Functional, Histological and Immunohistochemical Study on the Possible Effect of Vitamin D and Mesenchymal Stem Cells on Decreasing Bone-Remodeling Changes in Streptozotocin Induced Diabetic Rats

Original Article

Ashraf Kotb[1], Sara Adel Hosny[3], Ahmed Desoky[1], Fawzy Abd_ElTawab Abd_Ellatif[1], Rania Elsayed Hussein[4] and Asmaa Mohammed ShamsEldeen[2,1]

Department of Medical Physiology, Faculty of Medicine, [1]October 6 University, [2]Cairo University, Egypt

[3]Department of Histology, Faculty of Medicine, Cairo University, Cairo, Egypt

[4]Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Egypt

ABSTRACT

Background and Objectives: Diabetes mellitus (DM), is a metabolic disorder coupled with osteoporosis and elevated risk of bone fracture. This work aimed to clarify the impact of diabetes mellitus on bone remodeling changes and to study the role of vitamin D supplementation and mesenchymal stem cells (BM-MSCs) in decreasing bone remodeling changes in type II DM.

Material and Methods: 30 adult male albino rats were classified into the control group (n-6) and the main experimental group (n=24). All rats in experimental group were given high fat diet and a single injection of streptozotocin (STZ) (45 mg/kg) intraperitoneally (IP) then after 8 weeks they were divided into; Type II DM group; continued without treatment, Type II DM +vitamin D group; received vitamin D orally daily, Type II DM +BM-MSCs group; received a once intravenous injection of 3 × 106 BM-MSCs and Combined treated group; treated with combined BM-MSCs and vitamin D. At the end of the experiment, Blood samples were drawn to measure glucose level, reactive Oxygen species (ROS), lipid profile (FFA), and tumor necrosis factor (TNF-α.). Femur bone was dissected for biochemical, histological, immunohistochemical, morphometric, and statistical analysis.

Results: Mean serum levels of glucose, triglycerides, total cholesterol (TCH), ROS & TNF-α were significantly increased and associated with a significant decrease of HDL & FFA levels in Type II DM, Type II DM +vitamin D and Type II DM +BM-MSCs groups. In addition, the levels of Wnt/ b-catenin and BCL2 were significantly decreased, while sclerostin and BAX, and the area percentage of VEGF were increased, in Type II DM, Type II DM +vitamin D and Type II DM +BM-MSCs groups. Vitamin D and BM-MSCs encountered significant amelioration in biochemical markers and histological & immunohistochemical changes induced by STZ with a more obvious improvement in the combined treated group.

Conclusion: Combined treatment of vitamin D and BM-MSCs significantly improved diabetic bone turnover compared to both vit. D, and MSCs injection alone by alleviating the antioxidative and anti-inflammatory effects.

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Key Words: BAX/BCL2, bone turnover, diabetes, Wnt/ b-catenin, VEGF.

Corresponding Author: Sara Adel Hosny, MD, Department of Histology, Faculty of Medicine, Cairo University, Cairo, Egypt, **Tel.**: +20 10 0562 5595 , **E-mail:** dr.sara_adelh@hotmail.com

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INTRODUCTION

Vitamin D (vit D) is essential for bone health. Despite having different mechanisms of action, the vitamin D role in the regulation of bone homeostasis is still up for debate[1]. Multiple studies postulated vit D role in osteoblastic differentiation and, more lately, on mesenchymal stem cells (MSCs) regeneration-enhancing effects in reconstructive medicine[2]. 1,25(OH)2D3 is the active form of Vit D, which can induce differentiation of osteoblasts in dental bud stem cells that was confirmed by enhancing the in *vitro* exhibition of the typical osteoblastic markers and influencing the rate of mineralization[3].

Diabetes mellitus (DM) type II and its associated metabolic complications are a true economic health problem. The altered bone physiology is one of the consequences of chronically uncontrolled diabetes mellitus[4].

Complications such as osteoporosis and a higher risk of fragility have surfaced in both type I and II diabetic patients[5]. Recent studies have suggested enhanced

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activity of osteoclasts in DM but under certain conditions such as in osteoporosis. Other studies reported inhibition of osteoclast's function and ability for differentiation in a diabetic environment[2]. To better explain how diabetes affects bone remodeling, more research is required.

The state of chronic hyperglycemia induces the elevation of pro-inflammatory cytokines, such as interleukin 1-β, 6, 8, and tumor necrosis factor-alpha (TNF-α)[6]. High blood glucose levels together with the production of excess reactive oxygen species (ROS) exaggerate suppression of osteoblastic function and proliferation[7]. In addition, the state of hyperglycemia enhances production of sclerostin leading eventually to the adipogenic lineage of human BM-MSCs. The osteocyte's ability to differentiate into adipocytes is increased by inhibiting the Wnt/-catenin pathway[8]. On the contrary, Wnt/β-catenin signaling activation enhances the differentiation of osteogenic cells[9].

So, the current study was purposed aiming to clarify the effect of diabetes mellitus on bone remodeling changes and to study the role of vit D supplementation and mesenchymal stem cells in decreasing bone remodeling changes in type II DM.

MATERIALS AND METHODS

Experimental animals and groups

Thirty adult male albino rats aged approximately 10-12 weeks and of body weights (150 to 200 grams) were purchased and involved in this study. The animals were kept for a few days to acclimate to routine environmental circumstances in the Animal House, as regards temperature, humidity, and changes in dark/light cycles. The rats were left in cages made of wire mesh with unrestricted access to food and water. Then the rats were divided into 5 groups. This work was approved by the ethics committee of October 6 University, approval number; PMC-Me-2201006.

Group I; Control (n=6): This group declared the vehicle-treated rats. They were supplied with normal chow (12% of calories was derived from fat).

The main experimental group (n=24):

Induction of type II DM: The rats in the main experimental group consumed a high-fat diet (HFD) for two weeks in which fat accounted for 40% of calories prior to administering streptozotocin (STZ). Sigma-Aldrich provided the STZ powder (St Louis, MO, USA). At the time of injection, a freshly sterile sodium citrate buffer, pH 5-6 was prepared and used as a solvent for STZ. A dosage of the prepared solution was administered intraperitoneally (i.p.) 45 mg/kg, ~ 20 µl, and the same volume of the prepared buffer was injected into rats in the control group as a vehicle[10].

