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The Useful Role of Vitamin "D" and Coconut Oil in Attenuating the Disorders of Splenic T and B Cells in Diabetic Mice.

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ABSTRACT

The purpose of the present study is to evaluate the modulatory role of either vitamin D or coconut oil or both together in attenuating the splenic lymphocytes disorder (T and B cells) by using immunohistochemistry (IHC) in diabetic adult male mice (*Mus musculus*) induced by streptozotocin (STZ). The mice were divided into 7 equal groups (10 mice/each). The duration of the experiment was 4 weeks. Gp.I: normal control mice group without any treatments. Gp.II & Gp.III: non-diabetic mice groups received vitamin D orally in a daily dose of 500 IU (6.25 ml)/kg b.w/daily or coconut oil in a dose of 7.5 ml /kg b.w/daily, respectively. Gp.IV: diabetic mice group injected i.p. with a single dose of STZ dissolved in saline solution in a single dose of 200 mg/kg b.w to induce diabetes. Gps.V, VI & VII: diabetic mice given orally with vitamin D or coconut oil or both together in the same previous doses. The results recorded non- significant changes in the blood glucose and insulin levels of non-diabetic mice groups. A high significant increase in blood glucose level and a significant decrease in insulin of diabetic mice group as compared to the normal control ones. Diabetic mice group received the vitamin D only recorded a slight decrease in blood glucose level and a slight increase in insulin; while the diabetic mice received coconut oil alone or co-administered with vitamin D recorded a significant decrease in blood glucose levels and a significant increase in insulin as compared to diabetic group. IHC observations in the splenic tissues of mice group of normal control, non-diabetic group received vitamin D or coconut oil expressed normal strong immunoreactivity to T cells in the white pulp of splenic parenchyma and normal strong immunoreactivity to B cells in the lymphatic follicles between periarteriolar lymohoid sheaths (PALS) and marginal zone. The diabetic mice group expressed a marked depletion in these T & B cells. The diabetic mice groups given vitamin D or coconut oil or both together daily for 4 weeks expressed an obvious recovery and increment of immunoreactivity to T & B cells. By using hemocytometer, diabetic mice group recorded a marked decrease in the total number of splenocytes as compared to the normal control group, while the diabetic mice given vitamin D or/and coconut oil recorded an increase and recovery in the splenocytes number approximately near to normal control group. By using flow cytometer, the splenocytes recorded a marked decrease in the splenic $CD4^+$/ $CD8^+$ T-cells count of diabetic mice group and those given vitamin D only in comparison with the normal control group. After diabetic mice given coconut oil or coconut oil with vitamin D together; the splenocytes recorded an obvious recovery and increase in the number of$CD4^+$/ $CD8^+$ T-cells. In conclusion, the present results indicated that coconut oil alone or co-administered with vitamin D play an important role in the modulation and recovery of the splenic B and T lymphocytes and splenocytes count of diabetic mice to normal status more than those given vitamin D alone.

Keywords: Hyperglycemia, Spleen, IHC, T& B lymphocytes, Flow cytometer, Vitamin D, Coconut oil Mice.

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INTRODUCTION

Diabetes mellitus (DM) and its complications constitute a severe public health issue facing modern societies **(**1**).** It is characterized by disorders in carbohydrates, proteins and fat metabolism caused by complete or partial insufficiency of insulin secretion and/or insulin action (2,3). DM leads to hyperglycemia which later develops to micro and macro-vascular complications and becomes a major cause of death (4).

The spleen is an organ found in all vertebrates and acts primarily as a blood filter. The spleen plays important roles in regard to red blood cells and the immune system (5). It removes old red blood cells and holds a reserve of blood, which can be valuable in case of hemorrhagic shock, and also recycles iron. As a part of the mononuclear phagocyte system, it metabolizes hemoglobin removed from senescent red blood cells. The globin portion of hemoglobin is degraded to its constitutive amino acids and the heme portion is metabolized to bilirubin which is removed in the liver (6).

The spleen synthesizes antibodies in its white pulp and removes antibody-coated bacteria and antibody-coated blood cells by way of blood and lymph node circulation. The spleen is a center of activity of the mononuclear phagocyte system and can be considered analogous to a large lymph node, as its absence causes a predisposition to certain infections (7). A study published in 2009 using mice found that the red pulp of the spleen forms a reservoir that contains over half of the body's monocytes (8); these monocytes moving to injured tissue (such as the heart after myocardial infarction) turn into dendritic cells and macrophages while promoting tissue healing (9).

The spleen represents a large lymphatic tissue passed by re-circulating lymphocytes, which are able to promptly elicit specific T or B lymphocytes mediated immune reactions. The lymphocytes are stressed by diabetic toxicity starting with high levels of free radicals, increasing the levels of pro-inflammatory cytokines and ending by programmed cell death. Some microorganisms become more virulent in a high glucose environment (10).

Vitamin D refers to a group of fat-soluble steroids responsible for increasing intestinal absorption of calcium, magnesium, phosphate and zinc (11). There are several forms of vitamin D (vitamers). The two major forms are vitamin D2 (ergo-calciferol) and vitamin D3 (cholecalciferol). Vitamin D without a subscript refers to either D2 or D3 or both, these are known collectively as calciferol **(**12**)**. Vitamin D from the diet or dermal synthesis from sunlight is biologically inactive; activation requires enzymatic conversion (hydroxylation) in the liver and kidney. It is estimated that 80% - 90% of vitamin D in the body is produced through skin synthesis and the remaining by the ingestion of foods and supplements of this vitamin (13).

Some studies have shown that vitamin D is necessary for normal insulin secretion. Vitamin D may play a functional role on glucose tolerance through its effects on insulin secretion and insulin sensitivity. It has been demonstrated that the secretion of pancreatic insulin is inhibited by vitamin D deficiency, and this deficiency is related to glucose intolerance and DMT2 (14,15).

Coconut oil is the richest source of lauric acid. The high concentration of beneficial fats in coconut oil makes it helpful for digestion. Its antimicrobial properties can help fight irritation and infection in the gut from Candida (genus of yeasts and is the most common cause of fungal infections worldwide) (16,17).

Coconut oil acts as hormonal support as it contains specific fats that support the body’s natural hormone production and play an important role in immune support because it includes lauric acid, capric acid and caprylic acid which have antifungal, antibacterial and antiviral properties that make it beneficial for immune support (18). Antioxidant in coconut oil may enhance the sensitivity to insulin or otherwise may also reduce insulin resistance and injury to pancreatic beta cells by scavenging reactive oxygen species (ROS) in diabetic patients (19).

The aim of the present study is to evaluate the role of vitamin D or / and coconut oil in attenuating the disorders of blood glucose and insulin levels as T & B cells of the spleen in STZ diabetic adult male albino mice.

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MATERIALS AND METHODS

Animals:

Seventy adult male albino mice *(Mus musculus)*, aged 6-8 weeks and each weighing 25±2 g were obtained from Vacsera, Cairo. The animals were housed in plastic cages (10 per cage) for one week acclimatization under the same condition of temperature and natural dark-light cycle. Food and tap water were freely available to the animals throughout the experiment. All care and procedures adopted for the present investigation were in accordance with the approval of the Institutional Animal Ethics Committee of Tanta University and in accordance with recommendation of the proper care and use of laboratory animals.

