Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i3.4664

THERAPEUTIC INSIGHTS OF VITAMIN D IN THE MANAGEMENT OF TYPE 2 DIABETIC NEUROPATHY

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Abstract:
Background: Diabetes mellitus is derived from the Greek term diabetes, meaning siphon - to pass through, and the Latin word mellitus, meaning sweet. The most common consequence is neuropathy, namely distal symmetric polyneuropathy (referred to as diabetic neuropathy in this primer). Diabetic neuropathy is a loss of sensory function that begins distally in the lower limbs and is accompanied by discomfort and significant morbidity. Over time, at least 50% of diabetics develop diabetic neuropathy. The study aimed to identify the impact of oxidative markers (glutathione (GSH), advanced oxidation protein products (AOPP), advanced glycation end products (AGEs), malondialdehyde (MDA), and inflammatory biomarkers (interleukin-6, IL-6, TNF-α, and myeloperoxidase (MPO)) on the development of diabetic neuropathy along with routinely used biochemical parameters. Materials and Method: This was a case-control study. All the selected patients were screened and enrolled by convenient non-probability sampling technique at the social security hospital Lahore. Written Informed consent was obtained before enrollment of the study subjects. A total of 150 patients enrolled in the study, and they were divided into three groups, 50 subjects with type 2 diabetic neuropathy taking oral antidiabetics only and 50 diagnosed diabetic neuropathy (DN) subjects taking adjunct oral vitamin D therapy, 50 healthy individuals as a control group. Five mL of venous blood sample was taken from the antecubital vein of each participant. Statistical analysis was performed by GraphPad. The results of all variables were evaluated by using one-way ANOVA. Results: The mean value of biochemical parameters (WBCs, platelets, HbA1c, BSF, triglycerides, LDL, HDL, serum creatinine, and liver function tests, were increased in diabetics with neuropathy compared to the control and treated group. There was a significant improvement in these parameters after vitamin D therapy, the mean values of MDA, AGE, and AOPPs in type 2 diabetics with neuropathy were significantly increased compared to the control group and a significant decrease was noticed after vitamin D supplementation in the vitamin D group. GSH level was decreased in type 2 diabetics with DPN patients as compared to the control group. A significant increase in GSH was noticed in the vitamin D group. In addition, IL-6, TNFα, and MPO levels were

also increased in the case of diabetic neuropathy as compared to controls. However, a significant decrease in the levels was seen in the vitamin D group. Conclusions: ROS-mediated injuries in type 2 diabetics with neuropathy can be prevented by the restoration of an antioxidant defense system, through the administration of antioxidant agents and micronutrients like vitamin D as an adjunct therapy along with antidiabetics drugs insulin/oral antidiabetics. Moreover, increased levels of inflammatory mediators are responsible for enhancing inflammation in patients with diabetic neuropathy. Vitamin D helps to decrease the levels of inflammatory biomarkers in subjects with diabetic neuropathy.

Keywords: Type 2 diabetes; diabetic neuropathy; GSH; IL6;TNF-Alpha, AOPPS; MDA; MPO, AGEs

1.0. Introduction:

Diabetes mellitus is taken from the Greek word *diabetes*, meaning siphon - to pass through and the Latin word *mellitus* meaning sweet. A review of the history shows that the term "diabetes" was first used by Apollonius of Memphis around 250 to 300 BC. Diabetes mellitus is a chronic multifactorial metabolic disorder with complex pathology. It is distinguished by raised blood glucose levels or hyperglycemia, which results from abnormalities in either insulin production or insulin action or both at the same time. Hyperglycemia exhibits itself in different forms with a variable presentation and causes carbohydrate, fat, and protein metabolic abnormalities. Long-term hyperglycemia often causes different microvascular and macrovascular diabetic complications, which are mainly responsible for diabetes-associated morbidity and mortality. Hyperglycemia is the primary biomarker for establishing the diagnosis of diabetes as well (Banday, Sameer, & Nissar, 2020). Hyperglycemia and its associated carbohydrate, fat, and protein metabolic dysfunctions affect various organs of the body and disturb their normal functioning.

Diabetes is divided into two main types: type 1 Diabetes mellitus occurs due to the complete absence of insulin due to the destruction of pancreatic beta cells of Islets of Langerhans. The cause is autoimmunity while type 2 diabetes mellitus is characterized by defective function of beta cells due to defective receptors or the poor quality of insulin caused by toxic agents or viral disease (Sakran et al., 2022).

One of the most common and serious complications of DM is diabetic neuropathy (DPN) with an alarming increase in its prevalence particularly in developed countries. It is distinguished by morphological and functional changes in the body. Hyperglycemia, insulin resistance, and dyslipidemia are the main pathological reasons that are involved in causing nerve dysfunction and cellular death in diabetic neuropathy. These three factors activate different biochemical pathways such as the polyol pathway, hexosamine pathway, and loss of insulin signaling. This causes oxidative stress, inflammation, and dysfunction of mitochondria with altered gene expression. This altered metabolic environment not only affects nerves but also disturbs oxidant and antioxidant balance and there is increased production of mitochondrial reactive oxygen species (ROS). Hyperlipidemia causes the production of pro-inflammatory substances from adipocytes. These changes cause dyslipidemia leading to deranged levels of high-density lipoprotein (HDL), cholesterol, and lipoprotein levels of triglycerides. High levels of non-HDL cholesterol, as well as low levels of HDL cholesterol, have been significantly associated with an increased risk of neuropathy (Huma et al., 2021). Insulin deficiency influences the level of free fatty acid. There is a strong evidence that an ongoing cytokine-induced inflammatory response is closely associated with pathogenesis of diabetic neuropathy. One of the hypotheses related to the cause of diabetic complications is the role of oxidative stress and inflammation. Increased glucose level speeds up oxidative stress resulting in excessive changes of the structure and function of lipids and proteins by causing peroxidation and glycoxidation (Burgos-Morón et al., 2019). Therefore, increased glucose levels cause auto-oxidation and glycation of proteins and activates the polyol pathway (Khalid, Petroianu, & Adem, 2022). Furthermore, increased levels of glucose lead to the formation of free radicals which increase the reactive oxygen species

