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Role of Interleukin-6 (IL-6) and Indicators of Inflammation in the Pathogenesis of Diabetic Foot Ulcers.

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Abstract: It is relatively indicative that diabetic foot is characterized by definite inflammatory reaction as one of diabetes mellitus complications. On this basis, we seek by our study to examine the levels of interleukin-6 (IL-6), C-reactive protein (CRP) and fibrinogen in adult patients with diabetic foot ulcer and diabetic subjects with no foot ulcer and to validate the relationships between IL-6, CRP and fibrinogen in the diabetic patients with foot ulcers. IL-6 was measured by enzyme-linked immunosorbant assay (ELISA) in the serum samples from 31 patients with diabetic foot ulcers compared to 20 diabetic individuals with no foot ulcers. IL-6 levels were significantly higher in the serum of the patients with diabetic foot ulcers as compared with diabetic subjects with no foot ulcers. Results of IL-6 were correlated to the indicators of inflammation; C-reactive protein (CRP) and fibrinogen. The results of the current study revealed that the excessive presence of IL-6 might play a role in diabetic foot ulcer pathogenesis and development. In addition, our study demonstrated a strong correlation between the levels of IL-6, CRP and fibrinogen in the diabetic patients with foot ulcers.

Key words: Interleukin-6, C-reactive protein, fibrinogen, diabetic foot ulcer.

INTRODUCTION

Diabetic foot is one of the most feared complications of diabetes mellitus. The diabetic foot disease is associated with significant morbidity and mortality even to the endpoint amputation. Diabetes and its complications as foot ulcers may develop as a result of polneuropathy, ischaemia or subclinical inflammation causes or as a result of all. The term "diabetic foot" may consist of a mix of pathologies including diabetic neuropathy, peripheral vascular disease, Charcot's neuroarthropathy, osteomyelitis and foot ulceration. Diabetes is associated with 2-3 fold-increased risk of accelerated atherosclerosis. Hence those subjects with peripheral vascular disease are predisposed to poor wound healing (Rathur and Boulton, 2007).

Low-grade immune activation also represents an important risk factor not only for the development of Type II diabetes but also for several macrovascular complications (myocardial infarction and stroke) and microvascular complications (neuropathy and nephropathy). The status of the immune system may be relevant at several stages in the development of chronic wounds.

However immune activation may precede the incidence of a diabetic foot ulcer in the same way that it precedes the manifestation of Type II diabetes and coronary heart disease (Weigelt *et al*., 2009).

Because pro- and anti-inflammatory processes are crucial in the different phases of wound healing, it is possible that disturbances of the immune system interfere with tissue homeostasis and wound healing after the manifestation of ulcers and lead to the chronic, nonhealing wounds that are characteristic of diabetic foot syndrome (Weigelt *et al*., 2009).

Ulceration of the diabetic foot does not occur spontaneously, but usually follows some form of trauma, which may go unrealized by the patient. In some patients with diabetes, the local and systemic signs of inflammation may often be reduced as a result of the associated peripheral vascular disease. The infected foot can be painless as a result of neuropathy and thus may lead to delay in requesting medical attention (Urbanic-Rovan, 2005).

Human Interleukin 6 (IL-6) is a phosphorylated cytokine with 183 amino acids. It plays important roles in acute phase reactions, inflammation, haematopoiesis, bone metabolism, and cancer progression. IL-6, drives the acute inflammatory response, is almost solely responsible for fever and the acute phase response in the liver, and is important in the transition from acute inflammation to either acquired immunity or chronic inflammatory disease. It contributes to chronic inflammation in conditions such as obesity, insulin resistance, inflammatory bowel disease, inflammatory arthritis, and sepsis when dysregulated, often involving IL-6 trans-signaling (Wan-Wan and Micheal, 2007).

