Academic Sciences Asian Journal of Pharmaceutical and Clinical Research
Vol 5, Issue 2, 2012 ISSN - 0974-2441

Research Article

IMMUNOMODULATORY POTENTIAL OF HERBAL MEDICINE IN TYPE 2 DM PATIENTS AS EVALUVATED BY NEUTROPHIL PHAGOCYTIC INDEX , SERUM OPSONISATION AND LYMPHOCYTE PROLIFERATION RATE

VIDYA BERNHARDT[1], JANITA R. T. D'SOUZA*[2]

[1]Department of Biochemistry, Yenepoya Medical College, Yenepoya University, [2]Yenepoya Research Centre, Yenepoya University, Mangalore, Karnataka ,Email: pjanita@gmail.com

Received: 7 October 2011, Revised and Accepted: 21 Novenber 2011

ABSTRACT

Diabetes mellitus (DM) is a syndrome characterised by hyperglycemia and impaired carbohydrate, fat and protein metabolism due to insufficient action of insulin. Increased reactive oxygen production, decreased chemotaxis and phagocytosis, altered opsonization by complement 3 (C_3) and Immunoglobulin G (IgG) alters phagocytosis. T and B lymphocyte proliferation are found to be impaired in DM patients. Phagocytic index (PI), serum opsonization, T and B cell proliferation have been studied to evaluate effect of herbal medicine in DM patients. The study cohort included 111 type 2 DM patients with elevated HBA1c grouped as uncontrolled diabetes on antihyperglycaemics (51), diabetes under control with antihyperglycaemics (30), diabetes under control receiving herbal preparations (30). An age and sex matched control group without DM (30) was included. Whole serum opsonisation (PI WBO), IgG (PI IgG), C3 (PI C3) opsonisation, PI, proliferation of T and B cells were assessed. Results suggest that neutrophil functions are decreased in uncontrolled DM patients with decreased PI WBO, PI C3, PI IgG and T and B cell proliferation rate when compared to healthy controls suggesting that the neutrophil functions and innate immunity in these patients is reduced. In DM patients treated with anti hyperglycaemics and herbal preparations PI and B and T cell proliferation was restored. It was seen that the restoration was much more significant in patients receiving herbal preparations for blood glucose control. The study suggests that herbal preparations can act against altered neutrophil function, which may play a causative role in atherogenesis and atheroprogression and points out the potential importance of modulating neutrophilic and immune response as part of improved strategy for DM associated complications.

Key words: Diabetes mellitus, Herbal preparations, Immunomodulation, Innate immunity, T and B cell proliferation.

INTRODUCTION

Diabetes mellitus (DM) is a syndrome characterised by hyperglycemia and impaired carbohydrate, fat and protein metabolism due to insufficient action of insulin. It has been reported that oxidative stress contributes to cardiovascular complications and disease progression in DM patients predisposed by micro and macro vascular complications of DM[1]. It is known that the immune system is especially vulnerable to oxidative stress. Neutrophils produce reactive oxygen species (ROS) during respiratory burst as a defense against invading pathogens. Neutrophils from diabetic patients with high blood glucose show increased reactive oxygen metabolite production that may cause cell and tissue damage and also demonstrated subnormal activity in chemotaxis and phagocytosis. Several lines of evidence have suggested that impairment of phagocytic function of monocytes/macrophages contributes, at least in part, to the impaired resistance to infection in diabetes. Neutrophils represent a major mechanism of innate immunity and inflammation and can play a pivotal role in human atherosclerosis in DM led ischaemic heart disease (IHD)[2].

One of the other causes of atherosclerosis and IHD is reduced resorption of atherosclerotic plaques due to phagocytic dysfunction[3]. Chronic inflammation involves neutrophil dysfunctions and recent studies have revealed that atherosclerosis has several similarities to chronic inflammation. Atherosclerosis is a dynamic disease process that initiates and progresses under the influence of complex interactions of highly specialized cells including macrophages and also the T lymphocytes[4]. Findings also suggest that neutrophils from DM patients show opsonisation impairment (Complement 3-C3 and Immunoglobulin G-IgG)[5], decreased chemotaxis increased ROS that may cause cell and tissue damage decreased neutrophils phagocytic activity[6] which contributes to increased progression of atherosclerosis and IHD[7].

