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ORIGINAL ARTICLE

HISTOLOGICAL AND HISTOMORPHOMETRIC EVALUATION OF THE SYSTEMIC METFORMIN ADMINISTRATION ON BONE HEALING IN RABBITS

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Summary

Background: After injuries, infections, or tumor removal, endogenous healing depends on bone repair. Disorders of bone healing are difficult to treat in clinical settings. There are numerous induced methods for correcting bone abnormalities, such as the induced membrane technique, allogenic bone grafting, synthetic bone grafting, artificial joint replacement, and autologous bone grafting. However, the delivery of the bone graft and bone filling materials necessitates surgical implantation at the fracture site, which could cause edema, infection, and the development of heterotopic bone locally. Therefore, systemically administered osteogenic drugs will provide an excellent method for bone lesion healing. **Aim of the study:** to evaluate the systemic effect of metformin on bone healing after surgical induction of bony defect and to determine the amount of newly formed bone using histological, histomorphometric analysis, and the surface area measurement of newly formed bone. Also to study the safety of metformin administration at the administered dose for this purpose. **Materials and methods:** Twenty mature male New Zealand rabbits were separated into two groups, each including ten rabbits for the study. The same surgical procedure was performed on all rabbits. Two holes were made at the femur (3 mm in diameter and 3 mm in depth) and left empty. Metformin tablets were ground into a fine powder and the resultant powder was dissolved in 10ml of water to prepare a liquid dosage containing 50 mg /1ml of metformin. Metformin is administered orally to the rabbits through a feeding tube at a dose of 50 mg/kg body weight. Animals were euthanized at two-time intervals, 14 and 28 days. The femur was separated, sectioned preserved, and sent for histological analysis and histomorphometry. **Results:** The results revealed that there is an increase in new bone formation and bone-forming cells in the metformin-treated group. **Conclusion:** Metformin increases bone healing by increasing the number of bone-forming cells and the surface area of newly formed bone tissues and causes less inflammatory response at the site of a bone lesion. So it possesses an osteogenic effect.

Key words: Metformin; systemic; osteoblast; osteoclast; bone healing

Introduction

Bone accomplishes a variety of functions, such as serving as the primary supporting structure for the activity of muscles, ligaments, and tendons, providing mechanical support, and protecting vital tissues (1). The bone is one

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of the few tissues in the human body that can regenerate and repair without leaving scars. A complicated bone healing process is carried out after a bone injury to restore bone structure and function (2). After injuries, infections, or tumor removal, endogenous healing depends on the tightly controlled process of bone repair. Disorders of bone healing, such as non-union or significant bone abnormalities, continue to be difficult to treat in clinical settings (3).

Bone regeneration can occur through either endochondral ossification or intramembranous ossification processes. Mesenchymal stem cells (MSCs) immediately differentiate into osteoblasts during intramembranous ossification, which leads to the deposition of a mineralized extracellular matrix. This kind of healing is frequently observed in fractures that are tightly repaired, have a small fracture space, and include bone metaphysis. The standard stages of endochondral ossification which are inflammation, soft and then hard callus formation, and lastly remodeling of the fracture site are used to treat fractures in the diaphysis, which have poorer mechanical stability and a fracture gap (1).

Bone homeostasis constantly undergoes remodeling, including bone resorption by osteoclasts and bone formation by osteoblasts (4). Inflammation, proliferation, and bone remodeling are three closely related and overlapping stages of the healing of bones (5).

Malignant bone tumors and severe trauma can remove a significant portion of bone, resulting in massive bone deficiencies. There are numerous methods for correcting bone abnormalities, such as the induced membrane technique, allogenic bone grafting, synthetic bone grafting, artificial joint replacement, and autologous bone grafting. The size and location of the problem are two parameters that are taken into consideration when determining the treatment strategy (6).

The only FDA-approved treatments for long bone fracture non-unions and recalcitrant long bone non-unions, respectively, are bone morphogenetic protein-2 (BMP-2) and BMP-7 (7). However, the BMP supply needs surgical implantation at the fracture site, which could cause local problems such as edema, infection, and the creation of heterotopic bone. Additionally, to obtain therapeutic effects, the administration of supraphysiological doses is frequently necessary, which results in unfavorable side effects, clinical difficulties, and prohibitive and high costs (8).

Several medications with widely divergent indications that exhibit a pleiotropic spectrum of actions are used to target local and systemic regulation of bone metabolism. These include medications for hyperlipidemia (HMG-CoA reductase inhibitors), ACE inhibitors for hypertension, bisphosphonates for osteoporosis, proteasome inhibitors for cancer, and others (9).

Metformin belongs to a group of drugs known as biguanides. Biguanides are a significant class of oral hypoglycemic medications that work by decreasing resistance to the peripheral effects of insulin, increasing the density of low and high-affinity insulin receptors, and lowering the liver's ability for gluconeogenesis (10).

