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# Identification and characterization of bioactive factors in bladder submucosa matrix

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**Abstract**

In spite of long term clinical use of decellularized bladder submucosa matrix (BSM), little is known about the active factors within this material. In this paper, we analyzed the biological factors from the decellularized BSM using ELISA, Western blotting, and immunohistochemistry for the purpose of effective utilization of this material in the field of regenerative medicine. At least 10 growth factors, including VEGF, BMP4, PDGF-BB, KGF, TGFβ1, IGF, bFGF, EGF and TGFα were found to be preserved in the decellularized BSM . The existence of collagen (type 1, 2, 3, 4), laminin and elastin within the matrix was also demonstrated. The soluble BSM extracts showed a conspicuous effect on cell proliferation when added as a supplement in vitro. These findings demonstrate that growth factors and extracellular matrix in the BSM maintain valuable biological activity even after the decellularization and extraction processes, thus supporting the wide applicability of BSM in tissue regeneration. The identification and characterization of growth factors and extracellular matrix in the BSM is a prerequisite for understanding tissue regeneration using this scaffold.


*Keywords:* Growth factors; Bladder submucosa matrix (BSM); Extracellular matrix; Bioactive factors; Decellularized scaffold

## 1. Introduction

Naturally derived tissue matrices, such as small intestinal submucosa (SIS) or bladder lamina propria, commonly called ''bladder submucosa matrix'' (BSM), have been used as biomaterials for various reconstructive procedures, due to their biocompatibility and regenerative potentials [1–5]. Collagen-based tissue matrices are known to possess essential characteristics required for tissue regeneration. These include bioactive substances that are contained within the matrix, such as growth factors, adhesion molecules, modulators of coagulation and fibrinolysis, which are believed to enhance cell viability and tissue regeneration [6–8].

BSM either with or without cells has been used clinically for different purposes, including bladder reconstruction, hypospadias and urethral stricture repair [4,9]. BSM has been demonstrated to be an excellent biomaterial, since it can be processed easily, has good characteristics for tissue handling, and has the advantage of being ''off the shelf'' [10,11]. Moreover, BSM has been shown to be safe, biocompatible and readily integrated into surrounding tissues for regeneration when implanted in vivo [11–14]. Although it is assumed that bioactive factors contained within BSM may be responsible for its regenerative capability, none of the studies performed so far has attempted to identify the biologic factors that contribute to its regenerative characteristics. In this study we investigated the regenerative properties of BSM by defining the growth factors and proteins embedded within the matrix that may promote tissue regeneration.

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## 2. Materials and methods

### 2.1. BSM preparation

The mucosa (urothelium/suburothelium) of the porcine bladder was grossly removed by surgical delamination. The dissected tissue was cross-sectioned and using H&E stain, BSM was confirmed. The BSM was rinsed with water in a stirring flask (200 rpm) for 2 days at 4 °C, and subsequently treated with 0.03% trypsin for 1 h followed by a rinsing in 10% FBS with PBS overnight at 4 °C and finally treated with Triton X-100 (0.5%) and ammonium hydroxide (0.05%) in distilled water for 72 h at 4 °C. The solution was changed every day. After this washing step, a small piece of tissue was sampled for histology to confirm the levels of decellularization. The tissue was washed with distilled water for 2 days at 4 °C, frozen, lyophilized, pulverized under liquid nitrogen for subsequent protein extraction and cell proliferation assay. Decellularized BSM was fixed in 10% buffered formalin, processed and cut at 4–6 μm thickness for histo- and immunohistochemistry.

### 2.2. Extraction buffers

Three different types of extraction buffers (2 M urea, 0.5 M acetic acid or 0.1% Triton X-100, each containing 50 mM Tris–HCl (pH 7.4) and 0.1x protease inhibitor) were used for protein extraction from BSM. The pulverized BSM was suspended in 1% W/V of each buffer and stirred at 4 °C for various periods (0.5, 1, 3, 5 days). The mixture was centrifuged at 12,000*g* for 30 min at 4 °C and the supernatant was collected.

### 2.3. Total protein concentration

Molecular weight cut-off (MWCO) 9KDa concentrator (Pierce) was used to collect and concentrate the growth factors with molecular weights above 9 KDa. Low molecular weight growth factors under 9 KDa were processed using MWCO 3 KDa concentrator (Millipore). These samples were used for Western blotting.

### 2.4. Protein assay

The detergent compatible (DC) protein assay (BIO-RAD) was used for quantification of protein in the extract. Briefly, 500 μL of Reagent A was added into 100 μL of sample and vortexed. Subsequently, 4 mL of Reagent B was added into each test tube and vortexed immediately. After 15 min, the solution absorbance at 750 nm was determined. Various concentrations of bovine serum albumin (BSA) served as standards. The standards were prepared with the same buffer as the sample.

