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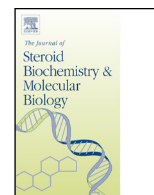

The protective effect of 1alpha, 25-dihydroxyvitamin d3 and metformin on liver in type 2 diabetic rats

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ABSTRACT

There is an accumulating evidence suggesting an immunomodulatory role of $1\alpha,25(OH)_2D3$. Altered $1\alpha,25(OH)_2D3$ level may play a role in the development of T2DM and contribute to the pathogenesis of liver diseases. Our study was designed to study and compare the effect of metformin and $1\alpha,25(OH)_2D3$ supplementation on liver injury in type 2 diabetic rat.

Sixty male Albino rats were divided into 5 groups; group 1: control rats. the remaining rats were fed high fat diet for 2 weeks and injected with streptozotocin (35 mg/kg BW, i.p.) to induce T2DM and were divided into: group 2: untreated diabetic rats, group 3: diabetic rats treated by metformin (100 mg/kgBW/d, orally), group 4: diabetic rats supplemented by $1\alpha,25(OH)_2D3$ (0.5 μg/kg BW, i.p.) 3 times weekly and group 5: supplemented by both $1\alpha,25(OH)_2D3$ and metformin. Eight weeks later, serum glucose and insulin levels were measured, HOMA IR was calculated, lipid profile, Ca2+, ALT and AST were estimated. Liver specimens were taken to investigate PPAR-α (regulator of lipid metabolism), NF-κB p65, caspase 3 and PCNA (proliferating cell nuclear antigen) and for histological examination.

The liver enzymes were elevated in the diabetic rats and the histological results revealed an injurious effect of diabetes on the liver. $1\alpha,25(OH)_2D3$, metformin and both drugs treatment significantly improved liver enzymes as compared to the untreated rats. The improvement was associated with a significant improvement in the glycemic control, lipid profile and serum Ca2+ with a significant reduction in NF-κB p65 and caspase 3 and increased PPAR-α, and PCNA expression as compared to the untreated group. $1\alpha,25(OH)_2D3$ induced a slightly better effect as compared to metformin. Both agents together had a synergistic action and almost completely protected the liver. Histological results confirmed the biochemical findings.

Our results showed a protective effect of $1\alpha,25(OH)_2D3$ and metformin on liver in diabetic rats as indicated by an improvement of the level of the liver enzymes, decreased apoptosis and increased proliferation and this was confirmed histologically, with modulating NFkB and PPAR-α. Both agents together had a synergistic effect.

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1. Introduction

Type 2 diabetes mellitus (T2DM) is one of the most common chronic diseases. Over the past three decades, its prevalence has more than doubled globally, therefore, it represents an eminent challenge to all nations [1]. Chronic complications of diabetes are the main causes of disability and mortality in diabetic patients [2].

A large spectrum of liver disease is documented in type 2 diabetic patients, including elevated liver enzymes, fatty liver disease, cirrhosis, hepatocellular carcinoma, and acute liver failure. The standardized mortality rate from uncompensated cirrhosis is higher than that from cardiovascular disease among diabetic patients [3].

Chronic hyperglycemia and insulin resistance induce an inflammatory response in the liver through accumulation of reactive oxygen species, which triggers the nuclear factor kappa

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beta (NF-κB) pathway [4]. Inflammation, together with disturbed systemic and hepatic fat metabolism, is the main causative factor underlying liver injury in diabetes [5]. Triglycerides accumulation within the hepatocytes is the main characteristic feature of fatty liver in T2DM [6].

Peroxisome proliferator-activated receptor α (PPAR-α), a ligand-activated transcription factor belonging to the nuclear receptor family, is highly expressed in the liver. It plays a crucial role in regulating hepatic lipid metabolism [7]. It has been reported that PPAR-α expression is decreased in diabetic liver [8].

Furthermore, caspase-3 is essential for the apoptotic process, being an effector caspase downstream of apoptotic pathways [9]. Haligur et al. found an increased apoptotic activity within the hepatocytes in streptozotocin induced diabetic rats and suggested that a possible mechanism of liver complications in diabetes may be linked to the increased apoptotic activity [10].

On the other hand, proliferating cell nuclear antigen (PCNA), a 36 kDa non-histone protein found in the nucleus, mediates DNA polymerase. Its expression has an extensive correlation with mitotic activity and can serve as a marker for cell proliferation [11,12].

1,25$(OH)_2$D3 receptors are disseminated in more than 38 tissues [13]. Animal studies demonstrated that vitamin D receptors (VDR) knockout mice showed spontaneous liver injury and fibrosis [14]. Also, VDR expression was found to be negatively correlated with the severity of non alcoholic fatty liver disease (NAFLD) [15].

Vitamin D insufficiency is frequent in patients with T2DM [16,17]. In fact vitamin D deficiency was related to the development of insulin resistance and many pathological complications of T2DM [18] and has been associated with an impaired glycemic control [19]. Mathieu et al. suggested that vitamin D deficiency had a detrimental effect to beta cell function and this caused glucose intolerance and predisposed to type 2 diabetes [20].

Many evidences suggested the key role of 1,25$(OH)_2$D3 in decreasing the risk of chronic diseases, including T2DM [21]. Administration of 1,25$(OH)_2$D3, led to diabetes prevention through immunomodulatory effects [20]. Other studies showed that vitamin D, as a lipid metabolism modulator, can correct dyslipidemia in overweight subjects [22,23]. Based on these information, it can be suggested that 1,25$(OH)_2$D3 supplementation may help in modulating the hepatic inflammation and lipid metabolism in T2DM rat models and protect against the liver injury associated with prolonged diabetes.

Metformin is the most widely used oral hypoglycemic agent as first-line therapy for T2DM and proved its efficiency in attenuating many complications of T2DM [24].

The aim of this study is to examine the possible effect of 1α,25 $(OH)_2$D3 supplementation on the diabetic state and liver functions, to compare the role of 1α,25$(OH)_2$D3 and metformin in providing protection for the liver in type 2 diabetic rat, and to explore the underlying mechanism for their actions.

2. Materials and methods

2.1. Animals

The study was in agreement with the approved guidelines and its protocol was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine Cairo University.

Sixty adult male Wistar Albino rats weighting 100–120 gm, were included in this study and were housed in wire mesh cages at comfortable temperature about 22 °C, normal light and dark periods in Physiology Department, Faculty of Medicine, Cairo University. The rats were provided with veterinary care by the Laboratory Animal House Unit and had a free access to rat chow[2] and water [25].