Verification of diabetes and care of diabetic rats: Fasting blood glucose level was monitored in rat tail using Accu-Chek Performa. The animals were subjected to estimation of fasting blood sugar (FBS) 3 days after injecting STZ, and the rats with FBS ≥ 250 mg/dl were involved in the present study and diagnosed to be diabetic. Continuous monitoring of FBS was done every week for 8 weeks to confirm the continuation of diabetic state. All diabetics were subjected to veterinary care that was delivered in the unit of the animal house. After 8 weeks, the following groups of rats were created from the main experimental group: (six in each).

Group II (n=6): (Type II DM) continued without treatment.

Group III (n=6): (Type II DM +vitamin D) In this group, rats received once daily for four weeks at a dose of 10 IU/Kg/day vitamin D. Week 8 after verification of the onset of DM vitamin D will be taken till the end of the experimental study.

Group IV (n=6): (Type II DM +BM-MSCs), in this group, rats were treated with bone marrow mesenchymal stem cells 8 weeks after verification of onset of DM, each rat received once intravenous injection of 3×10^6 BM-MSCs[11].

Group V (n=6): (Combined treated group) (Type II DM treated with BM-MSCs+ Vitamin D) The rats in this group were given a combined treatment of BM-MSCs and vitamin D[12].

All rats were subjected to euthanasia at the end of the study by cervical dislocation and tissue samples were obtained.

Samples collection and scarification

After terminating the experiment and immediately before scarification, blood samples were withdrawn from the rat tail vein using a syringe and placed in 10 ml Eppendorf tubes. After centrifugation, the plasma was separated and used for biochemical estimation of

- Blood glucose level.
- Total lipid profile: Triglycerides (TG), Total cholesterol (TCH), High density lipoprotein (HDL), Lipid profile (FFA).
- Reactive Oxygen species (ROS)
- Tumor Necrosis factor (TNF-α.).

Femur bones were separated and dissected (on both sides), with the left bone being used for biochemical estimation of:

- Wnt /β–catenin using western blot technique.
- Sclerostin, and Bax/BCL2 by polymerase chain reaction (PCR).

Histological: the right femur bones were subjected to histological preparation. Unstained sections were examined by fluorescent microscope as well as using Haemotoxylin and Eosin (H&E) stain, immunohistochemistry, cut sections were examined.

Estimation of Serum Glucose level using Rat Glucose assay kit

Rat Glucose Assay Kit, CATALOG# 81693. The Rat Glucose Assay Kit is based on a multi-step reaction. The final formed dye was monitored by measuring absorbance at 505nm and is directly proportionate to the glucose concentration in the rat specimen.

Estimation of Serum using Rat Free Fatty Acid (FFA) ELISA Kit

Cat No. MBS266907, Double Antibody Sandwich Technique is used by this kit. Characteristics of the tested antigen is the principle of this technique with more than two valances which can detect coated antibody and identification of antibody at the same time.

Estimation of Serum Rat Triglyceride (TG), total cholesterol (TCH), and high-density lipoprotein (HDL):

TG ELISA kit (Catalog Number: MBS726298), total cholesterol (Catalog No. ABIN772507), and high density lipoprotein (HDL) rat ELISA Kit (Catalog No: MBS266554), were used for quantitative detection of lipid profile factors. The intensity of color was measured spectrophotometrically in a microplate reader.

Estimation Of Serum Rat reactive oxygen species (ROS)

Catalog N:SL1189Ra, Sandwich-ELISA was used as the method. An antibody specific to ROS pre-coated the Microelisa strip plate provided in this kit.

Estimation of Rat TNF-α (Tumor Necrosis Factor-Alpha)

Catalog No: MBS2507393, this ELISA kit used the Sandwich-ELISA principle. A formed substrate solution from Avidin-HRP conjugate and biotinylated detection antibody was added to each well appeared. The formed color was blue only in those wells that contained Rat TNF-α. After termination enzyme-substrate reaction, then Spectrophotometric detection of the generated color occurred at a wavelength of 450 nm + 2 nm.

Detection of Wnt /B –Catenin using western blot technique

An established and popular method for identifying and analyzing proteins is immunoblotting. Bio BASIC INC provides RIBA lysis buffer PL005, used to extract protein. Tris-buffered saline with 3% bovine serum albumin (BSA) and Tween 20 (TEST) buffer was used to block the membrane. The blotting analysis using anti-Wnt (1:1000; obtained from Cell Signaling), and anti-β-catenin (1:2000; obtained from Cell Signaling) and were equalized to the internal housekeeping β-actin antibody (Cell signaling technology).

Detection of sclerostin, Bax, and Bcl2, gene expression in tissue homogenate by Real time qPCR

Tissue homogenate was handled for extraction of total RNA, then it was pursued by Reverse Transcriptase (for cDNA synthesis) and real time qPCR.

Tissue homogenate was used to extract the total RNA present in tissues using the SV total RNA isolation system (Thermo Scientific, USA). The yielded total RNA was specified spectrophotometrically at 260 nm. After that, it was utilized with a high-capacity cDNA reverse transcription kit to create cDNA (catalog NO #K4374966, obtained from Thermo Fisher Scientific, USA). The obtained cDNA including previously prepared samples were normalized to the internal control β-actin gene (Table 1).

