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Effects of Intermittent Teriparatide Administration on Bone Graft Healing and Local Simvastatin

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ABSTRACT

Introduction: Improving bone healing by various methods is one of the research areas of oral and maxillofacial surgery, as in the surgical branches dealing with hard tissue in medicine. Many methods, such as bone grafts, drugs, hormones, and tissue engineering applications, are used in bone reconstruction and rehabilitation. Moreover, improving bone healing is critical for better surgical outcomes. This study aimed to compare early and late period effects of intermittent teriparatide application on bone graft and local simvastatin application from histopathological, histomorphometric analysis, and biochemical aspects.

Materials and Methods: 24 New Zealand rabbits were divided into four groups. While experimental groups received intermittent teriparatide (30µg/kg), control groups were given sterile distilled water. A total of 3 defects were created on each rabbit's right and left tibia. Bone graft and local simvastatin were randomly applied to the opened defects, and one defect was left blank for control purposes. Rabbits were sacrificed on days 15 and 30 to examine early and late bone healing. Blood was drawn for biochemical analysis.

Results: The healing score and new bone development in teriparatide applied and grafted defects were statistically significant compared to all groups ($p<0.05$). A statistically significant difference was obtained in grafted defects compared to the control group in teriparatide-applied defects. Local simvastatin caused necrosis in both experimental and control groups. Teriparatide administration does not cause a statistically significant change in calcium, potassium, and parathyroid hormone biomarkers ($p>0.05$).

Conclusion: Better bone healing and bone graft healing in rabbits treated with teriparatide may encourage improved surgical outcomes in clinical practice.

Keywords: Teriparatide; Intermittent; Bone healing; Bone graft; Simvastatin.

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Introduction

Rehabilitation of oral and maxillofacial functionality and aesthetics is a priority for patients affected by various conditions such as tooth loss, infection, congenital skeletal disorders, trauma, and the removal of pathological lesions. Therefore, bone reconstruction in dentistry, oral and maxillofacial surgery, periodontology, orthodontics, endodontics, and daily clinical practice is significant. Current clinical approaches have various techniques, ranging from the traditional use of bone grafts to the most innovative regenerative procedures, such as tissue engineering [1]. Although autogenous grafts are accepted as the gold standard in repairing these defects, they have some disadvantages due to the secondary donor site, limited graft quantity, and unpredictability of graft healing. For this reason, various bone materials can be used, as well as pharmacological agents that increase bone healing [2].

In recent years, the use of teriparatide (TP) due to its anabolic effects has been used primarily in the treatment of osteoporosis patients. However, TP can also be used in the repair of bone defects. TP is a recombinant human protein consisting of the first 34 amino acid fragments of parathyroid hormone. It is the only Food and Drug Administration-approved drug for the treatment of osteoporosis due to its anabolic effects [3,4]. Simvastatin is the structural analog of HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) and belongs to the statin family. They were initially developed to treat hypercholesterolemia [5]. Mundy et al. drew attention to the osteoblast-stimulating effect of simvastatin in their mouse calvarial defect model. They reported that simvastatin could stimulate bone regeneration and formation. This can be explained by simvastatin's anti-inflammatory, osteoclast-inhibiting, and neovascularization properties [5,6]. This study compared the effects of applying teriparatide intermittently on bone defects treated with teriparatide and bone grafts. The study also aimed to show the systemic effects of intermittent teriparatide administration by monitoring PTH, Ca, and P biomarkers in the early and late stages. Healing was evaluated through histological and histomorphometric analysis.

