BACKGROUND: Disorders of bone healing still constitute real challenges in clinical care today. However, the bone filling materials delivery requires surgical implantation at the site of fracture, which may result in local complications. Therefore administered osteogenic drugs will provide an excellent method for bone lesion healing. Metformin osteogenic effect through increasing osteoblasts and decreasing osteoclasts

Aim of the study: to evaluate the systemic effect of metformin administration on bone healing at bony defect site by measuring the gene expression of osteocalcin and vitamin D receptors

Material and Methods: Twenty mature male rabbits were used and separated into two groups, ten in each group. Under general anesthesia, the same surgical procedure was performed on all rabbits. After the femur is surgically exposed, two holes 3 mm in diameter and 3 mm depth are prepared and left empty. The study lasts for 28 days. Metformin administered orally to the rabbits in a dose of 50 mg/kg for 28 days. Animals were sacrificed at two times intervals according to their groups at 14th and 28th day after surgery according to several studies. The femur isolated, sectioned, and bone specimens taken from the site of defect, the specimen placed in phosphate buffer saline until assessed for quantitative-PCR (QPCR).

Result: showed that there was an increase in the quantitative gene expression of both osteocalcin and vitamin D receptors in the metformin-treated group than in the control group in both study time periods.

Conclusion: Metformin increase bone healing and regeneration at the bone defect sites and enhance the process of osteogenesis and osseointegration more than the control untreated rabbits.

Keywords: Metformin, Gene expression, Bone healing, Bone defect.

Introduction

The structure and composition of the bone, a complex, hierarchically structured organ system, are significantly influenced by the demands placed on it by its function. Bone tissue is constantly undergoing turnover via coordinated activities by osteoblasts, osteoclasts, osteocytes, and their precursors. Through this process of bone remodeling, the bone organ system can respond relatively quickly to changes in metabolic and mechanical needs [1].

Bone has the ability to regenerate with a scar-free healing. Following bone injury, a complex bone healing process aimed to restore bone shape and function takes place. After injuries, infections, or tumor removal, endogenous healing depends on the tightly regulated process of bone repair. Disorders of bone healing, such as non-union or significant bone abnormalities, remain to be difficult to treat in clinical settings [2, 3].

Bone regeneration can occur through two ways either endochondral ossification or intramembranous ossification processes. Mesenchymal stem cells (MSCs) immediately differentiate into osteoblasts during intramembranous ossification, which leads to the deposition of mineralized extracellular matrix.
This kind of healing is frequently observed in fractures that are tightly repaired, have a small fracture gap, and include the bone metaphysis. Endochondral ossification occurs through steps include: inflammation, soft and then hard callus development, and remodeling of the fracture site are used to treat fractures in the diaphysis that have less mechanical stability and a greater fracture gap [4].

Bone homeostasis constantly undergoes remodeling including bone resorption and bone formation [5]. The process of bone healing involves three highly integrated and overlapping stages: inflammation, proliferation, and bone remodeling [6]. 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 [7].

Several medications with widely different indications that exhibit a pleiotropic spectrum of action are used to target local and systemic regulation of bone metabolism. These include antihyperlipidemic drugs such as (HMG-CoA reductase inhibitors), antihypertensive drugs (like ACE inhibitors), drugs for osteoporosis (bisphosphonates), drugs for cancer (proteasome inhibitors) and other drugs [8]. Metformin is a member of a group of drugs known as biguanides. Biguanides are a significant class of oral hypoglycemic medications that work by inhibition of the liver gluconeogenesis, improving the density of low and high affinity insulin receptors, and reducing resistance to the peripheral effects of insulin. The most often given oral antihyperglycemic medication for type 2 diabetes is metformin [9]. Metformin treatment for diabetes patients has been demonstrated to lower TNF-expression, The pharmacological action of metformin goes beyond mere glycemic control, decreasing markers of inflammation and contributing to the reduction of oxidative stress [10], with anti-inflammatory effect being confirmed, Metformin has been recently reported to provide anti-inflammatory effects in atherosclerosis through inhibition of transcription factor nuclear factor-kappa B (NF-κB) signaling in vivo and in vitro [11]. Under chronic periodontal inflammation, mesenchymal stem cells (MSCs) have greatly reduced osteogenic differentiation potential. A highly effective strategy to boost or restore MSCs osteogenic potential in an inflammatory environment remains an unmet goal [12].

