Effects of Vitamin D and Metformin on Diabetic Cardiomyopathy in Rats with Type 2 Diabetes Mellitus

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Abstract:

Aims/hypothesis: There is evidence that suggest a protective role of vitamin D in Diabetic cardiomyopathy (DCM). Our study was designed to compare the role of vitamin D and metformin on DCM in type 2 diabetic rats.

Methods: Sixty male rats divided into five groups; control, diabetic, diabetic + metformin, diabetic + vitamin D, diabetic + metformin and vitamin D. Echocardiographic measurement of systolic and diastolic functions was performed. Blood samples analysed for serum glucose, insulin, triglycerides, cholesterol, HDL, Ca2+, Brain natriuretic peptide and procollagen III. Gene expression of vitamin D receptor (VDR), Beclin I and SERCA 2 in cardiac tissue and histopathological examination were performed.

Results: Diabetic rats developed DCM with a significant decrease in VDR, beclin I and SERCAII gene expression in cardiac tissues of diabetic rats was recorded. Treatment with metformin, vitamin D or both improved the cardiac functions, glycemic control, and lipid metabolism and increased the expression of SERCA2, Beclin 1 and VDR. Histopathological examination showed collagen fibers deposition in hearts of diabetic rats that was decreased by treatment with metformin, vitamin D or both.

Conclusion: Vitamin D or metformin administration in combination to type 2 diabetic rats resulted in synergistic action as regard most of measured parameters.

Key words: Type 2 diabetes, cardiomyopathy, vitamin D, metformin, contractility, echocardiography

Introduction:

Diabetes mellitus (DM) is a common chronic disease, and its prevalence continues to increase worldwide. After the exclusion of coronary artery disease and hypertension, patients with DM remain associated with an increased incidence of heart failure.[1] Diabetic cardiomyopathy (DCM) is characterized by cardiac lipid accumulation, myocardial fibrosis and increased myocardial cell death, leading to left ventricular remodeling and hypertrophy, diastolic dysfunction, and ultimately systolic impairment.[2]

Vitamin D exerts pleiotropic effects on multiple systems and its deficiency is associated with many chronic disorders, including DM.[3] $1,25(OH)_2D$ has various cellular and molecular effects on the cardiovascular system and DM. Accumulating evidence suggests that $1,25(OH)_2D$ possesses anti-

inflammatory, anti-oxidative, anti-hypertrophic and anti-fibrotic properties. In addition, $1,25(OH)_2D$ may exert beneficial effects on cardiac metabolism, insulin secretion and sensitivity in diabetic cardiomyopathy.[4]

Metformin is believed to be the most widely prescribed anti-diabetic medication in the world. It improves peripheral insulin sensitivity and reduces hepatic glucose output, and thus helps in controlling hyperglycemia. Metformin has shown to upregulate cardiomyocyte autophagy that has role in prevention of diabetic cardiomyopathy in animal models.[5]

Our study designed to evaluate the potential beneficial effects and mechanisms of vitamin D on diabetic cardiomyopathy in type 2 diabetic rats. This study also attempted to elucidate the impact of metformin treatment in diabetic rats and the cumulative effect of vitamin D and metformin in comparison with each one alone.

Materials and Methods:

I-Experimental animals and study design:

The experimental steps, animal handling, sampling and scarification were done according to the *Guide for the care and use of laboratory animals*, Eighth edition (2011) and were approved by Ethical Committee of Physiology department, Faculty of Medicine, Cairo University.

60 adult male Albino rats weighing 100 to 120 g obtained from National Cancer Institute constituted the animal model for this study and have been divided into the following 5 groups (12 rats/group).

Control group: represent normal reference for the measured parameters. Rats were injected with vehicle alone.

Diabetic group: represent untreated type 2 diabetic rats. The diabetes was induced by administration of high fat diet (HFD) for 2 weeks[6] followed by an intraperitoneal (i.p.) injection of single low dose of streptozotocin (STZ) (35mg/Kg B.W.) (MP Biomedicals, USA), stored at $+4^oC$ and dissolved in sodium citrate buffer, pH 4.5.[7] Type 2 diabetes was proved by retro orbital blood samples taken 5 days after STZ injection for measuring serum glucose level. Rats above 11mmol/L were considered diabetics[8], and the rats below this value were excluded from the experiment.

Diabetes + Metformin group: Type 2 diabetes was induced and then the rats received oral metformin obtained as commercial tables (Cidophage 500 mg, CID pharmaceutical company). Tablet were dissolved in 50 ml of distilled water and given by oral gavage in a dose of 100mg/Kg B.W. for 8 weeks.[9]

Diabetic + Vit D: Type 2 diabetes was induced and then the rats received 1,25(OH)2D3 dissolved in castor oil and injected i.p., 3 times weekly for 8 weeks in a dose of 0.5μg/Kg B.W.[10]

Diabetes + Metformin + Vit D: Type 2 diabetes was induced and then the rats received daily metformin together with 1,25(OH)2D3 as described previously.

