

## Effect of Pentoxifylline Administration on an Experimental Rat Model of Femur Fracture Healing With Intramedullary Fixation

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### Abstract

**Background:** Globally, musculoskeletal injuries comprise a major public health problem that contributes to a large burden of disability and suffering. Pentoxifylline (PTX) has been originally used as a hemorheologic drug to treat intermittent claudication. Previous test tube and in vivo studies reported the beneficial effects of PTX on bony tissue.

**Objectives:** This study aims to evaluate the effects of different dosages of PTX on biomechanical properties that occur during the late phase of the fracture healing process following a complete femoral osteotomy in a rat model. We applied intramedullary pin fixation as the treatment of choice.

**Materials and Methods:** This experimental study was conducted at the Shahid Beheshti University of Medical Sciences, Tehran, Iran. We used the simple random technique to divide 35 female rats into five groups. Group 1 received intraperitoneal (i.p.) PTX (50 mg/kg, once daily) injections, starting 15 days before surgery, and group 2, group 3, and group 4 received 50 mg/kg, 100 mg/kg, and 200 mg/kg i.p. PTX injections, respectively, once daily after surgery. All animals across groups received treatment for six weeks (until sacrificed). Complete surgical transverse osteotomy was performed in the right femur of all rats. At six weeks after surgery, the femurs were subjected to a three-point bending test.

**Results:** Daily administration of 50 mg/kg PTX (groups 1 and 2) decreased the high stress load in repairing osteotomized femurs when compared with the control group. The highest dose of PTX (200 mg/kg) significantly increased the high stress load when compared with the control group ( $P = 0.030$ ), group 1 ( $P = 0.023$ ), group 2 ( $P = 0.008$ ), and group 3 ( $P = 0.010$ ), per the LSD findings.

**Conclusions:** Treatment with 200 mg/kg PTX accelerated fracture healing when compared with the control group.

**Keywords:** Pentoxifylline, Mechanical Phenomena, Biomechanics, Rats, Femoral Fractures

## 1. Background

Globally, musculoskeletal injuries comprise a major public health problem that contributes to a large burden of disability and suffering (1). The results from a US analysis of long-term follow-up data of a large population-based cohort have shown increased mortality long after the occurrence of fractures in adults (2). Donaldson et al. reported that fractures in England had an overall annual incidence rate of 3.6% (3). Therefore, treatment modalities that potentially augment the healing process might help in decreasing the rate of complications in the orthopedic population.

Pentoxifylline (PTX) was originally used as a hemorheologic drug to treat intermittent claudication, with mi-

nor side effects. Compared with other pharmaceutical agents, PTX is relatively inexpensive and has fewer side effects in the gastrointestinal tract (4). Previous in vitro and in vivo studies have reported positive effects of PTX on bony tissue (5, 6).

Recent studies reported the following positive effects of PTX including decrease in arthritis and associated periodontal co-morbidity in mice (7), relief of symptoms in chronic multifocal osteomyelitis, and management of osteoradionecrosis of the jaw (8). PTX has been used as a novel treatment for osteoradionecrosis of the temporal bone (9) and as a successful treatment of early zoledronic acid-related osteonecrosis of the jaw in cor-

ticosteroid-induced osteoporosis (OP) (10). This medication also had a protective effect on the growth plate in neonatal rats following long-term phototherapy (11), as well as resolution of pain and complete healing of mandibular osteoradionecrosis (12).

However, only a few studies have elucidated the effects of PTX on fracture healing (13-16). Bese et al. presented the case of a 63-year-old man with pelvic insufficiency fractures due to postoperative pelvic irradiation (radiotherapy) for rectal adenocarcinoma. He received treatment with PTX (400 mg) three times per day, and showed dramatic clinical improvement within six months and objective healing according to magnetic resonance imaging (MRI) results (13). Wei et al. reported the positive effect of PTX administration on fracture healing (14). Aydin et al. (15) investigated the effect of PTX on early stage fracture healing in an experimental animal model. In their study, radiological evaluation of the callus showed no significant differences between the control and PTX groups during the first, second, and third weeks, but at the end of the first week, histological callus formation was significantly more in the PTX group than in the control group. Aydin et al. (15) stated, however, that lack of biomechanical studies in their evaluations could be a limitation. Erken et al. (16) evaluated the effect of PTX on spinal fusion in a rabbit model. In their study, all rabbits received a single-level posterolateral, inter-transverse process fusion with an autologous iliac crest. The experimental rabbits were treated with intravenous PTX treatment at a dose of 100 mg/kg/day postoperatively, whereas the control rabbits received no PTX treatment. At nine weeks after surgery, their spines were tested by a manual palpation test, biomechanical testing, plain radiography, computed tomography (CT) scans, and histomorphometric analysis. The experimental group had significantly better results in most evaluations when compared with the control group. Erken et al. suggested that PTX might have a beneficial effect on spinal fusion (16).

