EXPERIMENTAL STUDY

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The effect of HIF stabilizer on distraction osteogenesis

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Objective: The aim of this study was to investigate the effect of an orally applicable hypoxia-inducible factor (HIF) stabilizer on distraction osteogenesis (DO) in a rat model.

Methods: The study included 24 Wistar albino rats undergoing osteotomy of the left tibia diaphysis. Rats were divided equally into experiment and control groups. Tibias were fixed using an external fixator. HIF stabilizer was administered to the experiment group. On the 5th postoperative day, distraction with increased rate (0.4 mm twice a day) was commenced and continued for 10 days. Histological and immunohistochemical evaluation was performed.

Results: Vascular endothelial growth factor levels of the experiment group were higher than those of the control group ($p<0.05$). The experiment group had slightly better intramembranous ossification quality than the control group on both Day 16 and 30. Endochondral ossification rates were better in the experiment group on Day 16.

Conclusion: Vascular endothelial growth factor levels increased and stimulated angiogenesis in the presence of HIF pathway activation by oral administration of HIF stabilizer during DO. The biomechanical features of the distraction and angiogenesis should be coupled to achieve adequate bone homeostasis.

Key words: Angiogenesis; distraction osteogenesis; HIF stabilizer; VEGF.

Bone regeneration and angiogenesis are coupled during skeletal development and fracture healing. Angiogenic factors and hypoxia have been shown to be necessary for appropriate bone regeneration and healing processes.[1,2] Distraction osteogenesis (DO) is an effective choice of surgery for many orthopedic disorders including congenital growth disturbance, posttraumatic bone deficiency, etc. Remodeling of preexisting vascular structures (angiogenesis) and new formation of vessels (vasculogenesis) are of the utmost importance for bone regeneration.[3,4] Angiogenesis and vasculogenesis are stimulated by complex molecular reactions. Hypoxia, another well-known angiogenic stimulator, enhances the expression of vascular endothelial growth factor (VEGF) through the mechanism which stabilizes the transcription factor hypoxia-inducible factor-1 (HIF-1). Vascular endothelial growth factor promotes blood vessel invasion in the newly formed bone regions.

Osteoblasts express increased levels of VEGF under hypoxic conditions. FG-2216 (butyl 10-undecenoate; formula: $C_{15}H_{28}O_2$) with a molecular weight of 240.38g/mol. functions as a prolyl-hydroxylase inhibitor and stabilizes HIF to achieve an increase in VEGF expression through HIF dependent pathway.

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Our study aimed to investigate the effect of an orally applicable HIF stabilizer (FG-2216) on a rat model with an increased DO rate.

Patients and Methods

Approval was obtained by the Uludağ University Animal Experimentation Ethics Committee (Report no: 2012-03/02). The study included 24 Wistar albino rats weighing between 250 to 400 g randomly divided into experiment and control groups. The left tibia midshaft of all rats were osteotomized and fixed with mini external fixators. Beginning on the 5th postoperative day, distraction of 0.4 mm was performed twice a day for 10 days.

The experiment group received 60 mg/kg/day butyl 10-undecenoate (FG-2216®; Sigma-Aldrich Corp., St. Louis, MO, USA) administration orally during the experiment period. Six rats from each group were sacrificed on the 16th and 30th days and tissue samples from the distraction gap were taken for histological and immunohistochemical evaluation. Equal rhythms of distraction were performed for both groups.

12 mg/kg 2% xylazine hydrochloride (Rompun®, Bayer HealthCare) and 80mg/kg ketamine hydrochloride (Ketalar®; Pfizer Inc., New York City, NY, USA) were applied intramuscularly for general anesthesia. Surgical fields were prepared using a 10% povidone iodine solution and covered with sterile drapes. Tibias were percutaneously pinned proximally and distally with 0.8 mm pins on each side and were transversely osteotomized through a longitudinal incision. Irrigation was done with physiological saline and skin was closed using 4/0 non-absorbable nylon monofilament sutures. Proximal and distal pins were fixed to the fixator. Rats were kept in separate cages and allowed independent cage activity. Paracetamol at a dose of 1 to 2 mg/kg/day was mixed with 100 ml of drinking water for early postoperative pain control. Animals were followed with daily wound care.