The first choice oral medication for type 2 diabetes is metformin. due to its inexpensive cost, reasonable safety, minimal risk of hypoglycemia, lack of weight gain, and few side effects [13]. Therefore, this study focuses on the effect of metformin on the bone.

Fluorescence-based quantitative real-time polymerase chain reaction (qPCR) analysis of gene expression is an important measure in many fields of biological research. The goal of qPCR is to «real-time» monitor the DNA polymerase-driven amplification process. A thermostable DNA polymerase enzyme is employed in a PCR reaction to create new DNA strands that are complementary to the target DNA sequence. The target sequence will be amplified in billions of copies at the conclusion of the PCR procedure (PCR amplicons). In contrast to conventional PCR, qPCR uses a fluorescent dye system and a thermocycler with fluorescence-detection capacity to detect the amplification of the PCR amplicons at the conclusion of each amplification cycle [14].

**Material and Methods**

**Experimental model**

Twenty adult male New Zealand rabbits ranging in weight from 1.75 to 2 kg and aged between 6 and 8 months were used. The animals were housed in regular conditions, under the same housing and feeding arrangements, receiving their water from the same source, and eating a standard diet (wheat and fresh vegetables). The University of Mosul’s College of Veterinary Medicine kept the rabbits in cages at its animal house. The animals will be 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) [15]. The study was performed according to the requirements of the institutional animal research ethics committee at 19/6/2022 (UoM.Dent/A.L.58/22).

**Medication**

The medication that used in this study is metformin tablets 500mg (the least dose available to control administered dose) produced by the well known German brand company (MERCK) under the name of (Glucophage). (Fig. 1).
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 500mg tablet was grinded alone; the resultant fine powder of this tablet was filled into a hard gelatin capsule to control the amount of drug (500mg 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 homogenous solution containing 500mg/10ml of the drug (Fig. 1). While metformin is essentially insoluble in organic solvents like acetone, ether, and chloroform, it is readily soluble in water [16]. Metformin liquid was administered to the rabbits orally in a dose of (50mg/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 divided into two groups. Each group consist of ten rabbits. The first group received no treatment (control group), while the other group received metformin daily single dose. Each group fatherly subdivided into two groups according to the sacrifice date at 14th and 28th day intervals.

**Group 1** (n=10) received no medication (control group). This group was additionally subdivided into two groups (5 rabbits for each period according to the sacrifice date at (14th and 28th) days.

**Group 2** (n=10) (systemically treated group). After the surgical procedure, metformin was administered orally in a once daily dose of (50mg/kg) body weight [17] using feeding tube. This group was subdivided into two groups (5 rabbits/period) according to the sacrifice date (14th and 28th day).

Preparation of animals for surgery
All the twenty rabbits were received anesthesia by intramuscular injection. Each rabbit was given mixed 40 mg/kg ketamine (KETALROM-50, Romvac company, SA) with xylazine (Holland) 4 mg/kg injection intramuscularly in the thigh muscle of the rabbit (Fig. 2) [18].

The incision site was shaved using electrical hair clipper, cleaned well and disinfected with povidone iodine 10% solution thoroughly and left to dry before incision. The animal covered with sterile towel except the site of operation (Fig. 3).

Animal surgical procedure
After twenty minutes the animal gain anesthesia [19], it was placed on the right lateral position on the operating table in a sterile environment. The surgery 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. The femoral bone was made visible as the two muscles parted. Hawarth periosteal elevator was used to lift the periosteum and expose the compact bone during blunt dissection (Fig. 4).

Two holes created in the exposed femur both of which with (3mm) diameter and depth using (2000) rpm low speed straight surgical hand piece with a 3mm round carbide bur attached and continuous normal saline irrigation (Fig. 5) [20].

The bone defects (holes) were 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 as shown in (Fig. 6).

Postoperative care of animals
After surgical procedure the animals were given [Oxytetracycline 20% injection-
Fig. 2. Intramuscular injection of anesthesia.

Fig. 3. Shaving and disinfecting the surgical site.