Different types of circulating immune cells, especially T lymphocytes are subjected to glucolipotoxicity due to chronic exposure to higher glucose[8]. The mitogenic lymphocyte proliferation in diabetic rats is seen to be decreased. Alterations in lymphocytes are a common finding in both type I and type II diabetes. Since activation of T lymphocytes plays a pivotal role in initiating immune response and cell-mediated cytotoxic activity, inhibition of lymphocyte activation by diabetic state could evoke a clinically relevant immunosuppressive effect[9]. Common hypertensive drugs cause elevation in cell calcium, which reduces cell proliferation, mainly B cell proliferation along with neutrophils phagocytic function[10].

Moreover atherosclerosis leading to IHD being a chronic inflammatory disorder of the vessel wall, defective immune responses influence disease progression[11].

Since the dawn of civilization herbal products have been used to maintain human health and as remedies for various diseases in several parts of the world. In recent years, there has been growing interest in complementary and alternative medicine. India has a rich history in traditional herbal medicine. Many herbal therapies have the potential of improving quality of life. Herbal therapies may be considered in the management of several diseases[12]. Various herbal formulations may modulate the cells involved in the immune response, including lymphocytes and monocytes. Many primary and secondary metabolites of plants have been described as able to interfere in the functioning of the immune system [13].

Herbal medicine is increasingly gaining greater acceptance due to greater advances in the understanding of the mechanisms by which herbs positively influence health and quality of life. Evaluation of herbal drugs for that could ameliorate immune dysfunction in DM patients is required with this aim we studied the phagocytic index of neutrophils, serum opsonisation capacity, T and B cell proliferation in DM patients and the influence of herbal preparations on the same.

MATERIALS AND METHODS

This prospective cohort study included 111 type 2 DM patients, Patients visiting on an outpatient basis at Tanvi Medical Center and Yenepoya Hospital, Mangalore, Karnataka, India were recruited for the study after obtaining their consent. All patients had normal lipid profile. (Total Cholesterol 183±26.8, LDL Cholesterol 110.4±16.8 and Triglycerides 167.3 ± 48.2). The duration of the diabetes ranged from 3 to 7 years. ECG reports and other clinical data and were collected for each patient The study was conducted after obtaining approval by the Ethics Committee.

The patients were classified into three groups. Blood glucose and glycated haemoglobin (HbAlc) were taken into consideration when grouping. All the recruited patients had normal lipid profile, were on

antihypertensives and were not taking any lipid lowering drugs. Exclusion criteria were known malignancies, surgeries in previous month, other existing chronic and acute diseases, severe hypertension, stroke, significant valvular disease, renal failure. Control subjects were age and gender matched.

Group 1 consisted of 51 patients with uncontrolled diabetes on antihyperglycaemics.

Group 2 consisted of 30 patients whose diabetes was under control with antihyperglycaemics.

Group 3 consisted of 30 patients whose diabetes was under control receiving herbal preparations consisting of seeds of *Syzygium cumini, Trigonella foenum-graecum, Curcuma longa* as the main ingredients.

Group 4 consisted of 30 healthy subjects with no history of DM and with normal fasting blood glucose and HBA1c values. This group served as control.

Chemicals and reagents

Concanavalin A (Con A), Lipopolysaccharide (LPS), 3- (4, 5-dimethylthiazol 2-yl)-2,5 Diphenyltetrazolium bromide (MTT), Sodium dodecyl sulfate

Sample Collection and Data Collection:

Venous blood (4 ml each) was collected from the patients in appropriate vacutainers, with and without anticoagulants. Samples were immediately transferred to the laboratory and were processed within 6 hours. Opsonization capacity, phagocytic index, T and B cell proliferation was measured through laboratory experiments. Blood glucose, HbAlc[15] and lipid profile assays were performed in all patients.

Separation of neutrophils for evaluation of phagocytic capacity:

Neutrophils were isolated by a method modified from Boyum[15]. Venous blood samples from the subjects was drawn into heparinized syringes and settled by gravity in 6% dextran (mol. wt. 70,000; Hi media chemicals, India) in normal saline (1:3). The leucocyte-rich plasma was withdrawn and centrifuged at 160g for 10 minutes. The pellet was re-suspended in Eagle's minimal medium; 6 ml of the cell suspension was carefully layered onto 3 ml of Ficoll-Paque (Hi media chemicals, India) and centrifuged at 160 g for 35 minutes. Mononuclear cells of the interface were removed. Residual erythrocytes in the pellet were lysed with ice-cold NH_4 Cl (0.87% in sterile water). After centrifugation at 160 g for 5 minutes, neutrophils were washed twice with Hanks balanced salt solution (HBSS). Number of neutrophils were adjusted to a final suspension such that 1ml contains $5x10^6$ neutrophils.