C-reactive protein (CRP) takes part in the systemic inflammatory response, acute injury, infection or other stimuli. CRP binds to specific molecular configurations typically present in case of cell death and additionally

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found on the surface of pathogens, and therefore CRP increases rapidly after tissue injury or infections and reflects the intensity of the inflammatory process (Mohamed *et al*., 2011).

Fibrinogen is a strong and independent predictor of myocardial infarction and stroke in non-diabetic subjects and elevated levels relate to macrovascular complications in diabetic patients.

Fibrinogen may be of particular importance in the context of acute phase activation, endothelial dysfunction and microalbuminuria. Microalbuminuria (a marker of endothelial dysfunction) predicts excess cardiovascular mortality and is associated with insulin resistance and fibrinogen in diabetic and non-diabetic individuals (Mills and Grant, 2002).

Aim of the Work:

We aim to measure systemic immune mediators, IL-6 concentrations in the sera of diabetic patients with/ without foot ulcers and to investigate the relationships between IL-6, CRP and fibrinogen in diabetic patients with foot ulcers.

Sample Collection:

All subjects who participated in this study were previously diagnosed diabetic patients. All cases were diabetic patients with diabetic foot ulcer at the time of sampling, while the control group was diabetic patients without foot ulcer. Samples were stored at -80ºC until analysis.

Subjects and Methods:

Subjects:

We involved 51 subjects to this study. Thirty one (31) diabetic patients with foot ulcers (13 females and 18 males, mean age 65.61 ± 7.48 years) were compared with 20 diabetic subjects with no foot ulcers considered as control group (8 females and 12 males, mean age 58.23 ± 5.35 years). All subjects who participated in this study were already previously diagnosed as type II diabetes mellitus cases. Clinically, the case group individuals had diabetic foot ulcer, which was defined as a full-thickness defect that required 14 or more days to heal.

Methods:

Interleukin-6 was determined by an ELISA; C- reactive protein (CRP) was evaluated by using immunoturbimetric assay and fibrinogen by immunonephelometric analysis.

Data Handling and Statistical Methods:

The statistical software package (SPSS version 10.0) was used for data management and analysis. Data were expressed as mean ± standard deviation. The data were subjected to the Kolmogorov- Smirmov test to determine the distribution and method of analysis. As most of the data was normally distributed continuous variables student's *t* test was used. To exam the correlation, Pearson's correlation coefficient's (*r*) were calculated by linear regression analysis.

p values < 0.05, < 0.01 and < 0.001 were considered significant, highly significant and very highly significant respectively.

Results:

Characteristics of subjects investigated are presented in Table (1). The mean duration of diabetes was (18.63 ± 2.45 years) for the case group, while for the control group the duration of diabetes was (10.45 ± 1.12 years). Table (2) demonstrates mean and ± SD values of IL-6, CRP and fibrinogen of the various studied groups. The mean CRP values for the diabetic patients with foot ulcers were (6.82 ± 0.48 mg/l) compared to the diabetic patients without foot ulcers were (1.86 ± 0.33 mg/l, $p < 0.01$). The mean fibrinogen values for the diabetic patients with foot ulcers were (6.45 ± 0.54 g/l) compared to the diabetic patients without foot ulcers were (4.03 ± 0.28 g/l, $p < 0.05$). Serum IL-6 levels (7.29 ± 0.51 pg/ml) were significantly higher in diabetic patients with foot ulcers compared to diabetic patients without foot ulcers (2.14 ± 0.26 pg/ml, $p < 0.01$).

Table (3) demonstrates the correlation between the IL-6 and levels of CRP and fibrinogen in the case group.