From the oral antihyperglycemic drugs, metformin is the most often recommended one for the management of type 2 diabetes. Metformin treatment for diabetic patients has been demonstrated to lower TNF expression (11), with documented anti-inflammatory action (12). Mesenchymal stem cells (MSCs) have significantly less osteogenic capacity when there is persistent periodontal inflammation. An essential objective is to develop a highly efficient approach to enhance or restore the osteogenic potential of MSCs in an inflammatory environment (13). Metformin is the first-line oral treatment for type 2 diabetes due to its inexpensive cost, reasonable safety, minimal risk of hypoglycemia, lack of weight gain, and few side effects (14). Therefore, this study focuses on the effect of metformin on bone healing.

Material and methods

Experimental model

In our investigation, 20 mature male New Zealand rabbits weighed between 1.75 and 2 kg, and those 6 to 8 months old were used. The animals were kept in uniform conditions, under identical housing and feeding arrangements, and were fed a conventional diet of wheat and fresh vegetables. Under the supervision of a veterinarian to monitor

their general and overall health. The rabbits were kept in their cages in the College of Veterinary Medicine's animal facility at the University of Mosul where the study was done. The animals will be euthanized at the end of the experiments using an overdose of anesthesia (ketamine 200 mg/kg) with xylazine (40 mg/kg) (15). The study was done under the institutional animal research ethics committee's guidelines. The study was performed according to the requirements of the institutional animal research ethics committee on 19/6/2022 (UoM.Dent/A.L.58/22).

Experimental Medication

The medication used in this study is metformin tablets 500 mg (Glucophage)R. Metformin tablet was prepared in the form of liquid by the fine grinding of the tablets to obtain a fine powder. Each 500 mg tablet was ground alone, and the resultant fine powder of this tablet was packed into a hard gelatin capsule to control the amount of the drug (500 mg per capsule). For the oral administration of the drug, the content of each capsule was dissolved in 10ml of distilled water with good vigorous shaking for at least two minutes to obtain a homogenous solution containing 500 mg/10ml or 50 mg/1ml of the drug. Metformin is freely soluble in water and is practically insoluble in organic compounds like acetone, ether, and chloroform (16). 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 (17).

Study design

Twenty healthy male rabbits were taken and divided based on weight randomization method into two groups (Figure 1):

- **Group #1:** (n = 10, control) received no drug and was further divided into two subgroups. Five rabbits in each according to the sacrifice date on the 14th and 28th days.
- **Group #2:** (n = 10, treated). After the surgical procedure, metformin was administered orally in a once-daily dose of 50 mg/kg body weight (17) using a feeding tube. This group was subdivided into two groups (5 rabbits/period) according to the sacrifice date (14th and 28th day).

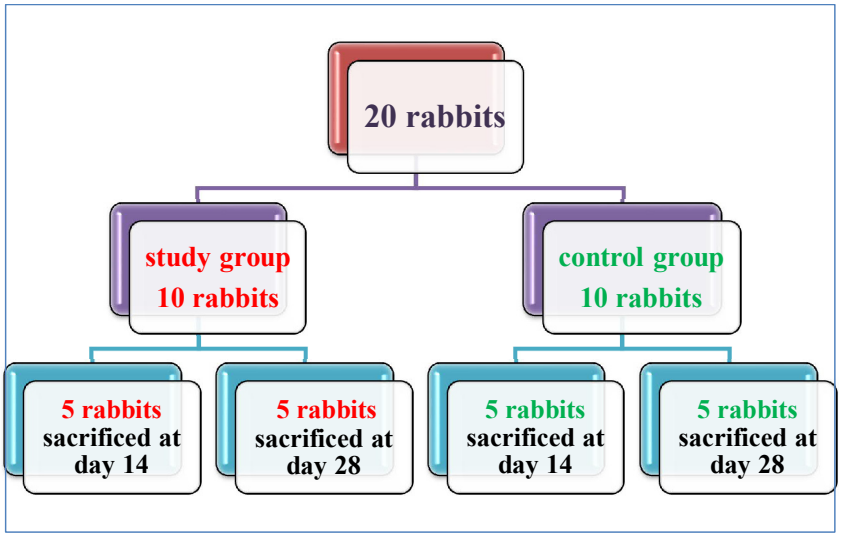

Figure 1. Experimental protocol for the studied groups.

Preparation of animals for surgery

All twenty rabbits received anesthesia by intramuscular injection. Each rabbit was given a mix of 40 mg/kg ketamine (KETALROM-50, romvac company, SA) with xylazine (Holland) 4 mg/kg injection intramuscularly

in the thigh muscle of the rabbit (18). The site of the operation was shaved using an electrical hair clipper. Then the site was cleaned well and disinfected with povidone-iodine 10% solution thoroughly and left to dry before incision. Now the animal is covered with sterile towels except at the site of operation.

Animal surgical procedure

The time required for the induction of anesthesia is about ten minutes to enter the non-surgical tolerance, but the surgical tolerance was started after twenty minutes, while initial recovery occurred after more than 60 minutes (19). Animals were placed in the right lateral position, covering the animal with a sterile towel except for the incision site, which was sterilized well with povidone-iodine. An operation was performed on the left femur bone. Without causing any muscle damage, a 1.5 cm incision was made over the femur bone toward its head using surgical blade number 15. The femoral bone was displayed as the two muscles parted. The periosteum was elevated, and the compact bone was exposed by blunt dissection using the Hawarth periosteal elevator. Two holes were made in the exposed femur using a 3 mm round carbide bur linked to a low-speed straight surgical handpiece with a dental engine spinning at 2000 rpm and continuous irrigation with normal saline. For all animals, the bone defect (holes) was left empty without any material. The surgical space dried well using sterile surgical gauze, then the wound was closed using a 3/0 black silk suture and rubbed well with povidone-iodine 10% disinfectant.

