2008). Therefore, many investigators have attempted to modify the surface property of poly(α-hydroxy ester) which would improve its cell-material interaction by modulating its hydrophilicity or introducing various functional groups (Zhu et al 2002, Ma et al 2005).

Generally, non-covalent immobilization and covalent immobilization have been used to modify the surface properties of biomaterials. The non-covalent method simply introduced biomolecules via hydrophobic interactions, but the affinity between the material and the biomolecules could weaken as time passed (Shin et al 2011). The covalent immobilization method was utilized to introduce varied chemical groups through plasma treatment, chemical etching or...
gamma ray irradiation (Cho et al. 2005, Park et al. 2005). These chemical groups can play a role as linkers for the immobilization of ECM proteins and cell adhesion peptides including fibronectin, collagen and Arg-Gly-Asp (RGD) peptides to improve cell behaviors on polymer biomaterials (Volcker et al. 2001, De Bartolo et al. 2007). Although functionalized biomaterials with ECM proteins or cell adhesion peptides show enhanced cell-material interactions, the covalent immobilization process is achieved through complicated steps that require time consuming work. The introduction of ECM proteins or cell adhesion peptides also carries a high cost (Suh et al. 2001, Ma et al. 2005).

Platelet-rich plasma had been widely known to facilitate healing of damaged tissue since it supplied various growth factors derived from the patient’s own blood (Marx et al. 1998, Marx 2001, Marx 2004). Therefore, many studies were carried out to investigate the effect of PRP incorporation on the modulation of in vitro cell behavior and in vivo animal defect models. Most studies reported the positive effects of PRP on in vitro proliferation of osteoblasts or osteoblast-like cells and mesenchymal stem cells (Lucarelli et al. 2003, Annunziata et al. 2005). PRP treatment on periodontal ligament cells and stromal cells facilitated differentiation as increased ALP activity and collagen type I gene expression (Gruber et al. 2002, Oprea et al. 2003).

PRP would be considered as a proper candidate for surface coating materials of synthetic polymers since the incorporation of PRP on biomaterials can provide sufficient growth factors as well as appropriate surface environments for the adhesion of anchoring dependent cells (Marx 2004). In our previous study, we suggested that the dynamic seeding method used for swine chondrocytes on PLGA (90:10) mesh induced homogenous distribution, but static or dynamic culture conditions did not affect in vitro cell proliferation or in vivo meniscus interface healing (Yoo et al. 2011). In our next study, we coated a tissue culture plate (TCP) surface with PRP using the centrifugal method and seeded human bone marrow stromal cells (hBMSCs) to investigate the cellular response after PRP coating. We found that hBMSCs were attached to fibrin networks which generated during PRP activation, and PRP coating of the TCP surface enhanced the proliferation and osteogenic differentiation of hBMSCs (Shin et al. 2012).

Based on the previous studies, we hypothesized that PRP pretreatment with different methods on PLGA (90:10) mesh (vicryl mesh” woven type) would provide different patterns of platelet adhesion, activation stages, and different surface topographies of fibrin networks, and finally, would modulate cell adhesion and proliferation on the surface of the PLGA mesh. In this study, we pretreated PLGA mesh with PRP using three different methods including simple dripping, dynamic oscillation, and the centrifugal method. We then determined the amount and activation stage distribution of incorporated platelets and the topographic change due to fibrin network formation in the presence or absence of calcium, an initiator of fibrin cross linking in PRP. Finally, we evaluated the adhesion and proliferation activities of both human chondrocytes (hCs) and hBMSCs on PRP-pretreated PLGA meshes using different preparation methods to investigate the effect of PRP amount, stage distribution, and topographic changes on cell behavior.

2. Materials and methods

2.1. Human PRP preparation and PRP treatment

Allogenic leukocyte-depleted PRP was provided by our hospital blood bank. The PRP contained 800–1100 platelets per nanoliter and was applied to experiments within 48 h after harvest. PRP was treated on 20 × 8 mm square shape of PLGA mesh (Vicryl™, Ethicon Inc, Somerville, NJ) using three different methods. (1) Simple dripping; the mesh was placed in a 6-well culture plate and 1000 μl of PRP was loaded into the wells which were then incubated for 35 min in a static condition at 37 °C. After treatment, residual PRP was removed and the same procedures were repeated for the opposite side of the mesh. (2) Dynamic oscillation; the mesh was placed in a 15 ml test tube, 5000 μl of PRP was added and the tube was incubated for 70 min with 40 rpm of dynamic oscillation at 37 °C. (3) Centrifugation; the mesh was placed in a 6-well culture plate, 1000 μl of PRP was loaded into the wells and the plate was centrifuged for 10 min at 150 g. After centrifugation, residual PRP was removed and the same procedures were repeated for the opposite side of the mesh. The mesh was incubated for an additional 50 min at 37 °C with agitation at 40 rpm. PRP coated PLGA meshes were immersed in 1 ml of Dulbecco’s modified Eagle medium (DMEM) containing 1.8 mM of calcium for 10 min at 37 °C to supply calcium and rinsed twice with phosphate-buffered saline (PBS) before cell seeding.

