Egypt. J. Vet. Sci. **Vol. 54,** No. 1, pp. 47-60 (2023)

Egyptian Journal of Veterinary Sciences
https://ejvs.journals.ekb.eg/

Evaluating the Systemic Effect of Metformin on Gene Expression of Osteocalcin and Vitamin D Receptors at Bony Defect in Rabbits

Raad M. Hussein[1] and Ghada A. Taqa[2*]
[1]*Ministry of Health, Kirkuk Health Directorate, Kirkuk, Iraq*
[2]*Department of Dental Basic Sciences, College of Dentistry. University of Mosul, Mosul, Iraq*

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.

Corresponding author: Ghada A. Taqa, E- mail.: ghadataqa@uomosul.edu.iq, Tel. 07702091219
(*Received* 27/07/2022; *accepted* 06/09/2022)
DOI. 10.21608/EJVS.2022.152617.1371

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 occur 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 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).

Fig. 1. Glucophage®(metformin 500mg) tablets.

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 14^{th} and 28^{th} 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 (14^{th} and 28^{th}) 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 (14^{th} and 28^{th} 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-

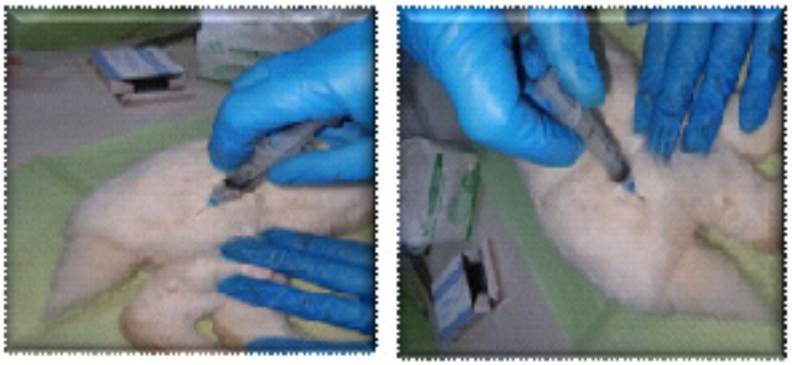

Fig. 2. Intramuscular injection of anesthesia.

Fig. 3. Shaving and disinfecting the surgical site.

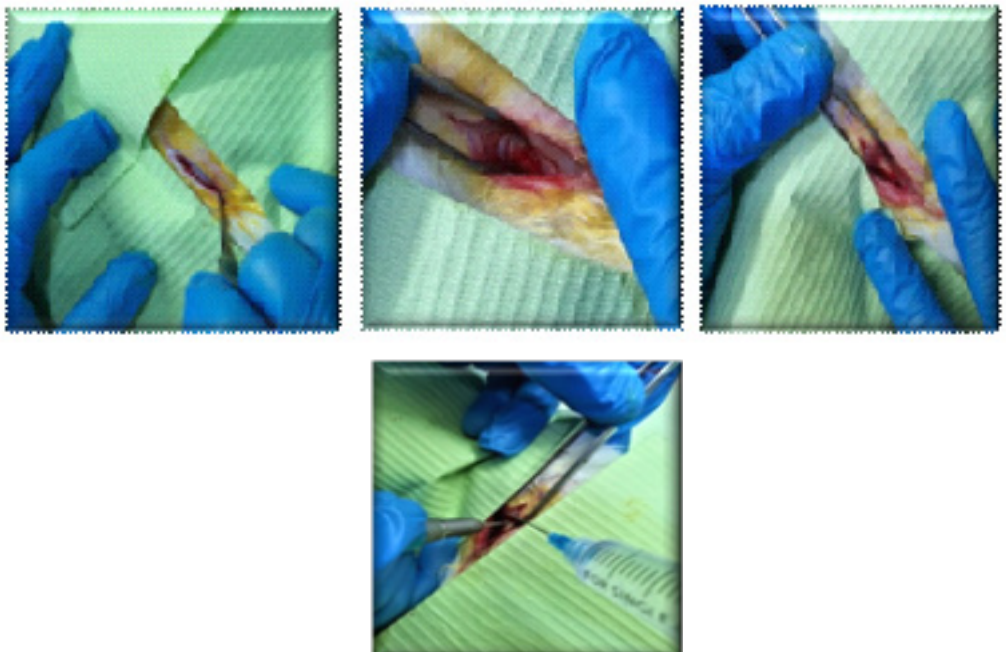

Fig. 4. Incision of the skin, separation of the two muscles, exposure of femur and making holes in the bone with continuous irrigation.