(ROS),finally leading to a fall in the activity of antioxidants—a primary factor in oxidative stress (Bhatti et al., 2022).
Activation of various mechanisms such as interleukins (IL-1β and 6), tumor necrosis factor (TNF-α), and pro-inflammatory chemokines are implicated in further progression of diabetic complications. Increase in glucose levels causes increase in levels of ROS and through the Bax–caspase pathway, activation of signaling pathways occurs. All these processes lead to decrease in electrochemical gradient by a leakage of mitochondrial cytochrome into the cytoplasm and this causes apoptosis. The role of inflammation and inflammatory cytokines in causing diabetic complications, especially DPN, has gained popularity in recent years. Markedly increased activity of T cells and an abnormal expression of T cell cytokines have been implicated in DPN (Feldman, Nave, Jensen, & Bennett, 2017).

Vitamin D deficiency has been reported to be linked with diabetic microvascular complications.The 25 (OH) D levels were found to be lower in patients with DPN, and multiple studies confirmed that vitamin D was an independent protective factor for DPN. Previous studies have noticed that vitamin D plays a vital role in the development of DPN, and vitamin D deficiency can be used as a predictor of DPN (Zhao, Xia, & Yin, 2021). Vitamin D has anti-inflammatory action. it inhibits cytokine production, which ultimately leads to suppression of chronic low-grade inflammation that is present in type2 DM (Zakhary et al., 2021). Recent evidence has suggested that Vitamin D can decrease oxidative stress. Meta-analysis performed by Sepidarkish et al. has reported that vitamin D supplementation significantly reduces levels of MDA, a primary biomarker of lipid peroxidation. In the same meta-analysis a positive effect on MDA was noticed only in subgroups with twice weekly doses of vitamin D that are between 100 000 and 200 000 IU per month. Vitamin D supplementation in diabetic patients leads to a significant fall in the level of oxidative stress markers and increase in total antioxidant status (Anandabaskar et al., 2017).

Vitamin D decreases BMI and Blood pressure in diabetic subjects. In addition it also reduces the levels of LDL,BSF, HbA1C,Triglycerides,LFTs,Creatinine, platelets and WBC in these patients (Saif-Elnasr, Ibrahim, & Alkady, 2017). In short we can say that vitamin D deficiency plays an important role in the development of DPN (Assy, Draz, Fathy, Hamed, & Neurosurgery, 2021) and its supplementation can delay or prevent DPN (Wei et al., 2020).

The purpose of the current study was to evaluate the role of oxidative markers like AOPP, AGEs, MDA, antioxidant like GSH and inflammatory biomarkers like IL-6, TNF-α, and MPO in diabetics with neuropathy taking oral antidiabetics compared to controls and type 2 diabetics with neuropathy taking oral adjunct vitamin D therapy. According to the findings all the stress and inflammatory markers were elevated and antioxidant levels were decreased in diabetic neuropathy group compared to control, which indicated the risk factor for progression of the disease. Among the microvascular complications, polyneuropathy affects up to 50% of adults with long-standing type 1 and type 2 diabetes.

Material and Methods

2.1. Study Participants

All the patients (150) were screened at the Social Security hospital in Lahore. Informed consent was obtained before being included in this study. A total of 150 patients were enrolled in the study divided into a diagnosed type 2 diabetic neuropathy group of 50 patients (D), a diagnosed diabetic neuropathy group (DN) Taking Vitamin D orally and 50 healthy individuals as a control group.

2.2. Sampling Technique

Convenient non-probability sampling technique was employed to select the study participants.

2.3. Inclusion and Exclusion Criteria

Participants who accepted to take part in the study were recruited. All diabetic neuropathy and healthy people affected with other diseases or under medication that can affect oxidative stress markers were excluded from the study. Diabetic patients were on a low-carbohydrate diet and treated with insulin. The control group was made up of healthy volunteers present either in the institute or in family. Moreover, none of the control individuals had a history of chronic infections or metabolic dysfunction such as hypertension, diabetes, and cancer.
None of the control subjects were taking any medication.

2.4. Determination of Malondialdehyde (MDA)

The method of Mansoor et al., 2022 (Mansoor et al., 2022) was used to determine the levels of MDA spectrophotometrically. Sodium Dodecyl Sulfate (SDS) (8.1%), Thiobarbituric acid (TBA), acetic acid (20%) with pH 3.5, n-Butanol, TBA (80%), and distilled water were used as reagents. During this protocol, a 200 µL serum sample was taken into the test tube and added SDS with a concentration of 8.1%. Then, 1.5 mL of acetic acid and 1.5 mL of TBA solution were added into the mixture. After that, distilled water was added and a 4.0 mL mixture was prepared. Then, the prepared mixture was heated in a water bath for 60 min at 90 °C, then chilled with tap water and 5.0 mL of n-butanol and 1.0 mL of distilled water were added. The mixture was shaken vigorously and centrifuged for 10 min at 4000 rpm. The upper layer was collected and an absorbance was taken in the spectrophotometer at 532 nm.