Neutrophil Phagocytosis:

For phagocytosis to take place add, to the separated neutrophils in phosphate buffered saline (PBS) + Bovine serum albumin (BSA) mixture, bacteria opsonised with complete serum, serum without IgG , serum without C3 as well as non opsonised bacteria in the ratio of 1:9. Incubate at 37°C for 30 minutes. Two smears were prepared and stained with Lieshman's stain and the number of bacteria engulfed by 200 neutrophils[16] were counted under a 100x microscope for phagocytosis.

Preparation of the bacteria was done as follows. *Staphylococcus aureus* was grown in nutrient agar under standard conditions and the suspension was washed in HBSS at pH 7.2 containing 0.1M mercaptoethanol, then diluted in HBSS and adjusted to the required quantity. Neutrophils and bacteria in the ratio of 1:9 was used to study opsonisation and phagocytic activity. The stock was stored for all experiments[17]. Opsonisation by IgG of the organism was done by inactivation of complement C3 as explained by Jan kallman et al[18].Opsonisation of the organism with C3 of the serum was done with salt chelating of IgG with a method slightly modified from the method previously mentioned[19]. Autologous serum was used in all experiments. *S. aureus* cells were centrifuged at 1200 rpm washed with PBS and then incubated with 500 µl of whole serum for evaluation of PI with serum opsonised bacteria (PI- WBO), 500 µl serum without IgG[19] for evaluation of PI with C3 opsonised bacteria (PI- C3) and 500 µl serum without C3[18] for evaluation of PI with IgG opsonised bacteria (PI-IgG). Kept at 37°C for 45 minutes with shaking. The cells were then centrifuged, pellets were washed and reconstituted in PBS.

Phagocytic capacity of the neutrophils was evaluated as PI calculated as follows: Phagocytic Capacity that is number of bacteria/Neutrophil, Phagocytic % which is %age of Neutrophils having phagocytic capacity and the PI which is the Phagocytic capacity times the Phagocytic % divided by 100. PI reveals the phagocytic function of the neutrophils.

T and B lymphocyte proliferation in response to mitogens in 48 hours lymphocyte culture:

Lymphocytes were separated by commercially available lymphocyte separation media (Hisep from Hi media, India) based on the principle of density gradient.

In order to study the T and B lymphocyte proliferation two specific mitogens Con A and LPS were used respectively. Cell proliferation assays were performed with 20µg Con A /mL and 100µg LPS/mL added to the cell cultures of lymphocytes at the beginning of the incubation period [20]. 5×10^5 lymphocytes were obtained and re-suspended in 200µL of RPMI-1640 medium mixed with 10% fetal calf serum, containing 5.6mM glucose, 2.0mM glutamine, and antibiotics (streptomycin 100 units/ml and penicillin 200 units/ml). Cells were then incubated in the 96-well plate at 37° C with 5% CO_2 for 48 hours.

MTT assay for cell proliferation

Cell proliferation was measured by the MTT assay. At the end of 44 hours of incubation of lymphocytes, 15µL MTT reagent (5mg/ml) was added. After 4 hours of incubation at 37°C, 150µL of 2% SDS solution was added into each well to dissolve the tetrazolium crystals. Following overnight incubation, the absorbance at 570nm was recorded in a microplate reader which is proportional to the rate of lymphocyte proliferation in vitro[21].

Statistical analysis

The data were analyzed by using Graph Pad Prism for Windows. The significance of differences was calculated by using one-way analysis of variance (ANOVA) followed by Tukey Kramer procedure for multiple comparisons. P value < 0.0001 was considered very highly significant (vhs), P < 0.001 was considered highly significant (hs) and P < 0.01was considered significant (sig).

RESULTS

The results are depicted as mean ± SD. Fasting blood glucose and HBA1c levels in the groups are given in Table 1 and PI, T and B cell proliferation in Table II. The statistical analysis is given in the graphs. When the difference between the groups differed with P value < 0.0001 the significance was referred to as very highly significant (vhs). When the difference between the groups differed with P value < 0.001 significance was referred to as highly significant (hs) when the difference between the groups differed with P value < 0.01 significance was referred to as significant (sig). The results of the study groups (Groups 1, 2 and 3) were compared with the control group also the study groups were compared with each other.

