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# Anti-Vascular Endothelial Growth Factor (VEGF) Antibody Ameliorates Cartilage Degradation in a Rat Model of Chronic Sports Arthritic Injury

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Data Interpretation D  
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**Background:** Although a relationship between vascular endothelial growth factor (VEGF) and articular cartilage degeneration has been reported, little is known regarding its role in articular cartilage injury induced by sports activities. In this study, we evaluated the role of VEGF in a rat model of chronic sports arthritic injury.

**Material/Methods:** Animals were divided into 3 groups: Control (n=10), Vehicle (chronic sports arthritic injury, n=10), and Bevacizumab (chronic sports arthritic injury treated with anti-VEGF monoclonal antibody Bevacizumab, n=10).

**Results:** No significant difference in body weight was observed following the establishment of chronic sports arthritic injury among these 3 groups. Compared with the Vehicle group, Bevacizumab exhibited improved structure of articular cartilage (revealed by HE staining), as well as elevated cartilage content (revealed by Safranin O staining). Moreover, altered cytokines were observed after Bevacizumab treatment, indicating the significant decrease in levels of interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , matrix metalloproteinase (MMP)-1, and MMP-3, and a clear increase in levels of transforming growth factor (TGF)- $\beta$ 1.

**Conclusions:** All these findings demonstrate that Bevacizumab treatment ameliorated cartilage degradation in rats subjected to chronic sports arthritic injury. Our results provide evidence supporting use of targeted therapy for VEGF in the clinical treatment of chronic sports arthritic injury.

**MeSH Keywords:** **Cartilage Diseases • Cartilage, Articular • Receptors, Vascular Endothelial Growth Factor**

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## Background

With the development of society, physical sports are becoming more and more popular, accompanied with more and more injuries resulting from sports. Articular cartilage injury is a common strain resulting from physical exercise; if not treated in time, it accelerates articular cartilage degeneration, which ultimately develops into osteoarthritis (OA) [1,2]. Articular cartilage is the connective tissue covering the articular surface, constituting an important component of the knee joint, with the function of shock absorption and reducing impact and friction [3]. Cartilage is non-vascular nerve and lymphatic tissue that lacks resident progenitor stem cells, and thus has little self-healing potential once injured [1,4]. Therefore, the treatment of articular cartilage injury has long been an important research topic.

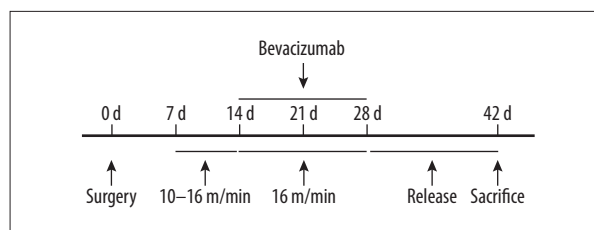
In recent years, vascular endothelial growth factor (VEGF), a multifunctional cytokine that mediates angiogenesis, was reported to be involved in articular cartilage degradation and to be a potential treatment option for OA patients. Pufe [5] stated that VEGF can be expressed in OA cartilage, but has almost no expression in normal articular cartilage. Zhang et al. provided evidence that VEGF downregulates chondrocyte activities, and reported that knockdown of VEGF promotes chondrogenesis and suppresses OA progression [6,7]. Kanata et al. suggested the possibility that VEGF and its receptors play vital roles in OA cartilage destruction through the upregulation of matrix metalloproteinases (MMPs), including MMP-1 and MMP-3 [8,9]. Ludin et al. also observed that in synovial hyperplasia there was increased cartilage calcification and subchondral bone sclerosis shortly after intra-articular administration of VEGF [10]. Nevertheless, the role of VEGF in articular cartilage injury induced by sports activity has not been studied.

In the present study, we established rat models of chronic sports arthritic injury, and then examined the expression of VEGF in articular cartilage and evaluated its effect on cartilage changes in knee joints. Subsequently, we investigated the effects of intra-articular injection of the anti-VEGF antibody Bevacizumab on cartilage degradation. We also detected some cytokines, including interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , MMP-1, MMP-3, and transforming growth factor (TGF)- $\beta$ 1, in knee synovial fluid of rats.

