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Hematological Changes in Diabetic Rats Receiving Melatonin, Vitamin D and Vitamin E are Not Reliable Indices of Inflammatory Changes

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A R T I C L E I N F O

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A B S T R A C T

Background: Finding a suitable model to study the effect of various treatments on diabetes will help to avoid undesirable effect on humans during empirical investigation. This study aims to evaluate the values of biomarkers such as Neutrophil-Lymphocyte Ratio (NLR), Platelet-Lymphocyte Ratio (PLR), and Lymphocyte-Monocyte Ratio (LMR) in monitoring diabetes in a rat model subject to treatment with melatonin, vitamin-D and vitamin-E.
Methods: Male albino rats (*n*=8-10 per group), normal and with diabetes were divided as follows into 12 groups: G1 normal fed, received no medications; G2 normal, treated with melatonin; G3 normal, treated with vitamin-E; G4 normal, treated with vitamin-D; G5 diabetic; received no medications; G6 diabetic, treated with insulin; G7 diabetic treated with melatonin; G8 diabetic, treated with melatonin and insulin; G9 diabetic, treated with vitamin-E; G10 diabetic, treated with vitamin-E and insulin; G11 diabetic, treated with vitamin-D and G12 diabetic, treated with vitamin-D and insulin. Two months post-treatment, hematological (NLR, PLR and LMR) and biochemical examination of glucose profile and oxidative stress status, were performed.
Results: NLR is significantly decreased on comparing G3 and G4 with G1, and significantly increased on comparing G9 with G1. On comparing G3 with G5 and G6, NLR is significantly decreased, but on comparing G9 with G5 and G6, NLR is significantly increased. However, PLR and LMR showed no statistically significant changes in all rat groups.
Conclusion: Hematological changes in diabetic rat model receiving melatonin, vitamin D and E are not reliable indices of inflammatory changes.

1. Introduction

Diabetes mellitus (DM) is a damaging chronic disorder, with growing prevalence globally [1]. The global rise in DM is primarily due to an increased incidence of type 2 diabetes (T2D). Likewise, the prevalence of type 1 diabetes (T1D) is also growing in similar manner to that of T2D. Although countries spend billions of dollars to treat diabetes and its complications, the incidence is still growing, causing significant health and socio-economic impact [2].

T2D is a multifactorial disorder, where inflammation is an important player in the pathogenesis of its complications [3]. Constituents of the immune system are changed in various tissues and organs in T2D [4]. These immunological disturbances include changes in the levels of certain cytokines and chemokines, as well as changes in the number and activation status of diverse leukocyte populations [4]. Several anti-inflammatory modulators have been suggested to play roles in the relationship between inflammation and diabetes. However, this area of research is not fully studied. Melatonin is an important multitasking hormone with fundamental clinical applications. It is a potent antioxidant and anti-inflammatory molecule, modulating both pro- and anti-inflammatory mediators in different pathological conditions such as obesity, hypertension [5] and diabetic complications [6]. Melatonin has a role to play in the regulation of glucose homeostasis and insulin secretion. Reduced in levels of melatonin have been observed in diabetic patients and such reductions could disturb melatonin functions and therefore contribute in the pathogenesis of diabetes. On the other hand, the lipid soluble vitamins D and E are potent antioxidants and have been used in various clinical settings. Reduced level of vitamin D and E appears to be linked to T2D and most of its complication reported to date. Both have been involved in various DM-associated disorders such as nephropathy, retinopathy and vasculopathy [7, 8]. They have been linked to inflammatory processes including regulation of immune cells [9, 10]. Moreover, they exert anti-inflammatory effects through regulating the generation of pro-inflammatory molecules through affecting cytokine production [11, 12].

