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Protective effects of Vitamin D and Metformin against Hematological, Biochemical and Histopathological Alterations in Induced Diabetic Rats

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ABSTRACT

Our study was designed to evaluate the association between vitamin D and diabetes mellitus in induced diabetic rats of some hematological and biochemical parameters as well as histopathological examination. The study was conducted on a total sixty male albino rats weighing (120-170 g , BW) , that were divided into two groups, the first group fed on normal diet, and the second group fed on high-fat diet for 2 weeks predispose cause to diabetic induction. Then intraperitoneal injection of Streptozotocin (STZ) to induce diabetes. The experimental rats randomly divided into six equals groups, as follow: 1st group (-ve control) non-supplemented non-diabetic, 2nd group diabetic untreated (DM) , 3rd group diabetic treated with vitamin D alone (DM+VD), 4th group diabetic treated with metformin (DM+ Met) , 5th group (DM+ VD+ Met) diabetic treated with vitamin D and metformin, 6th group (VD) rats supplemented with vitamin D. The treatment lasted for 4 weeks then whole blood, serum samples, and tissue specimens were collected for hematological, some serum biochemical analysis and histopathological examination respectively. Our findings revealed that the treatment of diabetic induced rats with both vitamin D and metformin had a significant effect on the hematological parameters except total leukocytic and lymphocytic counts, which were markedly increased. Moreover, a significant increase in the serum hepatic enzymes activities (ALT, AST, and ALP). Meanwhile, a significant reduction in TP, ALB, GLO, A/G ratio, Total Bilirubin , Direct Bilirubin and Indirect Bilirubin .

Keywords: Vitamin D; Diabetes Mellitus (DM); Streptozotcin (STZ); hematology, biochemical parameters and

INTRODUCTION

Diabetes Mellitus (DM) is a serious catabolic disorder, which incidence is globally increasing and considered as an epidemic disease characterized by either insulin insufficiency, or lack of body response to it (Alqasim et al., 2017).

Streptozotocin (STZ) is a 2-Deoxy-2-[(methyl nitroso amino) carbonyl] amino-D-glucopyranose, a synthetic antineoplastic agent chemically related to other nitrosoureas used in cancer chemotherapy (Akbarzadeh et al., 2007). Because of its cytotoxicity, it is used mainly in inducing Langerhans β-cell islets necrosis, as it decreases Nicotinamide-adenine dinucleotide (NAD) in beta cells (Yakhchalian et al., 2018), so is extensively used in inducing T2DM in animal models by low STZ dose injection after High Fat Diet (HFD) feeding as a predisposing cause. In the model of HFD/STZ, the state of obesity, insulin resistance and/or a period of an HFD stimulate glucose intolerance (Mohamed et al., 2016).

Metformin is abiguanide oral hypoglycemic agent. Caused decreases in endogenous glucose production by the liver and is being used to treat other conditions associated with insulin resistance (Gunton et al., 2003). It suppresses gluconeogenesis, reduces glycogenolysis, increases glucose turnover and enhances insulin-stimulated glucose uptake in skeletal muscle. In addition, metformin ameliorates insulin resistance, which is a key factor in the development of T2DM (Ajjan and Grant, 2006).

In fact, metformin showed beneficial effects in type 2 diabetes, including weight reduction, improved lipid profiles and enhanced endothelial function. (Cheng et al., 2005).

Metformin is a rather safe drug and its anti-hyperglycemic property has been generally attributed to reducing rate of intestinal absorption of carbohydrate, decreased hepatic gluconeogenesis and improvement of peripheral glucose utilization (Penicaud et al., 1989).

Vitamin D is a fat-soluble steroid hormone. It is a prohormone produced in the skin

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through the ultraviolet irradiation of 7-dehydrocholesterol. It is biologically inert and must be metabolized to 25-hydroxyvitamin D3 in the liver and then to 1, 25-dihydroxyvitamin D3 in the kidney before function (Bella et al., 2017). The active form (1,25dihydroxyvitamin D3) acts through a nuclear receptor to carry out its many functions, including calcium absorption, phosphate absorption in the intestine, calcium mobilization in bone, and calcium reabsorption in the kidney, also has several non-calcemic functions in the body (DeLuca, 2004).

Certainly, vitamin D has been playing a strong regulating role in the several systems including iron metabolism and erythrocyte production (Refaat et al., 2014).

Indeed, there was much evidence proposed that the circulating concentration of vitamin D was negatively related to the risk of liver disease (Rhee et al., 2013).

Thereby, the purpose of the present study was to investigate the association between vitamin D and induced type 2 diabetes mellitus in rats through evaluation of some hematological parameters (erythrogram as well as total and differential leukocytic count), Estimation of some selective serum biochemical parameters and histopathological examination of the hepatic tissues.