## Material and Methods

### Establishment of chronic sports arthritic injury model

Thirty healthy male Sprague-Dawley (SD) rats weighing 180–250 g were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). Animal

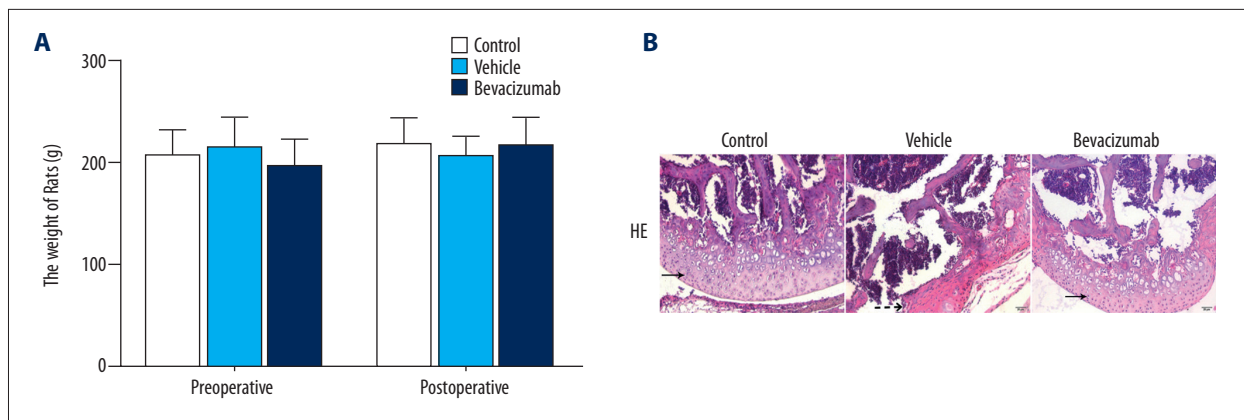


**Figure 1.** The experimental procedures are shown using a time schedule.

experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. The chronic sports arthritic injury model was established as previously described [11], with some alterations. SD rats were anaesthetized with 10% of chloral hydrate (0.4 mL/100 g weight) via intraperitoneal injection. Then, a medial patellar incision was made, the medial collateral ligament was cut, and knee joint cavity was opened, and the cruciate ligament was cut in front and in back with eye scissors. During the operation, the articular cartilage surface was not damaged. Then, bleeding was completely stopped, and sutured layer-by-layer (with intramuscular injection of penicillin sodium 200 000 units in case of infection). Seven days later, each group was trained to simulate athletic injury using an animal treadmill. From Day 8 to Day 14, the movement intensity gradually increased (the speed of the running platform increased from 10 m/min to 16 m/min). From Day 15 to Day 28, the training was kept at 16 m/min for 30 min (6 days once week, continuous 2 weeks). The rats were then released for 2 weeks (from Day 29 to Day 42), and were used for subsequent experiments. These rats were randomly assigned into 3 groups (n=10 for each): the Control group, the Vehicle group, and the Bevacizumab group. The Control group served as a blank control; the Vehicle group was established with a chronic sports arthritic injury; and the Bevacizumab group was established with chronic sports arthritic injury and then given intra-articular injection of 0.1 mL anti-VEGF monoclonal antibody Bevacizumab (Genentech, Inc., CA). Bevacizumab was administered continuously from Day 15 to Day 21, and then administered at one-day intervals from Day 22 to Day 28. The experimental procedures are shown in Figure 1, using a time schedule.

### Specimen preparation

At 42 days after the surgery, these rats were anaesthetized and decapitated to obtain right knee joints. Paraffin sections were prepared and used for routine pathological examinations. Briefly, isolated samples were fixed with 4% paraformaldehyde and decalcified in 10% EDTA solution (pH 7.4). After being washed with water, tissues were dehydrated with a graded series of ethanol, infiltrated with xylene, and then embedded in paraffin before being cut into 5- $\mu$ m-thick sections.