Samples taken from the distraction gap were fixed with 10% formaldehyde solution and decalcified in 10% formic acid. After dehydration and paraffin embedding, sections of 4 μm were taken and stained with hematoxylin and eosin (HE). Immunohistochemical staining protocol for VEGF was performed to demonstrate angiogenesis by using VEGF antibody (GeneTex®; GeneTex Inc., Irvine, CA, USA). Vascular endothelial growth factor scores were set according to the staining rate of the osteoblasts and osteocytes in the distraction gap. Scores were expressed as follows; 0 for none stained, 1 for 1 to 25% stained, 2 for 26 to 50% stained and 3 for more than 50% stained.

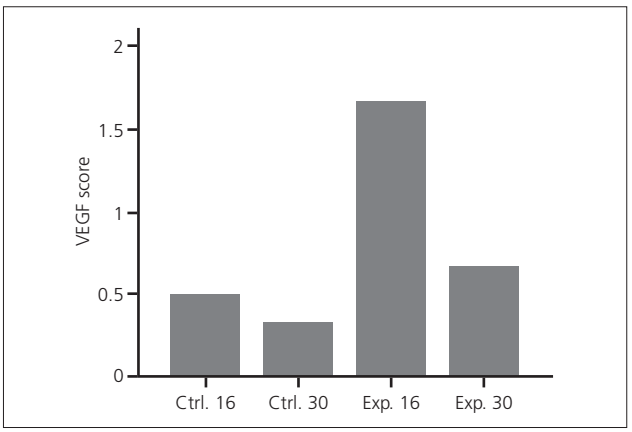

Fig. 1. Mean VEGF scores of both groups.

The IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA) software was used for statistical analysis of the research. The Pearson chi-square and Fisher's exact tests were used for comparison and values of $p<0.05$ were identified as statistically significant.

Results

No adverse effect was caused by oral administration of FG-2216. Two rats with pin tract infection were followed with daily wound care until the end of the experiment.

Intramembranous ossification was detected on Day 16 in 5 rats (83%) in the experiment group and 3 rats (50%) in the control group and on Day 30 in 4 rats (67%) in the experiment and 3 rats (50%) in the control group. Endochondral ossification was detected on Day 16 in 2 rats (33%) in the experiment group and in 1 (16%) in the control group and on Day 30 in 2 rats (33%) in the experiment group and 3 (50%) in the control group. Mean Huddlestone fusion score[5] was 6.5 in the experiment group and 5.16 in the control group. On Day 30, mean fusion score was the same in both groups.[5,33] Mean VEGF score was 1.67 and 0.5 on Day 16 and 0.7 and 0.3 on Day 30 for the experiment and control groups, respectively. Although mean VEGF scores were higher in the experiment group on both days, values were only significant for Day 16 ($p<0.05$) (Fig. 1, Table 1).

Discussion

Our study aimed to show the direct effects of an orally usable HIF stabilizer in a distraction osteogenesis model for the first time. In comparison with other studies on genetic manipulation and local application, our study presents a non-invasive chemical agent to stimulate angiogenesis. The administration of oral agents such as FG-2216 in DO will be of importance in the future. FG 2216 has previously been used for the treatment of anemia. Bern-

Table 1. Comparison of VEGF scores on days 16 and 30.

Rat	Control				Experiment			
	Day 16		Day 30		Day 16		Day 30	
	Score	Mean	Score	Mean	Score	Mean	Score	Mean
1.	1	0.5	1	0.3	1	1.7	1	0.67
2.	0		0		2		1	
3.	1		0		2		1	
4.	0		0		1		0	
5.	1		0		2		0	
6.	0		1		2		1	

hardt et al. administered oral FG-2216 to patients with chronic renal failure (CRF) and healthy volunteers and found increases of 30.8 times in erythropoietin (EPO) levels in the CRF group and 12.7 times in the volunteer group.[6] FG-2216 was also shown to provide a specific prolyl-hydroxylase inhibition and was thus proposed as a new treatment option to preserve myocardial function following elevation of HIF levels in myocardial infarction.[7] In another study, a prolyl-hydroxylase inhibitor was reported to decrease the proliferation of cancer cells and act as a neuroprotective agent.[8]

Previous studies have reported a broad spectrum of effective agents such as bisphosphonates, pamidronic acid, zoledronic acid, 25-dihydroxyvitamin D3, growth factor, fibroblast growth factor-b and insulin-like growth factor.[9-14] In addition, other studies have shown successful results in such agents' promotion of bone healing through transplantation of osteoblast to callus[15] and the use of demineralized bone matrix as a graft in DO models.

