A Study of Pain Threshold, Interleukins and NLR in Diabetic Polyneuropathy in a Selected Nigerian Population

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Summary: Pain serves a protective function and is often lost in chronic conditions such as painful diabetic neuropathy (PDN). This has been reported to be associated with ongoing inflammation. This study aims to investigate an association between body immune responses, neutrophil-lymphocyte ratio (NLR) and pain perception in DPN patients. Sixty volunteers were recruited for the study. 30 control and 30 diagnosed DPN patients were investigated. All subjects were trained and informed consents were obtained. The pain threshold was significantly (p<0.05) lower in DPN (23.48±1.19 sec) compared to control group (30.38±1.9 sec), there was significant lower NLR in DPN (1.27±0.09) compared to control group (1.93±0.1) and the serum level of IL6 (15.31±0.85 pg/ml) in DPN was significantly higher compared to control group (11.9±0.15 pg/ml), likewise the serum level of IL10 (13.26±2.78 pg/ml) in DPN is significantly higher compared to control group (6.59±1.07 pg/ml). This study showed that hyperalgesia seen in patients with DPN was independent of increased NLR, and increased IL6 & IL10 seen in this group of patients indicates need to further explore the role of immunological response in the pathogenesis and progression of DPN.

Keywords: Diabetes, inflammation, interleukins, pain threshold, polyneuropathy

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INTRODUCTION

Diabetes Mellitus (DM) is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. Fasting plasma glucose ≥ 7.0mmol/l (126mg/dl), 2hour plasma glucose≥ 11.1mmol/l (200mg/dl) after a 75g oral glucose load in a glucose tolerance test, or glycated haemoglobin (HbA1C) ≥ 4.8% (WHO, 2014). The World Health Organization estimates that 422 million (8.5 %) adults globally were living with diabetes in 2013 and around 80% of these people live in developing countries. Shaw et al. in 2010 reported that the prevalence of people living with Diabetes in Nigeria is 2,819,000 (3.9 %) of the over 174 million which is expected to hit 4.3% by 2030. Diabetic polyneuropathy (DPN) is the most common complication of diabetes mellitus, affecting up to 50% of patients “20% of them are presenting with pain” which usually present with one or more of these symptoms “tingling, shooting, numbness or burning pain sensation especially in the limbs” more frequently in the lower limbs (Dyck et al., 2011). Even though, DPN affect all peripheral nerves including pain fibres, motor neurons and the autonomic nervous system (Vinik and Mehrabian, 2004), typically sensory neuropathy is the first stage of DPN, and if not treated appropriately and strict blood glucose control is not implemented, it will precipitate to motor and autonomic disorders. Motor neuropathy will lead to loss of intrinsic muscle innervation causing different foot deformities (Cancelliere, 2016) like foot ulceration, Charcot neuroarthropathy, and lower-extremity amputation (Boulton et al., 2005). This is responsible for over 60% of non-traumatic amputations annually (Holzer et al., 1998). The relationship between diabetes mellitus and the common micro-vascular complications “Diabetic Retinopathy (DR), Diabetic polyneuropathy (DPN) and Diabetic nephropathy (DN)” has been studied to have more knowledge about the likely pathogenesis of these micro-vascular complications (Hewapathirana and Page, 2012). However, there are two main suppositions of the proposed mechanism of painful diabetic neuropathy: vascular and metabolic (Czyzyk, 1987), but the current hypothesis suggests that neuroimmune interactions actively contribute to the onset and persistence of pain in diabetes (Vinik et al., 2006; Bishnoi et al., 2011).
Total white blood cell count (TWC) is a basic, simple, cheap and readily available procedure. It is a good indicator of on-going inflammation in the body (Zahorec, 2001). On the other hand, the use of pro-inflammatory markers like c-reactive protein, IL 2, IL 6, TNF are expensive, time consuming and requires a lot of man power and expert techniques. Studies have shown a link between absolute neutrophil count (ANC) and the development and progression of DN (Virginia et al., 2012). Recently, the neutrophil-lymphocyte ratio (NLR), a novel potential marker to determine inflammation, has been demonstrated to be a higher sensitive factor than TWC, ANC or absolute lymphocyte count (ALC) in the prognostic outcomes in various medical conditions like cancer and cardiovascular diseases (Lee et al., 2012; Uthamalingam et al., 2011; Jie et al., 2015).

However little is known about the relationship between ANC, ALC and NLR, and DPN especially in this part of the world (Nigeria) and most importantly the role of immunological response if any in the pathogenesis of this disease.

In this study, the objectives were to investigate an association between systemic ANC, ALC, NLR, IL6, IL10 and pain perception of DPN patients in diabetic clinic of Ekiti-State University Teaching Hospital, Ado-Ekiti, Ekiti-State, South-West, Nigeria.