Table 1: Primers Sequence of sclerostin, Bax, Bcl2, and Beta-actin gene

Gene	Primer sequence
Sclerostin gene	Forward primer: 5′-TCCTCCTGAGAACAACCAGAC-3′, Reverse primer: 5′-TGTCAGGAAGCGGGTGTAGTG-3′.
Bax	Forward primer: 5′- GTT TCA TCC AGG ATC GAG CAG-3′ Reverse primer: 5′- CAT CTT CTT CCA GAT GGT GA-3′
Bcl2	Forward primer: 5′- CCT GTG GAT GAC TGA GTA CC-3′ Reverse primer: 5′- GAG ACA GCC AGG AGA AAT CA-3′
β-actin	Forward primer: 5′-AGGTCGGAGTCAACGGATTTGGT-3′ Reverse primer: 5′-CATGTGGGCCAT AGGTCCACCAC-3′

Histological examination

The dissected bone was fixed in 10% formalin for 48 hrs., then rinsed in water and placed in 18% EDTA for decalcification then handled for paraffin blocks. Serial portions were cut and subjected to testing for:

a. Fluorescent microscopic study: fluorescence microscopy examination of Type II DM +BM-MSCs group and combined treated group unstained sections to look for homing of PKH26-labeled BM-MSCs

b. Hematoxylin and eosin stain (H&E)[13].

c. Immunohistochemical staining using anti-VEGF.

Preparation, isolation, expansion, and labeling of mesenchymal stem cells (Figure 1):

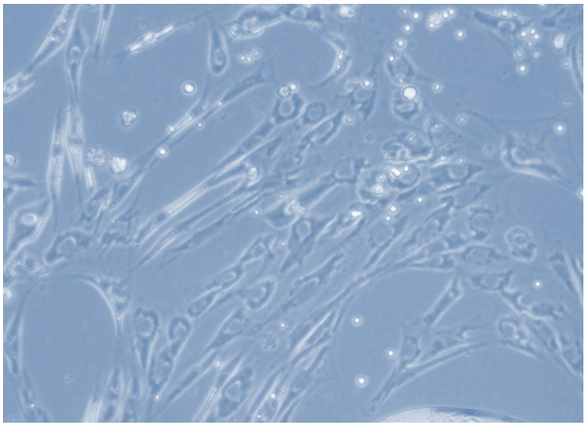


Fig. 1: A photomicrograph of unstained developing BM-MSCs

Under complete aseptic conditions in the cell culture unit, all experimental work was performed. Three rats were sacrificed by decapitation under general anesthesia for dissection of femur bones. Dulbecco's modified Eagle's medium (DMEM) was used to saturate the dissected bone, then under the hood, the epiphyseal ends were cut. Using a 20-gauge needle filled with DMEM, the bone marrow cavity was irrigated to retrieve the bone marrow plugs[14]. Then, the obtained samples were centrifugated to get the nuclear layer that contains stem cells. Then, this layer was aspirated and recentlyrifuged for separation of bone marrow mesenchymal stem cells. The resultant pellets were cultured in fetal bovine serum-containing full DMEM of 100 µg/ml and streptomycin sulfate of 100 µg/ml (Sigma-Aldrich, St. Louis, MO 63118, United States). Every 3 days, the cultural media was replaced with a new fresh one. After reaching 70% confluence, the stem cells were sub-cultured to get an adequate number of cells. The used cells were at the third passage at the time of transplantation. The obtained BM-MSCs were identified morphologically. To identify the in *vivo* homed BM-MSCs after transplantation, the stem cells were labeled with PKH 26 Red Fluorescent Cell Linker on the day of injection. A freshly made solution containing 2 106 PKH26 in an ethanolic dye. All steps of labeling are done according to manufacturer instructions. 1×10^7 cells/ml was added to a total volume of two microliters of working dye formed with the previous concentration[15].

Immunohistochemical staining[16]

Positively charged slides were used to mount the bone sections, deparaffinized, and rehydrated. Retrieving the antigen by using 10mM citrate buffer to boil tissue sections. Incubating the sections in hydrogen peroxide to block Endogenous peroxidase activity. Incubating the sections overnight with Anti-VEGF (ab2349) [A rabbit polyclonal antibody that can react with mouse and rat, Abcam plc, England].

Using a labeled avidin-biotin-peroxidase complex, the bound primary antibody was detected. Using diaminobenzidine (DAB) as a chromogen and Mayer's hematoxylin as counterstaining to recognize the immunohistochemical-staining. Specificity of immunohistochemical staining was established by processing negative control serial sections via using phosphate buffer saline to replace the primary antibody. For the positive tissue control of VEGF immunostaining, mouse skin tissue was used (brownish nuclear immunoreaction).

RESULTS

Biochemical results

1) Changes in levels of glucose and lipid profile in different studied groups (Table 2):

Type II DM group exhibited a marked increase in serum glucose, TG, TCH, and FFA levels and a relevant decrease in serum HDL in comparison to the control group. In Type II DM +vitamin D and Type II DM +BM-MSCs groups, serum glucose, TG, TCH, and FFA were significantly elevated in comparison with a control group and significantly reduced when compared to Type II DM group, while serum HDL level was markedly decreased in comparison with a control group and increased in comparison with Type II DM group. However, These statistics in Type II DM +vitamin D group and Type II DM +BM-MSCs group did not significantly differ from one another.

Serum glucose levels in combined treated group revealed a marked increase in comparison with the control group and a significant decline in comparison with Type II DM group, Type II DM +vitamin D group, and Type II DM +BM-MSCs group. While serum TG, TCH, and FFA levels were significantly decreased in comparison with groups Type II DM, Type II DM +vitamin D, and Type II DM +BM-MSCs. Otherwise, serum HDL was markedly elevated in comparison with Type II DM, Type II DM +vitamin D, and Type II DM +BM-MSCs groups. Moreover, the control group and combined treated group revealed no significant difference.

2) Changes in oxidative stress and inflammatory state in all studied groups (as shown in Table 3):

In Type II DM group, serum ROS and TNF-α levels were markedly increased in comparison with the control group. Type II DM +vitamin D group and Type II DM +BM-MSCs group revealed significant elevation of serum ROS and TNF-α compared to the control group and significantly reduced in comparison with Type II DM group with a significant decrease of TNF-α in type II DM +BM-MSCs group in comparison with Type II DM +vitamin D group and no significant difference in ROS level between the two groups. Whereas serum ROS and TNF-α exhibited a significant increase in the combined treated group in comparison with the control group and a marked decrease in comparison with Type II DM, Type II DM +vitamin D, and Type II DM +BM-MSCs groups.