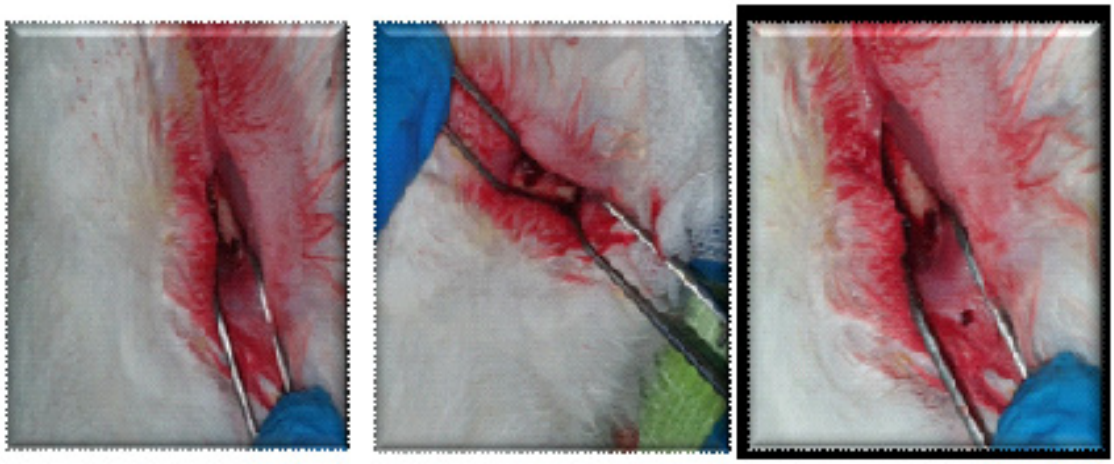

Fig. 5. Two holes were made in the femur bone of each of twenty rabbits.

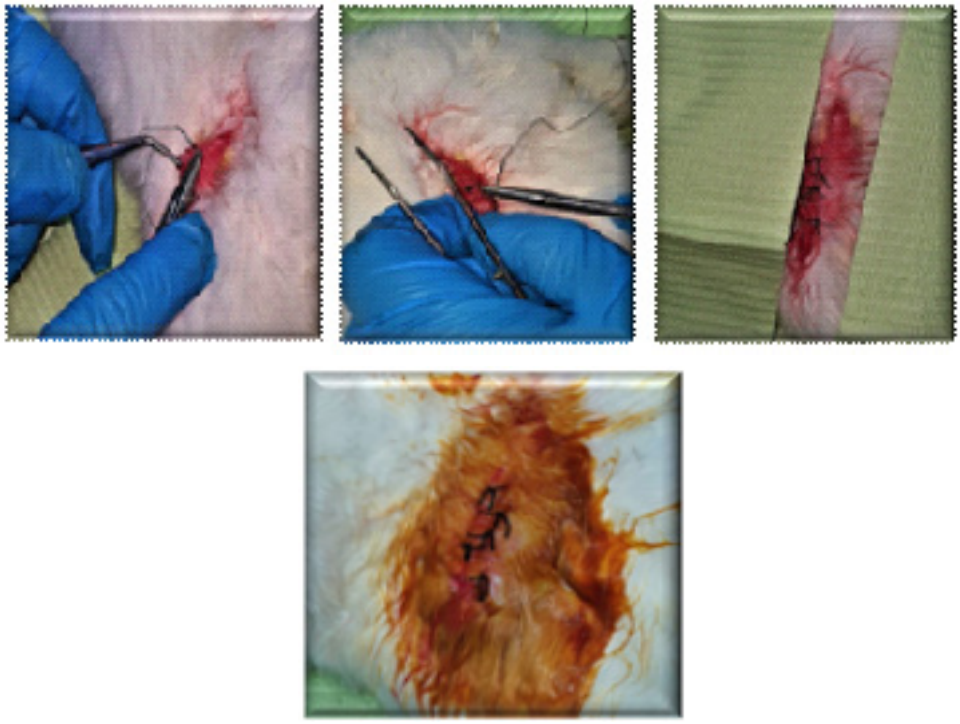

Fig. 6. Suturing and disinfection of the wound.