The rats were housed 6 rats per cage, acclimatized to the normal environmental conditions as regards dark/light cycle, degree of temperature, relative humidity with 12 h day: 12 h night cycle), and allowed free access to water.

Beginning on day 0, rats were fed either normal rodent chow (3% fat; 68.1% carbohydrate and 20.3% protein) in control group or HFD (60% fat; 19.2% carbohydrate and 20.8% protein) in the remaining groups.[11]

II-Experimental Measurements:
A-Echocardiographic Measurements:

After 8 weeks of treatment, cardiac functions were evaluated in vivo by echocardiography. The rats were anesthetized with an injection of ketamine hydrochloride (25 mg/kg, B.W. i.p.) and xylazine (5 mg/Kg B.W. i.p.).[12] The anterior chest hair was removed and the rats were secured to the surface of a warming table to maintain normothermia.[13]

Echocardiograms were performed with an echocardiography system equipped with a 10MHz phased-array transducer (**GE Healthcare's Vivid I U.S.A**). Two-dimensional short axis view of the left ventricle and M-mode tracings were recorded to measure ejection fraction (EF), fractional shortening

(FS), left ventricular dimension in diastole (LVIDd) was determined at the tips of the papillary muscle. Trans mitral Doppler flows (E and A velocities, and their ratio) were measured in an apical 4-chamber orientation with the sample volume placed at the tips of the mitral leaflet.[13]

On the next day and after overnight fasting, blood samples were taken for biochemical measurements and then rats were killed under anesthesia. Their hearts were removed and each heart was divided into 2 parts; one half for histopathological measurement and other half biochemical measurements.

B-Histopathological Examination:

Hearts were fixed immediately in a 10% neutral-buffered formalin solution and the left ventricles were processed for light microscopic study. Paraffin sections of 5 μm thickness were stained with H&E and Masson's trichrome.[14]

The area percentage of the collagen content was measured in Masson trichrome-stained sections. An objective lens of X10 in standard fields of 118476 mm^2 was chosen. Morphometric measurements were done in Cairo University-Research Park (CURP), Faculty of Agriculture, Cairo University using Leica Qwin 500 image analyzer computer system (Cambridge, England). The image analyzer comprised of a colored video camera; Panasonic wv. GP 210, colored monitor, hard disc of Leica IBM personal computer. They are connected to a BX41 Olympus microscope (Tokyo, Japan), LeciaQwin 500 software. The image analyzer was first standardized automatically to transform the measurement units (pixels) produced by the image analyzer program into actual micrometer units.

B-Biochemical Measurement:

Serum B-natriuretic peptide (BNP), Procollagen III (kits supplied from RayBio, USA) and insulin (kits supplied from Linco research, USA) were assessed by Enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions.

Serum glucose, cholesterol, triglycerides (TG) and calcium were assayed by conventional kits supplied by (Diamond Diagnostics, Hungary).

For the assessment of peripheral insulin resistance, homeostasis model assessment (HOMA) index was calculated as follows:

HOMA=glucose(mmol/dL) X insulin(μIU/mL)/22.5

HOMA values of more than 4.0 were taken as resistant.[15]

Gene expression of cardiac sarcoplasmic reticulum Ca2+-ATPase(SERCA II) ,vitamin D receptor (VDR)&beclin were evaluated by quantitative real time polymerase chain reaction (qRT-PCR) as follows:

a- RNA extraction and cDNA synthesis:

Total RNA was extracted from heart homogenate using Qiagen tissue extraction kit (Qiagen, USA) according to manufacturer's protocol. The purity (A260/A280 ratio) and the concentration of RNA were obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA). The total RNA (2 μg) was used for cDNA conversion using high capacity cDNA reverse transcription kit Fermentas, USA) following manufacturers instruction.

b- Real-time PCR

Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA) using the primers shown in Table 1. The qPCR assay with the primer sets were optimized at the annealing temperature. All cDNA were in duplicate and including previously prepared samples, and non-template control (water to confirm the absence of DNA contamination in the reaction mixture).

After the RT-PCR run the data were expressed in Cycle threshold (Ct). The PCR data sheet includes Ct values of assessed genes (for SERCA2, VDR & beclin 1 gene expression in heart tissue) and the house keeping gene, the gene that continuously and normally expressed in the cell (β-actin gene). Relative expression was calculated according to applied biosystem software.

D-Statistical Methods

Data was analyzed using the statistical package SPSS version 22. Data presented as mean and standard deviation for quantitative variables.

Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc Tukey test. Correlations between quantitative variables were done using Pearson correlation coefficient. P-values less than 0.05 were considered as statistically significant.[16]

Table 1: The oligonucleotide primers sequence of studied genes (in vivo).