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. It was given as a single daily dose of 0.5 ml/kg intramuscularly for three consecutive days from the operation day. Rabbits were placed alone until recovered from anesthesia and regained full consciousness, they also undergo twenty-four hours of supervision after the operation to monitor their general health, physical activity, and feeding.

Bone specimen preparation for histopathological examination

Samples for histological studies were taken from the hole site at the end of the experiment and fixed in neutral buffer formalin 10% for hours to one week. Fixation is done immediately after taking the sample for examination. It serves to preserve and immobilize the sample. It can be done by freezing or using a fixative solution. All fixed specimens are washed in moderately flowing tap water for at least 30 minutes. Do not rinse with water that is moving quickly, then fixed samples were decalcified in formic acid (10%) for three weeks, and change the solution periodically until the decalcification is complete. It can take 24 hours, several days, or even months, depending on the size of the specimens, then washing in phosphate buffer saline (PBS) to remove the used fixative. Decalcified samples were trimmed and washed again to remove formic acid, dehydrated in ascending grades of ethanol (70%, 90%, and 100%), cleared in xylene, infiltrated, and embedded in paraffin wax. Using a microtome, serial sections of 5 microns were cut, and they were then stained with hematoxylin and eosin (H and E) (20).

The procedure could be explained briefly as follows (21): Sections first are deparaffinized in two xylene changes, each for 10 minutes, and dehydrate in two changes of absolute alcohol, each for 5 minutes (alcohol 95 % for two minutes and 70 % for two minutes, gently wash with distilled water. Stain for 8 - 10 minutes in (Harris hematoxylin solution). Wash for 15 minutes under running water then differentiate for 30 seconds in 1% acid alcohol. Wash again for one minute under running water and rinse with 10 dips in 95 % alcohol. Counterstain for 30 seconds to 1 minute in eosin Y solution and dehydrate using two changes of 95% absolute alcohol, each lasting five minutes. Clear in two xylene changes that last five minutes for each and mount with xylene as a mounting medium.

Examination of prepared histopathological slides

The specimens were stored in 10% formaldehyde and then were processed to create slides stained with hematoxylin and eosin reagents and inspected under a light microscope at three power levels (40x, 100x, and 400x). The degree of repair of the bone defect was evaluated histopathologically using an Olympus CX31 light microscope.

Histomorphometry analysis

A histomorphometry analysis was done to count the number of osteoblasts, osteocytes, and osteoclasts for the new bone developed in the lesion location and to quantify the thickness of the trabecular bone. Scope Image 9.0 China, a USB 2.0 color digital camera was utilized for the measurement along with image-processing software. The software of the camera was calibrated to all lenses of the microscope with the aid of a 0.01 mm stage micrometer (ESM-11/Japan). By trapping the bony spicules within a field area of the field surface area = 5242701 μm^2 field area at 40x power level, the newly formed bone area was measured in square micrometers. By calculating the number of cells per 400X field (five fields for each group), the average number of osteoblasts and osteoclasts was determined (23). In these criteria, the number of osteoblast, osteoclast, and osteocytes was measured together with the surface area of newly formed bone.

Statistical Analysis

Statistical analysis was done using IBM SPSS Statistics 26.0 for all the results. Data were expressed as (means ± stander error). One-way ANOVA was used for analysis followed by Tukey's test for comparison between different groups of the study at two different time intervals. Statistical analysis comparisons were considered insignificant if ($p \geq 0.05$), significant at ($p \leq 0.05$), and p-value ($p \leq 0.001$) considered highly significant.

Results

Histopathological results

On day 14

In the control group, the site of the hole shows the poor formation of new trabecular bone (bonnie spicules), scanty and small areas of woven bone, and infiltration of inflammatory cells. Also, the surface area of the newly developed trabecular bone is much less than that in the metformin-treated group (Figure 2). In the metformin-treated group, the femoral bone hole is shown to have a well-developed new trabecular bone growth (bonnie spicules), modest infiltration of inflammatory cells, more woven bone and connective tissue than in the control group, fresh trabecular bone growth has a larger surface area (Figures 2).