MATERIALS AND METHODS

Animals and experimental design:

The experiment was conducted with 60 rats with 120-170 g were purchased from Zagazig laboratory animal unit. The animals were acclimatized for 2 weeks under standard laboratory conditions and had free access to standard rodent pellets diet and clean water ad-libitum. The animals were divided into 6 equal groups each one 10 rats control group was supplied with control diet for six weeks; rats injected I/P once with sodium citrate buffer and orally distilled water. Diabetes was induced in the other four groups (diabetic model groups), as following rats were supplied with high fat diet (HFD) for two weeks showed in table (1), followed by one doses of intraperitoneal (i.p) STZ injection (35mg/kg). Overnight fasting rats with Threshold value of FBS level more than > 300 mg/dl by 1 week of injection were considered diabetic. Then the groups classified as follow: diabetic non-treated group (DM), Vitamin D-treated group were supplied with high-fat diet (HFD) for 2 weeks, followed by STZ injection (35 mg/Kg), then fed on vitamin D-containing diet (1000 IU/Kg) for 4 weeks (DM+VD). Diabetic rats treated with metformin orally at dose of 100 mg/kg B.W daily for 4 weeks (DM+ Met) and, Diabetic rats treated with both vitamin D and metformin with their previous doses for 4 weeks (DM+ VD+ Met). The sixth group was supplied with control diet and vitamin D-containing diet (1000 IU/Kg) for 6 weeks. All rats after 4 weeks of treatment were weighted directly before sample collection.

Sample collection:

Blood samples were collected from all examined groups at the end of the 4^{th-} week

Table (1) Ingredients % of diet for rats.

Ingredients %	Experimental diets	
	Control	Diabetic
Yellow Corn (8.5%)	77	28.4
Soybean Meal (44%)	10	33
Corn glutein	2	7
Wheat bran	8	0
Vegetable oil	0	28
Minerals &vitamins premix	1	1
NaCl Salt	0.5	0.5
Dicalcium phosphate	1.7	1.7
Methionine	0.13	0.3
Lysine	0.26	0.1

post-treatment with vitamin D and /or metformin. From each rat three separate blood samples were collected from the medial canthus of the eye, the first sample was collected in Eppendorf tubes containing anticoagulant dipotassium salt of EDTA (0.5mg/ml blood), then gently mixed for hematological examination. The second blood sample was collected without anticoagulant and placed in a slant position for 20 minutes at room temperature, samples were stored in refrigerator for retraction of clot and centrifuged for 10 minutes at 3000 rpm to separate clear serum samples that carefully transferred to Eppendorf tubes to be stored at -20°C until used for biochemical estimation. As well as specimens from liver tissue were collected and fixed in 10% formalin for histopathological analysis, study.

Hematological parameters:

Using physiological diluting fluid and improved Neubauer double hemocytometer for manual counting of RBCs and leukocyte count. Furthermore, Blood indices (the mean corpuscular volume MCV (fl), mean corpuscular hemoglobin MCH (pg) and mean corpuscular hemoglobin concentration MCHC (%) values were calculated by standard formulae (Feldman et al., 2000). In addition, two-blood films were manually prepared from each sample, as soon as after collection of the blood sample, then stained by Giemsa stain, for further differential leukocytic count.

Serum Biochemical Analysis:

The serum ALT, AST and ALP activities as well as total and direct bilirubin were estimated according to Young (2001). Total protein determined according to Burtis et al. (1999) and albumin was done according to young (2001). Serum globulin was calculated by subtracting albumin from total protein and A/G ratio by dividing albumin to globulin according to Kaneko et al. (1997).

Histopathological study:

Specimens were collected from the liver fixed in 10% neutral buffered formalin , then were dehydrated and embedded in paraffin and were sectioned to be 5 micron in its thickness then stained with Hematoxyline and Eosin (Bancrrof et al., 1996).

Statistical analysis:

Data in tables and figures were expressed as means ± standard error of experimental study. All our data were statistically analyzed by SPSS version (20, USA). ANOVA was used to know differences between means of all groups using Duncan multiple comparison tests to determine the significant difference ($P < 0.05$) (Norusis, 2006).