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.

2. Fill the sample tube with 20 µl of Proteinase-K-solution (20 mg/ml), proper mixing by vortexing, and then incubate at (56 °C) 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 °C. 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 u sing 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, $MgCl_2$, dNTPs, a custom reaction buffer, a proprietary dsDNA-binding dye, and a low concentration of carboxy-X-rhodamine

TABLE 1. Primer sequences of Osteocalcin and VDR.

Genes	Forward sequence of primers	Reverse sequence of primers
GAPDH	ACATGCACAGGGTACTTCGA	TTACCCCAGCCTTCTCCATG
OSTEOCALCIN	CCGAAACATGGGGTGTGTCT	TGCCTTTCTCTGACCCCTAC
VDR	GATGCAGGGCTGTTTATGGG	GGCCTGCTTGCTGTTCTTAC

(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) .

ΔCT (Sample) = CT OSCN or VDR gene – CT GAPDH
ΔCT (Control) = CT control – CT GAPDH
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. ($p \geq 0.05$). As shown in Tables (2 and 3).

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

Osteocalcin	control	14 days							
Sample Name		Assay Name	mean CT	GAPDH	control(-ve)	ΔCT Sample	ΔCT control	ΔΔCT	2^-ΔΔCT
	S1	Oestocalcin	25.913	25.34876	25.54288	0.564245	0.194124	0.370121	0.773718
	S2	Oestocalcin	26.10056	25.34876	25.54288	0.751804	0.194124	0.55768	0.679394
	S3	Oestocalcin	25.93588	25.34876	25.54288	0.58713	0.194124	0.393006	0.761541
		Mean	25.98315	25.34876	25.54288	0.634393	0.194124	0.440269	0.738218
		SD						0.102323	0.051305

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

Osteocalcin	Metformin	14 days							
Sample Name		Assay Name	mean CT	GAPDH	control (-ve)	ΔCT Sample	ΔCT control	ΔΔCT	2^-ΔΔCT
	S1	Oestocalcin	25.8255	25.34876	25.54288	0.476745	0.194124	0.282621	0.822096
	S2	Oestocalcin	25.95881	25.34876	25.54288	0.610055	0.194124	0.415931	0.749536
	S3	Oestocalcin	25.93588	25.34876	25.54288	0.58713	0.194124	0.393006	0.761541
		Mean	25.90673	25.34876	25.54288	0.557976	0.194124	0.363853	0.777724
		SD						0.071276	0.038893

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($p \leq 0.05$). As shown in Tables (4,5).

Vitamin D receptors :

A- At 14 days :

VDR genes also express high value in the metformin group **(**0.056±0.01**)** more than that in the control group **(**0.048±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 ($p \geq 0.05$). Tables (7, 8).

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

Osteocalcin	Control	28 days							
Sample Name		Assay Name	mean CT	GAPDH	control(-ve)	ΔCT Sample	ΔCT control	ΔΔCT	2^-ΔΔCT
	S1	Oestocalcin	25.6856	25.34876	25.54288	0.336842	0.194124	0.142718	0.905811
	S2	Oestocalcin	25.98857	25.34876	25.54288	0.639812	0.194124	0.445688	0.734234
	S3	Oestocalcin	26.03527	25.34876	25.54288	0.686511	0.194124	0.492387	0.710848
		Mean	25.90314	25.34876	25.54288	0.554388	0.194124	0.360264	0.783631
		SD						0.189842	0.106455

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

Osteocalcin	Metformin	28 days							
Sample Name		Assay Name	mean CT	GAPDH	control(-ve)	ΔCT Sample	ΔCT control	ΔΔCT	2^-ΔΔCT
	S1	Oestocalcin	25.20141	25.34876	25.54288	-0.14734	0.194124	-0.34147	1.267046
	S2	Oestocalcin	25.23341	25.34876	25.54288	-0.11534	0.194124	-0.30947	1.23925
	S3	Oestocalcin	25.00982	25.34876	25.54288	-0.33893	0.194124	-0.53306	1.446994
		Mean	25.14821	25.34876	25.54288	-0.20054	0.194124	-0.39466	1.317763
		SD						0.120916	0.112776