2.5. Determination of Glutathione (GSH)

The levels of GSH were measured by the method of Maqbool t et al., 2019 (Maqbool et al., 2019) Commonly, GSH joins with nitrobenzoic acid and oxidized glutathione which consequently synthesizes chromophore TNB at the absorbance of 412 nm. This protocol required reagents including TCA (5%), DTNB (0.2 M), standard GSH (5%) and phosphate buffer (0.2 M). An amount of 0.1 mL of supernatant was prepared up to 0.2 M sodium phosphate buffer at 8.0 pH. Furthermore, the standard GSH was prepared for 2–10 moles. In addition, 2 mL of DTNB solution was added to the mixture and a yellow color then appeared. The absorbance was taken through a spectrophotometer at 412nm and GSH was expressed via nmol for every sample size.

2.6. Evaluation of Advanced Oxidative Protein Products (AOPPS)

The levels of AOPPs were determined by the protocol of Mansoor et al., 2022 (Mansoor et al., 2022). In this procedure, the serum sample was analyzed by semi-automated method. Moreover, the levels were measured on microplate reader through spectrophotometer. After this step, it was calibrated through chloramine T solution and then potassium iodide was added to take the absorbance to 340 nm. Plasma diluted PSB was added in the concentration of 200 mL in 96-well microtiter plates. The levels of AOPPs were expressed by micromoles per liter of chloramines.

2.7. Determination of Advanced Glycation Endproducts (AGES)

In vitro, AGE-HAS was performed according to (Mansoor et al., 2022), AGE-HSA was made by incubating HSA (type V; Sigma, St. Louis, MO, USA; 50 mg/mL) with 500 mM glucose in PBS for 65 days at 37 °C. TCA precipitated plasma proteins or AGE-HSA. It was then dissolved in 250 mL 0.01 M heptafluorobutyric acid (Sigma). Then, 4 mg plasma protein was injected into an HPLC apparatus (Waters Division of Millipore, Marlborough, MA, USA), 30.46 cm C18 Vydac type 218 TP (10 mm) (Separations Group, Hesperia, CA, USA). From 0 to 35 min, HPLC was designed with a 10% acetonitrile gradient. Pentosidine was eluted in approximately 30 min using 335 nm excitation and 385 nm emission fluorescence.

2.8. Determination of IL-6 and TNF-α by ELISA Kit Method

The levels of IL-6 and TNF-α were determined according to (maqbool et al., 2019) by the human available diagnostic ELISA kit method. The standard was prepared from 200 pg/mL and assessable

concentration of interleukins and TNF-α remained at 3 pg/mL. First of all, 100 µL of serum sample was added to the ELISA plate and incubated at room temperature for 120 min. After incubation, the plate was washed with washing buffer solution. After the removal of extra water from the ELISA plate, the plate was inverted on a paper towel. An amount of 100 µL of HRP conjugate solution was added into each well and incubated at room temperature for 1 h. The plate was washed again and dried on a paper towel for the removal of residual water. After that, the substrate was added into each well with a concentration of 100 µL and kept in dark room temperature for incubation for a period of 15 min. Later on, TMB was added with the amount of 100 µL into each well and placed for one hour. Finally, 50 µL of stop solution was added which provided the color perception during this reaction and showed the presence of TNF-α and interleukins in the serum sample of patientswith diabetic nephropathy. Finally, the absorbance was taken at the 460 nm wavelength by ELISA reader.

2.9. Determination of MPO by Using ELISA Kit Assay

Human Elisa Kit [ABCAM] was used to measure the levels of MPO. All the materials and reagents were prepared at room temperature. An amount of 50 µL of serum sample, standard and blank were put into their respective wells. After that, 50 µL of antibody mixture was added into each well and incubated at room temperature for 1 h. For the removal of the mixture, the microplate was washed with washing buffer and by tapping the plate on a paper towel for the removal of residual fluid from the plate. After washing, 100 µL of substrate was added into each well and the plate was placed for incubation at 37 °C. Again, the plate was washed with washing buffer and this process was repeated three times. After washing, 100 µL of stop solution was added into each well which generated a yellow shade color. Then absorbance was taken on ELISA plate reader at 450 nm.

2.10. Meaurement of Urinary Albumin and Urinary Albumin to Creatinine

The concentration of urinary albumin and urinary albumin to creatinine was determined using a Sequoia-Turner Digital Fluorometer, Model 450. For this analysis, the urine sample was collected from the subjects according to the study protocol. There are no particular instructions, such as special diet or fasting, that are required. The optimum specimen tube was selected (3–5 mL screw top cryogenic vial) for urine sample storage. All the materials and reagents were prepared at room temperature. Distilled water was sterilized, we then filtered the type 1 distilled water through sterile (115 mL), HCL (1 mol/L0), KOH (5 mol/L), KH2PO4 (0.003 mol/L), phosphate buffer saline stock solution (20X PBS), and buffer saline (IX PBS). Four vials were reconstituted with 5 mL of type 2 water and incubated for 1 h. After incubation, we added 2 mL of type 2 water and dialyzed against 0.003 mol/L KH2PO4 for four hours. After that, we divided the immunobeads into 6 bottles with a concentration of 250 mL and then incubated overnight. At the final stage, the absorbance was taken at 450 nm.