Gene symbol	Primers sequence
Rat Beclin-1	F:ATCCTGGACCGAGTGACCATTC; R:TCTCCTGAGTTAGCCTCTTCCTCC
Rat VDR	F-GCCCCTCATAAAGTTCCAGGTG R-GGATAGGCGGTCCTGAATGG
SERCA2a	F- CGAAAACCAGTCCTTGCTGAGGAT; R- TACTCCAGTATTGGCATGCCGA
GAPDH (rat)	F: 5'-ACCACAGTCCATGCCATCAC-3' R: 5'-TCCACCACCCTGTTGCTGTA-3'

Result:

I-CARDIAC FUNCTIONS ASSESSMENT BY ECHOCARDIOGRAPHY:

Table 2: Comparison between the echocardiographic parameters (Ejection Fraction%, Fractional Shortening%, E/A ratio % and LVIDd (mm)) among the studied groups.

Parameter	Group				
	Control	DM	DM+ Metformin	DM + Vit D	DM+ Metformin + Vit D
Ejection Fraction %	72.58± 2.49	47.64 ±5.21 [a]	71.65± 7.26 [b]	66.49± 6.74 [b]	77.01±10.20 [b]
%			50.4	39.57	61.65
Fractional Shortening %	36.12± 2.08	20.24 ±2.84 [a]	35.68± 6.22 [b]	31.92± 4.67 [b]	41.02±9.44 [b]
%			76.28	57.71	102.67
LVIDd (mm)	4±0.5	3.9± 0.7	3.2± 0.5	4.3± 1.1	3.8± 0.7
%			-17.95	10.26	-2.56
E/A ratio	1.88± 0.23	0.59± 0.15 [a]	0.46± 0.08 [a]	0.58± 0.11 [a]	0.48± 0.05 [a]
%			-22.03	-1.69	-18.64

DM: diabetes mellitus **LVIDd:** left ventricular internal diamention at end of diastole. Values are presented as mean ±SD; P< 0.05 is considered significant
[a]: statistically significant compared to corresponding value in control group (P<0.05)
[b]: statistically significant compared to corresponding value in DM group (P<0.05)
%: Percent change in treated groups compared to DM

As seen in **Table 2** there was a significant decrease of the ejection fraction and fractional shortening (P=0 for ejection fraction and 0.001 for fractional shortening) in diabetic group compared to control. Treatment with metformin or 1,25-(OH)2D3 or both in combination significantly increased ejection fraction and fractional shortening compared to the untreated diabetic group (P=0, 0.001, 0 respectively for ejection fraction and P=0.001, 0.012, 0 respectively for fractional shortening).

As regard LVIDd there was no significant difference in its value between all the studied groups.

On the other hand, the E/A ratio was significantly decreased in all groups (diabetic, diabetic + metformin, diabetic + vitD, diabetic + metformin + vitD, p=0 for all groups) compared to the control group.

II-HISTOPATHOLOGICAL ASSESSMENT:

Table 3: Comparison of the mean area percentage of collagen in µm² % among different studied groups

	Group				
	Control	DM	DM + Metformin	DM + Vit D	DM + Metformin +Vit D
Area percentage of collagen in µm² %	5.23 ± 1.24	23.54 ± 3.38 [a]	9.85 ± 3.5 [ab]	8.10 ± 1.56 [ab]	5.53 ± 1.41 [bcd]

DM:diabetes mellitus. Values are presented as mean ± SD; P < 0.05 is considered significant
[a]: statistically significant compared to corresponding value in control group (P<0.05)
[b]: statistically significant compared to corresponding value in DM group (P<0.05)
[c]: statistically significant compared to corresponding value in DM + metformin group (P<0.05).

d: statistically significant compared to corresponding value in DM + vitamin D group (P<0.05).

As shown in **Table 3**; the area percentage of collagen fibers of DM group was significantly (p=0) increased compared to control group. Administration of metformin or 1,25(OH)2D3 to diabetic rats resulted in significant (p=0.001) reduction the area percentage of collagen fibers as compared to DM group; however it was significantly higher (p=0.01) than control. Treatment of type 2 diabetes with both metformin and 1,25(OH)2D3 in combination produced a significant decrease (P=0) in the mean area percentage of collagen fibers as compared to diabetic group and restore it back to near control values.

Hematoxylin and eosin stained sections of control group **(Fig.1-A)** showed regular architecture of cardiomyocytes. They were short, branched and arranged in interlacing bundles with alternating light and dark bands. They had spindle shaped nuclei and abundant eosinophilic cytoplasm. Type 2 diabetic rats **(Fig 1-B1, B2)** had marked loss of the regular myocardial architecture affecting the cardiomyocytes; the cytoplasm showed intracellular vacuolations (V) and lost striations. The cytoplasm was slender indicating cardiomyocytes attenuation. The nuclei had karyolysis (K), pyknosis (arrow) or dark appearance. Edematous changes in the form of intercellular vacuolations (asterisk) and gapping were observed with increased interstitial and the perivascular collagen fibers with aggregates of inflammatory cells (I) were around the blood vessels and in the interstitial spaces.