Considering the positive cellular effects of PTX reported in previous *in vitro* and *in vivo* experiments, we propose that administration of PTX following a fracture may accelerate the healing process.

## 2. Objectives

This study aims to evaluate the effects of different dosages of PTX on femur fracture (complete osteotomy) with intramedullary fixation in an experimental rat model. We assessed the late phase of fracture healing (catabolic phase) with a biomechanical test. According to a literature review, such a study of the effects of PTX on rat femur fractures has not been performed to date. This study will provide further evidence on the probable positive effects of PTX and its application in fracture healing.

## 3. Materials and Methods

### 3.1. Animals and Study Design

This experimental study was conducted at the Shahid Beheshti University of Medical Sciences, Tehran, Iran. A total of 35 adult (three-month-old) female Wistar rats that weighed approximately 190 grams were obtained from the animal house at Pasteur Institute, Tehran, Iran. Animals were given a standard diet and tap water *ad libitum*, and housed in individual clean cages kept in a temperature-controlled room ( $23 \pm 1^\circ\text{C}$ ) with a 12:12 hours light/dark cycle. All procedures were approved by the medical ethics committee of the Shahid Beheshti University of Medical Sciences (protocol no. 1392-1-115-1159). Data were gathered in the research laboratory of the department of anatomy and biology at the Shahid Beheshti University of Medical Sciences during 2014.

The rats were divided into five groups of seven rats each by a simple random technique. The sample size for comparing five groups was based on a simple linear nomogram introduced by Day and Graham, with an effective and conservative size of 5.0, an approximate power of 0.9, and a significance level of 0.05 (17).

All tests and measurements were performed in a single-blind fashion following a standardized protocol. The five groups consisted of a vehicle/control group and four experimental groups. The vehicle/control group did not receive PTX. Group 1, as the first experimental group, received intraperitoneal (i.p.) injections of 50 mg/kg PTX (Sigma-Aldrich, St. Louis, MO, USA) once daily beginning 15 days before surgery. Group 2 received 50 mg/kg i.p. PTX immediately after surgery. Group 3 received a total daily dose of 100 mg/kg i.p. PTX (50 mg/kg at 9 a.m. and 5 p.m.) after surgery. Group 4 received a total daily dose of 200 mg/kg i.p. PTX (100 mg/kg at 9 a.m. and 5 p.m.) after surgery. The treatment continued for six weeks after surgery for all animals until killed. Complete transverse osteotomy of the right femur was performed for all animals.

### 3.2. Femoral Fracture Model (Complete Transverse Osteotomy)

Rats were anesthetized with 50 mg/kg ketamine hydrochloride (Rotex Medica, Tritteu, Germany) as an intramuscular injection along with 5 mg/kg diazepam (Jaber Ben Hayan Co., Tehran, Iran). After aseptic preparation with povidone iodine (Behvazan Co., Rasht, Iran), a 1-cm incision was made over the lateral aspect of the right thigh to expose the femur. First, we generated three to five partial transversal standardized osteotomies, circular deep to the central medullary canal on the midpoint of the femur. Osteotomies were made with a low speed drill (terminal, 1.0-mm diameter; Delab; Dental Fabrik-treffurt, Germany), after which the osteotomy site was broken manually and the bones were divided into two

parts. During the osteotomy procedure, the bones were irrigated with saline solution to avoid burning. An intramedullary fixation was performed using a 1.0-mm diameter stainless wire (Orthotec Co., Tehran, Iran). The fracture fragments were contacted and stabilized. A 3-mm gap between the edges of the fractures was constant for all rats. Wires were cut on the surface of the intercondylar groove of the femur to avoid restriction of motion at the knee joint. The muscles were sutured with 04 catgut (Supa, Tehran, Iran) and the skin with 04 nylon reversed cutting sutures (Figure 1). Animals received 50 mg/kg of ceftrax (Jaber ben Hayan, Co.) as antibiotic therapy before surgery, and at 24 and 48 hours after surgery. The animals were allowed unrestricted activity after recovery from anesthesia.