The rats were randomly divided into five groups, each group included twelve rats. **Group I, (Control group)** the rats of this group were used to provide normal control values for the parameters estimated. The remaining rats were fed a high fat diet[3] for 2 weeks before being injected with streptozotocin (STZ)[4] (35 mg/kg BW, ip.) to induce type 2 DM. After injection of STZ, the rats were given sugar in their drinking water for 24 h to prevent STZ induced fatal hypoglycemia resulting from immense pancreatic insulin release after its administration [26]. Five days later, the rats were anesthetized and blood samples were collected from the retro orbital plexus [27] and serum glucose levels were measured to confirm diabetes. Rats above 11 mmol/L were considered diabetics and the rats below this value were excluded from the experiment.

The diabetic rats were divided into 4 groups: **Group II, (DM group)**, these rats represent untreated type 2 diabetic rats which received vehicle solution. **Group III, (DM+ Metformin group),** this group involves type 2 diabetic rats treated with oral metformin[5] (100 mg/kg B.W.) dissolved in water, orally by gavage daily for 8 weeks [28]. **Group IV, (DM+ 1,25$(OH)_2$D3 group)** this includes type 2 diabetic rats supplemented by 1,25$(OH)_2$D3[6] (0.5 μg/kg B.W., i.p.) 3 times weekly for 8 weeks [29]. **Group V, (DM+ Metformin 1,25 $(OH)_2$D3 group),** they are type 2 diabetic rats which received 1,25 $(OH)_2$D3 supplementation (0.5 μg/kg B.W., i.p, 3 times weekly) plus metformin (100 mg/kg B.W., orally by gavage) daily for 8 weeks.

2.2. Experimental protocol

Eight weeks after treatment, and after an overnight fasting, the rats were anaesthetized and blood samples were withdrawn from the retro-orbital plexus by inserting capillary tubes [27]. Serum samples were separated and kept at −70 °C until used. The samples were used to measure serum glucose, insulin, calcium, HDL, cholesterol, triglycerides (TGs), ALT and AST levels and homeostasis model assessment (HOMA) index was calculated. After taking the blood samples, rats were culled and liver tissue samples were dissected and stored frozen at −80 °C in liquid nitrogen and used to measure PPAR-α expression by PCR and NF-κB p65 level using western blotting technique in the liver tissue. Samples from the large liver lobe were dissected and fixed in 10% formalin – buffered solution and were used for Immunohistochemical (IHC) staining for caspase 3 and PCNA, and H&E staining for histological analysis of liver injury.

2.3. Concise methods

2.3.1. ELISA and biochemical assessment

[2] laboratory chow ingredients (g/kg): contained 11.4% fat, 62.8% carbohydrate, and 25.8% protein (total 12.6 kJ/g) [30].

[3] High fat diet ingredients (g/kg): contained 58% fat from lard, 25.6% carbohydrate, and 16.4% protein (total 23.4 kJ/g) [30].

[4] streptozotocin (STZ) obtained from MP Biomedicals, stored at +4 °C, dissolved in sodium citrate buffer, pH 4.5. Each vial of sterilized STZ powder was dry-frozen, pale yellow, sterilized product and contains 1 g of STZ active ingredient.

[5] Metformin was obtained from pharmaceutical company (Cidophage, CID) in the form of tablets, each contains 500 mg. Metformin tablet was crushed and dissolved in distilled water with a concentration of 500 mg/5 mL.

[6] 1,25$(OH)_2$D3 was dissolved into olive oil so that each ml contain 0.2 mcg of 1,25 (OH)2D3 in a concentration 1:5.

Serum glucose, calcium, HDL, cholesterol, triglycerides (TGs), ALT and AST levels were determined by UV 2300 spectrophotometer using commercial kits diamond diagnostic. Homeostasis model assessment (HOMA) index was calculated as follows: HOMA = glucose (mmol/L) × insulin (μIU/mL)]/22.5 [31]. Insulin level was detected using ELISA kit supplied by (DRG, USA)

2.3.2. Detection of PPAR-α gene expression by quantitative real time PCR (qRT-PCR)

2.3.2.1. Total RNA Isolation and cDNA Synthesis. Total RNA was isolated from 30 mg tissue using the RNA purification Kit (Qiagen, USA) according to the manufacturer's instructions, the samples were treated with DNase I enzyme supported in the kit to avoid DNA contamination. Finally, Optical Density (A260/A280) and concentration were measured using Nanodrop 2000c (Thermo Science, USA). Reverse transcription reaction was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Reaction volume was 20 μL and components were used including 1 μg of total RNA as template, RevertAid RT 200 U/μL, RNase Inhibitor 20 U/μL, Random Hexamer prime, dNTP Mix 10 mM and Reaction Buffer 5×. Then, samples were incubated for 10 min at 25 °C, 60 min at 42 °C and 5 min at 75 °C.

2.3.2.2. qReal-Time PCR. Real-Time PCR reactions were performed via step one plus (Applied Biosystem, USA). Total PCR reaction volume was 25 μL including 12 μL of SYBR Green I PCR Master Mix (Qiagen,USA), 1 μL of forward and reverse oligonucleotide (400 nM), cDNA template (300 ng) and ddH2O. Thermal cycling program was performed for 30 s at 95 °C (first denaturation), following 5 s at 95 °C and 25 s at 60 °C for 40 cycles. Primers for PPARα were: forward 5′-AACCGGAACAAATGCCAGTA-3′ and reverse 5′-TGGCAGCAGTGGAAGAATCG-3′. GADPH was applied as reference gene, primers for GAPDH were: forward 5'-CTCCCATTCTTCCACCTTTG-3' and reverse: 5'-CTTGCTCTCAGTATCCTTGC-3'. The level of expression of PPARα was normalized relative to the expression of GAPDH mRNA in the sample using the ΔCt method. Relative differences in gene expression among groups were determined using the comparative Ct (ΔΔCt) method (which represents ΔCt values normalized relative to the mean ΔCt of control samples) and fold expression was calculated as using the comparative (2−ΔΔCt) method [32].