2.11. Measurement of Serum Creatinine

The level of serum creatinine is used to determine the performance of the kidneys and check the blood-filtrating capacity of the kidneys. Creatinine is a chemical compound that exists in the body as a waste product in urine. During analysis, serum, lithium heparin plasma, K2-EDTA TAPS buffer (30 mmol/L), creatinase (332 µkat/L), ascorbate oxidase (33 µkat/L), catalase (1.67 µkat/L) and HTIB (1.2 g/L) were used as reagents. We mixed all of the specimens and allowed them to make a clot after the addition of the serum sample. After that, we centrifuged the combine mixture for 10 min at 2000×. We preincubated the working reagent, standard and sample at room temperature. We adjusted the photometer with distilled water at zero absorbance. We prepared the working reagent with a concentration of 1.0 mL and used the sample/standard with a concentration of 100 µL. At the final stage, we recorded the absorbance at 510 nm with the spectrophotometer.

2.12. Measurement of Low Density Lipoprotein (LDL)

LDL cholesterol is catalyzed into fatty acid and free cholesterol through cholesterol esterase. Cholesterol oxidase oxidized the cholesterol into cholestone and hydrogen peroxide. This hydrogen

peroxide merges with 4-aminophenazone and phenol in the presence of peroxidase. As a result, a purple colored is generated and the color intensity expresses the concentration of cholesterol.

2.13. Determination of High Density Lipoprotein (HDL)

The concentration of HDL can be measured with the help of HDL cholesterol assay. Magnesium chloride (25 mM/L) and phosphotungstic acid (0.55 mM/L) were used as reagents. All of these reagents were added into a test tube with their respective concentrations and shaken vigorously. The mixture was then incubated at room temperature for 10 min. The supernatant was removed after centrifugation. The color intensity expressed the concentration of cholesterol liquid reagent.

2.14. Estimation of Triglycerides (TG)

Three test tubes were taken and named as blank, calibrator and assay tube. The buffer solution with concentration of 300 µL was added into watch test tube. Enzymes (lipase (≥1000 IU/L), glycerol 3 phosphate oxidase (≥3000 IU/L), POD (≥1700 IU/L) and glycerol kinase (≥600 IU/L)), buffer (magnesium chloride (9.8 mmol/L), chloro-4-phenol (3.5 mmol/L) and PIPES (100 mmol/L), 4-amino-antipyrine (PAP) 0.5 mmol/L, standard (triglycerides (200 mg/dL) and glycerol (2.8 mmol/L)), and adenosine triphosphate Na (1.3 mmol/L) were used as reagents during this assay. These contents were shaken and incubated at room temperature (10 min). The absorbance was taken with a spectrophotometer (546 nm). Triglycerides levels were expressed by unit mg/dl.

2.15. Evaluation of CBC (Complete Blood Count)

Complete blood count of the selected subjects was performed using an automated hematology blood analyzer by Sysmex (version. XP-2100).

2.16. Statistical Analysis

All data of experimental groups were expressed as mean ± SEM. For statistical analysis, group means were compared by one-way ANOVA and Bonferroni's test was used to identify differences between groups by using graph pad prism. A p-value less than 0.05 was considered significant from statistical analysis. Endnote was used to insert references.

3.0 Results

The current study was designed to investigate the effect of vitamin adjunct therapy on inflammatory cytokines, oxidative stress and biochemical parameters in patients suffering from diabetic neuropathy in age matched healthy subjects. The total number of individuals recruited into the study were 150, including 50 healthy control 50 diabetic neuropathy and 50 patients with type 2 diabetes and neuropathy taking adjunct vitamin D therapy. All the participants in the current study were matched for age, sex and body mass Index (BMI). The demographic profile of the patients and controls is summarized in Table 1 and Figure 1.

Table 1. Demographic profile of diabetics, diabetic nephropathy. Subject vs. healthy age matched control.

Variables	Controls	Diabetic neuropathy	Diabetic neuropathy	P-value
Age(Yrs.)	50.00±1.528	50.00±1.528	51;3±0.8819	>0.05
Weight(Kg)	58.33±0.8819	77.33±1.202	63.67±1.856	<0.05
BMI(Kg/m2)	21.67±0.8819	31.67±1.453	22.67±0.8819	<0.05
Systolic BP(mm/hg	107.7±1.453	148.3±4.410	127.3±1.453	<0.05
Diastolic BP(mm/hg)	74.00±0.5774	101.30±1.856	88.33±1.667	<0.05

