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Research Article

Evaluation of Intra-Articular Injection of Bevacizumab on the Prevention of Physeal Bar Formation in Type 4 Salter Harris Model in Rats: A Pilot Study

Zeinab Imani[1], Nesa Milan[1], Hossein Nematian[1], Leila Aghaghazvini[2], Niayesh Mohebbi[3], Mojtaba Sedaghat[4], Mohammad Bagher Ahadpour Sefidan[5], Alireza Mirbagheri[6], Mohammad Hossein Nabian[7,*]

[1] Medical Student, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[2] Associate Professor, Department of Radiology, Shariati Hospital, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[3] Assistant Professor, Department of Clinical Pharmacy, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[4] Associate Professor, Department of Community Medicine, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[5] Histological Technician, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran
[6] Assistant Professor, Department of Medical Physics and Biomedical Engineering, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran; Joint Affiliated with Research Center for Biomedical Technologies and Robotics (RCBTR), Advanced Medical Technologies and Equipment Institute (AMTEI), Tehran University of Medical Sciences, Tehran, Iran
[7] Assistant Professor, Center of Orthopedic Trans-Disciplinary Applied Research (COTAR), Tehran University of Medical Sciences, Tehran, Iran

[*]Corresponding author: Mohammad Hossein Nabian; Center of Orthopedic Trans-Disciplinary Applied Research (COTAR), Tehran University of Medical Sciences, Tehran, Iran. Tel: +98-9126305095, Email: dr.nabian@gmail.com

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Abstract

Background: This study was designed to achieve a new method as a preventive treatment for complications of growth plate fractures. In this study, we investigate the effect of intra-articular injection of anti-vascular endothelial growth factor (anti-VEGF) antibody bevacizumab on the repair process of articular cartilage in a type 4 Salter Harris injury model.
Methods: A Salter Harris injury was created on the proximal tibial growth plate of 14 rats by a 1.8 mm drill. The rats were randomly classified into two groups: group LD, administration of high-dose intra-articular injection of bevacizumab (250 μg), and group HD, administration of low-dose intra-articular injection of bevacizumab (50 μg) after injury. The rats were killed 2 months postoperatively and their tibia underwent micro-computed tomography (CT) analysis, histological assessment, and measurement of tibial bone length.
Results: Bony bar formation was observed in 71% of the samples in the high-dose group and in 100% of the low-dose group. Relative increase in physeal cartilage thickness (P = 0.007) and decrease in bony bar formation (P = 0.029) were observed significantly in the high dose group. There was no significant difference in tibia length between the two groups (P = 0.150).
Conclusion: Intra-articular administration of bevacizumab demonstrated positive restorative effects. We suggest this method of treatment due to its potential of improving cartilage repair and capability to be used as a main or adjacent treatment in osteochondral defects.

Keywords: Salter-Harris Fractures; Growth Plate Fracture; Vascular Endothelial Growth Factors; Bevacizumab; Orthopedics

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Background

Physeal fractures account for about 30% of all fractures in children and result in complications such as growth retardation, physeal bar formation, and angulation deformity (1). Phalanges are the most common site of fractures followed by distal radius, distal tibia, and distal fibula (2).

Several methods can be used to manage physeal injuries, including adipose tissue transplantation, evacuation of the damaged area, stretching the physis, corrective osteotomy, and hemiepiphysiodesis. The success rate of these treatments is between 18% and 36% (3). Common treatment methods are mostly used to repair complications. Recently, the course of studies has shifted to preventive methods to prevent the physeal bar formation.

Bevacizumab promotes cells to differentiate and proliferate into cartilage tissue (4-6). Previous studies showed that vascular endothelial growth factor (VEGF) is absent in the proliferative region of the normal physis (7). The hematoma in the fracture expresses a large amount of VEGF and its specific receptors, R1 and R2 on days 1 to 3 after injury. In the damaged area, collagen type 2, VEGF, and R1 receptor have the highest expression until the 7th day after injury, and vascular growth factor decreases over the 14th day (8). Based on this evidence, Chung and Xian (9) investigated the efficacy of using systemic bevacizumab on cartilage repair in distal tibial injury in rats. They showed the potential role of VEGF as a crucial angiogenic factor implicated in new blood vessel development that was important for the bony restoration of the injured growth plate.

Intravenous administration of bevacizumab has been proven to repair articular cartilage and growth plate cartilage repair (6), however the efficacy of bevacizumab on the growth plate cartilage repair has not been measured. In this experimental study, the aim is to study the efficacy and safety of intra-articular injection of bevacizumab in the distal tibia of rats.