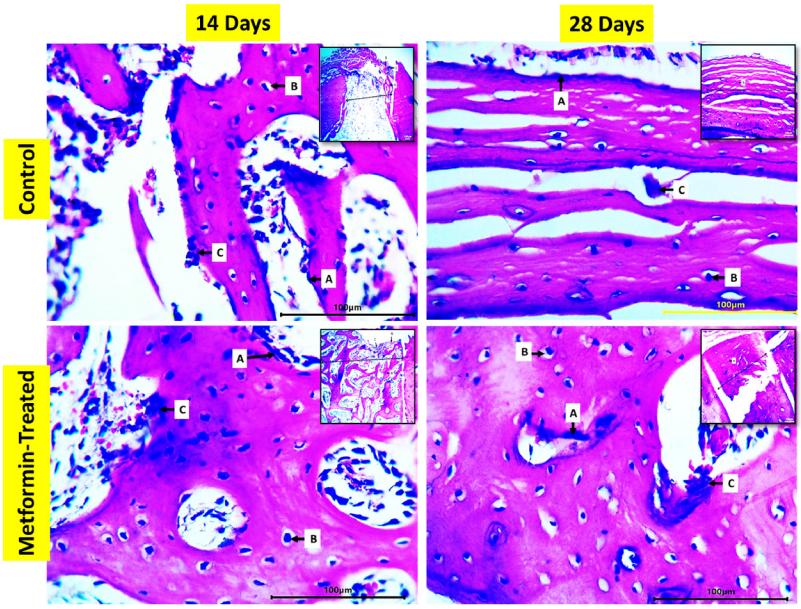

Figure 2. A representative image for histological findings of femur bone of the control versus a metformin-treated group over two-time points at 14 days and 28 days. H&E stain, scale bar 100µm.

On day 28

In the control group, the hole's area is seen with fully formed new trabecular bone development (bonnie spicules) without any signs of inflammation. The surface area of newly formed bone is greater than in the 14th day control group but less than in the 28th day of treatment group as shown in Figure 2. A very well-developed new trabecular bone formation (bonnie spicules) is present at the site of the hole in the group that received metformin treatment, with good or well-developed vascularity, without inflammation, and the greatest surface area of newly formed bone among all other groups (Figure 2).

Histomorphometric results statistical analysis

The number of osteoblasts

On day 14, the treatment group's osteoblast formation rate significantly increased more than the control group and the one-way ANOVA test showed that there was a highly significant difference between the groups during this period ($p \leq 0.001$).

In the 28th day period, the number of osteoblasts in the metformin-treated group decreased, and it was less than in the control group, but one-way ANOVA also showed a highly significant difference was found between these two groups in the 28 days ($p \leq 0.001$) (Table 1 and Figure 2).

Tukey's test in one-way ANOVA expressed that highly significant differences exist between all groups at both periods of the experiment.

Table 1. The number of osteoblasts per field (0.06 mm^2) in the control and treated group after 14 and 28 days of femur bone hole operation.

	14 days period	28 days period
Control group	31.5 ± 0.26	19.3 ± 0.56
Treat group	43.3 ± 0.46	16.5 ± 0.4
P value	0.000	0.000

The number of osteocytes

On day 14, there was an increment in the number of osteocytes found in the treatment group over that in the control group, and the one-way ANOVA test showed that there was a highly significant difference between the metformin-treated and the control groups during this period ($p \leq 0.001$).

On day 28, there was an increase in the number of osteocytes in the treatment group over the control group, and a highly significant difference was found between these two groups during the 28 days ($p \leq 0.001$) (Table 2 and Figure 2).

Table 2. The number of osteocytes per field (0.06 mm^2) in the control and metformin group at two-time intervals of the operation.

	14 days period	28 days period
Control group	21.8 ± 0.4	35 ± 0.18
Treat group	39.4 ± 0.42	50.6 ± 0.5
P value	0.000	0.000

Tukey's test in one-way ANOVA expressed that highly significant differences exist between all groups at both periods of the experiment.

The number of osteoclasts

On day 14, The osteoclast number found in the treatment group was slightly lower than in the control group, but according to the one-way ANOVA test which revealed no statistically significant difference between the groups at this time. ($p \geq 0.05$).

At 28 days period, the number of osteoclasts both in the treatment and the control group is approximately equal and one-way ANOVA test results revealed that there was no significant difference between these two groups at day 28, and ($p \geq 0.05$) (Table 3 and Figure 2).

Table 3. The numbers of osteoclasts per field (0.06 mm^2) in control and treated groups at 14 and 28 days.

	14 days period	28 days period
Control group	2.07 ± 0.20	1.04 ± 0.15
Treat group	1.36 ± 0.31	0.95 ± 0.22
P value	0.158	0.992

Tukey's test in one-way ANOVA expressed that a significant difference was found between the control group at 14 and 28 days ($p = 0.025$), and between the control group at day 14 and the treated group at day 28 where ($p = 0.014$).

The surface area measurements of new bone formation in cubic micrometers

The field surface area = 5242701 μm^2. It is observed that the entire surface area of newly formed trabecular bone increased significantly in the treated group after 14, and 28 days (Table 4 and Figure 2).

At 14 days

The total surface area in (μm^2) of newly formed trabecular bone in the treated group (185432 ± 1143) is much more than the control group (85855 ± 1405), and the one-way ANOVA test showed a highly significant difference existing at this period between the two groups ($p \leq 0.001$), (Figure 2).

At 28 days

Also, the treatment group exceeds the control group in the total surface area of newly formed bone. The surface area in the treated group was (237520 ± 6.86) greater than in the control group (226281 ± 7.10), but the one-way ANOVA test revealed that a non-significant difference was found between the metformin-treated and the control groups at this time of the experiment ($p = 0.124$), (Table 4 and Figure 2).

Tukey's test in one-way ANOVA expressed that a highly significant difference was found between the groups at both time intervals except that between the metformin-treated and the control group at day 28 where the difference is insignificant ($p \geq 0.05$).