**Figure 2.** Bevacizumab ameliorated articular cartilage lesions in chronic sports arthritic injury. Rats were assigned into Control (n=10), Vehicle (n=10), and Bevacizumab (n=10) groups. **(A)** There were no obvious differences in the weights of rats before and after the operation in each group. n=10 for each group. **(B)** Representative images of HE staining for articular cartilage in each group. In the Control group, articular cartilage surfaces appeared neat and smooth; in the Vehicle group, obscure cell layers and numerous necrotic cells were observed, only leaving the cell outlines; and in the Bevacizumab group, cell layers could be distinguished generally, but the cells were arranged in clusters, only leaving the cell outlines in some necrotic cells. Solid arrow indicates the cartilage surfaces and dotted arrow indicates the necrotic cells.

### Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from isolated samples using Trizol Reagent (Gibco, USA) according to the manufacturer's protocol. Then, RNA was quantified and reversely transcribed into cDNAs using the iScript cDNA synthesis kit (Bio-Rad, CA). The cDNAs were amplified through qRT-PCR using SYBR Green Realtime PCR Master Mix (TOYOBO, Japan). The relative expression level of VEGF was calculated using the  $2^{-\Delta\Delta Ct}$  method and normalized to the level of the internal control GAPDH.

### Western blot

The protein was extracted in RIPA lysis buffer (200  $\mu$ L, Thermo Scientific, USA). Equal amounts of protein from cell lysates were dissolved by 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore Corp., USA). After being blocked with 5% fat-free milk at room temperature for 2 h, the membranes were probed with primary antibody against VEGF (Genentech, Inc., CA) overnight at 4°C, and then incubated with secondary antibody horseradish peroxidase-conjugated IgG (Cell Signaling Technology, USA) at room temperature for 2 h. The protein was detected using an enhanced chemiluminescence kit (ECL kit, Pierce Biotechnology, IL) and the band intensity was quantified with software Image Pro Plus 4.5. GAPDH served as the loading control.

### Immunohistochemistry

The sections were dewaxed with xylene, rehydrated, and then incubated at room temperature for 15 min in 3%  $H_2O_2$  to quench endogenous peroxidase activity. Sections were then

