Original Research Paper

Effect of Plant Spices *(Thymus vulgaris, Murraya koenigii, Ocimum gratissimum, Piper guineense)* on Haemoglobin Glycation, Selected Enzymes and Red Blood Cell Indices in Alloxan-Induced Diabetic Rats

[1]Emmanuel Iroha Akubugwo, [2]Solomon Nwanze Ekoh, [3]victor Chibueze Ude*, [2]Jude Maduabuchi Kamah

[1]Department of Biochemistry, Faculty of Biological and Physical Sciences, Abia State University, PMB 2000, Uturu-Nigeria

[2]Chemical pathology Unit, Medical Laboratory Services, ESUT Teaching Hospital Parklane Enugu-Nigeria

[3]Department of Medical Biochemistry, College of Medicine, Enugu State University of Science and Technology, PMB 01660, Enugu-Nigeria

*Corresponding author E-mail: victor_chibueze@yahoo.co.uk, Tel: +234(0)8039461045

ABSTRACT

Aim: The aim of this study was to determine the effect of pant spices (*Thymus vulgaris, Murraya koenigii, Ocimum gratissimum and Piper guineense*) on hemoglobin glycation (HbA1c), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD) and red blood cell (RBC) indices in alloxan- induced diabetic rats.

Study Design: The animals were grouped into six of 5 rats each. Groups II, III, IV, V and VI were induced diabetes by intraperitoneal injection of alloxan monohydrate with a dose of 170mg/kg body weight. Group I was the control, group II diabetic control and group III to VI were the experimental group. Crude aqueous extracts (500mg/kg body weight) of each of the spices were orally administered to the rats.

Methodology: Blood samples were collected by cardiac puncture after fasting overnight and standard methods were used for the extraction of spices and, determination of biochemical and hematological parameters.

Place and duration of study: The study was carried out at Enugu State University Teaching Hospital from September 2012 to January 2013.

Result: The result showed that glycated hemoglobin, fasting blood sugar and lactate dehydrogenase decreased significantly ($P<0.05$) while glucose-6-phosphate dehydrogenase increased significantly ($P<0.05$) compared to diabetic control group but no significant difference ($P<0.05$) was observed

compared to normal control group. There were no significant differences ($P<0.05$) in red blood cell indices compared to diabetic control and normal control.

Conclusion: This study suggested that the spices extracts can be used to control diabetes and prevent its complications on antioxidant enzymes.

Keywords: lactate dehydrogenase, glucose-6-phosphate dehydrogenase, hemoglobin glycation, hyperglycemia, spices extract.

1. INTRODUCTION

Glycated hemoglobin is modified hemoglobin A. It is the product of a non-enzymatic condensation of glucose with hemoglobin [1]. The rate of glycation depends on the ambient glucose concentration which is usually high in diabetes mellitus. Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in the production and/or effectiveness of insulin. It is among the leading cause of death world-wide. The prevalence of this disease continues to rise in developing countries like Nigeria [2]. Despite the discovery of insulin over a century ago (which helped in improvement and management of diabetes), large gaps still remain in understanding and management of diabetes Mellitus. The lack of knowledge is particularly evident when we consider diabetic complications (such as retinopathy, neuropathy, nephropathy, cardiovascular diseases, etc.), which is a threat to human life, well-being of patients and also a great challenge to the clinician [3].

Diabetes complications usually arise as a result of heterogeneous, toxic, antigenic advanced glycation end products (AGEs) [4]. Knowledge about these AGEs is essential to provide basis for rational treatment and prevention, and to permit sober reflection, whether a careful replacement of insulin therapy can achieve this desired result [3].Hence, numerous studies have focused on the analysis of implicated glycated proteins or the AGEs modified circulating proteins [5].

An increase in oxidative stress may be due to an increase in oxidative process that produce oxidants or due to a decrease in antioxidant defense and proper activity of Glucose-6-phosphate dehydrogenase (G6PD) is required for adequate defense against oxidative stress and prevention of cell damage/death [6]. Increases in oxidative stress in hyperglycemic conditions have been suggested to be of central pathogenic importance [7]. It has also been hypothesized that under high glucose conditions impaired activity of G6PD would predispose cells to oxidant damage and cell death [6].