Induction of Diabetes mellitus (DM):

DM was induced in mice by intraperitoneal (i.p.) injection with a single dose of STZ dissolved in saline solution in a dose of 200 mg/kg of b.w. according to Deeds *et al.* (20). STZ was obtained from "Sigma Chemicals Co., St. Louis, Mo., USA".

Treatment:

1- Vitamin D was obtained from "Egyptian Group For Pharmaceutical Industries Co., Egypt." and administered orally (by a gastric tube).
2- Coconut oil was received from local pharmacy (Al-badawia Company for herbal and oil extraction, Mansoura, Egypt) and administered orally (by a gastric tube).

Experimental design:

After acclimatization, the mice were divided into 7 equal groups (10 mice for each); all were kept under the same conditions and received the same diet. Gp.I: normal control mice group without any treatments. Gp.II: non-diabetic mice group received vitamin D orally in a dose of 500 international units (6.25 ml)/kg of b.w daily for 4 weeks. Gp.III: non-diabetic mice group received coconut oil daily in a dose of 7.5 ml /kg of b.w for 4 weeks. Gp.IV: diabetic mice group injected i.p. with a single dose of STZ to induce diabetes. Gp.V: diabetic mice administered orally with vitamin D in a dose as in Gp.II.Gp.VI: diabetic mice given orally coconut oil in a dose as in Gp.III. Gp.VII: diabetic mice administered orally with both vitamin D and coconut oil at the same previous doses daily for 4 weeks.

Sample collection and serum separation:

At the end of the each period of the experiment, the animals were fasted for 13 hour, then anaesthetized by using diethyl ether, and then sacrificed. Blood samples were collected from all studied groups. The blood was allowed to clot at room temperature for 30 minutes before centrifugation at 1000 revolutions per minute (rpm) for 20 minute. Serum samples were collected into 1.5 ml epindorph tubes and stored at -20°C till be used to measure insulin. The spleen specimens were removed and processed for light microscopic and flow cytometric studies (21).

Immunohistochemical studies (IHC):-

The specimens of the splenic tissues were fixed in 10 % neutral buffered formalin for 24h. IHC avidin-biotin technique (22) and monoclonal antibodies (CD3 & CD20) against B and T cells expression respectively were used. CD3 and CD20 were received from Dako Carpinteria, 17A2U.S.A. (23).

Cell suspensions preparation

Splenic tissues were removed and washed in saline solution. Single-cell suspension and count of spleen were prepared according to Lutz *et al.* (24) and Diaz-Montero *et al.* (25). Briefly, splenocytes were isolated by dissociating spleen on 60 µm mesh Seives screens (Sigma, St. Louis, MO) and they were washed and diluted in supplemented RBMI-1640 media. Splenocytes viability and count analysis had been done by the trypan blue exclusion method using a hemocytometer.

Flow cytometry analysis

Splenocytes suspension was prepared and counted using a hemocytometer with trypan blue dye exclusion as described previously by Lutz *et al.* (24) and Diaz-Montero *et al.* (25). Briefly, suspension of spleen T-lymphocytes surface phenotypes were established with anti- mouse CD4 or anti- mouse CD8 (Bioscience, San Diego, CA, USA). T-lymphocytes were stained with the indicated conjugated monoclonal anti-mouse CD4 or CD8 antibody (10 µL/106 cells), and were incubated for 30 minutes at a room temperature in the dark and chill on ice for 1 min. The cells were washed twice with PBS and resuspended in 0.3 ml of PBS supplemented with 0.5% BSA and 0.02% sodium azide. Cells were then washed and acquired by flow cytometer (Sysmex-Partec Company, Germany). The CD4+ and CD8+ cell subsets analysis were phenotypically detected using Flow Jo software (Treestar, Ashland, OR, USA).

Calculation of the results:

The mean absorbance for each set of duplicate standards, control samples, subtract and the average zero standard optical density were calculated. The standard curve was plotted on log-log graph paper with standard concentration on the X-axis and absorbance on the Y-axis. The best-fit straight line through the standard points was drawn according to Bourne (26).

Image analysis:

Digital images were analyzed by a semi-quantitative system (Figi-Image J software, Java based application for analyzing images). The brown colour of B and T-cells was expressed in the spleen sections immunohistochemically ; the percentage of stained area(area fraction), per field area of B and T cells was determined by measuring six randomly photographed high-power fields (X400 magnifications) (27).

Statistical analysis:

Data were fed to the computer and analyzed using IBM SPSS® software package version 16.0, USA. Data were analyzed using numbers and percentages ± S.E. For normally distributed data, comparisons between the seven studied groups were analyzed using F-test (ANOVA) and Post Hoc test (LSD). Significance was obtained at $p< 0.05$ according to Diggle and Llang (28).

RESULTS

1. Effect of vitamin D and coconut oil on blood glucose levels:

The induction of diabetes in adult mice by STZ recorded highly significant increase in blood glucose level (Gp.IV) as compared to the normal control mice (Gp.I) (**$p \leq 0.001$); a significant increase in blood glucose of diabetic mice administered with vitamin D alone (Gp.V) (*$p \leq 0.05$), and non-significant increase in mice blood glucose values of diabetic mice given coconut oil only (Gp.VI) or co-administered with vitamin D and coconut oil (Gp.VII) as compared to the normal control mice ($p > 0.05$)**, (**Table 1& Graph 1**).**

2. Effect of vitamin D and coconut oil on insulin levels:

Diabetic mice recorded a significant decrease in blood serum insulin of STZ diabetic mice (Gp.IV) as compared to the normal control mice (Gp.I) (*$p \leq 0.05$), and non-significant decrease in blood serum insulin of the diabetic groups given vitamin D or/and coconut oil (Gps.V, VI and VII) as compared to the normal control mice ($p > 0.05$), (Table 2& Graph 2)**.**

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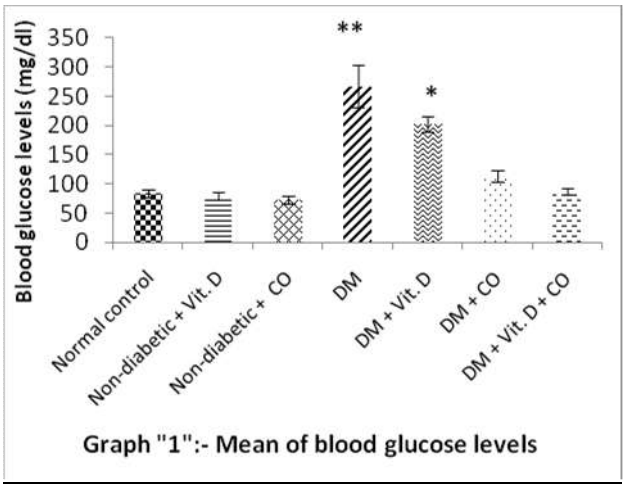
Table 1 & Graph 1: Effect of vitamin D or/and coconut oil on blood glucose levels:-