TABLE 1 SHOWS STUDY GROUPS WITH PARAMETERS - HBA1C, FBS

	No. of patients	HBA1c %	Fasting glucose mg/dl
Group 1- uncontrolled diabetes	51	11.37±2.63	219.49± 64.6
Group 2- diabetes controlled with anti-hyperglycaemics	30	5.9±0.82	105.7±20.2
Group 3 - diabetes controlled with herbal preparations	30	5.5±0.82	94.6±21.2
Group 4- Healthy controls	30	4.8±0.7	110.09±17.67

TABLE 2 SHOWS STUDY GROUPS WITH PARAMETERS PI, T AND B CELL PROLIFERATION

Groups	PI				T cell proliferation	B cell proliferation
	NO	WBO	C3 O	IgG O		
Group 1- uncontrolled diabetes	0.33±0.16	2.08±0.79	1.08±0.33	1.11±0.56	0.15±0.02	0.08±0.007
Group 2- diabetes controlled with anti-hyperglycaemics	1.04±0.27	4.94±0.86	2.33±0.73	2.58±0.60	0.54±0.16	0.09±0.03
Group 3 - diabetes controlled with herbal preparations	1.16±0.42	5.80±0.58	2.98±0.76	2.60±1.08	0.86±0.25	0.13±0.02
Group 4- Healthy controls	1.17±0.41	6.04±1.26	2.93±1.15	2.68±1.02	0.91±0.25	0.13±0.04

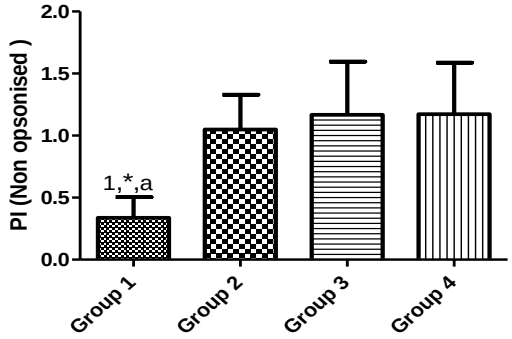

Figure 1 **PI (NO) of the study groups**
Values expressed as Mean ± SD in each group
1,*,a < 0.0001 vhs compared to group 2,
group 3 & control group 4 respectively
.Groups 2 and 3 do not differ significantly
No significant change seen in groups 2 and 3
when compared to control group 4

PI (NO) - Figure 1

Phagocytic index of the neutrophils without the influence of opsonins -PI (NO) - Figure 1

In group 1 when non opsonised bacteria was used to study the PI, PI decreased very highly significantly while compared to control subjects (p value >0.0001). There was no statistical difference between groups 2 and 3 when compared to control subjects. The PI in group 1 was very highly significantly lowered when compared to both groups 2 and 3 (p value >0.0001). Results suggest that neutrophil functions are decreased in uncontrolled DM patients. No statistical difference in PI between groups 2 and 3 was seen.

Phagocytic index of the neutrophils with the influence of opsonins present in the serum, PI (WBO) - Figure 2, PI (C3) - Figure 3, PI (IgG) - Figure 4

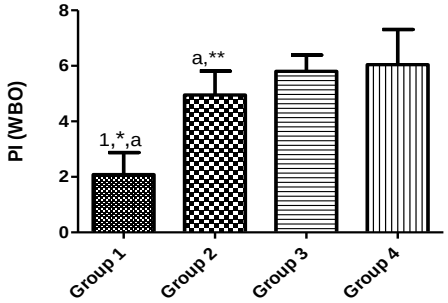

Figure 2. PI (WBO) of the study groups
Values expressed as Mean ± SD in each group
1,*,a < 0.0001 vhs compared to group 2,
group 3 & control group 4 respectively.
**.< 0.001 hs compared to group 3
No significant change between group 3 and
control group 4

PI (WBO) - Figure 2

Further to understand whether neutrophil function itself is decreased or whether phagocytic capacity is decreased due to the defect in opsonization, bacteria that was used to study the PI was opsonised with autologus serum. Results reveal that, in groups 1 and 2, PI (WBO) decreased with very highly significant difference, when compared to control subjects. There was no statistically significant difference in the values of PI (WBO) when group 3 was compared to control group. On comparison of group 1 with groups 2 and 3, in both the cases a very highly significant decrease in PI (WBO) (p value < 0.0001) was seen. Group 2 has a decreased PI (WBO) (p value < 0.01) when compared to group 3.