Fig. 4. Incision of the skin, separation of the two muscles, exposure of femur and making holes in the bone with continuous irrigation.
EV ALUATING THE SYSTEMIC EFFECT OF METFORMIN ON GENE EXPRESSION OF …

Fig. 5. Two holes were made in the femur bone of each of twenty rabbits.

Limoxin-200 LA® (Holland)] as a prophylactic antibiotic for wound healing, given as single daily dose of 0.5ml/kg intramuscularly for three consecutive days from the operation day. Rabbits were placed each alone until recovered from anesthesia and regain full consciousness, they also undergo twenty four hours supervision after operation to monitor their general health, physical activity and feeding.

Gene expression analysis

Tissue extraction protocol
1. Place up to 20 mg of tissue that has been cut into smaller pieces in a 1.5 ml microcentrifuge tube with 200 µl of Lysis Solution.

Fig. 6. Suturing and disinfection of the wound.

2. Fill the sample tube with 20 µl of Proteinase-K-solution (20 mg/ml), proper mixing by vortexing, and then incubate at (56 ºC) until the tissue is totally lysed. To make sure that it is distributed evenly during incubation, you can also put the sample tube in a water bath that is vibrating or on a platform that rocks. The amount of lysis time depends on the kind of tissue that is being treated. Overnight lysis had no impact on the preparation.

3. Rotate the tube downward to clear any droplets from the cap of sample tube.

4. (Optional RNase A treatment) If RNA-free genomic DNA is required, add the 20 µl of RNase A Solution (10 mg/ml, not supplied).

5. After adding 200 µl of the binding solution to the sample tube, thoroughly mix it with a pulse-vortex for 15 seconds.

6. Incubate for 10 minutes at 56 ºC. Longer incubation times have no impact on the quantity or caliber of the purified DNA.

7. Include (200 µl) of absolute ethanol and mix thoroughly using a pulse-vortex for (15) seconds. Once you’ve finished doing this, to remove the drops caught on the lid of the container, quickly spin it.

8. Carefully transfer the lysate into the upper reservoir of the spin column using a 2.0 ml collection tube without soaking the rim.

9. Remove the flow-through and connect the 2.0 ml collecting tube to the spin column after a minute of centrifuging at 13,000 rpm.

10. For 1 minute, centrifuge at 13,000 rpm while adding 500 µl of washing 1 solution using a collection tube to connect the spin column: Drain the flow through first, then insert the 2.0 ml collection tube into the spin column 1.

11. 500 µl of the Washing 2 Solution should be added. centrifuge for one minute at 13,000 rpm. Remove the flow through and put the 2.0 ml collecting tube into the spin column.

12. Dry the spin column by running a further 1 minute of 13,000 rpm centrifugation to remove any remnant ethanol.

13. Use the new 1.5 ml microcentrifuge tube, insert inside the spin column.

14. Pour 100 to 200 µl of the elution buffer solution into the spin column within the micro-centrifuge tube, and then leave it alone for at least a minute.

15. Centrifuge at 13,000 rpm for 1 minute to elute the genomic DNA.

---

**Primer design for genes (forward and the reverse primer sequence)**

One of the most important aspects of quantitative real-time PCR (qPCR) analyses’ performance and quality is the design of the primers since effective primer design is essential for accurate and reliable quantification. To locate possible primers for certain qPCR assays, primer design should follow several criteria[21]. The GAPDH gene was used as housekeeping gene. Osteocalcin and VDR gene primers were designed through the use of a well-known website software which is (NCBI), the primer sequences as in the following (Table 1):

**GoTaq® qPCR Master Mix:**

The second kit is for quantitative DNA detection which is (GoTaq Qpcr Master Mix) from (Promega Corporation USA).

The quantitative PCR(qPCR) reagent system GoTaq® qPCR Master Mix (a,b). This system includes a fluorescent DNA-binding dye (BRYT Green® Dye) that binds to double stranded DNA (dsDNA) and exhibits higher fluorescence amplification. All necessary components for qPCR are included in the easy-to-use, stable 2X formulation known as (GoTaq® qPCR Master Mix)except (sample DNA, primers and water).

