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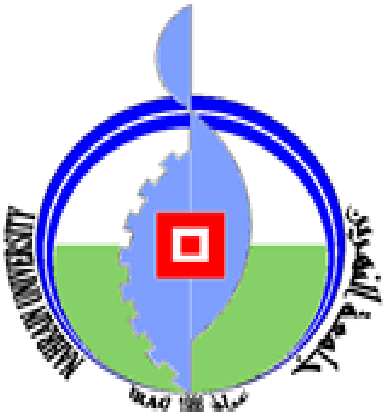

The Role of Oxidative Stress and Some Biochemical Aspects in Patients with Type II Diabetic Nephropathy

A Thesis

Submitted to the Council of Science College / Al-Nahrain University, In partial Fulfillment of the Requirements for the Degree of Master of science in Biotechnology

By

Ala'a Waleed Abbas

B.Sc. Biotechnology/College of Science/Al-Nahrain University (2014)

Supervised by

Prof. Dr. Waleed Hameed Yousif

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Supervisor Certification

I, certify that this thesis entitled **"The role of oxidative stress and some biochemical aspects in patients with type Π diabetic nephropathy"** was prepared by the student **"Ala'a Waleed Abbas "** under my supervision at the College of Science / Al- Nahrain University as a partial fulfillment of the requirements for the degree of Master of Science in Biotechnology.

Signature:

Name: **Dr.Waleed Hameed Yousif**
Scientific Degree: Professor
Date:

In view of the available recommendations, I forward this thesis for dissertation debate by the examining committee.

Signature:

Name: **Dr.Hameed M. Jasim**
Scientific Degree: Professor
Title: Head of Biotechnology Department

Committee Certification

We, the Examining Committee certify that we have read this thesis and examined the student in its content and according to our opinion, is accepted as a thesis for the Degree of Master of Science in Biotechnology.

Signature:

Name: **Dr. Saad S. AL-Dujaily**
Scientific Degree: Professor
Date:

(Chairman)

Signature:
Name: **Dr. Abbas Mahdi Rahmah**
Scientific Degree: Assistant professor
Date:

(Member)

Signature:
Name: **Dr. Rehab S. Ramadhan**
Scientific Degree: Assistant professor
Date:

(Member)

Signature:
Name: **Dr. Waleed Hameed Yousif**
Scientific Degree: Professor
Date:

(Member and Supervisor)

I, hereby certify upon the decision of the Examining Committee.

Signature:

Name: **Dr. Hadi M. A. Abood**
Scientific Degree: Professor
Title: Dean, College of Science
Date:


بسم الله الرحمن الرحيم
*رَبِّ اشْرَحْ لِي صَدْرِي
وَيَسِّرْ لِي أَمْرِي *وَاحْلُلْ عُقْدَةً
مِّن لِّسَانِي *يَفْقَهُوا قَوْلِي
صدق الله العظيم
سورة طه 25-28

Dedication

To my father……..

For his

unlimited care, guidance and

Sacrifices

To my lovely mother …….

For her

unlimited love and Kindness

To my dearest brothers and

sister ……

I dedicate this work

In gratitude for your

Support

Patience

Understanding

Ala'a

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At the beginning, thanks to great Allah, Lord of the whole creation who gave me the faith and strength to accomplish this work.

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Summary

This work was designed to study the role of oxidative stress and some biochemical aspects in nephropathy in type 2 diabetic patients.

Thirty – one type 2 diabetic nephropathy and thirty type 2 diabetes mellitus patients attending to the abd- Almajid private hospital between December 2015 to July 2016 were recruited for this study. For the purpose of comparison, twenty - eight healthy controls subjects matched for age were also included.

Random blood sugar, serum urea, serum creatinine, glycosylated hemoglobin, serum complements 3 and 4, serum malondialdehyde, serum glutathione and serum catalase activity were measured in blood. Microalbumin, creatinine and albumin / creatinine ratio were measured in urine.

The results of the present study showed an in increase in random blood sugar in diabetic and diabetic nephropathy patients at the ages of 45-80 years. No change was observed in serum urea concentration in diabetic patients, whereas an increase was found in diabetic nephropathy patients at the ages of 45-80 years. The findings also showed no change in serum creatinine concentrations in diabetic patients at the ages of 45-80 years and in diabetic nephropathy patients at the age of 45-56 years. Serum creatinine concentrations increased significantly in diabetic nephropathy patients at the ages of 57-80 years. The percentages of glycosylated hemoglobin increased in diabetic and diabetic nephropathy patients at the ages of 45-80 years. No changes were recorded in serum complements 3 and 4 concentrations in diabetic and diabetic nephropathy patients of all age groups. No changes were observed in serum malondialdehyde concentrations in diabetic and diabetic nephropathy patients at the age of

45-68 years, whereas a decrease in serum malondialdehyde concentration was noticed in diabetic and diabetic nephropathy patients at the ages of 69-80 years. Serum glutathione concentrations showed nonsignificant changes in diabetic and diabetic nephropathy patients at the ages of 45-68 years. At the age of 69-80 years, serum glutathione concentration decreased in diabetic and diabetic nephropathy patients. An increase in serum catalase activity was demonstrated in diabetic and diabetic nephropathy patients at the ages of 45-80 years. No change was observed in urine microalbumin concentration in diabetic patients , while an increase was seen in diabetic nephropathy patients. Urine creatinine concentrations showed no change in diabetic and diabetic nephropathy patients, with the exception of diabetic patients at the age of 45-56 years which demonstrated a decrease, and diabetic nephropathy patients at the age of 69-80 years which showed an increase. Albumin / creatinine ratios increased in diabetic patients at the age of 45-56 years, and diabetic nephropathy patients at the age of 45-80 years. No change was observed in albumin / creatinine ratio in diabetic patients at the ages of 57-80 years.