3) Changes in Wnt/ b-catenin, sclerostin, and Bax/ BCL2 in all studied groups (as shown in Table 4):

The tissue levels of Wnt and b-catenin in type II DM group demonstrated a significant decline with a significant increase in Sclerostin compared to the control group. In addition, type II DM +vitamin D group and Type II DM +BM-MSCs group exhibited a significant decline of Wnt and b-catenin with a significant increase of Sclerostin in comparison with control group. When compared to the Type II DM group, the Type II DM + vitamin D group and the Type II DM + BM-MSCs group showed a significant increase in Wnt and b-catenin levels associated with a significant decrease of Sclerostin. Additionally, compared to the control group, the combined treated group's Wnt and b-catenin levels were much lower, and elevated in comparison with Type II DM group, Type II DM +vitamin D group, and Type II DM +BM-MSCs group, while Sclerostin was significantly decreased in comparison with Type II DM, Type II DM +vitamin D, and Type II DM +BM-MSCs groups.

Type II DM group exhibited a significant decrease in Bcl2 level in bone associated with a significant increase of Bax compared to control group. Bcl2 level was significantly decreased in Type II DM +vitamin D, Type II DM +BM-MSCs, and V groups, and a significant increase in Bax in comparison with the control group. As opposed to the Type II DM group, the Bcl2 level was much higher and related to a considerable reduction in Bax. Additionally, the combined treated group's Bcl2 level was higher than that of the Type II DM + vitamin D and Type II DM + BM-MSCs groups, and this was linked to a much lower level of Bax than that of the Type II DM + vitamin D group. As opposed to the Type II DM group, Bcl2 level was much higher and related to a considerable reduction in Bax.

Histological results

1) Fluorescent microscopic results

Sections in Type II DM +BM-MSCs group and combined treated group exhibited the presence of labeled BM-MSCs in bone sections (Figures 2A,B respectively).

2) Hematoxylin and Eosin-stained sections

Examination of Longitudinal sections in femur of control group exhibited normal bone architecture in the form of Haversian system formed of Haversian canal surrounded with concentric layers of bone lamellae having osteocytes in lacunae (Figure 3A).

Sections in Type II DM group showed large, congested blood vessels, and widened some Haversian canals surrounded with irregular bone lamellae. Note some of the lacunae were empty with few osteocytes in their lacunae (Figure 3B).

However, Type II DM +vitamin D group exhibited Haversian system formed of widened Haversian canals surrounded with concentric bone lamellae and osteocytes in lacunae. Note the presence of some empty lacunae (Figure 3C).

While sections from Type II DM +BM-MSCs group and combined treated group demonstrated haversian canals surrounded by osteocytes in their lacunae with clear canaliculi (Figures D,E respectively).

3) Immunostained sections stained with VEGF (Figure 4)

Sections of Femur in the control group exhibited negative nuclear immunoreactivity to VEGF. However, section of bone in Type II DM group revealed a large number of nuclei immunostained by VEGF. On the other hand, Type II DM +vitamin D group and Type II DM +BM-MSCs group exhibited occasional VEGF immunostained nuclei, while the combined treated group showed negative VEGF immunoreactivity.

The mean area % of VEGF immunohistochemical stain in studied groups: (Histogram 1)

Comparing Type II DM group to control group, treated groups (Type II DM + vitamin D & Type II DM + BM-MSCs), and the combined treated group, Type II DM group showed an increase in mean area% of VEGF rise. Comparing the Vit D treated group to the control group and the combination treatment group, the mean area% of VEGF in the Vit D treated group significantly increased with a significant decrease as compared to Type II DM group and MSc treated group.

MSC treated group revealed an increase of mean area % of VEGF in comparison with control group, Vit D treated group and combined treated group with significantly reduce in compared to Type II DM group. The mean area % of VEGF in the combined treated group (V) exhibited a significant decrease in comparison to Type II DM group, Type II DM +vitamin D group and Type II DM +BM-MSCs group with no significant difference to control group.

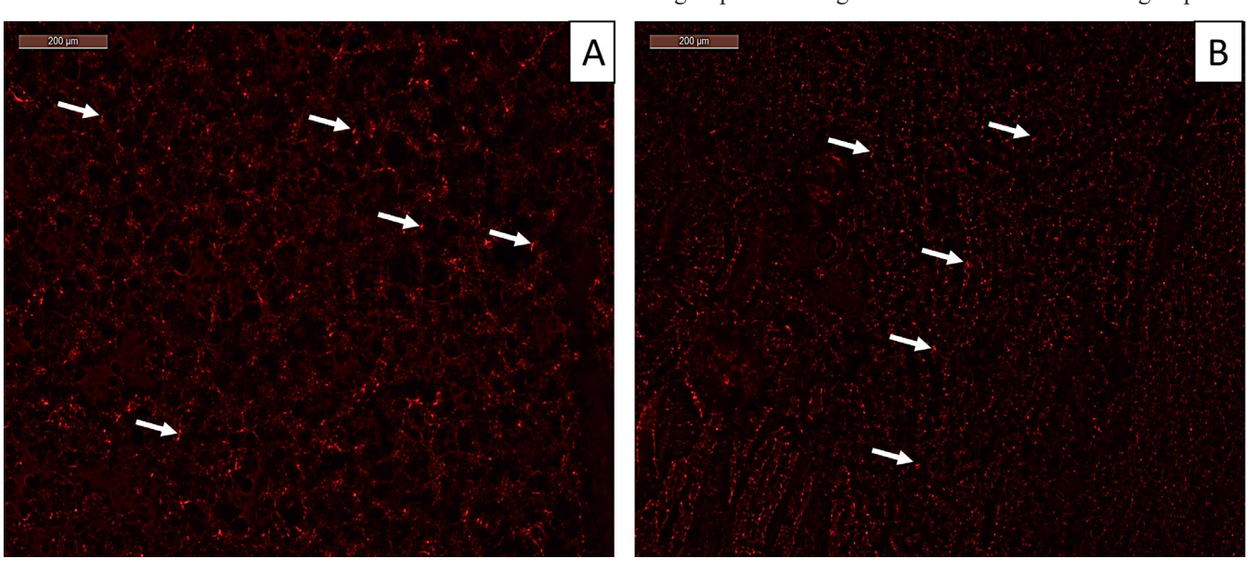


Fig. 2: A photomicrograph of microscopic fluorescent sections in the femur (100x):
A) Type II DM +BM-MSCs Group: showing PKH26 labeled BM-MSCs (white arrows).
B) Combined treated group: showing PKH26 labeled BM-MSCs (white arrows).

