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# The Injectable rhBMP-2-containing Collagen Gel for Tendon Healing in a Rabbit Extra-Articular Bone Tunnel Model

*Kwang-Il Lee, Ju-Woong Jang and Kwang-Won Lee*

## Abstract

This rabbit animal study has a hypothesis that the collagen gel, which is injectable easily, can be an effective carrier for recombinant human bone morphogenetic protein 2 (rhBMP-2) for the tendon healing in a bone tunnel. The cut upper long digital extensor tendon of each rabbit was inserted into the proximal tibia bone tunnel, and rhBMP-2 conjugated collagen gel was injected into the tendon-bone tunnel interface using a syringe. Biomechanical and histological performances were analyzed at 3 and 6 weeks after surgery. The collagen sol at room temperature was transformed to a gel at 37°C. The rhBMP-2 was slowly released from the collagen gel for more than 4 weeks. The in vivo experiment showed the enhanced new fibrocartilage and bone tissue formation at 6 weeks after injecting the rhBMP-2-containing collagen gel. The calcification and enthesis-like tissue were detected radiologically in the repaired tendon-bone junction. The viscous collagen gel-containing rhBMP-2 increased the fusion rate of the repaired tendon in the bone tunnel. This study showed that viscous collagen gel can be an effective carrier for rhBMP-2 for tendon healing in the bone tunnel. The rhBMP-2-containing collagen gel will be promising for tendon-bone interface healing in the future.

**Keywords:** rhBMP-2, tendon, bone tunnel, enthesis, ligament injury

## 1. Introduction

Tendon healing in the bone tunnel is the one of the most critical points for tendon repair [1]. Various types of tendon grafts such as peroneus longus, tibialis, gracilis, semitendinosus, and Achilles tendons can be transplanted for replacing the ruptured ligament/tendon tissues [2]. However, the failure rate after surgery has still remained the cause of poor recovery capacity, so more strategic studies are necessary [3]. One of the main reasons for poor healing is because the mechanical stresses keep affecting repaired tendon-bone tunnel junction [4]. The unique transitional tissue, the enthesis which is a fibrocartilage tissue, is generated with a connected region between tendon and bone tissue [5]. Thus, for successful tendon/ligament reconstruction, osteointegration of inserted tendon grafts in the bone tunnel is strongly recommended [6].

Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a well-known growth factor for new bone formation [7–9]. This promotes the differentiation of undifferentiated mesenchymal cells into chondrogenic or osteogenic lineages that support new bone formation [10, 11]. This differentiation also needs in the reconstructed tendon-bone tunnel region for postnatal enthesis formation. There have been many studies about an effective rhBMP-2 delivery system using collagen-based materials such as sponge and gel for sustained release [12–15]. In the previous studies, rhBMP-2 was used for bone-tendon interface healing using a collagen sponge [16, 17]. However, the collagen sponge system had a limitation of rhBMP-2 localization to the only targeted region and minimizing leakage from the bone tunnel [18]. For the enhanced delivery of rhBMP-2 into the targeted surgical sites, a viscous collagen gel may be useful. Another previous study had performed a comparative study of the osteogenic effects using two different rhBMP-2 delivery systems such as collagen sponge and collagen gel in a rat spinal fusion model [19]. The results showed that rhBMP-2 containing a collagen sponge had side effects of leakage BMP. To overcome this limitation, viscous collagen gel was applied to rabbit tendon-bone tunnel regions in this study.

The purpose of this translational study is to investigate whether the rhBMP-2-containing collagen gel can localize in the surgical site and improve new enthesis formation within reconstructed tendon-bone tunnel after the surgery.

## 2. Materials and methods

### 2.1 Conjugation of collagen gel and rhBMP-2

1% (w/v) collagen gel originated from porcine skin was mixed with 50 µg/ml rhBMP-2. This concentration had been confirmed from our previous rat and rabbit animal studies [20–23]. To check temperature dependency of collagen's sol-gel phase transition, the optical density of 1% collagen gel at 37°C was read at 313 nm in an absorbance microplate reader at 10, 20, and 30-minute time points. For the release kinetics analysis of rhBMP-2-containing collagen gel, it was plated on 12-well plate and incubated in 1 ml phosphate-buffered saline (pH 7.4) at 37°C for 28 days. At each time point of 1, 3, 5, 7, 14, and 28-day time points, each supernatant was collected and stored at –80°C until reading. Then, the rhBMP-2 was quantitated using an enzyme-linked immunosorbent assay kit and a cumulative release curve was plotted.

### 2.2 Animal study design and operative procedure

Healthy adult New Zealand White rabbits (n = 36, 3.0–3.5 kg) were used for this study. The animal treatment was followed by the Guidelines for Care and Use of Laboratory Animals, and this animal experiment was approved by the Committee of Experimental Animal Sciences. The rabbits were classified with three different groups: saline injection only (control group), collagen gel injection only without rhBMP-2 (collagen gel group), and rhBMP-2-conjugated collagen gel injection (rhBMP-2-collagen gel group). Rabbits were anesthetized with ketamine, 40 mg/kg IM; xylazine, 5 mg/kg IM.