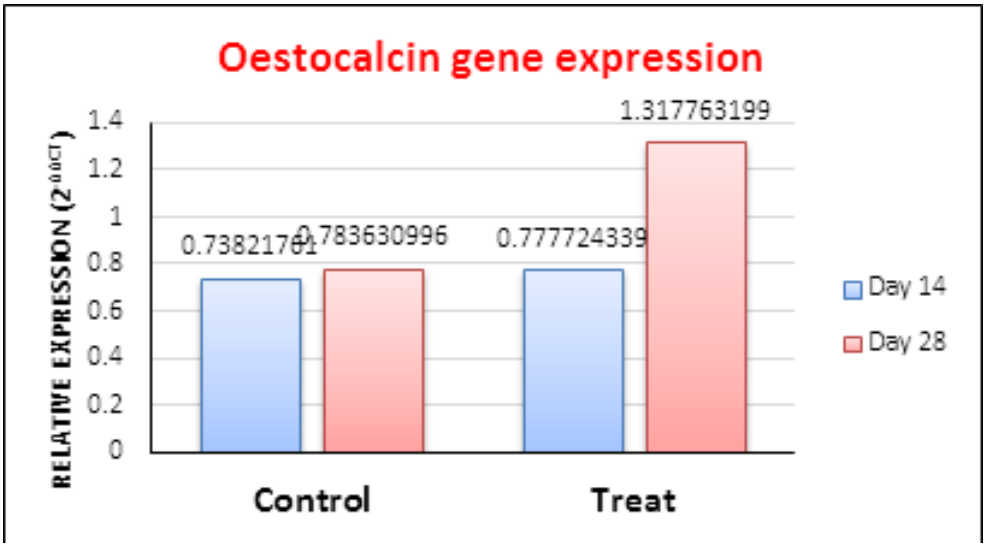

Fig. 7. The histogram of statistical analysis of osteocalcin gene expression.

B-At 28 days:

VDR genes also express high value in the metformin group (0.19±0.12) more than that in the control group (0.07±0.05). An independent sample T-test revealed a significant difference in the VDR genes between the control and metformin treated groups at the 28th day. ($p \leq 0.05$) (Tables 9, 10).

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.

VDR	Control	14 days							
Sample Name		Assay Name	Mean CT	GAPDH	Control (-ve)	ΔCT Sample	ΔCT control	ΔΔCT	2^-ΔΔCT
	S1	VDR	26.32555	24.75549	22.03101	1.570061	-2.72448	4.294543	0.050958
	S2	VDR	26.23656	24.75549	22.03101	1.481071	-2.72448	4.205553	0.0542
	S3	VDR	26.65656	24.75549	22.03101	1.901071	-2.72448	4.625553	0.040511
		Mean	26.40622	24.75549	22.03101	1.650734	-2.72448	4.375216	0.048556
		SD	0.221317	0	0	0.221317	0	0.221317	0.007154

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

VDR	Metformin	14 days							
Sample Name		Assay Name	Mean CT	GAPDH	Control (-ve)	ΔCT Sample	ΔCT Control	ΔΔCT	2^-ΔΔCT
	S1	VDR	25.99587	24.75549	22.03101	1.240387	-2.72448	3.964869	0.064041
	S2	VDR	26.11567	24.75549	22.03101	1.360179	-2.72448	4.084661	0.058938
	S3	VDR	26.50236	24.75549	22.03101	1.746868	-2.72448	4.47135	0.045081
		Mean	26.20463	24.75549	22.03101	1.449145	-2.72448	4.173627	0.05602
		SD	0.264702	0	0	0.264702	0	0.264702	0.009811

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

VDR	Control	28 days							
Sample Name		Assay Name	Mean CT	GAPDH	Control(-ve)	ΔCT Sample	ΔCT control	ΔΔCT	2^-ΔΔCT
	S1	VDR	25.99587	24.75549	22.03101	1.240387	-2.72448	3.964869	0.064041
	S2	VDR	25.00957	24.75549	22.03101	0.254079	-2.72448	2.978561	0.126871
	S3	VDR	27.00236	24.75549	22.03101	2.246868	-2.72448	4.97135	0.031877
		Mean	26.0026	24.75549	22.03101	1.247111	-2.72448	3.971593	0.074263
		SD	0.996411	0	0	0.996411	0	0.996411	0.048315

TABLE 10. VDR gene expression results at day 28 for the metformin group.