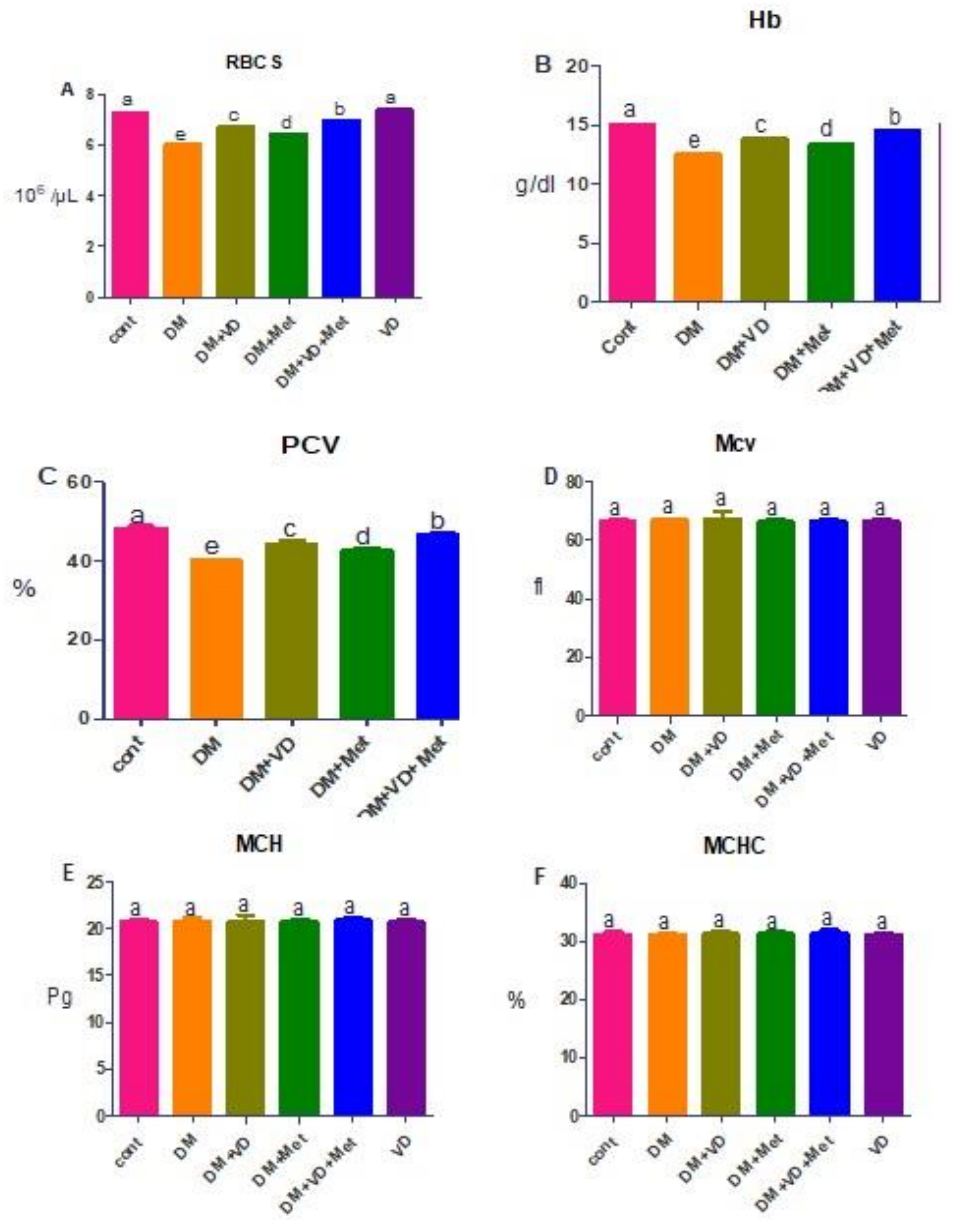


Figure (1): Erythrogram results (A)RBCS count, (B)Hb concentration, (C)PCV%, (D)MCV value, (E)MCH value, (F)MCHC % in different experimental groups at 4th week post treatment either with vitamin D and metformin in induced diabetic rats (mean ± SE).

RESULTS

A-Erythrogram:

The erythrogram results are presented in figure (1); Red blood cells count (RBCs 10^6/µl), hemoglobin concentration (Hb g/dl) and packed cell volume (PCV%) were significantly decreased in the DM group comparing with the control one. Meanwhile, there were significantly increased in all diabetic treated groups (DM+VD, DM+Met & DM+VD+Met) compared to the diabetic untreated one and they were significantly increased in vitamin D treated groups (DM+VD & DM+VD+Met) compared with metformin treated one. The above-mentioned parameters were insignificantly differed in the vitamin D treated group as compared to the control. The Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean

corpuscular hemoglobin concentration (MCHC) were insignificantly changed in all investigated groups.

B-Leukogram:

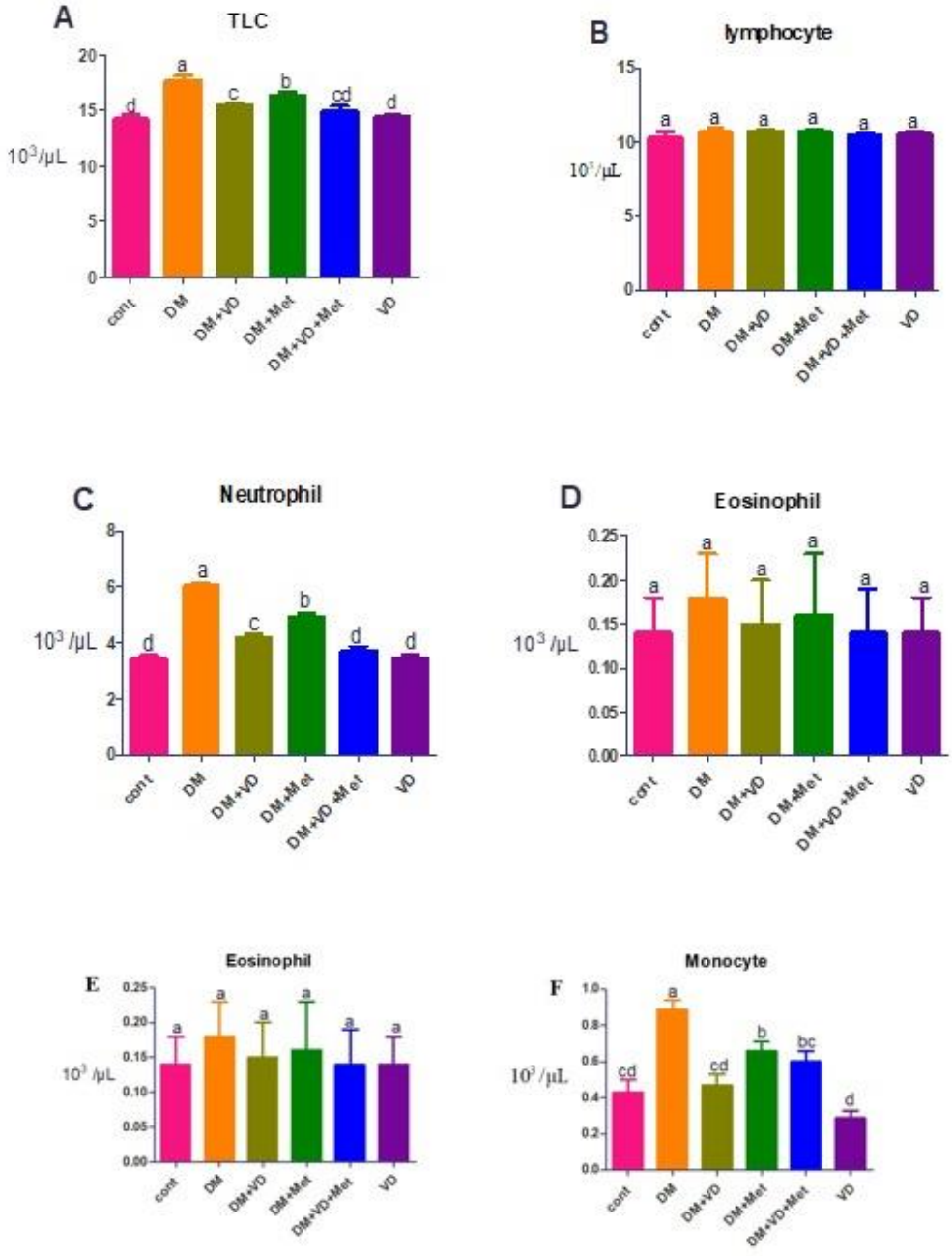


Figure (2): Leukogram results (A) TLC count, (B) Lymphocyte count, (C) Neutrophil count, (D) Eosinophil count, (E) Monocyte count in different experimental groups at 4th week post treatment either with vitamin D and /or metformin in induced diabetic rats (Mean ± SE).

The leukogram results showed a significant elevation in the total leukocytic, neutrophilic and monocytes counts in the DM group comparing with the control one. The elevation in the above mentioned parameters were significantly reduced upon the treatment with either vitamin D and/or metformin. Furthermore, there were significantly reduced in the vitamin D treated groups (DM+VD & DM+VD+Met) compared to the metformin treated one. The TLC and neutrophilic counts returned

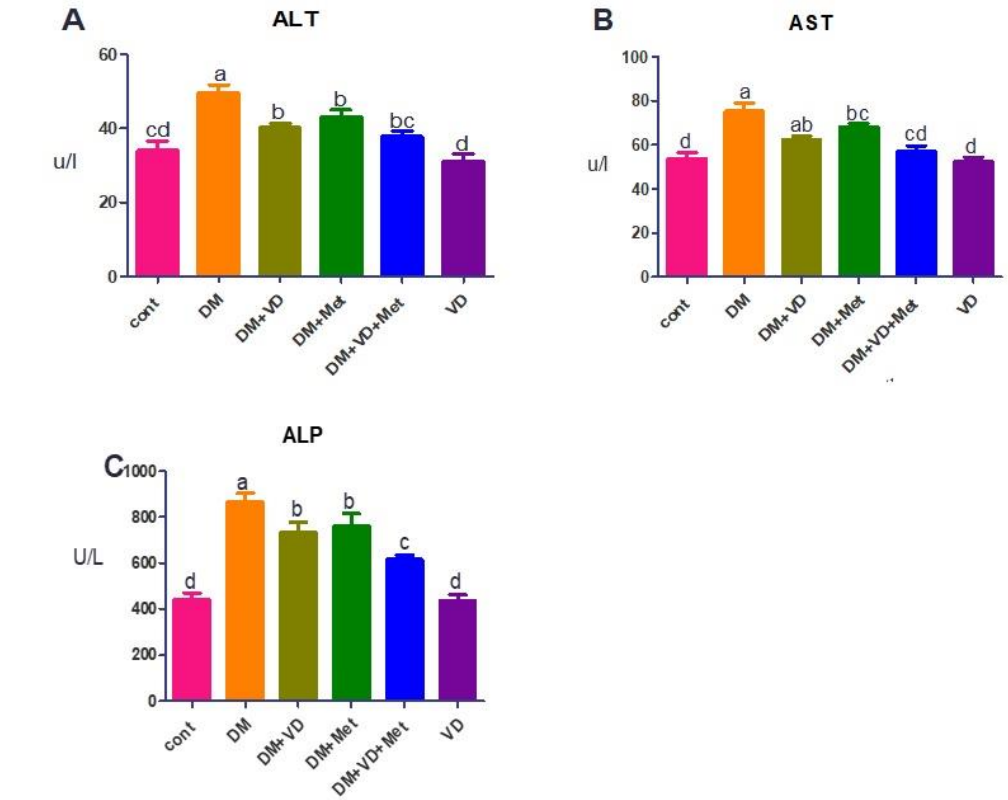


Figure (3): Liver enzyme activity(A)AST activity, (B)ALT activity, (C)ALP activity in different experimental groups at 4th week post treatment either with vitamin D and /or metformin in induced diabetic rats (Mean ± SE).

