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Activation of Platelet Rich Plasma by Soluble Canine Small Intestinal Submucosa Gel and Bovine Thrombin

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Abstract : This study evaluated the efficacy of soluble canine small intestinal submucosa gel in comparison to bovine thrombin in activating rabbit platelet rich plasma (PRP) by detecting growth factors. PRP from rabbits was activated by using soluble canine SIS gel, bovine thrombin, or both. The surface morphology of each group of samples was examined by scanning electron microscopy. The release of transforming growth factor (TGF)-β1 from each set of samples was measured over 7 days using enzyme-linked immunosorbent assay. The PRP-canine SIS gel group exhibited the highest total amount of released TGF-β1. However, there were no significant differences between any groups. The use of soluble type of canine SIS gel could be an effective alternative to bovine thrombin.

Key words **:** PRP (platelet-rich plasma) activation, soluble canine SIS (Small Intestinal Submucosa) gel, bovine thrombin, growth factor.

Introduction

Platelet-rich plasma (PRP) is defined as an autologous concentration of platelets in a small volume of plasma (13). PRP works via degranulation of α granules during activation; these granules contain a number of growth factors/cytokines (13). Application of these growth factors to a wound site can accelerate fibroblast DNA synthesis, upregulate collagen production, and mediate in collagen organization (6). The traditional method of PRP preparation involves isolating platelets followed by gel formation (fibrin clot) by using calcium chloride and bovine thrombin (9). The use of bovine thrombin has been associated with the development of antibodies to clotting factors V, XI, and thrombin, creating a risk of life-threatening coagulopathy (2,9,16).

Collagen also stimulates platelet release of growth factors, which promote cellular proliferation within and adjacent to the wound site (1). Collagen-based scaffolds combined with PRP have recently been used to stimulate repair in wound sites (1). Collagen-based gels have been used as three-dimensional scaffolds to study cell properties and develop tissue analogs, such as vasculature, skin, spinal cord, bone, and tendon (8,15).

Small intestine submucosa (SIS) is composed of extracellular matrix (ECM) is harvested and decellularized as biologic scaffolds. SIS is a collagen-based material, especially rich in type I collagen sheets. Moreover, growth factors such as basic fibroblast growth factor and transforming growth factor-β (TGF-β) have been documented in the SIS-ECM (19). These components are important in cell migration, proliferation, and differentiation, as well as cell-cell and cell-biomatrix interactions during the regenerative process of tissue remodeling (7).

However, SIS-ECM is usually characterized by a two-dimensional sheet. A soluble form of ECM gel could be applied via minimally invasive surgery, such as injection in three-dimensional spaces. In previous studies, We used canine SIS as a biologic scaffold for surgical reconstruction of defection part with promising results (10,11). Our hypothesis was that canine SIS gel would be effective for stimulating growth factor release from platelet granules, similar to collagen-based gel. The bovine thrombin and canine SIS gel mixture was designed to create a synergistic effect, as well as to provide an appropriate bioscaffold for stabilizing the PRP clots.

The purpose of this study was to investigate the biological materials (canine SIS gel and bovine thrombin) for their efficacy of PRP activators and to assess the release of growth factors. First, we prepared a soluble gel form from two-dimensional sheet canine SIS. We observed the activated PRP form with each PRP activator in combination, i.e., bovine thrombin, canine SIS, canine SIS mixed bovine thrombin, and PRP activator alone as canine SIS gel by scanning electron microscope (SEM). We evaluated the pattern of growth factor release from activated PRP for 7 days by enzyme-linked immunosorbent assay (ELISA).