2.3.3. Western blot analysis

Homogenization of tissues was done in ice-cold lysis buffer (20 mmol/L Tris HCl, 137.5 mmol/L NaCl, 1% Triton X-100 [pH 8.0]) containing protease inhibitors (10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride). Lysates were mixed with an equal volume of double-strength sample buffer (250 mmol/L Tris-HCl, 4% sodium dodecyl sulfate, 10% glycerol, 2% b-mercaptoethanol, 0.006% bromophenol blue [pH 6.8]), and then boiled for 10 min, mixed for 30 s, and centrifuged at 10,000 rpm for 10 min. The resulting supernatants were collected and separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes, and blocked in Tris-buffered saline (TBS) with (Tween 20; TBS-T buffer) containing 5% (wt/vol) nonfat dry milk. The membranes were then incubated with polyclonal rabbit anti-rat NFKb-p65 primary antibodies (1:1000 dilution; Thermo Scientific). Blots were subsequently washed in TBS-T and incubated with horseradish peroxidase–conjugated goat anti-rabbit secondary antibody (1:5000 dilution; Thermo Scientific). All incubations were done for 2 h at room temperature. The chemiluminescence Western blotting system was used for detection. Quantification of protein was performed using BoiRad soft ware. And the value was normalizes to beta actin.

2.3.4. Light microscopic study

The liver was excised and processed for histological and immunohistochemical studies. Liver specimens were kept in 10% solution formaldehyde solution (as a fixative) for 72 h. Tissues were then embedded in paraffin blocks. Sections of 5 μ thicknesses were obtained from the paraffin blocks and subjected to the following techniques:

1- Histological examination: using hematoxylin and eosin (H&E) [33]

2- Immunohistochemical staining: for Caspase-3 and PCNA antigens using the avidin-biotin peroxidase complex technique. The paraffin sections were dewaxed in xylene and rehydrated. The endogenous peroxidase activity was inhibited using 3% hydrogen peroxide then the sections were incubated with the primary antibodies. Caspase-3 antibody is a rabbit polyclonal antibody (ab 4051, Abcam, Cambridge, UK) and anti-PCNA antibody is a rabbit polyclonal antibody (ab 19166, Abcam, Cambridge, UK).

The specimens were washed with phosphate buffer. Then, the sections were incubated with biotinylated secondary antibody. The slides were incubated with labeled avidin- biotin peroxidase. The site of antibody binding was visualized with the chromogen 3.3'diaminobenzidine (DAB) and the slides were counterstained with hematoxylin [33]. The sections were examined and photographed using Canon digital camera attached to an IBM computer system.

Image analyser study:

The sections were examined using Leica Qwin 500 image analysis software on an IBM computer system. The mean area percentage of the immunopositive cells for caspase-3 and PCNA was measured using the grey measure menu in 10 non-overlapping fields in each specimen using an objective lens of magnification of 40 (a total magnification of 400 after grey calibration) using the color detect menu and in relation to the standard measuring frame of 7099.95 μm.

2.4. Statistical study

The quantitative data were first examined by Kolmogorov-Smirnov test for normality. Quantitative data are expressed as mean ±standard deviation. The data were collected and studied using analysis of variance (one way ANOVA) followed by post hoc Bonferroni test for pairwise comparisons among the experimental groups. Statistical tests were done using SPSS 17 (Statistical Package for Social Sciences), Windows Version 20, Chicago USA. Level of significance was considered at P-value < 0.05 [34].

3. Results

3.1. The liver functions and liver injury evaluation

3.1.1. ALT

The 1,25(OH)$_2$D3 supplementation, either alone or in combination with metformin, improved the ALT level as compared to the untreated diabetic group. Moreover, the combined treatment had no significant difference as compared to the control group, while the group treated with metformin only showed a non significant decrease in the level of ALT as compared to the untreated diabetic group (Table 1).

3.1.2. AST

All treated groups showed a significant decrease in the level of AST as compared to the untreated diabetic group. Moreover, the

groups supplemented with 1,25$(OH)_2$D3, either alone or in combination with metformin showed no significant difference as compared to the control group (Table 1).

3.1.3. Histopathological examination (H&E)

Photomicrograph of the liver sections of rats from the untreated diabetic group showed severe congestion, lost normal hepatic architecture, vacuolated hepatocytes, pyknotic nuclei and ghost nuclei, extravasated blood and lymphocytic infiltration. The group treated with metformin only showed a relatively normal architecture of the liver with few vacuolated hepatocytes and few pyknotic nuclei. Mild dilatation of the central vein and the sinusoids were noticed. The group supplemented with 1,25$(OH)_2$D3 alone exhibited a relatively normal architecture of the liver with few vacuolated hepatocytes and mild dilatation of the central vein and sinusoids, while the combined treated group had a preserved architecture of the liver with relatively normal hepatocytes (Fig. 1).

3.2. The glycemic state and insulin resistance evaluation

Table 2 showed that the blood glucose level, the insulin level and HOMA IR were significantly decreased in the treated compared to the untreated group ($p < 0.05$). Moreover, the combined treatment provided a better homeostatic control on glucose metabolism that the recorded values were comparable to the control values with no significant change from the control group.

There was no significant difference between the values recorded in the group treated with metformin compared with the group supplemented by 1,25$(OH)_2$D3 regarding the glycemic state and the insulin resistance, while, the combined treatment caused a significant better improvement in the glucose level as compared to the group treated with metformin only but showed no significant difference as compared to the group supplemented by 1,25$(OH)_2$D3 alone (Table 2).

3.3. The lipid profile and hepatic lipid metabolism evaluation

3.3.1. Systemic lipid profile

1,25$(OH)_2$D3 supplementation, either alone or in combination with metformin (group IV and group V respectively), induced a significant lowering effect on the TGs level as compared to the untreated diabetic group. Regarding the cholesterol and the HDL levels, all the treated groups showed a significant decrease and a significant increase respectively as compared to the untreated diabetic group. Adding 1,25$(OH)_2$D3 to the metformin treatment had an additional significant increasing effect on the level of HDL as compared to the metformin treated group (Table 3).

3.3.2. PPAR-α

All treated groups showed a significant increase in the level of PPAR-α as compared to the untreated diabetic group. Moreover, the 1,25$(OH)_2$D3 supplementation in combination with metformin improved PPAR-$\boldsymbol{\alpha}$ level with no significant difference as compared to the control group (Table 3).

3.4. The serum Ca level

Table 4 demonstrated that the serum level of Ca was significantly decreased in the non treated diabetic group as compared to the control group. On the other hand, the Ca levels in the groups of rats treated with either agents were not significantly changed as compared with the diabetic or the control groups, while the Ca levels in the group of rats received both drugs showed a significant increase in the level of Ca as compared to the non treated diabetic group and showed no significant difference as compared to the control group.