Lactate dehydrogenase (LDH) is a terminal glycolytic enzyme that plays an indispensible role in the inter conversion of pyruvate to lactate to yield energy under anaerobic condition [8]. Report showed that LDH activity can be altered by insulin, glucose, NADH, increase in mitochondrial membrane potential and, cytosolic free ATP and Ca^{2+} [9]. Increase in activity of LDH interferes with normal glucose metabolism and

insulin secretion in β- cells of pancreas and it is therefore responsible for insulin secretory defects in diabetes [10].

So many herbs and vegetables are known to have anti-diabetic effect but little is known about their ability to prevent or inhibit protein glycation and AGEs formation and, few reports are available on the effect of *T. vulgaris, M. koenigii, O. gratissimum, P. guineense* on G6PD and LDH. It is in a bid to assist in closing this scientific gap that this work was envisioned.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Spices

Fresh leaves of *P. guineense, T. vulgaris, O. gratissium* and *M. koenigii* were purchased from Ogbete Main Market Enugu-Nigeria. The Botanical identification was carried out at the department of Plant Science and Biotechnology, Faculty of Biological and Physical Sciences, Abia State University Uturu. The fresh leaves collected were sorted and all dead matter and unwanted particles were removed. The leaves were air dried for two weeks and grounded into powder using electric blender. The grounded powders were stored in air tight container labeled according to the different species of the spices and kept in the laboratory. A total of 5g of the grounded powder were weighed out from each container and soaked in separately 1000mls of distilled water for 12hrs at room temperature. The mixtures were filtered using Watman (NO 1) filter paper. The filtrates were dried in an incubator at a temperature of 40°C to produce a gel-like extracts which were diluted to 500mg/kg

2.2 Animal Treatment

Thirty (30) male albino rats weighing between 160-220g were used for the study. The rats were randomly placed into 6 groups (group I-VI) with 5 rats in each group. The animals were acclimatized for 7days before the commencement of the experiment and were all allowed free access to food (animal pellet) and water *ad libitum* throughout the experiment. Groups II to VI were induced diabetes by giving 170mg/kg body weight of alloxan monohydrate intraperitoneally. Five days after, fasting blood sugar were determined. Animals with blood glucose of 13mmol/l were used for the experiment and they were treated as follows.

Group I: The animals were non-diabetic and were given distilled water (placebo), and served as normal control.

Group II: The animals were diabetic and were given distilled water (placebo), and served as diabetic control.

Group III: The animals were diabetic and were given 500mg/kg body weight per day of *T. vulgaris* aqueous extract.

Group IV: The animals were diabetic and were given 500mg/kg body weight per day of *P. guineense* aqueous extract.

Group V: The animals were diabetic and were given 500mg/kg body weight per day of *M. koenigii* aqueous extract.

Group VI: The animals were diabetic and were given 500mg/kg body weight per day of *O. gratissium* aqueous extract.

Approval for animal studies was obtained from the animal ethic committee of Faculty of Biological Sciencies Ebonyi State University Abakaliki-Nigeria.

2.3 Collection and Analysis of Blood Sample

The animals were sacrificed after three months by anaesthetizing in a jar containing cotton wool soaked in chloroform. The blood samples were collected through cardiac puncture into K_2EDTA and plain tubes respectively. The blood samples in plain tubes were allowed to clot and were spun in Angular centrifuge (Techmel and Techmel, Engl. Model 80-2) at 3000rpm for 5min to obtain sera. The separated serum samples were stored in refrigerator at -4°C until the next day for the analysis, while the samples in K_2EDTA tubes were used for red blood cell (RBC) indices the same day the blood samples were collected.

The hemoglobin (Hb) estimation was carried out using cyanomethemoglobin method, pack cell volume (PCV) was determined using microhematocrit method and red blood cell (RBC) count was carried out using manual counting method [11]. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were derived using calculation method [11].

MCV was calculated in Femitoliters = PCV (%)/RBC (mm^3) ×10

MCH was calculated in picograms = Hb (g/dl)/RBC (mm^3) ×10

MCHC was calculated in gram per deciliter = Hb (g/dl)/PCV (%) ×100

Glucose oxidase enzymatic method was used to determine fasting blood sugar [12] and glycated hemoglobin in whole blood was determined using the method described by Nitin [13]. Spectrophotometric method and colorimetric were used to determine glucose-6-phosphate dehydrogenase (G6PD) and lactate dehydrogenase activity respectively [14].