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Materials and Methods

Canine SIS preparation and gel formation

German Sheperd military dogs were donated to the Veterinary Medical Teaching Hospital by animal body donation program. Canine SIS from the donated dogs was prepared as detailed previously (3,10). The decellularized SIS sheet was lyophilized and comminuted to a powder form (Fig. 1A). Then, 400 mg canine SIS powder and 25 mg pepsin (3,000-4,300 U/mg; Sigma) were mixed in 2 mL 8 M urea and 2 mL 8 M guanidine HCL. This solution was mixed using a solution of 8 mL Ham's F-10 medium and 1% Antibiotic-Antimycotic solution and constantly stirred for 48 h at room temperature (25°C). The resultant viscous solution of digested SIS or pre-gel solution had a pH of approximately 3.0-4.0. Pepsin was irreversibly inactivated by neutralization to pH 7.4 using 7.5% sodium bicarbonate. The canine SIS gels appeared white and were highly viscosity (Fig. 1B).

Rabbit PRP preparation

A PRP solution was produced in detail as described (11, 21) from 4 New Zealand white rabbits (8 weeks). Briefly, 5 mL whole blood was drawn from the marginal auricular vein into a sterile conical tube containing 0.75 mL sodium citrate. The blood was centrifuged at 2,500 rpm for 10 minutes to seperate the plasma and red blood cells. A second centrifugation step at 3,500 rpm and 4°C for 20 minutes was used to obtain PRP. The top layer, consisting of platelet-poor plasma (PPP), was discarded, and the platelet concentrate was resuspended in 500 μL plasma (PRP).

Culture conditions for PRP activation

PRP activation by bovine thrombin

Calcium gluconate (1 mL; 100 mg/mL Calmia; Korea United Pharmaceutical, Seoul, Korea) and 5,000 IU/mL bovine thrombin (Dirabine; Korea United Pharmaceutical, Seoul, Korea) were mixed. This mixture (50 μL) was added to PRP (450 μL) in a 1:10 (vol/vol) ratio for PRP clot formation.

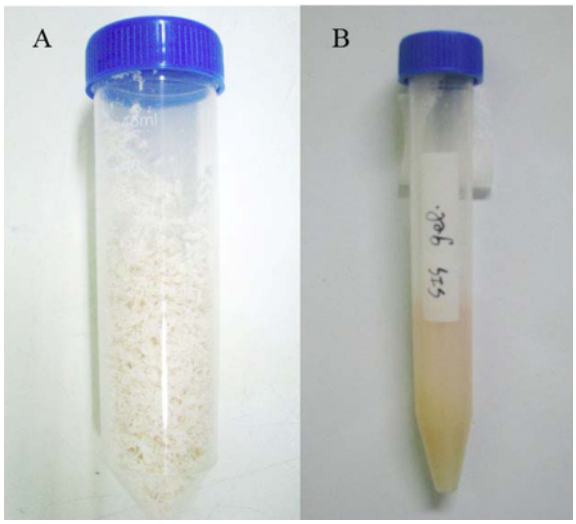

Fig. 1. Lyophilized canine SIS powder (A), and canine SIS gel (B).

PRP activation by canine SIS gel

Equal volumes of PRP (450 μL) and canine SIS gel (450 μL) were combined to create the PRP-canine SIS gel.

PRP activation by the mixture of canine SIS gel and bovine thrombin

PRP (450 μL) was added to the mixture of canine SIS gel (450 μL) and calcium gluconate/bovine thrombin solution (50 μL).

Canine SIS gel

Only canine SIS gel (450 μL) was placed on the plate to examine its form and detect released cytokines.

All mixtures were placed the wells of a sterile 24-well plate where it formed a gel. These samples were preserved in a CO_2 incubator at 37°C during the experiment.

SEM analysis

The surface morphology of each group was examined using SEM after 3 days in culture. The samples were fixed in cold 2 % glutaraldehyde and rinsed in PBS. Secondary fixation was completed in 2% osmium tetroxide (Sigma) in distilled water (DW) for 2 h at room temperature, followed by dehydration process through a graded series of ethanol (50-100%), and dried in a critical point dryer (HCP-2; Hitachi, Brisbane, CA). The samples were attached to aluminum SEM specimen mounting stubs and sputter coated with a gold palladium alloy using a Sputter Coater (E-1010; Hitachi). Finally, samples were examined using a scanning electron microscope (S-3500N; Hitachi). Images were taken at 5000 and 7,000× magnification.