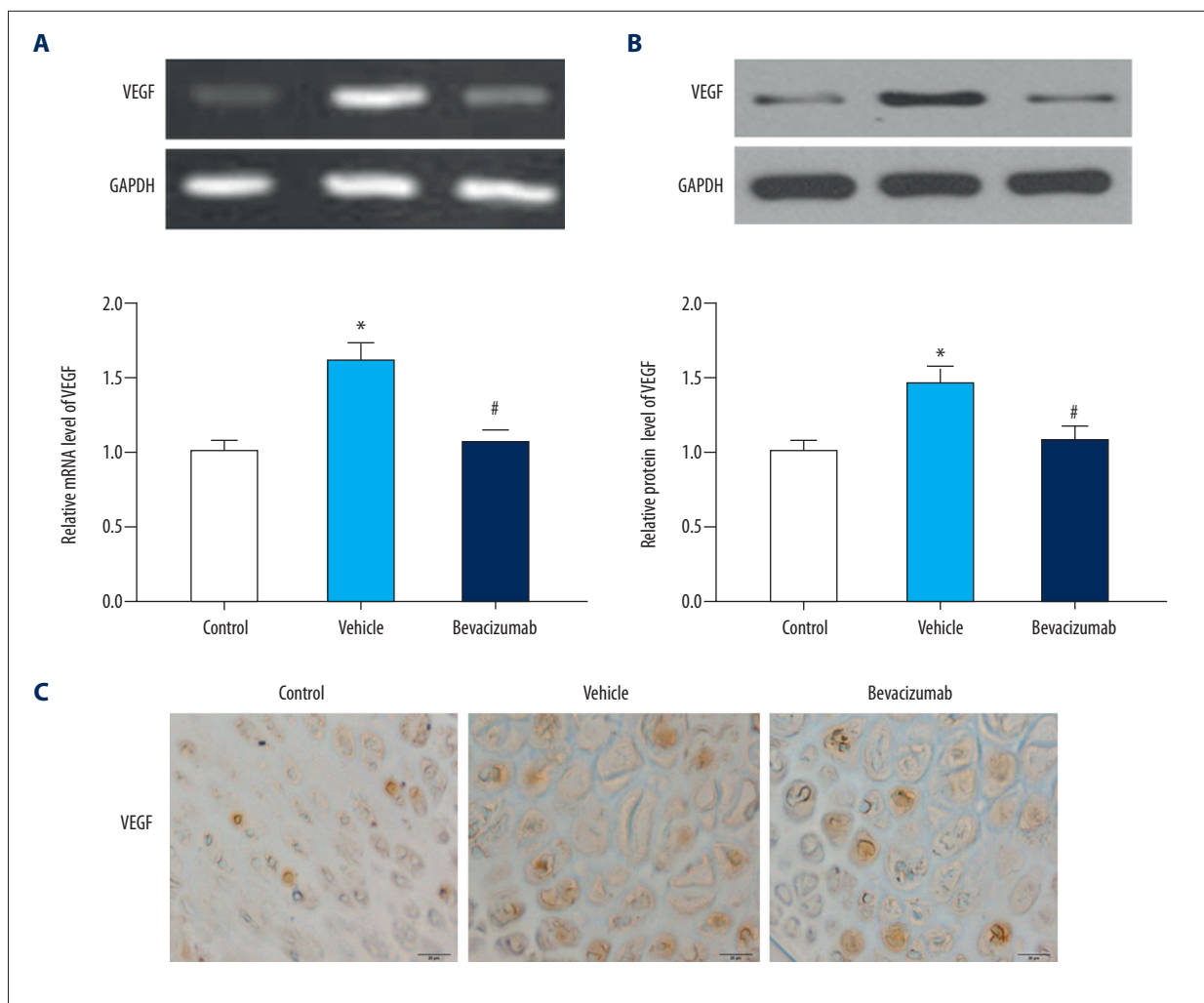
treated with trypsinization to retrieve the antigen. After blocking with normal goat serum at room temperature for 15 min, the sections were incubated with a primary antibody against VEGF (Genentech, Inc., CA) overnight at 4°C, followed by a biotinylated secondary antibody at room temperature for 30 min. Then, samples were stained with diaminobenzidine (DAB), counterstained with hematoxylin and eosin (HE), dehydrated, and then embedded in paraffin. The sections were analyzed using a microscope.

### Safranin O staining

The sections were dewaxed with xylene, rehydrated, and then stained with Safranin O to examine cartilage destruction. In brief, the sections were immersed in 0.5% Safranin O solution (pH 4.6, containing 0.1 mol/L sodium acetate) for 10 min. The sections were dehydrated, sealed, coverslipped, and photographed under a microscope. Then, the coverslips were removed within 24 h, and the samples were eluted with 0.6 mL elution (2 mol/L HCl, 50% ethanol). After centrifugation at 2000 $\times g$  for 10 min, the absorbance of supernatant was measured at 500 nm using a spectrophotometer. The sizes of specimens were the same and the locations of specimens in the cartilage were the same.

### Enzyme-linked immunosorbent assay (ELISA)

The levels of various cytokines (IL-1 $\beta$ , TNF- $\alpha$ , TGF- $\beta$ 1, MMP-1, and MMP-3) in knee synovial fluid of rats in each group were measured with an ELISA kit (R&D Systems, USA) according to the manufacturer's protocol.



**Figure 3.** Intra-articular injection of Bevacizumab inhibited the upregulation of VEGF in articular cartilage of the chronic sports arthritic injury. **(A)** The mRNA level of VEGF in articular cartilage of Control, Vehicle, and Bevacizumab groups was determined by qRT-PCR analysis.  $n=10$  for each group. **(B)** The protein level of VEGF in articular cartilage in each group was examined by Western blot analysis.  $n=10$  for each group. **(C)** The immunoreactivity for VEGF in articular cartilage in each group was consistent with the change at mRNA and protein levels of VEGF, demonstrated by immunohistochemistry. \*  $P<0.05$  vs. Control group and #  $P<0.05$  vs. Vehicle group.