Groups	Mean ± SE (mg/dl)
Group I (Normal control)	83.9 ± 6.14
Group II (Non-diabetic + Vit. D)	79.4 ± 6.25
Group III (Non-diabetic + CO)	72.9 ± 7.03
Group IV (DM)	267.27 ± 35.87**
Group V (DM + Vit. D)	202.2 ± 13.74*
Group VI (DM + CO)	112.6 ± 9.68
Group VII (DM + Vit. D + CO)	87.1 ± 4.88

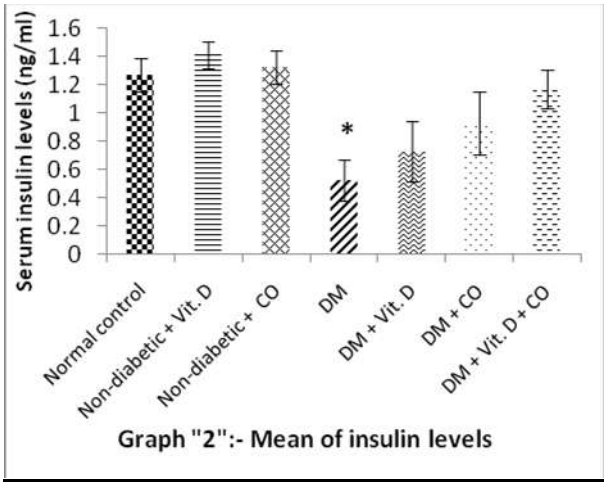


Graph "1":- Mean of blood glucose levels

Statistical significance was measured at p >0.05; *p ≤ 0.05;**p ≤ 0.001

Table 2 & Graph 2: Effect of vitamin D or/and coconut oil on insulin levels:

Groups	Mean ± SE (ng/ml)
Group I (Normal control)	1.27 ± 0.11
Group II (Non-diabetic + Vit. D)	1.41 ± 0.09
Group III (Non-diabetic + CO)	1.32 ± 0.11
Group IV (DM)	0.52 ± 0.14*
Group V (DM + Vit. D)	0.72 ± 0.21
Group VI (DM + CO)	0.92 ± 0.22
Group VII (DM + Vit. D + CO)	1.17± 0.13



Graph "2":- Mean of insulin levels

Statistical significance was measured at p > 0.05; *p ≤ 0.05

Immunohistochemical results:

CD3 (T-cells):-

The splenic tissues of normal control and non-diabetic mice groups received vitamin D in a dose of 6.25 ml /kg b.w/d or coconut oil in a dose of 7.5 ml /kg b.w/d for 4 weeks expressed normal strong immunoreactivity to T cells in the white pulp of splenic parenchyma (Figs. 1- 3). STZ diabetic mice group expressed a marked depletion of immunoreactivity to T cells in the white pulp of splenic parenchyma (Fig. 4). The diabetic mice groups given vitamin D or coconut oil or both together daily for 4 weeks expressed an obvious recovery and increment of immunoreactivity to T cells in the white pulp of splenic parenchyma (Figs. 5 - 7).

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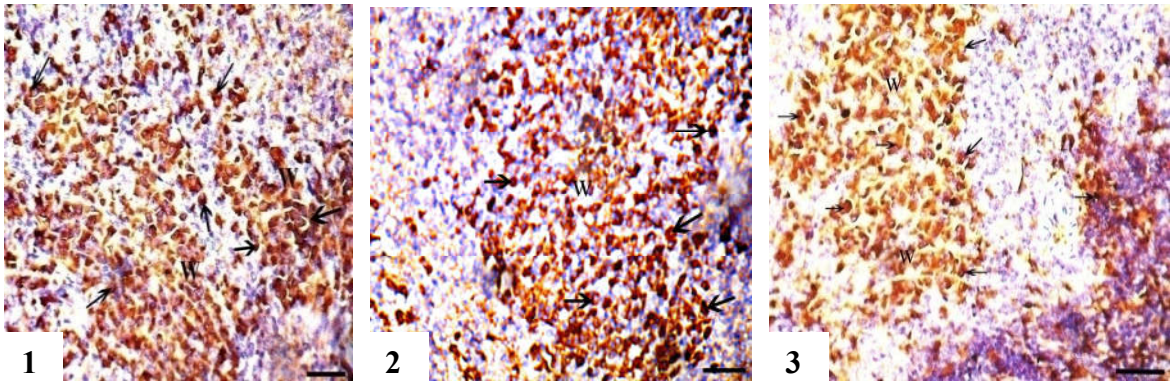

Fig (1-3): Sections of the spleens expressing the normal strong immunoreactivity to T cells (arrows) in the white pulp (W) of splenic parenchyma in normal control mice (Fig. 1), in non-diabetic mice received vitamin D daily for 4 weeks (Fig. 2), and in non-diabetic mice received coconut oil daily for 4 weeks (Fig. 3). CD3 immunostain, all; Bar = 6.25 µm.

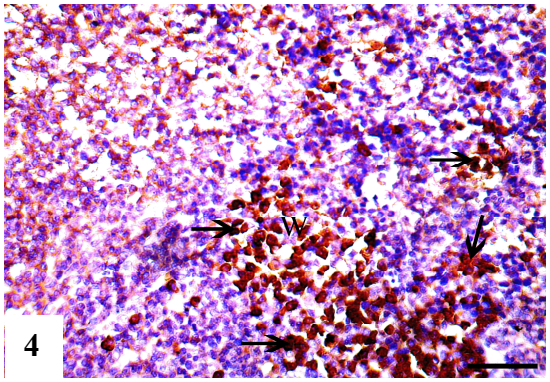

Fig (4): Section of the spleen of STZ diabetic mouse expressing a marked decrement of immunoreactivity to T cells (arrows) in the white pulp (W) of splenic parenchyma. CD3 immunostain, Bar = 6.25 µm.

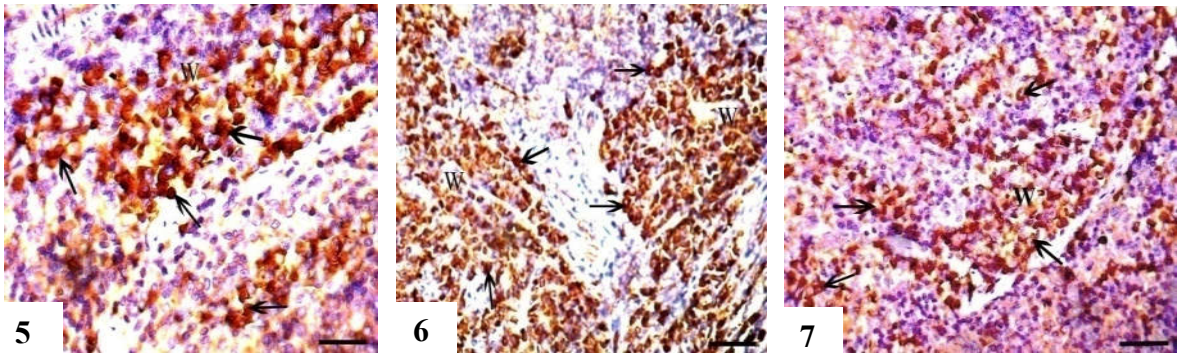

Fig (5-7): Sections of the spleen of the treatment of STZ diabetic mice stained with CD3 immunostain expressing an obvious recovery and increment of immunoreactivity to T cells (arrows) in the white pulp (W) of splenic parenchyma. Fig 5: diabetic mice received vitamin D daily for 4 weeks; Fig (6): diabetic mice received coconut oil daily for 4 weeks, and Fig (7): diabetic mice received vitamin D and coconut oil together daily for 4 weeks. CD3immunostain, all; Bars = 6.25 µm.

