HIGH PLASMA TNF-α LEVELS AND MONONUCLEAR CELLS iNOS AND TNF-α EXPRESSION AS RISK FACTORS FOR PAINFUL DIABETIC NEUROPATHY

Thomas Eko Purwata[1)], Ketut Suastika[2)],
A. A. Raka Sudewi[2)], Djoenaidi Widjaja[3)]

[1)]Postgraduate Program, Udayana University
[2)]Faculty of Medicine, Udayana University
[3)]Faculty of Medicine, Airlangga University
Email: thomaseko2000@yahoo.com

ABSTRACT

Painful Diabetic Neuropathy (PDN) is one of the most common and annoying complications of diabetes mellitus. The pathogenesis of PDN is complex and still unclear. Recently it has become clear that nitric oxide (NO) and proinflammatory cytokines play an important role in the pathogenesis and maintenance of pain in PDN. Based on this phenomenon, this study was conducted to investigate whether the cytokine tumor necrosis factor-alpha (TNF-α) and NO, in this case inducible Nitric Oxide Synthase (iNOS), play a role in PDN pathogenesis.

The study was carried in two steps. The first step was a cross sectional and the second step was a case-control study. The study was performed in 110 type-2 diabetic patients. The plasma TNF-α levels were determined by ELISA while the expression of TNF-α and iNOS in mononuclear cells were analyzed immunohistochemically.

Of 110 subjects, 59 patients suffered from Painful DN (case) and the remaining 51 patients were Painless DN (control). Cross sectionally, plasma TNF-α levels and immunoreactivity for iNOS and TNF-α were higher in patients with more severe pain in the Visual Analog Scale (VAS). There were statistically significant differences ($p < 0.05$) between mild and severe pain in regard to TNF-α level (15.24 pg/ml ± 5.42 vs. 20.44 pg/ml ± 10.34); to iNOS immunoreactivity (9.72 % ± 8.61 vs. 15.6% ± 11.84); and to TNF-α immunoreactivity (13.0 % ± 9. 48 vs. 20.44% ± 11.75).

The case control study showed that TNF-α had an odd ratio of 5.053 [CI 95% (2.241-11.392); $p < 0.001$]. TNF-α immunoreactivity of 4.125 [CI 95% (1.805-9.425); $p < 0.001$]; and iNOS immunoreactivity of 3.546 [CI 95% (1.613-7.795); $p = 0.002$].

There were correlations between TNF-α level, TNF-α and iNOS immunoreactivity and VAS with coefficient correlation: 0.330; 0.285 and 0.275 ($p < 0.05$) respectively.

It is concluded that Diabetic Neuropathy patients with high TNF-α levels, iNOS and TNF-α immunoreactivity of mononuclear cells have higher risk for painful DN than painless DN. The higher TNF-α level, iNOS and TNF-α immunoreactivity the more severe was the pain. This supports the hypothesis that TNF-α and iNOS have role in PDN pathogenesis. The results of this research could be applied as a basic for further research in pursuit of better management of PDN.

Keywords: Painful Diabetic Neuropathy; TNF-α; iNOS, Visual Analog Scale

1. Introduction

The Indonesian Diabetes Federation (Perkeni) has published new data indicating the enormity of the diabetes epidemic. Urbanization and changing of life style may account for this problem. About 14.7% prevalence of diabetes is in urban area and 7% is in rural area, in 2003 an estimated 8.2 million in urban and 5.5 million persons in rural area had diabetes (Perkeni, 2006). Uncontrolled diabetes potentially suffered from acute and chronic complications, disability, and death. The most common chronic complications of diabetes are dyslipidemia (67.0%), symptomatic diabetic neuropathy (51.4%) and erectile dysfunction/impotence (50.9%) (Tjokroprawiro, 1993). Diabetic neuropathy is a common complication of diabetes that can cause significant morbidity and mortality. Some 30% of hospitalized and 20% of community-dwelling diabetic patients has peripheral neuropathy; the annual incidence rate is approximately 2% (Duby et al, 2004).

Diabetic neuropathy (DN) can be painful or painless. The reason for this difference in clinical presentation is as yet unknown. The prevalence of painful diabetic neuropathy (PDN) in a population-based sample is 26.4 % (Davies, et al. 2006). PDN is difficult to treat as it does not respond to conventional analgesics. PDN can have a serious impact on quality of life, leading to severe depression and even suicide. Clinical manifestation of PDN may include burning, shooting (like 'electric shock' down the legs), lancinating (stabbing or knife like) and deep aching quality, allodynia and hyperalgesia especially in lower extremities (Benbow et al., 1998; Backonya, 2001; Meliala, 2004a). The pathogenesis of PDN is complex and still poorly understood. In the past, researches of neuropathic pain were focused on neuron and neurotransmitters so the treatment of neuropathic pain often unsatisfactory. Recently it has become clear that nitric oxide (NO) and proinflammatory cytokines play an important role in the pathogenesis and maintenance of pain in PDN. Neuroinflammation and neuroimmune activation occur following injury to peripheral nerves and nerve roots as well as in peripheral inflammatory models of persistent pain (Moalem et al., 2005; Koch, et al., 2007). The role of cytokine in the induction and maintenance of pain has been established in animal model, clinical research also gives evidence for the involvement of cytokine in painful and painless neuropathies (Sommer & Schafers, 2004). Peripheral nerve injury induces activation of resident immune cells as well as recruitment of inflammatory cells to the