Several biomarkers have been suggested to predict and monitor diabetes and other inflammatory disorders such as the neutrophil to lymphocyte ratio (NLR) [13, 14], platelet to lymphocyte ratio (PLR) [15] and lymphocyte to monocyte ratio (LMR) [16]. NLR has been used as a predictor of many diseases and conditions such as kidney function decline [17]. It has been suggested as a powerful and independent risk indicator for death rate in the elderly population [18]. NLR is associated with severe cholecystitis [19]. It may be used as a prognostic marker of recovery time in patients with Bell's palsy [20]. It can be used to predict peripheral arterial disease such as atherosclerosis [21] and some cardiovascular and renal disorders [22, 23]. NLR correlate positively with degree of limitation of joint mobility in patients with type 2 diabetes [24].

The NLR, PLR and LMR are used as biomarkers which can be easily computed from the blood picture and are also reproducible. Unlike many other inflammatory markers, they are low-cost and readily available and offer additional risk stratification beyond conventional risk scores. In current clinical practice the necessity to use new prognostic tools for monitoring inflammation and cardiovascular abnormalities in DM patients is emphasized. Whether the suitability of using these markers in models (other than human) which are subjected to various treatments for predicting and monitoring DM and its detrimental disorders is still not clear. The aim of the study is to evaluate the value of NLR, PLR and LMR in

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predicting and monitoring streptozotocin induced-diabetes in rat model subject to treatment with melatonin, vitamin D and E. Parameters such as glucose profile, oxidative stress status and total leukocyte count will be measured. Finding a suitable model, other than human, to study the effect of various treatment on diabetes indices will help to avoid undesirable effects on human during empirical investigation.

2. Materials and Methods

2.1 Induction of DM

DM was induced in rats intra-peritoneally by administering nicotinamide (230 mg/kg), 15 min before the single dose of streptozotocin (STZ) (65 mg/kg, i.p.) [25]. Control rats were treated with an equal volume of normal saline. STZ was prepared by dissolving STZ powder in saline with a sodium citrate buffer, pH 4.0. Blood glucose levels were measured to monitor the degree of diabetes by using standard diagnostic kits. Induction of DM was confirmed by recording glucose level before any treatment. Rats with proven hyperglycemia were used for further examination.

2.2 Groups and Treatments

Hundred and eight [108] male albino rats weighing 200-250 g were distributed into 12 groups: **G1** (*n*= 10) control rat with normal fed diet, with no additional treatment; **G2** (*n*= 10) normal rats received oral treatment with melatonin only (0.3 mg/kg); **G3** (*n*= 10) normal rats received oral treatment with vitamin-E only (40mg/kg); **G4** (*n*=10) normal rats received oral treatment with vitamin-D only (40 mg/kg); **G5** (*n*=9) diabetic rats, which received no additional treatment; **G6** (*n*=8) diabetic rat treated with insulin only; **G7** (*n*=10) diabetic rats received oral treatment with melatonin (0.3 mg/kg) only; **G8** (*n*=9) diabetic rats received oral treatment with melatonin (0.3 mg/kg) and insulin; **G9** (*n*=8) diabetic rats received oral treatment with vitamin-E (40 mg/kg) only; **G10** (*n*=8) diabetic rats received oral treatment with vitamin-E (40 mg/kg) and insulin; **G11** (*n*=8) diabetic rats received oral treatment with vitamin-D (40 mg/kg) only and **G12** (*n*=8) diabetic rats received oral treatment with vitamin-D (40 mg/kg) and insulin. The number of rats in diabetic groups were more than 10 rats per group. However, the variation in the number of samples some group, was due to death of some diabetic rats during the process. The length of the treatment was for eight weeks and the calculation of insulin dose was based on the weight of each rat and the level of its blood glucose.

2.3 Biochemical measurements

Following 2 months of treatment, blood samples were collected for biochemical examination of fasting blood sugar (FBS), hemoglobin A1c (HbA1c), fructosamine (FA), oxidized low density lipoprotein (Ox-LDL), total antioxidant capacity (TAC) and malondialdahyde (MDA) by the standard processes and available kits. Assays were performed through following the procedures of the kits and device guidelines, operation, calibration, and quality control.