### 3.3. Biomechanical Examination

At six weeks after surgery, all rats were killed by inhalation of chloroform (Merck Co., Germany) in a closed space. Right femurs were collected, wrapped in gauze previously soaked in physiologically balanced saline, and frozen at  $-20^{\circ}\text{C}$  for biomechanical testing. The specimens were slowly thawed at room temperature and kept moist throughout the handling and testing procedures.

We examined the biomechanical properties of the five bones from the groups. Bones were subjected to three-point bending on a material testing device (Zwick/Roell Z 2.5 H 15WN, Ulm, Germany) until they fractured. We performed biomechanical testing as follows. All bones were oriented similarly in the testing machine. Two loading points, 19-mm apart, were used to mount each bone and a press head was activated to compress the midline of the bone shaft until the fracture occurred. The compressive loading speed was 0.08 mm/s for the tests. Data were automatically recorded by the material testing device from the load-deformation curve and the values of the following biomechanical parameters were calculated: bending stiffness (N/mm), maximum force (N), high stress load ( $\text{N}/\text{mm}^2$ ), and energy absorption (N/mm).

Bending stiffness is the slope of linear proportion in the load-deformation curve and the ratio of loading deformation in the elastic region of the curve. Maximum force is the force needed to break a bone. High stress load is calculated by dividing the maximum force value by the surface area ( $\text{mm}^2$ ) of the bone at the osteotomy site. Of note, the transverse section of the bone was approximately triangular; therefore, we measured its height and breadth with a micrometer. In addition, we calculated the surface area. Energy absorption was defined as the amount of energy absorbed by the bone until breakage (18).

### 3.4. Clinical Observations

Rats were observed daily. We measured the body weights of animals in all groups at the beginning and end of the study with a fine balance (Pars Khzar Ind. Co., Rasht, Iran).

The presence or absence of a solid union at the osteotomy site was confirmed by manual palpation performed by a reviewer blinded to the group assignments at the time the animals were killed.

### 3.5. Statistical Analysis

All data are expressed as mean  $\pm$  standard error of mean (SEM) and standard deviation (SD).

Normal distributions of data were analyzed by the one-sample Kolmogorov-Smirnov test. The differences between treatment groups were tested by one-way analysis of variance (ANOVA). If significant differences were indicated, the differences between these two groups were tested by the least significant difference (LSD). A P value of  $<0.05$  was considered statistically significant. In addition to estimating the classical mean, SEM, and standard deviations, we also obtained bootstrap corresponding estimations using the bootstrap method of resample size 100.

## 4. Results

### 4.1. General Observations

There were no adverse effects, such as oral hemorrhage, vomiting, diarrhea, or dysentery, in any rat. We excluded 10 rats in total owing to poor fracture healing (non-union) or death after surgery. ANOVA showed no significant differences in body weight among the groups at the beginning and end of the study (Figure 2). However, at the end of the study, the body weight of animals in all groups increased when compared with the corresponding weight at the beginning of the study. The paired t-test showed significant increases in group 1 ( $P = 0.01$ ), group 2 ( $P = 0.023$ ), and group 4 ( $P = 0.047$ ).

### 4.2. Biomechanical Results

ANOVA showed significant differences in the biomechanical parameters across groups (Figure 3).