Image analysis of splenocytes T-cells:-

STZ diabetic mice and those given vitamin D alone recorded a significant decrease in the area of splenic T-cells (Gps.IV& V) as compared to normal control mice (Gp.I) (*p ≤ 0.05); non-significant decrease in the area of splenic T-cells of diabetic mice given coconut oil only (Gp.VI) as compared to normal control mice

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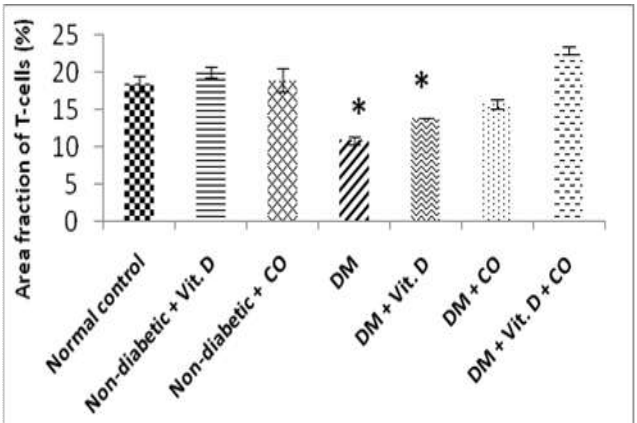
(p > 0.05),and non-significant increase in the area of splenic T-cells of diabetic mice given vitamin D and coconut oil (Gp.VII) as compared to normal control mice (p > 0.05) (Table 3& Graph 3).

Table 3 & Graph 3:-Mean of area fraction of T cells (%) in all mice groups

Groups	Mean ± SE (mg/dl)
Group I (Normal control)	18.48 ± 0.99
Group II (Non-diabetic + Vit. D)	20.03 ± 0.71
Group III (Non-diabetic + CO)	18.93 ± 1.55
Group IV (DM)	10.88 ± 0.46*
Group V (DM + Vit. D)	13.79 ± 0.03*
Group VI (DM + CO)	15.77 ± 0.65
Group VII (DM + Vit. D + CO)	22.91 ± 0.51

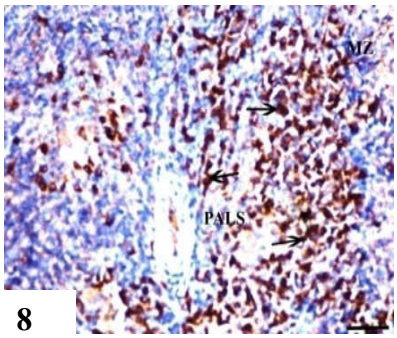


Graph "3":- Mean of area fraction of T-

Statistical significance was measured at p > 0.05; *p ≤ 0.05

1- CD20 (B- cells):-

The splenic tissues of normal control and non-diabetic mice groups given vitamin D or coconut oil expressed normal strong immunoreactivity to B-cells in the lymphatic follicles between periarteriolar lymphoid sheaths and marginal zone (Figs. 8 - 10).STZ diabetic mice group expressed a marked decrease of immunoreactivity to B-cells in the lymphatic follicles of the spleen(Fig. 11).The diabetic mice groups given vitamin D or/and coconut oil daily for 4 weeks expressed an obvious recovery and increment of immunoreactivity to B-cells in the splenic lymphatic follicles (Figs. 12 - 14).



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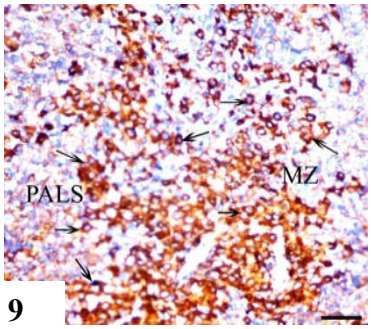

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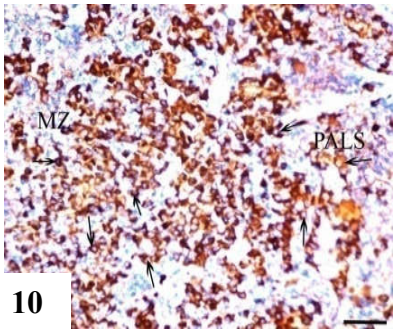

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Figs. (8-10): Sections of the spleen stained with CD20 immunostain expressing normal strong immunoreactivity to B-cells (arrows) in the lymphatic follicles between periarteriolar lymphoid sheaths (PALS) and marginal zone (MZ); in normal control mice (Fig 8), non-diabetic mouse received vitamin D daily for 4 weeks (Fig 9), non-diabetic mouse received coconut oil daily for 4 weeks (Fig 10). CD20 immunostain, all; Bars = 6.25 µm.

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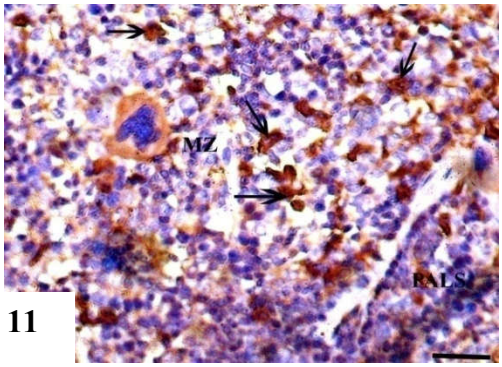

Fig (11): Section of the spleen of STZ diabetic mouse expressing a marked reduction of immunoreactivity to B-cells (arrows) in the lymphatic follicles between periarteriolar lymphoid sheaths (PALS) and marginal zone (MZ). CD20 immunostain, Bar = 6.25 µm.

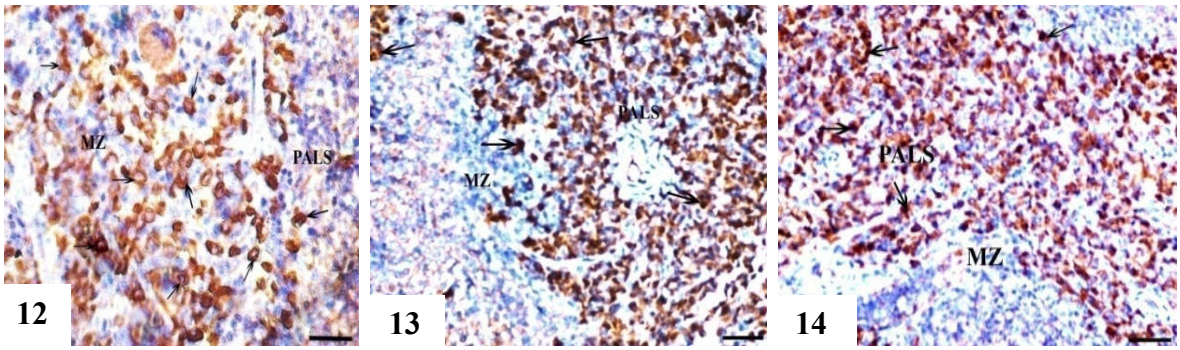

Fig (12-14): Sections of the spleens of the treatment of STZ diabetic mice stained with CD20 immunostain expressing recovery and increment of immunoreactivity to B-cells (arrows) in the lymphatic follicles between periarteriolar lymphoid sheaths (PALS) and marginal zone (MZ). Fig (12): diabetic mice received vitamin D daily for 4 weeks; Fig (13): diabetic mice received coconut oil daily for 4 weeks, and Fig (14): diabetic mice given vitamin D and coconut oil together daily for 4 weeks. CD20 immunostain, all; Bars = 6.25 µm.