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List of Abbreviation

Abbreviation	Meaning
ACE	Angiotensin converting enzyme
AER	Albumin excretion rate
AGEs	Advanced glycation end-products
ATP	Adenosine Triphosphate
ADP	Adenosine diphosphate
AKI	Kidney injury
CAT	Catalase
C3, C4	Complements 3 and 4
DN	Diabetes nephropathy
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
ESRD	End-stage renal disease
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EGFR	estimated glomerular filtration rate
FADH	Flavin adenine dinucleotide
GFAT	Glutamine fructose6-phosphate amino transferase
GSH	Reduced Glutathione
GSSG	Oxidized glutathione
GLDH	glutamate dehydrogenase
HbA_1c	Glycosylated hemoglobin
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl radical
IDF	International Diabetes Federation
Ig	Immunoglobulin
MALB	microalbuminuria
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
MAPK	Mitogen-Activated-Protein Kinase
NO	Nitric oxide
NO^-	Nitroxyl
NADPH	Nicotinamide adenine dinucleotide
NAC	N-acetylcysteine
NF-κB	Nuclear factor

List of Abbreviation

O.D	**Optical Density**
Ox-LDL	**Oxidized low-density lipoproteins**
$OONO^-$	**Peroxynitrite**
O_2^-	**Superoxide anion**
PKC	**Protein kinase C**
ROS	**Reactive oxygen species**
RNS	**Reactive nitrogen species**
RSNO	**Nitrosothiol**
RAGE	**Receptor for AGE binding**
TTAB	**tetradecyltrimethylammonium bromide**
TGF-β	**Transforming growth factor**
UDP	**Uridine-diphosphate**
UV	**Ultraviolet**
VEGF	**Vascular endothelial growth factor**
WHO	**World Health Organization**

Chapter One

Introduction and Literatures Review

1. Introduction and literature review.

1.1. Introduction.

The term diabetes mellitus (DM) describes a metabolic disorder of multiple etiologies characterized by disturbances of carbohydrate ,fat and protein metabolism resulting from defects in insulin secretion , insulin action or both (WHO, 1999). It is a major worldwide health problem predisposing to markedly increased cardiovascular mortality and serious morbidity and mortality related to the development of nephropathy , neuropathy and retinopathy (WHO, 2005).Three principal types of DM are recognized ; Type 1 DM(T1DM), Type 2DM (T2DM) and gestational diabetes. Type 1 diabetes mellitus is characterized by loss of the insulin- producing beta cells of the islets of Langerhans in the pancreas leading to a deficiency of insulin, while T2DM is characterized differently and is due to insulin resistance or reduced insulin sensitivity , combined with relatively reduced insulin secretion (American Diabetes Association, 2012). Due to increasing obesity, sedentary life style and dietary habits in both Western and developing countries, the prevalence of T2DM is growing at an exponential rate. The increase in T2 DM is also seen in younger people and in developing countries , and estimates in the Middle East and Africa revealed that the prevalence is high and set to increase dramatically during the next 18 years (WHO,2008; Li *et al*., 2009).The etiology of T2DM is not well-understood , although associated health risk factors are recognized ; for instance, a family history of diabetes age over 45 years , race or ethnic background, metabolic syndrome (also called insulin resistance syndrome), obesity, hypertension, and history of vascular disease such as stroke, abnormal cholesterol levels and history of gestational diabetes (American Diabetes Association , 2012) .Diabetic nephropathy is a major long-term complication of diabetes mellitus (Lu *et al*., 2013 ; Ribeiro *et al*., 2013). It develops in more than of 40% of patients in spite of glucose control(Gross *et al*., 2005). Oxidative

stress has been considered to be a pathogenic factor for diabetic nephropathy (Kumawat *et al*., 2013). Hyperglycemia is believed to activate oxidative stress resulting in proteinuria, (Schena and Gesualdo , 2005 ; Hans *et al*., 2002). It is suggested that increased oxidative stress through reduction of plasma antioxidants and increased lipid peroxidation could intensify mesangial cells susceptibility to free radical injury (Hans *et al*., 2002 ; Walti *et* al., 2002).The biological systems living in aerobic conditions are exposed to oxidants, either generated intentionally or as byproducts. Generally, these oxidants occur in two categories consisting of paramagnetic free radicals: reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS is a collective term used to describe the chemical species that are formed upon incomplete reduction of oxygen and includes superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$).In contrast, RNS refers to all the oxidation states and reactive adducts of nitrogenous nitric oxide synthase (NOS) products, from .nitric oxide (NO) to nitroxyl (NO^-), nitrosothiol (RSNO), and peroxynitrite ($OONO^-$) (D'Autreaux *et al*., 2007).ROS and RNS, previously considered to be toxic agents capable to damage molecules, have indeed critical biological functions essential for normal physiology. All these species are able to initiate or mediate many enzyme- and gene-dependent reactions in both physiological and pathophysiological processes. Overproduction or deficiency of ROS and/or RNS may result in impaired homeostasis and associated pathology. Thus, it is widely believed that multiple pathogenic mechanisms involve disequilibrium in the redox balance as the final common pathway ((D'Autreaux *et al*., 2007).

This study was suggested to fulfill the aim of: study the role of oxidative stress and some biochemical aspects in patients complaining from diabetic mellitus type 2 and nephropathy.

1.2 Literature review.

1.2.1 Definition of diabetes.

Diabetes is a systemic disease characterized by chronic hyperglycemia and disturbances in carbohydrate, lipid, and protein metabolism. It may present with characteristic symptoms such as thirst, polyuria, and weight loss, but its most severe manifestations are ketoacidosis or nonketotic hyperosmolaric coma. The symptoms are often vague or may even be absent altogether (Bennett *et al*., 2005). All over the world, the prevalence rates of diabetes are increasing, and in the latest IDF ATLAS, northern Europe had a 7% prevalence of diabetes in the adult population, while corresponding rates were already 8% in the US and 9% in China (Sicree *et al*., 2003).

1.2.2 Diabetic nephropathy.

1.2.2.1 Definitions.

Diabetic nephropathy is defined as a progressive increase in the urinary albumin excretion rate accompanied by increasing blood pressure and a relentless decline in the glomerular filtration rate with end-stage renal failure as the final endpoint (Marshall *et al*., 2003). Diabetic nephropathy is typically accompanied by retinopathy. Many people with diabetes do not necessarily progress to end-stage renal disease (ESRD), as they may die before then from cardiovascular disease (Borch-Johnsen *et al*., 1987). The different stages of diabetic nephropathy are classified according to the increase in the urinary albumin excretion rate in timed urine collections either overnight or during a 24-h period. Microalbuminuria is defined as an increase in the AER above normal (i.e. ≥ 20 μg/min or ≥ 30 mg/24 h). Proteinuria represents an increase in albuminuria of ≥ 200 μg/min or ≥ 300mg/24 h. When daily proteinuria exceeds 3 g, the patient is deemed to have nephrotic syndrome. The final stage of diabetic nephropathy is ESRD. To be classified as microalbuminuric or