3.5. Evaluation of the inflammatory marker NF-κB p65

Table 5 clarified that all treated groups showed a significant decrease in the level of NF-κB p65 as compared to the untreated diabetic group. Moreover the group supplemented with 1,25$(OH)_2$D3 alone exhibited a significantly better response as compared to the group treated with metformin only, and the combination of both agents resulted in a significant lower NF-κB p65 level as compared to the groups treated by each agent alone (Fig. 2).

3.6. Immunohistochemical results showing the apoptosis and the proliferation in the liver

3.6.1. Immunohistochemical staining of caspase-3 in rat liver

Immunohistochemical staining of caspase-3 in rat liver showed no expression of caspase-3 in the control group with a significant increase in caspase-3 immunoreactivity in the cytoplasm of hepatocytes in the non treated diabetic group; a significant reduction in caspase-3 immunoreactivity in the groups treated with only one agent, while the combined drugs treated group showed almost no expression of caspase-**3** (Table 6 and Fig. 3).

3.6.2. Immunohistochemical staining of PCNA in rat liver

Immunohistochemical staining of PCNA in rat liver showed low PCNA immunoreactivity in the nuclei of hepatocytes of the control group. A significant decrease in PCNA immunoreactivity was recorded in the untreated diabetic group as compared to the control group with a significant increase in PCNA immunoreactivity in the groups treated with only one agent, while, the combined drugs treated group showed a significant more positive PCNA immunoreactivity as compared to the groups treated by each agent alone and exhibited no significant change as compared to the control group (Table 6 & Fig. 4).

4. Discussion

Some previous studies showed that vitamin D plays a key role in modulating metabolic homeostasis, insulin secretion and insulin sensitivity [35]. Insulin resistance, the main problem in type 2 diabetes mellitus, is significantly involved in the development of the associated liver diseases [36]. People with T2DM often have lower circulating vitamin D levels than those without diabetes

Table 1
1,25$(OH)_2D_3$, metformin and both agents supplementation decreased liver enzymes levels in the diabetic rats.

Parameter	Group (I)	Group (II)	Group (III)	Group (IV)	Group (V)
ALT (U/L)	23.37 ± 5.69	78.27 ± 12.12*	63.42 ± 3.82*	43.5 ± 1.9$^{*,\#,\wedge}$	32.87 ± 4.62$^{\#,\wedge}$
AST (U/L)	15.82 ± 1.3	79.92 ± 6.64*	53.07 ± 7.24$^{*,\#}$	20.32 ± 2.85$^{\#,\wedge}$	21.25 ± 2.63$^{\#,\wedge}$

Values are shown as mean ± SD.
* significant change as compared to the mean value observed in group (I) ($P < 0.05$).
\# significant change as compared to the mean value observed in group (II) ($P<0.05$).
^ significant change as compared to the mean value observed in group (III) ($P<0.05$).