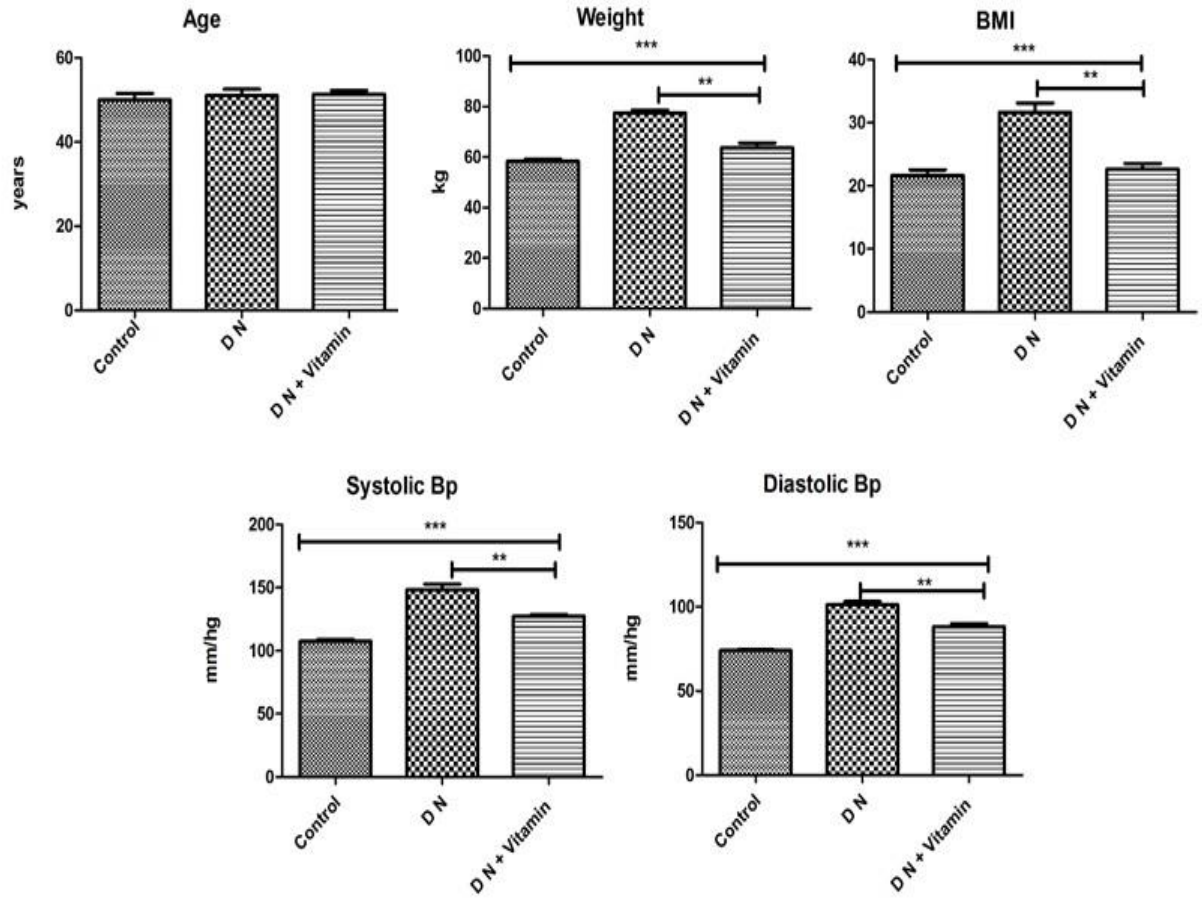

Figure 1. Age, weight, BMI, systolic and diastolic blood pressure of all the individuals. There is a significant difference between control and diseased group, an elevated level of BMI and weight can be observed between control vs. diseased group. A significant difference was also observed between patients on antidiabetics only and patients on adjunct vitamin D therapy while there is very less difference between control and treatment group. Where $p \leq 0.05$. **, *** (moderate, highly significant) represents the significant difference between groups.

Further biochemical parameters were performed, where levels of WBCs, platelets, Hb and HbA1c, BSF, triglycerides, LDL, HDL, serum creatinine, serum bilirubin, ALT and AST were observed. Biochemical parameters are shown in Table 2 and Figure 2.

Table 2. Biochemical profile of diabetics. Diabetic neuropathy subjects on antidiabetics only vs Diabetic neuropathy + on vitamin D

Variable	Control	Diabetic neuropathy	Diabetic neuropathy +vitamin D	P-value
ESR	5.333±0.333	12.00±1.155	6.333±0.333	<0.05
WBC	6.700± 0.4359	9.533±0.3180	7.333±0.3844	<0.05
Hb	12.50±0.2887	9.600±0.2082	12.07±0.1764	<0.05
BSF	91.33±1.856	173.3±9.563	131.3±1.856	<0.05
HbA1C	4.857±0.08686	7.533±0.4096	5.300±0.05774	<0.05
Serum Creatinine	0.5000±0.05774	1.467±0.1453	0.6667±0.08819	<0.05
HDL	75.67±2.848	43.33±4.055	66.33±1.202	<0.05
LDL	81.67±4.410	226.00±17.40	155±2.887	<0.05
Serum bilirubin	0.4667±0.08819	1.100±0.05774	0.7000±0.05774	<0.05
ALT	17.67±1.453	37.33±1.202	30.00±0.5774	<0.05
AST	18.33±1.202	37.33±1.764	27.33±1.453	<0.05