This formula include GoTaq® Hot Start Polymerase, MgCl₂, dNTPs, a custom reaction buffer, a proprietary dsDNA-binding dye, and a low concentration of carboxy-X-rhodamine.

---

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward sequence of primers</th>
<th>Reverse sequence of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ACATGCACAGGGTACTTCGA</td>
<td>TTACCCCAAGCCCTTCTCCATG</td>
</tr>
<tr>
<td>OSTEOCALCIN</td>
<td>CCGAAACATGGGGTGTGCTT</td>
<td>TGCCCTTCTCTGACCCCTAC</td>
</tr>
<tr>
<td>VDR</td>
<td>GATGCAGGGGCTGTTATGGG</td>
<td>GGCTGCTTGTGCTTTTAC</td>
</tr>
</tbody>
</table>

**TABLE 1. Primer sequences of Osteocalcin and VDR.**
EVALUATING THE SYSTEMIC EFFECT OF METFORMIN ON GENE EXPRESSION OF...

(CXR) reference dye (identical to ROXTM dye) yields the best results in qPCR tests. For use with instruments that need more reference dye than what is in the GoTaq® qPCR Master Mix, a separate bottle of CXR Reference Dye is provided.

Genomic study
Includes quantitative measurement of osteocalcin and vitamin D receptors DNA materials. DNA extracted as described above in details from bone tissues at the site of bony defect by using AddPrep Genomic DNA Extraction Kit. The osteocalcin and VDR genomic material determined by (qPCR) by using the (Go-Taq-qPCR master mix) produced by Promega and PCR max Eco machine. Replication reactions of the goal gene and household genes were performed for the samples. ΔΔCT calculated for comparison of genes between samples. Replication reactions were done for the genes of the study and household genes were done for all samples. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping genes were used as a control to calculate the ΔCT value. ΔΔCT calculated for comparison the results of gene expression between samples. The ΔCT value calculated for each sample as the difference in CT between the gene of interest and the household gene. ΔΔCT was measured as the difference between the ΔCT values of the study sample and the control sample. The osteocalcin and VDR receptor's genes in this study expressed as ΔΔCT(mean ± SD).

ΔΔCT = ΔCT (Sample) – ΔCT (Control)

Results
Bone sample assessment for quantitative PCR DNA detection of both osteocalcin and VDR through gene expression technique reveal that there is increase in both of bone osteocalcin and VDR representation in the metformin treated groups over than the control group (Fig. 7 and 8).

Osteocalcin

A-At 14 days
Osteocalcin genes expressed in larger quantity in the metformin group, where the mean± standard error was (0.78±0.04) more than the control group (0.74±0.05). The independent sample T-test demonstrated that the osteocalcin levels of the control and metformin groups did not differ significantly at the 14th day. (p ≥ 0.05). As shown in Tables (2 and 3).

TABLE 2. Osteocalcin gene expression results at day 14 for the control group.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Assay Name</th>
<th>mean CT</th>
<th>GAPDH</th>
<th>ΔCT (Sample)</th>
<th>ΔCT (Control)</th>
<th>ΔΔCT</th>
<th>2^−ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Osteocalcin</td>
<td>25.913</td>
<td>25.34876</td>
<td>0.564245</td>
<td>0.194124</td>
<td>0.370121</td>
<td>0.773718</td>
</tr>
<tr>
<td>S2</td>
<td>Osteocalcin</td>
<td>26.10056</td>
<td>25.34876</td>
<td>0.751804</td>
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<td>0.55768</td>
<td>0.679394</td>
</tr>
<tr>
<td>S3</td>
<td>Osteocalcin</td>
<td>25.93588</td>
<td>25.34876</td>
<td>0.58713</td>
<td>0.194124</td>
<td>0.393006</td>
<td>0.761541</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>25.98315</td>
<td>25.34876</td>
<td>0.634393</td>
<td>0.194124</td>
<td>0.440269</td>
<td>0.738218</td>
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<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td>0.102323</td>
<td></td>
<td>0.051305</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 3. Osteocalcin gene expression results at day 14 for the metformin group.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Assay Name</th>
<th>mean CT</th>
<th>GAPDH</th>
<th>ΔCT (Sample)</th>
<th>ΔCT (Control)</th>
<th>ΔΔCT</th>
<th>2^−ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
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<td>0.282621</td>
<td>0.822096</td>
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<tr>
<td>S2</td>
<td>Osteocalcin</td>
<td>25.95881</td>
<td>25.34876</td>
<td>0.610055</td>
<td>0.194124</td>
<td>0.415931</td>
<td>0.749536</td>
</tr>
<tr>
<td>S3</td>
<td>Osteocalcin</td>
<td>25.93588</td>
<td>25.34876</td>
<td>0.58713</td>
<td>0.194124</td>
<td>0.393006</td>
<td>0.761541</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>25.90673</td>
<td>25.34876</td>
<td>0.557976</td>
<td>0.194124</td>
<td>0.363853</td>
<td>0.777724</td>
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<td>SD</td>
<td></td>
<td></td>
<td></td>
<td>0.071276</td>
<td></td>
<td>0.038893</td>
<td></td>
</tr>
</tbody>
</table>
**B-At 28 days:**