VDR	Metformin	28 days							
Sample Name		Assay Name	Mean CT	GAPDH	Control (-ve)	ΔCT Sample	ΔCT control	ΔΔCT	2^-ΔΔCT
	S1	VDR	24.64323	24.75549	22.03101	-0.11226	-2.72448	2.612221	0.163547
	S2	VDR	23.65692	24.75549	22.03101	-1.09857	-2.72448	1.625913	0.324005
	S3	VDR	25.64971	24.75549	22.03101	0.894219	-2.72448	3.618702	0.081407
		Mean	24.64995	24.75549	22.03101	-0.10554	-2.72448	2.618945	0.189653
		SD	0.996411	0	0	0.996411	0	0.996411	0.123388

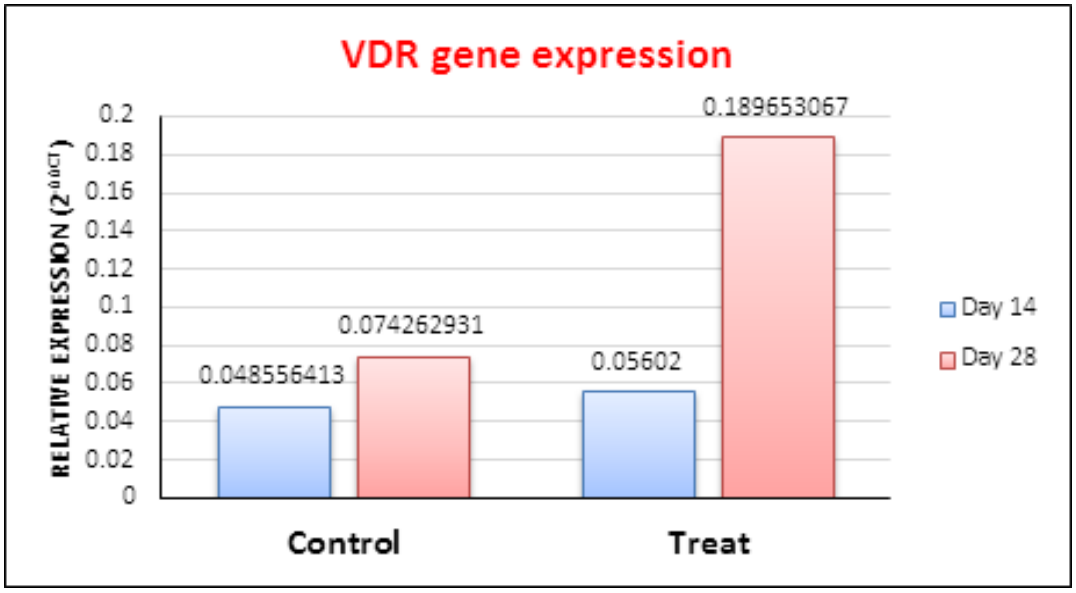

Fig. 8. The histogram of statistical analysis of the vitamin D receptors (VDR) gene expression.

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−ΔΔCT 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.

Acknowledgement

To all stuff in the department of dental basic sciences

Conflicts of interest

The authors declared no competing interests.

Funding Statements

None.

References

1. Morgan, E. F. and Gerstenfeld, L. C. . The bone organ system: form and function. In Marcus and Feldman's Osteoporosis. Academic Press , pp. 15-35(2021).
2. Schell, H., Duda, G. N., Peters, A., Tsitsilonis, S., Johnson, K. A. and Schmidt-Bleek, K. The haematoma and its role in bone healing. *Journal of Experimental Orthopaedics,* **4**(1), 1-11 (2017).
3. Bell, A., Templeman, D. and Weinlein, J. C. Nonunion of the femur and tibia: an update. *Orthopedic Clinics,* **47**(2), 365-375(2016).
4. Pajarinen, J., Lin, T., Gibon, E., Kohno, Y., Maruyama, M., Nathan, K. and Goodman, S. B. Mesenchymal stem cell-macrophage crosstalk and bone healing. *Biomaterials,* **196**, 80-89 (2019).
5. Naji, A. H., Taqa, G.A. and Al-Watter, W.A. The Effect of Xylitol on Osteoclastogenesis in Experimentally Induced Bone Defect in Rabbits. *Journal of Applied Veterinary Sciences,* **7** (1), 58–63(2022).
6. Medhat, D., Rodríguez, C. I. and Infante, A. Immunomodulatory effects of MSCs in bone healing. *International Journal of Molecular Sciences*, **20**(21), 5467(2019)
7. Hussein, A. A. and Taqa, G.A. The impact of natural calcium carbonate and Ubiquinone on bone mineral density in rabbits. *Journal of Applied Veterinary Sciences*, **6**(4), 15-22(2021)

