ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article / Araştırma Makalesi DOI: 10.5152/VetSciPract.2022.223353

Systemic Effect of Metformin on Bone Healing at Bone Defect in Rabbits Using Radiographical and Serum Alkaline Phosphatase Assessment

Kemik Defektli Tavşanlarda Metforminin Kemik İyileşmesi Üzerindeki Sistemik Etkisinin Radyografik ve Serum Alkalin Fosfataz Kullanılarak Değerlendirilmesi

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ABSTRACT

Clinical therapy for disorders of bone healing present serious challenges. The delivery of the bone filling materials necessitates surgical implantation at the fracture site, which could lead to local complications. As a result, taking osteogenic medications will offer a great way to heal bone lesions. Increased osteoblasts and decreased osteoclasts are 2 ways that metformin has an osteogenic impact. The aim of the study is to determine the systemic impact of metformin on bone healing at the site of a bony defect using radiographic and serum alkaline phosphatase testing. A total of 20 mature male rabbits, divided into 2 groups of 10 each for the treatment group and the control group, were utilized. All rabbits underwent identical surgical procedures under general anesthesia. Two holes that were 3 mm in diameter and 3 mm deep were created and left empty after the femur has been surgically exposed. The study was conducted over 28 days. The rabbits in the treatment group received 50 mg/kg of metformin orally daily for 28 days. The animals were sacrificed at 2 different time points, according to their groups, on the 14th and 28th day after surgery. Bone samples from the defect site of the femur were isolated, sectioned, assessed radiographically, and blood was drawn for serum alkaline phosphatase measurement. There was an increase in bone mineral density and osseointegration. In addition, serum alkaline phosphatase increased in the animals of the group treated with metformin than in the control group at both study time periods. Metformin increases bone healing and regeneration at the bone defect sites and enhances the process of osteogenesis and osseointegration more than the control untreated rabbits.

Keywords: Bone mineral density; bone defect; bone healing; metformin; serum alkaline phosphatase

ÖZ

Kemik iyileşme bozukluklarının klinik tedavisi ciddi zorluklar barındırır. Ancak, kemik dolgu malzemelerinin uygulanması yaralanma yerinde cerrahi implantasyon gerektirir, bu da yerel komplikasyonlara yol açabilir. Bu nedenle, osteojenik ilaçları almak kemik lezyonlarının iyileşmesine yardımcı olacaktır. Artan osteoblast ve azalan osteoklastlar metforminin osteojenik etkisini temsil eden iki yoldur. Çalışmanın amacı, metformin uygulamasının kemik defekti bölgesinde kemik iyileşmesi üzerindeki sistemik etkisini radyografik ve serum alkalin fosfataz testleri kullanarak belirlemektir. Çalışmada, toplam 20 yetişkin erkek tavşan onarlı iki gruba bölünerek kullanılmıştır. Tavşanların tamamı genel anestezi altında aynı cerrahi prosedüre maruz kaldılar. Femur cerrahi olarak ekspoze edildikten sonra üzerine 3 mm çapında ve 3 mm derinliğinde iki delik açıldı ve delikler boş bırakıldı. Çalışma 28 gün sürdü. Çalışma grubundaki tavşanlar, operasyonun ardından 28 gün boyunca günde 50 mg/kg oral metformin aldı. Hayvanlar araştırmadaki numaralarına ve gruplarına göre iki farklı zaman aralığında, operasyondan sonraki 14'üncü ve 28'inci günlerde sakrifiye edildiler. Femur defekt bölgesinden alınan kemik örnekleri, ayrıştırıldı, kesildi, radyografik olarak değerlendirildi ve serum alkalin fosfataz ölçümü için kan alındı. Metformin alan hayvanların kontrol grubuna kıyasla çalışma zamanlarının her ikisinde de kemik mineral yoğunluğu ve osseointegrasyon artışı olduğunu ve ayrıca serum alkalin fosfataz artışı olduğunu göstermiştir. Sonuç olarak, metformin, kontrol grubunda tedavi uygulanmayan tavşanlara kıyasla kemik defekti bölgesinde kemik iyileşmesini ve yenilenmesini artırmakta ve osteogenezis ve osseointegrasyon sürecini daha fazla güçlendirmektedir.