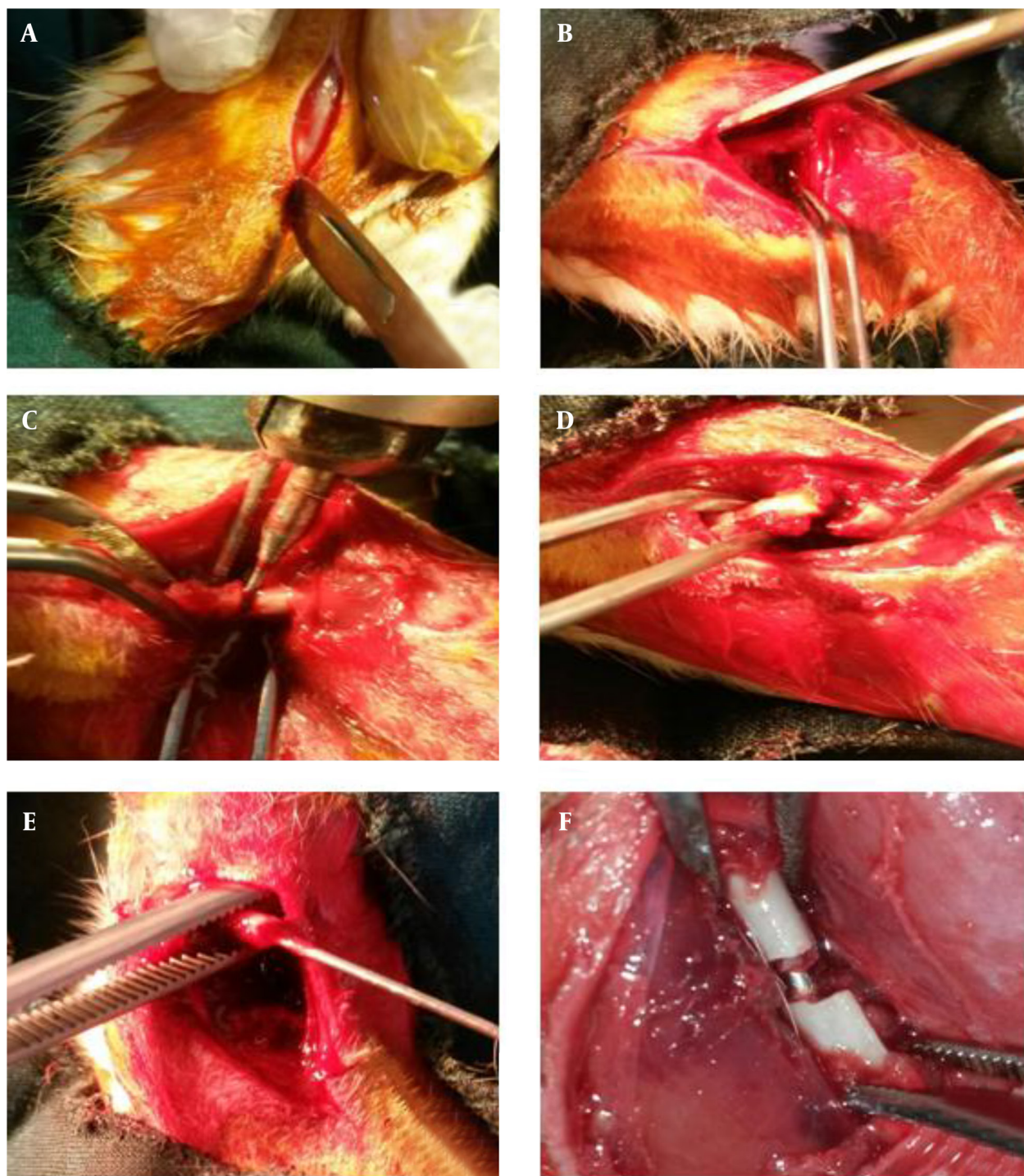
#### 4.2.1. Bending Stiffness (N/mm)

The group 1 that received 50 mg/kg of PTX had reduced bending stiffness compared with the control group. There was a significant increase in bending stiffness in group 4 (200 mg/kg) when compared with the control group, group 1, group 2, and group 3 (LSD test;  $P = 0.005$ ,  $P = 0.003$ , and  $P = 0.007$ , respectively).

#### 4.2.2. Maximum Force (N)

We observed decreased maximum force in group 1 and group 2 when compared with the control group. The maximum force was greater in group 4 when compared with group 2 and group 3 (LSD test;  $P = 0.036$ ,  $P = 0.010$ , and  $P = 0.010$ , respectively).

Figure 1. Different Steps of the Complete Osteotomy Operation



A, incision at the exposed femur's mid shaft; B, circular partial transversal standardized osteotomies; C, procedure with a low speed drill; D, complete fracture in the bone; E, insertion of a stainless wire; and F, reduction maintained with the stainless wire.