Osteocalcin genes expressed in larger quantity in the metformin group, where the mean±standard error was (1.32±0.11) more than the control group (0.78±0.11). An independent sample T-test revealed a significant difference in osteocalcin levels between the control and metformin groups (p ≤ 0.05). As shown in Tables (4,5).

**Vitamin D receptors:**

**A-At 14 days:**

VDR genes also express high value in the metformin group (0.05±0.01) more than that in the control group (0.04±0.007). An independent sample T-test results revealed no significant differences in the VDR genes between the control and metformin groups at the 14th day (p ≥ 0.05). Tables (7, 8).

---

### TABLE 4. Osteocalcin gene expression results at day 28 for the control group.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Assay Name</th>
<th>mean CT</th>
<th>GAPDH</th>
<th>ΔCT Sample</th>
<th>ΔCT control</th>
<th>ΔΔCT</th>
<th>2^-ΔΔCT</th>
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<tbody>
<tr>
<td>S1</td>
<td>Osteocalcin</td>
<td>25.6856</td>
<td>25.34876</td>
<td>0.336842</td>
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<td>0.142718</td>
<td>0.905811</td>
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<tr>
<td>S2</td>
<td>Osteocalcin</td>
<td>25.98857</td>
<td>25.34876</td>
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<tr>
<td>S3</td>
<td>Osteocalcin</td>
<td>26.03527</td>
<td>25.34876</td>
<td>0.686511</td>
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<td>0.492387</td>
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<td>Mean</td>
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<td>25.90314</td>
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<td>0.554388</td>
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<td>SD</td>
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<td></td>
<td>0.189842</td>
<td></td>
<td>0.106455</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 5. Osteocalcin gene expression results at day 28 for the metformin group.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Assay Name</th>
<th>mean CT</th>
<th>GAPDH</th>
<th>ΔCT Sample</th>
<th>ΔCT control</th>
<th>ΔΔCT</th>
<th>2^-ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Osteocalcin</td>
<td>25.20141</td>
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<td>S2</td>
<td>Osteocalcin</td>
<td>25.23341</td>
<td>25.34876</td>
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<tr>
<td>S3</td>
<td>Osteocalcin</td>
<td>25.00982</td>
<td>25.34876</td>
<td>-0.33893</td>
<td>0.194124</td>
<td>-0.53306</td>
<td>1.446994</td>
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<tr>
<td>Mean</td>
<td></td>
<td>25.14821</td>
<td>25.34876</td>
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<td>SD</td>
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<td></td>
<td></td>
<td>0.120916</td>
<td></td>
<td>0.112776</td>
<td></td>
</tr>
</tbody>
</table>

---

Fig. 7. The histogram of statistical analysis of osteocalcin gene expression.
**Discussion**

In the current study, the osteocalcin and vitamin D receptors (VDR) gene expression data both show higher values in the metformin treated group in comparison to the control group at 14 and 28 days. Osteocalcin (Ocn), the most prevalent non-collagenous protein in bone, is only expressed particularly in osteoblasts. By carboxylating three glutamic acids, Ocn gains a high affinity for Ca$^{2+}$ [22]. One of the most sensitive indicators of the osteogenic differentiation process is osteocalcin mRNA [23]. An increase in its activity can hasten the ALP process when paired with it, promoting osteoblasts’ formation of bone [24].