Anahtar Kelimeler: Metformin, BMD, serum alkalin fosfataz, kemik iyileşmesi, kemik defekti

Received/Geliş Tarihi: 29.09.2022
Accepted/Kabul Tarihi: 20.12.2022
Publication Date/Yayın Tarihi: 29.12.2022

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Cite this article: Mahmood Hussein R, Abdulrahman Taqa G. Systemic Effect of metformin on bone healing at bone defect in rabbits using radiographical and serum alkaline phosphatase assessment. *Vet Sci Pract*. 2022; 17(3), 91-97.

Atıf: Mahmood Hussein R, Abdulrahman Taqa G. Kemik defektli tavşanlarda metforminin kemik iyileşmesi üzerindeki sistemik etkisinin radyografik ve serum alkalin fosfataz kullanılarak değerlendirilmesi. *Vet Sci Pract*. 2022; 17(3), 91-97.

INTRODUCTION

Bones provide a structural function and cover vital organs. In addition, bones serve as storage for minerals and growth factors as well as a site for the creation of blood cells. Their physiological function is closely linked to the presence of stem cells, which are crucial regulators of cell activities.[1]

One of the most frequent traumatic injuries is a bone fracture. Although the majority of minor fractures recover within a few weeks, 5% to 10% of long bone fractures take 6–8 months to heal properly. Current therapies, including autologous bone transplants, intramedullary nails or fixation plates, and bone morphogenetic protein (BMP)-based therapy do not always enable full bone restoration.[2]

Bone regeneration can occur either by endochondral ossification or by intramembranous ossification processes. Mesenchymal stem cells (MSCs) immediately differentiate into osteoblasts upon intramembranous ossification, which leads to the deposition of mineralized extracellular matrix. Endochondral ossification goes through stages such as inflammation, development of soft to hard callus, and remodeling of the fracture site.[3]

Bone is a highly dynamic tissue. Maintaining homeostatic bone metabolism and skeletal strength requires a series of coordinated steps known as "bone remodeling."[4] Clinical judgments, radiographic analyses, and serological tests are used to evaluate bone healing.[5] Malignant bone tumors and serious trauma can remove a considerable amount of bone, resulting in severe bone defect. In addition, bone abnormalities that necessitate bone augmentation treatments are typically present with fractures sustained from high energy trauma and osteoporotic fractures.[6]

Several drugs with very different indications and exhibiting a pleiotropic spectrum of actions are used to target the local and systemic regulation of bone metabolism. These include antihyperlipidemic drugs such as (HMG-CoA reductase inhibitors), antihypertensive drugs (such as ACE inhibitors), drugs for osteoporosis (bisphosphonates), cancer drugs (inhibitors proteasome), and other drugs.[7] Metformin works by preventing the production of hepatic gluconeogenesis, increasing the density of low and high affinity insulin receptors, and lowering resistance to the peripheral effects of insulin.[8] Metformin treatment for diabetic patients has been shown to reduce TNF expression. The pharmacological action of metformin goes beyond simple glycemic control, decreasing the markers of inflammation, and contributing to the reduction of oxidative stress with a confirmed anti-inflammatory effect.[9] In the setting of chronic periodontal inflammation, MSCs have a significantly reduced potential for osteogenic differentiation. A highly effective strategy to stimulate or restore the osteogenic potential of MSCs in an inflammatory environment remains an unrealized goal.[10]

Metformin belongs to a group of drugs known as biguanides, which work well both alone and in conjunction with other hypoglycemic medications. Metformin is typically well accepted, has few adverse effects, and is relatively inexpensive.[11] Therefore, this study focuses on the effect of metformin on bone regeneration.

During the maturation and mineralization phases of the newly formed osteoid, alkaline phosphatase (ALP), which can be detected in serum, is secreted into extracellular fluid by osteoblasts. However, only around 50% of the ALP activity in healthy people's blood is derived from bone, with the remaining 50% largely coming from the liver. The bone-derived isoform (BALP) can be more specifically detected by some techniques.[12]

MATERIAL AND METHODS

Experimental Model

Twenty adult male New Zealand rabbits weighing 1.75 kg to 2 kg and aged between 6 and 8 months were utilized. The animals were sacrificed at the end of the experiments (at 14 and 28 days) using an overdose of general anesthesia (ketamine 200 mg/kg + xylazine 40 mg/kg)[13]. The study was carried out in agreement with the Institutional Animal Research Ethics Board's guidelines as of June 19, 2022 (UoM.Dent/A.L.58/22).