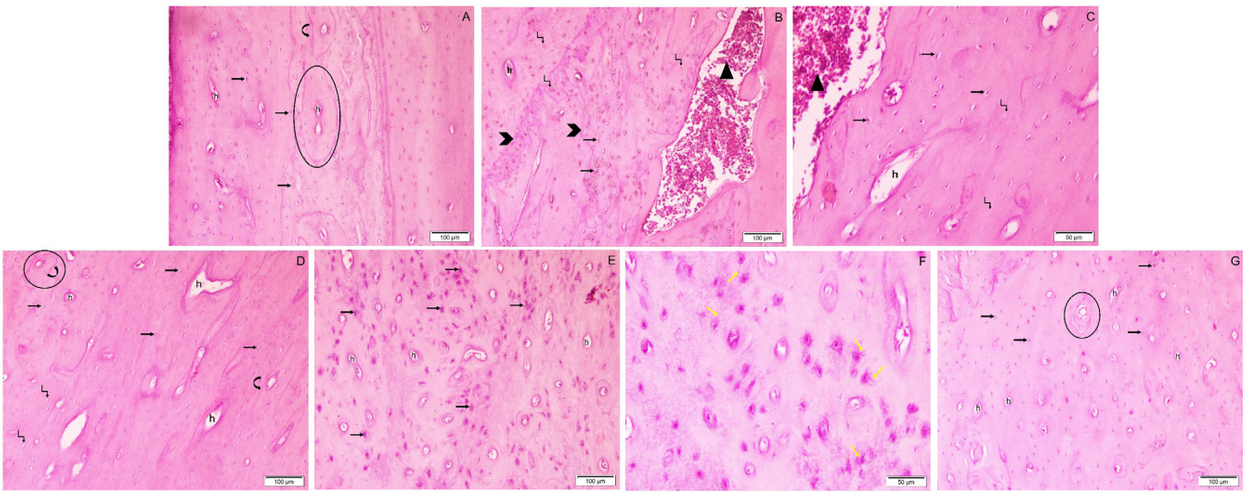


Fig. 3: A photomicrograph of H&E-stained longitudinal sections in the femur (magnification A,B,D,E,G100x; C 200x; F 400x):
E) Control group shows the Haversian system (encircled structure) formed by the Haversian canal (astrex) surrounded with concentric layers of bone lamellae (curved arrow) and osteocytes in lacunae (arrows).
B, C) Type II DM group exhibiting large, congested blood vessels (triangle) and occasionally Haversian canals (Asterix) surrounded with irregular bone lamellae (arrowheads). Note some of the lacunae are empty (kinked arrows) with few osteocytes in their lacunae (arrows).
E) Type II DM +vitamin D group demonstrating Haversian system (encircled structure) formed of widened haversian canals (Asterix) surrounded with concentric bone lamellae (curved arrow) and osteocytes in lacunae (arrows). Note the presence of some empty lacunae (kinked arrows).
E, F) Type II DM +BM-MSCs group revealing haversian canals (Asterix) surrounded by osteocytes in their lacunae with clear canaliculi (arrows).
G) combined treated group exhibiting Haversian system (encircled structure) formed of haversian canal (Asterix) and osteocytes in their lacunae (arrows).

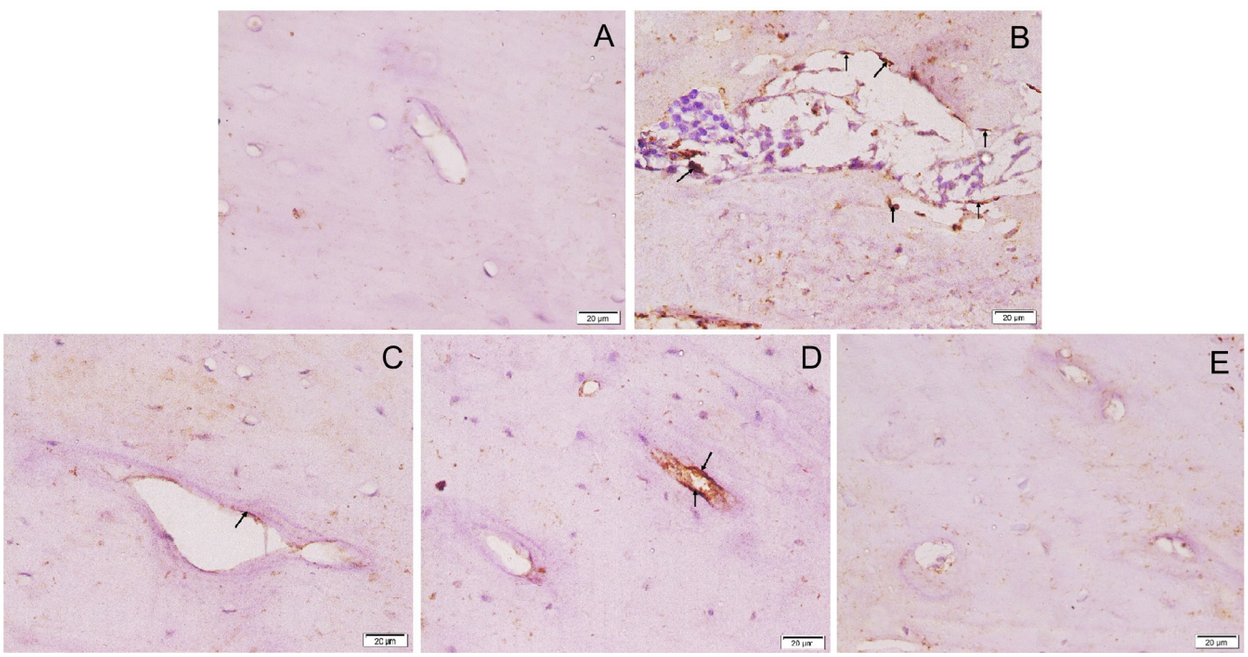


Fig. 4: A photomicrograph of VEGF immunostained longitudinal sections in the femur (400x):
A) Control group reveals negative nuclear immunoreactivity to VEGF.
B) Type II DM group showing a large number of nuclei (arrows) immunostained by VEGF.
C) Type II DM + vitamin D group exhibiting occasional nuclei (arrows) immunostained by VEGF.
D) Type II DM +BM-MSCs group showing few nuclei (arrows) immunostained by VEGF.
E) combined treated group revealing negative nuclear VEGF immunoreactivity.