Materials and Methods

This study included 24 male New Zealand rabbits with complete skeletal development, weighing 2500-3500 g, at an average of 9 months. The study protocol was approved by the Istanbul University Animal Experiments Local Ethics Committee (Reference Number: 2013/128). Experiments were conducted in the Istanbul University Experimental Medicine Research Institute (DETAE) laboratories. Rabbits were housed in suitable cages at 21±1°C with 12 hours of light/12 hours of darkness. They had free access to standard laboratory feed and water. They were fed ad libitum with standard laboratory feed. The rabbits were divided into four groups. In the 1st group, intermittent (every other day) teriparatide (Forsteo®, Fegerscheim, France) was administered, and rabbits were sacrificed on the 15th day to examine the early healing. Blood samples were collected on 0. and 15th days. In the 2nd group, teriparatide was not administered, and the same volume of subcutaneous distilled water was injected intermittently. Rabbits were sacrificed on day 15 to examine early healing. Blood samples were collected on 0. and 15th days. In the 3rd group, intermittent teriparatide was administered, and rabbits were sacrificed on the 30th day to examine the late healing. Blood samples were collected on 0., 15th and 30th days. In the 4th group, teriparatide was not administered, and the same volume of subcutaneous distilled water was injected intermittently. Rabbits were sacrificed on day 30 to examine late healing. Blood samples were collected on 0., 15th and 30th days (Figure 1a).

The teriparatide (Forsteo®, Fegerscheim, France) used in the experiments contains 250 micrograms of teriparatide per 1 ml of injectable solution. The dose adjustment was made to be 30μg/kg. The preparation with the cold chain was kept in the refrigerator throughout the experiment. Simvastatin tablets with a diameter of 5 mm were produced in the Department of Pharmaceutical Technology of the Faculty of Pharmacy of Istanbul University. The production method of the tablets is as follows: First, 100 mg of simvastatin was weighed, and 200mg of avicel Ph 101 was added and mixed. 1% magnesium stearate was added to this mixture and the same way mixed. The prepared mixture was turned into tablets containing 3mg of simvastatin using a 5 mm punch in a tablet compression machine. Later, the pills were transferred to Gamma-Pak® sterilization company. Bovine source (BioOss®, Geitslich Sons Ltd Wolhusen, Switzerland) was used as bone graft material. Experimental animals were fasted for 12 hours before the operation. General anesthesia was provided by intramuscular injection (IM) of 35mg/kg Ketamine HCl (50mg/ml Ketalar®, Parke Davis) and 10mg/kg Xylazine HCl (23.32mg/ml Rompun®, Bayer). Tibia area is shaved, disinfected with an iodine solution, and covered with sterile drapes (Figure 2a). Local an-

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esthesia of the area was provided subcutaneously with a 2ml volume of 40mg/ml articaine HCl and 0.012mg/ml epinephrine containing anesthetic (Ultracain® DS Forte). To reach the tibia region, a longitudinal skin, subcutaneous and periosteal incision of 4cm was made in the leg with two defects and 2.5cm in the leg with a single defect. With blunt dissection, the medial surface of the tibia was exposed, and soft tissues were excluded. After careful elevation of the periosteum, a 2mm deep defect, including the cortex and medulla layers of the bone, was opened with a standard trephine bur with an outer diameter of 6mm under saline irrigation (Figure 1b,c).

In tibias with two defects, a gap of 5mm was left between the defects. Due to its lipophilic feature, the simvastatin tablet is placed with a single bone defect so that it does not pass through the bone marrow and affect the other defect. On the side where two standard defects were created (randomly right or left tibia), a bone graft was placed in one defect, and the other was left blank for control (Figure 1b,2d). Then, the subcutaneous tissues were closed with 3.0 half-round Pegasorb (Dogsan, Istanbul, Turkey) (Figure 2e) and the skin tissues with 3.0 atraumatic sharp silk (Dogsan, Istanbul, Turkey) sutures (Figure 2f). Post-operatively, rabbits received intramuscular tetracycline (50mg/kg). Medical gauze and plasters were applied to prevent fractures.