The rabbits underwent an operative procedure for an extra-articular tendon-bone healing model at the rerouted long digital extensor tendon. The knee joint was accessed through a lateral para-patellar incision. The long digital extensor tendon was identified and then detached from its insertion at the lateral femoral condyle by sharp dissection. The free tendon was tied with 3-0 Vicryl. Then, the

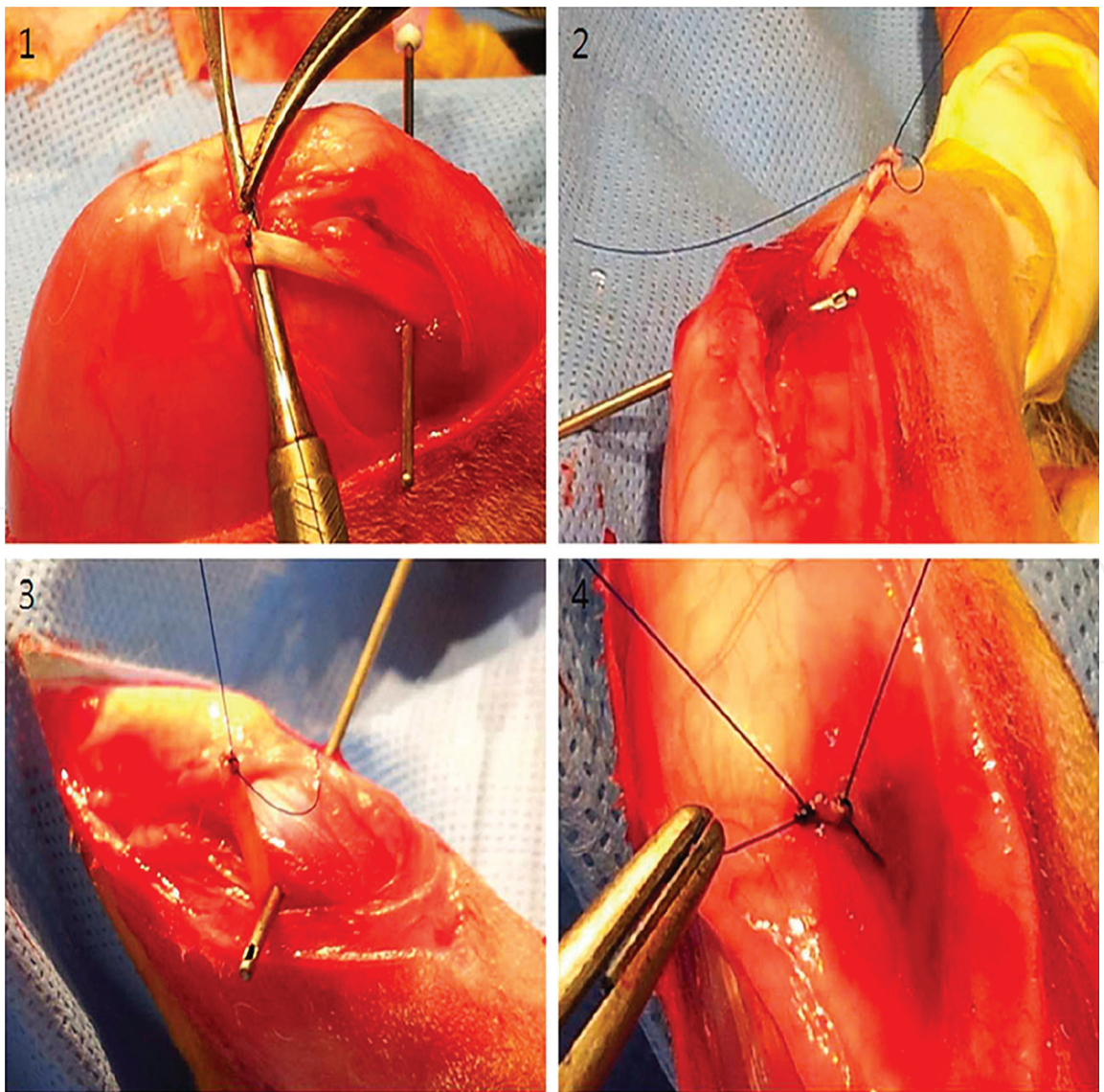


anterior tibia muscle was retracted laterally. A bone tunnel was created in the proximal tibia metaphysis with 30° angle relative to the long-bone axis using a drill (diameter: 2 mm). The finalized bone tunnel size after drilling was an average of  $2.09 \pm 0.04$  mm-diameter and  $5.13 \pm 0.05$  mm-length, which was measured by scanned microcomputed tomography images. The cut long digital extensor tendon was relocated. It was pulled manually through the bone tunnel and sutured to the periosteum and soft tissue at the medial proximal tibia with 3-0 nylon (**Figure 1**).

Each 200µl of rhBMP-2-containing collagen gel was injected into the tendon-bone tunnel junction. The joint capsule, fascia, and subcutaneous tissue were closed with 3-0 Vicryl and skin was closed with 3-0 nylon.

### 2.3 Analysis of three-dimensional computed tomography (CT) and bone mineral density (BMD)

The BMD and mineralized tissue ingrowth inside the tendon-bone tunnel junction were quantified by using CT system. Specimens were scanned perpendicular to the long-bone axis covering the entry and exit of the bone tunnel. The sections were reconstructed using the 3D software. To quantify the amount of newly formed mineralized tissue over time, the regions of interest (ROI) was chosen and



**Figure 1.**  
*Operative procedure of the long digital extensor tendon sutured to the periosteum and soft tissue of rabbit medial tibia.*

reconstructed using the 3D software. After thresholding, the BMD (mg/cm<sup>3</sup>) of the mineralized tissue inside the tendon-bone tunnel junction was calculated.

