Prevention of arthrofibrosis by monoclonal antibody against vascular endothelial growth factor: A novel use of bevacizumab in rabbits

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KEYWORDS
Bevacizumab; Vascular endothelial growth factor; Fibrosis; Joint; Induced arthrofibrosis

Summary
Background: Prevention of arthrofibrosis by different drugs and surgical techniques is an essential issue in modern orthopedics.
Hypothesis: Intra-articular injection of bevacizumab can reduce arthrofibrosis on the rabbit’s stifle joint model.
Materials and methods: Arthrofibrosis was induced in the right stifle joint of thirty male New Zealand white rabbits by removing the cortical bone of the medial femoral condyle under general anesthesia. The rabbits were randomly divided into three equal groups. The control group received intra-articular injection of saline; the one-injection group received a single dose of bevacizumab (2.5 mg/kg), and the two-injection group received two intra-articular injections; the operation day and 14 days later. Forty-five days after surgery, animals were sacrificed. The severity of fibrosis was assessed based on the range of motion of the joint, a macroscopic adhesion score, and histopathologic variables such as the number of fibroblasts and of inflammatory cells, collagenous matrix deposition, synovial hyperplasia, granulation tissue formation, vascular proliferation, and presence of giant cells.

This study was carried out in: Research Center for Bone and Joint Diseases, Chamran Hospital, Shiraz University of Medical Sciences, Shiraz, Iran; Stem Cell and Transgenic Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

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Introduction

Arthrofibrosis, defined as abnormal fibrous tissue hyperplasia within and around a joint, is one of the most significant complications after intra-articular injuries, surgeries, infection, and joint arthroplasties [1–3]. The most disabling clinical findings are persistent loss of joint motion, pain, and muscle weakness [4]. Intra-articular adhesions in the knee joint are particularly disabling. Arthrofibrosis can lead to progressive osteoarthritis by provoking drastic changes in knee kinematics [2,5].

Prevention of arthrofibrosis is the best strategy [4]. Several surgical approaches [4,6] and agents such as botulinum toxin [7], hyaluronan [8], chitosan [9], proteoglycan decorin [10], anti-inflammatory drugs [11], mitomycin C [12], and barrier effect of hyaluronic and amniotic membrane [13] have been suggested to reduce arthrofibrosis. The efficacy of other drugs such as enoxaparin was not proven [14].

The physiological mechanism of intra-articular fibrosis still remains unclear; however, inflammatory process and immune response play a crucial role [2,3]. Recently, control of cytokines and inflammatory factors has been investigated as possible means to reduce arthrofibrosis [15,16]. Vascular endothelial growth factor (VEGF), the most potent mediator of vascular regulation [17], has multiple effects on adhesion formation. Apart from angiogenesis, VEGF facilitates vessel permeability, inflammatory cells migration, deposition of fibrinous exudates, and subsequent formation of the extracellular matrix with collagen deposition [2,18]. Moreover, it has been shown that VEGF is released by fibroblasts and monocytes in the tumor stromal compartments [19]. Expression of VEGF in peritoneal and pelvic adhesions [20–23], proliferative diabetic retinopathy [24], progressive idiopathic pulmonary fibrosis [25], rheumatoid arthritis, psoriasis, and arthrosclerosis [26] was shown.

Bevacizumab (Avastin) is a recombinant humanized monoclonal lg G1 antibody targeting VEGF. It could disrupt fibroblast proliferation, inhibit collagen gel contraction ability, and induce fibroblast death at high concentrations in in vitro model of wound healing in primary human Tenon’s capsule [27]. Also, the expression of transforming growth factor β1 (TGF-β1), major fibrogenic factor, was suppressed by bevacizumab in alkali-burned mouse cornea [28]. Moreover, bevacizumab was significantly able to delay the development of arthritis in mice and prevent intraperitoneal adhesions [18,29].