2.4 Statistical Analysis

Values were represented as mean ± S.D. Data was analyzed with analysis of variance (ANOVA) and group means were compared using Duncan's multiple range tests at $P<0.05$.

3. RESULT

Table1 showed the blood glucose of rats before and after feeding with spices extracts. Before feeding with the spices extracts, the sugar level of the experimental groups of rats showed no significant difference (P>0.05) compared to diabetes control group but, after treating with spices extracts, the sugar level of experimental groups showed significant decrease (*P*<0.05) compared to diabetes control group.

Table 1. Effect of aqueous extract of the spices on blood glucose of alloxan induced diabetic rats measured in mmol/l.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
FBSa	5.29±1.20[a]	22.08±2.94[b]	21.82±4.83[b]	21.28±4.86[b]	20.98±1.52[b]	22.10±1.14[b]
FBSb	5.32±1.21[a]	23.49±2.0[d]	7.72±2.66[b]	9.66±2.98[b]	8.13±1.73[b]	10.99±2.1[c]

Values are mean ± standard deviation of five determinations. Values with different superscript alphabet are significantly different at *P*<0.05. FBSa=fasting blood sugar level after alloxan-induction of diabetes. FBSb = fasting blood sugar after treatment with the spices for 3 months.

The effects of aqueous extract of the spices on glycated haemoglobin (HbA1c) are as shown in table 2. Glycated haemoglobin of experimental groups showed a significant (*P*<0.05) decrease compared to the diabetes control group.

Table 2. Effect of aqueous extract of the spices on glycated hemoglobin (HbA1c) of alloxan induced diabetic rats measured in percentage (%)

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI
HbA1c	2.8±2.059[a]	5.61±1.65[b]	2.96±0.26[a]	2.85±0.08[a]	2.81±0.18[a]	3.11±0.09[a]

Values are mean ± standard deviation of five determinations. Values with different superscript alphabet are significantly different at *P*<0.05.

Table 3 represents the effect of aqueous extracts of the spices on selected enzyme activities. It was observed that LDH activity showed a significant (*P*<0.05) decrease compared to diabetes control while G6PD showed significant (*P*<0.05) increase compared to diabetes control

Table3. Effect of aqueous extract of the spices on selected enzyme activities of alloxan induced diabetic rats measured in U/L

Enzymes	Group I	Group II	Group III	Group IV	Group V	Group VI

LDH	90.40± 28.68[a]	178.20± 17.11c	107.20± 12.13[a]	87.80± 19.88[a]	91.60± 26.17[a]	128.80± 29.45[b]
G6PD	1.57±0.94[b]	0.54±0.38[a]	1.30±0.21[b]	1.28±0.15[b]	1.36±0.25[b]	1.29±0.32[b]

Values are mean ± standard deviation of five determinations. Values with different superscript alphabet are significantly different at $P<0.05$.

Table 4 showed the effect of aqueous extract of the spices on Red Blood Cell (RBC) indices. The result of the red blood cell indices showed no significant ($P<0.05$) difference when compared to both normal control group and diabetic control group.

Table 4. Effect of aqueous extract of the spices on Red Blood cell (RBC) indices of alloxan-induced diabetic rats

RBC Indices	Group I	Group II	Group III	Group IV	Group V	Group VI
PCV (%)	42.40±4.62 [a]	40.80±4.92[a]	41.20±1.79[a]	39.80±1.92[a]	39.90±6.38[a]	43.80±2.68[a]
Hb (mg/dl)	13.84±0.94[a]	14.14±0.80[a]	13.74±0.42[a]	13.76±1.08[a]	13.10±1.90[a]	13.22±0.89[a]
RBC($\times 10^{12}$/l)	5.46±1.49[a]	5.70±1.25[a]	5.86±0.43[a]	4.88±0.75[a]	4.86±0.26[a]	4.82±0.69[a]
MCH (pg)	26.66±6.30[a]	25.48±3.91[a]	24.22±1.92[a]	26.15±2.13[a]	24.60±4.82[a]	28.64±5.11[a]
MCHC (g/dl)	33.12±1.50[a]	35.68±3.18[a]	33.04±1.11[a]	32.66±1.05[a]	32.70±1.68[a]	31.66±1.37[a]
MCV (fl)	81.54±15.43[a]	72.54±11.33[a]	76.50±10.72[a]	80.44±10.21[a]	76.80±14.71[a]	87.64±14.05[a]

Values are mean ± standard deviation of five determinations. Values with different superscript alphabet are significantly different at $P<0.05$.