MATERIALS AND METHODS

Human Subjects

Sixty (60) volunteers were recruited for the study based on the recommendation of Voorhis and Morgan (2001). Thirty (30) healthy volunteers were randomly selected in the community and 30 volunteer patients with presence of symptoms or signs of peripheral nerve dysfunction in people with diabetes type 2 after other possible causes have been excluded (Zeng et al., 2017) were selected consequentially from diabetic clinic in Ekiti State University Teaching Hospital.

Protocol: These individuals were older than 20 years, known diabetic neuropathic patient presenting with minimum of two symptoms (pain plus any other) and Vibration Perception Threshold (VPT) was also measured, using a biothesiometer, (Model: Vibrometer-VPT; Serial No: V117093366; Made by Diabetic Foot Care India Pvt Limited, India) which was applied to six assigned locations at the dorsum of the foot and the average value of these measurements calculated to define the presence of diabetic neuropathy on each foot with a cut off VPT of more than 25 volts for the diagnosis of loss of protective sensation, this was carried out at diabetic clinic in Ekiti-State University Teaching Hospital, Ado-Ekiti. They were recruited, trained on what they should expect during the study and informed consent was obtained. Demographic features of the subjects are shown in Table 1

Subjects were excluded if they had other neurological disorders (such as shingles and fibromyalgia), infection, psychiatric illness, myocardial infarction, cancer, HIV, blood diseases that affect neutrophils and lymphocytes counts (e.g. leukaemia) or if they were unable to give written informed consent.

All subjects underwent the following procedures: history taking, physical examination, sub-maximal effort tourniquet test, biochemical analysis. All procedures were performed in the morning after an overnight fast. Approval (Protocol number: EKSUTH/A67/2016/12/005) was obtained from the Research and Ethical Review Committee of the Ekiti State University Teaching Hospital, Ado Ekiti, Ekiti State, Nigeria.

Sub-maximal effort tourniquet test: The ischemic pain testing (sub-maximal effort tourniquet test) was based on the method described by Plesan et al. (2000). A blood pressure cuff was placed around the non-dominant upper arm of the subject’s (on the brachia artery). The cuff pressure was increased to 20mmHg above the subject’s systolic blood pressure. With the pressure maintained, subject performed a hand grip exercise on an elastic ball. The subject closes his/her eyes for the entire procedure to minimize distraction and time cues. Subjects were then asked to indicate when they first detected (feel) the pain and when they could no longer tolerate the pain (to a maximum of 300 seconds). Once pain tolerance was reached, the pressure curve was immediately deflated and endpoints were measured in seconds with the process performed 3 times and average of the readings documented (Plesan et al., 2000).

Pain threshold assessment: The pain threshold is defined as the point between being “about to be painful” and “just became painful” and the time taken for this to occur is recorded in seconds. The process is performed 3 times and the average is documented

BIOCHEMICAL ANALYSIS

Determination of glycated haemoglobin level: This assay employs the chromatography technique. The non-glycasylated haemoglobin, which consists of the bulk of haemoglobin, has been designated HbAo. A haemolysed preparation of whole blood is mixed continuously for 5 minutes with a weakly binding cation-exchange resin. The labile fraction is eliminated during the haemolysate preparation and during the binding. During this mixing, HbAo binds to the ion exchange resin leaving GHb free in the supernatant. After the mixing period, a filter separator is used to remove the resin from the supernatant. The percentage glycasyalted haemoglobin is determined by measuring absorbances of the ratio of the absorbances of the glycosylated haemoglobin (Ghb) and the Total haemoglobin fraction (Thb). The ratio
of the absorbances of GHb and THb of the control and test is used to calculate the percentage GHb of the sample (Jeppsson et al., 1986).

**Determination of full blood count (FBC) and differential white blood cell count:** Blood samples were collected at the cubital vein, separated in two different sample bottles. EDTA bottle was used for the full blood count analysis and the remaining blood sample collected in a plane bottle was centrifuged at 1000 rev/min for 5 minutes, serum separated into another plane bottle then store at -70°C in the refrigerator for the analysis of IL10, IL6 and CRP. All values of full blood count (FBC) and its differential white blood cell counts (ANC, ALC) were measured by an automated haematology analyzer (Sysmex kx-21n).

**Determination of neutrophil lymphocyte ratio (NLR):** NLR was calculated as the ratio between the ANC and the ALC both obtained from the same blood sample of each subject.