Samples of the blood were collected in ethylene diamine tetra acetic acid (EDTA; Sigma-Aldrich) tubes for assessment of HbA1c in the same day. For biochemical measurements, serum was taken after drawing of blood in plain tubes which were left to coagulate for 30 minutes, followed by centrifugation for 15 min at 3000 revolutions per minute. Aliquots (1ml) were divided into small Eppendorf tubes for the measurement of FBS, FA, Ox-LDL, TAC and MDA. Serum samples were then preserved at -80 °C for later examination.

FBS and HbA1c were measured by using the standard procedures and available kits in a totally automated system (COBAS integra 400 plus; Roche Diagnostics). The calibration of the machine was made by using calibrator for this system made by Roche Diagnostics. Assessment of fructosamine was made by using reagent set (POINTE SCIENTIFIC, Inc, Canton, Michigan, USA). The collective antioxidant activities of all vitamins, proteins, lipids, glutathione, uric acid, and others were assessed by measuring TAC, which was performed by using rat TAC ELISA Kit from MyBioSource, Inc. Assessment of lipid peroxidation in rat serum was made by measuring MDA by using TBARS ELISA assay kit from rat (MyBioSource, Inc). The MDA reaction byproduct was measured calorimetrically at 530–540 nm and the concentration of MDA was expressed in μM. Sensitivity, linearity and precision of the used kits were assessed by the manufacturers. The % coefficient of variation of all kits was less than 5%.

2.4 Hematological Examination

EDTA anticoagulated blood samples were handled through the Coulter Gen.S (Beckman Coulter, Miami, Fla) for complete blood cell count (CBC) and automated differential leukocyte count (differential). Calibration and quality control of the Coulter apparatus were done by using S-cal and 3 levels of 5C control (both from Beckman Coulter), respectively, according to the manufacturer's instructions.

2.5 Statistical analysis

Descriptive statistics and one-way ANOVA were used to compare the concentration of the metabolic parameters between the 12 groups. *P* value of < 0.05 was considered as statistically significant. All statistical methods were performed using SPSS for windows (version 20, SPSS Inc.).

3. Results

The obtained results were statistically analyzed, and tabulated in table (1). When comparing the results of all groups with group 1, the following results were obtained: Glucose was significantly increased ($p < 0.05$) in all groups except group 2. HbA1c was significantly increased ($p < 0.05$) in groups 3, 4, 5, 6 and 7. Fructosamine was significantly increased ($p < 0.05$) in all groups except group 2 and 9. TAC was significantly increased ($p < 0.05$) in groups 2, 7, 8, 10 and 11. Oxidized LDL and MDA showed non-significant changes in all groups. As shown in figure 1, NLR is significantly decreased on comparing group 3 and 4 with group 1, and significantly increased on comparing group 9 with group 1. On comparing group 3 with group 5 and group 6, NLR was significantly decreased, but on comparing group 9 with group 5 and group 6, NLR was significantly increased. However, PLR and LMR showed no statistically significant changes as shown in figures 2 and 3 respectively.

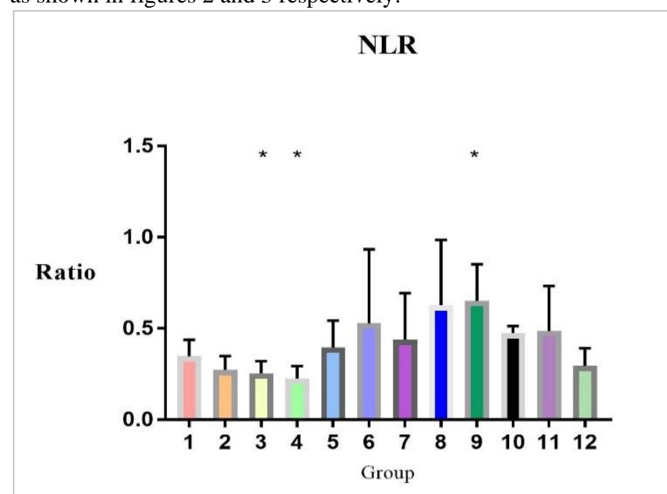

Figure 1: Effect of melatonin, vitamin D and vitamin E (*n*=8-10) on NLR. Results are presented as mean±SD and *P* values of less than 0.05 ($P<0.05$) are considered significant. The star symbol (*) indicates a statistically significance difference ($P<0.05$) when compared with G1.