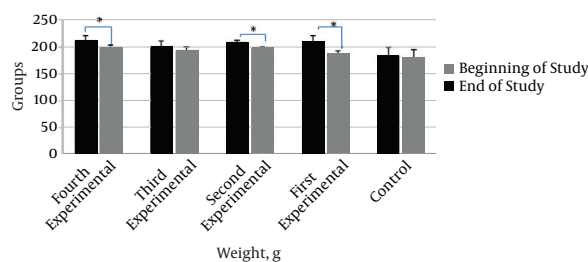
### 4.2.3. High Stress Load (N/mm<sup>2</sup>)

High stress load was lower in group 1 and group 2 than in the control group. However, group 4 (200 mg/kg PTX) showed a significantly increase in the high stress load when compared with the control group (P = 0.030), group 1 (P = 0.023), group 2 (P = 0.008), and group 3 (P = 0.010), per the LSD findings.

### 4.2.4. Energy Absorption (N/mm)

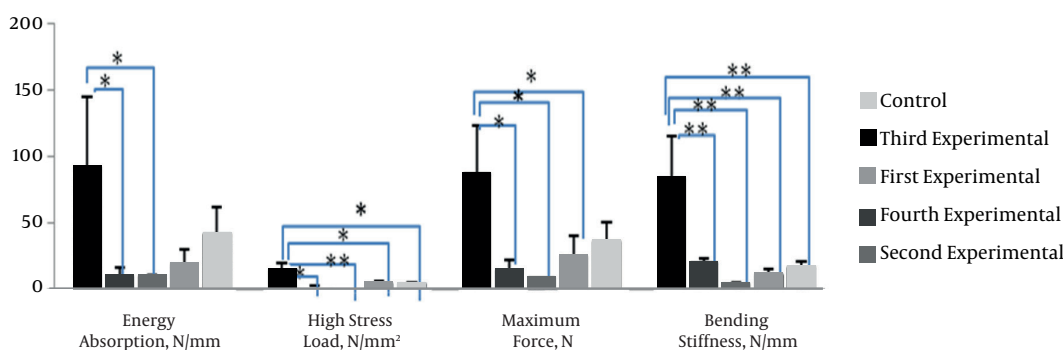
We observed decreased energy absorption in group 1 and group 2 when compared with the control group. There was increased energy absorption in group 4 when compared with group 2 and group 3 (LSD test; P = 0.039 for both) (Table 1).

**Figure 2.** Mean ± SEM for the Weight of Rats of Groups 1- 4



ANOVA revealed no significant differences in body weight across groups at the beginning and end of the study.

**Figure 3.** Mean ± SEM of Biomechanical Properties in the Five Study Groups



There were significant increases in the biomechanical properties in group 4 when compared with the other groups.

**Table 1.** Mean ± SD of Biomechanical Properties in the Five Rat Groups and Mean ± SEM Estimated by the Bootstrap Method<sup>a,b</sup>

Variables	Group 1 Control	Group 2 PTX, BS, 50 mg/kg	Group 3 PTX, S, 50 mg/kg	Group 4 PTX, 100 mg/kg	Group 5 PTX, 200 mg/kg
<b>Bending stiffness, N/mm</b>					
Mean ± SD	16.6 ± 10	10.3 ± 11	4 ± 1.6	18.9 ± 11	83.1 ± 72.4
Bootstrap mean ± SEM	16.6 ± 3.9	10.3 ± 4.1	4 ± 0.6	18.9 ± 4.6	83.1 ± 30.7
<b>Maximum force, N</b>					
Mean ± SD	35.7 ± 34	24.4 ± 36	7.8 ± 1.9	13.7 ± 17.7	86.5 ± 82.1
Bootstrap mean ± SEM	35.7 ± 11.3	24.4 ± 12.6	7.8 ± 0.2	13.7 ± 6.5	86.5 ± 33.8
<b>High stress load, N/mm<sup>2</sup></b>					
Mean ± SD	4 ± 3.7	3.4 ± 5.3	1.2 ± 0.32	1.8 ± 2.3	14.2 ± 13.9
Bootstrap mean ± SEM	4 ± 1.2	3.4 ± 1.9	1.2 ± 0.1	1.8 ± 0.8	14.2 ± 5.8
<b>Energy absorption, N/mm</b>					
Mean ± SD	41 ± 47.2	18.8 ± 25	8.9 ± 4.8	9.1 ± 16.4	91.3 ± 118.9
Bootstrap mean ± SEM	41 ± 16.1	18.8 ± 8.6	8.9 ± 2.0	9.1 ± 7.0	91.3 ± 46.1

<sup>a</sup>Abbreviations: BS, before surgery; PTX, pentoxifylline; S, surgery.

<sup>b</sup>There are no differences in mean ± SD values between the routine method and bootstrap method. Significant differences are shown in Figure 3.