nerve. Injury of a peripheral nerve initiates an inflammatory cascade in which mast cells residing in the nerve are the first to be activated. A variety of immune mediators are released, which exert algesic actions by acting directly on nociceptors, or indirectly via the release of other mediators. There is increasing knowledge of the intracellular cascades that are activated in nociceptors by some mediators Tumor Necrosis Factor (TNF)-α, Interleukin-1β, Interleukin-6; NO, prostaglandins (PG), nerve growth factor (NGF), cyclooxygenase 2, which ultimately either activate or sensitize these neurons. Nerve injury also initiates Schwann cell de-differentiation and the release of several algesic mediators such as pro-inflammatory cytokines, NGF, PGE2 and ATP. This cocktail of mediators serves as a mechanism of enhanced inflammatory response in the injured nerve and contributes to neuropathic pain (Moalem & Tracey, 2005; Thacker et al., 2007). TNF- α has been shown to be directly involved in the production of pain in several models of nerve injury. Injury-induced increases in TNF-α mRNA and protein expression have been observed to correlate with the development of allodynia/hyperalgesia in several neuropathic pain models. The effect of TNF-α on neurons seems to be mediated, directly and/or indirectly, by the phosphorylation of extracellular regulated kinase and p38 mitogen activated protein kinase (Schafers et al.,2003), translocation of NFκB to the nucleus and activation of COX-2-dependent prostanoid release (Dinarello, 1999). The phosphorylation of p38 mitogen activated protein kinase may mediate mechanical allodynia via a modulation of tetrodotoxin-resistant Na channels (Jin & Gerean, 2006). TNF-α also activates NFkB (*Nuclear Factor kappa B*) for initiation of NOS (*Nitric Oxide Synthase*) and NO production whereas NO is a pain neurotransmitter (Lowenstein, 1994). Previous data suggest that the clinical application of specific agents that suppress the production and/or activity of TNF-α may inhibit the development and exacerbation of chronic diabetic complications. Studies have revealed that an increased concentration of TNF-α correlates well with increased concentrations of NF-κB. Inhibition of serum TNF-α and NO levels with insulin and its combinations with resveratrol and curcumin contribute in attenuating diabetic neuropathic pain, since TNF-α plays an important role in the development of diabetic neuropathy (Sharma, et al., 2007). The role of NO in pathogenesis of PDN seems to be via up-regulation of iNOS as a result of chronic inflammation. There was reciprocal interactions between NO and PG, high levels NO

increased PDN and iNOS inhibitor attenuated PDN. With this background, the present study was designed to explore the plasma level of TNF-α and mononuclear cells iNOS and TNF-α expression as risk factors for PDN.

2. Patients and Methods

2.1 Study subjects

A hundred and ten type-2 diabetic patients whom fulfilled inclusion criteria were enrolled in this study (61 women and 59 men, mean age 54.1, range 37-65 years). Patients were recruited consecutively at the outpatient Clinic of Neurology and Internal Medicine Sanglah Hospital Denpasar from 1 October 2008 until 31 July 2009. The patients had a means duration of diabetic 5.30 years (2-10 years). Of 110 subjects, 59 patients suffered from Painful DN (case) and the remain 51 patients were Painless DN (control). The clinical characteristic of subjects and comparison of case and control group are given in Table 1 and 2 .

2.2 Inclusion criteria

Cross sectional study : type-2 diabetic patients, age between 20-65 years, duration of diabetes 2-10 years since diagnosis established, patients live in Bali, signed written informed consent. Case control study: same with cross sectional study criteria plus Painless DN for control and Painful DN for case.

2.3 Exclusion criteria

Exclusion criteria included : ongoing local and systemic infection (documented with a blood cell count and erythrocyte sedimen rate), active ulcer in the leg, patients under treatment of glyceryl trinitrate or sildenafil , herbal or synthetic medication that contain anti oxidant or anti inflammation and patients suffering another neuropathic pain.

To minimize confounding factors for blood cytokine levels, the study conditions were standardized by applying the following exclusion criteria: heavy physical activity in the last 3 days, food intake within 60 minutes before blood sampling, alcohol consumption up to 24 hours before the examination, and any current infectious disease or fever.

2.4 Method

2.4.1 Study protocol

The study was carried in two step. The first step was a cross sectional and the second step was a case-control study. In cross sectional study, type-2 diabetic patients who visited outpatients Neurology and Internal Medicine Clinic Sanglah Hospital were performed anamnesis, neurological, laboratory and ENMG (Electromyoneurography). The diagnosis of a DN was based on a typical history and a thorough neurologic and ENMG examination . Laboratory studies included erythrocyte sedimentation rate, whole blood, blood sugar, HbA1C, ureum, creatinin and liver function test. A complete electrophysiologic assessment with standard nerve conduction studies in motor and sensory nerves at upper and lower extremities was performed in all diabetic patients. Nerve conduction studies revealing low compound muscle action potentials and sensory nerve action potentials or low conduction velocities.