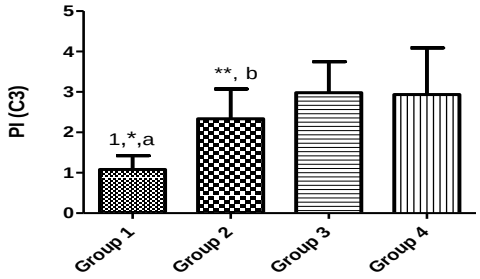

Figure 3. PI (C 3) of the study groups
Values expressed as Mean ± SD in each group
1,*,a < 0.0001 vhs compared to group 2,
group 3 & control group 4 respectively.
**.< 0.001 hs compared to group 3
b.< 0.01 sig compared to group 4
No significant change between group 3 and
control group 4

PI (C3) – Figure 3

Further with a aim to understand the whether it is the C3 opsonin is defective, the bacteria that was used to study the PI was opsonised with IgG depleted serum. Results reveal that, in groups 1 and 2 PI (C3) decreased with very highly significant difference (p value >0.0001), and significant difference (p value >0.01) when compared to control subjects. There was no statistical difference between group 3 and control group. On comparison of group 1 with groups 2 and group 3 a very highly significantly decreased (p value >0.0001) in PI (C3) was seen. Group 2 has a decreased PI (C3) (p value < 0.001) when compared to group 3 .

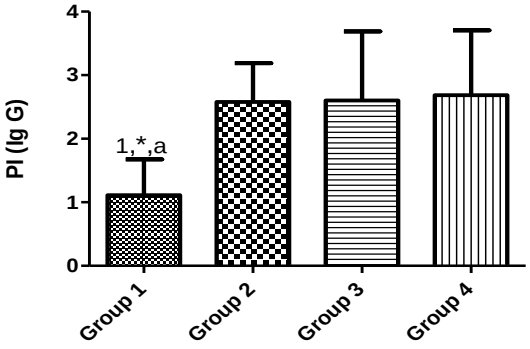

Figure 4. PI (IgG) of the study groups
Values expressed as Mean ± SD in each group
1,*,a < 0.0001 vhs compared to group 2,
group 3 & control group 4 respectively.
.Groups 2 and 3 do not differ significantly
No significant change seen in groups 2 and 3
when compared to control group 4

PI (IgG) – Figure 4

Further with an aim to understand whether it is the IgG opsonin that is defective, bacteria that was used to study the PI was opsonised with C3 inactivated serum. In group 1 PI (IgG) decreased with very highly significant difference (p value >0.0001) compared to control subjects and when compared to group 2 and group 3. In groups 2 and 3 PI (IgG) was not statistically different than the control group. The PI (IgG) values were similar in group 2 and 3.

The results are suggestive that the opsonisation capacity has been decreased in uncontrolled DM patients and also DM patients who are on anti hypergycelmic drugs. Only opsonisation by IgG is similar in patients receiving anti hyp[ergycemic drugs and herbal preparation for blood glucose control

T and B cell proliferation in response to mitogens -Figures 5 & 6

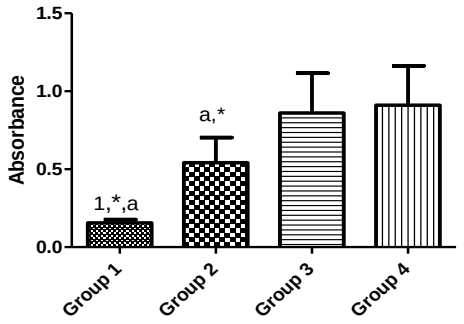

Figure 5. T cell proliferation in response to Con A of the study groups

Figure 1 **PI (NO) of the study groups**
Values expressed as Mean ± SD in each group
1,*,a < 0.0001 vhs compared to group 2,
group 3 & control group 4 respectively
*.< 0.0001 vhs compared to group 3
No significant change between groups 3
and control group 4

T cell proliferation – Figure 5

T cell proliferation rate was decreased (p value >0.0001) in both groups 1 and 2 when compared to control group. There was no statistical difference between group 3 and control group 4. On comparison of group 1 with groups 2 and 3, group 1 showed a very highly significant decrease (p value >0.0001) in T cell proliferation rate. Values differed between groups 2 and 3 and in group 2 the T cell proliferation rate was lesser were compared to group 3 (p value >0.0001).