TGF- ß1 detection in activated PRP medium

For growth factor detection, all samples were placed in 24-well plates and allowed to form a gel. Interval release of TGF-ß1 from the sample media was measured at 12 h and at 1, 3, 5, and 7 days. Dulbecco's modified Eagle's medium (DMEM, 500 μL; Sigma-Aldrich, St. Louis, MO) mixed with 2% antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO) was poured into each sample. At each time point, the spent medium was exchanged for fresh medium. The collected DMEM samples (500 μL) were stored in cryotubes in a –80°C freezer. The release of TGF-ß1 in DMEM was determined using the commercially available Quantikine colorimetric sandwich enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN).

Statistical analysis

All data within groups were combined and expressed as the mean ± standard error (SEM). The data was analyzed via analysis of unpaired t-tests or nonparametric Kruskal-Wallis tests to compare results from each group at each time point.

A P-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL).

Results

Surface morphology

Activated PRP with bovine thrombin

SEM micrographs demonstrated the platelet fibrous morphology and the interaction between bovine thrombin and nanofibers *in vitro*. (Fig. 2A).

Canine SIS gel

SEM micrographs demonstrated the structure of the canine SIS gel was composed of oriented SIS collagen fibers. Three-dimensional pores between chopped canine SIS fibers were interconnected and distributed throughout the structure (Fig. 2B).

PRP-canine SIS gel interaction and PRP-canine SIS gel and bovine thrombin mixture

Platelets adhered and spread across the surface of the canine SIS fiber network. The interspace between SIS fibers was filled with activated platelet, giving the samples a mud-plastered appearance (Fig. 2C). In canine SIS gel mixed with bovine thrombin samples, the activated platelets mass interacted and integrated with the surrounding canine SIS fibers, similar to the canine SIS gel group (Fig. 2D). However, the PRP mass in the direction of SIS fiber orientation, formed a much smaller three-dimensional porous structure than the nanofibrous structure.

Cytokine release from PRP prepared

The released TGF-ß1 for SIS gel was not detected by the ELISA kit (Fig. 3). The PRP-canine SIS gel group had the highest mean value of TGF-ß1 (total amount: 1915.33 ± 230.96 pg/mL); the PRP-thrombin group had the second highest value (total amount: 1563.55 ± 359.53 pg/mL), and the mixed PRP-canine SIS gel and thrombin group (total amount: 1322.97 ± 329.56 pg/mL) had the lowest value. In all groups, the patterns of TGF-ß1 release were similar. The amount of released TGF-ß1 was strongest in the first 12 h and gradually decreased with persistent release over the 7 days experiment. However, there were no significant differences in total the TGF-ß1 release between any groups and at all time points.

Discussion

Biological scaffolds have a number of applications, including physical supports for the creation of functional tissues and protective gels to aid in wound healing for localized hormone-delivery therapies (20). Many studies have sought to develop more sophisticated growth factor delivery mecha-