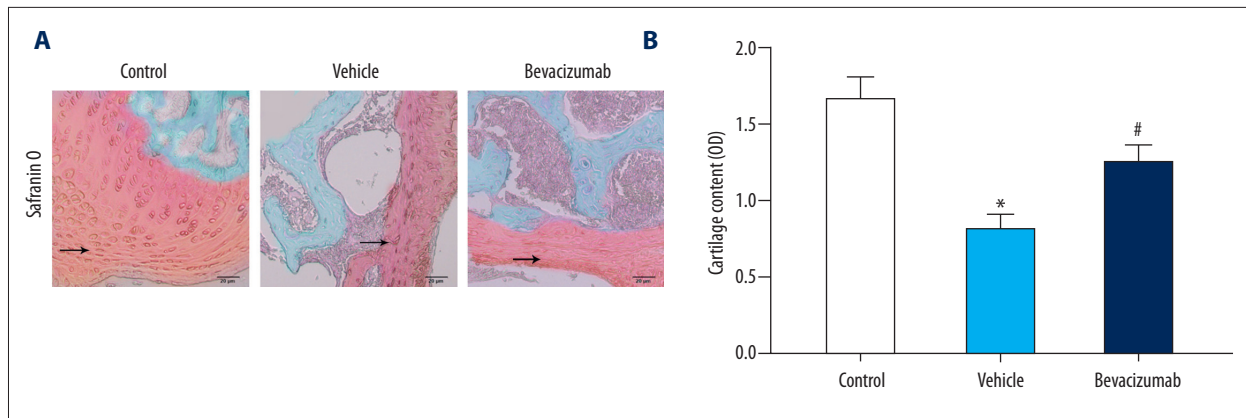
### Statistical analysis

Statistical analysis was performed with SPSS 17.0 (SPSS Inc, USA). Each experiment was conducted as least 3 times. Data are shown as mean  $\pm$  standard deviation (SD). Statistically significant differences between multiple groups were determined using one-way ANOVA, followed by the Tukey's test.  $P<0.05$  was considered statistically significant.

### Results

#### Bevacizumab inhibited the upregulation of VEGF induced by chronic sports arthritic injury

Thirty male rats were randomly assigned into Control ( $n=10$ ), Vehicle ( $n=10$ ), and Bevacizumab ( $n=10$ ) groups as described in Material and Methods. As shown in Figure 2A, there was no significant difference in the body weight of rats following the establishment of chronic sports arthritic injury among these 3 groups. We also checked the expression of VEGF in articular cartilage of these 3 groups through qRT-PCR and Western blot analysis. As shown in Figure 3A and 3B, VEGF expression in the Vehicle group was significantly up-regulated compared with



**Figure 4.** Bevacizumab reversed the reduction of cartilage content in chronic sports arthritic injury. **(A)** Representative images of Safranin O staining for articular cartilage in Control, Vehicle, and Bevacizumab groups. Solid arrow indicates the cartilage surfaces. **(B)** Absolute quantification of cartilage content was performed by measuring the light absorption (optical density) of Safranin O using a spectrophotometer. \*  $P < 0.05$  vs. Control group and #  $P < 0.05$  vs. Vehicle group.

the Control group, while Bevacizumab significantly reversed the elevated VEGF induced by sports arthritic injury at mRNA and protein levels. The results of immunohistochemical analysis for VEGF further confirmed the changes in VEGF expression in these 3 groups (Figure 3C). Our results show that VEGF expression was up-regulated in the chronic sports arthritic injury, which was attenuated by intra-articular injection of Bevacizumab.

#### Bevacizumab ameliorated articular cartilage lesions in chronic sports arthritic injury

HE staining was performed to evaluate the effect of Bevacizumab on articular cartilage. The results revealed that chronic sports arthritic injury resulted in articular cartilage lesions, while Bevacizumab further improved the structure of articular cartilage (Figure 2B). These results suggest that Bevacizumab ameliorated articular cartilage lesions induced by chronic sports arthritic injury.