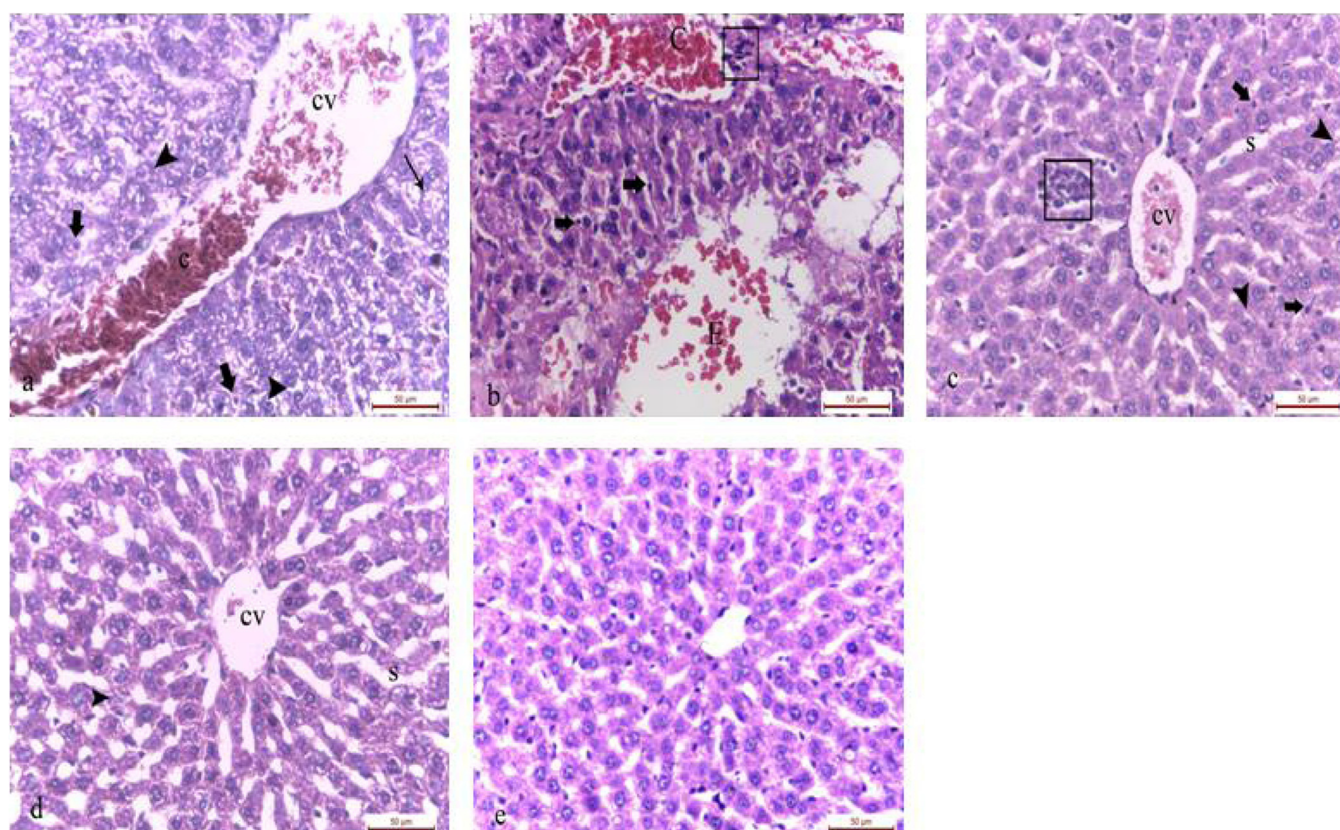

Fig. 1. $1,25(OH)_2D_3$, Metformin and both agents Supplementation Preserved Liver Architecture in the Diabetic Rats. Hx&E; X 400.
Photomicr. ograph of sections of liver of rats from:
a (untreated diabetic group) showing severe congestion, lost normal hepatic architecture, vacuolated hepatocytes, pyknotic nuclei and ghost nuclei;
b (untreated diabetic group) showing extravasated blood, marked congestion, lymphocytic infiltration and pyknotic nuclei;
c (diabetic rats treated by metformin) showing relatively normal architecture of liver with few vacuolated hepatocytes, few pyknotic nuclei, mild dilatation of central vein and the sinusoids were noticed;
d (diabetic rats supplemented with $1,25(OH)_2D_3$) showing relatively normal architecture of liver with few vacuolated hepatocytes and mild dilatation of central vein and sinusoids;
e (diabetic rats supplemented with both agents) showing preserved architecture of the liver with relatively normal hepatocytes. (C) congestion; (arrow head) vacuolated hepatocytes; (thick arrows) pyknotic nuclei; (thin arrow) ghost nuclei; (E) extravasated blood; (rectangle) lymphocytic infiltration; (cv) central vein; (s) sinusoids

Table 2
$1,25(OH)_2D_3$,metformin and both agents supplementation improved the glycemic state and insulin resistance in the diabetic rats.

Parameter	Group (I)	Group (II)	Group (III)	Group (IV)	Group (V)
glucose (mmol/L)	5.39 ± 0.46	$14.62 \pm 3.01^{*}$	$10.92 \pm 1.28^{*,\#}$	$10.65 \pm 2.42^{*,\#}$	$7.19 \pm 1.52^{\#,\wedge}$
insulin (μIU/mL)	10.69 ± 1.39	$21.45 \pm 4.14^{*}$	$14.68 \pm 1.84^{\#}$	$16.28 \pm 2.34^{*,\#}$	$14.65 \pm 1.74^{\#}$
HOMA IR	2.54 ± 0.2	$13.69 \pm 2.62^{*}$	$7.15 \pm 1.36^{*,\#}$	$7.78 \pm 2.41^{*,\#}$	$4.73 \pm 1.4^{\#}$

Values are shown as mean ± SD.
* significant change as compared to the mean value observed in group (I) ($P < 0.05$).
\# significant change as compared to the mean value observed in group (II) ($P<0.05$).
^ significant change as compared to the mean value observed in group (III) ($P<0.05$).

[37], and this was believed to contribute to an increased risk of diabetic complications and mortality.

Thus this study was designated to evaluate the effects of $1,25(OH)_2D3$ supplementation, compared with the classic hypoglycemic drug, metformin, on insulin resistance in T2DM and their potential protective effect on the liver in T2DM.

For this purpose, T2DM was induced in sixty male Albino rats by high fat diet for 2 weeks, followed by STZ injection. Eight weeks after induction of T2DM, a significant damage was reported in the liver that was indicated histologically by disruption of the hepatic architecture and a significant elevation of the liver enzymes, ALT and AST, as compared to the control rats. The most common cause of a mild elevation of serum ALT is non alcoholic fatty liver disease (NAFLD) [38], the most prevalent liver disease in T2DM [3].

4.1. Effect of induction of diabetes on glycemic state, lipid metabolism, inflammation, apoptosis and proliferation

In the present study, we recorded the development of hyperglycemia, insulin resistance with systemic dyslipidemia in the form of a significant increase in the serum level of triglycerides, cholesterol and decrease in the serum level of HDL in the diabetic rats compared to the control group.

Insulin inhibits hormone sensitive lipase and so in cases with insulin resistance, there would be an increased mobilization of free fatty acids from the peripheral stores, resulting in an increase in serum lipids [39].

Although, no significant fatty changes was observed in the liver by histological examination, hepatic lipid metabolism was

Table 3
$1,25(OH)_2D_3$, metformin and both agents supplementation improved systemic lipid profile and hepatic lipid metabolism in the diabetic rats.

Parameter	Group (I)	Group (II)	Group (III)	Group (IV)	Group (V)
TGs (mg/dL)	95.65 ± 7.77	144.15 ± 15.25*	127.32 ± 8.02*	126.55 ± 6.67*,#	117.33 ± 8.01*,#
cholesterol (mg/dL)	153.77 ± 8.07	211.5 ± 11.99*	186.22 ± 7.21*,#	177.02 ± 16.77*,#	175.93 ± 13.09*,#
HDL (mg/dL)	61.27 ± 2.77	28.08 ± 3.62*	38.92 ± 4.64*,#	42.02 ± 4.95*,#	48.73 ± 3.3*,#,^
PPAR-α	1.06 ± 0.04	0.21 ± 0.03*	0.6725 ± 0.12*,#	0.7975 ± 0.07*,#	0.8425 ± 0.15#

Values are shown as mean ± SD.
* significant change as compared to the mean value observed in group (I) ($P < 0.05$).
# significant change as compared to the mean value observed in group (II) ($P<0.05$).
^ significant change as compared to the mean value observed in group (III) ($P<0.05$).

Table 4
Serum Ca (mg/dl) level in the different rat groups.

Parameter	Group (I)	Group (II)	Group (III)	Group (IV)	Group (V)
Ca (mg/dL)	9.65 ± 1.2	6.27 ± 1.22*	8.27 ± 1.2	8.32 ± 0.6	9.02 ± 1.51#

Values are shown as mean ± SD.
* significant change as compared to mean value observed in group (I) ($P < 0.05$).
# significant change as compared to mean value observed in group (II) ($P<0.05$).

significantly disturbed in the diabetic rats compared to the control group as shown by the significant decrease in PPAR-α in the liver tissue. PPAR-α is one of the major regulators of fatty acid oxidation, utilization and storage. In addition to the disturbed lipid metabolism at the level of the liver, there was a systemic dyslipidemia. We can say that there is an imbalanced systemic and hepatic fat metabolism with excess uptake and synthesis of fatty acids by the liver.

This disturbed fat metabolism is closely related to insulin resistance that causes lipolysis and increases the circulating free fatty acids [40]. These fatty acids are then taken up by the liver as an energy source. Failure of proper utilization, due to the decrease of the enzymes regulating their metabolism (PPAR), causes an increased production and release of reactive oxygen species [41] and induces a proinflammatory cytokines production [42].

This was confirmed in our study by a significant increase in the proinflammatory cytokine regulator NFkB p65. The lipotoxicity together with the developed inflammatory state cause an abnormal gene expression, resulting in a state of cellular dysfunction and cellular death.