Rabbits in the first and third experimental groups were injected subcutaneously with 30μg/kg TP every other day, and the control groups (second and fourth groups) were injected subcutaneously with the same volume of sterilized distilled water (because it is the solvent of the parathormone analog) on the same days. The first and second groups were sacrificed 15 days after surgery, and the third and fourth groups 30 days after surgery, using 135mg/kg sodium pentothal intraperitoneally. Tibias were collected, cleared from soft tissues, and fixed in a 10% formaldehyde solution. Samples decalcified for histopathological examination were embedded in paraffin blocks. Samples stained with Hematoxylin-Eosin were evaluated under the light microscope. In histopathological examinations, inflammation, fibrous tissue formation, necrosis, and new bone formation were examined comparatively. For biochemical analysis, 4ml of blood was drawn from the marginal ear vein on days 0, 15, and 30. After the blood taken from the rabbits was centrifuged at 4000 rpm for 10 minutes (Hettich Universal 32), the separated serums were stored at -20°C until the number of tests was completed. Calcium, phosphorus, and parathormone were examined as biochemical markers. Statistical analyses were performed using SPSS 15.0 for Windows. Descriptive criteria; are presented as mean and percentage distribution. The conformity of the data to the normal distribution was checked with the Kolmogorov-Smirnov test. Since parametric conditions could not be met to determine mean differences between groups, the Mann-Whitney U test was used in independent groups, and the Wilcoxon signed-rank test was used in dependent groups. Chi-square analysis (Fischer's exact test when necessary) was used to compare dichotomous variables. The significance level was taken as $p<0.05$.

Results

According to the biochemical marker results, no statistically significant difference was found between the TP (–) and TP (+) groups in terms of percentage changes in calcium, potassium, and PTH levels between the beginning and end of the experiment ($p>0.05$) (Details in Figure 3a). In the histological examination, evaluation and comparison were made regarding inflammation, necrosis, and fibrosis in the early and late-stage groups. When TP (-) and TP (+) groups were compared in both early and late periods, no significant difference was found between the groups ($p>0.05$) (details can be seen in Figure 3b). In the histomorphometric examination, no significant difference was found regarding "early and late healing scores" and "new bone scores" in defects where simvastatin was placed. Necrosis has been observed in defects treated with simvastatin. However, there was a significant difference between the TP (+) and TP (-) groups of defects that were left blank and grafted both in the early and late periods ($p<0.05$). TP (+) groups were significantly higher. In addition, a significant difference was found between the TP (+) and TP (-) groups of the defects that were left blank in the early and late periods. "Bone healing scores" and "new bone scores" were statistically significantly higher in TP (+) groups (Figure 3c,d).

A statistically significant difference was found when the bone healing in both early and late period graft placed groups were compared with the control groups ($p<0.05$) (Figure 3c,d). In the evaluation of histological findings, the defect wall is shown with blue arrows in the early healing image of the TP (+) graft group. The yellow arrows indicate the Retzius line between the defect wall and the graft. Graft healing is observed in the area marked with the red arrow [Figure 4A]. Necrosis was observed in the early healing of the TP (-) simvastatin group. No new bone formation was observed

[Figure 4B]. The new bone area is marked in red in the early healing image of the TP (-) control group. The parts marked with blue arrows are the defect walls. The parts indicated by the white arrow are the medulla of the bone (Figure 4c). Graft healing is marked in red in the late recovery image of the TP (+) graft group. The new bone has formed around the graft in the places indicated by the orange arrows (Figure 4d). In the late recovery image of the TP (-) graft group, the graft areas are marked with a "G". The medulla of the bone is shown with white arrows. Defect walls are marked with blue arrows (Figure 4e). In the late healing image of the TP (-) simvastatin group, areas of necrosis are marked with yellow arrows (Figure 4f).

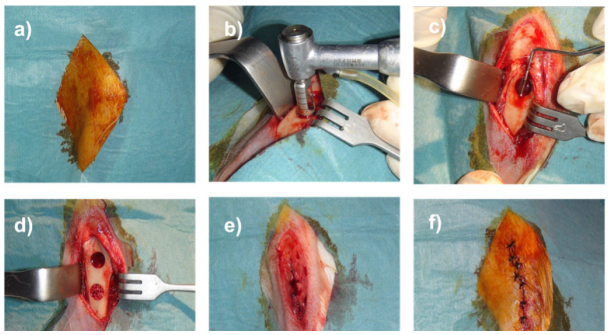


Figure 1. a) Details of experimental groups b) Description of tibial defects.