The present randomized experimental trial in rabbits was designed to assess the efficacy of intra-articular injection of bevacizumab in reducing arthrofibrosis and tested the effect of two injections in comparison to a single injection. We hypothesized that intra-articular injections of bevacizumab would reduce arthrofibrosis in the rabbit’s stifle joint model, and that repeating injection would optimize its effect.

Materials and methods

Animals

The experiment protocol was approved by Ethics Committee of Shiraz University of Medical Sciences. The study was conducted on thirty mature male New Zealand white rabbits weighing 3.2 kg (2.7–4.2 kg). They were kept under standard laboratory conditions (temperature 20 °C–22 °C, relative humidity, 14 hours light and 10 hours dark) in 60 × 60 × 60 cm cages under specified pathogen-free conditions with free access to water and food at Laboratory Animal Unit.

Surgery

Anesthesia was induced by intramuscular injection of 10 mg/kg ketamine (Ketalar, Parke Davis) and 8 mg/kg xylazine (Rompun, Bayer AG, Leverkusen, Germany). A pre-operative dose of intramuscular cefazolin sodium (50 mg/kg) was injected for infection prophylaxis. All surgical procedures were performed under aseptic condition by a single surgeon. Induction of arthrofibrosis was performed as previously described in the literature [16,30]. Arthroscopy was performed through a medial parapatellar approach. The medial gutter of the femoral condyle was exposed. Using a template marking a 10 × 5 mm surface, the cortical bone was removed by a dental burr at a depth of 3 mm, thus exposing the cancellous bone. After irrigation, capsule and skin were sutured by nylon. Following surgery, the joint was fixed in full flexion by a trans-articular 1.6 mm diameter Kirschner wire for 45 days.

The animals were randomly assigned to one of three groups of 10 rabbits. Animals of the one-injection and
the two-injection groups were given 2.5 mg/kg [18] bevacizumab (Avastin. Roche 100 mg/4 mL, Basel, Switzerland) diluted with 0.9% normal saline to the extent of 0.8 mL volume. Two weeks later, under local anesthesia, the second dose of intra-articular bevacizumab (2.5 mg/kg) was injected in the stifle joint of the two-injection group rabbits. An identical volume of sterile normal saline was injected in the control group.

After the procedure, intramuscular injection of buprenorphine 0.01 mg/kg three times daily [31] and acetaminophen (2 mg/kg) dissolved in 100 mL drinking water was prescribed for 5 days as analgesics. Moreover, three doses of cefazolin were injected intramuscularly. Forty-five days after surgery, the rabbits were sacrificed with a high dose (200 mg/kg) of pentothal.

### Macroscopic evaluation

Trans-articular Kirschner wire was gently removed after sacrificing the animal and the range of joint motion was evaluated with an orthopedic goniometer by one of the co-authors who was blinded to group allocation. Then, the joint was opened using a surgical knife. The macroscopic evaluation was done using a modified visual scoring system (Table 1) as described by Rothkopf et al. [7,16,30,32].

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No adhesion</td>
<td>0</td>
</tr>
<tr>
<td>Weak, mild adhesions eliminated by minimal manual traction</td>
<td>1</td>
</tr>
<tr>
<td>Moderate adhesions eliminated by usual manual traction</td>
<td>2</td>
</tr>
<tr>
<td>Dense adhesions eliminated by knife</td>
<td>3</td>
</tr>
</tbody>
</table>

### Microscopic evaluation

The stifle joints were excised while preserving all connective tissues. The samples were fixed in 10% neutral formalin. The tissue samples were embedded in paraffin blocks. Sections for histopathological evaluation were cut in 6 μm transverse sections perpendicular to the femoral axis and stained according to standard laboratory procedures with hematoxylin-eosin (H&E). Two expert pathologists blinded to the injection protocol examined the pathologic slides. The number of fibroblasts in ×400 magnification of the microscope together with inflammatory cells, collagenous matrix deposition, synovial hyperplasia, granulation tissue formation, vascular proliferation, and presence of giant cells were investigated and scored 0, 1, 2, and 3 for absent, mild, moderate, and severe states of these variables in each tissue section, respectively [7,33,34].