8. Rothe, R., Schulze, S., Neuber, C., Hauser, S., Rammelt, S. and Pietzsch, J. Adjuvant drug-assisted bone healing: Part III–Further strategies for local and systemic modulation. *Clinical Hemorheology and Microcirculation*, **73**(3), 439-488(2019)

9. Bucher, S., Bauduceau, B., Benattar-Zibi, L., Bertin, P., Berrut, G., Corruble, E. and Becquemont, L. Primary care management of non-institutionalized elderly diabetic patients: The S. AGES cohort–Baseline data. *Primary Care Diabetes*, **9**(4), 267-274(2015)

10. Andrews, M., Soto, N. and 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*, **140**(11), 1377-1382(2012).

11. Koh, S. J., Kim, J. M., Kim, I. K., Ko, S. H. and Kim, J. S. Anti-inflammatory mechanism of metformin and its effects in intestinal inflammation and colitis-associated colon cancer. *Journal of Gastroenterology and Hepatology*, **29**(3), 502-510(2014).

12. Ren, C., Hao, X., Wang, L., Hu, Y., Meng, L., Zheng, S. and Sun, H. Metformin Carbon Dots for Promoting Periodontal Bone Regeneration via Activation of ERK/AMPK Pathway. *Advanced Healthcare Materials*, **10**(12), 2100196(2021).

13. Huang, W., Castelino, R. L. and Peterson, G. M. Metformin usage in type 2 diabetes mellitus: are safety guidelines adhered to? *Internal Medicine Journal*, **44**(3), 266-272(2014).

14. Kuang, J., Yan, X., Genders, A. J., Granata, C. and Bishop, D. J. An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PloS one*, **13**(5), e0196438 (2018).

15. De Lima Santos, A., da Silva, C. G., de Sá Barreto, L. S., Leite, K. R. M., Tamaoki, M. J. S., Ferreira, L. M. and Faloppa, F. A new decellularized tendon scaffold for rotator cuff tears–evaluation in rabbits. *BMC Musculoskeletal Disorders*, **21**(1), 1-12(2020).

16. Kumar, A. B., Mohan, S., Sakthi, R. S. and Ramkanth, S. A review on clinical pharmacokinetic and pharmacodynamic profile of metformin. *Clin. Pharmacokinet.,* **50**(2),81-98(2011). doi: 10.2165/11534750-000000000-00000

17. Ikewuchi, C. C., Ikewuchi, J. C. and Ifeanacho, M. O. Restoration of plasma markers of liver and kidney functions/integrity in alloxan-induced diabetic rabbits by aqueous extract of Pleurotus tuberregium sclerotia. *Biomedicine & Pharmacotherapy*, **95**, 1809-1814(2017).

18. Ahirwar, L. K., Kalra, P., Sharma, S., Mohamed, A., Mittal, R., Das, S. and Bagga, B. Linezolid shows high safety and efficacy in the treatment of Pythium insidiosum keratitis in a rabbit model. *Experimental Eye Research*, **202**, 108345(2021).

19. Jiron, J. M., Mendieta Calle, J. L., Castillo, E. J., Abraham, A. M., Messer, J. G., Malphurs, W. L. and Aguirre, J. I. 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*, **58**(1), 40-49 (2019).

20. Nguyen-Thanh, T., Nguyen-Tran, B. S., Cruciani, S., Dang-Cong, T. and Maioli, M. A rabbit femoral trochlear defect model for chondral and osteochondral regeneration. *Acta Veterinaria Brno*, **91**(3), 293-301(2022).

21. Rodríguez, A., Rodríguez, M., Córdoba, J. J. and Andrade, M. J. Design of primers and probes for quantitative real-time PCR methods. In PCR primer design (pp. 31-56). *Humana Press,* New York, NY.2015)).