Patients who diagnosed DN and fulfilled inclusion criteria were recruited in case-control study and were asked to assess their pain intensity on visual analog scale (VAS), with 0 representing no pain and 10 representing the worst pain imaginable. Patients were attributed to two groups according to their VAS: 1–5 mild pain and 6–10 severe pain. Patients also examined blood test for plasma TNF-α level by ELISA and iNOS and TNF-α expression in mononuclear cells immunohistochemically.

2.4.2 ELISA

Blood for TNF-α analysis was drawn in the morning, stored at 4° C, centrifuged at 1500 rpm **f**or 10 minutes and the plasma was stored at – 20 ° C until assayed. The presence of TNF-**α** in plasma was detected by commercially available ELISA Kit (R&D system, Wiesbaden, Germany).

2.4.3 Immunohistochemistry

Detection TNFα and iNOS expresson in mononuclear cells

Approximately 3 ml blood was collected from DN patients. The blood was centrifuged at 1500 rpm for 10 minutes and the plasma was collected into small aliquots. Buffy coat layer at the interface was collected into a clean centrifuge tub**e.** Red blood cells present in

the buffy coat were lyzed using 0.8 % NH4Cl for 1 minute and leucocytes were pelleted by centrifugation as above. Leucocytes were washed twice by repeated centrifugation and a thin smear of leucoytes at the density of 1 million per 1 ml PBS was prepared on poly-L-lysine microscope slides. After air dried, the leucocytes were fixed with cold acetone for 20 minutes.The endogenous peroxidase of the leucocytes was then inactivated by 3% H_2O_2 in PBS for 30 minutes at room temperature. After blocking with 2% skim milk in PBS, monoclonal antibodies (MAb against TNF-α (biodesign international) and against iNOS (Santa Cruz biotechnology) was added into leucocytes smear on microscope slides. After three time washes with PBS (pH. 7.4) and incubated for 1 hour at room temperature. The bound mAbs were the detected by biotinylated goat anti-mouse IgG (Biodesign International) and avidin-horse radish peroxidase (Sigma Co, USA). The expression of TNF-α and iNOS were then visualized by adding Diazinobenzidine (DAB) substrates (Sigma Co, USA, 50 mg/50 ml PBS containing 0.07% H2O2). The mononuclear cells expressed iNOS and TNF-α were count under 450 x manigtude microscope by observation of 100 cells.

2.4.4 Cut off point

Cut off point for risk factors plasma TNF-α levels, iNOS and TNF-α expression in mononuclear cells are median of the risk factors respectively. Cut of point of HbA1C is 7 %.

2.4.5 Statistical analysis

Data obtained from this study comprised descriptive analysis, non parametric One sample Kolmogorov-Smirnov test, bivariate correlation test, stepwise linear regression test, cross tab (chi-square test and odds ratio) and logistic regression test. The data were analyzed using SPSS software, version 15 with significant values $p < 0.05$.

Table 1 Characteristic of Diabetic Neuropathy Patients

Variable	N	Range	Mean ± SD
Age (years)	110	37-65	54,11 ± 7,64
Gender			
• Women	61		
• Men	49		
Duration of DM (years)	110	2-10	5,30 ± 3,05
PDN			
• Yes	59		
• No	51		
Fasting Blood sugar (mg %)	110	65 - 363	167 ± 70
Blood sugar 2 hourspp (mg%)	110	102 - 588	255 ± 105
HbA1C (%)	110	3,30 - 18,60	9,69 ± 3,49
Plasma TNF-α (pg/ml)	110	5,40 - 56,64	15,06 ± 7,01
TNF-α expressión (%)	110	0 – 40	12,43 ± 10,36
iNOS expressión (%)	110	0 – 38	9,22 ± 9,33

SD = standard deviation ; N = total sample; TNF = Tumor Necrosis Factor .
iNOS = inducible Nitric Oxide Synthase *;* HbA1C = Glycosylated Hemoglobine

Table 2. Comparison Clinical Data of Painful DN (Case) and Painless DN (Control)

Characteristic	Case Mean ± SD	Control Mean ± SD	P
Age (years)	53,97 ± 8,26	54,27 ± 6,94	0,834
Duration of Diabetes (years)	5,31 ± 3,13	5,29 ± 2,99	0,985
Fasting Blood Sugar (mg %)	168,96 ± 73,02	165,31 ± 69,14	0,789
Blood Sugar 2hourspp (mg %)	259,37 ± 109,51	251,96 ± 100,77	0,714
Hb A1C (%)	10,40 ± 3,61	8,88 ± 3,18	**0.023**
Plasma TNF-α (pg/ml)	17,44 ± 8,23	12,30 ± 3,76	**< 0,001**
iNOS expression (%)	12,21 ± 10,43	5,77 ± 6,41	**< 0,001**
TNF-α expression (%)	16,15 ± 11,05	8,13 ± 7,56	**< 0,001**

TNF = Tumor Necrosis Factor ; SD = standard deviation; p = significant level.

iNOS = inducible Nitric Oxide Synthase; HbA1C = Glycosylated Hemoglobine

3. RESULT

3.1 Characteristic of mononuclear cells that expressed TNF-α dan iNOS

The mononuclear cells expressed TNF-α and iNOS are characterized by brownish cytoplasma and violet-blue of nucleus as depicted in Figure 1 and 2.