Image analysis of splenic B cells:-

STZ diabetic mice recorded a highly significant decrease in the area of splenic B-cells (Gp.IV) as compared to normal control mice (Gp.I) (**p ≤ 0.001); significant decrease in the area of splenic B-cells of diabetic mice given vitamin D only (Gp. V) as compared to normal control mice (Gp.I) (*p ≤ 0.05); non-significant decrease in area of splenic B-cells of diabetic mice given coconut oil only (Gp.VI) as compared to normal control mice ($p > 0.05$),and non-significant increase in area of splenic B-cells of diabetic mice co-administered of vitamin D and coconut oil (Gp.VII) as compared to normal control mice ($p > 0.05$) (Table 4& Graph 4).

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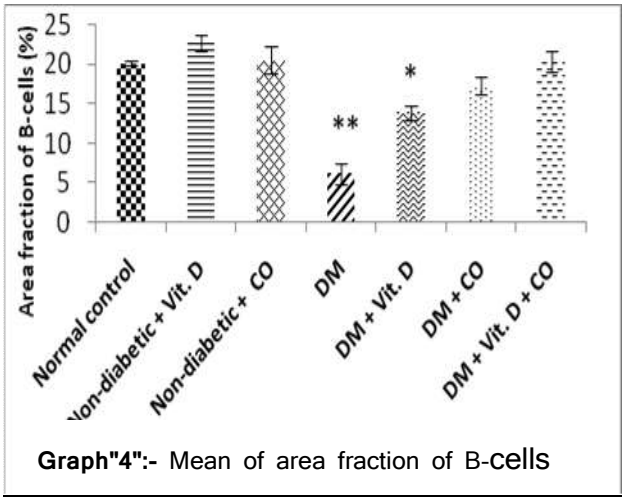
Table 4 & Graph 4:-Mean of area fraction of B-cells (%)

Groups	Mean ± SE (mg/dl)
Group I (Normal control)	20.15 ± 0.29
Group II (Non-diabetic + Vit. D)	22.75 ± 0.99
Group III (Non-diabetic + CO)	20.56 ± 1.80
Group IV (DM)	6.18 ± 1.34**
Group V (DM + Vit. D)	13.92 ± 0.92*
Group VI(DM + CO)	17.30 ± 1.13
Group VII (DM + Vit. D + CO)	20.35 ± 1.35



Graph"4":- Mean of area fraction of B-cells

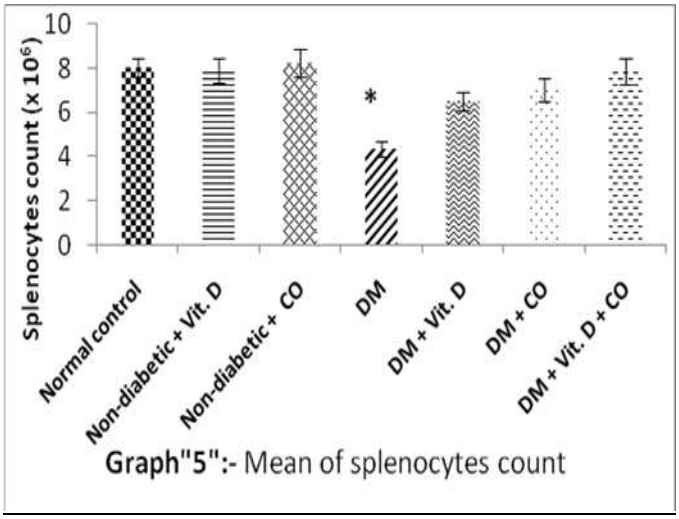
Statistical significance was measured at p > 0.05; *p ≤ 0.05;**p ≤ 0.001

Effect of vitamin D or/ and coconut oil on splenocytes count:

The induction of diabetes in adult mice by STZ caused significant decrease in splenocytes count (Gp.IV) as compared to the normal control mice (Gp.I) (*p ≤ 0.05) and non-significant decrease in splenocytes count of diabetic mice administered with vitamin D or/and coconut oil (Gps.V, VI and VII) (p >0.05) in comparison to the normal control mice **(**Table 5& Graph 5**).**

Table 5 & Graph 5:-Mean of splenocytes count.

Groups	Mean ± SE ($x10^6$)
Group I (Normal control)	8.03 ± .42
Group II (Non-diabetic + Vit. D)	7.91 ± .54
Group III (Non-diabetic + CO)	8.24 ± 0.63
Group IV (DM)	4.36 ± 0.36*
Group V(DM + Vit. D)	6.50 ± 0.43
Group VI (DM + CO)	7.03 ± 0.51
Group VII (DM + Vit. D + CO)	7.88 ± 0.57



Graph"5":- Mean of splenocytes count

Statistical significance was measured at p >0.05; *p ≤ 0.05

Flow cytometry data of spleen:

a- Effect of vitamin D or/and coconut oil on splenic $CD4^+$/ $CD8^+$ T-cells:-

The induction of diabetes in adult mice by STZ recorded highly significant decrease in splenic $CD4^+/CD8^+$ T-cells count in Gp.IV as compared to group I (**p ≤ 0.001); a significant decrease in splenic

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$CD4^+/CD8^+$ T-cells count in Gp.V as compared to Gp.I (*p ≤ 0.05), and non-significant decrease in splenic $CD4^+/CD8^+$ T-cells count in Gps.VI and VII as compared to Gp.I (p >0.05). The splenic $CD4^+$ is a T-helper and $CD8^+$ is a T-killer (Fig. 15 &Graph 6).