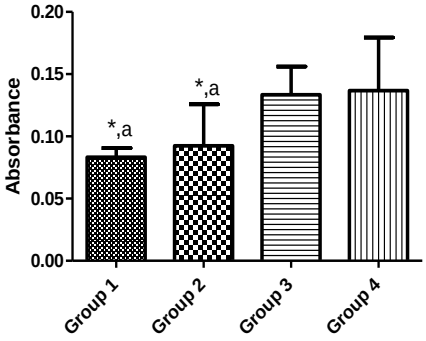

Figure 6.. B cell proliferation in response to LPS of the study groups

Values expressed as Mean ± SD in each group
*, a < 0.0001 vhs compared to group 3 &
control group 4 respectively
Groups 1 and 2 do not differ significantly
No significant change between group 3
and group 4 was observed

B cell proliferation – Figure 6

B cell proliferation rate was decreased (p value >0.0001) in both groups 1 and 2 when compared to control group. There was no statistical difference between group 3 and control group 4. On comparison of group 1 and group 2 with group 3, group 1 and 2 showed decreased B cell proliferation rates (p value >0.0001)

DISCUSSION

The present investigation suggests decreased neutrophil phagocytic capacity in type 2 DM patients when compared to control subjects. PI (NO), PI (WBO), PI (C3) and PI (IgG) are found to be greatly decreased in group 1 patients whose diabetes was not under control . In DM patients whose blood glucose is controlled phagocytic capacity of neutrophils is ameliorated suggesting that PI is dependent on mean glucose value in the blood [22]. In group 2 patients whose diabetes was under control with antihyperglycaemics the PI (NO), PI (WBO), PI (C3) and PI (IgG) was found to be lowered as compared to controls but was more than that of group 1 patients. In group 3 patients whose diabetes was under control using herbal preparations the PI(NO), PI (WBO), and PI (C3) was almost as similar as the control group suggesting that the herbal preparations were exerting a therapeutic effect on the innate immune system thereby ameliorating the phagocytic functions of the neutrophils and the serum opsonization capacity, additionally T and B cell proliferation rate which was lowered in groups 1 and 2 when compared to healthy controls was increased in group 3 suggesting that the innate immunity in patients treated with herbal preparation is enhanced.

Neutrophils play an essential role in the host inflammatory response. In diabetic patients chemotactic activity of neutrophils is significantly lower than in the cells from healthy controls leading to a decrease in PI[23], decreased bactericidal activity[24] and impairment of phagocytosis [25]. DM lead oxidative stress affects the neutrophils functions, oxidative stress causes lipid peroxidation and alters the structure of cell membrane lipids, and this depresses phagocytosis and cell viability[2]. Patients with DM also display significant increment in the basal levels of calcium in neutrophils which is associated with marked impairment in their phagocytosis[26]. One of the commonest complication in DM are atherosclerotic lesions which are characterized by the presence of a large number of macrophages, proper phagocytic function is required. Improper removal of these macrophages from the tissue results in necrosis which causes adverse inflammatory reaction and severe complications such as plaque rupture and thrombosis. This effect could be due to the dysfunction of the macrophages of atherosclerotic tissue wherein they become less efficient phagocytes[27]. Even though C3 and IgG levels are higher in DM patients [28], enhanced PI is not observed, this may be due to the decreased activity of the neutrophils itself. The decrease in the overall opsonization capacity in DM patients may be due to gylcosylation of the opsonins [29].

Proliferation of lymphocytes which is impaired in response to mitogen could be due to potential increase in basal levels of intracellular calcium in the lymphocytes of diabetic patients. Similar decrease in proliferation B lymphocytes was reported in other clinical conditions such as in chronic renal failure where elevation in intracellular calcium of B cells is associated with impairment in their proliferation and in antibody production[30]. Suppression of cellular immunity and reduced mitogenic response to several antigens has been reported in experimental diabetic animals as well[31]. In DM altered functions of different types of circulating immune cells, T lymphocytes and B lymphocytes has also been reported [8, 32]. Moreover common hypertensive drugs cause elevation in cell calcium, which reduces cell proliferation, mainly B cell proliferation, along with neutrophil phagocytic function[10, 28].

DM lead oxidative stress is responsible for immune cell dysfunctions[10]. The redox imbalance in DM patients extends to circulating lymphocytes, as shown by Rosemari Otton et al[33]. Experimental evidence suggests that antioxidant supplementation may reduce oxidative stress in diabetics and thus can modulate immune cells[34].