2.4 Biomechanical testing

Rabbit knee joints including the tendon-bone tunnel site were collected. To analyze the tensile mechanical properties, tensile strength was measured by a universal testing machine. The specimen was fixed vertically on a 5000 N load-cell, and tensile strength was measured by pulling each specimen at a load displacement rate of 10 mm/min. The failure load and ultimate strength (N) were recorded.

2.5 Histologic and histomorphometric analyses

Rabbit knee joints were collected and fixed in a neutralized formalin solution for 2 days and decalcified in 10% formic acid until being cut. The specimens were sliced into 4-um-thickness in an orientation parallel to the bone tunnel, and each section was stained with Masson’s trichrome, and visualized using an optical microscope. Healing of the tendon in the bone tunnel was graded histomorphologically by two blinded observation methods.

Histomorphometric analysis was assessed for tendon healing in the bone tunnel. Quantitative histomorphometric analysis was performed by two blinded observations, which apportioned 0–3 points based on histomorphologic criteria, representing fibrocartilage formation, new bone formation, and tendon graft bonding to adjacent tissue (Table 1).

Characteristic	Points
Fibrocartilage formation	
Abundant	3
Moderate	2
Slight	1
None	0
New bone formation	
Abundant	3
Moderate	2
Slight	1
None	0
Tendon graft bonding to adjacent tissue	
75–100%	3
50–75%	2
25–50%	1
0–25%	0

**Table 1.**  
*Histomorphometric analysis to assess healing of the tendon within the bone tunnel (full score = 9 points).*

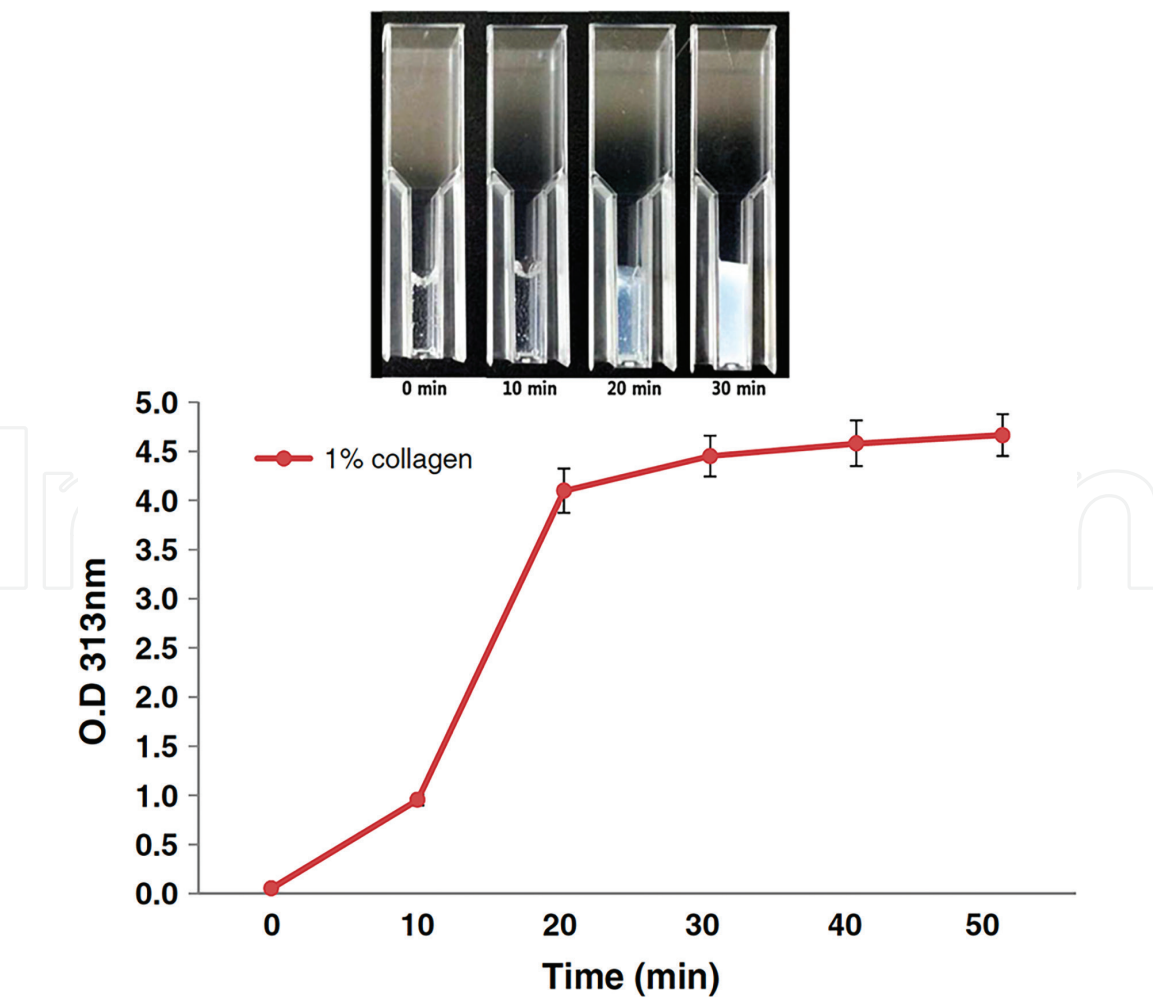
### 2.6 Statistical analysis

The data were averaged from at least triplicated samples. The same experiments were repeated for three times to ensure the reproducibility of the methods used. All statistical analyses were performed using SPSS version 15.0. The post hoc Scheffé test was used to analyze the significant difference between groups, with significance levels at \* $p < 0.05$  and \*\*  $< 0.01$ .