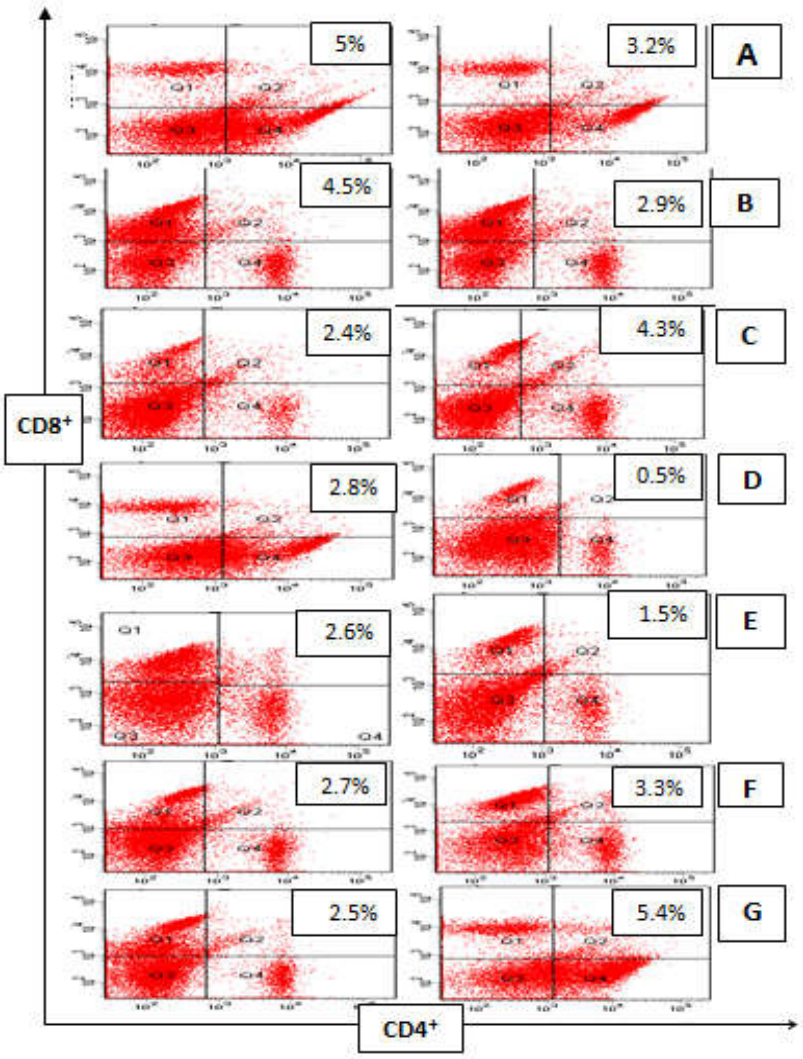
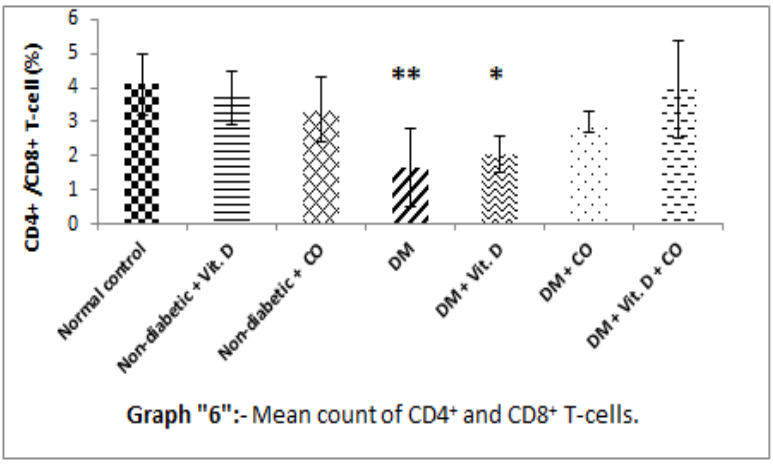


Fig (15): A representative flow cytometric analysis of $CD4^+$ and $CD8^+$ T-cells count in the mice groups of normal control (A), non-diabetic received vitamin D (B), non-diabetic received coconut oil (C), diabetic mice (D), diabetic mice administered orally with vitamin D (E), diabetic mice taken orally coconut oil (F) and diabetic mice given orally both vitamin D and coconut oil (G).



Graph "6":- Mean count of $CD4^+$ and $CD8^+$ T-cells.

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Effect of vitamin D or/and coconut oil on splenic CD4$^+$ T- helper cells /anti Ly-6G:-

The diabetic mice recorded a significant decrease in splenic CD4$^+$ T-helper cells/Ly-6G count (Gp.IV) as compared to control group I (*p ≤ 0.05); a highly significant decrease in splenic CD4$^+$T-helper cells/Ly-6Gcount in Gp.V as compared to Gp.I (**p ≤ 0.001) and non-significant decrease in splenic CD4$^+$T-helper cells / Ly-6G count in Gps.VI and VII as compared to Gp.I (p >0.05) (Fig.16 & Graph 7).

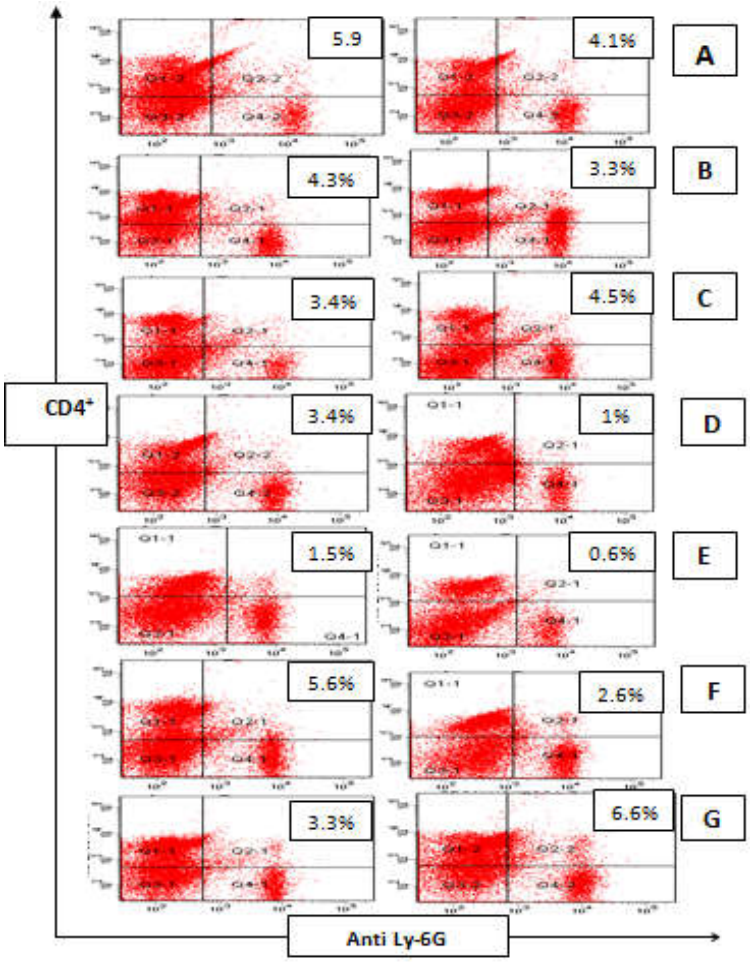
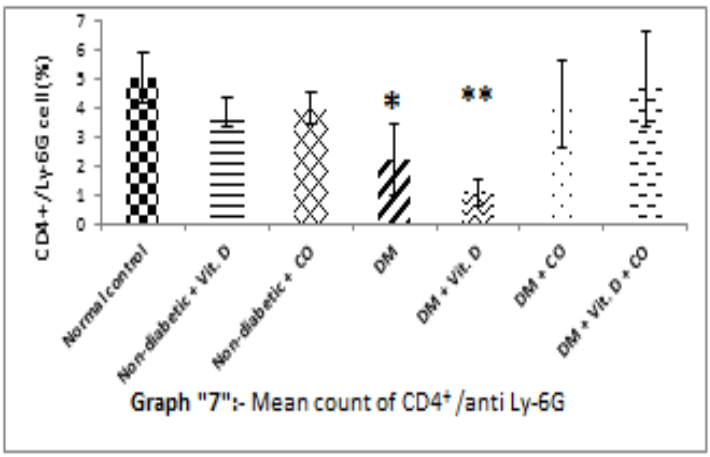


Fig (16): A representative flow cytometric analysis of CD4$^+$ T-helper cells and anti Ly-6G count in the mice groups of normal control (A), non-diabetic received vitamin D (B), non-diabetic received coconut oil (C), diabetic mice (D), diabetic mice administered orally with vitamin D (E), diabetic mice taken orally coconut oil (F) and diabetic mice given orally both vitamin D and coconut oil (G).