## 5. Discussion

Our results indicate that repairing osteotomy femurs from rats treated with 50 mg/kg of PTX had decreased biomechanical properties when compared with the control group. However, the high dose of PTX, i.e., 200 mg/kg, significantly increased the biomechanical properties in repairing osteotomized femurs when compared with the control group.

Bone regeneration is a complex, well-orchestrated physiological process during bone formation, which can be seen during normal fracture healing. Regeneration is involved in continuous remodeling throughout the adult life (19). The fracture repair process involves the interaction of numerous molecular factors, cell lineages, and tissue types (20). The process is biochemically complex and energy dependent (21). These biological processes allow for an impressive feat of engineering: an elastic soft callus is progressively replaced by a more rigid and mineralized callus. During this reparative phase, the healing process is exposed to the risk of re-fracture (20).

The mechanism of action of PTX in bone formation is an important area of research. Thus far, the precise roles of PTX in bone remodeling are not fully understood. A number of previous studies have shown positive effects of PTX administration in bony tissue *in vitro* (5, 6, 22). Tsutsumimoto et al. observed that PTX enhanced BMP-4-induced chondro/osteogenic differentiation in C3H10T1/2 and ST2 cells. They observed similar effects when dibutyryl- cyclic adenosine monophosphate (cAMP) and forskolin were added. The results indicated that cAMP might potentiate the action of BMP-4 on osteoprogenitor cells, and highlighted the possibility that phosphodiesterase inhibitors could be used as therapeutic agents to enhance bone formation through this effect (5). Kinoshita et al. treated vertebrae and tibiae from mice with PTX or rolipram. Analyses of bone densitometry and bone histomorphometry showed that both PTX and rolipram increased bone mass in normal mice, mainly through the acceleration of bone formation (6). Yao et al. designed a study to determine whether rolipram (a selective phosphodiesterase inhibitor) could prevent and restore bone loss in ovariectomy-induced OP in rats. These rats were treated with vehicle, PGE-2, alendronate, or rolipram. Certain doses of rolipram prevented OP, whereas other doses restored ovariectomy-induced cancellous and cortical bone loss in the long bones and lumbar vertebra. Dynamic bone histomorphometry suggested that these beneficial effects were achieved by partial maintenance of the elevated bone formation; furthermore, it reduced bone turnover (22).

The bio-stimulatory effects of PTX have numerous potential clinical applications and this is a medical research topic of interest. OP is the most common bone disease that results in increased fractures among elderly people (23). It is well known that osteoporotic fractures decrease quality of life and increase mortality rates in the older population (24). However, currently, there are few effec-

tive therapies and medications available for long-term treatment and prevention of this chronic disease (25). PTX administration can promote bone formation and inhibit bone resorption, thus facilitating bone remodeling. This technique may be a potential therapy for the treatment of OP in patients.

PTX administration in bone can reduce osteoclast activation, increase osteoblast differentiation, enhance bone morphogenetic protein (BMP)-2, and induce new bone formation (26). According to a number of *in vitro* studies in osteoblastic cells, the elevated levels of intracellular cyclic AMP enhance their bone-forming activities (27, 28). PTX, a xanthine derivative similar to other methylated xanthine derivatives, is a competitive non-selective phosphodiesterase inhibitor that raises intracellular cAMP (25, 29). Previous studies have shown that PTX may be useful for the treatment of numerous bone diseases and disorders such as prosthetic loosening, bisphosphonate-associated osteonecrosis, and radiotherapy-related chondrocyte apoptosis (26). There are a number of positive effects with PTX administration on fracture healing in animal models (14, 15). Therefore, we have hypothesized that administration of PTX *in vivo* at an adequate dosage will have the potential to provide enough energy to increase bone anabolism (21) and accelerate the catabolic stage of the fracture healing process in femoral fractures in rats treated by intramedullary pin fixation. Our results have shown that 50 mg/kg of PTX decreased the biomechanical properties of repairing osteotomized femurs when compared with the control group. Aydin et al. reported that radiological evaluation of the callus did not reveal any significant difference between the control and PTX-treated groups in the first, second, and third weeks after surgery. Callus formation in PTX-treated group was better than that in the control group in the third week after surgery. They concluded that 50 mg/kg of PTX could be used to accelerate fracture union during the early phases (15). On the other hand, PTX inhibited fracture union in the later stages, presumably owing to its anti-inflammatory effect. Non-steroidal anti-inflammatory drugs have been shown to delay fracture healing (28). This should be considered during the clinical use of PTX (15). According to Aydin et al. (15), the lack of biomechanical studies in their evaluations could be considered a study limitation. Histomorphometric parameters and biochemical markers of bone metabolism in animal studies only indicate decreased bone formation and minimal changes in bone resorption. These parameters are less important for OP-associated fractures and investigations in orthopedic surgery. Histological studies do not give direct information about the mechanical strength of the bone. The ultimate reason for bone fracture following minimal trauma is reduction in mechanical strength (29). Although bone densitometry is often used as a surrogate to evaluate bone fragility, direct biomechanical testing of the bone undoubtedly provides more information about mechanical integrity (30). To eval-