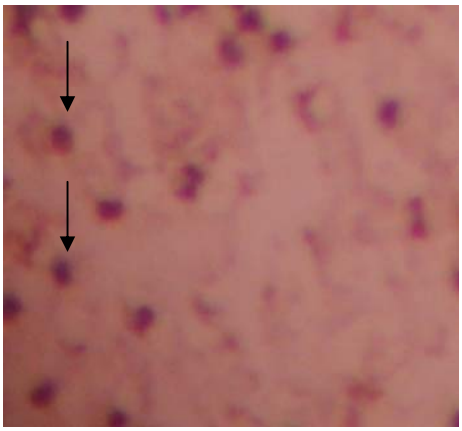

Fig 1. Expression of TNF-α in mononuclear cells

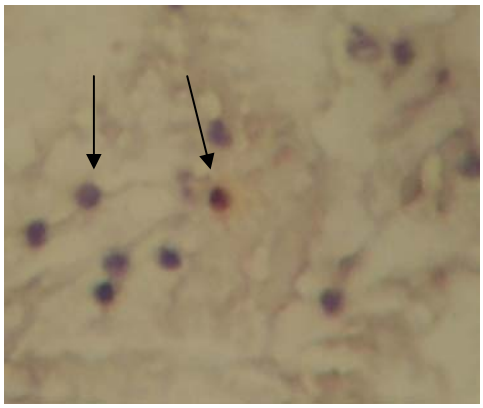

Fig.2. iNOS expression in mononuclear cells

3.2 Cross sectional Study

3.2.1 Comparison of risk factors of PDN according pain intensity

Of 110 subjects, 59 patients suffered from Painful DN which consist of 34 mild pain and 25 severe pain. Plasma TNF-α levels, iNOS and TNF-α expression were higher in patients with more severe pain in the VAS ($p < 0.05$) as shown in Figure 3.

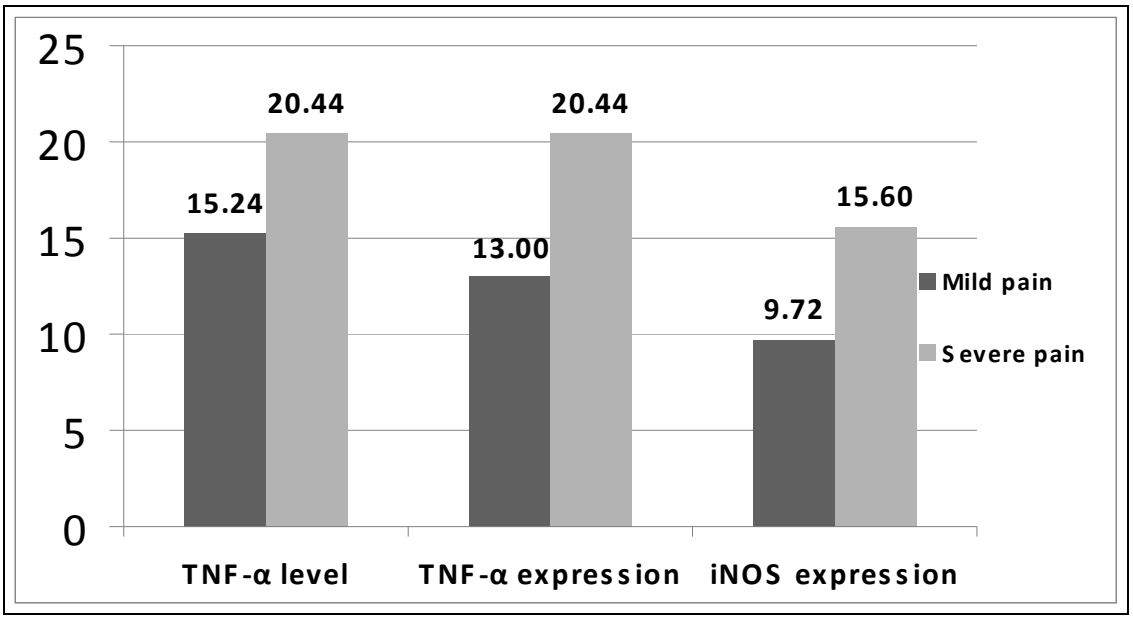

Figure 3
Plasma TNF-α level, TNF-α and iNOS expression in mononuclear cells according to pain intensity.

Plasma TNF-α level, TNF-α and iNOS expression of severe pain intensity were statistically significant higher than mild pain intensity patients (means 20.44 ± 10.34 pg/ml vs 15.24 ± 5.42 pg/ml ; 20.44 ± 11.75 % vs 13.00 ± 9.48 % and 15.60 ± 11.84 % vs 9.72 ± 8.61% ; $p < 0.05$).

3.2.2 Correlation between pain intensity and some risk factors of PDN

Correlation between pain intensity and some risk factors PDN as age, duration of diabetes, blood sugar level, HbA1C, plasma TNF-α level, TNF-α and iNOS expression are given in Table 3.