4.2. Effect of $1\alpha,25(OH)_2D3$ or metformin treatment on the liver function and structure

Early administration of $1\alpha,25(OH)_2D3$ or metformin to the diabetic rats significantly decreased the liver enzymes as compared to the untreated diabetic rats. The structural architecture was preserved with a significant decrease in apoptosis and a significant increase in cellular proliferation. This was confirmed histologically by the significant change in the caspase-3 and PCNA expression in the treated groups compared with the untreated group.

The mechanism of this protective effect of $1\alpha,25(OH)_2D3$ was studied and can be explained according to the results obtained in many ways. First, $1\alpha,25(OH)_2D3$ improved glucose metabolism and decreased insulin resistance. Second, $1\alpha,25(OH)_2D3$ improved fat metabolism systemically and at the level of hepatocyte. Third, $1\alpha,25(OH)_2D3$ modulated the inflammatory reactions, thus decreased apoptosis and enhanced proliferation of the liver cells.

Table 5
$1,25(OH)_2D_3$, metformin and both agents supplementation decreased the inflammation in the diabetic rats.

Parameter	Group (I)	Group (II)	Group (III)	Group (IV)	Group (V)
NF-κB p65	1.03 ± 0.03	9.95 ± 0.68*	5.45 ± 0.5*,#	3.61 ± 0.69*,#,^	2.4 ± 0.22*,#,^,~

Values are shown as mean ± SD
* significant change as compared to the mean value observed in group (I) ($P < 0.05$).
# significant change as compared to the mean value observed in group (II) ($P<0.05$).
^ significant change as compared to the mean value observed in group (III) ($P<0.05$).
~ significant change as compared to the mean value observed in group (IV) ($P<0.05$).

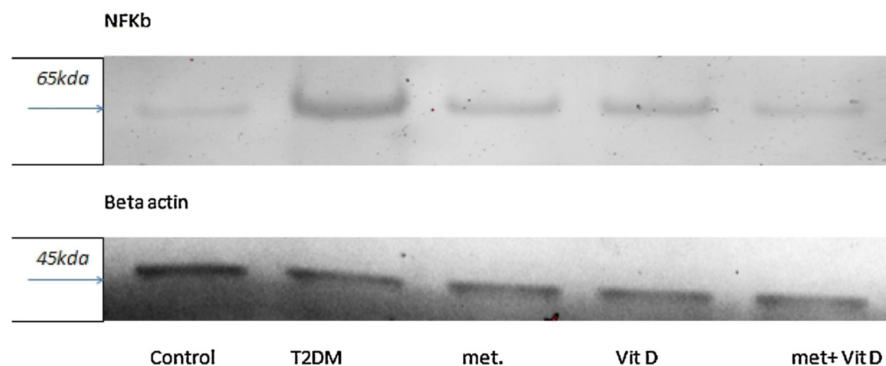

Fig. 2. $1,25(OH)_2D_3$, Metformin and both agents Supplementation Decreased the Expression of NF-k B p65 Protein in the Diabetic Rats.
The results of Western blot revealed that the expression of NFKb p65 protein was high in diabetic group compared with normal controls. The expression was lower in diabetic group receiving metformin or vit D and further lower expression on receiving both agents. Beta-actin was used as a loading control.

Table 6
$1,25(OH)_2D_3$, metformin and both agents supplementation decreased apoptosis and increased proliferation in the diabetic rats.

Parameter	Group (I)	Group (II)	Group (III)	Group (IV)	Group (V)
Caspase-3	1.46 ± 0.05	1119.17 ± 279.68*	2.59 ± 0.64#	3 ± 0.73#	1 ± 0#
PCNA	6.44 ± 0.51	0.78 ± 0.26 *	2.75 ± 0.29 *,#	3.5 ± 0.58*,#	5.5 ± 0.71 #,^,~

Values are shown as mean ± SD.
* significant change as compared to the mean value observed in group (I) ($P < 0.05$).
# significant change as compared to the mean value observed in group (II) ($P < 0.05$).
^ significant change as compared to the mean value observed in group (III) ($P < 0.05$).
~ significant change as compared to the mean value observed in group (IV) ($P < 0.05$).