Graph "7":- Mean count of CD4$^+$ /anti Ly-6G

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Effect of vitamin D or/ and coconut oil on splenic $CD8^+$ T-killer cells / anti Ly-6G:-

The induction of diabetes in adult mice by STZ of splenic $CD8^+$ T-killer cells/ Ly-6G count was caused highly significant decrease in splenic $CD8^+$ T-killer cells/Ly-6Gcount in Gp.IV as compared to group I (**p ≤ 0.001); a significant decrease in splenic $CD8^+$ T-killer cells/Ly-6G count in Gps.V and VI as compared to Gp.I (*p ≤ 0.05) and non-significant decrease in splenic $CD8^+$ T-killer cells / Ly-6Gcount in Gp.VII as compared to Gp.I(p > 0.05) (Fig. 17 & Graph 8).

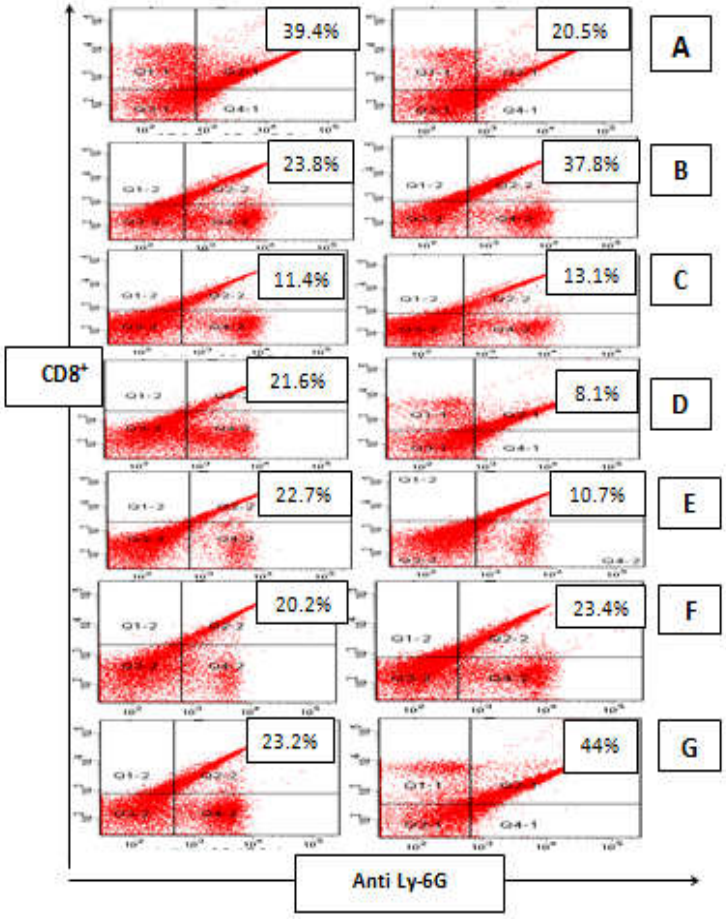
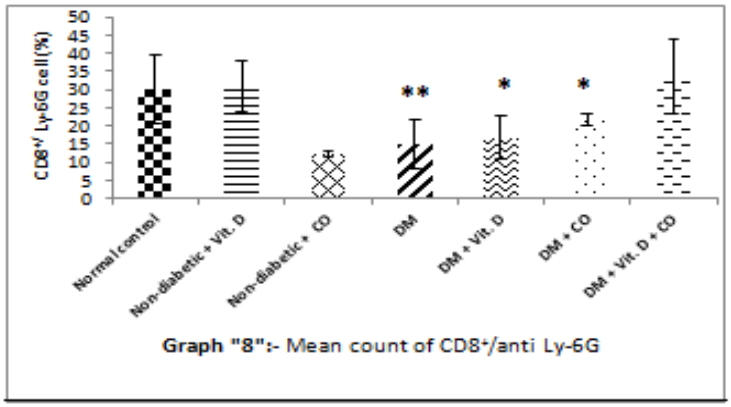


Fig (17): A representative flow cytometric analysis of $CD8^+$ T-killer cells and anti Ly-6G count in the mice groups of normal control (A), non-diabetic received vitamin D (B), non-diabetic received coconut oil (C), diabetic mice (D), diabetic mice administered orally with vitamin D (E), diabetic mice taken orally coconut oil (F) and diabetic mice given orally both vitamin D and coconut oil (G).



Graph "8":- Mean count of $CD8^+$/anti Ly-6G

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DISCUSSION

Diabetes mellitus (DM) is a metabolic disorder and caused by complete or partial insufficiency of insulin secretion (2, 3). DM is also called hyperglycemia which later develops to micro and macro-vascular complications (4).DM can be induced by Streptozotocin (STZ). STZ is glucosamine-nitrosourea compound which is toxic to the insulin-producing beta cells of the pancreas in mammals. It is used in medicine for treating certain cancers of the islets of Langerhans and is used in medical research to produce an animal model for hyperglycemia by causing damage to the DNA(29, 30, 31).

The present results of non-diabetic adult mice groups recorded non-significant changes in the blood glucose and insulin levels. A highly significant increase in blood glucose levels and a significant decrease in insulin were recorded in STZ-diabetic mice group as compared to the normal control ones. Diabetic mice received vitamin D only recorded a slight decrease in blood glucose levels and a slight increase in insulin; while the diabetic mice given coconut oil alone or co-administered with vitamin D recorded a significant decrease in blood glucose levels and a significant increase in insulin as compared to diabetic group.

The disorders in insulin secretion leads to various metabolic aberrations in type 2 DM, spanning from hyperglycemia due to defective insulin-stimulated glucose uptake and up-regulated hepatic glucose production, along with dyslipidemia, which includes impaired homeostasis of fatty acids, triglycerides and lipoproteins (32,33). The expression of insulin secreting cells (β-cells) of pancreatic rats by using monoclonal anti-insulin markedly demonstrated the destruction and evident reduction of β-cells immune-reaction (34).

Hypovitaminosis D is associated with insulin resistance leading to DM as vitamin D that seems to affect the glucose homeostasis. Vitamin D inhibits the inflammatory responses caused by cytokines, diminishes stress in the pancreatic Langerhans β-cells which in turn avoids pancreatic cellular apoptosis. Along with these discoveries on a cellular level, there are possibilities that vitamin D could have a role in the prevention of the beginning of insulin resistance (35,36).