Medication

The drug used in this study is metformin tablets 500 mg (the smallest dose available to control the administered dose)

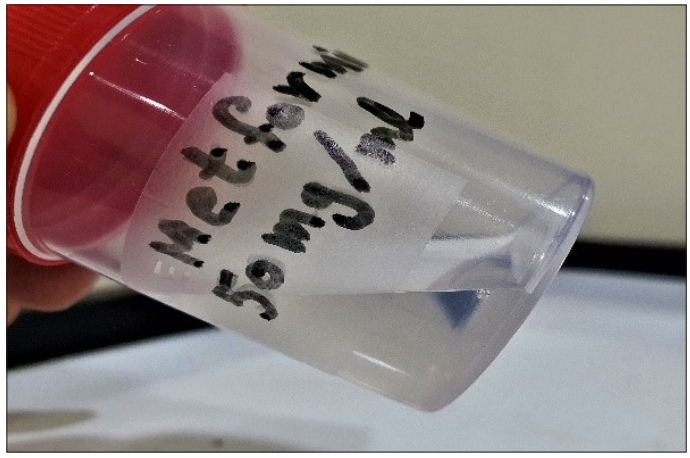

Figure 1. Glucophage®(metformin 500 mg) tablets / prepared liquid metformin.

produced by the well-known German brand company, MERCK under the trade name, Glucophage.

Metformin in Iraq and most of countries is available as tablets only. Therefore, we were prepared it in the form of liquid by fine grinding of the tablets to obtain fine powder. Each 500 mg tablet was ground alone; the resultant fine powder of this tablet was filled into a hard gelatin capsule to control the amount of drug (500 mg per capsule). For oral administration of the drug, the content of each capsule was dissolved in 10 mL of distilled water with vigorous shaking for at least 2 mins to obtain homogenous solution containing 500 mg/10 mL of the drug (Figure 1). Though metformin is essentially insoluble in organic solvents like acetone, ether, and chloroform, it is readily soluble in water.[14] Metformin liquid was administered to the rabbits orally in a dose of 50 mg/kg once daily using a feeding tube and pushed through a graduated syringe to give the exact and accurate dose.[15] The rabbits in the treatment group received 50 mg/kg of metformin orally daily for 28 days.

Study Design

Group 1: Control group that received no medication (n = 10). This group was subdivided into 2 groups of 5 rabbits for each period, according to the sacrifice date (day 14 and 28 post-surgery).

Group 2: Treatment group that was systemically treated with metformin (n = 10). After the surgical procedure, metformin was administered orally in a once daily dose of (50 mg/kg) body weight [15] using feeding tube. This group was subdivided into 2 groups of 5 rabbits for each period, according to the sacrifice date (day 14 and 28 post-surgery).

Preparation of Animals for Surgery

All the 20 rabbits received anesthesia by intramuscular injection. Each rabbit received an intramuscular injection of a mixture of ketamine (KETALROM-50), 40 mg/kg and xylazine (Holland), 4 mg/kg.[16]

Animal Surgical Procedure

After 20 mins, the animal gained anesthesia,[17] and the surgery was performed on the left femur bone.

Two holes, 3 mm in diameter and 3 mm deep, were created in the exposed femur diaphysis, using 2000 rpm low speed straight surgical hand piece with a 3 mm round carbide bur and continuous normal saline irrigation (Figure 2).[18]