22. Komori, T. What is the function of osteocalcin?. *Journal of Oral Biosciences,* **62**(3), 223-227(2020).

23. Zhu, L. and Xu, P. C. Downregulated LncRNA-ANCR promotes osteoblast differentiation by targeting EZH2 and regulating Runx2 expression. *Biochemical and Biophysical Research Communications,* **432**(4), 612-617(2013).

24. Eleniste, P. P., Patel, V., Posritong, S., Zero, O., Largura, H., Cheng, Y. H. and Bruzzaniti, A. Pyk2 and megakaryocytes regulate osteoblast differentiation and migration via distinct and overlapping mechanisms. *Journal of Cellular Biochemistry*, **117**(6), 1396-1406(2016).

25. Li, H., Meng, D., Zhang, X. and Yuan, D. Effect of psoralen on the expression of PPARγ, osteocalcin, and trabecular bone area in rabbits with steroid-induced avascular necrosis of the femoral head. *Journal of Orthopaedic Surgery and Research*, **14**(1), 1-8(2019).

26. Rendina-Ruedy, E., Guntur, A. R. and Rosen, C. J. Intracellular lipid droplets support osteoblast function. *Adipocyte,* **6**(3), 250-258(2017).

27. Karsenty, Gerard, and Eric N. Olson. "Bone and muscle endocrine functions: unexpected paradigms of inter-organ communication." *CelPress,* **164**(6), 1248-1256(2016). https://doi.org/10.1016/j.cell.2016.02.043

28. Qi, G., Yu, K., Feng, Y., Zhang, Y., Shao, Q., Yu, M. and Jiang, Z. 1α, 25-dihydroxyvitamin D3 promotes early osteogenic differentiation of PDLSCs and a 12-year follow-up case of early-onset vitamin D deficiency periodontitis. *The Journal of Steroid Biochemistry and Molecular Biology,* **208**, 105805 (2021).

29. Jang, W. G., Kim, E. J., Bae, I. H., Lee, K. N., Kim, Y. D., Kim, D. K. and Koh, J. T. Metformin induces osteoblast differentiation via orphan nuclear receptor SHP-mediated transactivation of Runx2. *Bone,* **48**(4), 885-893(2011).

30. Wang, P., Ma, T., Guo, D., Hu, K., Shu, Y., Xu, H. H. and Schneider, A. Metformin induces osteoblastic differentiation of human induced pluripotent stem cell-derived mesenchymal stem cells. *Journal of Tissue Engineering and Regenerative Medicine*, **12**(2), 437-446 (2018).

31. Zheng, L., Shen, X., Ye, J., Xie, Y. and Yan, S. Metformin alleviates hyperglycemia-induced apoptosis and differentiation suppression in osteoblasts through inhibiting the TLR4 signaling pathway. *Life Sciences*, **216**, 29-38(2019).

32. Li, H., Gou, Y., Tian, F., Zhang, Y., Lian, Q., Hu, Y. and Zhang, L. Combination of metformin and exercise alleviates osteoarthritis in ovariectomized mice fed a high-fat diet. *Bone*, **157**, 116323(2022).

33. Gupta, A. A., Kheur, S., Badhe, R. V., Raj, A. T., Bhonde, R., Jaisinghani, A. and Patil, S. Assessing the potential use of chitosan scaffolds for the sustained localized delivery of vitamin D. *Saudi Journal of Biological Sciences*, **28**(4), 2210-2215(2021).

34. Aoshima, Y., Mizobuchi, M., Ogata, H., Kumata, C., Nakazawa, A., Kondo, F. and Akizawa, T. Vitamin D receptor activators inhibit vascular smooth muscle cell mineralization induced by phosphate and TNF-α. *Nephrology Dialysis Transplantation*, **27**(5), 1800-1806(2012).

35. Schröder, M., Riksen, E. A., He, J., Skallerud, B. H., Møller, M. E., Lian, A. M. and Reseland, J. E. Vitamin K2 Modulates Vitamin D-Induced Mechanical Properties of Human 3D Bone Spheroids In Vitro. *JBMR plus*, **4**(9), e10394(2020).

36. Olivares-Navarrete, R., Sutha, K., Hyzy, S. L., Hutton, D. L., Schwartz, Z., McDevitt, T. and Boyan, B. D. Osteogenic differentiation of stem cells alters vitamin D receptor expression. *Stem Cells and Development*, **21**(10), 1726-1735(2012).