### Statistical analysis

Analysis was done using SPSS, version 18.0 for windows (SPSS Inc. Chicago, IL, USA). Continuous variables such as range of joint motion and number of fibroblasts were compared using student’s t test. Chi² test or Fisher’s exact test were used for nonparametric values including microscopic variables and macroscopic visual scores. Analysis was done between the control group and the one-injection group and between the one-injection group and the two-injection group respectively. P value less than 0.05 was considered as significant.

### Results

Of the 30 rabbits, two died without any specific cause during the survey (one from the control group and the other from the one-injection group). We excluded a rabbit from the two-injection group because of a septic arthritis. Finally, there were 27 rabbits (nine rabbits in each group). There was a skin necrosis exposing the patellar tendon in a rabbit of the two-injection group, but the joint remained proof and covered by the retinaculum. Interestingly, this sample had a macroscopic adhesion score of zero. Loosening of Kirschner wires occurred in 17 rabbits without fixation loss. Mean and standard deviation of different histological and macroscopic variable scores in each group are shown in Table 2.

### Comparison of the control group and the one-injection group

No statistically significant difference was found between the two groups in terms of range of joint motions and macroscopic adhesion score. The mean range of motion in the control group and the one-injection group were 58.3 and 65.5°, respectively (P = 0.222). The mean macroscopic adhesion score was 2.2 in the control group and 1.2 in the one-injection group (P = 0.067).

There were statistically significant differences between the microscopic aspects of the two groups, except granulation tissue (P = 0.347). The cellularity including inflammatory cells infiltration and fibroblast numbers was higher in the control group (P < 0.05). The number of fibroblasts in the microscopic high power field was higher in the control group (mean = 152.7 in the control group vs. 101.1 in the one-injection group with P = 0.003). Also, giant cell formation was more prevalent in the control group (P = 0.015). The vascular density was less in the one-injection group (P = 0.036) and also collagen deposition (P = 0.037) (Figs. 1 and 2).

### Comparison of the one-injection group and the two-injection group

The macroscopic criteria of these two groups were statistically different. The mean macroscopic adhesion score was 1.2 in the one-injection group vs. 0.2 in the two-injection group (P = 0.012). The mean range of motion in the two-injection group was 97.7° vs. 65.5° in the one-injection group (P = 0.001).
Table 2  Mean and standard deviation of macroscopic and histopathologic variables in different groups.

<table>
<thead>
<tr>
<th>Group: mean (SD)</th>
<th>Range of motion (Degrees)</th>
<th>Macroscopic adhesion score</th>
<th>Cellularity</th>
<th>Number of fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.3 (10.6)</td>
<td>2.2 (.6)</td>
<td>2.6 (.5)</td>
<td>152.7 (37.8)</td>
</tr>
<tr>
<td>One-injection</td>
<td>65.5 (13.3)</td>
<td>1.2 (.6)</td>
<td>1.5 (.5)</td>
<td>101.1 (23.1)</td>
</tr>
<tr>
<td>Two-injections</td>
<td>97.7 (18.0)</td>
<td>.2 (.4)</td>
<td>1.3 (.7)</td>
<td>60.0 (22.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group: mean (SD)</th>
<th>Collagen deposition</th>
<th>Granulation tissue</th>
<th>Vascular proliferation</th>
<th>Inflammatory cells</th>
<th>Giant cells</th>
<th>Synovial hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.6 (.5)</td>
<td>2.4 (.7)</td>
<td>2.5 (.5)</td>
<td>2.4 (.5)</td>
<td>1.7 (.4)</td>
<td>2.5 (.5)</td>
</tr>
<tr>
<td>One-injection</td>
<td>1.8 (.6)</td>
<td>2.0 (.7)</td>
<td>1.6 (.7)</td>
<td>1.7 (.4)</td>
<td>1.1 (.3)</td>
<td>1.4 (.5)</td>
</tr>
<tr>
<td>Two-injections</td>
<td>1.0 (.5)</td>
<td>1.1 (.6)</td>
<td>.5 (.5)</td>
<td>1.1 (.3)</td>
<td>.5 (.7)</td>
<td>.5 (.7)</td>
</tr>
</tbody>
</table>

SD: standard deviation.