proteinuric, the patient's AER must exceed the upper limit in at least two of three urine collections. Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) in Western countries.It is characterized by accumulation of mesangial cells, collagen IV, fibronectin and laminin. Accumulation of these substances activates the oxidative stress cascade and results in overproduction of ROS. As in the eye, mesangial cells are essential to preserve normal function also in the kidney(Tracy *et al*., 1997) By contrast, in diabetic patients accumulation of AGEs can induce TGF-β synthesis, thereby leading to extracellular fibrosis. Studies have demonstrated a role for the Src pathway in this process. Src is a non-receptor tyrosine kinase activated by receptor tyrosine kinases such as EGFR. Different stimuli, such as ROS or high glucose levels, can induce Src-dependent EGFR transactivation, thus resulting in increased synthesis of MAPK and collagen IV (Koenig *et al*., 1999). Glycation of some proteins,such as laminin and collagen IV, can also alter vascular permeability to albumin, thus contributing to renal damage. In addition, high glucose may decrease the expression of mitochondrial anti-oxidants, such as manganese superoxide dismutase, with consequent impairment of the electron chain and overproduction of ROS. Oxidative stress, in turn, is a stimulus for the synthesis of mitochondrial DNA. Because mitochondria are the main intracellular source of ROS, an increase in their number amplifies cell exposure to ROS and increases kidney damage (Visser *et al.*, 1999).

1.2.2.2 Pathogenesis of diabetic nephropathy.

The specific pathology of diabetic nephropathy is restricted mainly to the renal glomeruli and the tubular interstitium (Gnudi *et al*., 2003). Histologically, the hallmarks of diabetic nephropathy include thickening of the glomerular basement membrane and an increase in the fractional volume of the mesangium (Mauer *et al*., 1981). Expansion of the glomerular mesangium correlates closely with a reduced renal function and the development of proteinuria (Mauer *et al*., 1984). During the progression of the disease, mesangial expansion typically presents as nodular glomerular lesions. For diabetic nephropathy, these pathognomonic lesions bear the name Kimmelstiel-Wilson nodules according to their first description by Kimmelstiel and Wilson (Kimmelstiel and Wilson 1936).The tubulointerstitial injury appears to be closely associated to the glomerular pathology. Interstitial expansion is related to renal dysfunction, proteinuria, and mesangial expansion (Remuzzi *et al*.,1998).

1.2.2.3 Risk factors of the diabetic nephropathy.

1.2.2.3.1 Hyperglycemia.

Poor glycemic control has been shown to contribute to the development of micro- and macroalbuminuria. Intensive glucose control, HbA1c 7% vs 9%, reduces the risk for progression from normo- to microalbuminuria in patients with type 1 diabetes (Diabetes Control and Complications Trial Research Group 1993).Hyperglycemia has also been linked to many deleterious processes in renal tissue. High glucose in the mesangial cells induces cell hypertrophy and increases the extracellular matrix deposits by stimulating the expression of various genes and protein secretion, such as collagen and fibronectin (Ayo *et al*.,1990 ; Ayo *et al*.,1991). Furthermore, hyperglycemia has been shown to stimulate the transforming growth factor (TGF)-β system, and the induction of this system is considered to be one of the main determinants of hypertrophy of the mesangial and tubular cells in diabetic nephropathy (Wolf and Ziyadeh

1999).Sustained hyperglycemia leads to enhanced non-enzymatic protein glycation, which represents the increased covalent binding of glucose to proteins. The process of glycation progresses via relatively stable ketoamines, products of Amadori, to stable advanced glycation end-products (AGEs).AGEs accumulate in the tissues over the lifetime of the protein (Raj *et al*., 2000). In patients with diabetes, AGEs accumulate in renal glomeruli and tubuli (Makino *et al*., 1995).AGEs have been shown to affect properties of extracellular matrix proteins leading to matrix rigidity and mesangial expansion (Lee *et al*., 1997).The enzyme aldose reductase via the polyol pathway, reduces glucose to sorbitol. In chronic hyperglycemia sorbitol accumulates in many tissues, including the renal glomeruli and tubuli. Some have suggested that this accumulation of sorbitol is deleterious to the renal tissue by disturbing cellular osmoregulation and by changing the cellular redox potential (Greene , 1988 ; Greene *et al*., 1987). In addition, inhibition of the enzyme aldose reductase has been shown to prevent a glucose-induced increase in TGF-β1 production and protein kinase C (PKC) activity in human mesangial cells (Ishii *et al*., 1998). The hexoasamine pathway, another of the intracellular pathways of glucose metabolism, also appears to be related to diabetic complications (Schleicher and Weigert 2000). Activation of this pathway during hyperglycemia has been linked to diabetic nephropathy through its end-product, N-acetylglucosamine, which in turn is associated with increased TGF-β1 expression (Kolm-Litty *et al*., 1998).Hyperglycemia is also associated with oxidative stress through the increased production of reactive oxygen species (Giugliano et al., 1996 ; Nishikawa *et al*., 2000). Oxidative stress and its concomitant, reactive oxygen species, are not only recognized as one of the most important components in the pathogenesis of diabetic microvascular complications, but also as a possible unifying mechanism in the pathogenesis of both microvascular and macrovascular complications (Baynes and Thorpe 1999 ; Brownlee , 2005). High glucose in diabetic cells induces the production of superoxide,which is

deleterious to the cells by activation of the polyol and hexosamine pathways, the formation of AGEs, and the activation of PKC (Brownlee , 2005).

1.2.2.3.2 Proteinuria.

Proteinuria is a key factor in diabetic nephropathy and a predictor of progression to ESRD, and has even been suggested as an important factor in promoting the progression of diabetic nephropathy (Remuzzi *et* al., 1997 ; Thomas *et al*., 2001). Excessive protein overload leads to tubulointerstitial damage by inducing the release of chemokines and endothelin (Wang *et* al., 1997 ; Zoja *et al*., 1995). The beneficial effect of ACE-inhibition in diabetic nephropathy has at least partly been associatedwith its effect on proteinuria (Kasiske *et al*., 1993).

1.2.2.3.3 Blood pressure.

Blood pressure rises in parallel with the increase in the urinary albumin excretion rate, and if blood pressure goes untreated, over 80% of the patients with proteinuria will have blood pressure exceeding 140/90 mmHg. It thus comes as no surprise that hypertension is an essential component of the clinical picture in patients with ESRD (Marshall , 2003).