Table 2: changes in serum glucose and lipid profile in all studied groups

	Group I	Group II	Group III	Group IV	Group V
Glucose (mmol/l)	5.4±0.39	17.85±1.22 *	9.25±0.85 *#	9.37±0.54 *#	6.99±0.56 *#$0
TG (mg/dl)	100±12	203.67±12.91 *	130.83±5.19 *#	144.17±9.06 *#	103.67±6.15 #$0
TCH (mg/dl)	139.83±8.52	266.83±24.84 *	172.17±7.78 *#	159.83±4.17 #	131.17±5.98 #$0
HDL (mg/dl)	67.17±6.59	27±4.2 *	40.5±2.81 *#	43±4.43 *#	53.5±3.08 #$0
FFA (UG/ML)	2.1±0.45	9.33±0.91 *	4.66±0.42 *#	5.08±0.4 *#	2.68±0.51 #$0

Values are presented as mean ±SD
*: statistically significant compared to corresponding value in group I (P<0.05)
#: statistically significant compared to corresponding value in group II (P<0.05)
$: statistically significant compared to corresponding value in group III (P<0.05)
0: statistically significant compared to corresponding value in group IV (P<0.05)

Table 3: Changes in oxidative stress and inflammatory state in all studied groups:

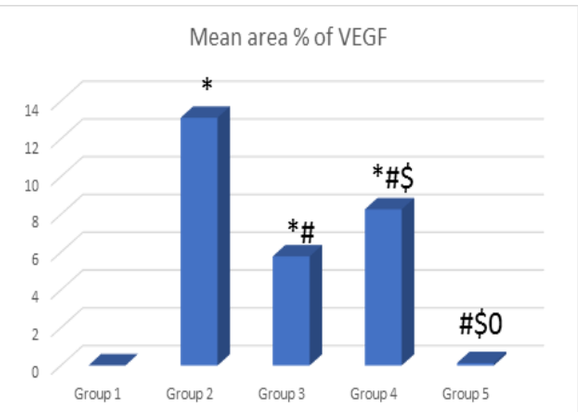
	Group I	Group II	Group III	Group IV	Group V
ROS (nmol/ML)	23±2.42	129.77±6.2 *	70.9±5.1 *#	75.67±9.72 *#	41.2±3.49 *#$@
TNF-α (mg/dl)	2.63±0.39	13.82±0.96 *	9.95±0.45 *#	8.47±0.46 *#$	8.15±0.78 *#$

Values are presented as mean ±SD
*: statistically significant compared to corresponding value in group I (P<0.05)
#: statistically significant compared to corresponding value in group II (P<0.05)
$: statistically significant compared to corresponding value in group III (P<0.05)
@: statistically significant compared to corresponding value in group IV (P<0.05)

Table 4: Changes in Wnt/ b-catenin, sclerostin, and Bax/BCL2 in all studied groups:

	Group I	Group II	Group III	Group IV	Group V
Wnt (relative expression)	1.02±0.04	0.38±0.03 *	0.53±0.03 *#	0.62±0.04 *#$	0.84±0.04 *#$@
β- catenin (relative expression)	1.01±0.01	0.37±0.04 *	0.49±0.02 *#	0.65±0.03 *#$	0.78±0.05 *#$@
Sclerostin (relative expression)	1.02±0.01	6.22±0.69 *	3.78±0.38 *#	2.83±0.34 *#$	1.42±0.17 #$@
BCL2 (relative expression)	1.02±0.02	0.25±0.06 *	0.7±0.04 *#	0.65±0.06 *#	0.82±0.05 *#$@
Bax (relative expression)	1.03±0.04	6.32±0.84 *	2.99±0.05 *#	2.95±0.16 *#	2.26±0.24 *#$

Values are presented as mean ±SD
*: statistically significant compared to corresponding value in group I (P<0.05)
#: statistically significant compared to corresponding value in group II (P<0.05)
$: statistically significant compared to corresponding value in group III (P<0.05)
@: statistically significant compared to corresponding value in group IV (P<0.05)



Histogram 1: Histogram presenting the mean area percent of VEGF immunohistochemical stain in studied groups.

DISCUSSION

The long-term exposure to hyperglycemia causes changing bone metabolism and impairment of its micro-architecture. These changes cause an impairment of osseous healing and increase the fracture risk[2].

In this study, type II DM was induced by injecting streptozotocin (STZ) after starting the intake of 40% of calories as fat [high-fat diet (HFD)] for 2 weeks[10]. Our main findings were increased serum glucose levels and lipid profile, ROS, and TNF-α levels in Type II DM in comparison with control. When compared to the control group, the Wnt/b-catenin and BCL2 levels in the DM group were significantly lower, while compared to the control group, the diabetic group had higher levels of sclerostin and BAX. Type II DM is associated with increased

oxidative stress, which underlies the promotion of type II DM complications. Elevated oxidative stress is thought to be a detrimental component that contributes to insulin resistance, decreased glucose tolerance, dyslipidemia, and beta-cell dysfunction, all of which contribute to the eventual development of type II diabetes mellitus[17]. It is widely known that the generation of reactive oxygen species (ROS) is mostly influenced by hyperglycemia[18].