A highly significant positive correlation was detected in the case group between the IL-6 and the levels of CRP and fibrinogen ($r = 0.994$, $p < 0.001$, $r = 0.964$, $p < 0.001$ respectively). (Figure 1, Figure 2 respectively)

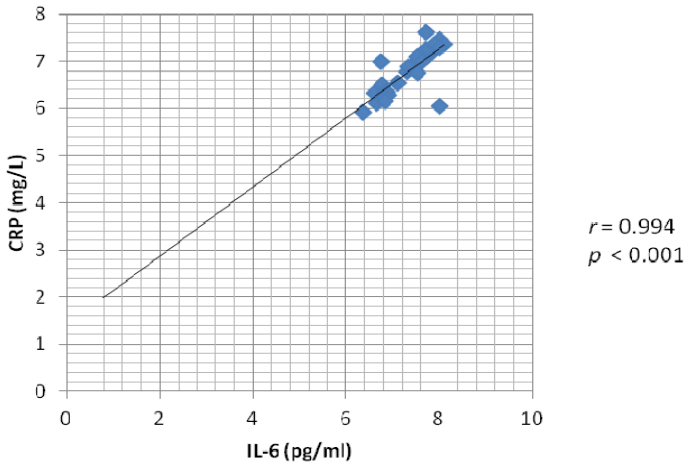

Fig. 1: The correlation between the serum levels of IL-6 and CRP in the diabetic patients with foot ulcers.

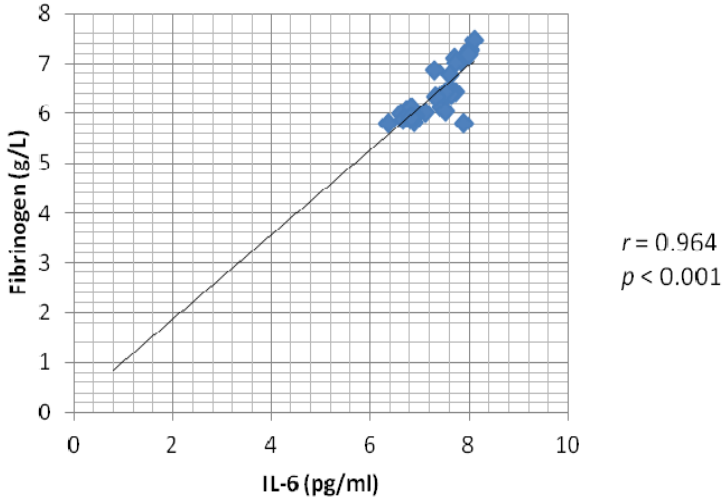

Fig. 2: The correlation between the serum levels of IL-6 and Fibrinogen in the diabetic patients with foot ulcers.

Table 1: Clinical characteristics of studied subjects.

	Controls N= 20	Cases N= 31
Gender: Female Male	 8 12	 13 18
Age (years)	58.23 ± 5.35	65.61 ± 7.48
Duration of diabetes Mellitus (years)	10.45 ± 1.12	18.63 ± 2.45

Table 2: CRP, Fibrinogen and IL-6 concentrations of diabetic patients with foot ulcers (Cases) compared diabetic subjects without diabetic foot (Controls).

	Controls N= 20	Cases N= 31	*p* values
CRP (mg/l)	1.86 ± 0.33	6.82 ± 0.48	$p < 0.01$
Fibrinogen (g/l)	4.03 ± 0.28	6.45 ± 0.54	$p < 0.05$
IL-6 (pg/ml)	2.14 ± 0.26	7.29 ± 0.51	$p < 0.01$

Table 3: Demonstrates the correlation between IL-6 and levels of CRP and Fibrinogen in the diabetic patients with foot ulcers.

	CRP (mg/l)	Fibrinogen (g/l)
IL-6 (pg/ml)	$r = 0.994$ $p < 0.001$	$r = 0.964$ $p < 0.001$

Discussions:

Several researchers have claimed the association of Type II (non-insulin- dependent) diabetes mellitus with increased blood concentrations of markers of the acute-phase response, including: sialic acid, serum amyloid A, cortisol, CRP and the main cytokine mediator of the response, interleukin-6. Long-term cytokine-mediated acute-phase reaction has been proven to occur in Type II diabetes and is part of a wide-ranging innate immune response (Pickup and Crook, 1998).