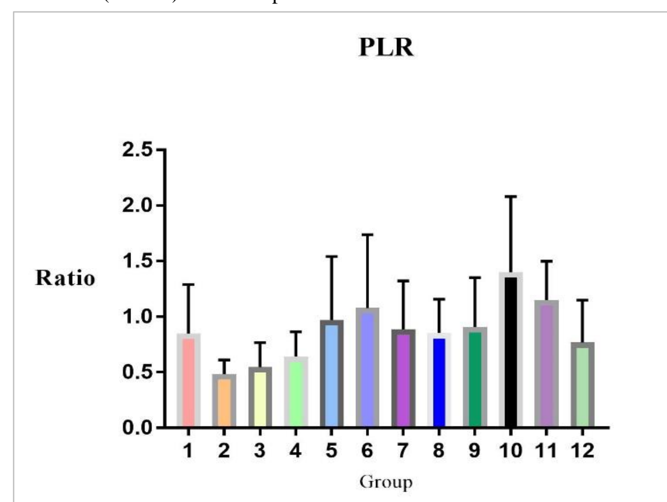

Figure 2: Effect of melatonin, vitamin D and vitamin E (*n*=8-10) on PLR. Results are presented as mean±SD.

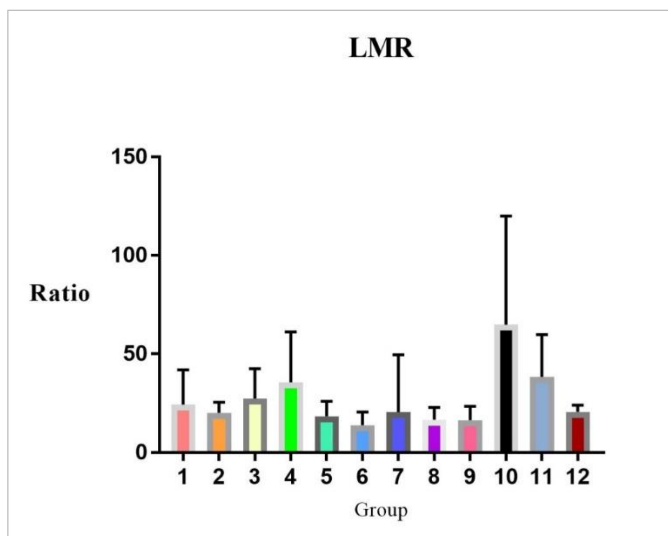

Figure 3: Effect of melatonin, vitamin D and vitamin E (*n*=8-10) on LMR. Results are presented as mean±SD.

Table 1: Effect of melatonin vitamin D and E on normal and diabetic rats.