4. DISCUSSION

Diabetic complications results from non-enzymatic glycation of proteins. Glycation inhibitors are part of the ongoing research. Hence, the inhibitory effect of some spices on hemoglobin glycation shown in table 2. A decrease in glycosylated hemoglobin when diabetic rats were treated with *M. koenigii* was observed by Arulselvan and Subramanian [15]. It was also reported that the peak of glucose tolerance curve correlates with glycation and with the improvement of glycemic control, glycated hemoglobin decreased [16].

This work revealed that *T. vulgaris* is a better hypoglycemic agent followed by *P. guineense* as shown in table 1. On the other hand, *P. guineense* is a better inhibitor of hemoglobin glycation than *T vulgris* (table 2). This is an indication that reduction in HbA1c by the spices may not be only as a result of its hypoglycemic effect, but might have inhibitory action on other step(s) of amadori reaction. The extract might act as a competitive inhibitor at the committed step of protein glycation. The initial step in hemoglobin glycation is the binding of sugar and amino group of protein. This reaction can be inhibited by any compound that is capable of competing with the binding site [17]. Hyperglycemia produces free radicals which are known to stimulate protein glycation [18]. Researcher has reported that free radicals and trace elements are promoters of AGEs formation [19]. Flavonoid, saponin, alkaloid, tannin are both antioxidant and chelating agent and can block protein glycation by oxidizing the free radicals [19, 20, 21, 22]. The extracts can promote proteolysis of AGEs because cellular proteolysis of AGEs produces AGEs peptide and AGEs – free adducts and if released into the plasma can be excreted in the urine [23].

The result of this work showed a significant decrease ($P<0.05$) in the level of LDH activity when diabetic control rats were compared with experimental groups. The high level of the serum LDH level in the diabetic rats might be as a result of lack of inactive insulin and lack or inhibition of insulin activity which encourages tissue catabolism. This is in agreement with the work carried out by Celik et al. [24}, who reported increase in serum lactate dehydrogenase level in diabetic patients. However, it contradicts the work of Oliver et al. [25], who reported non-significant difference in the LDH activity of diabetic and non-diabetic rats. The reversal effect of LDH activity by the spices as shown in table 3 indicates the ability of the spices to induce better utilization of glucose and this may be attributed to the phytochemical constituents of the spices. The reduction in LDH activity may be by regulation of pyruvate and NADH thereby promoting the mitochondrial oxidation of glucose and the protective effect of the spices may be an indication of the ability of the extract to prevent any leakage of marker enzyme [10].

Increase in oxidative stress may be due to increase in the processes that produce oxidant or decrease in antioxidant defense [26]. Glucose-6-phosphate dehydrogenase, the first and the rate limiting enzyme in the pentose phosphate pathway, is regarded for its antioxidant defense system because it produces reduced nicotinamide adenine dinucleotide phosphate (NADPH), the cells principal reductant. It has been shown that G6PD deficiency has a positive association with diabetes mellitus [27]. Hyperglycemia leads to an increase in cyclic adenosine monophosphate (cAMP) via cAMP-dependent phosphokinase A (PKA). This causes phosphorylation and inhibition of G6PD activity leading to decreased NADPH, increased reactive oxygen species and then cell damage/death [6, 28]. This research showed increase in the activity of G6PD after treatment with spices (table 3). The enhanced activity correlates with the hypoglycemic effect of the spices. *T. vulgaris* and *M. koenigii* which were better hypoglycemic agents enhanced the activities of the enzyme better than *P. guieneense and O. gratissimum.*

Our result showed little increase in the pack cell volume of *M. koenigii* and *P. guieneense* and slightly lowered hemoglobin level, but these values had no significant ($P<0.05$) difference, when diabetic control rats were compared with experimental groups and normal control group respectively. This work differed from the work of Tanko et al. [29], who showed significant increase in the PCV of diabetic patients. However increase in PCV level of diabetic patients is more pronounced in electronically determined PCV and in patients of glucose level above 40mmol/l [30]. The electronically determined PCV depends on the optically measured mean cell volume (MCV) to determine both PCV and MCHC. The observed non-significant difference may be attributed to the used of manual hematocrit centrifuge which is not affected by tonicity of red blood cells and/or viscosity of whole blood as a result of high glucose level.