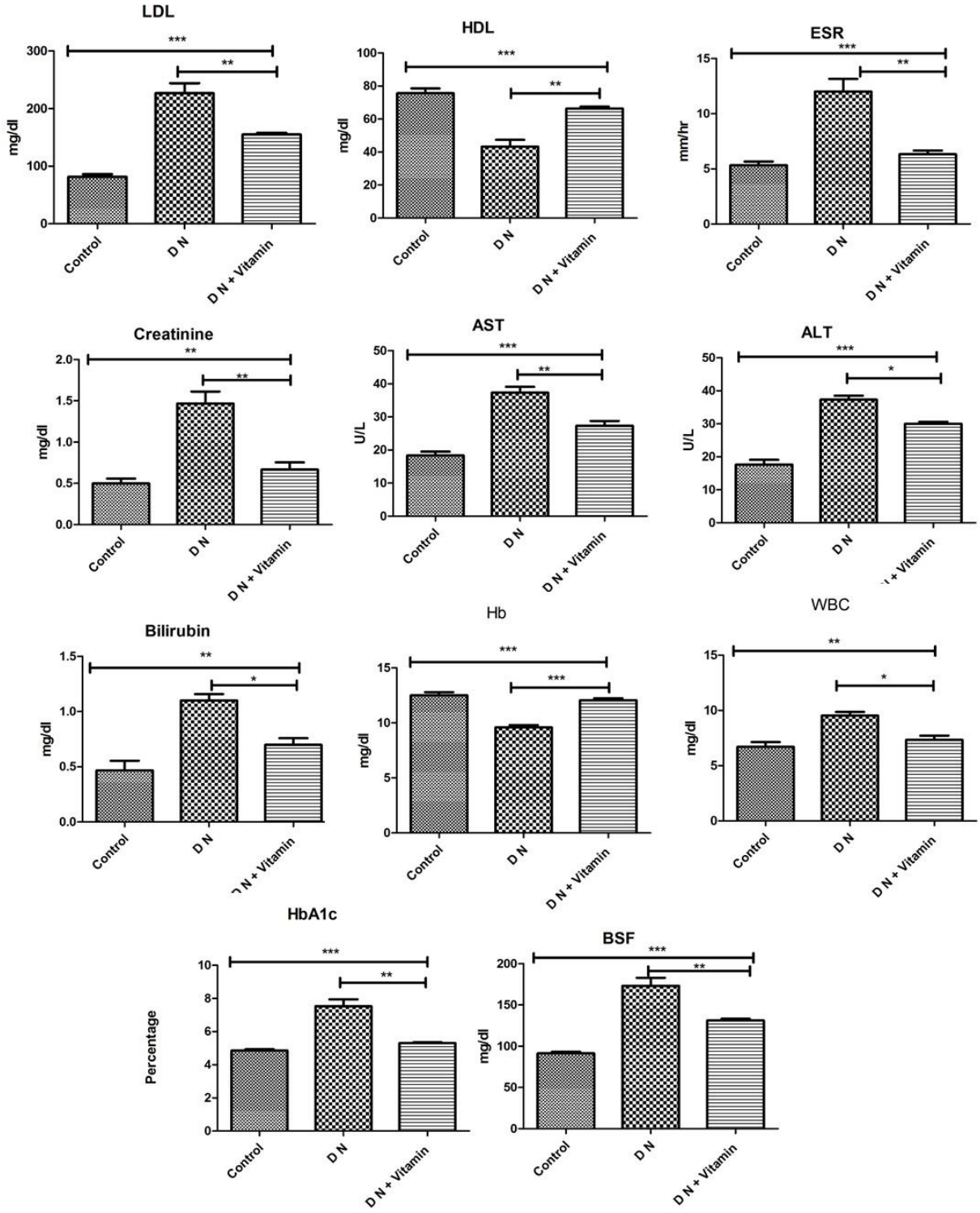

Figure 3. Hb, WBCs, Hb1Ac, HDL, LDL, triglyceride, serum creatinine, serum bilirubin,ALT and AST of all the individuals. There is a significant difference between control and diseased group between all the groups while vitamin D treated group showing reduced level of all the parameters and there is a minor difference compared to control. Where $p \leq 0.05$. *, **, *** (less, moderate, highly significant) represents the significant difference between groups.

An increased level of all the stress markers and inflammatory markers while the decreased level of antioxidant GSH was observed in diabetic neuropathy compared to control while very little difference in between control and diabetic neuropathy was observed. The levels of IL-6, MDA, AGEs, AOPPs, MPO were significantly increased in the patients with type 2 diabetes, as compared to control individuals while very slite difference between control and treatment group was observed. The data

analysis of TNF-α and MPO showed statistically significant enhanced levels in the diabetic group comparison with normal and treatment group as shown in the Table 3 and Figure 3.

Table 3. Levels of circulating stress markers, inflammatory cytokines in controls,diabetic neuropathy group on antidiabetics only and the group on adjunct oral vitamin D therapy.

Variable	Control	Diabetic neuropathy	Diabetic neuropathy +vitamin D	P-value
MDA	3.367±0.2028	6.167±0.1764	4.800±0.1528	<0.05
GSH	156.1±1.784	125.3±1.784	148.4±1.438	<0.05
AGEs	1.377±0.3712	13.57±0.779	3.800±0.4366	<0.05
AOPPs	17.76±1.399	28.20±1.155	20.33±0.7513	<0.05
MPO	4.480±0.5516	27.32±0.6116	7.663±0.4449	<0.05
IL-6	14.35±0.4856	115.6±0.4410	21.04±1.111	<0.05
TNF-α	0.1533±0.0088	28.95±0.7497	1.470±0.04359	<0.05