Administration of metformin improved the histopathological changes induced by type 2 diabetes mellitus **(Fig. 1-C)**; cardiomyocytes were comparable to the control with spindle shaped nuclei and alternating light and dark band. Few cardiac muscle fibers showed nuclear changes as pyknosis or karyolysis. Also 1,25-(OH)2D3 therapy **(Fig. 1-D);** improved the pathology as cardiomyocytes were seen comparable to the control with spindle shaped nuclei and abundant eosinophilic cytoplasm without edematous changes or inflammatory cells. Supplementation of 1,25(OH)2D3 plus metformin **(Fig.1-E)** markedly ameliorated the histopathological changes induced by type 2 diabetes mellitus with normal distribution of the cardiomyocytes with no remarkable pathological findings. Muscle fibers were comparable to the control**.**

Masson trichrome stain **(Fig.2)** revealed normal distribution of interstitial collagen fibers in control group (**Fig. 2-A)**. In type 2 diabetic group the interstitial collagen fibers were markedly increased in **(Fig 2-B)**. Therapy with metformin or 1,25(OH)2D3 caused minimal increase of the interstitial collagen fibers when compared to the control group **(Fig.2-C, D)**. Rats received simultaneous therapy with both drugs showed normal distribution of the interstitial collagen fibers **(Fig.2-E).**

III-BIOCHEMICAL ASSESSMENT

a) Cardiac biomarkers:

As demonstrated in **Table 4**:

-Serum level of BNP was significantly (P=0) increased in type 2 diabetic rats compared to control. Administration of metformin, 1,25(OH)2D3 or both drugs simultaneously caused significant (P=0) decrease of serum level of BNF compared to type 2 diabetic group with the percent change highest in group received both drugs, but therapy failed to restore the serum level back to normal.

-Serum level of procollagen III significantly (P=0) increased in type 2 diabetic group compared to control. Metformin administration to diabetic rats didn't cause significant change in serum level of procollagenIII compared to diabetic group. However 1,25(OH)2D3 therapy caused significant (P=0.001) decrease in serum procollagen III compared to control. Co-administration of metformin and 1,25(OH)2D3 caused significant (P=0) decrease of serum procollagen III compared to diabetic group without significant difference compared to control group (P=0.2).

Table 4: Serum Biochemical measurements in the studied groups

Serum biochemical parameters	Group				
	Control	DM	DM + Metformin	DM + Vit D	DM + Metformin + Vit D
BNP (picogm/ml)	0.56± 0.10	2.06± 0.24 [a]	0.93± 0.23 [ab]	0.90± 0.13 [ab]	0.73± 0.17 [ab]
%			-54.85	-56.31	-64.56
procollagen III (ngm/ml)	1.05± 0.15	6.90± 1.92 [a]	5.61± 0.58 [a]	4.19± 1.65 [ab]	2.55± 0.47 [b c]
%			-18.7	-39.28	-63.04
Glucose (mmol/l)	5.40± 0.46	14.62 ± 3.01 [a]	10.92± 1.28 [ab]	10.66± 2.43 [ab]	7.19± 1.52 [bcd]
%			-25.31	-27.09	-50.82
Insulin (µ IU/L)	10.69± 1.39	21.45 ± 4.14 [a]	14.68± 1.84 [b]	16.28± 2.34 [ab]	14.65± 1.74 [b]
%			-31.56	-24.1	-31.7
HOMA IR	2.54± 0.20	13.69 ± 2.62 [a]	7.15± 1.36 [ab]	7.78± 2.41 [ab]	4.73± 1.40 [b]
%			-47.77	-43.17	-65.45
Ca (mg/dl)	9.65± 1.20	6.27± 1.22 [a]	8.27± 1.20	8.32± 0.60 [b]	9.02± 1.51 [b]
%			31.9	32.7	43.86
Triglycerides (mg/dl)	95.65± 7.77	144.15±15.25 [a]	127.32 ±8.02 [ab]	126.55 ±6.67 [ab]	117.33± 8.02 [ab]
%			-11.68	-12.21	-18.61
Cholesterol (mg/dl)	153.77 ±8.07	211.50±12.00 [a]	186.22 ±7.21 [ab]	177.02 ±16.77 [ab]	175.93± 13.10 [ab]
%			-11.95	-16.3	-16.82
HDL (mg/dl)	61.27± 2.77	28.08 ±3.62 [a]	38.92± 4.64 [ab]	42.02± 4.95 [ab]	48.73± 3.30 [abcd]
%			38.6	49.64	73.54

DM:diabetes mellitus, BNP:brain natriuretic peptide, HOMA-IR: Homeostatic model of insulin resistance; HDL: High density lipoprotein. Values are presented as mean ±SD, P < 0.05 is considered significant

[a]: statistically significant compared to corresponding value in control group (P<0.05)

[b]: statistically significant compared to corresponding value in DM group (P<0.05)

[c]: statistically significant compared to corresponding value in DM + metformin group (P<0.05).