Other reports demonstrated that chronic low-grade inflammation and activation of innate immunity are involved in the pathogenesis of Type II diabetes. Studies showed not only that the systemic concentration of acute-phase proteins and cytokines are elevated in Type II diabetic patients, but that these immune abnormalities may precede Type II diabetes by several years (Herder *et al*., 2005).

Studies for cellular innate immunity showed decreased cellular functions as chemotaxis, phagocytosis, killing of polymorphonuclear cells and monocytes/ macrophages in diabetics compared to cells of controls. Better regulation of diabetes mellitus lead to an improvement of these cellular functions (Suzzanne and Andy, 1999).

An accumulating body of evidence suggests that inflammation may pay a crucial intermediary role in the pathogenesis of diabetes, thereby linking it with a number of commonly coexisting condition thought to originate through inflammatory mechanisms (Aruna *et al*., 2001).

Pickup and Crook, 1998 suggested that Type II diabetes is an acute phase disease in which increased concentrations of cytokines are secreted from cells such as macrophages, adipose tissue and endothelium, under the influence of stimuli such as over nutrition, perhaps in those predisposed to having an unregulated response because of increasing age, or genetic or fetal metabolic preprogramming (Pickup and Crook, 1998).

Through the action of cytokines on the brain, liver, endothelium, adipose tissue and else where, this process could be a major contributor to the biochemical and clinical features as glucose intolerance, dyslipidaemia, insulin resistance, hypertension, central obesity, accelerated atherosclerosis and also provides a mechanism for many other abnormalities seen in Type II diabetes, including those in blood clotting, the reproductive system, metal ion metabolism, psychological behavior and capillary permeability (Pickup and Crook, 1998).

Pickup and Crook, 1998 also stated that the long term hypersecretion of IL-6 and TNF-α may impair beta-cell insulin secretion which has been investigated as an aetiological mechanism for islet cell destruction in Type I diabetes. Furthermore prolonged exposure to marginally raised -cytokine concentrations may produce different effects on the beta-cells of the pancreas in Type II diabetes (Pickup and Crook, 1998).

Cytokines, mainly IL-1, IL-6 and TNF-α, act on the liver to promote the release of acute-phase proteins which are atherosclerotic risk factors: such as fibrinogen, stimulate leptin release from adipose tissue, and act on the brain to release adrenocorticotropic hormone (ACTH) and thus cortisol. The latter may contribute to obesity, hypertension and insulin resistance that may occur in Type II diabetes (Pickup and Crook, 1998).

Proinflammatory cytokines produced at the sites of diabetic complications or by the diabetic process itself may also exacerbate atherosclerosis by acting on the endothelium, smooth muscle cells and macrophages. The atherosclerotic plaque itself produces cytokines, which further exacerbate the process of atherosclerosis locally but also cause an increase in circulating acute-phase proteins, many of which are themselves risk factors for atherosclerosis (Pickup and Crook, 1998).

Our study aimed to compare systemic immune mediators such as acute-phase proteins- CRP, fibrinogen and cytokine IL-6 representing different aspects of the immune system, in diabetic patients with and without ulcer.

In patients with foot ulcer, the mean levels of both acute- phase proteins, CRP and fibrinogen, were significantly elevated compared with those in patients without foot ulcer. Similarly, mean levels of cytokine IL-6 in patients with the foot ulcer were significantly higher.

These findings were in accordance with the results of Weigelt and coworkers, 2009 who suggested that patients with foot ulcers experience an upregulation of different components of the immune system (Weigelt *et al*., 2009).

Muller *et al*., 2002 confirmed that with the development of diabetic complications, a substantial rise of systemic IL-6 is found. Hence analysis of the association between cytokine concentrations and Type II diabetes has to cope with diabetic complications as a possible confounder (Muller *et al*., 2002).