Methods

This study was approved by the Institutional Research Ethics Committee School of Medicine, Tehran University of Medical Sciences (Approval ID: IR.TUMS.CHMC.REC.1398.078) and performed in accordance with the Guidelines on Animal Use of Tehran University of Medical Sciences.

Animal Care Conditions: 14 Wistar rats weighed 100-150

grams were obtained from Pasteur Laboratory of Iran. During the experiment, the rats were kept in the Research Center of Neural Repair (RCNR) in the University of Tehran with a cycle of 12 hours of light and 12 hours of darkness, humidity of 40-60, temperature of 16-21 °C, and in standard cages with free access to water and food.

Study Design: This was a pilot study performed on 14 Wistar rats and designed to estimate the effective and safe dose of intra-articular injection of bevacizumab. The method applied was based on establishing the animal model of type 4 Salter Harris (10) and intra-articular injection of the drug on the first day after surgery. The animals were divided into two groups (7 rats each group); the first group with a dose of 250 µg of bevacizumab (high dose) and the second group with a dose of 50 µg of bevacizumab (low dose). The measurement of the tibia length, micro-computed tomography (CT) analysis, and histological assessment were performed after 60 days.

Salter Harris Injuries Model: A mixture of 10% animal ketamine (80 mg/kg) and 2% xylazine (8 mg/kg) was used intraperitoneally for anesthesia (10). In addition, 0.05 mg/kg bupropion was used intraperitoneally for three consecutive days after surgery to reduce postoperative pain.

The animal model of type 4 Salter Harris was established according to the protocol proposed by Erickson et al. (10). A 3-cm skin incision was made from the medial-anterior aspect of tibia to the medial femoral condyle. The skin and subcutaneous tissue were removed. Knee was flexed to expose tibial tuberosity and growth plate 1 cm below the patella. The muscles on the medial-anterior side of the proximal tibia was dissected. A cortical window was drilled on the exposed aspect of the tibia using a micro drill and a 1.8 mm Steinmann pin (Figure 1). The joint was washed out by intra-articular injection of normal saline. Each animal was injected 2.5 cc of normal saline and 60 mg/kg of cefazolin as postoperative care. The rats were randomly assigned to two groups and were monitored in the recovery room until they regained consciousness.

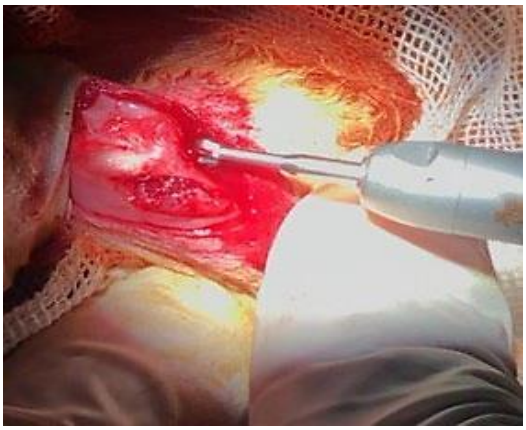

Figure 1. Drilling a cortical window on the medial-anterior aspect of the proximal tibia

Intra-Articular Injection of Bevacizumab: 24 hours after the surgery, the rats were anesthetized again to receive bevacizumab. The lower limb of the rats was placed in a flexion position. Then, they were injected intra-articularly 250 µg of bevacizumab with brand name of Stevant 100/4 mg (Aryogen Pharmaceutical Company, Iran) in the high-dose group and 50 µg in the low-dose group into the patella tendon at a 90-degree angle in the right knee (11) (Figure 2).

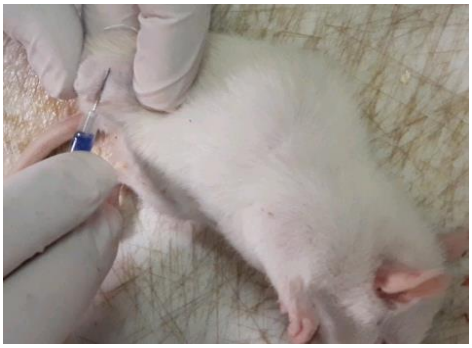

Figure 2. Intra-articular injection of bevacizumab

Measurements of Tibial Length: On the 60th day after the surgery, the rats were euthanized carbon dioxide inhalation. Full length tibia was removed and measured from the knee joint to the ankle joint with a caliper ruler (with an accuracy of 0.02 mm). The harvested tissue was fixed in the 10% neutral buffered formalin (NBF, PH. 7.26).