### 3. Results

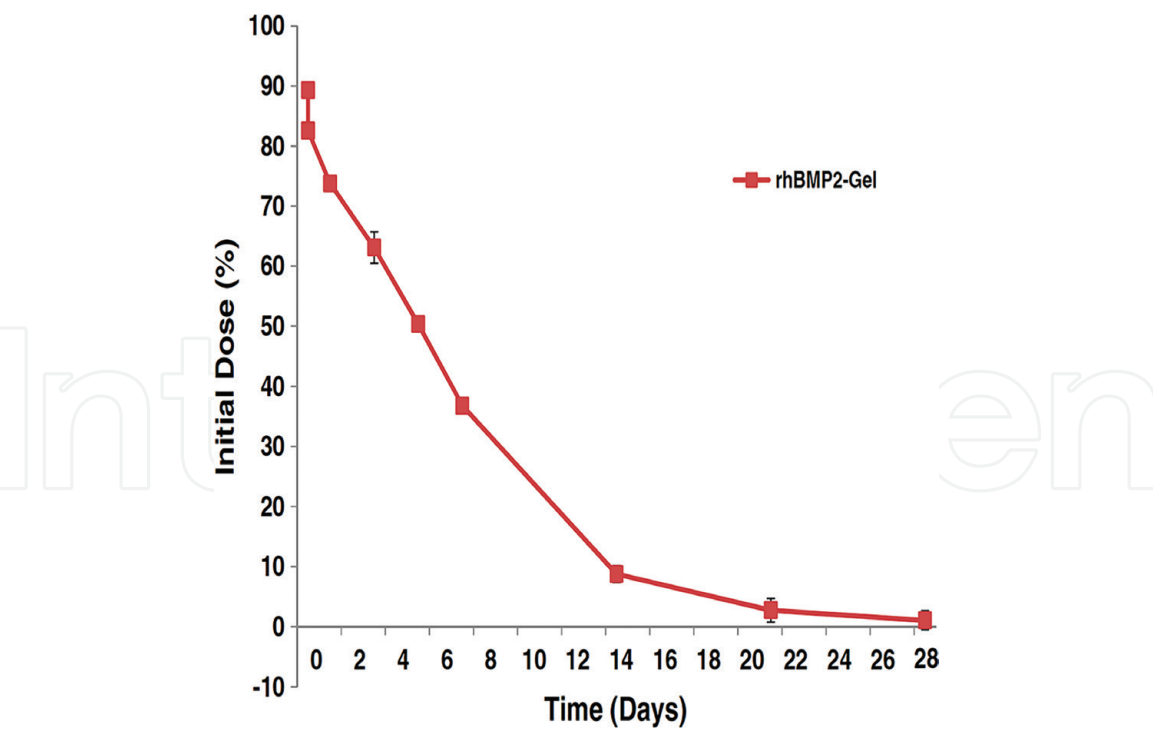
The turbidity of collagen gel at 37°C was significantly increased between the time points of 10 (OD: 0.953) and 20 (OD: 4.099) minutes (**Figure 2**). The half release from the total rhBMP-2 quantity from the collagen gel was done within 5 days from incubation. And the rest half was released slowly for over 28 days when it was released 89.3% totally. The rhBMP-2 conjugated collagen gel showed a sustained release phase (**Figure 3**).

In the 3D-CT analysis, new bone formation was detected at the interface between the tendon and tibia bone tunnel in the rhBMP-2-collagen gel group after 3 weeks even though the normal distal epiphyseal plate of rabbits has a limited cancellous bone. However, the control and collagen gel groups did not show new bone formation (**Figure 4**). At 6 weeks after the surgery, the rhBMP-2-collagen gel group