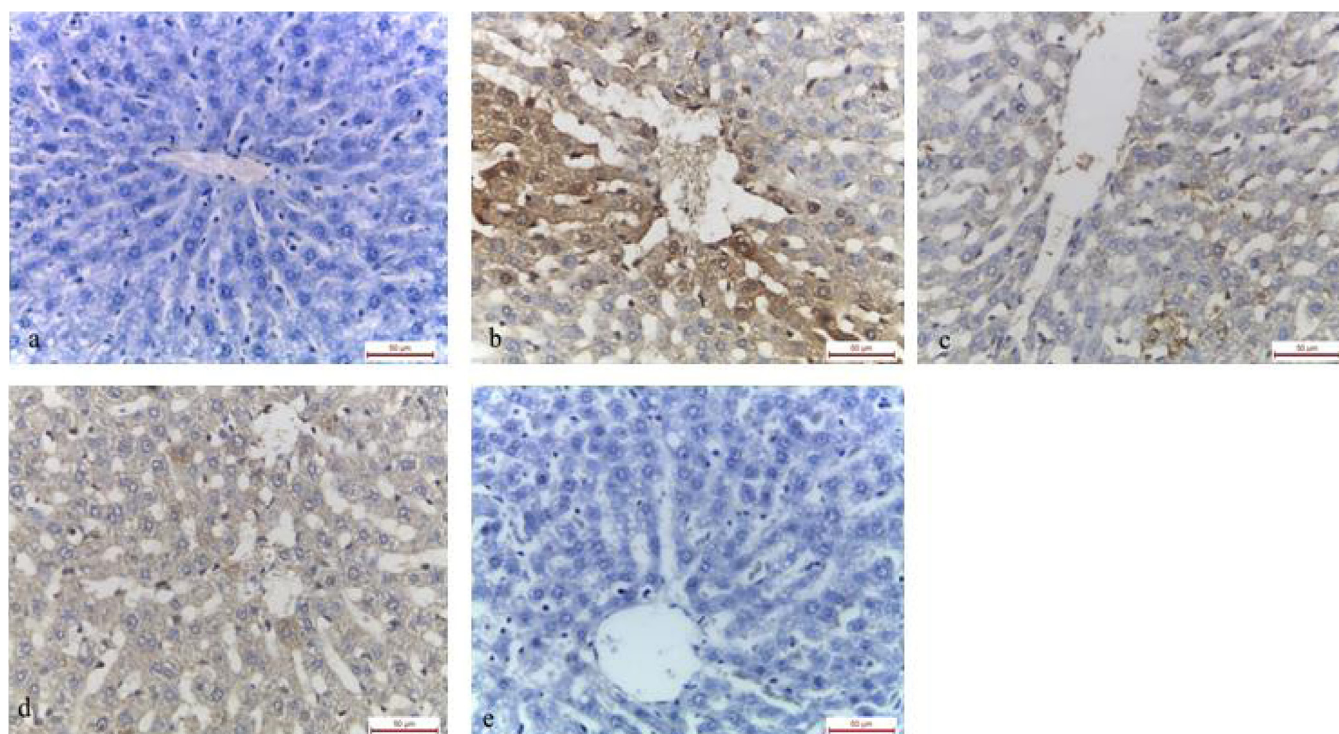

Fig. 3. $1,25(OH)_2D_3$, Metformin and both agents Supplementation Decreased liver apoptosis in the Diabetic Rats. caspase-3 × 400.
Immunohistochemical staining of caspase-3 in rat liver from:
a (control group) showing no expression of caspase-3;
b (untreated diabetic group) showing marked increase in caspase-3 immunoreactivity in the cytoplasm of hepatocytes;
c (diabetic rats treated by metformin) showing reduction in caspase-3 immunoreactivity;
d (diabetic rats supplemented with $1,25(OH)_2D_3$) showing reduction in caspase-3 immunoreactivity;
e (diabetic rats supplemented with both agents) showing no expression of caspase-3.

4.3. Effect of 1α,25(OH)$_2$D3, metformin and both agents treatment on the glycemic control

First mechanism suggested for the protective action of 1α,25 $(OH)_2D3$, is through decreasing insulin resistance known to be a major causative factor in the pathogenesis of steatosis. Insulin resistance increases lipolysis, and causes dyslipidemia [43].

The increase in insulin sensitivity in response to $1,25(OH)_2D3$ supplementation may be through suppression of the chronic inflammatory condition associated with insulin resistance together with increased expression of the insulin receptor and/or proteins of the insulin-signaling cascade [44]. A previous study identified a vitamin D response element on the human insulin receptor gene promoter [45], increasing the transcription of the insulin receptor gene.

Additionally, it was reported that $1,25(OH)_2D3$ stimulates glucose utilization by a direct regulation of phosphatidylinositol 3-kinase activity [46], an essential insulin regulator known to be impaired in diabetes [47]. In their in vitro study, Manna and Jain, showed that $1,25(OH)_2D3$ treatment of adipocytes up-regulated GLUT4 receptor expression and translocated it to the cell surface [48]. Moreover, $1,25(OH)_2D3$ controls the calcium flux through the membrane of both the β cells and the peripheral insulin-target tissues increasing the insulin secretion and sensitivity [49].

Metformin contributed to the glycemic control through increasing insulin receptor expression and tyrosine kinase activity [50]. Maida et al. have reported an acute increase in plasma levels of glucagon-like peptide 1 (GLP-1) and islet incretin receptor gene expression as a result of metformin administration [51], additionally, metformin decreases hepatic glucose production by inhibiting gluconeogenesis [52].

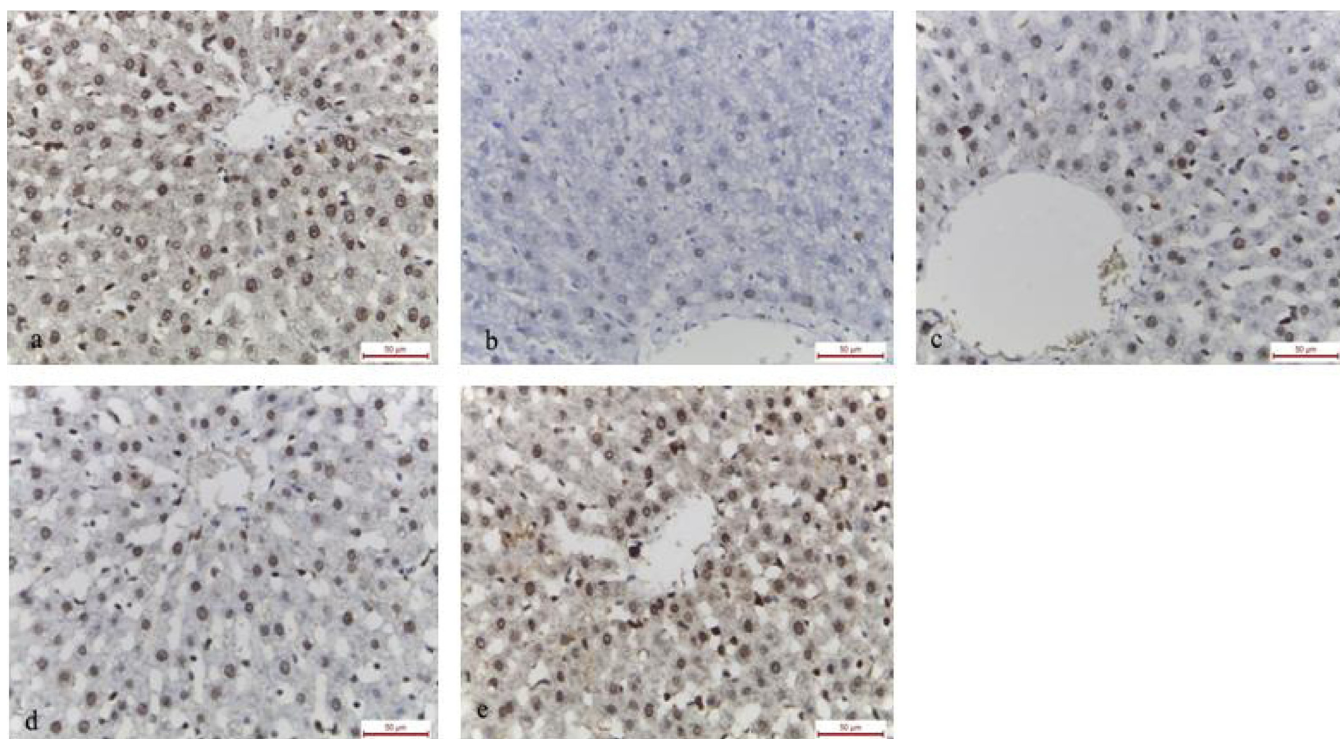

Fig. 4. $1,25(OH)_2D_3$, Metformin and both agents Supplementation Increased liver proliferation in the Diabetic Rats. PCNA x 400.
Immunohistochemical staining of PCNA in rat liver from:
a (control group) showing positive PCNA immunoreactivity in the nuclei of hepatocytes;
b (untreated diabetic group) showing marked decrease in PCNA immunoreactivity;
c (diabetic rats treated by metformin) showing increase in PCNA immunoreactivity;

4.4. Effect of 1α,25(OH)$_2$D3, metformin and both agents treatment on lipid metabolism

In the current study, $1,25(OH)_2D3$, metformin, or both drugs supplementation, controlled the systemic dyslipidemia and improved the lipid profile, compared with untreated diabetics. In accordance with these results, previous studies reported that vitamin D supplementation induced a significant reduction in the serum total cholesterol concentrations and improved the lipid profiles [53,54].