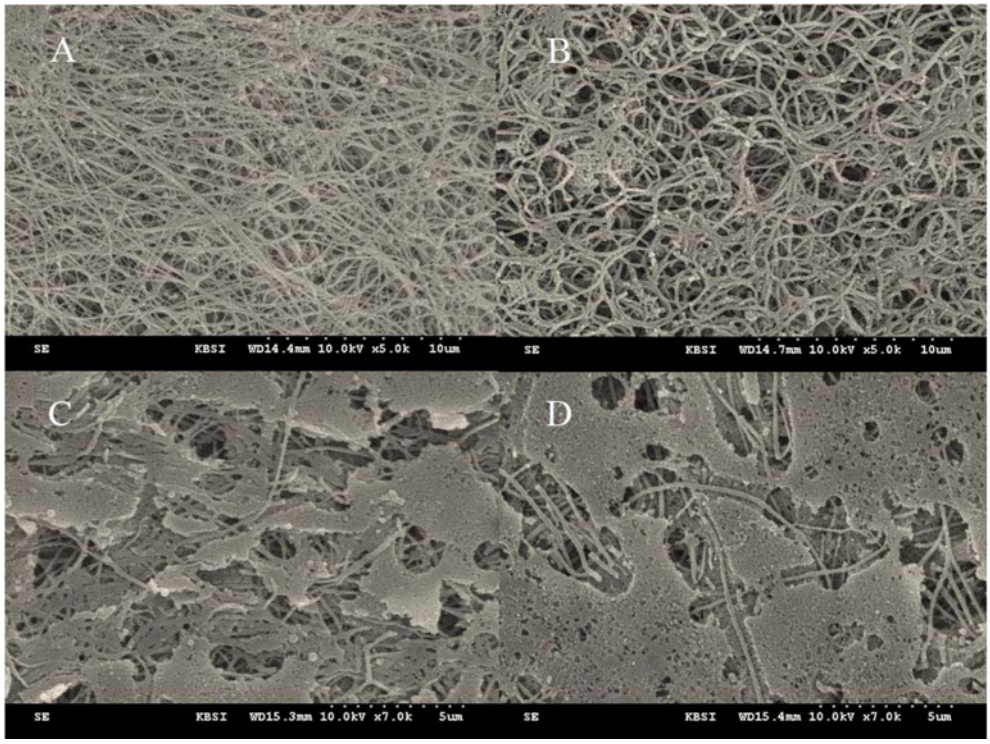

Fig. 2. Surface morphology examination by scanning electron microscopy. PRP activation by bovine thrombin, × 5000 (A); canine SIS gel, × 5000 (B); PRP activation by canine SIS gel, × 7000 (C); and PRP activation by the mixture of canine SIS gel and bovine thrombin, × 7000 (D).

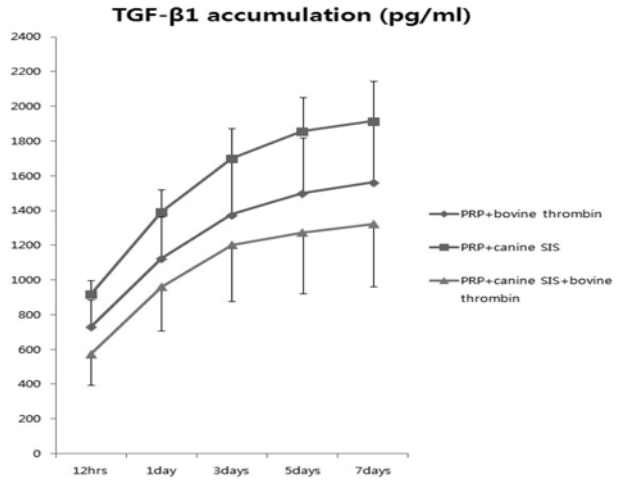

Fig. 3. The accumulation of TGF-ß1 release for 7 days from each group. The PRP-canine SIS gel group presented the highest yield of TGF-ß1 and the PRP-canine SIS and bovine thrombin mixed group had the lowest value. However, the differences between groups were insignificant.

nisms to mimic the endogenous profiles of growth factor production during natural tissue morphogenesis or regeneration (14,20). However, some studies of growth factor applications still remain inferior; the researchers suggest their results may due to the mode and timing of their delivery, poor control over the subsequent distribution of the growth factors locally and systemically, and an insufficient local concentration for the required time frame during regeneration (14).