37. Wang, Q., Zhou, X., Jiang, J., Zhang, P., Xia, S., Ding, Y. and Wang, Q. Relationship between serum 25-hydroxyvitamin D3 levels and severity of chronic periodontitis in type 2 diabetic patients: A cross-sectional study. *Journal of Periodontal Research*, **54**(6), 671-680 (2019).

38. Patil, V. S., Mali, R. S. and Moghe, A. S. Evaluation and comparison of Vitamin D receptors in periodontal ligament tissue of Vitamin D-deficient chronic periodontitis patients before and after supplementation of Vitamin D3. *Journal of Indian Society of Periodontology*, **23**(2), 100-105(2019).

39. Mahdi, A., Cortese-Krott, M. M., Kelm, M., Li, N. and Pernow, J. Novel perspectives on redox signaling in red blood cells and platelets in cardiovascular disease. *Free Radical Biology and Medicine*, **168**, 95-109(2021).

40. Ding, L., Sui, X., Yang, M., Zhang, Q., Sun, S., Zhu, F. and Cao, J. Toxicity of cooking oil fume derived particulate matter: Vitamin D3 protects tubule formation activation in human umbilical vein endothelial cells. *Ecotoxicology and Environmental Safety*, **188**, 109905 (2020).

تقييم التأثير الجهازي للميتفورمين على التعبير الجيني للاوستيوكالسين ومستقبلات فيتامين د في العيب العظمي في الأرانب

رعد محمود حسين [١] و غادة عبد الرحمن طاقة [٢]
[١] وزارة الصحة - مديرية صحة كركوك - كركوك - العراق
[٢] قسم العلوم الأساسية لطب الأسنان - كلية طب الأسنان - جامعة الموصل - العراق

الخلفية: لا تزال اضطرابات التئام العظام تشكل تحديات حقيقية في الرعاية السريرية اليوم. ومع ذلك ، فإن تسليم مواد حشو العظام يتطلب زرع جراحي في موقع الكسر ، مما قد يؤدي إلى مضاعفات موضعية. لذلك فإن العقاقير المكوّنة للعظم ستوفر طريقة ممتازة لشفاء آفات العظام. تأثير الميتفورمين المنشأ للعظم من خلال زيادة بانيات العظم وتقليل ناقضات العظم. **الهدف من الدراسة**: تقييم التأثير الجهازي لإعطاء الميتفورمين على التئام العظام في موقع الخلل العظمي عن طريق قياس التعبير الجيني لمستقبلات أوستيوكالسين وفيتامين د. **المواد وطرائق العمل**: تم استخدام عشرين ذكور أرنب ناضج وقسموا إلى مجموعتين ، عشرة في كل مجموعة. تحت التخدير العام ، تم إجراء نفس الإجراء الجراحي على جميع الأرانب بعد تعريض عظم الفخذ جراحيًا ، وتم تحضير فتحتين بقطر ٣ مم وعمق ٣ مم وتركها فارغة. تستمر الدراسة لمدة ٢٨ يومًا. يتم إعطاء الميتفورمين عن طريق الفم للأرانب بجرعة ٥٠ مجم / كجم لمدة ٢٨ يومًا. تم التضحية بالحيوانات على فترات مرتين وفقًا لمجموعاتها في اليوم الرابع عشر والثامن والعشرين بعد الجراحة وفقًا لعدة دراسات. تم عزل عظمة الفخذ ، مقطوعة ، وعظام مأخوذة من موقع الخلل ، العينة الموضوعة في محلول ملحي الفوسفات حتى يتم تقييمها من أجل PCR الكمي (QPCR). **النتيجة**: أظهرت أن هناك زيادة في التعبير الجيني الكمي لكل من مستقبلات أوستيوكالسين وفيتامين د في المجموعة المعالجة بالميتفورمين مقارنة بالمجموعة الضابطة في فترتي الدراسة. **الاستنتاج**: يزيد الميتفورمين من التئام العظام وتجديدها في مواقع عيوب العظام ويعزز عملية تكون العظم والاندماج العظمي أكثر من السيطرة على الأرانب غير المعالجة.

الكلمات المفتاحية : الميتفورمين ، التعبير الجيني ، التئام العظام ، عيب العظام..