Micro CT: In this study, we used an in vivo X-ray micro-CT scanner (LOTUS-in Vivo, Behin Negareh Co., Tehran, Iran) (12). LOTUS-in Vivo has a cone beam micro-focus X-ray source and a flat panel detector. In order to obtain the best possible image quality, the X-ray tube voltage and its current were set to 80 kV and 105 µA, respectively, and frame exposure time set to 2 seconds. The total scan duration was 28 minutes and the slice thicknesses of the reconstructed images was set to 50 µm. All the protocol settings process was controlled by the LOTUS-inVivo-ACQ software. The acquired three-dimensional (3D) data was reconstructed using LOTUS in Vivo-REC by a standard Feldkamp, Davis, Kress (FDK) algorithm (13). In the micro-CT images from the physeal cartilage and its surrounding area, the diameter of the physis in the injured area, the diameter of the physis in the growth plate, the bone repair rate, and the density of the bone tissue were measured.

Histopathology: The animals were euthanized 60 days after the operation and the harvested tissue (implanted site) was fixed in the 10% NBF (PH. 7.26) for 48 hours. The harvested samples were decalcified using 10% ethylenediaminetetraacetic acid (EDTA) for 28 days, then processed and embedded in paraffin. The 5-µm thick sections were prepared and stained with hemtoxylin and eosin (H&E) and Masson's trichrome (MT). The histological slides were evaluated by the independent reviewer, using light microscopy (Olympus BX51, Olympus, Tokyo, Japan). The growth plate of each slide was classified as normal (no disruption) or physeal defects (bone/cartilage). The images of the slides were assessed using computer software Image-Pro Plus® V.6 (Media Cybernetics Inc., Silver Spring, USA).

Statistical Analysis: The statistical analysis was performed using SPSS software (version 20, IBM Corporation, Armonk, NY, USA). The comparisons between the low dose and high dose groups were performed using independent sample t-test. The level of significance was set to less than 0.05.

Results

All of the 14 rats were survived for two months and no complications were observed.

Tibial Length Measurement: The mean tibial length was 32.88 mm in the high dose group and 32.83 mm in the low dose group, with no significant difference between the two groups (P = 0.150).

Micro-CT: On the 60th day after the injury, the bone tissues were examined. Bone trabeculae were formed in 100% of the samples in the low-dose group, however in the high dose group, they were formed in 71% of the samples. Two of the high dose group samples did not form trabeculae in 60 days, which was not significant (P = 0.120).

The physis diameter at the injury site was significantly greater in the high dose group (P = 0.004) than the low dose group (Figure 3). The diameter of the growth plate was significantly higher in the high dose group (P = 0.007). There was no significant difference between the two groups in terms of bone fusion, bone density, and bone repair.

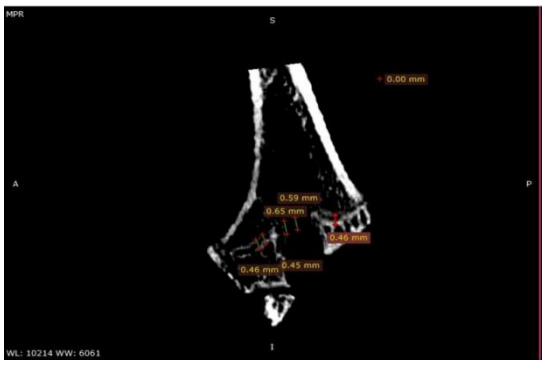

Figure 3. Micro-computed tomography (CT) image of a tibia bone sample from high dose group

Histology: New bone formation was seen in both groups. The defect area was completely filled with bony tissue in the low-dose group. The mean size of the destructed physis was 2.3 ± 0.2 mm in the low dose group and 1.2 ± 0.2 mm in the high dose group. Stained images showed osteochondral defects in the high-dose group and the amount of new bone formation was significantly lower in the high dose group compared to the low dose group (P = 0.029) (Figure 4). In general, this treatment does not show any side effects or severe inflammatory reactions and the higher-dose group showed a greater protective effect on the growth plate after the Salter Harris fracture.