On the other hand, osteocalcin and calcium ions work well together to encourage the formation and growth of new bone. Apoptosis

---

**TABLE 7. VDR gene expression results at day 14 for the control group.**

<table>
<thead>
<tr>
<th>VDR</th>
<th>Control 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Name</td>
<td>Assay Name</td>
</tr>
<tr>
<td>S1</td>
<td>VDR</td>
</tr>
<tr>
<td>S2</td>
<td>VDR</td>
</tr>
<tr>
<td>S3</td>
<td>VDR</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 8. VDR gene expression results at day 14 for the metformin group.**

<table>
<thead>
<tr>
<th>VDR</th>
<th>Metformin 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Name</td>
<td>Assay Name</td>
</tr>
<tr>
<td>S1</td>
<td>VDR</td>
</tr>
<tr>
<td>S2</td>
<td>VDR</td>
</tr>
<tr>
<td>S3</td>
<td>VDR</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 9. VDR gene expression results at day 28 for the control group.**

<table>
<thead>
<tr>
<th>VDR</th>
<th>Control 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Name</td>
<td>Assay Name</td>
</tr>
<tr>
<td>S1</td>
<td>VDR</td>
</tr>
<tr>
<td>S2</td>
<td>VDR</td>
</tr>
<tr>
<td>S3</td>
<td>VDR</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 10. VDR gene expression results at day 28 for the metformin group.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Assay Name</th>
<th>Mean CT</th>
<th>GAPDH</th>
<th>Control (−ve)</th>
<th>ΔCT Sample</th>
<th>ΔCT control</th>
<th>2^−ΔΔCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>VDR</td>
<td>24.64323</td>
<td>24.75549</td>
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<td>-2.72448</td>
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<tr>
<td>S2</td>
<td>VDR</td>
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<td>24.75549</td>
<td>22.03101</td>
<td>-1.09857</td>
<td>-2.72448</td>
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<tr>
<td>S3</td>
<td>VDR</td>
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<td>24.75549</td>
<td>22.03101</td>
<td>0.894219</td>
<td>-2.72448</td>
<td>3.618702</td>
</tr>
<tr>
<td>Mean</td>
<td>VDR</td>
<td>24.64995</td>
<td>24.75549</td>
<td>22.03101</td>
<td>-0.10554</td>
<td>-2.72448</td>
<td>2.618945</td>
</tr>
<tr>
<td>SD</td>
<td></td>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>0.123388</td>
</tr>
</tbody>
</table>

Fig. 8. The histogram of statistical analysis of the vitamin D receptors (VDR) gene expression.

of osteoclasts and osteoblast differentiation are positively correlated with osteocalcin activity. By turning osteoclasts into blood, one of the key players in bone endocrinology is osteocalcin, or bone-carboxyglutamic acid protein, a substance expressed and secreted only by osteoblasts. Osteocalcin is one of the hormones that controls bone production, regulates the pathogenesis of osteoblasts and osteoclasts as well as the activity of adipocytes. After protein synthesis, the mature peptide initially goes through a number of splicing processes before being γ-carboxylated at three residues, creating a peptide with a strong affinity for bone and the extracellular matrix. But because of the low pH in the osteoclast resorption compartments, osteocalcin is furtherly decarboxylated, reducing its affinity for bone and causing the release of uncarboxylated osteocalcin into the bloodstream [25]. Therefore, high levels of osteocalcin expression have the ability to significantly reduce adipocyte differentiation, raise bone matrix, and reinforce sclerotin [26]. Osteocalcin is activated through decarboxylation, a reaction that occurs in the bone resorption lacunae, also recent study demonstrated that osteocalcin is a bone-derived hormone that regulates β-cell proliferation, insulin expression, and insulin secretion in mice and in humans. They also suggest that the hormonally active form of osteocalcin is the decarboxylated one, this has been subsequently confirmed through genetic means [27].

The nucleotide sequences of the osteogenic differentiation gene, which includes (Runx2, ALP, BGLAP, BMP, and OCN) and VDR levels,
also indicate the degree of osteogenesis. The 2−△△CT method was used to determine the gene expression. The GAPDH CT data were used to average and calibrate all of the CT results [28].