2.2. Isolation and primary culture of human chondrocytes

Human articular chondrocytes (hCs) were isolated from macroscopically normal cartilages obtained from the femoral heads of ten patients (five men and five women; 45–68 years of age) who underwent total hip arthroplasties. The chondrocytes were obtained after receiving informed consent and the approval of our Institutional Review Board (IRB number H-1009-012-331). The cells from the ten donors were mixed to minimize donor-dependent variations. Full-thickness cartilage was minced and digested at 37 °C with 0.2% protease (Type XIV; Sigma-Aldrich, St. Louis, MO) for 1 h, followed by 0.2% collagenase (Type IA; Sigma-Aldrich) for 3 h. After digestion, the undigested cartilage fragments were removed using a 70 μm nylon sieve. Isolated chondrocytes were washed, plated in tissue culture dishes at a density of approximately $3 \times 10^4$ cells cm$^{-2}$, and grown in...
monolayer culture in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 units mL$^{-1}$ of penicillin-streptomycin (Sigma-Aldrich), and 25 μg mL$^{-1}$ of L-ascorbic acid (Sigma-Aldrich) at 37 °C in a humidified atmosphere of 5% CO$_2$. The medium was changed twice a week. Cells were passaged at 80 ~ 90% confluency with the use of 0.05% trypsin-EDTA (Gibco BRL). At 80 ~ 90% confluency after the first passage, the chondrocytes (P1) were harvested, mixed, and used.

2.3. Isolation and primary culture of hBMSCs

Primary hBMSCs were isolated from fresh bone marrow obtained from the iliac crest of patients during total hip arthroplasty. To collect the cells, bone marrow aspirates were washed with Dulbecco’s phosphate-buffered saline (DPBS) (GibcoBRL®, Gaithersburg, MD, USA) and the supernatant was discarded after 10 min of centrifugation at 900 × g. These steps were repeated twice. The collected cells were resuspended in DPBS at a final density of 4 × 10$^6$ cells mL$^{-1}$. A 5 mL aliquot was layered over 1.073 g mL$^{-1}$ of Percoll solution (Pharmacia®, Piscataway, NJ, USA) in a 50 mL conical tube and centrifuged for 30 min at 1100 × g. Nucleated cells were collected at the interface and plated in 150 mm culture dishes at a density of 2 × 10$^6$ cells cm$^{-2}$ with MSC culture medium containing Dulbecco’s modified Eagle medium-low glucose (DMEM-LG) (GibcoBRL$^+$), and 1% Anti-Anti (Antibiotic-Antimycotic, penicillin G sodium 100 U mL$^{-1}$, streptomycin 100 μg mL$^{-1}$, and amphotericin B 0.025 μg mL$^{-1}$; GibcoBRL$^+$). The cells were maintained in 5% CO$_2$ at 37 °C and the media was replaced every 3~4 d.

2.4. FE-SEM observation

The morphology of PRP treated PLGA meshes was observed under field emission scanning electron microscope (FE-SEM; JSM-7401F, JEOL Ltd., Japan) and platelet adhesion was confirmed. For quantification of adhered platelets on the PLGA meshes, we captured five images from one mesh at different sites under ×3000 magnification (126.31 × 94.74 = 11966.21 μm$^2$ per image). Each image was printed at 18 × 24 cm (height × width) and divided into nine fields. The number of platelets within all regions from one image (total five images per mesh) was then counted. The average number of adhered platelets per image was used to calculate the total number of adhered platelets on the mesh (160 mm$^2$). We classified adhered platelets by morphological appearance according to the method described by Ko et al (1993). The percentage of each stage was calculated by dividing the number of total adhered platelets.