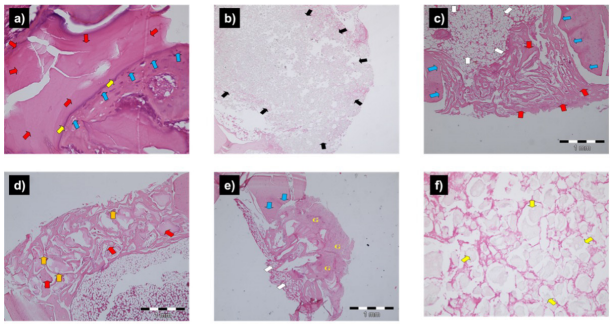


Figure 2. a) Preparation of the surgical site b) Preparation of standardized defects c) Standardized Defects (6mm) d) While one defect was left empty, a graft was placed in the other defect e) Suturing of the deep tissues f) Suturing of the skin.

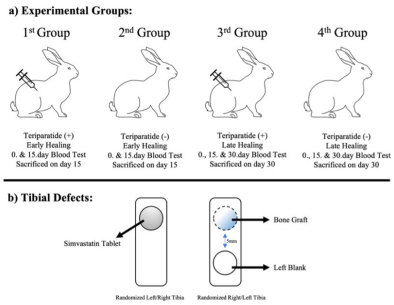


Figure 3. a) Biochemical marker results b) Histological examination of inflammation, necrosis, and fibrosis in the early and late-stage groups c) Comparison of early and late bone healing scores) d) Comparison of early

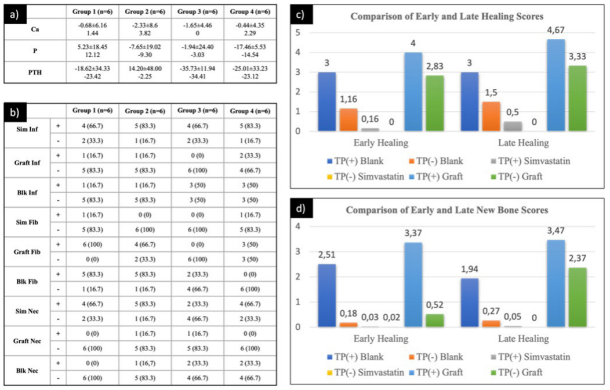


Figure 4. a) Histologic view of 15th day TP(+) Bone Graft Group (200x). b) 15th day TP(-) Simvastatin Group (40x). c) 15th day TP(-) Control Group (40x). d) 30th day TP(+) Bone Graft Group (40x). e) 30th day TP(-) Bone Graft (40x). f) 30th day TP(-) Simvastatin Group (400x).

Discussion

Our aim in this study was to examine the effects of intermittent TP application on the healing of bone graft and simvastatin-applied defects. Current studies have reported that TP applied at different doses and intervals has an anabolic effect on bone [3,7-12]. In the literature, various experimental animal models, such as rats, guinea pigs, and rabbits, have been used for biomaterial and animal studies to increase bone formation [13,14]. In our study, the rabbit experimental model was preferred because the rabbit tibia is larger than the rat and guinea pig tibia, allowing more than one open defect. In addition, thanks to this advantage, the risk of fracture is reduced, and the risk of morbidity decreases at the same rate. Using only male rabbits, the experiment was intended to be unaffected by hormonal changes. Rabbits similar in weight and age were pre-

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ferred. Matos et al. reported that a 2-week period was sufficient for early recovery, and mature lamellar bone was seen at the end of 4 weeks in their experimental modeling study of rabbits. In our research, sacrificed tibiae were dissected by administering 135mg/kg sodium pentothal intraperitoneally on the 15th day in the early healing groups and on the 30th day in the late healing groups [15]. Various studies have shown intermittent or daily TP injections to have an anabolic effect on fracture and graft healing, implant osseointegration, distraction osteogenesis, and spinal fusion in different animal models.