### 2.5. Polyacrylamide gel electrophoresis

Ten microgram of protein were mixed with sample buffer and heated for 10 min at 95 °C. The samples were then loaded onto a 15% or 20% polyacrylamide gel and electrophoresed. The composition of 5 mL of 15% acrylamide lower gel was 1.25 mL of water, 2.5 mL of 30% acrylamide, 1.25 mL resolving buffer, 65 μL of 10% APS and 6.5 μL of TEMED. For the upper gel, 3.1 mL of water, 0.6 mL of 30% acrylamide, 1.25 mL stacking buffer, 75 μL of 10% APS and 7.5 μL of TEMED were used. Pre-stained protein ladder (Invitrogen) was used as size markers.

### 2.6. Coomassie stain and Silver stain

The gel was treated with Coomassie solution (Bio-Rad) for 1 h. The gel was rinsed with distilled water for 30 min and stored in water. Silver stain (Bio-Rad) was also carried out for detecting proteins in polyacrylamide gels after electrophoresis. The gel was placed in the fixative enhancer solution and gently agitated for 20 min. Then the gel was rinsed in 400 mL deionized distilled water for 10 min with gentle agitation. The gel was rinsed for an additional 10 min. Using Staining Solution for 20 min, bands were detected. The gel was placed in Stop Solution for 15 min, and then stored in high purity water.

### 2.7. Verhoeff stain

Paraffin embedded tissue sections (size was 5 μm) were deparaffinized, rehydrated with distilled water and stained in freshly prepared Verhoeff's mixture for 15 min. After washing, the slides were differentiated using 2% ferric chloride solution. The differentiation was monitored continuously under the microscope until the nuclei and fine elastic fibers were black. The slide washed in water, then in 95% alcohol for 5 min to remove iodine coloration, counter stained with Van Gieson stain for 3 min, and then subsequently dehydrated with alcohols.

### 2.8. Immunohistochemistry

Tissue sections in a cryostat cut into 5 μm and fixed to 4% formaldehyde for 30 min at RT. After rinsing with PBS 3 times, they were treated with 3% hydrogen peroxidase for 30 min at RT. Tissue sections were blocked in 10% serum for 30 min and rinsed with PBS 3 times. Primary antibody solutions for collagen type 1 (Southern biotech, 1310-01), collagen type 2 (Southern biotech, 1320-01), collagen type 3 (Southern biotech, 1330-01), collagen type 4 (Southern biotech, 1340-01) and laminin (Sigma, L9393) were treated for 24 h at 4 °C. After washing with PBS 3 times, the sections were treated with secondary antibody with biotinylated IgG (VECTOR BA 9200, BA 5200, BA1000) for 1 h at RT, washed with PBS. Immunolabeling was done using the avidin–biotin detection system. The sections were count stained with Gill's hematoxylin.

### 2.9. Western blot

After polyacrylamide gel electrophoresis, the separated proteins were transferred to PVDP (MILLIPORE, Immobilon-P and -$P^{SQ}$) in 1x Tobin buffer (250 mM Tris base, 1.92 M glycine, 1% SDS, pH 8.3) using semi-dry transfer at 100 mA/gel for 1–3 h. Blots were blocked with 5% nonfat dry milk or BSA (PIERCE) in 1x TBST (20 mM Tris–HCl, 200 mM NaCl, 0.2% Tween-20, pH 7.5) for 1 h at 4 °C. Then, they were incubated in primary antibody dilutions for VEGF (Santa Cruz Biotechnology, SC507), BMP4 (Santa Cruz Biotechnology, SC12721), KGF (Santa Cruz Biotechnology, SC7882), PDGF-B (Santa Cruz Biotechnology, SC7878), TGFβ1 (Abcam ab25121), IGF1 (Santa Cruz Biotechnology, SC9013), bFGF (Santa Cruz Biotechnology, SC7911), EGF (Santa Cruz Biotechnology, SC275), TGFα (Calbiocam, GF10), and NGF (Chemicon, AB1526SP) at 4 °C overnight. The blots were washed and treated with secondary antibodies (cell signaling #7076 or #7074) labeled with horseradish peroxidase for 1 h at RT. After the final washing, the PVDF was incubated with chemiluminescence reagent (PerkinElmer) and exposed to hyperfilm-ECL (Amersham Life Science INC.).

### 2.10. Enzyme-linked immunosorbent assay (ELISA)

For the quantitification of various growth factors present in the extraction solution, ELISA was performed using VEGF (Quantikine DVE00), BMP4 (Quantikine DBP400), PDGF-BB (Quantikine DBB00), KGF (Quantikine DKG00), TGFβ1 (Quantikine DB100B), IGF1 (Quantikine DG100), bFGF (Quantikine DFB50), EGF (Quantikine DEG00) and TGFα (Quantikine DTGA00) ELISA kit. The optical density was determined using a microplate reader (EL × 800, BIO-TEK INSTRUMENTS, Inc.).

### 2.11. Cell proliferation assay

To evaluate the effect of bioactive molecules contained within the BSM on cell proliferation, Swiss 3T3 fibroblast were cultured at various