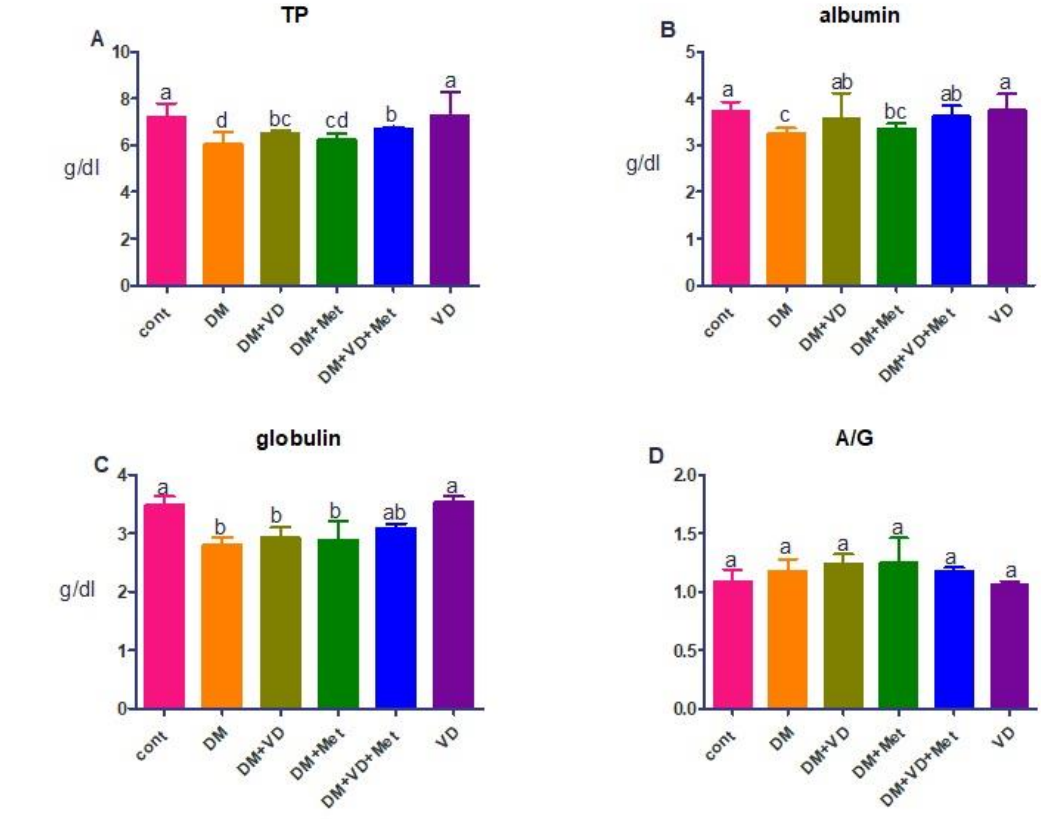


Figure (4): (A)TP level, (B)Albumin level, (C)Globulin Level, (D)A/G ratio level in different experimental groups at 4th week post treatment either with vitamin D and /or metformin in induced diabetic rats (Mean ± SE).

treated groups (DM+VD+Met) meanwhile monocyte insignificantly varied in (DM+VD) group compared to the control. Lymphocytes ($10^3/\mu L$) and eosinophils counts ($10^3/\mu L$) were insignificantly changed in all experimental groups. Additionally, the other leukogram component insignificantly affected in VD treated group (figure 2).

C-Biochemical Results:

1-Liver function biomarkers

Our result as illustrated showing a significant increase in the serum activities of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in the DM group compared to the control one. In contrast, their activities were reduced significantly in the DM+VD, DM+Met and DM+VD+Met groups compared to the DM non treated group except AST activity in DM+VD group insignificantly differed and the significant reduction was recorded in the combined treated group (figure 3).

DM non-treated group showed a significant reduction in the serum total protein (TP), albumin (Alb) and globulin (Glo) levels comparing with the control group. The DM+VD and DM+VD+Met had higher TP and Alb levels and insignificantly varied in the DM+Met compared to the DM group. The serum globulin level was insignificantly differed in all diabetic treated groups compared to the DM non-treated one. Concerning A/G ratio, it was insignificantly changed in all investigated groups (figures 4, 5, 6 & 7 respectively). Vitamin D alone (VD) insignificantly affect all liver function biomarkers with respect to the control group.