The bone mass is negatively regulated by Sclerostin by blocking osteoblast genesis. Anti-sclerostin antibody therapy increases bone mass in healthy monkeys, rodents, and rats with type II diabetes, according to in *vivo* investigations[4]. For osteoblast differentiation, the bone morphogenetic pathway and Wnt signaling are essential. Reduction of Wnt activity in osteogenic cell lines is associated with declined differentiation of osteogenic cells when incubated in high glucose concentrations[19]. One of the key mechanisms that control the differentiation of MSCs into adipocytes as opposed to osteoblasts is Wnt signaling. In type II DM, elevated PPAR signaling, which is largely Wnt-dependent, causes bone marrow obesity by increasing adipogenesis while decreasing osteogenesis[20].

In our study, bone tissues of Type II DM group showed large, congested blood vessels and widened Haversian canals surrounded with irregular bone lamellae. These findings were described by a previous study that stated that uncontrolled diabetic patients exhibited immature woven bone, vascular architectures, and sinusoidal vessels present proximally to the defect border[21]. Diabetes alters bone homeostasis by blocking osteoblastic development and inducing osteoblastic death[22].

In addition, previous studies stated that osteoclastic bone resorption is regulated with osteoblasts. Indeed, acidic pH is needed for bone resorption to decarboxylate proteins via osteoclasts. Therefore, altering insulin signaling in osteoblasts causes the induction of glucose metabolism in a way that is dependent upon bone resorption[23]. Diabetes also prevents mesenchymal cells from proliferating and differentiating, decreasing osteoblast mineralization and increasing osteoclastic activity[24].

In this study, type II DM induced osteocyte apoptosis leaving empty lacunae. This finding was detected by[25] who induced type II DM by high fat diet revealing empty lacunae with the death of osteocytes.

Apoptosis of osteocytes is an event that proceeds resorption of osteoclast in bone remodeling. Apoptosis of osteocytes is induced by diabetes. It has been found recently that advanced glycation end products, accumulated during DM, stimulate p53, bax, and caspase-3 via a forkhead box protein O1-dependent pathway to cause cellular apoptosis[26].

Angiogenesis is a vital process that occurs to generate capillaries from preexisting vascular beds under the controlling mechanism of the pro-angiogenic factors such as vascular endothelial growth factor (VEGF)[27].

In the present study large number of nuclei were immunoassayed with overexpression of VEGF associated with a significant increase of mean area percent in type II DM group compared to the control group. This could be clarified by previous studies[28] which stated that diabetes creates hypoxic media inducing VEGF expression and abnormal angiogenesis.

The principal effect of Vit D is concerned with skeletal homeostasis and minerals[29]. Minimal levels of Vit D lead to osteopenia and a higher risk for bone resorption by osteoclasts. These outcomes are the direct controlling effects of vit D.[30]. Active form from vit D accelerated commitment of human dental pulp extracted-MSCs with the subsequent osteoblastic maturation[3].

The potential ameliorative impact of vitamin D on the levels of the serum lipid profile (TC, TG, and FFA) is confirmed in our study where there was a significant decrease in all these parameters in type II DM +vitamin D group compared to Type II DM group except for HDL, which was significantly elevated in Type II DM +vitamin D group in comparison with Type II DM group. These results were in accordance with our data[31]. Increased generation of ROS, increased inflammation, increased lipid peroxidation, and decreased antioxidant levels are all associated with diabetes[32]. There is recent evidence that vit. D can lessen oxidative strain[18]. The marked decrease in TNF-α, and ROS levels in diabetic rats after treatment with vit. D in group Type II DM +vitamin D compared to diabetic rats in Type II DM group is an important finding[32]. In addition, Wnt/ b-catenin and BCL2 levels are significantly increased associated with a significant reduction of sclerostin and Bax levels in type II DM +vitamin D group compared to DM untreated group.

In Chen's work, the osteogenic differentiation of MSCs is influenced by Vit. D. They stated that mineral matrix deposition and expression of typical osteoblastic markers during MSCs osteogenic differentiation was enhanced by active form of vit D[33].

In the present experiment, glucose level was significantly reduced after treatment with vit. D which was also reported by[31].

In the present study vitamin D treatment in Type II DM +vitamin D group exhibited Haversian system formed of widened haversian canals surrounded with concentric bone lamellae and osteocytes in lacunae apart from some empty lacunae. It has been reported that Vit D induces alkaline phosphatase activity and increases expression of collagen type I during osteoblastic proliferation and differentiation[3,34].

Vitamin D has been shown to improve osteogenesis/ angiogenesis coupling through increased VEGF levels, restore the equilibrium between osteoblast genesis and osteoclast genesis, and correct biomechanical parameters and mineral metabolism in rat bones[35]. This was detected in Type II DM +vitamin D group where mean area % of

VEGF was significantly elevated in control group but markedly reduced in Type II DM group.

In our study, serum glucose level was decreased in the group of combined treatment in comparison with the DM group. Blood glucose level is lowered by MSCs in STZ-diabetic rats[31]. In addition, the treatment of diabetic rats with MSCs optimizes the lipid profile pattern. This finding agrees with[36]. Moreover, the circulating TNF-α and ROS in diabetic rats following treatment with MSCs was significantly decreased compared to untreated diabetic animals. Our study confirms a significant increase in Wnt/ b-catenin and BCL2 Levels and a significant decrease in sclerostin and Bax levels in the Type II DM +BM-MSCs group in comparison with Type II DM group.

The Wnt signaling pathway plays an essential role in bone remodeling by promoting osteoblastic proliferation and decreasing apoptosis[37]. Increased Wnt/-catenin pathway is essential for enhancing MSC differentiation into osteoblasts and inhibiting their differentiation into adipocytes, which allows MSCs to induce the production of new bone and boost mineralization, which strengthens bone[38]. This was detected in our study after homing of PKH26 labeled BM-MSCs in bone sections of Type II DM +BM-MSCs group and the histological examination revealed normal bone architecture which could be explained by the secretion of biologically active factors by MSCs as growth factors, cytokines, and chemokines that promotes healing and repairing of tissues. Furthermore, MSCs increased the osteoprotegerin/ receptor activator of nuclear factor-kappa B ligand (OPG/RANKL ratio), by limiting the formation of osteoclasts and bone resorption by raising OPG levels while lowering RANKL levels[39,40].