#### Bevacizumab reversed the reduction of cartilage content induced by chronic sports arthritic injury

Tissue sections in each group were stained with Safranin O to detect cartilage content. As shown in Figure 4A, the Control group displayed uniformly colored articular cartilage and smooth surfaces, while the Vehicle group showed structurally obscure articular cartilage and erosive, rough, rugged, and cracked surfaces. Calcification of cartilage, the reduction of matrix chromophilic capability, and partial failure of staining were also observed in the Vehicle group. The Bevacizumab group exhibited smoother surfaces and improved staining compared with the Vehicle group. Given that the concentration of Safranin O in the cartilage matrix is proportional to the level of proteoglycans, the major components of articular cartilage [12], we measured the absorbance of Safranin O

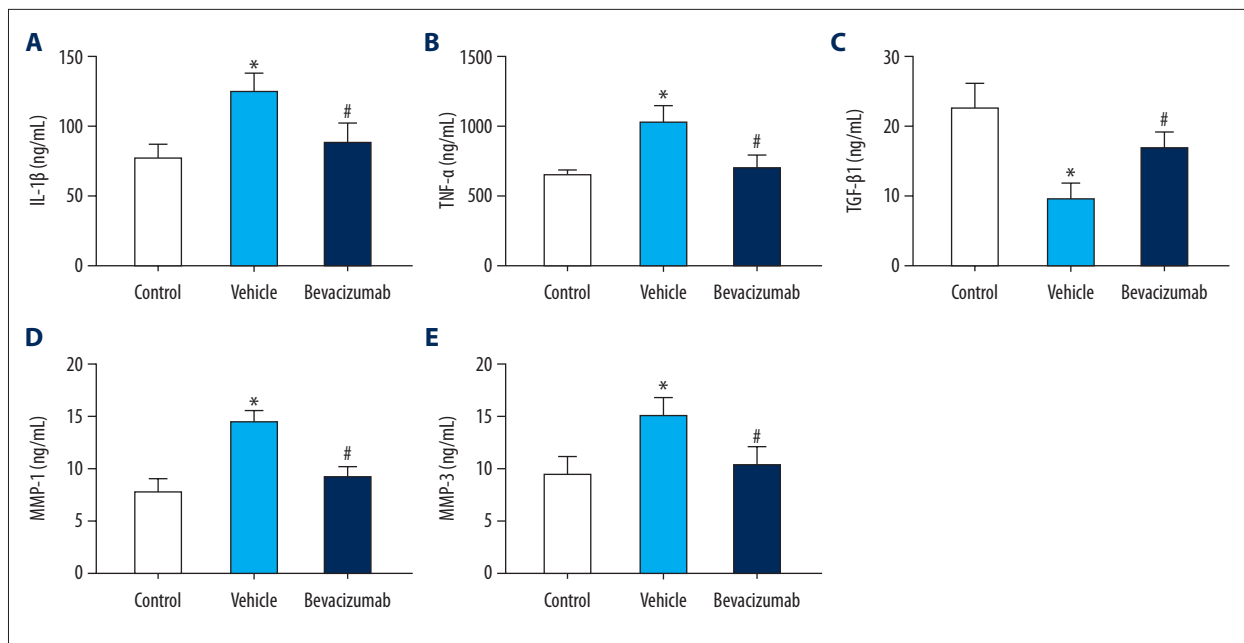
using a spectrophotometer to perform absolute qualification of cartilage content. We found that cartilage content in the Vehicle group was significantly decreased compared to that in the Control group, and was reversed by intra-articular injection of Bevacizumab (Figure 4B).

#### Bevacizumab inhibited the upregulation of IL-1 $\beta$ , TNF- $\alpha$ , MMP-1, and MMP-3, but reversed the down-regulation of TGF- $\beta$ 1

As shown in Figure 5A, 5B, 5D, 5E, ELISA results demonstrated that the levels of IL-1 $\beta$ , TNF- $\alpha$ , MMP-1, and MMP-3 in knee synovial fluid of rats were significantly up-regulated in the Vehicle group, but the Bevacizumab group showed decreased levels of these 4 cytokines. However, TGF- $\beta$ 1 exhibited the opposite results (Figure 5C).