Group	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
FBS (mg/dl)	98.29	113	125.7*	125*	145.57*	146.37*	142.8*	144*	148*	142.7*	147.5*	130.3*
SD	8.2	23.66	11.23	14.98	7.19	9.13	9.94	6.0	2.28	6.66	0.71	16.44
HbA1c (%)	4.12	4.03	4.77*	4.6*1	4.76*	4.9*	4.59*	4.34	4.83	4.17	4.6	4.17
SD	0.378	0.61	1.08	0.46	0.583	0.58	0.465	0.346	0.859	0.503	0.01	0.651
Fructosamine (mmol/l)	0.494	0.46	0.68*	0.87*	0.91*	1.17*	1.04*	0.95*	0.75	0.92*	0.88*	0.72*
SD	0.036	0.188	0.071	0.218	0.258	0.353	0.28	0.164	0.381	0.33	0.008	0.108
TAC (ng/ml)	7.249	7.924*	7.229	7.188	7.433	7.426	7.813*	7.784*	7.233	8.1*	8.65*	7.693
SD	0.4189	0.6181	0.438	0.5495	0.3024	0.7472	0.5195	0.3624	0.197	0.472	0.3977	0.8109
Ox- LDL (ng/ml)	48.1	63.1	49.2	52.5	61.1	54	46.9	49.7	54.8	45	52.5	45.7
SD	3.13	16.09	5.61	9.47	15.33	6.7	2.81	4.57	5.64	3.61	2.12	4.73
MDA (nmol/l)	135.6	120	134.6	111.8	127	124	160.3	114.44	157.8	217	131	154
SD	26.416	16.036	30.35	28.87	55.79	26.43	72.20	21.06	59.71	152	61.42	35.58
TLC (10/ul)	979	1321	997	1120	965	903	989	1223*	778	540*	803	1086
SD	309	251	295	191	437	224	393	392	235	63	375	371
NLR	0.347	0.273	0.254 * +^	0.225*	0.397	0.528	0.439	0.626	0.651 * +^	0.475	0.486	0.295
SD	0.091	0.076	0.067	0.068	0.146	0.405	0.254	0.359	0.201	0.039	0.246	0.096
PLR	0.846	0.483	0.546	0.641	0.969	1.077	0.887	0.853	0.908	1.399	1.150	0.766
SD	0.440	0.128	0.220	0.221	0.570	0.658	0.434	0.303	0.443	0.679	0.346	0.382
LMR	24.3	20.2	27.4	35.7	18.4	13.8	20.6	16.7	16.3	64.8	38.4	20.7
SD	17.6	5.3	15.1	25.5	7.5	6.7	29.1	6.2	7.0	55.1	21.5	3.3

Table. 1 Effect of administration of melatonin, vitamin D and vitamin E on the level of FBS, % of HbA1c, fructosamine, TAC, oxidized-LDL, malondialdahyde (MDA), Total Leukocyte Count, Neutrophil-Lymphocyte Ratio (NLR), Platelet-Lymphocyte Ratio (PLR), Lymphocyte-Monocyte Ratio (LMR) in diabetic rats. Symbol represented by *, +, and ^ indicates statistically significance difference ($P<0.05$) when compared with G1, G5 and G6 respectively. The value of each parameter represented by the mean in one row and the standard deviation (SD) in the following row. The groups (n=8-10 per group) are divided as follows: G1 normal fed rats, which received no medications; G2 normal treated with melatonin; G3 normal treated with vitamin-E; G4 normal treated with vitamin-D; G5 diabetic, which received no medications; G6 diabetic treated with insulin; G7 diabetic treated with melatonin; G8 diabetic treated with melatonin and insulin; G9 diabetic treated with vitamin-E; G10 diabetic treated with vitamin-E and insulin; G11 diabetic treated with vitamin-D and G12 diabetic treated with vitamin-D and insulin.