Table 3. Correlation between pain intensity and some risk factors of PDN

	Pain intensity	
Variable	R	p
Age	-0,144	0,275
Duration of Diabetes	-0,101	0,448
Fasting blood sugar darah	0,196	0,136
Blood sugar 2 hours post prandial	0,177	0,180
Hb A1C	0,138	0,298
Plasma TNF-α	**0,330**	**0,011***
iNOS Expression	**0,285**	**0,029***
TNF-α expression	**0,275**	**0,035***

TNF = Tumor Necrosis Factor ; HbA1C = Glycosylated Hemoglobine
iNOS = inducible Nitric Oxide Synthase ; r = coefficient correlation
p = significant level; * statistical significant

Correlation between pain intensity and age, duration of diabetes, fasting blood sugar, blood sugar 2 hpp and HbA1C were not statistically significant ($p > 0.05$) where as correlation between pain intensity and plasma TNF-α level, TNF-α and iNOS expression are statistically significant with $r = 0.330$, 0.285 and 0.275 ($p < 0.05$).

To investigated correlation between plasma TNF-α, iNOS dan TNF-α expression in mononuclear cells as risk factors of PDN , a bivariat correlation test was conducted **.** There were positive correlation between 3 risk factors above that statistically significant. ($p < 0,05$). Correlation between plasma TNF-α and iNOS expression was moderate ($r = 0,348$) , correlation between plasma TNF-α and TNF-α expression also moderate ($r = 0,389$), where as correlation between TNF-α and iNOS expression was strong ($r = 0,705$). Linear regression test showed that those 3 risk factors of PDN altogether have effect on pain intensity by 22.6 %, ($p < 0.05$) while their individual effect was not statistically significant.

3.3 Case control study

3.3.1 Risk of high plasma TNF-α level to PDN event

The odds ratio of high plasma TNF-α level to PDN event were 5.053, Confidence Interval (CI) 95% (2.241-11.392), it indicated that DN patients with high plasma TNF-α level had risk 5.053 times higher of getting PDN than low plasma TNF-α level ($p < 0.001$).

3.3.2 Risk of high TNF-α expression in mononuclear cells to PDN event

The odds ratio of high TNF-α expression to PDN event were 4.125, Confidence Interval (CI) 95% (1.805-9.425), it indicated that DN patients with high TNF-α expression had risk 4.125 times higher of getting PDN than low TNF-α expression.($p < 0.001$).

3.3.3 Risk of high iNOS expression in mononuclear cells to PDN event

The odds ratio of high iNOS expression to PDN event were 3.546, Confidence Interval (CI) 95% (1.613-7.795), it indicated that DN patients with high iNOS expression had risk 3.546 times higher of getting PDN than low TNF-α expression.($p < 0.002$)

3.3.4 Risk of high HbA1C level to PDN event

The odds ratio of high level HbA1C to PDN event were 2.380, Confidence Interval (CI) 95% (0.996-5.688) and $p > 0.05$. it indicated that high HbA1C level were not increasing risk of getting PDN than low plasma HbA1C level.

3.3.5 The role of risk factors to PDN event

To investigate the role of some risk factors which categorically have significant odds ratio to PDN event, such as plasma TNF-α levels, iNOS and TNF-α expression; a forward stepwise (conditional) logistic regression test was conducted. All of 3 risk factors above had a role to PDN event. The odds ratio of plasma TNF-α , iNOS and TNF-α expression were : 5.053 and 3.735 ($p < 0.05$) (Table 4)

Table 4. The role of plasmaTNF-α , iNOS and TNF-α expression to PDN event

Variable	B	Exp(B)	95% CI	P
1.Plasma TNF-α	1,620	5,053	2,241 - 11,392	< 0,001*
2.TNF-α expression	1,318	3,735	1,587 - 8,,787	0,003 *

B = beta ; CI = Confidence interval ; * statistically significant.

3.4 Summary of Study Result

In overall, the study result could be summarized as follow : cross sectionally the higher plasma TNF-α level, iNOS and TNF-α expression in mononuclear cells the more severe was the pain. There were positive correlation between pain intensity and plasma TNF-α level, iNOS and TNF-α expression, also moderate to strong correlation each other between 3 risk factors above.

In case-control study : plasma TNF-α as a risk factor was consistently and to be the most important risk factor of PDN, both direct and indirect by increasing expression of TNF-α and iNOS. TNF-α expresión appeared to be the second most important risk factor after plasma TNF-α and also both direct and indirect increasing the risk of PDN via plasma TNF-α level and iNOS expression.

4.Discussion

4.1 Diabetic Neuropathy as chronic complication of diabetes

In this study of 110 subjects whom fulfilled cross sectional inclusion criteria all subjects also fulfilled inclusion criteria for case control study, 59 subjects suffered from Painful DN and 51 subjects suffered from Painless DN. All diabetic patients who have been suffering diabetes for 2 years or more in this study suffered DN, this finding supported the reference that DN as complication of diabetes has already present early stage of hyperglycemia process before clinical diagnosis (Soliman, 2004)

4.2 . Comparison of risk factors of PDN according pain intensity

Comparison of plasma TNF-α and expression of iNOS and TNF-α according pain intensity were depicted in Fig. 3. There were tendency the higher plasma TNF-α level, iNOS and TNF-α expression in mononuclear cells the more severe was the pain, this

differences statistically significant ($p < 0.05$). These results are in agreement with previous study of cytokine expression in nerve Suralis biopsy specimen of neuropathy patients. Patients with painful neuropathies showed a stronger TNF-α immunoreactivity compared with patients with nonpainful neuropathy (0.949 ± 0.047 vs 1.010 ± 0.053, $p < 0.05$).(Empl et al.,2001). Study Uceiler et al. (2007) reported patients with a painful neuropathy had about twofold higher TNF-α mRNA ($p < 0.0001$) and protein levels ($p = 0.009$) than healthy control subjects and about twofold higher IL-2 and TNF mRNA ($p = 0.03$; $p = 0.001$) and protein levels ($p = 0.04$) than patients with painless neuropathy.