## Discussion

Emerging evidence suggests that VEGF is involved in articular cartilage degradation-related diseases. VEGF was reported to be highly expressed in synovial rheumatoid arthritis (RA) cells [13]. Leonardi et al. [14] proposed that the coordinated expression of VEGF and its receptors in hypertrophic chondrocytes exerts an autocrine effect of maintaining chondrocyte survival and regulating cell proliferation and differentiation. In the present study, we established rat models of chronic sports arthritic injury, in which obscure cell layers and numerous necrotic cells were observed, while articular cartilage surfaces appeared neat and smooth in the Control group, suggesting that the sports arthritic cartilage models were established successfully. Then, we assessed the expression of VEGF in articular cartilage of the Control and Vehicle groups. Our results showed that VEGF expressions was significantly higher in the Vehicle group



**Figure 5.** The levels of various cytokines IL-1 $\beta$  (A), TNF- $\alpha$  (B), TGF- $\beta$ 1 (C), MMP-1 (D), and MMP-3 (E) in knee synovial fluid of rats in each group were measured by ELISA. Bevacizumab dramatically reversed the elevated levels of IL-1 $\beta$ , TNF- $\alpha$ , MMP-1, and MMP-3 in the Vehicle group, while the change of TGF- $\beta$ 1 level was the opposite of changes found in levels of other cytokines. n=10 for each group. \*  $P < 0.05$  vs. Control group and #  $P < 0.05$  vs. Vehicle group.

compared to in the Control group, indicating that VEGF may be involved in the pathological process of articular cartilage injury.

To further confirm the role of VEGF in the cartilage degradation induced by chronic sports arthritic injury, the rats were given intra-articular injection of the anti-VEGF monoclonal antibody Bevacizumab. We found that Bevacizumab significantly down-regulated the high expression of VEGF in the Vehicle group, and HE and Safranin O staining showed that the degeneration of articular cartilage in the Vehicle group was ameliorated by Bevacizumab. Our results further verify the involvement of VEGF in sports articular cartilage injury and the ameliorating effect of Bevacizumab on cartilage degradation.

Joint diseases are typically characterized by inflammatory processes that lead to pathological changes in the articular tissues, including cartilage degradation and the release of components into the synovial fluid [15]. A number of cytokines appear to induce the expression of VEGF, such as IL-1 $\beta$ , IL-6, prostaglandin E, epidermal growth factor (EGF), and TNF- $\alpha$  [16–20]. Among these, IL-1 $\beta$  and TNF- $\alpha$  were reported to be elevated in OA synovial fluid and play a dominant role in inflammatory damage and cartilage destruction [21,22]. IL-1 $\beta$  was reported to act as an inducer of OA [23]. IL-1 $\beta$  and TNF- $\alpha$ , as pro-inflammatory cytokines, are suspected to cause OA cartilage damage through enhancing the expression of MMPs in chondrocytes in an autocrine/paracrine manner [24,25]. MMPs are capable of degrading all types of cartilage extracellular matrix

proteins and are therefore important in erosion, proteolysis, and destruction of cartilage [9,26]. The increased production of MMP-1 and MMP-3 by VEGF was reported in endothelial cells [27,28], and vascular smooth muscle cells [29]. VEGF contributes to the cartilage destruction in OA by the enhanced expressions of MMP-1 and MMP-3 [9]. In the present study, we also examined the expression of some representative cytokines in knee synovial fluid of rats. The concentration of IL-1 $\beta$ , TNF- $\alpha$ , MMP-1, and MMP-3 were significantly higher in the Vehicle group than in the Control group, and Bevacizumab obviously reversed the high levels of these cytokines induced by sports articular cartilage injury, indicating that Bevacizumab exerts an anti-inflammatory effect through down-regulating inflammatory factors. We also found that the down-regulation of TGF- $\beta$ 1 in the Vehicle group was significantly reversed by Bevacizumab. Considering that TGF- $\beta$ 1 plays an important role in the repair of cartilage injury [30], and stimulates the production of proteoglycans [31], we speculated that Bevacizumab reverses the reduction of cartilage content through up-regulating TGF- $\beta$ 1.

## Conclusions

Intra-articular anti-VEGF monoclonal antibody Bevacizumab injection ameliorated articular cartilage lesions and reversed the reduction of cartilage content in a rat model of chronic sports arthritic injury. This may provide a novel therapeutic option for ameliorating cartilage degradation induced by chronic sports arthritic injury.

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