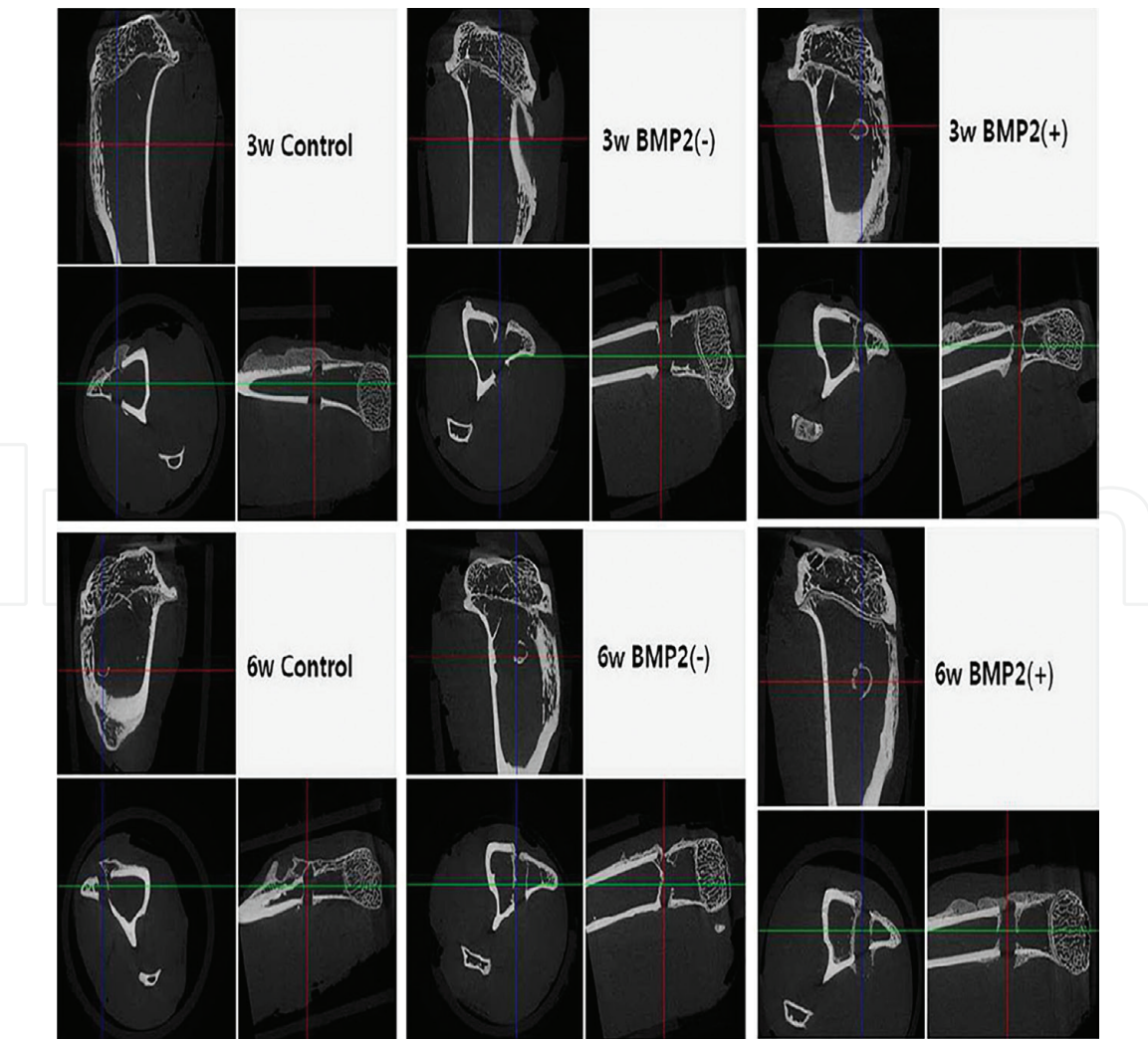


**Figure 2.** Ultraviolet turbidity of 1% collagen gel at each time point, dependent on temperature; average of triplicates at each time point, which is 0, 10, 20, and 30 min at 37°C incubation.





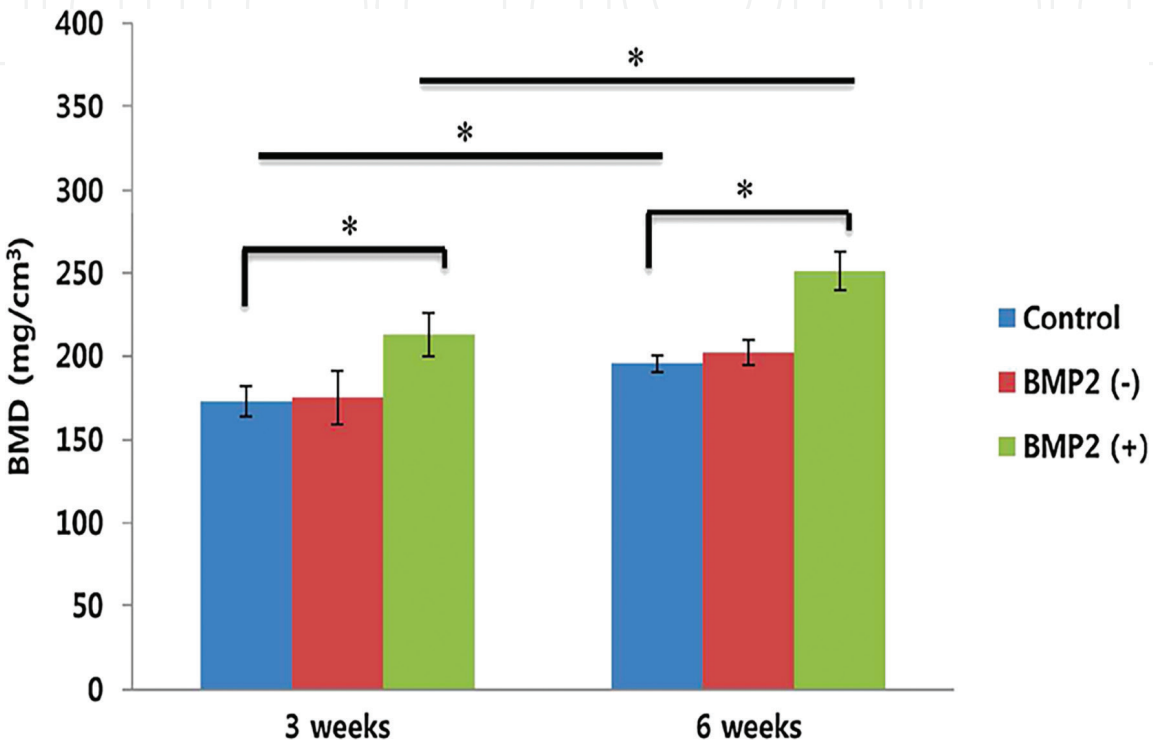
**Figure 3.**  
*Release profile of rhBMP-2 from 1% collagen solution; average of triplicates at each time point for 4 weeks.*