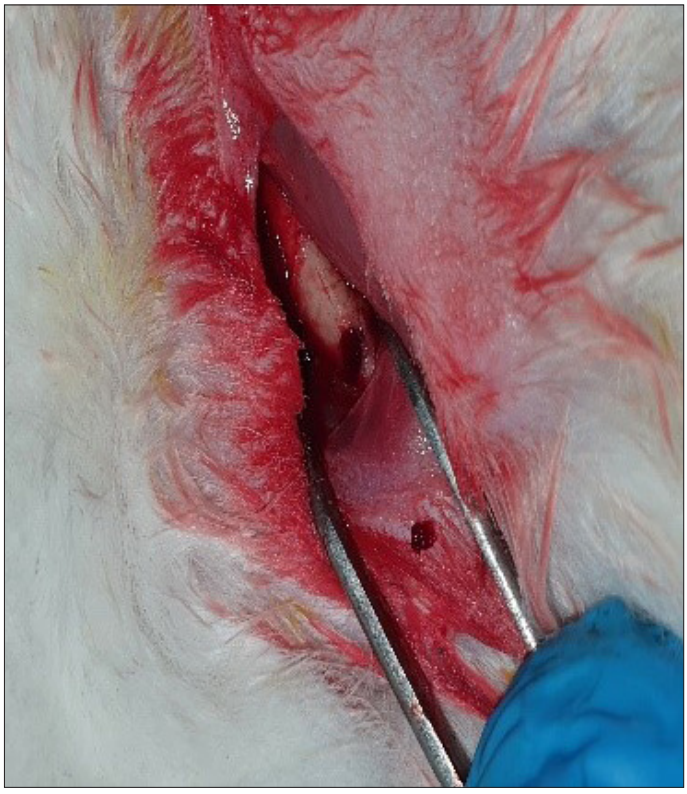

Figure 2. Two holes were made in the femur bone of each of the 20 rabbits.

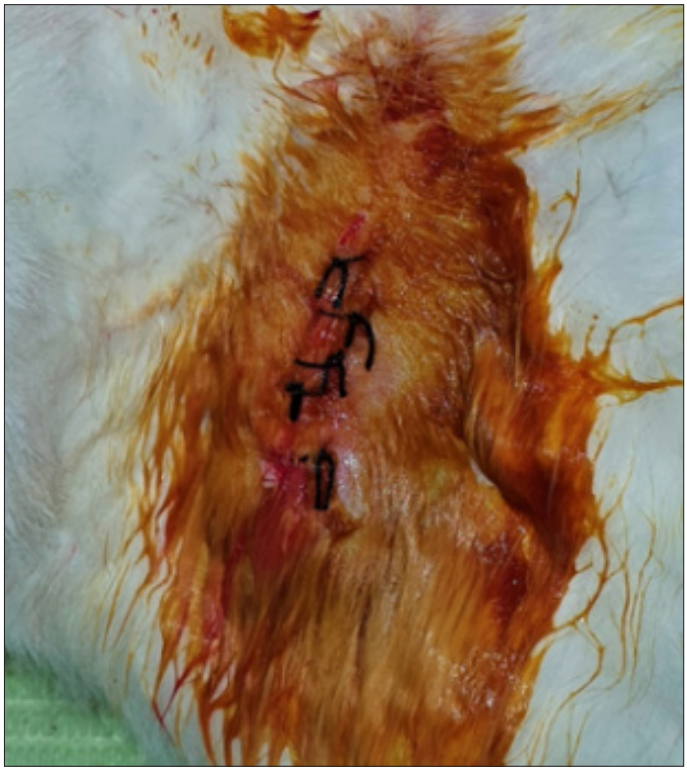

Figure 3. Suturing and disinfection of the wound

The bone defects (holes) were left empty without any material. The wound was closed using a 3/0 black silk suture and rubbed well with povidone iodine 10% as shown in (Figure 3).

Postoperative Care of Animals

After the surgical procedure, the animals were given oxytetracycline 20% injection (Limoxin-200 LA® [Holland]) as a prophylactic antibiotic for wound healing, administered as a single daily dose of 0.5 mL/ kg intramuscularly for 3 consecutive days from the operation day.

Criteria of the Study

Biochemical Criteria

Blood Sample Preparation

After the surgery, on days 14 and 28, the animals were sacrificed and blood was drawn directly from the jugular vein. The serum was then separated and stored until it was used for analysis of serum ALP.

Determination of Serum Alkaline Phosphatase for Rabbits Using the Spectrophotometer

Materials Supplied:

The whole kit was stored in a refrigerator at temperatures between 2°C and 8°C until the time of analysis, during which the kit should reach to room temperature (20°C–25°C).