2.5. Evaluations of cell response

To evaluate the cell response, each type of PRP coated mesh was plated in non-adhesive 6 well plates and cells were seeded onto meshes at a density of 2 × 10$^4$ cells/well. We examined the cell attachment of hCs and hBMSCs on meshes after 4 h of culture by using CCK-8 assay (Dojindo Molecular Technologies, INC., MD, USA). Briefly, cultured cells were washed with DPBS for the assay, then a mixture of medium and CCK solution (100:10 ratio) was treated to each well. After 3 h of incubation at 37 °C, we measured absorbance using a microplate reader (Spectramax M5, Versamax; Molecular Devices) at 450 nm wavelength. The cell number was calculated using a standard curve. CCK-8 assay was also performed after days 1 and 5 of culture to measure the proliferation rate of cells.

2.6. Statistical analysis

For quantification of platelets, platelets were counted from 5 different regions per mesh. Five samples were used for cell attachment and proliferation analysis. All data were reported as average ± standard deviation and were compared across groups by using Student’s t-test. The comparison was for a separately performed condition with or without calcium.

3. Results

Figure 1 shows the structure and morphology of PLGA woven mesh (figure 1). The fibers showed defined woven structures with smooth surfaces and the average diameter of the fibers was approximately 15 μm. We
J-H Lee et al prepared PRP treated PLGA woven mesh using six different methods including simple dripping (SD), dynamic oscillation (DO), and centrifugation (CE) with or without calcium treatment. We compared the density of adhered platelets on PRP treated PLGA meshes (figure 2). In the SEM image, only few adhered platelets were found on the SD mesh (figure 2(a)). Treatment of calcium on PRP is well known as an initiator of platelet activation which leads to the formation of a fibrin network. We supplied calcium by treating the culture media after completing PRP coating on PLGA meshes. The calcium treatment on the SD mesh enhanced the adhesion of platelets on the PLGA mesh (figure 2(b)). The amount of platelets on the DO mesh was similar to the number of platelets on the SD mesh and some platelets were observed between fibers (figure 2(c)). The treatment of calcium on the DO mesh resulted in the formation of connections among platelets on the mesh (figure 2(d)). The CE mesh had a larger amount of adhered platelets on the surface than the other meshes (figure 2(e)). A similar number of platelets on the calcium treated CE mesh showed lots of connections with fibrin fibers (figure 2(f)).

The numbers of adhered platelets on each mesh was analyzed using FE-SEM images (figure 3). A similar number of platelets were observed on SD and DO mesh without calcium treatment. On the other hand, the number of platelets on CE mesh without calcium treatment was six folds larger than the other two groups. Calcium treatment of SD and DO meshes induced significant increase of platelet adhesion compared to treatments without calcium. The calcium treated CE mesh had the highest number of adhered platelets. These results demonstrated that the centrifugal coating method was more effective in introducing large numbers of platelets onto PLGA mesh than the

**Figure 2.** FE-SEM images (×1000 magnification) of adhered platelets on meshes after PRP coating using simple dripping (a), (b), using dynamic oscillation (c), (d) and using centrifugation (e), (f).

**Figure 3.** Quantification results of adhered platelets on each type of mesh. The data are presented as the mean ± SD of seven samples (n = 7). *p < 0.01, compared with simple dripping without Ca, **p < 0.01, compared with dynamic oscillation without Ca, †p < 0.01, compared with simple dripping with Ca, ‡p < 0.01, compared with dynamic oscillation with Ca.
other coating methods. Moreover, platelets were captured on PRP treated PLGA mesh by additional treatment of calcium.

As shown in figure 4, the platelets on the SD and DO mesh were very small with round shapes and were separated individually (figures 4(a) and (c)). However, the platelets on the CE mesh were connected to each other with slightly spread morphologies (figure 4(e)). The platelets were large and flat on all types of meshes with calcium treatment (figures 4(b), (d) and (f)). The fibrin network was more increased on DO and CE meshes, which trapped the platelets inside.

Activation stages of the platelets were divided into five levels based on FE-SEM images and according to categories described in a previous report by Ko et al (1993). They defined the five stages of platelets from inactivated to activated states as round, dendritic, spread-dendritic, spreading and fully spread. In figure 5, the stage distribution of platelets on SD and DO without calcium treatment contained over 70% of

![Figure 4](image_url) Magnified FE-SEM images (×3000 magnification) of adhered platelets on meshes after PRP coating using simple dripping (a), (b), using dynamic oscillation (c), (d) and using centrifugation (e), (f).