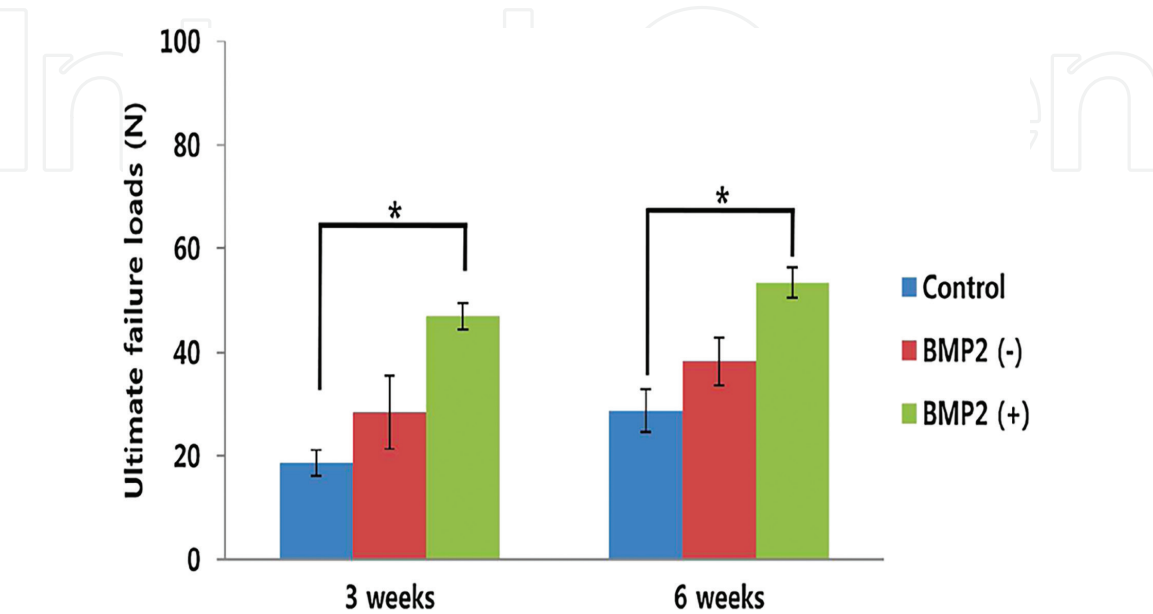
**Figure 4.**  
*3D CT images of the enthesis generated by transfer of the toe flexor or rhBMP-2<sup>+</sup> or rhBMP-2<sup>-</sup> bone complex to the proximal tibia at 3 and 6 weeks.*

showed higher new bone formation than the other groups. In addition, the BMD of the rhBMP-2-collagen gel groups was significantly higher than the control group at 3 and 6 weeks after the surgery (**Figure 5**). The BMD of the collagen gel group was slightly higher than the control group; however, it was not significant.

In the biomechanical test result, the ultimate failure load of the rhBMP-2-collagen gel group was significantly higher than the other groups at 3 and 6 weeks (**Figure 6**). After 3 weeks, the ultimate failure load of the rhBMP-2-collagen gel group was 2.5-fold higher than the control group. After 6 weeks, the thBMP-2-collagen gel group was 1.8-fold higher than the control group. However, there was no significance between the collagen gel group and control group.



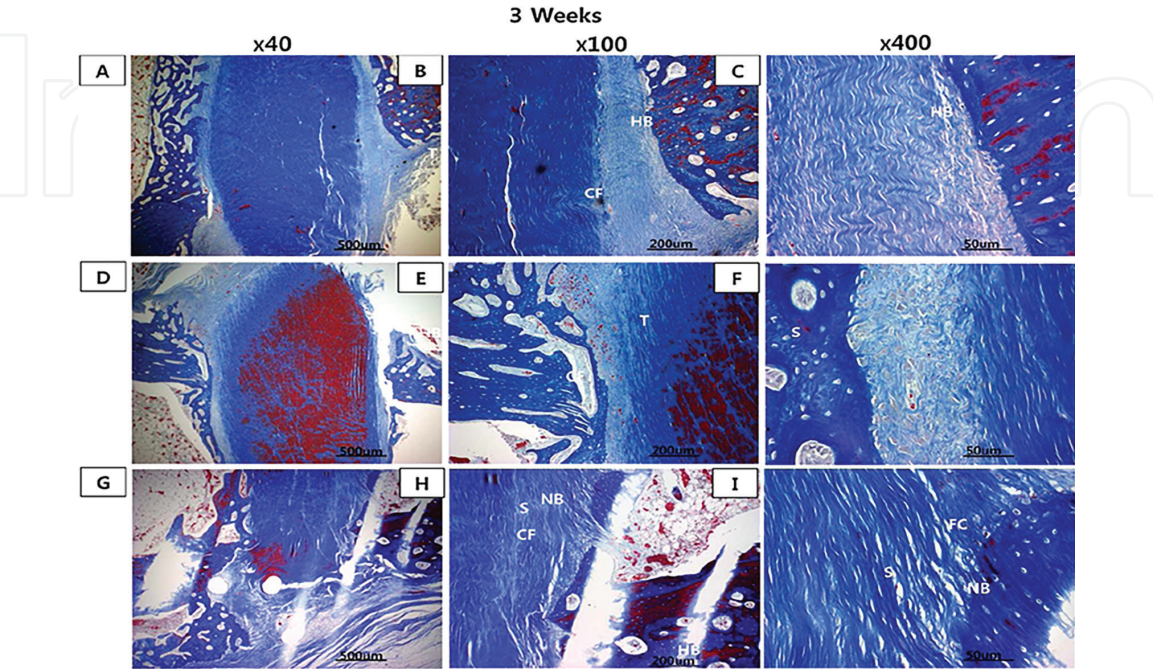
**Figure 5.** Bone mineral density of the enthesis generated by transfer of the toe flexor or rhBMP-2<sup>+</sup> or rhBMP-2<sup>-</sup> bone complex to the proximal tibia at 3 and 6 weeks.