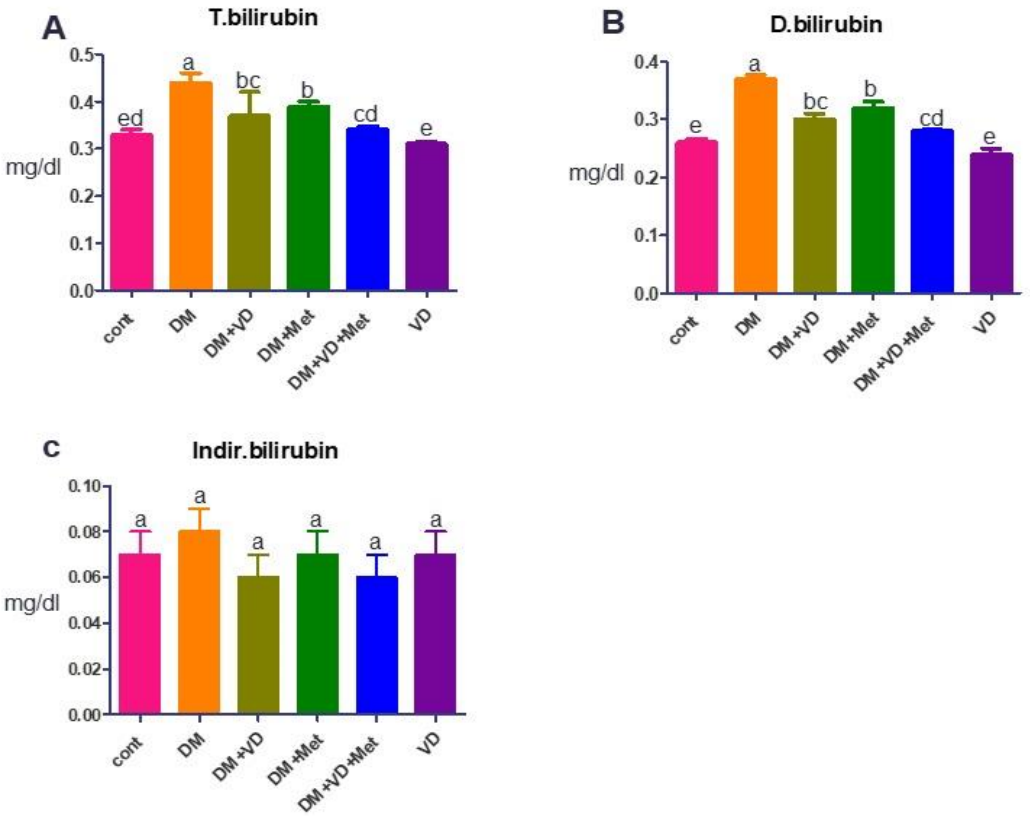
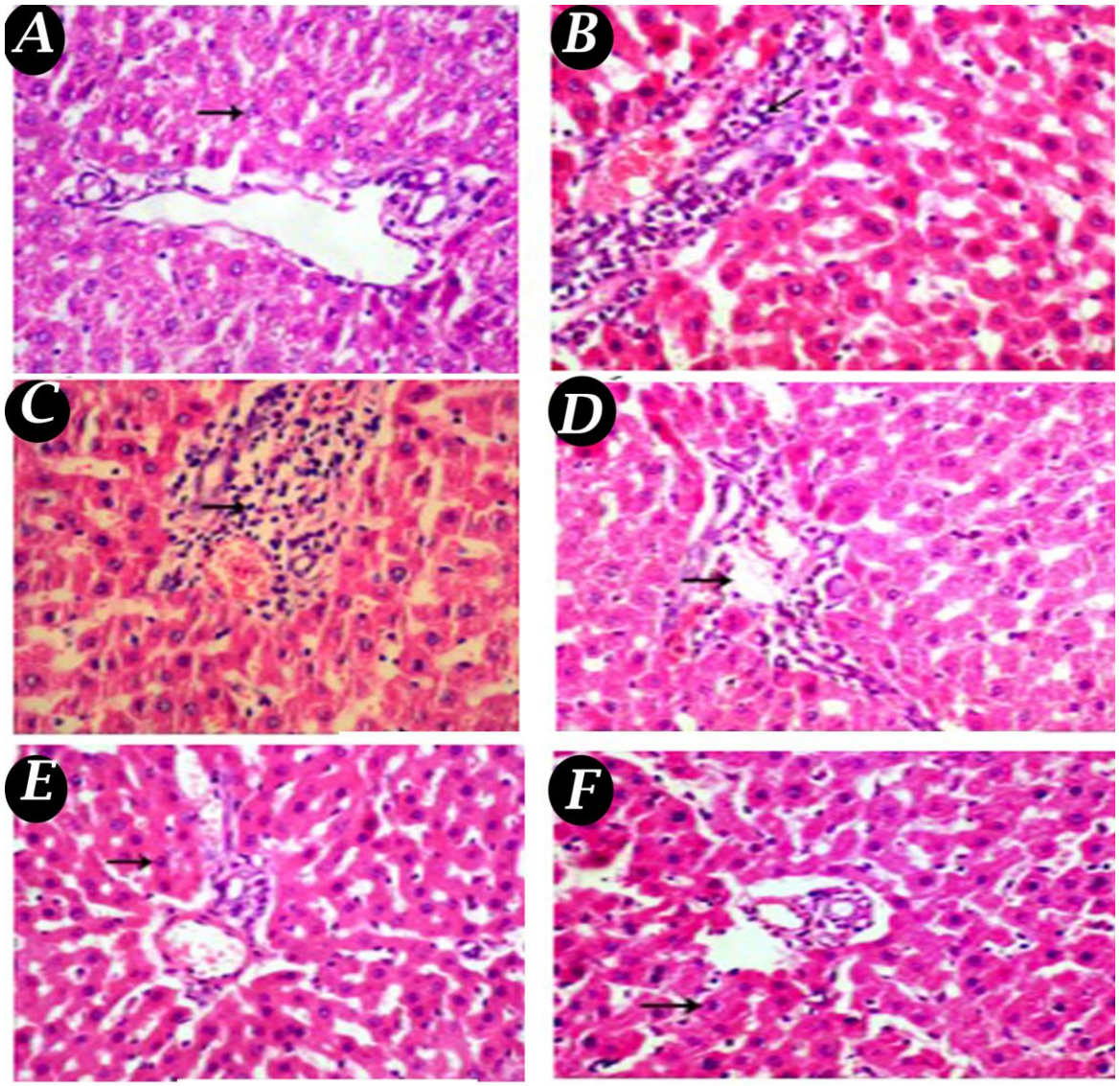


Figure (5) Bilirubin level (A)T. bilirubin, (B)D. bilirubin level, (C)indirect bilirubin level in different experimental groups at 4th week post treatment either with vitamin D and /or metformin in induced diabetic rats (Mean ± SE).