**Determination of serum interleukin 6 (IL6):** This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to Interleukin 6 (IL6) has been pre-coated onto a microplate. A competitive inhibition reaction was launched between biotin labelled IL6 and unlabelled IL6 (Standards or samples) with the pre-coated antibody specific to IL6. After incubation the unbound conjugate was washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. The amount of bound HRP conjugate was reverse proportional to the concentration of IL6 in the sample. After addition of the substrate solution, the intensity of colour developed was reversed proportional to the concentration of IL6 in the sample.

**Determination of serum interleukin 10 (IL10):** This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to Interleukin 10 (IL10) has been pre-coated onto a microplate. A competitive inhibition reaction was launched between biotin labelled IL10 and unlabelled IL10 (Standards or samples) with the pre-coated antibody specific to IL10. After incubation the unbound conjugate was washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. The amount of bound HRP conjugate was reverse proportional to the concentration of IL10 in the sample. After addition of the substrate solution, the intensity of colour developed was reversed proportional to the concentration of IL10 in the sample.

**STATISTICAL ANALYSIS**
All data were expressed as the Mean ± SEM; the effects of the varied intervention of each of the groups were tested for homogeneity using Independent-Samples T test using SPSS version 20 software with the level of significance set at p ≤ 0.05. A difference between two means was considered to be statistically significant when p < 0.05.

**RESULTS**

**Effect of diabetic polyneuropathy on glycated haemoglobin**
The results showed that the diabetes neuropathic group (6.60±0.26 %) has significantly higher HbA1C level when compared to the control (4.32±0.68 %) with the p <0.01 (figure 1).

**Effect of diabetic polyneuropathy on pain threshold**
The pain threshold was significantly (p<0.05) lower in DPN (23.48±1.19 seconds) compared to control group (30.38±1.9 seconds) (figure 2).

**Effect of diabetic polyneuropathy on serum interleukin 6 (IL6)**
The IL6 level (15.31±0.85 pg/ml) in DPN is significantly (p<0.01) higher compared to normal group (11.94±0.15 pg/ml) (figure 3).

<table>
<thead>
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<th>Demographic features of subjects</th>
<th>CONTROL</th>
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<tr>
<td></td>
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<td>Male = 10</td>
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<tr>
<td>Mean age (years)</td>
<td>51.7±1.72</td>
<td>58.17±1.6</td>
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**Figure 1:** HbA1C level among the Control group and Diabetic neuropathy (DPN) patients. Values are expressed in Mean± SEM, *p < 0.01

**Figure 2:** Pain Threshold among the Control group and Diabetic neuropathy (DPN) patients using ischaemic-induced pain test. Values are expressed in Mean± SEM, *p < 0.01
Diabetic polyneuropathy and pain perception

Effect of diabetic neuropathy on serum interleukin 10 (IL10)
The level of IL10 (13.26±2.78 pg/ml) in DPN is significantly higher compared to control group (6.59±1.07 pg/ml) with the p<0.03 (figure 4).

Effect of diabetic polyneuropathy on total white blood count (TWBC)
There was no significant difference in the TWBC in control group (6.71±0.41 10^9/l) and DPN group (6.19±0.33 10^9/l) with the p>0.05, (figure 5).

Effect of diabetic polyneuropathy on absolute neutrophil count
There was significant reduction in the neutrophil count in DPN (3.1±0.2 10^9/l) when compared to normal group (4.02±0.3 10^9/l) with the p<0.01 (figure 6).

Effect of diabetic polyneuropathy on absolute lymphocyte count
There was significantly higher lymphocyte count in DPN (2.7±0.19 10^9/l) when compared to normal group (2.12±0.12 10^9/l) with the p<0.01 (figure 7).

Effect of diabetic polyneuropathy on neutrophil lymphocyte ratio (NLR)
NLR was significant (p<0.01) lower in DPN (1.27±0.09) when compared to normal group (1.93±0.1) (figure 8).

DISCUSSION

The present study investigated the association between systemic ANC, ALC, NLR, IL-6, IL-10 and pain perception of DPN patients, with a view to understanding the likely role of adaptive immune system as well as inflammatory responses in the pathogenesis of diabetic neuropathy.

The result showed that, there is significantly lower pain threshold in DPN group compared to the control group indicating hyperalgesia which is consistent with previous study (Farmer et al., 2012). This finding is accompanied by significantly higher glycated haemoglobin (HbA1c) level in DPN group. HbA1c is a clinical parameter used to measure three months average plasma glucose concentration which makes it a better clinical tool for monitoring of the glucose control of these patients when compared to their fasting blood sugar or random blood sugar level. The
higher glycated haemoglobin seen in DPN group indicates prolong hyperglycemia is associated with increase in intracellular glucose in nerves cells and its supporting tissues in the central nervous system, it is reported that hyperglycemia leads to hyper-saturation of normal glycolytic pathway with extra glucose shunted to polyol pathway and then converted to sorbitol and fructose by enzymes aldose reductase and sorbitol dehydrogenase with resultant increase in nerve cell damage and gene dysregulation (Arikawa, 2007).