Table 4. The total surface area of new trabecular bone formed in control and treated groups at two-time intervals (14 and 28 days).

	14 days period	28 days period
Control group	85855 ± 1405	226281 ± 7.10
Treat group	185432 ± 1143	237520 ± 6.86
P value	0.000	0.124

Discussion

The results of this study show that systemic metformin exhibits more osteogenic effects, a larger surface area of a new bone formation, and a healing tendency in the metformin-treated group at both time intervals, the 14th and

28th days of treatment. This result is in agreement with a recent study that demonstrated that metformin promoted osteogenesis in UC-MSCs (UC is one of the most important sources of mesenchymal stem cells) (24). Also, results in parallel with numerous studies have shown that metformin regulates bone marrow homeostasis and metabolism (25). It protected against glucose-damaged osteoblasts, encouraged their differentiation and mineralization of MSCs and osteoblasts produced from induced pluripotent stem cells, and even directly contributed to osteoblast proliferation (26).

This study showed a decrease in the number of osteoclasts in the metformin-treated group, this result may belong to the ability of metformin to suppress osteoclast differentiation and explain how it has osteogenic effects (27). Osteoblast and osteoclast cells are responsible for bone remodeling, where there is resorption and then mineralization occurs. The receptor of nuclear factor-B (RANK) ligand (RANKL) and osteoprotegerin (OPG) are the two main factors that control osteoclastogenesis, the process by which osteoclasts (bone-resorbing cells) are formed. The osteoblast produces RANKL, which binds to the pre-osteoclast RANK to form a fused polykaryon, a multinuclear cell that develops into a mature and functional osteoclast (28).

On the contrary, (OPG), which is expressed by osteoblasts as well, is a RANKL fake receptor and can thus stop osteoclastogenesis (29). The formation, activation, differentiation, and survival of osteoclasts can all be directly affected by the inhibition brought on by OPG. Since osteoblasts produce both RANKL and OPG, it follows that osteoblasts are essential for maintaining balanced bone remodeling and for ensuring a balanced RANKL/OPG ratio required (30).

Metformin has the ability to the polarization of the macrophages and the inhibition of inflammatory activation (31). Metformin treatment for diabetes patients has been demonstrated to lower TNF- expression, (12) with verified anti-inflammatory action (13).

According to some research, metformin promotes preosteoblast and MSC differentiation, which has an osteogenic effect (32). This study showed that metformin has the ability of pro-osteogenic action, which highlights its potential application in bone regeneration medicine as a medication to enhance bone tissue's ability for self-renewal and as a catalyst for osteoblast lineage differentiation of adipose-derived multipotent stromal cells (33). The mechanisms that explain the metformin osteogenic effect may be explained by both in vivo and in vitro studies, which showed that metformin had an osteogenic impact that may have been mediated through Runx2 (Runt-related transcription factor 2) and activation of AMPK (adenosine monophosphate-activated protein kinase) (34). The mesenchymal stromal cells (MSC) produce osteoblasts, which build up bones during osteogenesis. The differentiation of MSC can either produce adipocytes or osteoblasts, depending on the body's signal. Increased bone production can result from the differentiation of MSC into osteoblasts, which is facilitated by AMPK stimulation. Runx2 is necessary for the maturation of chondro-cytes and the proper development of the skeleton mediates these effects through activating AMPK which will start the Runx2 pathway (35). Additionally, because AMPK is inhibited, less alkaline phosphatase, osteocalcin, and type 1 collagen are produced, all of which are necessary for the development of healthy bones. Furthermore, it is demonstrated that AMPK inhibition will result in reduced Runx2 and alkaline phosphatase cellular activity (36).

Runx2 has been reported to have two main activities: promoting osteogenesis and inhibiting adipocyte differentiation. It has been found to play an essential role in MSC osteogenic differentiation ability (37). As determined by western blotting, it is confirmed that metformin might directly stimulate AMPK phosphorylation (38).

Several receptors, such as adenosine monophosphate-activated protein kinase (AMPK), are involved in metformin's effects on bone quality. Therefore, AMPK is essential for bone regeneration (35). Metformin also inhibits advanced glycation end product (AGE), which can raise bone turnover (39).

The differentiation of the osteoblasts and the formation of bone may be significantly influenced by metformin administration, which acts as an AMPK activator. This may be related to metformin's indirect or combinatorial effects, as well as the direct effects of the AMPK signaling pathway, Runx2/AMPK signaling pathway, and their interactions (40). Further studies and research are required to confirm the effect of systemic administration of metformin on the healing of bone lesions. The results of a recent study showed that metformin monotherapy was superior

to other antidiabetic treatments for reducing oxidative stress and enhancing antioxidant defenses and as a result, offer protection from harm brought on by oxidative stress during diabetes and its side effects (41).