A significant elevation in both total and direct bilirubin were observed in the diabetic non-treated group comparing with the control one. Meanwhile, they were down regulated significantly in all diabetic treated rats comparing with the DM non-treated group but not return to their normal value except total bilirubin in the DM+VD+Met group insignificantly differed as compared to the control one. Statistically, there was no significant difference in the indirect bilirubin level in between all tested groups (figure 8).

Histopathological finding:

Liver histopathology displays normal portal area with normal hepatocytes (arrow) in control group, vitamin D treated group and diabetic group treated with both vitamin D and metformin (Photos 1, 6, 5 respectively). On the other hand, In the DM group liver showing a lympho-histiocytic infiltrate in portal area (arrow) (Photo 2). While diabetic group treated only by vitamin D, liver



Hepatic micrograph (H&E 400x) of the a) Control group at the end of 4th week shows normal portal area with normal hepatocytes (arrow). B) Diabetic group at the end of 4th week displays lympho histiocytic infiltrate in portal area (arrow).C) DM+VD group at end of 4th week displays mild edema infiltrate in portal area (arrow) with normal hepatocytes. D) DM+ Met group at the end of 4th week displays mild edema infiltrate in portal area (arrow) with normal hepatocytes. E) DM+ VD+ Met group at the end of 4th week displays normal portal area with normal hepatocytes (arrow). F) VD group at the end of 4th week displays normal portal area with normal hepatocytes (arrow)

displays mild edema infiltrate in portal area (arrow) with normal hepatocytes (Photo 3). In addition, in DM+ Met treated group liver showing mild edema infiltrate in portal area (arrow) with normal hepatocytes (Photo 4).

DISCUSSION

Our erythrogram results clarified that, induced diabetic untreated group suffered from significantly decrease in RBCs count, Hb concentration and PCV % with no significant change in blood indices which revealed normocytic normochromic anemia due to chronic inflammation, that may be returned to the increase in non-enzymatic glycosylation of erythrocyte membrane proteins and oxidation of these glycosylated membrane proteins. As well the hyperglycemia in DM caused an increase in the production of hydrogen peroxides, which act as free oxygen radicals and its effect on bone marrow progenitor cells and effect of TNF-α and their effect on bone marrow (Nasirian et al., 2017; Jothi et al.,2016; Oyedemi et al., 2011). Our results in accordance with Mahmoud et al. (2013) who recorded that HFD for 2 weeks then STZ (35 mg/kg) I/P injection cause a significant decrease in RBC count, Hb concentration, PCV%, and blood indices. But our finding disagree with Mojani et al. (2014) who reported insignificant differences in hemogram results between STZ diabetic rat and control, this difference may be due to the injection of diabetic group by 110 mg nicotinamide I/P then after 15min injected by 65 mg/kg, B.W, I/P STZ.

Our result showed an improvement of the CBC picture in diabetic rats treated with vitamin D either alone or in combination with metformin which go parallel with Refaat et al. (2014) results who observed that oral treatment with vitamin D at a dose (500 IU/rat/day) for 5 weeks correct the erythrogram picture compared by diabetic untreated group. In addition, our erythrogram results may show some improvement in the CBC picture with metformin treatment which reflected by increased RBCs count, that could be referred to diminish in the lipid peroxide level in RBC membrane causing reduce of the non-enzymatic glycosylation of membrane proteins (Mans and Aburjai,2019). Our results may agree with Irshaid et al. (2011) who observed a significant elevation in the mean value of PCV followed by oral administration of metformin at a dose (14.2 mg/Kg) for six weeks to diabetic rats.

The significant increase in leukocyte count in induced diabetic rats without treatment compared by the control could be explained by the stimulation of the immune system as progression of diabetes. Which, agree with Luong et al. (2005) who observed that in STZ induced diabetic rats several types of immune cells including antigen-presenting cells (APCs such as macrophages and dendritic cells), CD4+ and CD8+ T cells and B cells that infiltrate the pancreatic islet cells and this immunological events might trigger self-destruction of the pancreatic β-cells. Also, this result agrees with Usoh and Akpan (2015) who mentioned that I/P STZ injection at one dose (55mg/kg/BW) for 28 days had significantly higher level of WBCs count.

Moreover, in diabetic untreated rats which may be explained as DM was accompanied by elongate circulation time of neutrophil and monocyte and low circulation time of lymphocyte, which elevate the occurrence of infection as systemic inflammation, stress and pancreatic β-cell dysfunction resulted from insulin resistance and the development of T2DM (Harinarayan, 2014).