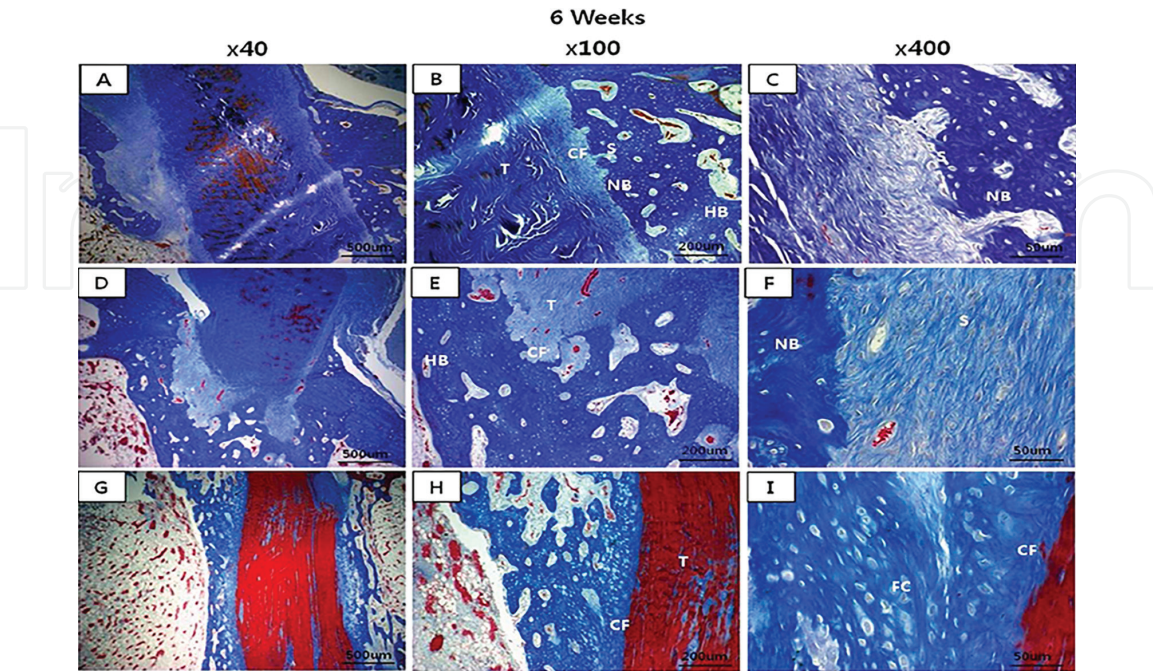
**Figure 6.** Ultimate failure loads of the enthesis generated by transfer of the toe flexor or rhBMP-2<sup>+</sup> or rhBMP-2<sup>-</sup> bone complex to the proximal tibia at 3 and 6 weeks.



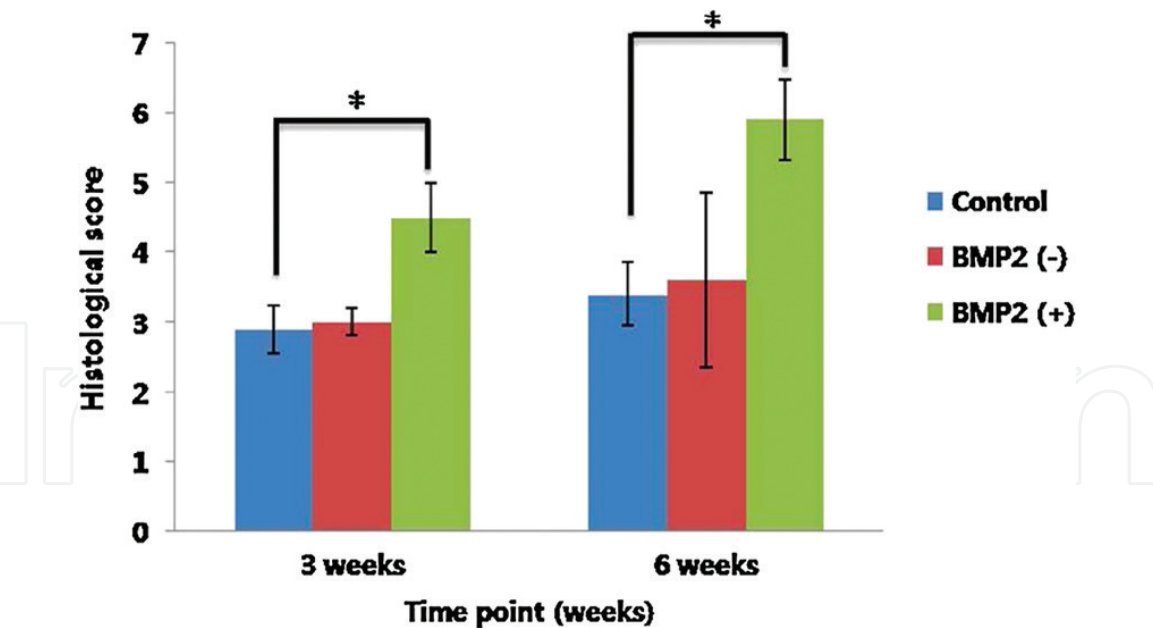
In the histology results, Masson's trichrome staining showed that collagen fibers and fibrous cartilage were widely detected in the tendon-bone interface of the rhBMP-2-collagen gel group at 3 weeks. The new bone was partly detected between the tendon and host bone (**Figure 7**). After 6 weeks, there was increased fibrous cartilage and new bone tissues between the tendon and the bone. Moreover, the new Sharpey-like fibers were detected in the rhBMP-2-collagen gel group (**Figure 8**).