Metformin may improve bone quality by lowering blood sugar decreasing hepatic gluconeogenesis and improving insulin sensitivity and secretion. This process is thought to occur independently of AMPK and is currently understood to involve hepatocyte-based fructose 1,6-biphosphatase inhibition (40). Metformin's effect on bone in our results may also belong to its favorable effects on lipid profile, liver function, and bone mineral density as well as bone turnover (42). Metformin was shown to improve liver metabolism during glucocorticoid excess, which in turn could have positive effects on bone health (43). Metformin has been shown in preventing osteoporosis-related fractures. It modulates bone quality through numerous receptors, including adenosine monophosphate-activated protein kinase (AMPK), which is of most importance for bone regeneration (44). Metformin also inhibits advanced glycation end product (AGE), which may promote bone turnover (39). Despite the aforementioned effects of metformin bone-inducing effects via mesenchymal stem cells, there might be variation between different studies due to variations in the response of stem cells according to localized oxygen concentration because the differentiation itself is oxygen-dependent (45, 46) and responsive to the localized milieu of present immunomodulatory cytokines (47, 48). In addition, Metformin can suppress osteoclastogenesis in cell cultures in a dose-dependent manner by reducing the expression of RANKL and by stimulating the expression of OPG. Systemic metformin application positively affects osseointegration by increasing the percentages of bone filling (BF) ratios. Statistically significant differences in BF were detected between the dental implants of the control and metformin treatment groups during the four-week osseointegration period (49). The ability of metormin to lower glucose levels without causing hypoglycemia is a key benefit of the medication. Additionally, this drug normally has few side effects and is well-taken. Gastrointestinal problems like nausea and diarrhea are frequent metformin side effects Metformin has also been shown to prolong life in animal models and decrease overall mortality in humans (50).

Study limitations

The limitations of this study represented by that liquid dosage form or topical preparations of metformin is not available in Iraq and most countries therefore we prepared it daily at the time of the study before each dosing process. The number of sample animals in each group was little than desired for a more accurate study analysis, despite we obtained good and expressive results.

Conclusion

The administration of metformin systemically can induce bone regeneration, clear and quick results was obseved at the early stages (14 days) of the study. Systemic metformin increased bone healing by increasing the number of osteoblasts, osteocytes, bone matrix deposition, osteoid, and new trabecular bone formation, it counteracts the effect of hyperglycemia on bone tissue, reducing oxidative stress and enhancing antioxidant defenses and as a result, offer protection from harm brought on by oxidative stress during diabetes and its side effects. It also exerts an anti-inflammatory effect at the site of bone lesions and counteracts the deteriorating effect of excessive inflammatory response at the lesion or fracture site.

Acknowledgments

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Conflicts of interest

The authors declared no competing interests.

Adherence to Ethical Standards

This study followed the ethical committee's instructions and guidelines and received ethical approval no. UoM.Dent/A.L.58/22.

References

1. Pajarinen J, Lin T, Gibon E, et al. Mesenchymal stem cell-macrophage crosstalk and bone healing. Biomaterials. 2019;196:80-9. https://doi.org/10.1016/j.biomaterials.2017.12.025
2. Schell H, Duda GN, Peters A, et al. The hematoma and its role in bone healing. Journal of experimental orthopedics. 2017;4(1):1-1. https://doi.org/10.1186/s40634-017-0079-3
3. Bell A, Templeman D, Weinlein JC. Nonunion of the femur and tibia: an update. Orthopedic Clinics. 2016;47(2):365-375. https://doi.org/10.1016/j.ocl.2015.09.010
4. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003 May;423(6937):337-342. https://doi.org/10.1038/nature01658
5. Medhat D, Rodríguez CI, Infante A. Immunomodulatory effects of MSCs in bone healing. International Journal of Molecular Sciences. 2019;20(21):5467. https://doi.org/10.3390/ijms20215467.
6. Aoki K, Saito N. Biodegradable polymers as drug delivery systems for bone regeneration. Pharmaceutics. 2020;12(2):95. https://doi.org/10.3390/pharmaceutics12020095.
7. Ratko TA, Belinson SE, Samson DJ, et al. Bone Morphogenetic Protein: The State of the Evidence of On-Label and Off-Label Use (Internet). https://europepmc.org/article/nbk/nbk284865
8. Wang P, Ma T, Guo D, et al. Metformin induces osteoblastic differentiation of human induced pluripotent stem cell-derived mesenchymal stem cells. Journal of tissue engineering and regenerative medicine. 2018;12(2):437-446. https://doi.org/10.1002/term.2470
9. Rothe R, Schulze S, Neuber C, et al. Adjuvant drug-assisted bone healing: Part III–Further strategies for local and systemic modulation. Clinical Hemorheology and Microcirculation. 2019;73(3):439-488. https://doi.org/10.3233/CH-199104
10. Kobayashi M, Yamazaki K, Hirao K, et al. The status of diabetes control and antidiabetic drug therapy in Japan—a cross-sectional survey of 17,000 patients with diabetes mellitus (JDDM 1). Diabetes research and clinical practice. 2006;73(2):198-204. https://doi.org/10.1016/j.diabres.2006.01.013
11. Andrews M, Soto N, Arredondo M. Effect of metformin on the expression of tumor necrosis factor-α, Toll-like receptors 2/4 and C reactive protein in obese type-2 diabetic patients. Revista medica de Chile. 2012;140(11):1377-1382. https://doi.org/10.4067/s0034-98872012001100001
12. Koh SJ, Kim JM, Kim IK, et al. Anti-inflammatory mechanism of metformin and its effects in intestinal inflammation and colitis-associated colon cancer. Journal of gastroenterology and hepatology. 2014;29(3):502-510. https://doi.org/10.1111/jgh.12435
13. Ren C, Hao X, Wang L, et al. Metformin Carbon Dots for promoting periodontal bone regeneration via activation of ERK/AMPK pathway. Advanced Healthcare Materials. 2021;10(12):2100196. https://doi.org/10.1002/adhm.202100196
14. Huang W, Castelino RL, Peterson GM. Metformin usage in type 2 diabetes mellitus: are safety guidelines adhered to? Internal medicine journal. 2014;44(3):266-272. https://doi.org/10.1111/imj.12369
15. de Lima Santos A, da Silva CG, de Sá Barreto LS, et al. A new decellularized tendon scaffold for rotator cuff tears–evaluation in rabbits. BMC Musculoskeletal Disorders. 2020;21(1):1-2. https://doi.org/10.1186/s12891-020-03680-w
16. Merkhan MM. Effect of metformin, glibenclamide, and insulin on lipid profile in type 2 diabetic patients. Tikret Journal of Pharmaceutical Sciences. 2013;9(2).
17. Ikewuchi CC, Ikewuchi JC, Ifeanacho MO. Restoration of plasma markers of liver and kidney functions/integrity in alloxan-induced diabetic rabbits by aqueous extract of Pleurotus tuberregium sclerotia. Biomedicine & Pharmacotherapy. 2017;95:1809-1814. https://doi.org/10.1016/j.biopha.2017.09.075
18. Ahirwar LK, Kalra P, Sharma S, et al. Linezolid shows high safety and efficacy in the treatment of Pythium insidiosum keratitis in a rabbit model. Experimental Eye Research. 2021;202:108345. https://doi.org/10.1016/j.exer.2020.108345.
19. Jiron JM, Mendieta Calle JL, Castillo EJ, et al. Comparison of isoflurane, ketamine–dexmedetomidine, and ketamine–xylazine for general anesthesia during oral procedures in rice rats (Oryzomys palustris). Journal of the American Association for Laboratory Animal Science. 2019;58(1):40-49. https://doi.org/10.30802/AALAS-JAALAS-18-000032
20. Garcia-Donas JG, Dalton A, Chaplin I, et al. A revised method for the preparation of dry bone samples used in the histological examination: Five simple steps. Homo. 2017;68(4):283-288. https://doi.org/10.1016/j.jchb.2017.07.001.