[d]: statistically significant compared to corresponding value in DM + vitamin D group (P<0.05).

%: Percent change in treated groups compared to DM .

b) Glycemic control Markers:

As demonstrated in **Table 4** there was significant (P=0) increase in serum glucose in type 2 diabetic group compared to control group. Administration of metformin or 1, 25(OH) 2D3 to diabetic rats caused significant (P=0.02) decrease of serum glucose compared to untreated diabetic group but it is still significantly (P=0) increased compared to control group. The group received metformin and 1,25(OH)2D3 showed significant (P=0.014) decrease of serum glucose level compared to untreated diabetic group and without significant difference (P=0.51) compared to control group.

Serum insulin level Insulin resistant index; HOMA-IR and showed significant (P=0) increase in untreated type 2 diabetic group compared to control. Treatment with metformin, 1,25(OH)2D3 or both drugs resulted in significant (P=0.001, 0.01, 0.001 respectively) decrease in serum insulin and HOMA-IR compared to untreated diabetic group with the least insulin resistance in group treated with metformin and 1,25(OH)2D3.

c) Lipid profile Markers:

As shown in **Table 4**: Serum TG and cholesterol showed significant (P=0) increase in untreated diabetic group compared to control. Treatment with metformin or 1,25(OH)2D3 or both drugs caused significant (P=0.04,0.03, 0.001 respectively for TG and P=0.009, 0, 0 respectively for cholesterol) decrease of serum TG and cholesterol compared to untreated diabetic group. Treatment with metformin or 1,25(OH)2D3 or both drugs caused significant (P=0.001, 0, 0 respectively) increase of serum HDL compared to untreated diabetic group but still significantly decreased (P= 0) compared to control group.

d) Calcium homeostasis Markers:

As concluded from **Tables 4 and 5**: serum calcium level was significantly (P=0) decreased in untreated type 2 diabetic group compared to control. Treatment with metformin didn't cause significant difference (P=0.052) in serum calcium level compared to untreated diabetic group. However; administration of 1,25(OH)2D3 alone or with metformin caused significant (P= 0.045, 0.004 respectively) increase in serum calcium compared to untreated diabetic group but still below the serum level of the control.

Cardiac expression of vitamin D receptors and SERCA2 showed significant decrease (P=0) in untreated type 2 diabetic group compared to control group. Treatment with metformin or 1,25(OH)2D3 or both drugs simultaneously caused significant increase of vitamin D receptor and SERCA2 expression compared to untreated diabetic group (P=0) with percent increase highest in the group that treated with both drugs simultaneously.

e) Autophagy Marker:

Table 5: Gene expression in cardiac tissue among the studied group

	Group				
	Control	DM	DM + Metformin	DM + Vit D	DM + Metformin + VitD
Vit D receptors	1.03± 0.05	0.17± 0.04	0.61± 0.15 [ab]	0.74± 0.16 [ab]	0.80± 0.14 [ab]
%			258.82	335.29	370.59
SERCA2	1.04± 0.04	0.26± 0.11 [a]	0.64± 0.11 [ab]	0.73± 0.13 [ab]	0.72± 0.18 [ab]
%			146.15	180.77	176.92
Beclin-1	1.03± 0.04	0.16± 0.03 [a]	0.58± 0.16 [ab]	0.71± 0.16 [ab]	0.90± 0.10 [acd]
%			262.5	343.75	462.5

DM:diabetes mellitus.
Values are presented as mean ±SD, P < 0.05 is considered significant
[a]**: statistically significant compared to corresponding value in control group (P<0.05)**
[b]**: statistically significant compared to corresponding value in DM group (P<0.05)**
[c]**: statistically significant compared to corresponding value in DM + metformin group (P<0.05).**
[d]**: statistically significant compared to corresponding value in DM+vitamin D group (P<0.05).**

%: Percent change in treated groups compared to DM

As shown in **Table 5** there was significant decrease (P=0) in cardiac expression of Beclin-1 expression in untreated diabetic group compared to control. Treatment with metformin, 1,25(OH)2D3 or both drugs simultaneously caused significant increase of Beclin-1 expression (P=0) compared to untreated diabetic group, however only simultaneous administration of metformin and 1,25(OH)2D3 increased the expression to level close to the control group (P=0.28).