This work did not agree with the work of Thomas et al. [31], who showed decrease in Hb of diabetic patient. This disagreement could be explained on the basis that end stage organs like kidney are yet to be involved. The kidney produces the hormone, erythropoietin which initiates erythropoiesis and untreated hyperglycemia causes renal dysfunction leading to non-production of the hormone and eventual anemia.

5. CONCLUSION

The present work showed that the administration of the aqueous extract of the Spices (*T. vulgaris, M. koenigii, O. gratissimum and P. guineense*) on alloxan- induced diabetic rats led to significant ($P<0.05$) decrease in blood glucose level, LDH and glycated hemoglobin while a significant ($P<0.05$) increase was observed in G6PD and no significant difference ($P<0.05$) was found in RBC indices. Therefore it can be deduced from the result that the species possess hypoglycemic properties, inhibit hemoglobin glycation and prevents other diabetic complications.

COMPETING INTERESTS

Authors have declared that no competing interest exists.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration of the Authors. Authors EIA and SNE designed the study and wrote the protocol. Authors VCU, SNE and SMK managed the literature search and wrote the first draft of the manuscript. Author VCU performed the statistics. Authors EIA and ESN managed the analyses of the study. All the authors read and approved the manuscript.

CONSENT

Not applicable.

REFERENCES

1. Chandellia HB, Krishnaswamy PR. Glycated hemoglobin. Current Science. 2002;83(12):1522-1532.

2. Gill G. Practical management of diabetes in tropics. Tropical Doctor. 1990;20(1):4-10.

3. Brownlee M. Glycation products and the pathogenesis of diabetic complications. Diabetes Care. 1992;15(12)1835-1843.

4. Brownlee M, Ceremi A, Vassa H. Advance glycation end products in tissue and the biochemical basis of diabetic complications. New England Journal of Medicine. 1988;318(20):1315-1321.

5. Makita Z, Viassara H, Rayfield E, Cartwright K, Freidman E, Roddy R, Ceremi A, Bucala R. Hemoglobin AGE: A circulating marker of advanced glycosylation. Science. 1992; 258(5082):651-653.

6. Zhang Z, Apse K, Pang J, Stanton RC. High glucose inhibits Glucose-6-phospahte dehydrogenase via C-AMP in aortic endothelia cells. The Journal of Biological Chemistry. 2000;275(51): 40042 – 40047.

7. Giugliano D, Ceriello A,Pasolisso G. oxidative stress and diabetic vascular complication. Diabetes Care. 1996; 19(3):257-267.

8. Kavanagh KL, Elling RA, Wilson DK. Structure of *Toxoplasma gondii* LDH1: active site difference from human lactate dehydrogenase activity and the structural basis for efficient $APAD^+$ use. Biochem. 2004;43:879-889.

9. Ainscow EK, Zhao C, Rutter GA. Pivotal role of lactate dehydrogenase activity in the control of mitochondrial metabolism in MIN6 beta cells. Diabetogia. 1999;42:A140

10. Reddy SR, Lavany T, Narasimhulu G, Reddy KS. Effect of *Pimpella tirupatiensis* on oxidative enzymes in STZ-induced diabetic rat kidney. Iranian ournal of Pharmaceutical Research. 2012;11(1):277-286.

11. Ochei A, Kolhatkar A. Medical Laboratory Science: Theory and Practical. New Delhi India Tata, McGraw Company; 2008.

12. Trinder P. Determination of blood glucose using 4-aminophenazone, J Clin Pathol. 1969;22:246.

13. Nitin S. HbA1c and factors other diabetes mellitus affecting it. Singapore Medical Journal. 2010;51(8):616-622.

14. Kachmar JF, Moss DW. Enzymes In*:* Tietze NW editor. Fundamentals of Clinical Chemistry. 11th ed. Philadelphia: W.B Saunders Company; 2009.