1.2.3 Reactive oxygen species in diabetes mellitus.

There is a bulk of evidence demonstrating that mitochondrial ROS (predominantly superoxide anion) overproduction is involved in diabetes and diabetic complications, even though it is difficult to identify the exact site of ROS formation in the mitochondria. Earlier work suggested that glucose can directly stimulates ROS overproduction (Du *et al*., 1999), but it was later shown that high glucose activates various enzymatic cascades in mitochondria, including activation of NADPH oxidase, uncoupling of NO synthases and stimulation of xanthine oxidase [Du *et al*., 1999 ; Du *et al*., 2000). Glycated proteins can also be the promoters of ROS formation (Mullarkey *et al*., 1990),

thus suggesting that different sources may be responsible for ROS overproduction and oxidative stress in diabetes. The exact role of mitochondria is not completely clear. For some time, mitochondria have been considered the major source of ROS in diabetes and diabetic complications, but Martens *et al.* have demonstrated that HG might actually suppress mitochondrial superoxide formation in metabolically responsive pancreatic β-cells (Martens *et al*., 2005). Similarly, Herlein *et al.* have shown that there is no excess of superoxide production by complexes I and III from mitochondria of streptozotocin diabetic rats (Herlein *et al*., 2009). In addition, Hou *et al.* have reported significant ROS generation under low glucose conditions in mouse β-cells, which is prevented by the ROS scavengers *N*-acetylcysteine (NAC) and manganese(III)tetrakis(4-benzoic acid) porphyrin (Hou *et al*., 2008). Other studies assert an increase of the number of mitochondria. Although their role seems to be controversial, mitochondria are the main source of ROS and further studies are required to deeply analyze their action.

1.2.4 oxidative stress and insulin resistance.

A large number of studies have provided evidence for the pivotal role of oxidative stress in insulin resistant states such as obesity, the metabolic syndrome and type 2 diabetes (Schaffer *et* al., 2012 ; . Stadle , 2012) Thus, ROS overproduction is an important trigger for insulin resistance and a relevant factor in the development of type 2 diabetes (Houstis *et al*., 2006). Again, mitochondria and NADPH oxidase are considered the major sources of ROS overproduction, given that mitochondrial superoxide production is a common feature in models of insulin resistance in adipocytes, myotubes and mice. Several animal studies have been performed to investigate the role of increased oxidative stress in insulin resistant states. In obese mice, increased H2O2 generation by adipose tissue can be observed prior to the onset of diabetes (Furukawa *et al*., 2004). This event is accompanied by decreased mRNA levels

of SOD, catalase and glutathione peroxidase and all these changes are exaggerated by the development of diabetes. Obesity and related insulin resistance are frequently associated with increased accumulation of lipids (triglycerides) in the liver. Increased lipid peroxidation markers have thus been observed in the liver of animal models of diabetes and obesity (Svegliati-Baroni *et al*., 2006). Evidence of systemic oxidative stress includes detection of increased circulating and urinary levels of the lipid peroxidation product F2-isoprostane (8-epi-prostaglandin F2α) in both types 1 and 2 diabetes (Davi *et al*., 2003 ; Monnier *et al*., 2006), as well as in obesity. Remarkably, this marker correlates with blood glucose levels and glucose variability, and ameliorates following therapeutic interventions (Davi *et al*., 1999).

1.2.5 Antioxidant deficiency.

In addition to overproduction of oxidant agents, higher oxidative stress may be due to the reduction of plasma antioxidant capacity. Even though Savu et al. have reported higher levels of antioxidants in patients with uncomplicated type 2 diabetes (Savu *et al*., 2012), a number of studies have shown a reduction of plasma antioxidant capacity occurring already even in the early phase of the disease. Another well recognized antioxidant agent is uric acid, which plays its role in two different ways: it promotes superoxide dismutase activity and enhances the action of ascorbate. Lower level of blood and urinary uric acid have been detected in women with type 1 diabetes, in whom uric acid reduction was associated with increased oxidative stress (Pitocco *et al*., 2008). Oxidation-induced alterations in molecules involved in insulin signaling are also associated with impaired insulin action, as shown in a rat model of oxidative stress induced by inhibition of glutathione biosynthesis. In this model, the drop in tissue levels of glutathione, a major cellular antioxidant, was associated with increased oxidative stress and impaired glucose homeostasis (Bashan *et al*., 2009).

1.2.6 Oxidative stress and other diabetic complications.

Type 2 diabetes is the leading cause of blindness, non-traumatic lower-limb amputation and chronic kidney disease (Gao *et* al., 2010 ; Centers for Disease Control and Prevention , 2011). Many experimental models of both types 1 and 2 diabetes exhibit increased ROS generation, triggered in large part by high glucose (Huang *et al*., 2012). In the development of diabetes, high glucose triggers the overproduction of superoxide and H2O2, which, in turn, determine a decline in the antioxidant systems, directly damage many biomolecules; increase lipid peroxidation and results in insulin resistance (Schaffer *et al*., 2012). Mullarkey et al. have proposed that glycated proteins enhance superoxide production and lipid peroxidation compared to non-glycated ones, thus suggesting that increased protein glycation accelerates lipid damage in diabetes (Mullarkey *et al*., 1990). Subsequent studies, however, have shown that the most important sources of ROS under hyperglycemic conditions are mitochondria and NADPH oxidases, whose overproduction of ROS causes hyperglycemia-induced damage through the following mechanisms:

1. Activation of the polyol pathway, probably by means of consumption of NADPH, an important scavenger of ROS (Chung *et al*., 2003).

2. Increase in intracellular advanced glycation end-products (AGEs) formation, stemming from non-enzymatic reaction of glucose and other glycating compounds with proteins (Wautier and chmidt , 2004 ; Candido *et al*., 2003) .

3. Increased expression of the receptor for AGEs and its activating ligands: the receptor for AGE binding (RAGE) induces the production of ROS, which in turn activates the pleiotropic transcription nuclear factor NF-κB, causing multiple pathological changes in gene expression (Stitt *et al*., 1997 ; Nishino *et al*., 1995).

4. Increased PKC activation: PKCs are a family of at least 11 isoforms that can phosphorylate various target proteins [134]. Persistent and excessive activation of several PKC isoforms has been implicated in the decreased NO production in smooth muscle cells and has been shown to inhibit insulin-stimulated expression of eNOS in cultured endothelial cells. Activation of PKC by high glucose also induces expression VEGF, thereby enhancing permeability in vascular smooth muscle cells (Inoguchi *et al*.,1992 ; Ganz and Seftel 2000) .

5. Activation of the hexosamine pathway: hyperglycemia and insulin resistance-induced excess of fatty acid oxidation contributes to the pathogenesis of diabetic complications by increasing the flux of fructose-6-phosphate into the hexosamine pathway. Fructose 6-phosphate is the substrate for the rate-limiting enzyme of the glutamine: fructose6-phosphate amidotransferase (GFAT) pathway, which converts fructose 6-phosphate into glucosamine 6-phosphate that is in turn converted into uridine-diphosphate (UDP)-N-acetylglucosamine. Inhibition of GFAT may block the hyperglycemia-induced increases in the transcription of both TGF-α and TGF-β1 (Du *et al*., 2000). In addition, specific inhibitors of aldose reductase activity, AGE formation, RAGE ligand binding, PKC activation and hexosamine pathway flux may ameliorate diabetes-induced abnormalities in cell culture or animal models (Brownlee , 1995).