The increased VEGF activity in the experiment group revealed the effect of FG-2216. Vascular endothelial growth factor scores were better just after the termination of distraction as the distraction phase is considered the dominant period for angiogenesis in the distraction gap in correlation with new bone formation. Our results suggest that the application of a periodic HIF stabilizer may shorten the total distraction time. In addition, activation of VEGF through the HIF pathway includes EPO stimulation which might act as an indirect blood perfusion accelerator on the distraction gap leading to better conditions for new cell recruitment. Wan et al. reported loss of bone volume in mice without HIF as the upper levels of the angiogenesis pathway is dominated by HIF.[16] In addition, local administration of VEGF was found to be effective for angiogenesis in some animal fracture healing and bone deficiency models. Fassbender et al. used the angiogenesis inhibitor fumagillin in a study of DO in rats and reported a remarkable distraction gap at the osteotomy level that had gone to atrophic nonunion in 84 days.[17]

Although the stimulation of proliferative and biosynthetic cell functions by a mechanotransduction phenomenon was not clearly understood, it has been accepted that the longer the mechanical stress, the stronger the signal production for new bone formation. Distraction rate and frequency are important at this point. The microenvironment of the distraction gap would be maintained under conditions where frequency is increased and the rate is kept constant as increasing the frequency of mechanical stimulation will induce new bone and vessel formation.[18] We preferred a distraction rate of 0.8 mm/day which is slightly higher than the usual rate in rats. There are studies about the distraction rate and rhythm but none have described an optimum value for practice in rodents. Paccione et al. reported that distraction of 1 mm/day in rat mandibula resulted in poor angiogenesis and bone regeneration while a distraction rate of 0.5 mm/day showed excellent results.[19] Moreover, it was shown that rapid distraction led to poor angiogenesis in a rabbit DO model.[20]

Clinical and experimental studies have shown that damage to the periosteum, new developing vessels and bone regeneration resulting in delayed union and nonunion may be caused by distraction of more than 1 mm/day.[19] We detected increased VEGF activation due to the administration of FG-2216 but also noticed that activation of VEGF by the HIF stabilizer could not balance the lack of appropriate biomechanical conditions affecting DO. What we set up for distraction rhythm affected the microenvironment negatively and led to poor bone formation by creating a general blockage. However, we detected increased VEGF activity in the distraction gap. We believe that the low quality of ossification and

high rate of distraction gap encountered in our DO model were related to the increased distraction rate due to the early progression of poorly organized granulation tissue with little blood supply caused by fast distraction rates.[20] As distraction continues, the granulation tissue, fibrocartilage and cysts fill up the distraction gap and relatively less bone formation is observed. Decreased osteoblast activity and the presence of cartilage and cysts intervene with bone formation in the distraction gap result in nonunion.[21-23] Every periodic attempt at distraction creates tension on the osteotomized sides of the bone and produces mechanical stress on the regenerate. It can be considered that increasing the frequency with a constant daily distraction rate would maintain a stable tension around the gap, creating less damage to the soft tissue surrounding the regenerate. In this study, the use of a fast distraction rate with low frequency (twice a day) altered the mechanical microenvironment and caused negative effects on the regenerate.

In conclusion, in the presence of FG-2216, VEGF levels tend to increase through the HIF pathway in order to stimulate angiogenesis. Vascular endothelial growth factor stimulation alone could not balance the negative biochemical conditions created by fast distraction, resulting in poor new bone formation. The stimulation of VEGF activity in distraction osteogenesis using an oral agent is encouraging for clinical procedures of extremity lengthening surgery in the future. New studies are required to investigate the interactions between the stimulation of angiogenesis and different biomechanical conditions in experimental DO models.

Conflicts of Interest: No conflicts declared.

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