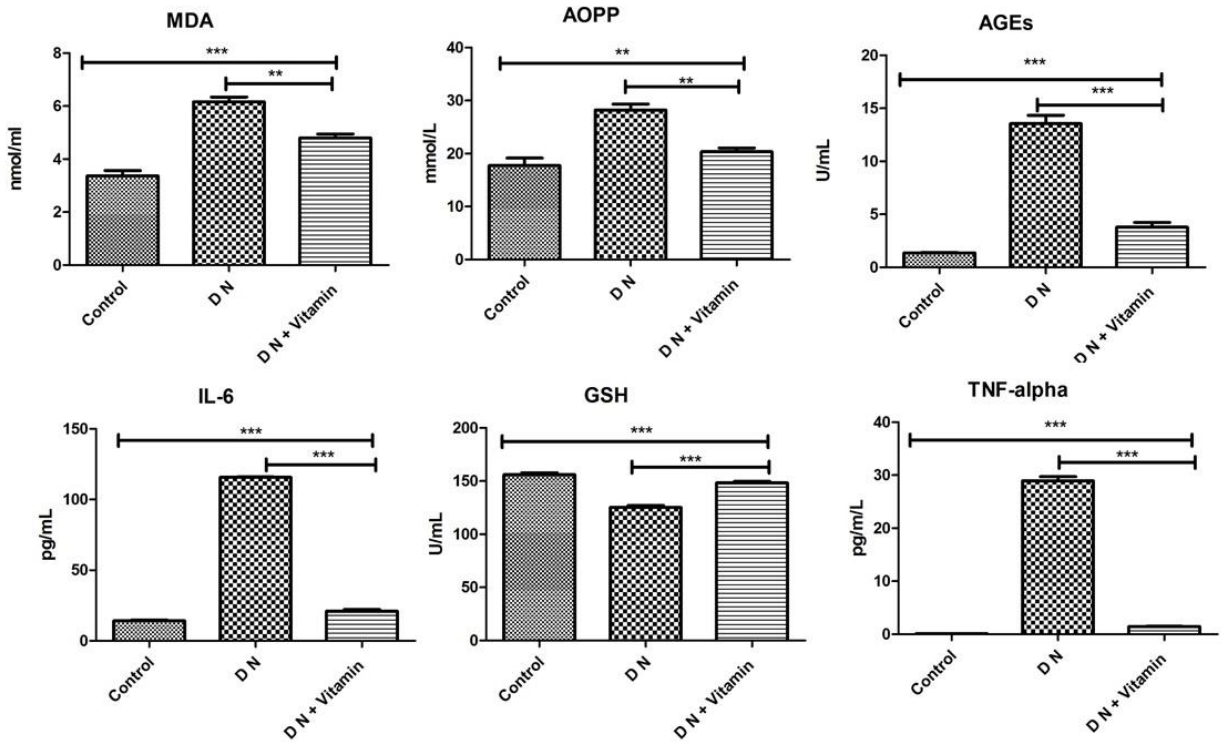

Figure 3. Levels of AOPPS, AGEs, GSH, MPO, MDA, TNF-alpha and IL6 of all the individuals.

There is a significant difference between control and diseased groups. Elevated levels of all the stress markers and decreased levels of GSH can be observed in the diseased group compared to control while the stress markers are highly increased in the diabetic neuropathy group. In vitamin D group the levels of GSH is increased significantly. Where $p \leq 0.05$. **, *** (less, moderate, highly significant) represents the significant difference between groups.

4.0. Discussion:

Diabetic peripheral neuropathy (DPN) is a disabling, extremely frequent complication of type 2 diabetes mellitus (T2DM). Hyperglycemia is an important cause of DPN. Different epidemiological studies have demonstrated that family history, raised blood pressure, dyslipidemia, and obesity are the major risk factors for diabetic neuropathy. Other risk factors include smoking and raised levels of glycosylated hemoglobin. Hyperglycemia causes imbalance of the local metabolic environment triggered by oxidative stress and inflammation and followed by remodeling of tissues. This is the main cause of neuropathy (Iacobini, Vitale, Pesce, Pugliese, & Menini, 2021).

The prevalence of type 2 diabetes is increasing at a very fast pace. Clinical studies have suggested that there is an association between serum levels of vitamin D and glycemic control and improvement

in diabetic neuropathy have been noticed with vitamin D supplementation even when therapy is given for a short time (Al Ali, Ashfaq, Sharif-Askari, Abusnana, & Mussa, 2023). As the previous studies have reported that Oxidative stress triggered by hyperglycemia plays an important role in the pathogenesis of DPN, we evaluated oxidative stress biomarkers and inflammatory biomarkers in addition to routine biochemical parameters which are routinely performed in labs to check diabetes and diabetic neuropathy and observed improved levels of Hb,WBCs, HbA1c, fasting glucose, triglycerides, LDL, HDL, and serum creatinine after adjunct oral vitamin D therapy. We found elevated MDA levels in the diabetic neuropathy group as compared to controls and type 2 diabetes taking adjunct oral vitamin D. As mentioned in a previous study, MDA is formed by lipid peroxidation and there is up regulation of MDA in diabetes. A study by (Lee et al., 2018) found increased production of MDA in Diabetics and reported that In subjects with T2DM, treatment with vitamin D3 (500 IU twice a week for 12 weeks) showed reduction in MDA and increased GSH levels as shown in previous study (Decroli, Manaf, Syahbuddin, Syafrita, & Dillasamola, 2019; Khalili et al., 2022; Román-Pintos, Villegas-Rivera, Rodríguez-Carrizalez, Miranda-Díaz, & Cardona-Muñoz, 2016) . D Macakova et al 2021 enrolled 49 type 2 diabetic patients, with moderate to severe polyneuropathy of lower limbs, and a control group ,29 patients without microvascular complications.The level of oxidative markers (advanced glycation end-products—AGEs, glycation products of AOPP proteins, MDA malondialdehyde and oxidized LDL), parameters of metabolic control and parameters of vascular wall stiffness were measured by sphygmomanometry. And found higher vascular stiffness in type 2 DM as compared to healthy individuals and that this correlates with the incidence of microvascular and macrovascular complications of diabetes. a correlation of oxidative markers like AOPP with vascular stiffness was noticed in patients with diabetic neuropathy. In the current study a significantly higher level of AOPPs in DN group was found compared to controls and vitamin D supplementation significantly reduced the levels of AOPP.