It has now been established that the different pathogenic mechanisms described above stem from a single hyperglycemia-induced process, the overproduction of superoxide by the mitochondrial electron-transport chain (Nishikawa *et al*., 2000 ; Wallace , 1992). Thus, in cells with high intracellular glucose concentration, there is more glucose-derived pyruvate to increase the flux of electron donors (NADH and FADH2) into the electron transport chain. Coenzyme Q donates the electrons to molecular oxygen, hence leading to generation of superoxide anions. The mitochondrial isoform of the enzyme SOD, by contrast, degrades this oxygen free radical to H2O2, which is then

converted to H2O and O2 by other enzymes (Korshunov *et al*., 1997). Dynamic changes in mitochondrial morphology are associated with high glucose-induced overproduction of ROS (Yu *et al*., 2006).

1.2.7 Complement fixation and immune complex deposition in diabetic nephropathy.

There are emerging data that activation of the complement cascade may contribute to human diabetic nephropathy (stergaard *et* al., 2005 ; Watanabe *et* al., 1987).Hyperglycemia-induced intracellular generation of reactive oxygen species activates complement (stergaard *et* al., 2005). Another route for complement activation is immune complex deposition. Ainsworth and colleagues examined 16 kidney biopsies from type 1 and type 2 diabetic patients with diabetic nephropathy, and were among the first to observe complement and immune complex deposition in both the glomerular basement membrane and mesangium.Based on the immunofluorescence findings the authors concluded that the morphologic alterations observed in diabetic glomerulopathy might be mediated by immune mechanisms (Ainsworth *et al*., 1982).Circulating immune complex levels have also been associated with the development of albuminuria and proliferative retinopathy in patients with diabetes, suggesting immunoglobulin (Ig) deposition can promote tissue injury (Atchley *et* al., 2002 ; Di Mario *et al*.,1986). In both adults and children with diabetes significantly higher levels of circulating IgG immune complexes correlate with microalbuminuria, suggesting that elevated levels of circulating immune complexes are associated with the development of early diabetic nephropathy (Nicoloff *et al*., 2004). Circulating immune complexes have also been detected in several rodent models of diabetes at higher titers than controls and are associated with increased glomerular deposition (Watanabe *et* al., 1987; Abrass , 1984). In these models glomerular deposition of Ig and C3 has been correlated with renal injury (Thiele and McDonald 1989 ; Fujita *et al*., 1999) Several

pathogenic mechanisms of immune complex deposition in diabetic renal injury have been proposed. Glomerular and tubular basement membrane thickening may involve an antigen-antibody reaction that stimulates resident cells to proliferate (Nicoloff *et al*., 2004). Moreover, certain immune complexes stimulate mesangial expansion by inducing collagen production (Abdelsamie *et al*., 2011). Immune complexes may also promote glomerular macrophage accrual. For example, in diabetic mice increased accumulation of macrophages in the glomeruli correlates with an increased glomerular deposition of IgG (Chow *et al*., 2004). The immune complex activated complement cascade was directly tested in a study using human antibodies to oxidized low-density lipoproteins (Ox-LDL) collected from sera of patients with type 1 diabetes. Human anti-Ox-LDL antibodies incubated with complement fragments *in vitro* form immune complexes and trigger activation of the classical complement pathway. Moreover, Ox-LDL immune complexes induce inflammatory cytokine release from macrophages *in vitro*(Saad *et al*., 2006).

1.2.8 Malondialdehyde(MDA).

Malondialdehyde (MDA) is a substance produced during polyunsaturated fatty acid peroxidation which has been detected in the serum of patients with T2D and correlate with the progression of disease(Del Rio *et* al., 2005 ; Kamper *et al*., 2010). MDA is known to have toxic influence on cell membrane structure. It can modulate signal transduction as well as modify proteins and DNA(Srivastava *et al*., 2002). diabetic nephropathy is associated with high blood glucose level and oxidative stress (significant increase in MDA level).

1.2.9 Catalase (CAT).

Catalase is a homotetrameric enzyme found in the tissues of almost all mammals, and demonstrating the highest activity in the liver and erythrocytes. Within the cell, catalase is localized primarily in the peroxisomes and

mitochondria. The principal role of catalase lies in the degradation of H_2O_2, produced with support of peroxisomal oxidases. In the erythrocytes, catalase constitues the first line of defense against H_2O_2 (Mueller *et al*., 1997).In the situations of normal H_2O_2 concentration, the reaction goes towards H2O2 conversion into water and oxygen:

Catalase + 2 H_2O_2 → Compaund I + H_2O

Compaund I + H_2O_2 → Katalaza + H_2O + O_2

The second H_2O_2 molecule serves as the donor of hydrogen ion.In the situations of low H_2O_2 concentration and in the presence of small molecular electron donors, catalase may act as peroxydase as well (Ghadermarzi and Moosavi-Movahedi,1996). Catalase forms a firm bond with NADPH, which can prevent the accumulation of inactive forms of the enzyme slowly created when catalase is exposed to hydrogen peroxide. These effects of NADPH are evident in low concentrations in the cell as well (Kirkman *et al*., 1987).Numerous diseases can be accompanied with altered catalase activity. Reduced catalase activity has been documented in diabetes mellitus (DM), malignant diseases, Down syndrome, as well as in regenerating tissues and in experimental conditions of nephrotoxicity (Djordjević *et al*., 2000). Since catalase has a predominant role in the control of H2O2 concentration (Mueller *et al*., 1997; Gaetani *et al*., 1996), which has been shown to damage pancreatic cells and inhibits insulin activity (Murata *et al*.,1998; Tiedge *et al*., 1998; Jorns *et al*., 1999), it is believed that catalase in that manner protects cells from the harmful action of H2O2 (Murata *et al*.,1998).