Reagents:

1- Vial R1 (substrate-buffer):

Disodium phenyl phosphate 5 mmol/L

Carbonate-bicarbonate buffer pH 10 50 mmol/L

Stabilizer

2- Vial R2 (standard):

Phenol corresponding to 20 U king and kind

3- Vial R3 (blocking reagent) (Toxic)

4-amino antipyrine 60 mmol/L

Sodium arsenate 240 mmol/L

4- Vial 4 (dye reagent)

Procedure

Reagents and specimens were at room temperature. Tubes were prepared as shown in Table 3.

Reagent 1 was prepared and incubated for 5 mins at 37°C. Reagent 2 was prepared and was let to stand exactly 15 mins at 37°C.

The reagents were mixed and then incubated for 10 mins at room temperature and were stored away from light. Read absorbances of the blank specimen, standard, and assay at 510 nm against reagent blank. Coloration was stable for 45 mins away from light.

Radiological Criteria

At the time of animal sacrificing, the site of bone defect (hole) and around it in the femur bone were taken directly for radiographical examination. To assess the degree of bone development, the specimens were examined using a digital radiography machine.

In this criterion, the linear measurement was not used, instead the densitometric analysis were used to measure the bone mineral density at the desired area in the hole. (Figure 4).

RESULTS

Serum Alkaline Phosphatase

On day 14, serum ALP was measured for both groups. That of the control group was used as a reference to compare with the

Figure 4. Radiographical machine, sensor, and control.

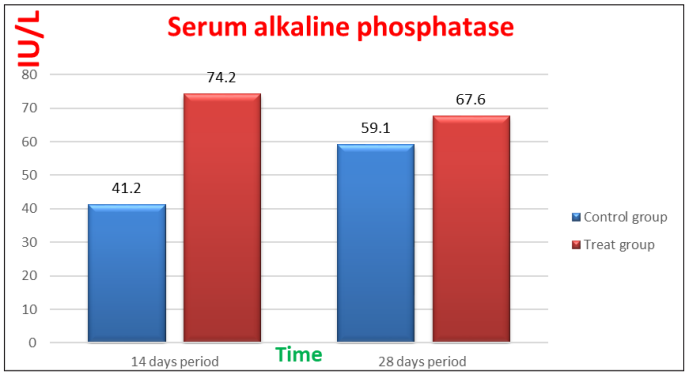

Figure 5. The histogram of the statistical analysis of serum alkaline phosphatase measured in (IU/L).

Table 1. Alkaline phosphatase levels at both groups at different periods.

Group Time	14 days period	28 days period
Control group	41.2 ± 0.73	59.1 ± 0.9
Treat group	74.2 ± 1.53	67.6 ± 1.36
P value	.0001	.0001

treatment group. An independent sample *t* test (using IBM SPSS 26) showed a highly significant difference in serum ALP at day 14 between the control group (41.2 ± 0.73) IU/L and the treatment group (74.2 ± 1.53) IU/L ($P \leq .001$), as shown in Table 1 and Figure 5.

Independent sample *t* test showed highly significant difference in serum ALP on day 28 between the control group (59.1 ± 0.9) IU/L and the treatment group (67.6 ± 1.36) IU/L ($P \leq .001$), as shown in Table 1 and Figure 5.

Radiological Results

In this criterion, densitometric analysis were used to measure the bone mineral density at the desired area in the hole. Also, gross radiographical assessment was used to observe the effect between various groups. Radiological criteria are a good indication of new bone formation at the site of the hole and for comparison between the holes of different sites.

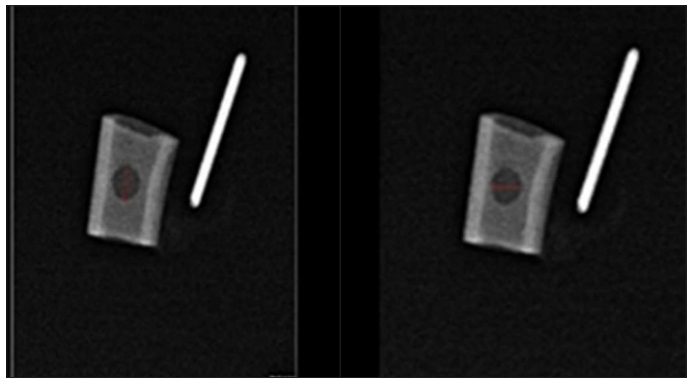

Figure 6. Densitometric study of the femur bone hole in the control group after 14 days.