There was a significant positive correlation between serum calcium and ejection fraction, fractional shortening and E/A ratio (r=.509, P=.004; r=.452, P=.012; r=.434, P=.017respectively) and a significant positive correlation between vitamin D receptor expression in the heart and ejection fraction, fractional shortening and E/A ratio (r=.745, P=0; r=.699., P=0; r=.541, P=.002 respectively)

Also there was significant positive correlation between cardiac expressions of Beclin-1and ejection fraction, fractional shortening and E/A ratio (r=.689, P=0; r=.626, P=0; r=.505, P=.004 respectively).

Figure (1): Photomicrograph of Hematoxylin and Eosin staining in the studied groups (X400).
A: Control group, **B1 and B2:** DM group, **C:** DM + metformin group, **D:** DM + Vit D, **E:** DM + Metformin + Vit D
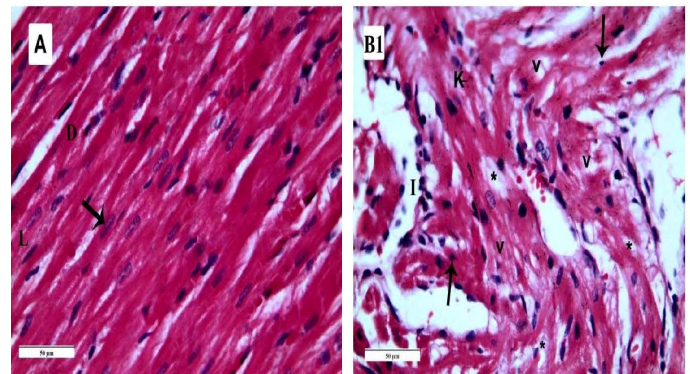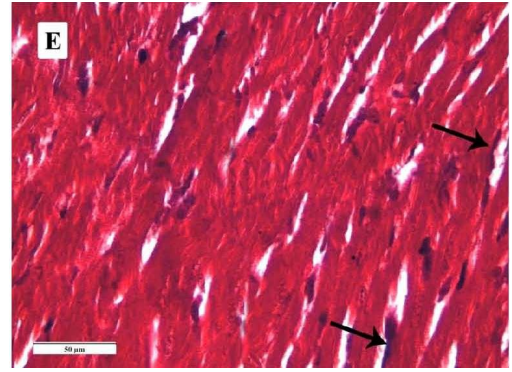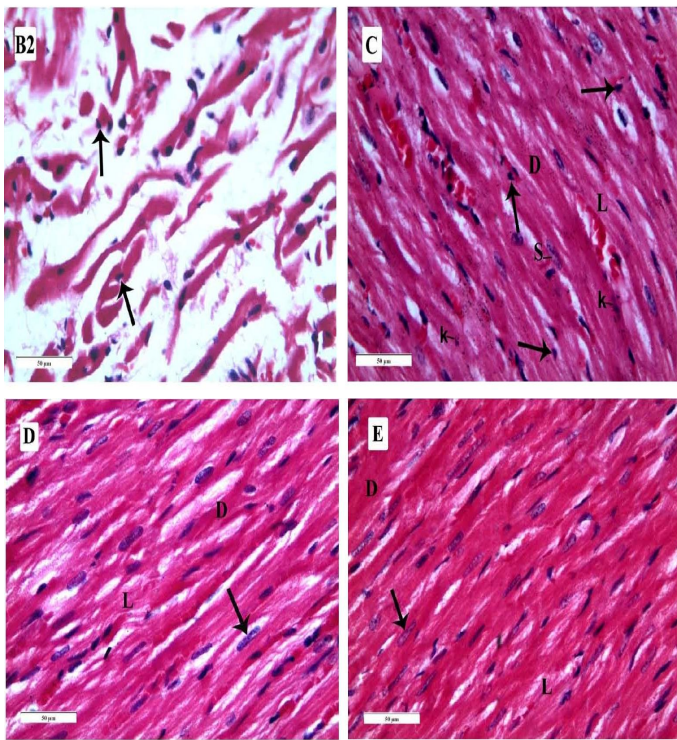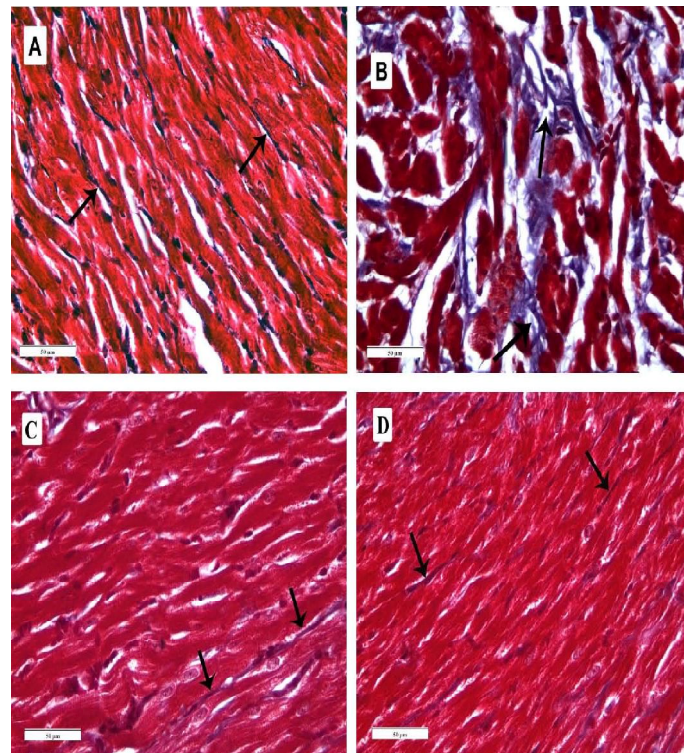
Figure (2): Photomicrogtaph of Masson Trichrome staining in the studied groups (x400)
A: Control group, **B:** DM group, **C:** DM+metformin group, **D:**DM+Vit D, **E:**DM+Metformin+Vit D