By regulating adenosine monophosphate-activated protein kinase enzyme (AMPK), metformin may promote osteoblast differentiation by transactivating genes. It has been demonstrated that AMPK dramatically increases the expression of the important osteogenic genes, including osteocalcin (OC) [39].

Metformin increased collagen synthesis, osteocalcin production, and extracellular calcium deposition in bone marrow mesenchymal cells in vitro, possibly by upregulating the expression of Runx2 [30]. By controlling AMPK, metformin may promote osteoblast differentiation by transactivating genes by AMPK regulation. It has been demonstrated that AMPK dramatically increases the expression of the important osteogenic genes, including osteocalcin (OC)[29]. Metformin also increased alkaline phosphatase (ALP) and osteocalcin (OCN) secretion, enhanced BMP-2 expression and improved bone mineral density (BMD) [31]. Together with exercise metformin increased the concentration of osteocalcin and decreased the serum concentration of IL-1β and glucose [32].

A common bone modulator in regenerative medicine is vitamin D [33]. Vitamin D has been shown to improve osteogenesis in primary murine osteoblasts and MC3T3-E1 cell lines in a number of investigations [34]. Mechanical studies revealed that vitamin D caused an osteosphere to be stiffer than the control. It has been suggested that low vitamin D intake has a deleterious impact on fracture risk [35].

According to reports, vitamin D affects osteoblasts via membrane-binding protein and vitamin D receptors (VDR) [36]. One of the elements that regulate nuclear transcription is VDR. Increased osteoclast activity, bone loss, and even periodontal disease may be the results of any VDR gene mutation. There has been prior evidence linking VDR and periodontitis [37]. According to a study, genetic VDR mutations may reduce the effectiveness of vitamin D supplements [38].

Reactive oxygen species (ROS) may be produced at higher levels in individuals with gestational DM due to reduced vitamin D levels or altered vitamin D receptor activation [39]. Due to increased activation of vitamin D receptors and decreased oxidative stress, vitamin D may improve the amount and bioavailability of NO [40].

**Conclusion**

The use of metformin systemically increase the level of bone formation markers such as the gene expression of both osteocalcin and vitamin D receptors in the metformin treated animals over than that of the control group at two different time intervals.

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**Conflicts of interest**

The authors declared no competing interests.

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**References**


تقييم التأثير الجهازي للميتفورمين على التعبير الجيني للإوستيوكالسين ومستقبلات فيتامين D في العيب العظمي في الأرانب

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الخلفية: لا تزال اضطرابات النمو العظمي تشكل تحديات حقيقية في الرعاية المبكرة. ومع ذلك، فإن تسيير علاج هذه الحالات يتطلب زرع جراحي في موقع الكسر، مما قد يؤدي إلى مضاعفات موضعية. لذلك فإن العقاقير المكونة للعظم ستوفر طريقة ممتازة لشفاء آفات العظام. تأثير الميتفورمين المنشأ للعظم من خلال زيادة بانيات: تقييم التأثير الجهازي لإعطاء الميتفورمين على التئام العظام.

الهدف من الدراسة

評価結果: تم استخدام عشرين أرنبًا وقعت تحت التخدير العام، تم إجراء نفس الإجراء الجراحي على جميع الأرانب بعد تعريض عظم الفخذ جراحيًا، وتم تحضير عينة من الموقع العضلي بعد)، وتُحضر في مروم بالماء على فترات محددة. يتم إعطاء الميتفورمين عن طريق الفم للآرانب بجرعة 28 مجم / كجم لمدة 3 بناءً على دراسات. يتم عزل عظم الفخذ، وPCR (QPCR) ثم نتائج التعبير الجيني. 

النتائج: أظهرت هذه الدراسة أن هناك زيادة في التعبير الجيني للإوستيوكالسين ومستقبلات فيتامين D في المجموعة المعززة بالميتفورمين مقارنة بالمجموعة القياسية. يزيد الميتفورمين من التئام العظام وتحقيق المجموعة المعززة بالعظام. عملي ثبت مفعول الميتفورمين على الأرانب غير المعززة.

الكلمات المفتاحية: الميتفورمين، التعبير الجيني، النمو العظمي، عيب العظام.