Increased basal levels of intracellular calcium in the lymphocytes of DM patients can lead to decreased response to mitogenic stimuli. The antioxidants present in the herbal preparation may be responsible in bringing about immune modulation observed in our study. Several medicinal plants have been reported to possess strong antioxidant activity. The health benefits of these plants are related to their antioxidant activity[35]. Antioxidants could modulate the functions of the neutrophils, their opsonising capacity and T and B cell proliferation response. Herbal preparations are said to affect the immune reactions through their anti–inflammatory actions. In most cases the therapeutic efficiency of these plants may, in part, be mediated via their influence on the immune response[36].

This study demonstrated that herbal formulations could re-establish the neutrophil functions and the lymphocytes proliferation capacity pointing out that they can restore innate immune functions in DM patients. This enhancement of immune functions can also decrease the risk of IHD. Lower risk for IHD for DM patients taking herbal preparations is that they may act against altered PMN function, which may play a causative role in atherogenesis and atheroprogression and suggests the potential importance of modulating neutrophilic and immune response with herbal preparations as part of a novel, improved strategy for protecting against complication of DM by modulating the immune functions.

REFERENCES

1. Lawrence HY, Frans JTW, Deborah AC, Janice AD, Eugene JB, Raymond T et al. Cardiac Outcomes After Screening for Asymptomatic Coronary Artery Disease in Patients With Type 2 Diabetes. JAMA. 2009; 301(15):1547-1555.
2. Marin DP, Bolin AP, Macedo RCS, Sampaio SC, Otton R. ROS production in neutrophils from alloxan-induced diabetic rats treated in vivo with astaxanthin. International Immunopharmacology 2011; 11: 103-109.
3. John RG, Keith FK. Development of the Lipid-Rich Core in Human Atherosclerosis. Arterioscler Thromb Vasc Biol 1996; 16:4-11.
4. Watanabe T, Haraoka S, Shimokama T. Inflammatory and immunological nature of atherosclerosis. Int. J. Cardiol 1996; 54 (Suppl) : 51-60.
5. Emmanuelle C, Alan Hl. Identification of Two Distinct Mechanisms of Phagocytosis Controlled by Different Rho GTPases. Science 1998; 282: 27.
6. David B, David ER. The phagocytic activity of polymorphonuclear leukocytes obtained from patients with diabetes mellitus. J.lab.clin med 1964; 64:1
7. Leonardo L, Gianni C, Maria AA, Andrea Si, Egidio L. Oxidative Metabolism of Polymorphonuclear Leukocytes and Serum Opsonic Activity in Chronic Renal Failure. Nephron 1989; 51:44-50.
8. Marhoffer W, Stein M, Maeser EF, Federlin K. Impairment of polymorphonuclear leukocyte function and metabolic control of diabetes. Diabetes Care 1992; 15: 256–260.
9. Otton R, Soriano FG, Verlengia R, Curi R. Diabetes induces apoptosis in lymphocytes.J. Endocrinol 2004; 182: 145–156.
10. Alexiewicz JM, Kumar D, Smogorzewski M, Klin M, Massry SG. Polymorphonuclear leukocytes in non-insulin dependent diabetes mellitus: Abnormalities in metabolism and function. Ann Intern Med 1995;123:919-924.
11. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N. Engl. J. Med 2005 ;352:1685–1695.
12. Ahmad R, Javed S, Bhandari U. Antiapoptotic potential of herbal drugs in cardiovascular disorders: An overview. Pharmaceutical Biology 2010; 48(4): 358–374.
13. Paul EL, Lunardelli A, Caberlon E, Oliveira CB, Santos RCV, Biolchi V et al. Anti-inflammatory and immunomodulatory effects of Baccharis trimera aqueous extract on induced pleurisy in rats and lymphoproliferation in vitro. Inflammation 2009; 32:6.
14. Bunn, H.F. Evaluation of glycosylated hemoglobin in diabetic patients. Diabetes 1981; 30(7):613–617.
15. Boyum A. Isolation of mononuclear cells and granulocytes from human blood: isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation, and sedimentation at 1 g. Scand J Clin Lab Invest 1968; 97:S77-S89.