15. Arulselvan, P, Subramanian S. Effect of *Murraya koenigii* leaf extract on carbohydrate metabolism studied in streptozoticin Induced diabetic rats. International Journal of Biological Chemistry. 2007;1:21−28.

16. Koenig RJ, Peterson CM, Kido C. Haemoglobin A_1c as an indicator of the degree of glucose intolerance in diabetes. Diabetes.1976;25:230-232.

17. Khataimi m. Role of pyridoxal phosphate/pyridoxine in diabetes: Inhibition of non-enzymatic glycosylation. Annals of the York Academy of Science.1990;585:502- 504.

18. Vinson JA, Howard TB. Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients, Journal of Nutritional Biochemistry. 1996; 7: 659 – 663.

19. Kaiserova H, Simunek T, Vander-vijgh WJ, Bast A, Kvasinckova E. Flavonoid as protectors against doxorubicin cardotoxicicity: role of iron chelation, antioxidant activity and inhibition of carboxyl reductase. Biochemica Biophysica Acta. 2007;1772(9):1065-1074.

20. Dearlove RP, Greenspan P, Hartle DK, Swanson RB, Hargrove J. Inhibition of protein gylcation by extracts of culinary herbs and spice. journal of medicinal foods.2008; 11(2):275-281.

21. Takagi Y, Kashiwagi A, Tanaka Y, Asahina T, Kikkawa R, Shigeta Y. Significance of fructose-induced protein oxidation and formation of advanced glycation end product. Journal of Diabetes Complications. 1995;9:87 – 91.

22. Halliwel B. Dietary polyphonols; good, bad, or indifferent for your health. Cardiovascular Research. 2007;73(3):341- 347.

23. Gugliucci A, Mehlhaff K, Kinugasa E, Ogata H, Hermo R, Schulze J, Kimura S. Paraoxonase-1 concentration in end stage renal disease patients increase after hemodialysis: Correlation with low molecular age- adduct clearance. Clinica Chemica Acta. 2007;377(1-2) 213 – 220.

24. Celik I, Yegin E, Odabasoglu F. Effect of experimental diabetes mellitus on plasma lactate dehydrogenase and glutamic oxalocetatic transminase level in rabbits. Turkey Journal of Biology. 2002;26:151-154.

25. Oliver RC, Tervonen T, Flynn DG, Keenan KM. Enzyme activity in clavicular fluid in relation to metabolic control of diabetes and other periodentals risk factors. Journal of Periodentol. 1993;64(5):358 – 362.

26. Stanton RC. Diabetic nephropathy and oxidative stress. United States Endocrine Disease. 2006;65-67.

27. Adinortey M B, Owusu RK, Galyuon IKA, Ekloh W, Owusu I, Larbi D A. G-6-PD deficiency, a potential risk factor for development of diabetes mellitus. Journal of Medicine and Medical Science. 2011;2(8):1017−1021.

28. Xu Y, Osborne BW, Stanton RC. Diabetes causes inhibition to Glucose-6-phosphate dehydrogenase via activation of PKA, which contributes to oxidative stress in rat kidney cortex. American Journal of Physiology – Renal Physiology. 2005;289(5): 1040 – 1047.

29. Tanko Y, Mabrouk MA, Adelaiye AB, Fatihu NY, Musa KY. Antidiabetic and some haematological effect of ethylacetate and n-butanol fraction of indigofera pulchra extract on alloxan induced diabetic wister rat. Journal of Diabetics and Endocrinology. 2011;2(1): 1 – 7.

30. Straucheen JA, Alston W, Anderson J, Gustafson Z, Fajardo LF. Inaccuracy in automated measurement of hematocrit and corpscular indices in the presence of hyperglycemia. Blood. 1981;57(6):106–1067.

31. Thomas MC, Macisaac RJ, Tsalamandris C, Mohyneaix L, Goubina I, Fulcher G, Yue,D, Jerunis G. Anaemia in patients with Type I diabetes. The Journal of Clinical Endocrinology and Metabolism. 2004;89(9):4359 – 4363.