Our study showed plasma TNF- α level in PDN had nearly 1.5 fold higher than Painless DN (17.44 ± 8.23 pg/ml vs 12.30 pg/ml ± 3.76 pg/ml, $p < 0.001$) and TNF-α expression in PDN had nearly twofold higher than Painless DN (16.15 ± 11.05 vs 8.13 ± 7.56 % , $p < 0.001$). Doganay et al.(2002) investigated relation between the stages of DR (Diabetic Retinopathy) highest values obtained in patients with PDR (Proliferative Diabetic Retinopathy). Taken together, the mean serum NO, sIL-2R, IL-8 and TNF-α levels increased with the stage of DR and the highest levels were found in patients with PDR. In our study the highest levels of TNF-α (56.64 pg/ml) and iNOS (38%) and TNF-α expression (40%) also were found in patients with PDN. Pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IFN-γ, TNF-α) in the plasma correlate with increasing pain intensity. Chronic pain patients show a significant increase in plasma levels of NO in comparison to healthy controls.(Koch et al.,2007). Another study also showed correlation between pro inflammatory cytokine and pain intensity or severity of the disease in Juvenile rheumatoid arthritis (Chen et al., 2004) and fibromyalgia (Gur et al., 2002).

4.3 Correlation between pain intensity and some risk factors of PDN

There were positive correlation between pain intensity and plasma TNF-α level, iNOS and TNF-α expression, also moderate to strong correlation each other between 3 risk factors above. Linear regression test showed that those 3 risk factors of PDN altogether have effect on pain intensity by 21.5%, ($p < 0.05$) while their individual effect was not statistically significant. The contributed of plasma TNF-α level, iNOS and TNF-α expression in pathogenesis PDN were 21.5 % beside this there were still 78.5% another factors play a role in pathogenesis PDN.

Correlation between pain intensity and age, duration of diabetes, fasting blood sugar, blood sugar 2 hpp and HbA1C were not statistically significant ($p > 0.05$), it indicated that factors above were not proven increased PDN event.

4.4. Odds ratio of risk factors to PDN event

Chi-square test revealed odds ratio of high plasma TNF-α , high TNF-α expression to OR high of iNOS expression to PDN were 5.053, 4.125 and 3.546, $p < 0.001$ respectively. Forward stepwise (conditional) logistic regression test showed all of 3 risk factors above had a role to PDN event. The odds ratio of plasma TNF-α , iNOS and TNF-α expression were : 5.053, 2,869 and 4.327 , $p < 0.05$ respectively (Table 4). In contrast odds ratio of high level HbA1C to PDN event were 2.380 , $p > 0.05$. it indicated that high HbA1C level were not increasing risk of getting PDN than low plasma HbA1C level.

4.5 Summary of PDN Pathophysiology

According to this research and supported by some other investigations, a pathophysiological relationship can be made between TNF-α, iNOS and PDN as depicted in figure 2

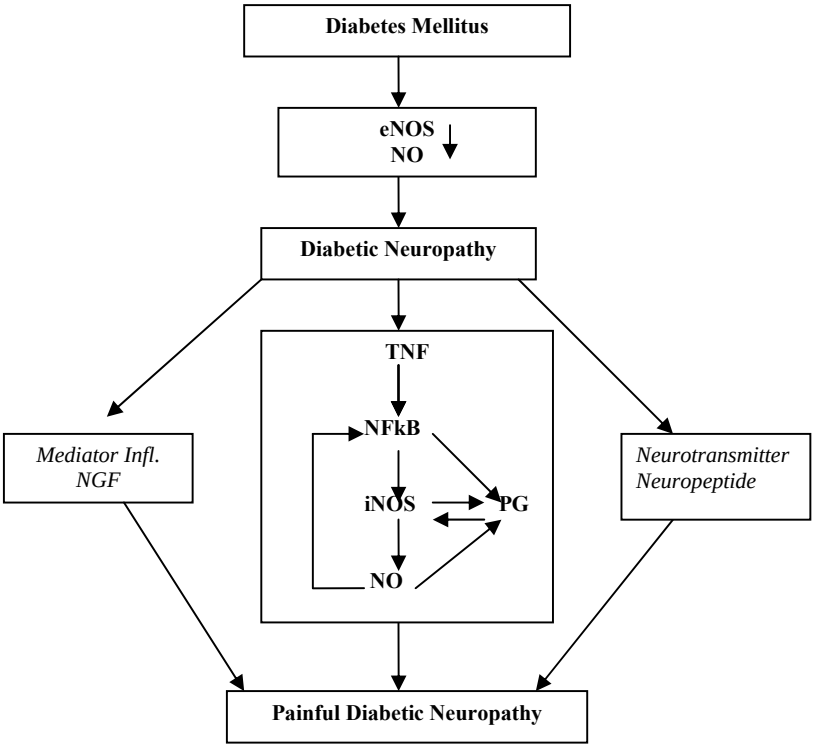

Figure 4
Pathophysiological relationship between TNF-α, iNOS and PDN

Prolong hyperglycemia in diabetic patients can causes diabetic pain through many mechanisms, including metabolic, oxidative stress, immunologic, AGEs, and vascular. All of those mechanisms relate closely with decreased eNOS which is followed by decrease in NO as eNOS is one of isoenzyme forming NO. NO are needed for vasodilating, inhibiting platelet aggregation and proliferation of smooth muscle (De Catarina et al., 1995). Decrease in NO will result in vessel endothelium disruption followed with nerve fiber disruption.