**Figure 7.** Masson's trichrome staining of the enthesis generated by transfer of the toe flexor or rhBMP-2<sup>+</sup> or rhBMP-2<sup>-</sup> bone complex to the proximal tibia at 3 weeks: (A–C) control; (D–F) collagen only; (G–I) collagen with rhBMP-2; CF, collagen fibers; FC, fibrocartilage; HB, host bone; NB, new bone; S, Sharpey-like fibers; and T, tendon.



**Figure 8.** Masson's trichrome staining of the enthesis generated by transfer of the toe flexor or rhBMP-2<sup>+</sup> or rhBMP-2<sup>-</sup> bone complex to the proximal tibia at 6 weeks: (A–C) control; (D–F) collagen only; (G–I) collagen with rhBMP-2; CF, collagen fibers; FC, fibrocartilage; HB, host bone; NB, new bone; S, Sharpey-like fibers; and T, tendon.



**Figure 9.**  
*Histological score of the enthesis generated by transfer of the toe flexor or rhBMP-2<sup>+</sup> or rhBMP-2<sup>-</sup> bone complex to the proximal tibia at 3 and 6 weeks.*

In the histomorphometric analysis results, the histologic score about enthesis formation in the rhBMP-2-collagen gel group was significantly higher than the other groups (**Figure 9**). Moreover, the results after 6 weeks were higher in score in the rhBMP-2-collagen gel group than the score in the other groups after 3 weeks.

#### 4. Discussion

The healing process after tendon or ligament reconstruction needs stable enthesis generation at the interface between the inserted tendon and drilled bone tunnel, which is one of the most important conditions [4, 24]. rhBMP-2 may be useful as a growth factor for new bone formation by inducing differentiation of osteoprogenitor cells to osteoblasts [25]. For the effective soft tissue healing, rhBMP-2 application will be the ideal method for new bone formation between the inserted tendon and the drilled bone tunnel. However, rhBMP-2 requires a carrier for embedding itself [26, 27]. It is important to establish the rhBMP-2 delivery system with immobilization for the sustained release in the surgical site. The immobilization of rhBMP-2 can enhance the host cell infiltration into the surgical site and stimulating cellular activity for new tissue formation [28, 29].

We used viscous and elastic collagen gel for the minimum loss and sustained release of rhBMP-2. Collagen gel is easy to inject and biocompatible for drug delivery. Collagen gel also has been employed as a scaffold in tissue engineering and regenerative medicine [30]. When the collagen gel is injected into the surgical site, it will be easy to use due to its viscous solution state. After its implantation, the gel becomes semisolid at body temperature. This thermos-sensitive state can make the injected gel stable and good for sustained release of a growth factor for soft tissue reconstruction.

In this study, a rabbit extra-articular bone tunnel model was used to investigate the tendon/ligament healing in the drilled bone tunnel. We developed an advanced viscous rhBMP-2-conjugated collagen gel for the soft tissue reconstruction. This gel system will be useful as a void filler between the tendon graft and host bone tunnel.

Collagen showed temperature-responsive gelation at the body temperature. This demonstrates that collagen gel can be effective for the stable filling into the surgical



site and rhBMP-2 will be effectively and slowly released from the stable gel without any loss by irrigation during the surgery. The phase transformation will also affect the degradation rate of the collagen gel and the time course of stimulation of osteogenesis [18].

In vivo test results showed that the rhBMP-2-collagen gel group increased the fusion rate between the grafted tendon and host bone tunnel. BMD analysis results also showed the enhanced new bone formation by rhBMP-2.

In conclusion, the injectable rhBMP-2-containing collagen gel induced earlier and more new bone formation at the tendon-bone tunnel interface. This study demonstrated that the mixture of the rhBMP-2 and collagen gel can accelerate the healing process of the grafted tendon in the host bone tunnel. The clinical use of the injectable rhBMP-2-collagen gel will be promising for the enhancement of tendon/ligament reconstruction in the future.

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## Author details

Kwang-Il Lee<sup>1,2\*</sup>, Ju-Woong Jang<sup>2</sup> and Kwang-Won Lee<sup>3</sup>

<sup>1</sup> The Scripps Research Institute, La Jolla, CA, United States

<sup>2</sup> The Institute of Biomaterials and Medical Engineering, Cellumed Co., Ltd., Seoul, Republic of Korea

<sup>3</sup> Department of Orthopedic Surgery, Eulji University Hospital, Daejeon, Republic of Korea

\*Address all correspondence to: [jasonklee77@gmail.com](mailto:jasonklee77@gmail.com)

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