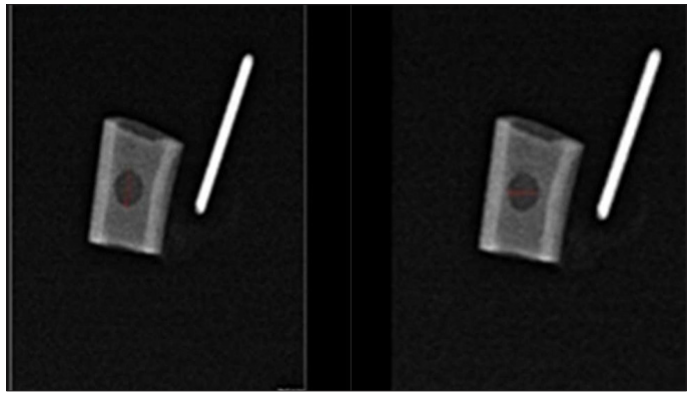

Figure 7. Densitometric evaluation of the treated group's 14-day femur bone hole.

Table 2. Bone mineral density mean ± standard error at different time periods.

Group Time	14 days	28 days
Control group	134.5 ± 1.5	160.5 ± 1.7
Treated group	135.75 ± 1.3	171.25 ± 0.85
P	0.085	0.001

On day 14, independent sample *t* test showed that there was no significant difference ($P \geq .05$) in bone mineral density (BMD) between the control group (134.5 ± 1.5) the metformin-treated group (135.75 ± 1.3). The treatment group slightly exceeded the control group in BMD, and there was gross observable difference in radiopacity between these 2 groups, as shown in Table 2, and Figures 6 and 7.

On day 28, obvious difference was observed in gross radiopacity between the control and treatment groups. In addition, there was a significant difference in BMD at day 28 between the control group (160.5 ± 1.7) and the treatment group (171.25 ± 0.85) ($P \leq .05$), as shown in Table 2 and Figures 8 and 9.

DISCUSSION

In this study, serum ALP levels indicate that the metformin-treated group experienced an increase in the level of enzyme more than the control group during both time intervals of 14 and 28 days. Serum ALP can be used as an indicator to measure the activity of bone formation. Studies suggest that assessing markers of bone formation such as serum ALP throughout the healing of fractures could increase the accuracy of the evaluation of the

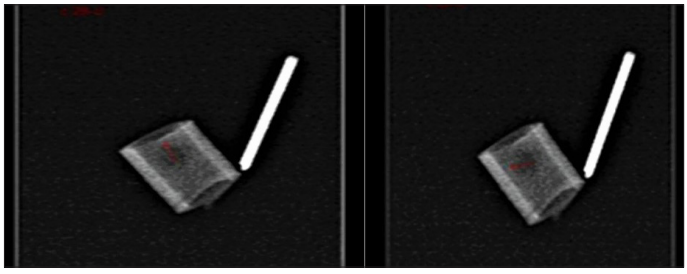

Figure 8. Densitometric analysis of the femur bone hole of the control group at 28 days period.

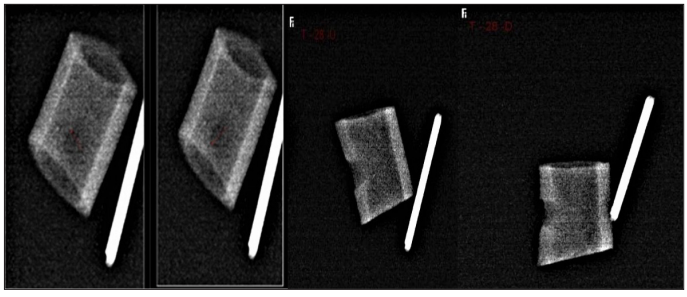

Figure 9. Densitometric analysis of the femur bone hole of the treated group at 28 days period.

Table 3. Manual procedure of serum alkaline phosphatase measurement.