1.2.10 Glutathione (GSH).

Glutathione is one of the most important nonenzymatic antioxidant in mammal cells. In the cell, it is most commonly found as a thiol – in a reduced form – and less as a disulfide – in an oxidized form (Toyokuni, 1996; Hann *et al*., 1990; Natarajan,1995).GSH concentration in the cell is determined by the control of enzymes involved in its synthesis, availability of synthesis precursors, intensity of GSH depletion for cellular detoxification processes, interorgan GSH distribution, as well as GSH regeneration in the so called glutathione redox cycle. Erythrocytes represent a unique transport system for glutathione and its conjugates. In contrast to other cells, GSH appears in erythrocytes in several intermediary metabolic forms. In physiologic conditions, reduced GSH form appears in the highest percentage. The other GSH form is oxidized glutathione, occurring in the process of nonenzymatic oxidation or oxidation mediated by GSH-peroxidase. The third erythrocyte form is the disulphide form of glutathione bound to proteins and non-protein sulphydril compounds.Glutathione S-conjugates (occurring via the action of glutathione S-transferase) are the fourth potential intracellular-intermediary form of GSH in the erythrocytes. Erythrocytes take up toxic molecules from the plasma, to be excreted back into plasma after conjugation with GSH. Further detoxification of S-conjugates continues in the liver and kidneys; nontoxic compounds are then excreted from the organism via the bile or urine. GSH is involved in many cellular functions. It is thought that during the evolution, depending on the cellular metabolic needs, glutathione is engaged in the regulation of different processes. Though the role of glutathion has been commonly associated with the protection of the cell from active free radicals, glutathione is involved in many other processes such as detoxification from xenobiotics, synthesis of nucleic acids and proteins, cell signaling, proliferation, and differentiation (Djordjević *et* al., 2000). However, its essential role lies in the protective antioxidant

system. GSH, GSH-transferase and GSH-reductase constitute an antioxidant system of glutathione, in which GSH-reductase is required for the reduction of oxidized glutathione and consequential glutathione recycling in the so called glutathione redox cycle. Depletion of GSH in the cell increases its sensitivity to oxidative damage (Rizzardini *et al*., 2003). In contrast, accumulation of GSH in the cell, especially in the mitochondria, can prevent neural apoptosis caused by ischemia (Li *et al*., 2002) and excitotoxicity (Kobayashi *et al*., 2000). The involvement of GSH in the limitation of prooxidative cell status, has as the ultimate consequence deceleration of aging, atherogenesis, mutagenesis, and carcecogenesis (Anghileri and Thouvenot ,1997; Vostreis *et al*., 1988).

1.2.11 Creatinine.

Creatinine is produced in muscle metabolism as the end product of creatine phosphate and is subsequently excreted principally by way of the kidneys, predominantly by glomerular filtration (Taylor , 1989).Blood assays for creatinine constitute the most commonly used measure of the presence and progression of CKD. Thus, the level of the serum creatinine in a subject is a general reflection of the level of kidney function. With kidney disease and loss of nephrons, the level of serum creatinine would therefore show an upward trend. Conversely, with improving kidney function, such as that seen following acute kidney injury (AKI), the level of serum creatinine will then trend downwards.Accordingly, serum creatinine is the most widely used assay to measure the presence and progression of CKD (Levey , 1990).

1.2.12 Microalbuminuria.

Diabetic kidney disease or nephropathy is the most common cause of end stage renal disease (ESRD) or kidney failure in the Western World. One of the earliest markers of diabetic nephropathy is the presence of small amount of the protein albumin in the urine. This is called MAU or microalbuminuria (urinary albumin excretion of 30-300 mg/24 hours). Microalbuminuria may progress over a span of a number of years to overt nephropathy characterized by the presence of larger amounts of the protein albumin leaking through the kidneys' filter mechanism into the urine. This is called macroalbuminuria (urinary albumin > 300 mg/24 hours). The presence of macroalbuminuria indicates more serious kidney disease. Progression to ESRD or kidney failure may then occur within several years. Once overt kidney failure has developed 2 year survival is approximately 50%(Canadian Diabetes Association , 2003 ; Rose and Bakris , 2004).

Chapter Two

Materials and Methods

2. Materials and Methods.

2.1 Materials.

2.1.1 Equipment.

The used equipment and their sources are presented in table (2-1).

Table (2-1): The used equipments and their sources.

Name of equipment	Company	Origen
Automatic micropipettes	Dragon MeD	China
Automatic micropipettes (0.5- 10)	Eppendorf Research plus	Germany
Blood Mixer	KJMR2	China
Centrifuge	Remi	India
Comblizyer (urinelyzer)	Human	Germany
Disposable gel and clot activator tube	AFCO-DISPO	Jordan
EDTA K2(2.5 ml)	AL-Hanof factory	Jordan
Eppendorf	CETOTEST	China
Plastic disposable syringes; 5ml	QJECT	Qater
Printer	EPSON	UK
Refrigerator and freezer (-20 ˚C)	LG	Korea
Cobas cIII	Roche	Germany
HumanReaderHS	Human	Germany
Combiwash	Human	Germany

2.1.2 Kits.

The used chemicals and their sources are mentioned in Table (2-2).

Table(2-2):The used chemicals and their sources.

Name of chemical	Company
Blood urea kit	Roche
Serum creatinine kit	Roche
Glucose kit	Roche
Glycosylated hemoglobin (HbA1c) kit	Roche
Urine strip (COMNBINA13)	Human
Complement kit (C3,C4)	LTA
Catalase kit	Bioassay Technology Laboratory
Glutathione kit	Bioassay Technology Laboratory
Malondialdehyde kit	Bioassay technology Laboratory

2.2 Subjuct.

2.2.1 Type2Diabetic nephropathy patients.

The study was conducted on 31 type 2 diabetic nephropathy patients, their ages (45-80 years). The patients having microalbuminuria or elevated serum creatinine were randomly selected from those attending the Al-fajer laboratory at Abd-almajid Private Hospital between December 2015 to July 2016.

The information about the age, the presence of hypertension and having treatment were documented from the patients.

2.2.2 Type2 Diabetic mellitus patients.

The study also included 30 type 2 diabetes mellitus (T2DM) patients, their ages (45-80 years). They were randomly selected from those attending the Al-fajer laboratory at Abd-almajid Private Hospital between December 2015 to July 2016.

The information about the age, the presence of hypertension and having treatment were documented from the patients.

2.2.3 Control subjects.

For the purpose of comparisons, 28 healthy controls subjects comparable to diabetes mellitus and diabetic nephropathy patients in respect to age (45-80) year. The controls were selected among subjects who were apparently healthy in terms of being non-diabetic, with no other endocrine disorders or metabolic kidney diseases and were free of acute illness or infection at time of sampling.

2.3 Collection of samples.

2.3.1 Collection of blood samples.

Five ml of blood were obtained from patients and control subjects by venipuncture, using a 5 ml disposable syringe, 3ml of blood and dispensed in a gel tubes and centrifuged at 3000 rpm for 10 minutes to collect serum. The serum was divided into aliquots in Eppendorf tubes for measuring the random blood sugar (RBS), Blood urea , serum creatinine, complement(C_3,C_4), catalase, malondialdehyde and glutathione and stored in deep freezer at (-20◦C), other 2 ml were collected into EDTA tubes for measuring HbA1c.