Discussion:

Diabetic cardiomyopathy is characterized by both systolic and diastolic dysfunction, due to reduced contractility and decreased compliance.[1] In the current study we found that diabetes decreased ejection fraction and fractional shortening and that decrease was improved by metformin, vitamin D or simultaneous administration of both drugs. In accordance to our echocardiographic results, *Zhao et al.*[17] study indicated that diastolic and systolic myocardial performance was depressed in the diabetic rats.

The echocardiographic findings in the current study were supported by the structural changes recorded by the histopathological evaluation that indicated irreversible damage. In agreement with our results, *Radovits et al.*[18] demonstrated collapse of myofibers and myocardial degeneration in the LV myocardium of diabetic rats. Myocardial glucotoxicity, lipotoxicity, overproduction of reactive oxygen and nitrogen species in DM result in derangement of cardiac structure and functions.[19]

The diagnosis of DCM was further confirmed by the significant increase serum level of BNP and procollagen III in the untreated group. This was in agreement with previous studies.[20,21]

Our results reported a significant increase in serum glucose, insulin level and HOMA- IR value compared to control group. The high energy diet induces insulin resistance in peripheral tissues, and this resulted in compensatory hyperinsulinemia due to enhanced β-cell secretion[22] and low dose STZ

injection makes partial dysfunction of beta cell to suppress insulin secretion.

Moreover; our results revealed a significant increase serum level of triglycerides, cholesterol and decrease serum level of HDL in diabetic group compared to the control group, this is in agreement with a study by *Patel and Goyal.*[23]

The hypertriglyceridemia in the DM due to mobilization of free fatty acids from peripheral deposit, as insulin inhibits hormone sensitive lipase.[24] Lipotoxicity impairs myocyte calcium handling with subsequent disruption of myocardial mechanics.[25]

Our results demonstrated a significant decrease in serum $Ca2^+$ and SERCA2 expression in cardiac tissue of diabetic rats compared to the control. In accordance to our results, **Wang** *et al.*[26] showed reduction of SERCA2a level in diabetic rats. However; *Fredersdorf et al.*[27] *showed* that SERCA2a mRNA levels were higher in diabetic rats compared to non-diabetic animals.

PI3-kinase/Akt signaling regulates intracellular calcium thorough potentiation of L-type calcium channel function and up-regulation of SERCA2a expression and activity while cardiac insulin resistance blunts phosphatidylinositol-3-kinases/AKT (PI3-kinase/Akt) signaling and impairs calcium handling.[28]

We showed an attenuation of autophagy process in diabetic rats as indicated by a significant decrease beclin I expression in cardiac tissue. In agreement with our results previous studies showed that autophagy was inhibited in diabetic animals, obesity and metabolic syndrome.[28] However; *French et al.*[29] showed that there was unchanged cardiac autophagy in mice fed a high fat diet. Autophagy may antagonize ventricular hypertrophy by increasing protein degradation and when autophagy is depressed, it impairs removal of damaged structures.[30]

Metformin or vitamin D therapy in the current work improved the cardiac function parameters measured by echocardiography. This was in agreement with previous studies by *Xiao et al.*[32] and *Lee et al.*[31]

Treatment of diabetic rats with metformin decreased the mean area percentage of collagen fibers as compared to the untreated diabetic group, however it did not return back to normal. *Xiao et al.*[32] demonstrated the cardio protective effects of metformin due to the inhibition of collagen synthesis by cardiac fibroblasts.

In the present work, treatment of type 2 diabetic rats with metformin resulted in a significant decrease in serum level of procollagen III compared to the untreated diabetic group but couldn't reach the control value. Previous studies showed that metformin reduced vascular remodeling, attenuates cardiac collagen, increases nitric oxide production and inhibits protein synthesis induced by angiotensin II in cardiomyocytes.[33]

At the present work, supplementation of vitamin D markedly improved the histopathological changes and decreased the mean area percentage of collagen fibers. *Rahman et al.*[34] showed that paricalcitol regulate fibrotic genes including matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), fibronectin and collagen type III.