Statistically significant differences were found in microscopic variables except granulation tissue ($P=0.099$). The number of fibroblasts observed in the high power field of the microscope was higher in the one-injection group (101.1 vs. 60.0 in the two-injection group, $P=0.002$). The number of inflammatory cells was significantly lower in the two-injection group ($P=0.015$). Also, the giant cell formation and synovial hyperplasia were higher in the one-injection group ($P=0.026$ and $P=0.026$, respectively). The vascular density was lower in the two-injection group ($P=0.028$) and also collagen deposition ($P=0.039$) (Figs. 2 and 3).

Discussion

Arthrobiosis provoked by intra-articular surgery or trauma is a serious problem. Its pathogenesis involves inflammatory and immune responses. Inhibition of specific cytokines and growth factors activities were shown to prevent adherence formation by neutralizing the fibroblast growth factor-2 as described by Fukui et al. [15]. In another rabbit model [16], it was shown that adhesions were prevented by continuously injecting a blocking antibody of TGF-β, a growth factor inducing the differentiation of fibroblasts from peripheral blood mononuclear cells [35,36]. Blocking VEGF, which is one of the most active mediators of angiogenesis and vascular permeability [17], was thought to have the same effect. In fact, neutralizing VEGF using bevacizumab over a short time interval (1 to 2 weeks) was shown to limit extra-osseous adherence formation [37,38]. Although a single injection may have some histological effects, Chung et al. [39] recommended repeated injections of intra-vitreal bevacizumab in patients with proliferative diabetic retinopathy, so as to optimize its effect.

Our study showed that intra-articular injections of bevacizumab reduced joint fibrosis in the rabbit. The number of rabbits in each group was small, however the results reached significance. Second, the stifle joints were fixed only by trans-articular Kirschner wire without casting, thus explaining the high rate of wire loosenning, which may have slowed down the process of fibrosis formation. Despite these limitations, histopathological observations supported the

Figure 1  Inflammatory cell infiltration, fibroblasts, (green arrow) and giant cells (yellow arrow) with more collagenous matrix deposition (blue arrow) and blood vessels (black arrow) in the control group (H&E × 200).

Figure 2  The cellularity including fibroblasts (green arrow), collagenous material (blue arrow), and blood vessels (black arrow) are greater in the one-injection group in comparison to the two-injection group (H&E × 200).
Bevacizumab protocol concerning confirmed injections. Effects of two-injection regimen on articular fibroblast proliferation is demonstrated in Figure 3. (H&E × 200). Fibroblasts are shown with green arrow, blood vessels with black arrow, collagenous matrix with blue arrow.

The initial hypothesis and confirmed the effect of bevacizumab. Because the adhesion maturation process could be affected by the remaining circulating bevacizumab up to 6 weeks [18], we re-injected bevacizumab 2 weeks after the first injection (i.e. at the end of its half-life [40]) to increase its activity and thus obtained improved clinical outcome. Skin necrosis, which was seen in one rabbit in the two-injection group, was reported as a serious side effect of bevacizumab due to its selective therapeutic effect targeting VEGF [41,42]. However, a suboptimal skin suture was incriminated in this particular case because the underlying tissues had already healed and the joint was not exposed. Therefore it seems reasonable to recommend two successive injections.

In conclusion, two intra-articular injections of bevacizumab diminished fibrosis formation in the rabbit as confirmed by histological and also clinical findings. Although further investigations should be first undertaken in big animals, it could be ultimately planned to apply the same protocol in clinical practice and hoped to obtain the same effects in human orthopaedic surgery.

Disclosure of interest
The authors declare that they have no conflicts of interest concerning this article.

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References