2.3.2 Collection of urine samples.

Urine was collected two times by spot urine collection. The urine was collected into sterile cups without any blood or urinary tract infection.

2.4 Methods.

2.4.1 Blood glucose determination using glucose kit.

2.4.1.1 Principle of the method.

Enzymatic reference method with hexokinase.

Hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate by ATP.

$$\text{Glucose + ATP} \xrightarrow{\text{HK}} \text{G-6-P + ADP}$$

Glucose-6-phospate dehydrogenase oxidizes glucose-6-phosphate in the presence of NADP to gluconate-6-phosphate. No other carbohydrate is oxidized. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and is measured photometrically.

$$\text{G-6-P + NADP} \longrightarrow \text{gluconate-6-p + NADPH + H}$$

2.4.2 Blood urea determination using blood urea kit.

2.4.2.1 Principle of the method.

Kinetic test with urease and glutamate dehydrogenase.

Urea is hydrolyzed by urease to form ammonium and carbonate.

$$\text{Urea + } H_2O \xrightarrow{\text{Urease}} 2\ NH_4^+ + CO_3^{2-}$$

In the second reaction 2-oxoglutarate react with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD for each mole of urea hydrolyzed.

$$NH_4^+ \text{ +2-oxoglutarate + NADH} \xrightarrow{\text{GLDH}} \text{L-glutamate + } NAD^+ + H_2O$$

The rate of decrease in the NADH concentration is directly proportion to the urea concentration in the specimen and is measured photometrically.

2.4.3 Creatinine determination using creatinine kit.

2.4.3.1 Test principle.

This kinetic colorimetric assay is based on the Jaffé method. In alkaline solution, creatinine forms a yellow-orange complex with picrate. The rate of dye formation is proportional to the creatinine concentration in the specimen. The assay uses "rate-blanking" to minimize interference by bilirubin. To correct for non-specific reaction caused by serum/plasma pseudo-creatinine chromogens, including protein and ketones, the results for serum or plasma are corrected by -18 µmol/L (-0.2 mg/dL).

Creatinine + picric acid $\xrightarrow{\text{Alkaline pH}}$ yellow-red complex

2.4.4 Glycosylated hemoglobin determination using HbA1c kit.

2.4.4.1 Principle of the method.

This method uses TTAB (tetradecyltrimethylammonium bromide) as the detergent in the hemolyzing reagent to eliminate interference from leukocytes (TTAB does not lyse leukocytes). Sample pretreatment to remove labile HbA1c is not necessary.

All hemoglobin variants which are glycated at the β-chain n-terminus and which have antibody-recognizable regions identical to that of HbA1c are measured by this assay. Consequently, the metabolic state of patients having uremia or the most frequent hemoglobinopathies (HbAS, HbAE) can be determined using this assay.

The hemoglobin A1c (HbA1c) determination is based on the turbidimetric inhibition immunoassay (TINIA) for hemolyzed whole blood.

2.4.5 Malondialdehyde determination using ELISA kit (MDA).

2.4.5.1Test principle.

This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Malondialdehyde (MDA). Add Malondialdehyde (MDA) to the wells, which are pre-coated with Malondialdehyde (MDA) monoclonal antibody and then incubate. After that, add anti MDA antibodies

labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Malondialdehyde (MDA) are positively correlated.

2.4.5.2 Assay procedure.

1. Standard solutions were diluted as follow:

40nmol/ml	Standard No.5	120µl Original Standard + 120µl Standard diluents
20nmol/ml	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
10nmol/ml	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
5nmol/ml	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
2.5nmol/ml	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

2. The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3. Sample injection: 1) Blank well: no sample, anti MDA antibody labeled with biotin or streptavidin-HRP is added to comparison blank well except chromogen solution A & B and stop solution while taking the same steps that follow. 2) Standard solution well: Add 50µl standard and streptomycin-HRP 50µl (biotin antibodies have united in advance in the standard so no biotin antibodies are added.) 3) Sample well to be tested: Add 40µl sample and then 10µl MDA antibodies, 50µl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃ for 60 minutes.

4. Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.

5. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.

6. Color development: Add 50µl chromogen solution A firstly to each well and then add 50µl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.

7. Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).

8. Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

9. According to standards' concentrations and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding sample. Special software could be employed to calculate as well.

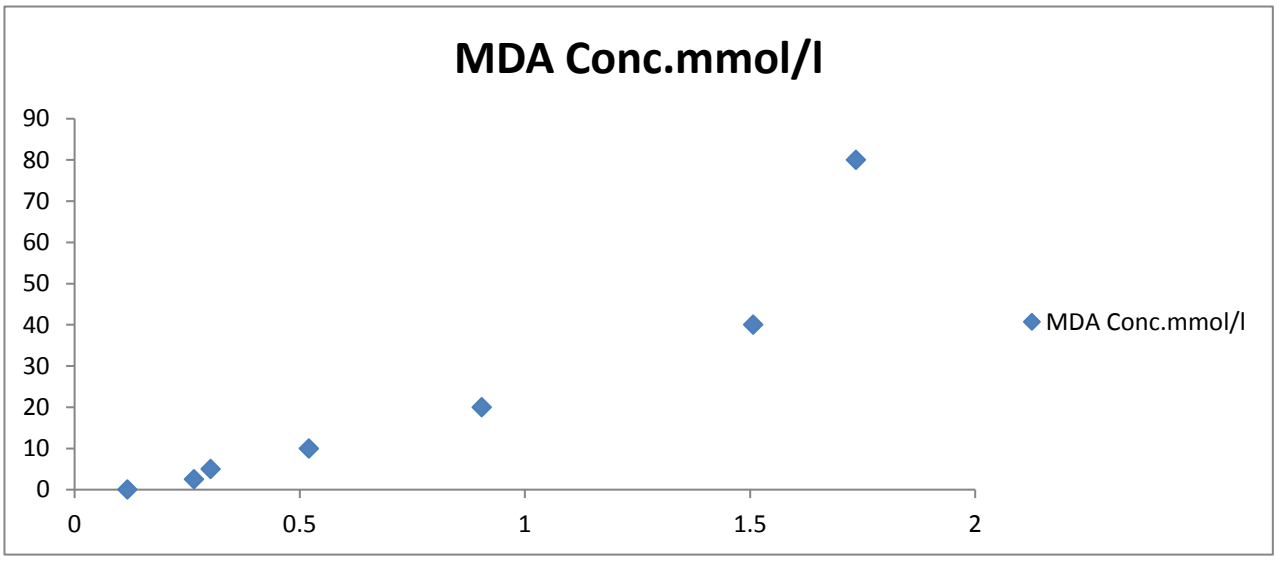

Figure 2.1 Standard curve of MDA.