Although there is no standardization regarding the doses, it is generally given in 25-40μg/kg doses. It is crucial to look at biochemical markers in hormone studies. Because the given hormone should not have a negative effect systemically, it should only show the desired effect. Tang ZL et al. gave 25μg/kg intermittent TP injections to Japanese white rabbits with mandibular defects. X-ray imaging, histomorphometric analysis, and biochemical markers were evaluated. As a result, they reported that new bone formation was earlier in the experimental group than in the control group. They also reported that bone-specific alkaline phosphatase (bALP) and osteoprotegerin (OPG) values slightly increased after the operation. However, there was no significant difference between the control and experimental groups at the end of the experiment [10].

Sugiura T et al. applied 40μg/kg TP in their study on 18 rats with an osteoporosis model. They observed that the microstructural parameters peaked at the end of 4 weeks in the experimental group in which TP was applied. Osteocalcin (OC) was evaluated as a biochemical marker, and they reported no significant difference between the experimental and control groups [11]. Qui et al. considered biochemical markers with m-CT in their study on 38 osteoporotic female Sprague-Dawley rats. They observed higher fusion bone volume and cortical thickness in the experimental group administered 30μg/kg TP. They reported significantly higher N-terminal peptide (NTP) and OC values as biochemical markers [12]. In our study, in the biochemical tests we performed on the 0th, 15th, and 30th days, we found that although the PTH, calcium, and potassium levels increased slightly at first, there was no significant difference between the control groups at the end of the experiment. In our hypothesis, we did not expect intermittent TP administration to change biochemical markers. No significant difference was found between the values taken at the beginning of the experiment in the early and late periods. Yukata et al. reported that intermittent TP application increased bone healing, accelerated callus formation, and increased biomechanical strength in bone in rats with genetic COX-2 deficiency and healing disorders [16]. In our study, faster callus formation was observed histologically in the TP groups in the early recovery period. In addition, histomorphometrically, new bone formation was found to be statistically significant in TP (+) groups (except the simvastatin group). The literature has reported that bone grafts support healing and help bone formation, especially in large defects. Few studies have examined the relationship between TP and bone graft healing. Pelled et al. reported that TP increased bone allograft integration in segmental mandibulectomy [17]. Kuroshima et al. investigated the effectiveness of rhPTH in the healing of grafted and ungrafted extraction sockets. As in our study, TP application was made intermittently and systematically, and as a result, they found that bone healing was significantly different in grafted and TP-treated animals. They observed that the TP-treated control group formed more bone than the TP (-) control group[18]. In parallel with our study, the best bone healing and new bone formation scores were obtained in TP (+) and grafted defects in our study, and a statistically significant difference was found in early and late healing compared to other groups. The study found a significant difference in the new bone formation and bone healing scores between the TP (+) control group and the TP (-) control group during both the early and late stages of healing. There was no statistically significant difference between the TP (+) control group and the TP (-) graft group regarding early and late recovery scores. A statistically significant difference was found in the TP (+) control group compared to the TP (-) graft group in the early healing of new bone formation. However, no significant difference was found in the late healing period.

Many researchers have reported that local application of simvastatin increases bone formation [19,21]. However, some researchers have stated that local simvastatin application is ineffective on bone healing and may even have a negative effect [22,23]. Looking at the literature, there are very few studies in which PT and simvastatin were applied and compared systemically [24,25]. Tao et al. investigated the effects of systemic simvastatin and PT applications on bone formation in their study and stated that combined use contributed more to bone formation [24]. Leiyang et al. reported that combined use increased bone formation more in a similar study on mice [25]. When the literature is reviewed, no study compares locally applied simvasta-

tin with systemically applied TP. This makes our study unique.

Conclusion

TP and bone grafts are used for different purposes in clinical practice. Our study shows that TP application can be an alternative additional application for bone regeneration and reconstruction, with the correct doses being standardized with more studies. Also, studies on using TP in healing bisphosphonate-induced necrosis may be an alternative to solving the problems in this area.

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Conflict of Interest

There is no conflict of interest to declare.

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