21. Ahmed HN, Ghada AT, Wael T. The Effect of Xylitol on Osteoclastogenesis in Experimentally Induced Bone Defect in Rabbits. Journal of Applied Veterinary Sciences. 2022;7(1):58-63. https://doi.org/10.21608/JAVS.2021.104726.1114
22. Abdo FS. Effects of Combination of Platelet Rich Plasma and OSTEON Material in Rabbits Bone Healing (A comparative study). Al-Rafidain Dental Journal. 2014;14(1):90-100. https://doi.org/10.33899/rden.2014.89258
23. Mohammed ZS, Taqa GA, Sulaiman MS. Evaluating the local and systemic effects of silicon dioxide on healing of mandibular bone in rabbits. InAIP Conference Proceedings 2022 (Vol. 2547, No. 1, p. 020006). AIP Publishing LLC. https://doi.org/10.1063/5.0116015
24. Shen M, Yu H, Jin Y, et al. Metformin facilitates osteoblastic differentiation and M2 macrophage polarization by PI3K/AKT/mTOR pathway in human umbilical cord mesenchymal stem cells. Stem Cells International. 2022;2022. https://doi.org/10.1155/2022/9498876
25. Zhou R, Ma Y, Qiu S, et al. Metformin promotes cell proliferation and osteogenesis under high glucose conditions by regulating the ROS AKT mTOR axis. Molecular Medicine Reports. 2020;22(4):3387-3395. https://doi.org/10.3892/mmr.2020.11391
26. Zhao R, Yang R, Cooper PR, et al. Bone grafts and substitutes in dentistry: A review of current trends and developments. Molecules. 2021;26(10):3007. https://doi.org/10.3390/molecules26103007.
27. Son HJ, Lee J, Lee SY, et al. Metformin attenuates experimental autoimmune arthritis through reciprocal regulation of Th17/Treg balance and osteoclastogenesis. Mediators of inflammation. 2014. https://doi.org/10.1155/2014/973986
28. Crockett JC, Rogers MJ, Coxon FP, et al. Bone remodeling at a glance. Journal of cell science. 2011;124(7):991-998. https://doi.org/10.1242/jcs.063032
29. Chen X, Wang Z, Duan N, et al. Osteoblast–osteoclast interactions. Connective tissue research. 2018;59(2):99-107. https://doi.org/10.1080/03008207.2017.1290085
30. Owen R, Reilly GC. In vitro models of bone remodeling and associated disorders. Frontiers in bioengineering and biotechnology. 2018;6:134. https://doi.org/10.3389/fbioe.2018.00134
31. Qing L, Fu J, Wu P, et al. Metformin induces the M2 macrophage polarization to accelerate wound healing via regulating AMPK/mTOR/NLRP3 inflammasome singling pathway. American journal of translational research. 2019;11(2):655.
32. Bahrambeigi S, Yousefi B, Rahimi M, et al. Metformin; an old antidiabetic drug with new potentials in bone disorders. Biomedicine & Pharmacotherapy. 2019;109:1593-1601. https://doi.org/10.1016/j.biopha.2018.11.032.
33. Śmieszek A, Tomaszewski KA, Kornicka K, et al. Metformin promotes osteogenic differentiation of adipose-derived stromal cells and exerts pro-osteogenic effect stimulating bone regeneration. Journal of clinical medicine. 2018;7(12):482. https://doi.org/10.3390/jcm7120482.
34. Zhen D, Chen Y, Tang X. Metformin reverses the deleterious effects of high glucose on osteoblast function. Journal of Diabetes and its Complications. 2010;24(5):334-344. https://doi.org/10.1016/j.jdiacomp.2009.05.002.
35. Seetharaman R, Mahmood A, Kshatriya P, et al. An overview of stem cells in tissue regeneration. Current pharmaceutical design. 2019;25(18):2086-2098. https://doi.org/10.2174/1381612825666190705211705.
36. Chai X, Zhang W, Chang B, et al. GPR39 agonist TC-G 1008 promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Artificial Cells, Nanomedicine, and Biotechnology. 2019;47(1):3569-3576. https://doi.org/10.1080/21691401.2019.1649270.
37. Chava S, Chennakesavulu S, Gayatri BM, et al. Novel phosphorylation by AMP-activated kinase regulates RUNX2 from ubiquitination in osteogenesis over adipogenesis. Cell death & disease. 2018;9(7):1-6. https://doi.org/10.1038/s41419-018-0791-7.
38. Sun R, Liang C, Sun Y, et al. Effects of metformin on the osteogenesis of alveolar BMSCs from diabetic patients and implant osseointegration in rats. Oral Diseases. 2022;28(4):1170-1180. https://doi.org/10.1111/odi.13808.
39. Gonnelli S, Caffarelli C, Giordano N, et al. The prevention of fragility fractures in diabetic patients. Aging Clinical and Experimental Research. 2015;27(2):115-124. https://doi.org/10.1007/s40520-014-0258-3
40. Jiating L, Buyun J, Yinchang Z. Role of metformin on osteoblast differentiation in type 2 diabetes. BioMed Research International. 2019;2019. https://doi.org/10.1155/2019/9203934.
41. Almulathanon AA, Mohammad JA, Fathi FH. Comparative effects of metformin and glibenclamide on the redox balance in type 2 diabetic patients. Pharmacia. 2021;68(2):327-332. https://doi.org/10.3897/pharmacia.68.e63365