Dysfunction or lesion in peripheral nerve fiber leads to membrane remodelling and hyperexcitability which in turn cause diabetic pain. Lesion in primary afferent nerve fiber will induce activation of immune resident cell and recruitment of inflammatory cells to the inflamed cell. Then inflammation cascade takes place as activated mast cells release TNF-α which sensitizes nociceptor and contribute to neutrophils and macrophages recruitment. In nerve cells, neutrophils and macrophages produce and secrete neurotransmitter, neuropeptide and inflammatory mediators such as TNF-α,

Prostaglandin E2 (PGE2), bradykinin, serotonin, histamine and NGF. Those cocktail mediators activate nociceptor directly, cause sensitization and contribute to neuropathic pain, which result in pain, spontaneously or primary hyperalgesia (Moalem & Tracey, 2006).

NO role is thought to be through increased iNOS following chronic inflammation in diabetic pain. Strong evidences support a reciprocal relationship between NO and PGs biosynthetic pathways (Mollace et al., 2005). NO and PGs interaction occurs in some levels. NO directly influences COX expression and PGs biosynthesis. Conversely, AA and its metabolites, which produced by COX isoform, are also have effect on NO biosynthesis. NO ability to directly activate COX-2 is supported by evidences that NO increases purified COX-2 recombinant enzymes. NO is also influential to COX-2 enzymes activities by post transcription and translation process which make macrophages increase their PGs production. There are evidences that NO also activate COX-2 (Mollace et al., 2005). NO release by cNOS in basal condition deactivates iNOS by inhibition of NFkB signal. NFkB signal is one of the signaling pathways for iNOS expression by means of extracellular mediators, including TNF-α (proinflammatory cytokine) and endotoxin (Chao et al., 1997). In low concentration NO, NFkB is maintained inactive (Togashi et al., 1997), while in high concentration NO, NFkB is not suppressed by NO so that it activates iNOS by producing combination anion superoxide from peroxynitrite . NFkB is a strong COX-2 inducer and inhibitory role of NO in COX-2 is mediated by NFkB inhibition. This mechanism seems to be the basic NO inhibitory effect in PG biosynthesis (Mollace et al., 2005). Physiologically, iNOS is not expressed constitutively on mammal cells. Its expression is induced by proinflammatory agents such as bacteria LPS or cytokines. NO is produced by many type of cells in peripheral tissues after inflammation occurred. Once expressed, iNOS will produce NO in a large amount for a long period (Nathan, 1992).

The finding of our study support Law of Pain hypotheses from Omoigui (2007) which states that every process of pain derived from inflammation and its responses. High NO is related with PDN while iNOS inhibitor can decrease PDN symptoms (Meller et al., 1994; Alba et al., 2006; Sharma et al., 2007). Evidence that TNF-α and iNOS have important roles in pathogenesis PDN can lead to a new therapy strategy, that is by

modulating NO pathway and neuroimmune system. Modulation can be achieved by specifically inhibiting iNOS, antibody cytokine receptors and TNF-α.

4.6 Novelty

Plasma TNF-α level, iNOS and TNF-α expression in mononuclear cells have role in PDN pathogenesis. There is a spesific correlation between iNOS and TNF-α expression in mononuclear cells, in which both are thought to have important roles in PDN pathophysiology.

4.7.Conclusion

Diabetic Neuropathy patients with high TNF-α levels, iNOS and TNF-α immunoreactivity of mononuclear cells have higher risk for painful DN than painless DN. The higher TNF-α level, iNOS and TNF-α immunoreactivity the more severe was the pain. This supports the hypothesis that TNF-α and iNOS have role in PDN pathogenesis. The results of this research could be applied as a basic for further research in pursuit of better management of PDN.

Alba, J.D., Clayton, N.M., Collins, S.D., Colthup, P., Chessell,I., Knowles, 2006. GW 274150, a novel and highly selective of the iNOS, shows analgesic effects in rat models of inflammatory and neuropathic pain. Pain, 120 (1-2) : 170-81.

Backonja, M. 2001. Managing Painful Diabetic Neuropathy.Hospital Practice Magazine.Philadelphia : The McGraw-Hill Companies p 81-88.

Benbow, S.J., Wallymahmed M.E., MacFarlane, I.A. 1998. Diabetic peripheral neuropathy and quality of life. The Quarterly J. Med , 91(11): 733-7.

Chao, C.C., Lokensgard, J.R., Sheng, W.S., Hu, S. and Peterson, P.K. 1997. IL-1-induced iNOS expression in human astrocytes via NF-kappa B. *Neuroreport,* 8: 3163-3166.