This study investigated the application of canine SIS gel as an alternative to bovine thrombin for triggering PRP activation. Our team focused on timing growth factor release to be triggered by the repair process. We detected TGF-β1 from PRP and compared groups for 7 days *in vitro*. The results showed that canine SIS gel was as effective as thrombin in stimulating TGF-β1 release at 1-7 days. Growth factor release evaluations showed that PRP activation with only canine SIS gels were more effective than bovine thrombin in TGF-β1 release.

PRP activation causes α granules to fuse to the platelet cell membrane (degranulation) where some of growth factors (e.g., PDGF, IGF, TGF β, and VEGF) are transformed to their bioactive state by the addition of histones and carbohydrate side chains (17). Especially, TGF-β1 is released by platelet degradation during the initial inflammatory phase of bone healing, which affect proliferation of fibroblasts, marrow stem cells, and preosteoblasts (5,8). TGF-β1 therefore represents a means for sustaining and amplifying osteoblastic activity during bone healing and may represent one of the most important platelet-released factors (8).

Structural biocompatibility is affected by the physical morphology of a scaffold, primarily its architecture and the dimensions of its building components (18). Previous studies have shown that cell proliferation is influenced by the architectural scale of the structure and that adhesion is affected by the topography of the material (12). The dimensions of the building components of a scaffold are important factors in regulating cell activities and acting as a reservoir for bioactive molecules such as growth factors (18,20). This study characterized the form of three-dimentsional ECM scaffold-derived canine SIS gel, PRP-bovine thrombin, PRP-canine SIS gel, and PRP and canine SIS gel mixed with bovine thrombin by SEM.

Collagen-platelet interaction mediates coagulation and platelet aggregation, raising the possibility that collagen lies upstream of thrombin generation and may thus be the primary physiologic agonist for platelets (4). Instead of collagen, we considered canine SIS gel and canine SIS gel mixed with bovine thrombin for PRP activation. There was no significant difference between groups; the canine SIS gel with bovine thrombin group produced the least amount of cytokine. The decrease in pore size with activated factors refutes our suggestion that the various composite application appears to be unsuitable for cytokine release. Abreu *et al*. suggested that increasing the collagen density of the collagen-platelet scaffolds did not result in higher levels of PDGF-AB release (1). This phenomenon might cause filling of the pores with composite formulations (activation factor), and generate an irregular interconnective network with canine SIS.

We found that canine SIS gel could be substituted for bovine thrombin as a PRP activator. This study could be used as the formation for new guidelines as a PRP activation. These findings suggest canine SIS gel may provide a safe, readily available alternative to bovine thrombin in the clinical applications of PRP. Further studies are underway to evaluate healing in the presence of PRP and PRP activation factor *in vivo*.

Acknowledgements

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개 소장점막하 겔과 소 트롬빈을 이용한 혈소판풍부혈장의 활성화 연구

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요 약 : 본 연구는 개 소장점막하 조직을 겔화시킨 것과 일반적으로 사용하는 소 트롬빈, 그리고 이 두 가지 요소를 혼합한 것을 혈소판풍부혈장의 활성화 인자로 적용하여, 혈소판에서 분비되는 성장인자를 7일간 in vitro 실험을 실시하였다. 또한 전자현미경으로 각 그룹 별 샘플의 표면을 관찰하여, 측정한 성장인자와의 상관관계를 연구하였다. 그 결과, 그룹별로 유의적인 차이는 존재하지 않았지만, 성장인자인 (TGF)-β1이 가장 많이 분비된 그룹은 혈소판풍부혈장을 개 소장점막하조직 겔로 활성화시켰을 때였다. 본 연구 결과를 통해, 혈소판풍부혈장을 활성화하기 위한 요소로 개 소장점막하조직 겔은 소의 트롬빈을 대체할 만한 인자로 여겨지며, 추후 임상에서 다양하게 적용할 수 있을 것으로 기대된다.

주요어 : 혈소판풍부혈장, 개의 소장점막하조직겔, 소 트롬빈, 성장인자