	Reagent blank	Specimen blank	Standard	Assay
Reagent R1	2 mL	2 mL	2 mL	2 mL
Incubated for 5 mins at 37°C				
Specimen				50 µL
Reagent 2 (standard)			50 µL	
Let to stand exactly 15 mins at 37°C				
Reagent R3	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Mixed well				
Reagent R4	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Specimen		50 µL		
Demineralized water	50 µL			

bone healing stage and enable early identification of patients at risk for developing delayed union or nonunion.[19] This supports the choice of serum ALP as a marker in our study.

Because of its ease of use in individuals with normal liver function, total serum ALP is a frequently applied marker of bone metabolism. ALP levels also suggest normal osteoblast activity and new bone development.[20]

This study uses serum ALP as an indicator of bone healing by comparing its level to that of the control group (baseline), despite the fact that there are few studies establishing the role of this enzyme in the evaluation of the healing process following fractures. The bone-isoenzyme of ALP (BsALP), which is believed to be a more accurate marker of bone formation, was not available at our center and had a high cost. The healing of fractures is highly associated with serum ALP levels. Tissue-nonspecific ALPs make up the majority of the enzyme fraction circulating in serum, which is of therapeutic importance.[21]

The circulating enzyme in healthy people is mostly derived from liver and bone. The hydrolysis of organic phosphate esters that are present in the extracellular space is catalyzed by a group of isoenzymes called ALPs, which are found on the outer layer of the cell membrane. The cofactors magnesium and zinc are essential for this enzyme. Though ALPs are present in a variety of body regions and exhibit a variety of physiochemical properties, they are actual isoenzymes because they catalyze the same biochemical reaction. More than 80% of the ALP in serum is released from the liver and bone, with small amounts from the intestines.[22]

Serum total ALP activity is frequently used as a biochemical marker of osteoblast function, however, because of contribution of its activity, it lacks great specificity. It is particularly derived from the liver. About half of the serum ALP activity in healthy people comes from bone and the other half from the liver.[19]

The results of our study in which there was an increase in serum ALP at both time intervals of the study, seems to correspond with a study on humans after orthognathic surgery in which ALP and bone-specific ALP were used as markers of bone formation and osteoblastic activity. Enzyme levels significantly decreased 1 day and from 1 day to 1 week, respectively, after surgery. They then increased to their maximum values by 1 month before gradually returning to preoperative levels by six months.[23]

In agreement with this study, the biochemical parameters were assessed during fracture healing in 7 dogs with femoral fractures stabilized using string of pearls locking plates, in another study. Serum ALP was tested on the day prior to surgery, as well as on days 15 and 45 after the surgical operation. The results of a one-way ANOVA statistical analysis showed that there was a highly significant difference between the 3 intervals. The 15th postoperative day showed the highest value, whereas the 45th postoperative day showed the lowest value.[24]

Metformin significantly stimulated the gene and protein expression of essential osteoblastic transcription factors such as Runt-related transcription factor2 (RUNX2), enhanced ALP activity, and mineral deposition.[25] The increase in ALP enzymatic activity is a result of metformin's ability to stimulate cell proliferation and promote osteogenic differentiation. The stimulation of cells' proliferative activity and their differentiation into osteogenic cells appear to be solely dependent on metformin concentration, and this is according to one of the key findings of *in vitro* research that examine metformin-related osteogenic activity[26] and the dose used in our study exhibit osteogenecity.

Metformin increases BMP-2 expression through increasing ALP and osteocalcin (OCN) secretion, and this will increase BMD. Through the transactivation of genes via adenosine monophosphate-activated protein kinase (AMPK) regulation, metformin promotes osteoblast differentiation. The major osteogenic genes such ALP have been demonstrated to be expressed more strongly when AMPK is activated.[27]

All the previously mentioned studies agree with the results of the present study, which confirms the role of systemic metformin administration at a dose of 50 mg/kg on increasing serum ALP levels at 14 and 28 days after the surgical induction of bone defect.

It is observed in this study that the animals treated with metformin showed greater bone growth and mineralization than the control group. Despite the small and insignificant difference between the 2 groups on day 14, there was a significant difference between metformin and the untreated group on day 28.