The reduction in blood glucose in STZ diabetic mice after treated with coconut oil daily for 3 weeks has been recorded by Siddalingaswamy *et al.* (19). Moreover, the virgin coconut oil alleviates hyperglycemia and improves glucose tolerance probably by its antioxidant effect which consequently leads to improvement of insulin secretion (37).

Coconut oil is a source of to cotrienols, capric acid, caproic acid, and lauric acid which are natural antioxidants. These substances act as scavengers of damaging oxygen free radicals that have been suggested to play an important role in aging, atherosclerosis, cancer, and Diabetes mellitus (38,39,40). Lauric acid in coconut oil has insulin- tropic properties (41). In diabetic patients, antioxidants may play a vital role in improving insulin response to the loaded glucose and may reduce insulin resistance (42).

The immunohistochemical microscopic observations in the present study exhibited normal strong immunoreactivity to T-cells (by using CD3 immunostain) in the white pulp of splenic parenchyma and B-cells (by using CD20 immunostain) in the lymphatic follicles between periarteriolar lymphoid sheath (PALS) and marginal zone of the spleen sections of normal control and non-diabetic mice received vitamin D or/and coconut oil. STZ diabetic mice expressed a marked decrement of immunoreactivity to splenic T and B cells, while those given vitamin D or coconut oil or both together daily for 4 weeks expressed an obvious recovery and increment of immunoreactivity to T and B-cells.

In accordance, Gaulton *et al.* (43) and Koulmanda *et al.* (44) reported that STZ was directly toxic for lymphocytes, inducing apoptosis *in vitro* and was responsible for early depletion of blood and spleen T and B lymphocytes *in vivo*. Moreover, Luo *et al.* (45); Long and Buckner (46) reported that STZ-induced diabetes is associated with a higher frequency of T regulatory cells (T regs) in the blood and secondary lymphoid organs and reduced numbers of T-cells in the blood.

Immunity to allogeneic insulinoma cells could be reduced by diabetes-induced immune-suppression (47).The chronic effects of diabetes on immunity are well recognized. In contrast, the acute effects of diabetes

have not been established. Diabetogenic β-cells toxic drugs such as STZ have been associated with direct and rapid immunity suppression (44, 48).

Hyperglycemia is associated with the increment of corticosteroids level causing a rapid depletion in thymocytes and splenic T-cells followed by homeostatic T-cell proliferation. However, the exact mechanism leading to the observed immune-suppression after STZ administration especially the effect on different lymphocyte subpopulations, including T-regs is still unknown(49).This observation is in line with the decrease in CD8+ T-cell numbers in the peripheral blood of STZ-treated mice. Because T cells are known to play a critical role in pancreatic β cells autoimmunity and rejection direct T cells depletion induced by STZ administration may contribute to the better Langerhans islet engraftment described after transplantation (50,51). STZ is not the only cause depletion of T and B-cells in diabetic mice but also in various animal models of diabetes (52,53).

Flow cytometric observations in the present study exhibited a marked decrease in mice splenic $CD4^+$/$CD8^+$ T-cells and $CD4^+$/anti Ly-6G of STZ diabetic mice group and those given vitamin D for 4 weeks as compared to normal control group. After the STZ diabetic mice groups taken coconut oil alone or co-administered with vitamin D daily for 4 weeks, an obvious recovery was observed in splenic mice $CD4^+$ $CD8^+$ T-cells and $CD4^+$ anti Ly-6G. While splenic mice $CD8^+$ T-cells and anti Ly-6G of non-diabetic mice group received coconut oil daily for 4 weeks, diabetic group and those received vitamin D daily for 4 weeks expressed significant decrease as compared to normal control group. A marked improvement of $CD8^+$ T-cells and anti Ly-6G was observed in the diabetic mice received coconut oil alone or co-administered with vitamin D daily for 4 weeks.

Similar results were observed by Nekoua *et al.* (54) who reported that, naive $CD4^+$ T-cells were declined in the patients with DMT2 which may be associated with adaptive immune activation and chronic inflammation during the pathogenesis of DMT2. Adaptive immune system especially T lymphocyte plays a pivotal role in the pathogenesis of DMT2. $CD8^+$ T-cells were crucially involved in evoking inflammatory cascades in obese adipose tissue and essential for induction of macrophage activation and migration to adipose tissue by secreting MCP1, MCP-3, and RANTES (regulated on activation, normal T-cell expressed and secreted(55,56).

The results of the present study illustrated that STZ diabetic mice group recorded a marked decrease in the number of splenocytes as compared to normal control group, while the diabetic mice given vitamin D or/and coconut oil daily for 4 weeks expressed an obvious increase in the number of splenocytes as compared to STZ diabetic mice group. These results agreed with Hashish and Kamal (57) who found that diabetes caused decrease in the body weight and degeneration of splenocytes in diabetic albino rats treated with STZ.

High levels of blood sugar cause depletion of oxidant defense system (58,59). Excessive reactive oxygen radicals activate the process of apoptosis by activating caspase-3 and Bcl-2 (B-cell lymphoma 2) suppression (60). Splenic lymphocytes are very sensitive to apoptotic signals. Apoptosis results in the release of cytochrome c from mitochondria and activation of a specific class of cytoplasmic enzymes known as caspase (61).Caspase-3 is the key inducer of apoptosis. This activated caspase-3 destroys numerous cellular structures, leading to cell death (62).

Moreover, the Bcl-2 family proteins are major antagonist of apoptosis which presents in mitochondria that has been shown to inhibit cytochrome c release and protect against oxidative-induced apoptosis (63). DM increases the expression of caspase-3 immunoreactivity in the spleen and decreases the expression of Bcl-2. Also, Mellado and Aguilar (64) recorded that the high glucose concentration enhances release of pro-inflammatory cytokines that mediated apoptosis of rat pancreatic islet cells.

Coconut oil is composed of antimicrobial medium chain fatty acids, and therefore can play an important role with the immune system in fighting microscopic invaders. Coconut oil is ideal for immune suppressed individuals. For this reason, it is now being studied as a treatment for HIV / AIDS patients whose immune systems are severely compromised (65).

Many studies have reported the effectiveness of dietary unsaturated fatty acids on proliferation of lymphocytes (66,67), synthesis of cytokines (53,56, 68), activation of phagocytosis, pro-coagulant activity of monocytes and elimination of bacteria from the spleen (64,69, 70).

Eicosanoids are signaling molecules made by the enzymatic or non-enzymatic oxidation of poly-unsaturated fatty acids which participate as important mediators and therefore may alter immune function. Their production may be changed due to modification of membrane fluidity and regulation of gene expression seems to play an important role in immune system modulation. Phospholipids from plasma membrane and eicosanoids metabolism are directly involved in the immune system modulation and dietary lipids may modulate the effects attributed to these substances due to the inability of inhibitors to promote lymphocytes proliferation (71,72).

In conclusion, the disorders in blood glucose and insulin levels as well as decrement in B and T lymphocytes of spleen by IHC and counting by flow cytometer in diabetic mice were restored to normal status after administration of coconut oil alone or co- administered with vitamin D, i.e. they are stronger anti-hyperglycemic effects than those given vitamin D only.

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