![Figure 5](image_url) Stage distribution of adhered platelets on each type of mesh.
platelets that were round and dendritic, meaning they were in the inactivated stage. On the CE mesh, platelets in the round and round-dendritic stage portion were relatively decreased while those in the spread-dendritic stage had increased to 65%. Calcium treatment on meshes resulted in dramatic changes in the distribution. Over 90% of platelets on calcium treated CE mesh were in the spreading and fully spread stages. These results showed that the treatment of calcium after the coating of PRP on PLGA mesh facilitated fibrin network formation as well as triggered the activation of adhered platelets on meshes.

The number of adhered hCs after 4 h on SD and DO were similar but the amount of adhered hCs on CE was significantly higher than the other groups (figure 6). Calcium treatment of meshes enhanced cell adhesion only on CE but not on SD or DO. The proliferation rate of hCs was compared after 5 d of culture on each mesh (figure 7). The proliferation rates of hCs on SD, DO and CE without calcium treatment were very similar and were also similar to the calcium treated groups. These results indicated that the amount of platelets and their stage distribution on PLGA meshes modulate only the initial adhesion of hCs but not proliferation.

Next, we cultured hBMSCs for 24 h and a CCK-8 assay was performed to determine initial cell adhesion (figure 8). The number of adhered hBMSCs on SD, DO and CE did not show statistical differences and the calcium coating did not enhance cell adhesion on the three types of meshes. The proliferation rate of hBMSCs within 1 to 5 d on SD and DO were similar but the rate on CE was significantly higher than the other groups (figure 9). On calcium treated meshes, the proliferation rate on CE was the highest out of the three groups whereas the rate on SD and DO were similar. These results indicated that the amount of platelets and their...
stage distribution on PLGA meshes did not modulate the initial adhesion of hBMSCs, but that proliferation could be enhanced by PRP coating via the centrifugal method.

4. Discussion

Regenerative medicine has emerged as a hot issue for effective regeneration of damaged tissue and many types of biomaterials have been developed for tissue engineering (Shin et al 2003). In this study, PLGA was chosen as a promising material for study due to its biocompatibility and safety which approved by the FDA. However, the surface of PLGA is hydrophobic and coated with stearic acid, which causes limited cell–material interactions (Shin et al 2008). Therefore, we applied PRP on PLGA meshes as a coating material to improve cell–material interactions using three different coating methods including simple dripping, dynamic oscillation, and centrifugation.

PRP is known as an autologous growth factor cocktail, therefore it has been widely applied to in vitro and in vivo studies as well as in clinical treatment of various disorders including spinal fusion, total knee arthroplasty, and chronic elbow tendinosis patients (Weiner et al 2003, Berghoff et al 2006, Mishra et al 2006). PRP was also added into the media as a culture supplement. Most of studies used a 10% volume ratio to culture media and examined cell behaviors including cell adhesion, proliferation and differentiation (Lucarelli et al 2003, Annunziata et al 2005, Mishra et al 2009, Xie et al 2012). The studies reported the positive effect of PRP on cell proliferation over 7 d for various types of cell including adipose-derived stem cells, bone-marrow-derived stem cells, stromal stem cells and periodontally related cells. They also reported that PRP treatment enhanced...
osteogenic differentiation of stem cells, which showed significant increases in ALP activity and calcium deposition (Lucarelli et al 2003, Annunziata et al 2005).

However, it is difficult to keep the liquid form of PRP in a specific site for a long period. To improve this limitation, PRP was used as scaffolds made by gel formation or freeze-drying (Van den Dolder et al 2006, Nakajima et al 2012, Diaz-Gomez et al 2014). PRP was applied to biomaterials made of synthetic polymers such as polycarbonate (PCL) electrospun fiber mesh by simply soaking the materials in PRP solution for 4 h at 4°C and then freeze-dried (Diaz-Gomez et al 2014). Freeze-drying after coating with PRP resulted in a sponge-like structure of PRP on fibrous scaffolds and prevented the loss of growth factors within PRP. The implantation of PRP-PCL scaffolds showed significantly improved angiogenesis in chicken chorioallantoic membrane (CAM). Yu et al applied PRP on the same material, PLGA mesh (Vicryl mesh® woven type), by 3 min of immersion in PRP and subsequent freeze-drying (Nakajima et al 2012). Their method for PRP application was similar to the simple dripping method in this study. They indirectly estimated the number of adhered platelets on the mesh by comparing the number of platelets in PRP before and after immersion of the PLGA mesh. The results demonstrated that about 95% of platelets were adhered onto the PLGA mesh. However, the adherence pattern was different from our SEM observation of the same mesh with a lower density of adhered platelets.