uate fracture repair, biomechanical evaluations have been used. Wei et al. reported a positive effect of 200 mg/kg PTX on fracture healing. During tibial fracture, there was chronic up-regulation of TNF-alpha, IL-1-beta, and IL-6 mRNA and protein levels in hindpaw skin. PTX administration significantly reduced the mRNA expression and cytokine protein levels for these cytokines. PTX also inhibited nociceptive sensitization along with some vascular changes. There were insignificant effects on most bone-related parameters measured in these studies (14).

These previous results are supported by the current study results that showed a positive effect of 200 mg/kg PTX on biomechanical properties of a complete osteotomy model in rats during the late stage (remodeling phase) of fracture healing. Our results showed a dose-dependent effect of PTX administration in the fracture-healing model in female rats. PTX, at 50 mg/kg, decreased the mechanical properties of repairing osteotomized femurs, whereas 200 mg/kg of PTX significantly increased the mechanical properties of repairing osteotomized femurs. Low-dose PTX administration may alter the inflammatory reaction in tissue repair and delay the fracture healing process. In addition, it inhibited fracture union in the later stages and decreased the biomechanical properties of bone repair. Understanding the mechanism of this function would require additional research. However, the high dose of PTX, 200 mg/day, provided potent energy needed by the repairing bone to accelerate the fracture healing process. Thus, PTX-treated rats showed increased biomechanical values in bone healing when compared with the control group. Similar results were reported by Erken et al. (16) who used a compressive test machine to evaluate spinal (vertebral body) bone strength. They reported that radiological fusion grading, biomechanical testing, volume of the fusion mass, and trabecular bone rate had a significant advantage with 100 mg/kg PTX administration during posterolateral lumbar spinal fusion (16). It appeared that the dose response of repairing trabecular bone to PTX administration was different from that of repairing cortical bone.

Infection is a major cause for non-union of a fracture. Infection is not always evident clinically or by bacteriological analyses. If untreated, non-union may occur (30). Fracture non-union in the pediatric population can occur from childhood to adolescence, and is often due to underlying causes such as neurofibromatosis or osteogenesis imperfecta. Although less commonly seen, non-union may also occur in the otherwise healthy pediatric population (31).

## 5.1. Strengths and Limitations

### 5.1.1. Limitations

- We did not use additional evaluation methods such as histochemistry and gene expression analyses.
- The sample size was small (n = 5) for each studied group.

### 5.1.2. Strengths

- We used a biomechanical evaluation method that provided direct information about the mechanical strength of the bone.
- We tested three different dosages of PTX.
- The strongest point of this study is that it generated positive results regarding optimal dosages of PTX that could be successfully used in compromised bone repair situations, such as in patients with OP and diabetes mellitus. These results should be verified in future studies.

## 5.2. Conclusion

Biomechanical strength of the repairing tissue in the fracture healing process in a complete osteotomy rat femur model was accelerated by treatment with 200 mg/kg PTX when compared with no treatment. Additional research with histological, biochemical, and molecular techniques is needed in healthy and osteoporotic rats.

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## Footnotes

**Authors' Contribution:** Mohammad Mahdi Vashghani, Reza Masteri Farahani, Mohammad Reza Abbasian, Mohammad Noruzian and Ataroalsadat Mostafavinia contributed to data collection. Ramin Pouriran contributed to editing the paper. Seyed Kamran Ghorishi performed statistical analysis. Arefe Aryan contributed to the materials and methods section and data collection. Mohammad Bayat designed and directed the project, conducted data analyses, interpreted the results, and wrote the manuscript.

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