4. Discussion

Numerous studies that have connected systemic inflammation with vascular disease indicated that chronic inflammation promotes the development and progression of micro- and macro-angiopathic complications in patients with diabetes. Total white blood cell count (TWBC) is a crude but sensitive inflammatory biomarker, which can be measured in the laboratory easily and routinely and is a cost-effective investigation. Increased neutrophil count is seen in thrombus formation and ischemic diseases. The NLR in complete blood count is studied in several cardiac and noncardiac abnormalities as an inflammatory biomarker and is used for prediction of diagnosis such as acute myocardial infarction (MI), stroke, and heart failure. [26, 27]. Diabetic Nephropathy in T2DM has an inflammatory pathogenesis with many inflammatory markers being implicated, such as interleukin-1 (IL1), IL6, IL8, transforming growth factor beta 1, tumor necrosis factor-alpha (TNF-α), and cytokines [28, 29]. However, their measurements are not performed routinely due to cost and effort reasons. In this respect, NLR has emerged as a novel surrogate marker. NLR is a marker of chronic inflammation that displays a balance of two constituents of the immune system; neutrophils, which are the nonspecific inflammatory mediator which form the first line of protection, while lymphocytes are the regulatory or defensive constituent of inflammation [30]. Interestingly, NLR has been found to have a positive relation with not only the presence but also the severity of metabolic syndrome [31]. Shiny *et al*. have shown that NLR is correlated with increasing severity of glucose intolerance and insulin resistance and can be used as a prognostic biomarker for macro- and micro-vascular complications in patients with glucose intolerance [32].

An increased NLR represents leukogram shifts toward a higher level of neutrophils or a lower level of lymphocytes, or both, from the basal innate immunity. The pathological development of NLR in DM is still unknown. Circulating levels of neutrophils have been reported to be higher [33, 34] or lower [35, 36] in patients with T1D. This disagreement was thought to be due to studies performed on different stages of DM progression, or due to several unrecognized causes (such as infections and mild chronic diseases linked to inflammation) that could change the NLR in clinical studies, or simply that neutrophils may not be the main cause of the increased NLR. This phenomenon has been confirmed by other studies, where the rate of lymphocyte apoptosis was considerably higher in T2D patients as compared to a healthy population [37]. These observations suggest that higher NLR values may simply be due to a fall in lymphocyte numbers caused by hyperglycemia-induced oxidative stress and cellular apoptosis.

A DM experimental model could be applied to examine this assumption. In comparison to clinical studies, experimental models have the benefit of using controlled experimental designs to systematically measure the diagnostic value of NLR during the progression of DM. Regardless the discrepancy between white blood cell indices in humans and rat model [38], the changes in ratios of leucocytic count still could be a reliable marker for inflammation and diabetic complications.

Increased NLR but not the absolute numbers of white blood cells is the measure that should be stressed upon as mentioned. Our study shows variable changes in NLR in different groups of the study regardless of the diabetic state or the antioxidant given. While it shows decreased NLR in normal rats taking vitamins E and D as compared to normal placebo rats, it exhibits an increase in diabetic rats taking vitamin E only as compared to normal placebo rats. Again, diabetic rats with or without insulin administration show decreased NLR compared to normal rats taking vitamin E, and an increased NLR compared to diabetic rats taking vitamin E. It is evident that most NLR ratios are either decreased or increased regardless of the administration of the different antioxidants in diabetic rats, treated or not, nor in normal rats in our study. Other white blood indices like PLR, LMR are

not consistent and/or reliable. NLR in different groups are not correlated to the state of oxidative stress as observed by changes in oxidized LDL and / or antioxidant parameters. This clarifies that our model of diabetic rats for this study is either not a good model for our study or there are multiple interfering parameters that could not be avoided and lead to changes in white blood indices. Many of the exclusion criteria should be regarded for correct interpretation of changes in these parameters. Among these criteria are ischemia of any organ (heart, kidney, lung), heart failure, active infection, severe tissue damage, acute hemorrhage, acute poisoning, cancer, blood diseases. All these factors could have been shown to affect neutrophils and lymphocytes [39]. To conclude, our study still confirms the multiplicity of factors that influence the NLR due to different stages of the pathological progress of DM, or from multiple unrecognized factors and could not be relied upon for interpretation of diabetic complications in rat model.

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6. Ethical approval and Declaration statement

The experimental procedures were all approved by the University of Umm Al-Qura Research Ethical and Institutional Review Board. The study complied with the protocols and guidelines of the National Institute of Health, USA. Procedures were also conducted according to the policy of public health service on the use of laboratory animals (NIH, 2002).

The authors declare that they have no competing interests.

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