The production of calcium and phosphorus-rich deposits in mineralized extracellular matrix is another sign of metformin's osteogenic activity as determined by an *in vitro* model.[28]

It appears that metformin's anti-adipogenic properties may be linked to its protective effects on bone tissue.[29]

Independent of age, BMI, or GFR, metformin administration is linked to a decreased incidence of osteopenia and osteoporosis, particularly in the female population.[30]

Other animal studies that examined the impact of metformin on bone and supported our findings revealed that metformin can raise bone mineral density and lessen bone loss in rats. In addition, both diabetic and nondiabetic rats treated with metformin had better bone repair.[31]

A recent study examined the effectiveness of metformin and glucosidase inhibitors on BMD in patients with type 2 diabetes mellitus (T2DM) and explored its mechanism of action in treating osteoporosis induced by T2DM, thereby providing references for the prevention and treatment of osteoporosis. It is obvious from this study's data and results that metformin increases the amount of bone formed and plays a critical role in preventing osteoporosis.[32] By reducing systemic inflammation and encouraging the production of osteoclasts, metformin can prevent osteoporosis.[33] Also, by enhancing BMP-2 expression and raising ALP and OCN secretion, it increased BMD.[34] Metformin promotes stimulation of osteopontin (OPN) and lowers RANKL expression in osteoblasts in rats with ovariectomies; according to Mai *et al*., osteogenesis was controlled by AMPK through OPN, whereas adipogenesis was suppressed.[35]

There is expectation that metformin can help prevent fractures induced by osteoporosis. Through a variety of receptors, including AMPK, which is crucial for bone regeneration, metformin regulates bone quality. In addition, metformin blocks the production of advanced glycation end products (AGEs), which may enhance bone turnover because AGEs inhibit osteoblasts (bone-forming cells) and activate osteoclasts.[36]

These data demonstrate that more complicated metabolic processes, particularly in T2DM, are the cause of fragility. The generation of AGEs and reactive oxygen species is induced by hyperglycemia. These substances may lead to improper bone homeostasis through oxidative stress.[37]

Our radiographic findings were consistent with a study showing that systemic metformin use improves osseointegration by raising bone filling percentages. During the 4-week osseointegration period, statistically significant variations in bone filling were found between the dental implants in the control and metformin-treated groups.[38]

Metformin has been proven in numerous studies to have anti-inflammatory and antioxidative stress effects on a number of disorders, including rheumatoid arthritis, neuropathic pain, renal problems, and Ankylosing spondylitis.[39] Therefore, metformin as it seems counteracts the oxidative stress and inflammation at bone lesion sites and promote osteogenesis.

Metformin use consistently increases the level of bone formation markers such as serum ALP, bone mineral density, and greater radiopacity observed by radiographic evaluation in metformin-treated animals compared to that of the control group at 2 different time intervals.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Institutional Animal Research Ethics Board's (Date: 19.06.2022 Decision No: UoM.Dent/A.L.58/22).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - H.R.M., T.G.A.; Design - H.R.M., T.G.A.; Supervision - T.G.A.; Resources - H.R.M.; Materials - H.R.M.; Data Collection and/or Processing - H.R.M.; Analysis and/or Interpretation - H.R.M.; Literature Search - H.R.M.; Writing Manuscript - H.R.M.; Critical Review - T.G.A.

Declaration of Interests: The authors have no conflicts of interest to declare.

Funding: The authors declared that this study has received no financial support.

Etik Komite Onayı: Bu çalışma için etik komite onayı Hayvan Hakları Etik Kurulu'ndan alınmıştır (Tarih: 19.06.2022, Karar No: UoM.Dent/A.L.58/22).

Hakem değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Fikir - H.R.M., T.G.A.; Tasarım - H.R.M., T.G.A.; Denetleme - T.G.A.; Kaynaklar - H.R.M.; Malzemeler - H.R.M.; Veri Toplanmasıve/veya İşlemesi - H.R.M.; Analiz ve/veya Yorum - H.R.M.; Literatür Taraması - H.R.M.; Yazıyı Yazan - H.R.M.; Eleştirel İnceleme - T.G.A.

Çıkar Çatışması: Yazarlar çıkar çatışması bildirmemişlerdir.

Finansal Destek: Yazarlar bu çalışma için finansal destek almadıklarını beyan etmişlerdir.

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