42. Pernicova I, Kelly S, Ajodha S, et al. Metformin to reduce metabolic complications and inflammation in patients on systemic glucocorticoid therapy: a randomized, double-blind, placebo-controlled, proof-of-concept, phase 2 trial. The lancet Diabetes & endocrinology. 2020;8(4):278-291. https://doi.org/10.1016/S2213-8587(20)30021-8
43. Zhu X, Yan H, Chang X, et al. Association between non-alcoholic fatty liver disease-associated hepatic fibrosis and bone mineral density in postmenopausal women with type 2 diabetes or impaired glucose regulation. BMJ Open Diabetes Research and Care. 2020;8(1):e000999. http://dx.doi.org/10.1136/bmjdrc-2019-000999
44. Bahrambeigi S, Yousefi B, Rahimi M, et al. Metformin; an old antidiabetic drug with new potentials in bone disorders. Biomedicine & Pharmacotherapy. 2019;109:1593-1601. https://doi.org/10.1016/j.biopha.2018.11.032.
45. Merkhan MM, Shephard MT, Forsyth NR. Physoxia alters the human mesenchymal stem cell secretome. Journal of Tissue Engineering. 2021;12:20417314211056132. https://doi.org/10.1177/20417314211056132.
46. Forsyth NR, Steeg R, Ahmad M, et al. Mimicking Physiological Oxygen in Cell Cultures. InCell Culture Technology 2018 (pp. 129-137). Springer, Cham. https://doi.org/10.1007/978-3-319-74854-2_8.
47. Chen L, Merkhan MM, Forsyth NR, et al. Chorionic and amniotic membrane-derived stem cells have distinct and gestational diabetes mellitus independent, proliferative, differentiation, and immunomodulatory capacities. Stem Cell Research. 2019;40:101537. https://doi.org/10.1016/j.scr.2019.101537
48. Shephard MT, Merkhan MM, Forsyth NR. Human Mesenchymal Stem Cell Secretome Driven T Cell Immunomodulation Is IL-10 Dependent. International Journal of Molecular Sciences. 2022;23(21):13596. https://doi.org/10.3390/ijms232113596.
49. Yıldırım TT, Dündar S, Bozoğlan A, et al. The effects of metformin on the bone filling ration around of TiAl6Va4 implants in non diabetic rats. Journal of Oral Biology and Craniofacial Research. 2020;10(4):474-477. https://doi.org/10.1016/j.jobcr.2020.07.012.
50. Bonnet F, Scheen A. Understanding and overcoming metformin gastrointestinal intolerance. Diabetes, Obesity and Metabolism. 2017;19(4):473-481. https://doi.org/10.1111/dom.12854