Chen, D.Y., Lan, J.L., Lin, F.J., Hsieh, T.Y. 2004. Proinflammatory cytokine profiles in sera and pathological tissues of patients with active untreated adult onset Still‘s disease. J Rheumatol , 31(11): 2189–98.

De Caterina, R., Libby. P., Peng, H.B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone, M.A. Jr., Shin, W.S., and Liao, J.K. 1995. NO decreases cytokine-induced endothelial activation. NO selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Investig,* 96: 60-68.

Dinarello, C.A. 1999. Overview of inflamatory cytokines and their role in pain. In : Watkins, L.R., Maier, S.F (Eds), Cytokines and Pain. Basel : Birkhauser Verlag, p. 1-39.

Dogonay, S., Evereklioglu, C., Turkoz,Y., Servinc, A., Mehmet, N., Savli. 2002. Comparison of serum NO, TNF-α, sIL-2R, IL-6 and IL-8 levels with grades of retinopathy in patients with diabetes mellitus. Eye, 16 : 163-170.

Empl, M., Renaud, S.,Erne,B., et al. 2001. TNF-alpha expression in painful and nonpainful neuropathies. Neurology, 56 :1371-1377.

Gur, A.,Karakoc., Nas, K., Cevik,R., Denli, A., Sarac,J. 2002. Cytokine and depression in cases with fibromyalgia. J Rheumatol, 29:358-61.

Jin, X., and Gerean R, W. 2006. Acute p38-Mediated Modulation of Tetrodotoxin-Resistent Sodium Channels in Mouse Sensory Neuron by Tumor Necrosis Faktor-α. J Neuroscience, 26 (1) : 246-255.

Koch, A. , Zacharowski1, K., Boehm, O., et al. 2007. Nitric oxide and pro-infl ammatory cytokines correlate with pain intensity in chronic pain patients. Inflamm.res , 56 : 32-37.

Lowenstein, C.J. 1994. Nitric oxide : A Physiologic Messenger. Ann Int Med, 120 :3:227-237.

Meller, S.T., Cummings, C.P., Traub, R.J., Gebhart, G.F. 1994. The role of nitric oxide in the development and maintenance of the hyperalgesia produced by intraplantar injection of carrageenan in the rat. Neuroscience , 60(2): 367–74.

Meliala, L.2004a. Terapi Rasional Nyeri : Tinjauan Khusus Nyeri Neuropatik..Aditya Media.Yogyakarta, hal 15-54.

Moalem, G., Tracey, D.J. 2006. Immune and inflammatory mechanisms in neuropathic

Mollace ,V., Muscoli, C., Rotiroti, D., and Nisticom G. 1997. Spontaneous induction of nitric oxide- and prostaglandin E2-release by hypoxic astroglial cells is modulated by interleukin 1 beta. *Biochem Biophys Res Commun,* 238**:** 916-919.

Nathan, C. 1992. NO as a secretory product of mammalian cells. *FASEB J* 6**:**3051–3064.

Perkeni. 2006. Konsensus Pengelolaan dan Pencegahan Diabetes Melitus tipe 2 di Indonesia. Jakarta : PB Perkeni .

Sharma,S.,Chopra, K., Kulkarni, S.K. 2007. Effect of Insulin and its Combination with Resveratrol or Curcumin in Attention of Diabetic Neuropathic Pain : Participation of Nitric Oxide and TNF-alpha. Phytother.Res,21 : 278-283.

Soliman, E .2004.Diabetic Neuropathy.e Medicine September 20.

Sommer, C., Scha¨fers, M.2004. Mechanisms of neuropathic pain: the role of cytokines. Drug Discov Today, (1): 4 : 441-446.

Thacker, M.A., Clark, A.K., Marchand, F., McMahon, B.S. 2007. Pathophysiology of Peripheral Neuropathic Pain: Immune Cells and Molecules. Anesth Analg.105:838–47.

Tjokrorpawiro, A. 1993. GIGULOCHIPS (Sindrome-11) : Faktor-faktor Penentu Kualitas Pembuluh Darah (Aspek Klinik resistensi Insulin). Disajikan pada Simposium Cardiology Update III , Jakarta, 14-15 Mei.

Togashi ,H., Sasaki, M., Frohman, E., Taira, E., Ratan, R.R., Dawson, T.M. and Dawson ,V.L. 1997. Neuronal (type I) nitric oxide synthase regulates nuclear factor kappaB activity and immunologic (type II) nitric oxide synthase expression. *Proc Natl Acad Sci USA* ,94**:** 2676-2680 .

Uceyler, N., Rogausch, JP., Toyka, KV., Sommer, C. 2007. Differential expression of cytokines in painful and painless neuropathies. Neurology, 69 :42-49.

Uceyler,N., Sommer,N. 2007. Cytokine Profiles in Patients with Chronic Widespread Pain. In : De Leo, JA., Sorkin, L.S., Watkins, LR.(eds). Immune and glial regulation of Pain. Seattle : IASP Press :417-424.

Uceyler,N., Sommer,N. 2008.Status of Immune Mediators in Painful Neuropathies. Current Pain and Headache Report 12: 159-164.