16. Niemailtowski M, Nonnecke BJ, Targowski SP. Phagocytic activity of milk leukocytes during chronic Staphylococcal Mastitis. J. dairy science 1988; 71:780-787.
17. Muniz-Junqueira MI, Peçanha LMF, Silva-Filho VL, Cardoso MCA, Tosta CE. Novel mirotechnique for assessment of postnatal maturation of the phagocytic function of neutrophils and monocytes. Clinical and Diagnostic Laboratory Immunology 2003; 10: 1096-1102.
18. Kallman J, Scholln J, Schalen C, E Ann, K Erik.Impaired Phagocytosis and Opsonisation towards Group B Streptococci in Preterm. Neonates. Dis Child Fetal Neonatal Ed 1998; 78: 46-50.
19. Tofte RW, Peterson PK, Schmeling D, Brake J, Kim Y, Paul Q. Opsonisation of four bacteroides Species: Role of classical Complement pathway and Immunoglobulins. Infection and immunity 1980; 27:784-792.
20. Duc HT, Nakagawa S, Rucay P, Righenzi S, Voisin GA. Differential modulation of the in vitro lymphocyte activation pathways by soluble and solubilized placental substances, Am. J. Reprod. Immunol 1990; 24: 73–79.
21. Van de Loosdrecht AA, Beelen RH, Ossenkoppele GJ, Broekhoven MG, Langenhuijsen MM. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloidleukemia. J. Immunol Methods 1994; 174(1-2): 311-320.
22. Jakelić J, Kokić S, Hozo I, Maras J, Fabijanić D. Nonspecific immunity in diabetes: hyperglycemia decreases phagocytic activity of leukocytes in diabetic patients. Med Arh 1995; 49(1-2): 9-12.
23. Mowat AG, Baum J. Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. N Engl J Med 1971; 284:621-627.
24. Sanchez A, Reeser JL, Lau HS, Yahiku PY, Willard RE, McMillan PJ et al .Role of sugars in Human Neutrophilic Phagocytosis, The American Journal of Clinical Nutrition 1973; 26(11):1180-1184.
25. Van Oss CJ. Influence of Glucose Levels on the In Vitro Phagocytosis of Bacteria by Human Neutrophils. Infect Immun 1971; 4(1): 54-59.
26. Lijnen P, Fagard R, Petrov V. Cytosolic calcium and lymphoproliferative response during calcium antagonism in men. Eur J Clin Pharmacol 1999; 54: 911-915.
27. Cathcart MK, Morel DW, Chisolm.GM. Monocytes and Neutrophils oxidize low density lipoprotein making it cytotoxic. J. Leukoc. Biol 1985; 38:341-350.
28. Mistry K, Kalia K. Non enzymatic Glycosylation of IgG and their urinary excretion in patients with diabetic nephropathy. Indian journal of clinical biochemistry 2008; 23 (3):159-165.
29. Muscari A, Bozzoli C, Puddu G, Sangiorgi Z, Dormi A, Rovinetti C, Descovich G, Puddu P. Association of serum C3 levels with the risk of myocardial infarction; The American Journal of Medicine 1998;4: 357-364.
30. Alexiewicz JM, Klinger M, Pitts TO, Gaciong Z, Linker-Israeli M, Massry SG. Parathyroid hormone inhibits B cell proliferation: Implications in chronic renal failure. J Am Sot Nephrol 1990; 1: 236-244.
31. Otton R, Carvalho CR, Mendonca JR, Curi R. Low proliferation capacity of lymphocytes from alloxan-diabetic rats: involvement of high glucose and tyrosine phosphorylation of Shc and IRS-1. Life Sci 2002 ; 71: 2759–2771.
32. Casey J, Sturm CH Jr: Impaired response of lymphocytes from non-insulin-dependent diabetics response of lymphocytes from non-insulin-dependent diabetics to staphage lysate and tetanus antigen. J Clin Microbial 1982; 15: 109-114.

33. Otton R, Popp MD, Paola Bolin A, Dos Santos RDCM, Polotow TG, Coccuzzo Sampaio S et al. Astaxanthin ameliorates the redox imbalance in lymphocytes of experimental diabetic rats. Chemico-Biological Interactions 2010 ; 186 : 306–315.
34. Arnlov J, Zethelius B, Riserus U, Basu S, Berne C, Vessby B et al. Serum and dietary beta-carotene and alpha-tocopherol and incidence of type 2 diabetes mellitus in a community-based study of Swedish men: report from the Uppsala Longitudinal Study of Adult Men (ULSAM) study. Diabetologia 2009; 52: 97–105.
35. Fugh-Berman A. Herbs and dietary supplements in the prevention and treatment of cardiovascular disease. Prev Cardiol 2000; 3: 24–32
36. Amirghofran, Z., M. Azadbakht, and M. H. Karimi. Evaluation of the immunomodulatory effects of five herbal plants. J. Ethnopharmacol 2000; 72:167–172.