In the present study, we performed analysis of different stress markers, inflammatory cytokines, and antioxidants along with biochemical parameters and demographic data in type 2 diabetic neuropathy compared to control and diabetic neuropathy group treated with Vitamin D. In the current study, GSH levels were found to be highest in the control group and treated group, as compared to the DPN patients. (Lutchmansingh et al., 2018) reported that in DPN patients there is an imbalance in prooxidant/antioxidant levels. The ROS decreases the enzymatic activity of glutathione peroxidase which decreases GSH levels in DPN markedly. As compared to non-diabetic healthy controls, patients with T2DM have glutathione deficiency, especially if they have microvascular complications as well. This could be due to decreased synthesis and increased utilization by non-glycemic mechanisms. The results of the above mentioned studies are comparable with our study results where a decreased GSH level was observed in the DPN group. Several clinical studies have shown the same results as our study and estimated that the levels of advanced glycation end products (AGEs) were significantly highest in DPN patients compared to the control group. (Khalid et al., 2022) reported that persistent hyperglycemia in type 2 diabetics causes initiation and progression of glycation reaction which leads to formation of advanced glycated end products.(AGEs). This causes oxidative stress and diabetic complications micro and macrovascular.A study by (Omidian et al., 2019) analyzed the association of serum AGEs with diabetic complications. Same was observed in our case where and increased level was observed in Diabetic neuropathy compared to control and Vitamin D group.

The present study estimated the levels of Myeloperoxidase in the study groups. It was observed that levels were raised in the DN group as compared to the controls and vitamin d induced subjects showed a decrease in its levels. A study by (Xu et al., 2017) reported that Myeloperoxidase and reactive oxygen species are released during the activation of increased neutrophils, which may lead to excessive oxidative stress and persistent inflammation. This cascade in inflammatory response will eventually lead to an increase in neutrophils.

We found that levels of IL-6 were significantly higher in the DPN group on antidiabetics only as compared to the controls and with type 2 diabetes with DPN taking adjunct oral vitamin D, also we observed that the levels of IL-6 in DPN group were double the levels found in the controls, (Xiaohua

et al., 2021) investigated the role of IL-6 in diabetic neuropathy and found out that IL-6 has a proinflammatory effect in its pathophysiology.

In the present study, TNF-α (pg/mL) was also estimated in three groups. The levels were highest in the DPN group as compared to the healthy controls. According to previous studies, the serum concentrations of TNF-α levels were increased in the type 2 diabetes group but were highest in the type 2 DPN group. A study carried out by (Mu et al., 2017) suggested that TNF-α may serve as an independent risk factor for neuropathy in patients with type 2 diabetes. All these studies correlate with the results of our study. (El Hajj, Walrand, Helou, & Yammine, 2020) carried out a study on 88 Labanese non-Obese patients with T2DM .Subjects who had deficiency and insufficiency of vitamin D, were divided into one of two groups randomly, a treatment group taking 30,000 IU cholecalciferol weekly for a period of six months, and a placebo group. Serum levels of TNF-α and Interleukin-6 (IL-6) were the primary outcomes. In addition serum concentrations of fasting blood glucose (FBG), HbA1C, (25(OH) D) were measured. The vitamin D group showed higher serum levels of (25(OH) D) ($p < 0.0001$), and a significant decrease in TNF-α concentrations ($p < 0.0001$) as compared to placebo group. (Pinzon, Wijaya, & Veronica, 2021) reported that treatment of diabetic polyneuropathy will definitely have a better outcome if the modalities used for treatment are well targeted. Although good glycemic control may be the best prevention of DPN, it can develop in spite of the treatment of diabetes. Such targeted modalities can be achieved by the knowledge of powerful antioxidants. Inhibitors of oxidative stress and inflammation can be a useful targets for therapy. Therefore, in this article we focused on the basic mechanisms of oxidative stress caused in diabetes mellitus by ROS formation and different signaling pathways that are responsible for the initiation of different downstream signaling cascades that finally lead to changes in the function and structures of nerves. ROS-mediated injuries that lead to diabetic neuropathy can be avoided by restoring the antioxidant defense system. This can be achieved by administring antioxidant and anti-inflammatory agents like vitamin D.

5.0. Study Limitations

A limitation of this study is the cross-sectional design, which only provides the basis for associations and does not evaluate the 'cause and effect' relationship between elevated circulatory stress markers and inflammatory markers.

6.0. Strength of the Study

Diabetic neuropathy is a major challenge in the field of medicine. In this study we focused on the critical signaling pathways and pathophysiology involved in DPN. A large amount of evidence now exists to prove that several proinflammatory cytokines and oxidative stress markers are known to be involved in its mechanism. Furthermore, vitamin D can play a vital role in reducing inflammation and oxidative stress in addition to improving biochemical parameters.

7.0. References:

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