It is also suggested that nerve injury may be due to immune responses as shown by significantly higher serum level of IL-6 and IL-10 in DPN group when compared to the normal subjects. The relationship between the pain behaviour noticed in these patients and their serum level of IL-6 is best explained in the context that IL-6 influences transduction, conduction, and transmission of the nociceptive signal, resulting in prolonged or permanent signalling to the brain’s cognitive centres in the absence of a painful noxious or non-noxious stimulus. This cytokine is synthesized by the neurons, microglia and the astrocyte cells after nerve injury in the peripheral nerves, in dorsal root ganglia (DRG) and in the spinal cord (Olivera et al., 2011).

According to Hirota et al. (1996) spinal IL-6 mRNA, spinal IL-6, microglial and astrocyte activation, and pain behaviour did not differ in rats that sustained an injury at L5 either proximally or distally to the DRG. They also noted that a significant amount of IL-6 receptors (IL6R and glycoprotein 130 (gp130)) on cell membranes increases under this condition, suggesting a physiological role of IL-6 in pain behaviour in experimental rats. The findings in this study is also in agreement with that of Muller et al. (2002) which reported that with the development of diabetic complications, a substantial rise of systemic IL-6 was found.

IL-10, produced by helper T (Th2) cells, is a well-known immune regulatory cytokine, which regulates T cells and monocytes/macrophages (D’Andrea et al., 1993). The results from this study showed tremendous increase serum level of IL-10 in patients with DPN when compared to the control hereby suggesting the role of adaptive immune system. This supports the hypothesis that DPN is a chronic pathology that has a strong correlation between the body immune system and the pain noted in these patients (Vinik et al., 2006). IL-10 inhibits pro-inflammatory cytokines, especially TNF, IL-1, and IL-6, produced by activated macrophages and monocytes, stimulating endogenous production of anti-inflammatory cytokines. Besides, IL-10 suppresses the pro-inflammatory functions of antigen presenting cells (APCs) by antagonizing expression of co-stimulatory molecules, the release of pro-inflammatory cytokines and, in general, APC maturation (Langenkamp et al., 2000). This then suggest a positive relationship between the serum level of IL-10 and the pain behaviour seen in PDN. This relationship seen in the result (figure 4) suggests that the body attempt to contain the effects of the pro-inflammatory markers (e.g. IL-2, IL-6 and others) by the production and release of IL-10 which function is part of the general effort of the body to self-contain the extent of the inflammatory process in situations when the exposure to a given insult is in continuum as seen in DPN patients.

To further support the hypothesis that DPN may be due to an immunological and not just inflammatory response, our results showed that, there were significantly reduced Neutrophil count and higher Lymphocyte count in DPN group compared to the control group although both results are within the normal acceptable range according to Dacie and Lewis (2011). These range are 2.0–7.0 x 10⁹/l (40–80%) for neutrophil and lymphocytes 1.0–3.0 x 10⁹/l (20–40%) of Total White Blood Count (TWBC). Even though our result suggested that the neutrophil count is close to the lower margin of normal and lymphocytes count is also close to the upper limit of the normal control range. However, worthy of note is their relative high level of lymphocyte count supporting the role of the immune system in the later stage of this pathology.

Clinicians are looking for less expensive, less elaborate and accurate techniques that can be used in the assessment and as projective tool in clinical managements of various medical illnesses especially in the developing and under-developed world (Guo et al., 2015). NLR is one of these techniques that is relatively cheap, and readily assessable in most clinical institutions. NLR has been used to screen out many patients that have sensorineural hearing loss (Sukhija et al., 2007), diabetic retinopathy (Ulu et al., 2013), adverse cardiac events (Azab et al., 2013) and diabetic nephropathy (Azab et al., 2012) resulting from the vascular complications of diabetes via inflammation pathways. The fact that the present study demonstrate hyperalgesia with significantly lower NLR in DPN group compared to the control group suggests that DPN may occur with or without ongoing inflammatory process.

This study has shown that though patients with DPN are more susceptible to pain as shown by their pain threshold, NLR may not be elevated but reduced in them and focus should shift to the immunological roles and not just inflammation in the pathogenesis and progression of DPN, knowing that sensory neuropathy (e.g. with abnormal pain sensation) is the first stage of DPN which if well managed will halt the progression of DPN. Further studies will be required to fully understand and if possible isolate the likely antibody resulting in the pathogenesis and the progression of DPN and other micro-vascular complications of diabetes mellitus, as this will aid more efficient clinical management of DPN.
REFERENCES


Diabetic polyneuropathy and pain perception