Our results revealed that treatment of diabetes with metformin resulted in a significant decrease in serum level of BNP and procollagen III compared to untreated diabetic group but couldn't reach the control value. *Wang et al.*[35] agreed with our results as the mean BNP in the metformin treated group was increased in the heart tissue of the heart failure model but was still lower than that in the control group.

In the present study, treatment of diabetic rats with vitamin D reduced the BNP and procollagen III as compared with the untreated rats. In accordance with our results *Tamez et al.*[36] showed that treatment with paricalcitol reduced BNP serum level.

Searching for the protective mechanism of action of vitamin D and metformin, our results demonstrated that treatment of diabetes with metformin or vitamin D resulted in decrease in serum level of glucose and insulin together with HOMA IR compared to untreated diabetic group. Our results

agree with previous studies.[37-39] Metformin exerts its anti-hyperglycemic effect primarily through enhancement of GLUT-4 gene expression and inhibition gluconeogenesis.[38]

The proposed mechanisms for vitamin D's role in improving glucose homeostasis and metabolism include stimulating insulin secretion and improving beta-cell function of the pancreas in T2DM.[40] Additionally, 1, 25-(OH) 2D3 attenuates the expression of pro inflammatory cytokines and NF-Kb (Nuclear factor) activity.[41]

Wesuggest that the protective effect of metformin and vitamin D can be explained partially by the improvement of glycemic control, reducing the state of insulin resistance, and modifying lipid metabolism.

Metformin have been reported to reduce total cholesterol, LDL-cholesterol and triglycerides in type 2diabetes.[42] In disagreement with our results *Akyuz et al.*[24] reported that HDL levels increased by metformin treatment, however, it had no effect on cholesterol levels.

Abdelghany and Ibrahim[43] showed that supplementation of diabetic rats with low levels of 1, 25 (OH) 2 vitamin D, resulted in a significant decrease in total cholesterol, LDL-C and a slight non-significant decrease in triglyceride, while HDL-C was significantly raised as compared to the values before treatment. Vitamin D inhibits cholesterol synthesis and suppresses lipolysis in adipocytes.[44]

Further mechanisms explaining the cardioprotective role of metformin and vitamin D may be through improving Ca2+ homeostasis in the cardiomyocytes. The present results showed decreased serum Ca compared to control while treatment of diabetes with metformin or vit D or both in combination increased the serum Ca back to control level.

Meems et al.[45] reported that presence of 1, 25(OH) 2D3 increases efficiency of the absorption of renal calcium and of intestinal calcium.

Restoration of the normal autophagy may be a mechanism by which metformin and vitamin D improve the cardiac functions in diabetic rats. In agreement with our results *Xie et al.*[30] demonstrated that metformin enhanced Beclin1 protein expression in diabetic mice. Also; vitamin D3 up-regulates Beclin1 to trigger autophagy and they explained that vitamin D3 stimulates autophagy via down-regulation of mTOR protein levels, the principal negative regulator of autophagy.[46]

The present work showed that metformin, 1, 25(OH) 2D3 or both increased VDR gene expression in cardiac tissue but couldn't reach the control value. *Peeyush et al.*[47] Restoration of disrupted vitamin D receptor expression was seen with vitamin D3 treatment to diabetic rats via its up regulation.

Conclusion and Recommendations:

DM is associated with increased glucotoxicity, lipotoxicity, insulinresistance, decreased Ca2+ level and SERCA gene expression and depressed autophagy contribute to the development of diabetic cardiomyopathy. Metformin or vitamin D, each treatment separately attenuated the deleterious effects of disturbed metabolism and autophagy. Using both agents in combination gives synergistic effects thus ameliorating the structural and functional damage of diabetic cardiomyopathy. Vitamin D is promising for treating diabetic cardiomyopathy especially if given in addition to the classical hypoglycemic treatment, metformin.

We recommend further evaluation of markers of glucotoxicity, Lipotoxicity and autophagy using techniques that detect protein expression rather than gene expression.

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Contribution Statement:

Doaa M. El Sayed: she contributed to the experimental work, data collection, shared in

writing and revision of the manuscript and approved the final version.

Shaimaa N.Amin: Participated to conception and study design, revision of the collected data, writing and revision of the manuscript and approved the final version.

Hanan D. Yassa: Participated through the histologicalassessment performed in the study, shared in writing the manuscript,and approved the final version.

Laila A.Rashed: Participated through the biochemical assessment performed in the study, shared in writing the manuscript, and approved the final version.

Nashwa El Tablawy: Participated to conception and study design, revision of the collected data, shared in writing, revision of the manuscript and approved the final version.

SamahElattar: Participated to conception and study design, revision of the collected data, shared in writing, revision of the manuscript and approved the final version.

Compliance with Ethical Standards:

All authors declare that; there is no conflict of interest.

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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