Our finding revealed that treatment with vitamin D regulates the above-mentioned parameters, which confirm anti-inflammatory role of vitamin D as the endogenous vitamin D has been shown to regulate vital functions in each organ including suppression of inflammation and oxidative stress. In addition, vitamin D simultaneously promoted several anti-

oxidant enzymes and down regulated a panel of pro-inflammatory cytokines (e.g. IL-1β, TNF-α) (BaSalamah et al., 2018).

Metformin treated rats showed a decrease in TLC, neutrophil, and monocyte as metformin leads to control hyperglycemia of diabetic rat so decrease the inflammation and stress of diabetes. The same reported by ElKarim et al. (2017) who narrated a significant decrease in total leukocytic count in comparison with control group by the administration of metformin at dose 300 mg/kg, B.W orally for 30 days.

Our results clarify a significant elevation in serum activities of ALT, AST, and ALP in induced diabetic untreated model compared with control which may be referred to dysfunction in liver cells, and leakage of these enzymes from the liver cytosol into the bloodstream (Al-Logmani and Zari, 2009; Ohaeri, 2001; Navarro et al., 1993) these results confirmed by our histopathological results. Our results in agreement with Nwozo et al. (2016) who found that Serum ALT, AST, ALP were significantly elevated in diabetic rats induced by a single I/P injection of STZ at a dose (50 mg/kg, B.W) when compared to control rats.

Vitamin D either solely or combined with metformin showed protective action on hepatic cells manifested by reduced activities of serum ALT, AST, and ALP. Vitamin D supplementation lowered the risk of hepatic decompensation by decreasing several inflammatory cytokines in patients with advanced liver fibrosis and cirrhosis (El-Boshy et al., 2019). Also, our results in agreement with Ning et al. (2015) who postulated that treatment with vitamin D(orally at 0.03 µg/kg /day) for 8 weeks in STZ diabetic rats (35 mg/kg B.W byI/P injection) significantly restored the elevated ALT, AST, and ALP activities close to the control value compared with diabetic untreated group.

The observed ameliorative effects of vitamin D on the alteration of liver function tests, that may be participated in its Anti-diabetic actions through improving the hepatic metabolism (Abdel-Rehim et al., 2018) also metformin prevented liver damage (Yanardag et al., 2005). These findings were supported by our histological findings Which in agreement with Yanardag et al. (2005) and Nam et al. (2018) who explained that Serum ALT, AST activities were significantly declined in diabetic STZ rats treated orally with metformin at a dose (25 mg/kg) for 28 days or (50 mg/kg) compared to the diabetic control respectively.

The present study revealed that, the diabetic induced rats showed a significant reduction in the total protein, albumin, and globulin levels all over the experimental period. This decline may be attributed with the inhibited oxidative phosphorylation processes, which lead to a decrease of protein synthesis, an increase in the catabolic processes and reduction of protein absorption (Al-Logmani and Zari, 2009). Also those findings may referred to the hyperglycemia, which, cause an increase in the generation of free radicals by glucose auto-oxidation. The increment of free radicals may lead to oxidative damage and subsequently, liver cell damage, causing leakage of hepatic enzymes that responsible for protein synthesis, in addition to that damage which caused in the proximal tubules cause leakage of albumin resulted in microalbuminuria (Al-Logmani and Zari, 2009 : Tojo et al., 2001).

Our results in agreement with Al-Logmani and Zari (2009) who narrated that, serum total protein and albumin levels decreased significantly by the induction of streptozotocin. In reverse, Akileshwari et al. (2014) corroborated that, STZ diabetic rats at single dose (35mg/kg/BW) for 12 weeks significantly increased the level of albumin compared to control rats.

Our data revealed that, the levels of total protein, albumin and globulin were significantly increased in (DM+ VD, DM+ Met, DM+VD+Met) groups compared with untreated diabetic group. These finding can

be referred to the hepatoprotective role of vitamin D either combined with met or alone, by its anti-inflammatory role , which may be occurred through decreasing the level of some inflammatory cytokines such as (TNF-α and NF-kb) (Harinarayan, 2014). As well, treatment with metformin might have protective activity due to its effect against cellular leakage and loss of functional integrity of the cell membrane in hepatocyte and reduced hepatocellular inflammatory reaction and necrotic changes (Kabil et al., 2015).

In our investigation, the induced diabetic untreated group showed a significant elevation in both total and direct bilirubin. This may be attributed to hepatic damage that confirmed by our histopathology, also bilirubin is a product that used in clinical practice as a marker for the hepatobiliary disease. Furthermore, it recognized as an endogenous antioxidant so it may have a protective role via suppressing oxidative stress in the patients with type 2 diabetes mellitus, as oxidative stress is an important pathogenic factor in the development of diabetic vascular complications (Inoue et al., 2018).

Referring to our data, the administration of vitamin D and /or metformin for 4 weeks showed significantly decrease on the level of both total and direct bilirubin, which may be due to their hepatoprotective action as showed in our histopathology, also their role control of hyperglycemia, caused a decrease in the production of hydrogen peroxides.

CONCLUSION

The data from our study indicated that supplementation of vitamin D to rats suffer from type 2 diabetes mellitus significantly improve hemogram picture and improve liver damage and suppress liver stress than treatment with drug only. From these result, it can be concluded the protective and effective role of vitamin D against diabetes mellitus complication .

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