We cannot directly compare the adhesion of platelets result with their quantification results due to the difference in presentation units used. Although we treated PRP for 60 min by simple dripping whereas they immersed for only 3 min, the adhesion density of platelets in their SEM images was similar with our results. They explained that the adhesion of platelets on PLGA mesh is from spontaneous adsorption which may be caused by the positive charge of the PLGA mesh. The results showed that the length of treatment time was not important for the adhesion of platelets on PLGA mesh when using the simple dripping method.

In our previous study, we used the dynamic oscillation method to seed and culture cells for homogenous distribution (Yoo et al 2011). Dynamic oscillation during cell seeding resulted in homogenous distribution of cells as expected but did not affect cell proliferation. We applied a dynamic oscillation method for the homogenous distribution of platelets during PRP coating on PLGA meshes. However, the effect of dynamic oscillation on the distribution of platelets was relatively smaller than for cell distribution.

Another of our previous studies showed the result of adhesion, proliferation and differentiation of hBMSCs on PRP coated tissue culture plates (TCP) using the centrifugation method (Shin et al 2012). We observed abundant fibrin networks around platelets triggered by the addition of calcium after PRP coating. In this study, we applied the same procedures to coating PLGA meshes and found significantly higher amounts of adhered platelets than with the other coating methods. Although we cannot directly compare adhered platelets in this study with those in the previous study, the centrifugation method was the most efficient at introducing platelets onto PLGA mesh compared to other coating methods.

Platelets can be activated by the addition of calcium and the morphology of platelets can be divided into five stages of activation including round (R), dendritic (D), spread-dendritic (SD), spreading (S), and fully spread (FS) (Allen et al 1979, Ko et al 1993, Frank et al 2000). Our results showed that more than 90% of platelets were in R, D or SD stages when calcium treatment was absent after PRP coating. On the other hand, additional calcium treatment after PRP coating led to a significant transition of platelet stages from inactivated (R, D, SD) to activated stages (S or FS) in three types of PRP coated PLGA meshes. Activated platelets release various growth factors including TGF-β1, PDGF-AB and IGF-1 which can modulate cell behaviors (Pierce et al 1989, Kanno et al 2005). Fibrin network formation was also induced by platelet activation. Platelet-rich fibrin (PRF) has a complicated architecture composed of natural fibrin matrixes with concentrated platelets and leucocyte cytokines. PRF can provide a mechanically supportable matrix for cell growth and also growth factors can be protected from proteolysis for relatively longer periods (Wu et al 2012). In the current study, we exposed PRP-coated PLGA meshes to calcium containing culture media to trigger the clotting process. The process can lead to the formation of a thin layer of ‘platelet-rich fibrin’ on PLGA meshes which could provide a favorable environment for cells.

The initial adhesion and proliferation aspects of chondrocytes and hMSCs were different on PLGA mesh coated using different PRP coating methods. The initial adhesion of chondrocytes was highest on the PLGA mesh that was coated using the centrifugation method, whereas the proliferation rates on the three types of PLGA meshes had no statistically significant differences. The result demonstrated that the initial adhesion but not the proliferation of hCs on PLGA mesh was affected by the number of adhered platelets and that platelet activation was related to 3D fibrin network structure formation. In our previous study, we already examined the adhesion of hCs after 24 h on PLGA mesh coated with PRP using centrifugation (Kwak et al 2014). The result showed that a significantly higher amount of cells adhered to the PRP coated PLGA mesh than to the non-coated PLGA mesh. Therefore, we applied the PRP coated PLGA mesh as a cell carrier for the regeneration of meniscal tear and successfully regenerated the tear after 6 weeks.

On the other hand, hMSCs showed similar initial adhesion on the three types of PLGA meshes but the proliferation rate was significantly higher on the PLGA mesh coated using the centrifugation method. We postulated that the proliferation of hMSCs may
be affected by growth factors from platelets during the culture period rather than by environmental condition.

5. Conclusion

In conclusion, PRP coating of PLGA mesh using the centrifugation method showed significantly higher initial cell adhesion of hCs and proliferation of hBMSCs compared with simple dripping and dynamic oscillation. The results demonstrated that the centrifugation method can be considered as a promising coating method for introducing PRP onto PLGA polymeric material which could improve cell-material interaction using a simple method.

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