Their actions on the lipid profile with the significant correction of systemic dyslipidemia can be explained by their ability to increase insulin sensitivity, secretion and augmenting its action [55]. Insulin has an important role in regulating fat metabolism as it increases β-hydroxy-β-methylglutaryl coenzyme A reductase activity decreasing cholesterol synthesis [56].

Beside its effect on insulin, $1,25(OH)_2D3$ directly increases adipocyte fatty acid synthase expression and activity, stimulates glycerol-3-phosphate dehydrogenase activity, and inhibits of lipolysis [57]. These direct actions of $1,25(OH)_2D3$ may be through its modulation of calcium ion concentration in the adipocyte, an important factor in regulating lipid metabolism.

Through improving lipid metabolism and correcting dyslipidemia, both agents separately or in a combined form were able to protect the liver against injury caused by lipotoxicity.

An important finding in the current study is that, in addition to its systemic action on the lipid profile, $1,25(OH)_2D3$ up-regulates the expression of PPAR-α, promoting hepatic fat utilization. Ning et al. [8], showed that $1,25(OH)_2D3$ improves hepatic fat metabolism, promotes β-oxidation and results in decreased TG levels through increasing the PPAR-α expression in the liver tissue. They suggested that these actions may be through its role in modulating the inflammatory response, and that $1,25(OH)_2D3$ may also act as a PPAR-α agonist. Also, metformin was shown to be related to PPAR-α regulation [51].

4.5. Effect of 1α,25(OH)$_2$D3, metformin and both agents treatment on inflammation

$1,25(OH)_2D3$ is known to have an anti-inflammatory effect [58]. In the present study, either of $1,25(OH)_2D3$ or metformin was able to switch off the inflammatory reactions by significantly decreasing NFκB p65 expression in the liver tissues. NFκB is a major regulator of the immune, inflammatory and stress responses [59]. $1,25(OH)_2D3$ was shown to up-regulate the inhibitor of NFκB (IκB-α) by increasing mRNA stability and decreasing IκB −α phosphorylation [60].

Again, metformin inhibits mitochondrial ROS production; therefore, it can attenuate the inflammatory response [61]. Metformin can exert an anti-inflammatory effect by inhibiting NF-kB by blocking the PI3K-Akt pathway [62].

4.6. Effect of 1α,25(OH)$_2$D3, metformin and both agents treatment on apoptosis and PCNA

Multiple interconnecting mechanisms can explain the protective effects of $1,25(OH)_2D3$ or metformin on liver in the diabetic rats. The net result of all these protective mechanisms is decreasing hepatocyte loss and increasing their proliferative power in an attempt to compensate for the potential injury.

In the current study, $1,25(OH)_2D3$ significantly decreased the expression of the proapoptotic factor caspase 3 and increased the

expression of PCNA in the treated groups compared with the untreated diabetic rats.

In the liver of STZ-induced diabetic rats, hyperglycemia enhances the inflammatory process and ROS production with consequent activation of caspase-3 that ultimately induces apoptosis [63].

Either 1,25$(OH)_2$D3 or metformin, via increasing the insulin sensitivity and improving the glucose metabolism, leads to a decrease of ROS production, an inhibition of the inflammation and a decrease in caspase-3 activity [64].

Additionally, Tourigny et al. [65], suggested that pro-caspase-3 expression could be vitamin D dependent and Asensio-Lopez et al. [66] reported that metformin decreased the expression of the caspase-3 enzyme and prevented its activation.

Regarding the cellular proliferation marker PCNA, it appears that our study was the first one to show the increased expression of PCNA by 1,25$(OH)_2$D3 supplementation.

Most previous studies reported that 1,25$(OH)_2$D3 had an antiproliferative action by inhibiting PCNA expression, suppressing the malignant growth [67], cardiovascular hypertrophy [68] and airway remodeling [69]. Similar results were reported for metformin [70,71].

On the other hand, few studies suggested a stimulatory effect of 1,25$(OH)_2$D3 on PCNA expression in the protection from injury and during the repair process. Kim et al., [72] reported an increased tubular cell proliferation as determined by increased PCNA expression when the rat kidneys were preconditioned with vitamin D. Domingues-Faria et al., [73] reported that vitamin D depletion reduced PCNA protein expression resulting in skeletal muscle atrophy in old rats.

The relation and interaction between 1,25$(OH)_2$D3 and PCNA expression appear to be more complicated than just stimulation or suppression, It appears to be a sort of regulation that the level of PCNA in resting cells is low, while it is considerably increased in multiplying and transformed cells to allow repair [74] and it appears that 1,25$(OH)_2$D3 in this case through immune-modulation can stimulate the expression of PCNA and enhances the repair, however, in cases of over expression associated with malignant growth, hypertrophy or abnormal growth, 1,25$(OH)_2$D3 has a suppressing action on the PCNA expression.

5. Conclusion

In conclusion, we can suggest from the results of the present study that 1,25$(OH)_2$D3 as well as metformin can have beneficial effects in protection against liver injury in T2DM. These effects may be through the modulation of glucose metabolism, correcting the disturbed lipid profile and hepatic PPARα. Each drug was able to ameliorate insulin secretion and sensitivity, attenuating the inflammatory response and promotes hepatocyte survival via inhibiting apoptosis and stimulation of cellular proliferation and repair. Our preliminary results demonstrated that the beneficial effects of 1,25$(OH)_2$D3 on hepatic function is comparable or even in some aspects exceeds the effect of the classical antidiabetic drug, metformin, and the utilization of both agents together may represent a promising therapeutic agent for augmenting their value in the management of the liver pathology associated with T2DM.

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