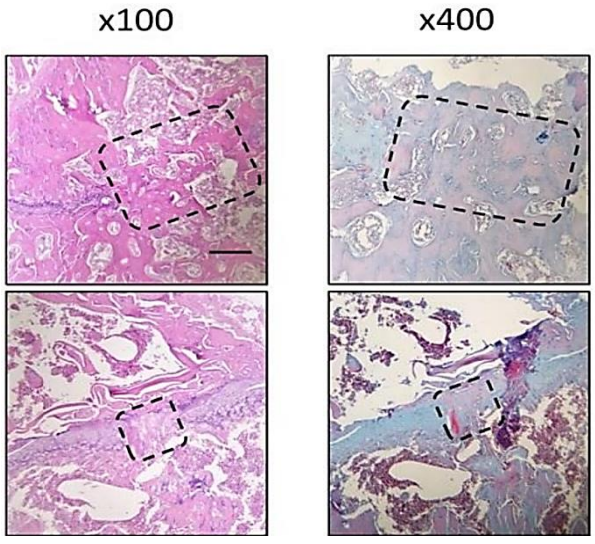

Figure 4. Histological findings of osteochondral defect; Dotted lines: Disruption of the growth plate, hemtoxylin and eosin (H&E) and Masson's trichrome (MT) staining. In the upper row images, the level of the physis in the lower dose group shows that most areas are filled with trabeculae. Lower row images show physis and the regular cartilaginous layer of the growth plate in the higher dose group.

Discussion

In this pilot study, animal model of type 4 Salter Harris was established following intra-articular injection of bevacizumab 24 hours later. They were randomly divided into the two high-dose group receiving 250 µg and low-dose group receiving 50 µg. All the rats survived for two months and none of them showed any complications. This study estimated the effect of bevacizumab in local administration by micro-CT and general and specific histological assessments and limb length measurements. The results showed reduced incidence of physeal bar formation, decreased bone bar volume, and increased growth plate thickness in the high dose group.

Due to the common side effects of systemic injection and the limitation in increasing the dose of the drug in this type of injection, we decided to conduct a study with local injection. It is noteworthy that due to the lack of access to the effective dose range in local injection and its safety and efficacy in local injection, the need for a pilot study was obvious, resulting in the present study.

Previous studies demonstrated the effect of intravenous administration of bevacizumab on the repair of cartilage defect (6). In a similar study, intraperitoneal injections of bevacizumab resulted in a bone bar formation in all samples, but the ossification rate was significantly reduced in the treatment group (4). The average bone length was reduced compared to the control group.

The micro-CT assessment results in our study showed that increasing the dose of the bevacizumab decreased the incidence and volume of bone bar formation, which is consistent with the findings of previous studies (9). Moreover, the relationship between the physeal thickness and drug dose was assessed in the current study, indicating increment in the bevacizumab dose, which led to the increased physeal thickness both in the injured area and the uninjured area, which is similar to the study conducted by Nagai et al. (6). In this study, 20 18-week-old Japanese white rabbits weighing approximately 3 kg were divided into two groups after surgical induction of the cartilage defect model; the intervention group (treated with 100 mg intravenous bevacizumab in two doses; the first dose on the first day after injury and, the second dose on the 14th day after injury) and the control group. The results demonstrated that the intravenous administration of bevacizumab helps to better articular cartilage repair (6). However, in the present study, bevacizumab was administered locally and the results were the same as those for the systemic use in the cartilage repair.

Previous studies revealed that the evidence for the use of VEGF in bone formation was effective (14). However, in our study groups, due to the fivefold increased dose of anti-VEGF, no significant difference was observed between the two groups in terms of bone repair.

In this study, no significant relationship was observed between increasing the dose and increasing the lack of bone repair between the two groups. However, the healing process in both groups was longer than the healing process mentioned in previous articles (6, 15, 16). Evaluation of the course of bone bar formation in previous studies showed that on the 14th day, the small bony trabeculae and on the 28th day, the bone tissue replaced the injured area, and the complete healing of the bone occurred on the 60th day. However, in our study, on the 60th day after the injury, complete repair of the bony tissue was not still observed in the micro-CT images.

On the 60th day, the tibial length was measured, but no

difference in longitudinal growth was observed between the two groups. However, in previous studies, limb length was decreased compared to that of the control group (4).

Unfortunately, due to the existing limitations, including the limited financial resources and sanctions, the study of gene expression, immunohistochemistry, and design of control groups for this study was not possible.

Conclusion

Intra-articular injection of anti-VEGF antibody bevacizumab in a type 4 Salter Harris model results in enhancing the repair process of articular cartilage and reducing bony repair. It can be concluded that this method of treatment could be useful to enhance restorative effects of present regenerative medicine approaches. It is suggested that in another study, while increasing the test groups in different doses, adding the control group, genetic and immunohistochemistry evaluations and increased duration of the study be assessed. To study the bone bar and close the growth plate in the micro-CT images, the possibility of the effect or ineffectiveness of the drug should be expressed with more certainty.

Conflict of Interest

The authors declare no conflict of interest in this study.

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