Additionally, MSCs promote angiogenesis by secreting angiogenic, mitogenic, and antiapoptotic substances such as VEGF and insulin-like growth factor-1 (IGF-1). The latter stimulates cellular proliferation and suppresses apoptosis in addition to its angiogenic function[41]. This was proved in this study where MSCs induced /angiogenesis by VEGF release promoted osteoblastic activity and repairing the bone, Thus VEGF is markedly reduced in Type II DM +BM-MSCs group when compared to Type II DM group with a marked increase in comparison with control and Type II DM +vitamin D groups. Moreover, H&E sections demonstrate clear canaliculi which is explained by[42] who recently hypothesized that osteocytes send multiple bone canaliculi in the bone matrix during bone regeneration, similar to cerebral neural networks.

In the current study, the combined treatment with MSCs and vit. D was better than each one alone. Moreover, MSCs can exert different effects, it may help to regenerate cells[43]. Additionally, vitamin D influences MSCs directly and powerfully by encouraging their differentiation[44]. Vitamin D promotes the development of osteoblasts and stem cells (MSCs) thus promoting their abilities to enhance cellular regeneration and bone repair[45]. This is consistent with our findings where histological examination exhibited normal bone architecture with the restoration of VEGF levels which is significantly reduced in comparison with Type II DM group, Type II DM +vitamin D group, and Type II DM +BM-MSCs group and no significant difference with the control group.

CONCLUSION

In conclusion, the biological plausibility of using vitamin D in type II diabetes management-induced bone turnover becomes of high quality. The ability of MSCs to reduce inflammation and oxidative stress could promote cell survival, which would lessen or stop the degeneration brought on by type II DM. Compared to an injection of vitamin D or MSCs alone, the combination of MSCs with oral vitamin D appears to contribute to the improvement of diabetic-induced bone turnover in rats.

Further studies are needed to know if MSCs combined with vitamin D can be used in prophylactic therapy in diabetic high-risk persons.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة وظيفية ونسيجية وكيميائية مناعية عن التأثير المحتمل لفيتامين د والخلايا الجذعية اللحمية المتوسطة على تقليل تغييرات إعادة تشكيل العظام في الجرذان المصابة بداء السكري التي يسببها الستربتوزوتوسين

اشرف قطب[١]، ساره عادل حسني[٣]، احمد دسوقي[١]، فوزي عبدالتواب عبداللطيف[١]، رانيا السيد حسين[٤]، اسماء محمد شمس الدين[١،٢]

قسم الفسيولوجيا الطبية كلية الطب جامعة[١] ٦ أكتوبر و[٢]جامعة القاهرة، مصر

[٣]قسم الأنسجة، كلية الطب، جامعة القاهرة، القاهرة، مصر

[٤]قسم الكيمياء الحيوية الطبية والبيولوجيا الجزيئية، كلية الطب، جامعة القاهرة، مصر

الخلفية والأهداف: داء السكري هو اضطراب استقلابي مرتبط بهشاشة العظام وزيادة خطر الإصابة بكسور العظام. هدفت هذه الدراسة إلى توضيح تأثير داء السكري على تغيرات إعادة تشكيل العظام ، ودراسة دور مكملات فيتامين د والخلايا الجذعية اللحمية المتوسطة في تقليل تغييرات إعادة تشكيل العظام في النوع الثاني من داء السكري.

المواد والطرق: تم تصنيف ٣٠ ذكور جرذ ألبينو بالغ إلى: المجموعة الأولى (المجموعة الضابطة) والمجموعة التجريبية الرئيسية. تم تغذية جميع الفئران في المجموعة التجريبية بنظام غذائي عالي الدهون وحقنت مرة واحدة بالستربتوزوتوسين (٤٥ مجم / كجم) داخل الصفاق ثم بعد ٨ أسابيع تم تقسيمها إلى ؛ المجموعة الثانية استمرت دون علاج ، المجموعة الثالثة ؛ تناول فيتامين د عن طريق الفم يوميًا ، المجموعة الرابعة ؛ تلقي حقنة في الوريد من ٣ × ١٠ من الخلايا الجذعية اللحمية المتوسطة والمجموعة الخامسة ؛ يعالج بالخلايا الجذعية اللحمية المتوسطة وفيتامين د. في نهاية التجربة ، تم جمع عينات الدم لقياس مستوى الجلوكوز ونسبة الدهون وأنواع الأكسجين التفاعلي وعامل نخر الورم. تم تشريح عظام الفخذ من أجل التحليل الكيميائي الحيوي ، النسيجي ، المناعي ، المورفومتري والإحصائي.

النتائج: تم زيادة متوسط مستويات المصل من الجلوكوز والدهون الثلاثية والكوليسترول الكلي وأنواع الأكسجين التفاعلية وعامل نخر الورم بشكل ملحوظ مع انخفاض معنوي في مستوى البروتين الدهني المرتفع ومستوى الأحماض الدهنية الحرة في المجموعات الثانية والثالثة والرابعة. بالإضافة إلى ذلك ، انخفضت مستويات Wnt / b-catenin وخلية سرطان الغدد الليمفاوية بشكل ملحوظ ، بينما زاد sclerostin و BAX في النسبة المئوية لعامل النمو البطاني الوعائي في المجموعات الثانية والثالثة والرابعة. واجه فيتامين د والخلايا الجذعية اللحمية تحسنًا ملحوظًا في الواسمات البيوكيميائية والتغيرات النسيجية والنسيجية المناعية التي يسببها الستربتوزوتوسين مع تحسن أكثر وضوحًا في المجموعة المشتركة (المجموعة الخامسة).

الخلاصة: العلاج المشترك لفيتامين (د) والخلايا الجذعية الوسيطة أدى إلى تحسن كبير في معدل دوران العظام الناتج عن مرض السكري مقارنة بحقن الخلايا الجذعية الوسيطة أو فيتامين (د) وحده عن طريق التخفيف من التأثيرات المضادة للأكسدة والمضادة للالتهابات.