IL-6 is a major proinflammtory cytokine produced in a variety of tissues; cells known to express IL-6 include $CD8^+$ T cells, synoviocytes, adipocytes, osteoblasts, endothelial cells, neutrophils, monocytes, eosinophils, and pancreatic islet beta cells. IL-6 production is generally correlated with cell activation and is normally kept in control by glucocorticoids, catecholamines and secondary sex steroids (Alberli *et al*., 2005).

IL-6 modulates bone resorption and is a major effector in inflammatory joint destruction in rheumatoid arthritis. It contributes to atherosclerotic plaque development and destabilization (Alberli *et al*., 2005).

Major components of the IL-6 associated immune network, are the acute- phase proteins for which the chief inducer of production in hepatocytes is IL-6. Individual concentrations of acute-phase proteins closely correlated with those of IL-6 and each other (Muller *et al*., 2002).

This observation shows the biological relevance of mildly increased concentrations of IL-6 in Impaired glucose tolerance (IGT) and Type II diabetes as these changes are accompanied by considerable increases of acute-phase protein production (Muller *et al*., 2002).

The observation that IL-6 regulated immune mediators are increased not only in Type II diabetes but also in IGT clearly dissociates immune changes from overt hyperglycaemia and points to a contribution of IL-6 and acute-phase proteins to pathogenic events prior to the onset of Type II diabetes. These statements concur with recent reports of increased CRP concentrations in individuals with insulin resistance syndrome and of increased CRP and IL-6 in incident Type II diabetes (Muller *et al*., 2002).

Aruna and co-authors, 2001 suggested that IL-6 and CRP both are sensitive physiological markers of subclinical systemic inflammation, and are associated with hyperglycemia insulin resistance and overt Type II diabetes. Recently it has been postulated that Type II diabetes may represent a disease of the innate immune system (Aruna *et al*., 2001). Our study is in agreement with these reports as we found a strong positive correlation between the levels of IL-6 and CRP in the case group; diabetic patients with foot ulcers.

CRP is a principal downstream mediator of the acute phase response and is primarily derived via IL-6 dependent hepatic biosynthesis. It is a member of the pentraxin family of oligomeric proteins involved with pattern recognition in innate immunity. Reported immunoregulatory functions of CRP include enhanced of leucocytes reactivity, complement fixation, modulation of platelet activation, and clearance of cellular debris from sites of active inflammation (Aruna *et al*., 2001).

CRP plasma levels may increase up to 1,000-fold in response to acute and chronic inflammatory conditions, making it a useful measure of inflammation in a wide range of physiological and pathological conditions. CRP can mediate activation of monocytes and macrophages via IL-6, TNF-α and other cytokines, and assist in the complement pathway (Alexander *et al*., 2000).

A relationship between fibrinogen and insulin has been demonstrated in glucose tolerant women and subjects with abdominal obesity (Vaan Gaal *et al*., 2006). Although the biological mechanism responsible for these associations is still unclear, but may involve the release of proinflammatory cytokines such as TNF-α and IL-6, which, in addition to fibrinogen, have, been shown to correlate with insulin resistance (Mills and Grant, 2002). Our result showed that IL-6 is significantly correlated to fibrinogen in the case group; diabetic patients with foot ulcers.

Weigelt *et al*., 2009 suggested a parallel upregulation of IL-6, CRP and fibrinogen is biologically possible, because IL-6 is known to increase the release of both acute-phase proteins, which is reflected in the high correlations between the systemic levels of these markers (Weigelt *et al*., 2009).

Conclusion:

We found that the significantly high IL-6 in diabetic patients with foot ulcer may be fundamental in the development of the ulcer, associated increase in CRP and fibrinogen may indicate an essential relation between IL-6 and the indicators of inflammation in diabetic patients with foot ulcer.

Recommendations:

Proper understanding of the pathogenesis of the diabetic foot ulcer may help prevention and treatment of the ulcer and thus may cause reduction in the morbidity and mortality of one of the diabetic complications.

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