2.4.6 Glutathione determination using ELISA kit (GSH).

2.4.6.1 Test principle.

The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human glutathione (GSH) in samples. Add glutathione (GSH) to monoclonal antibody Enzyme well which is pre-coated with Human glutathione(GSH) monoclonal antibody, incubation; then, add glutathione (GSH) antibodies labeled with biotin, and combined with Streptavidin-HRP to form immune complex; then carry out incubation and washing again to remove the uncombined enzyme. Then add Chromogen Solution A, B, the color of the liquid changes into the blue, And at the effect of acid, the color finally becomes yellow. The chroma of color and the concenthumanion of the Human Substance glutathione (GSH) of sample were positively correlated.

2.4.6.2 Assay procedure.

1. Standard solutions were diluted as follow:

24ng/ml	Standard No.5	120µl Original Standard + 120µl Standard diluents
12 ng/ml	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
6ng/ml	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
3 ng/ml	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
1.5ng/ml	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

2. The quantity of the plates depends on the quantities of to-be-tested samples and the standards. It is suggested to duplicate each standard and blank well. Every sample shall be made according to your required quantity and try to use the duplicated well as possible.

3. Inject samples: 1. Blank well: don't add samples and GSH-antibody labeled with biotin, Streptavidin-HRP, only Chromogen solution A and B, and stop solution are allowed; other operations are the same.1. Standard wells: add standard 50µl, Streptavidin-HRP 50µl (since the standard already has combined biotin antibody, it is not necessary to add the antibody); 3. To be test wells: add sample 40µl, and then add both GSH-antibody 10µl and

Streptavidin-HRP 50µl. Then seal the sealing memberance, and gently shaking, incubated 60 minutes at 37 ℃.

4. Confection: dilute 30 times the 30×washing concentrate with distilled water as standby.

5. Washing: remove the memberance carefully, and drain the liquid, shake away the remaining water.

6. Add chromogen solution a 50µl, then chromogen solution B 50µl to each well. Gently mixed, incubate for 10 min at 37℃ away from light.

7. Stop: Add Stop Solution 50µl into each well to stop the reaction(the blue changes into yellow immediately).

8. Final measurement: Take blank well as zero , measure the optical densit (OD) under 450 nm wavelength which should be carried out within 15min after adding the stop solution.

9. According to standards' concentration and the corresponding OD values, calculate out the standard curve linear regression equation, and then apply the OD values of the sample on the regression equation to calculate the corresponding sample's concentration. It is acceptable to use kinds of software to make calculations.

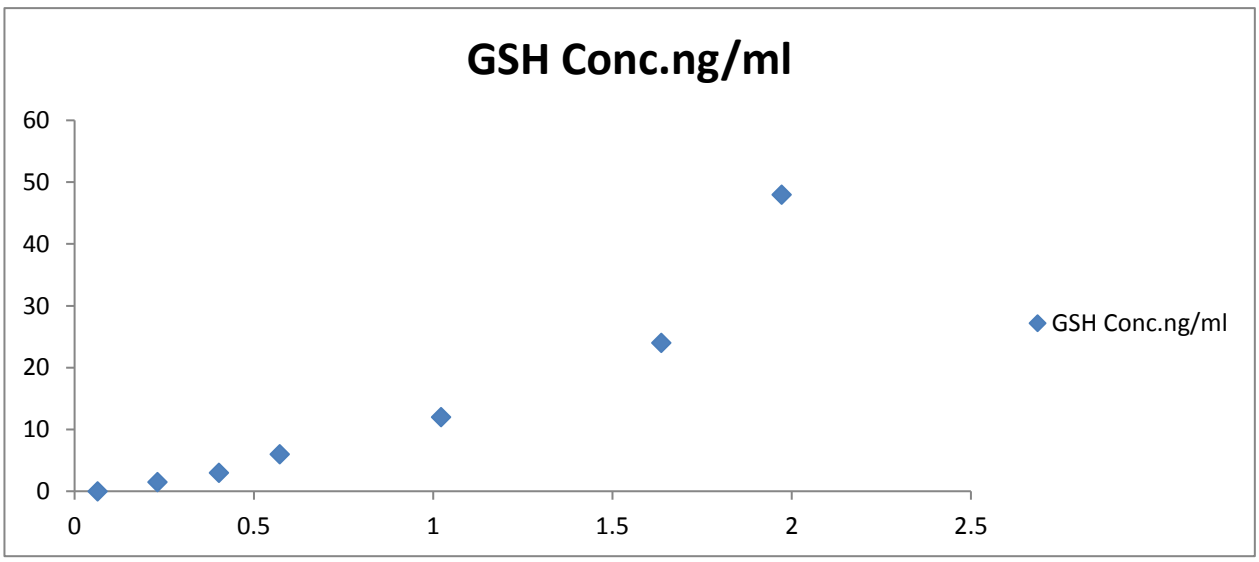

Figure 2.2 Standard curve of GSH.

2.4.7 Catalase determination using ELISA kit (CAT).

2.4.7.1Test principle.

This kit uses enzyme-linked immune sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Catalase (CAT). Add Catalase (CAT) to the wells, which are pre-coated with Catalase (CAT) monoclonal antibody and then incubate. After that, add anti CAT antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. Remove unbound enzymes after incubation and washing. Add substrate A and B. Then the solution will turn blue and change into yellow with the effect of acid. The shades of solution and the concentration of Human Catalase (CAT) are positively correlated.

2.4.7.2 Assay procedure.

1. Dilution of standard solutions: (This kit has a standard of original concentration, which could be diluted in small tubes by user independently following the instruction.):

400KU/L	Standard No.5	120µl Original Standard + 120µl Standard diluents
200KU/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
100KU/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
50KU/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
25KU/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

2. The number of stripes needed is determined by that of samples to be tested added by that of standards. It is suggested that each standard solution and each blank well should be arranged with three or more wells as much as possible.

3. Sample injection: 1) Blank well: no sample, anti-CAT antibody labeled with biotin or streptavidin-HRP is added to comparison blank well except chromogen solution A & B and stop solution while taking the same steps that follow. 2) Standard solution well: Add

50µl standard and streptavidin-HRP 50µl (biotin antibodies have united in advance in the standard so no biotin antibodies are added. 3) Sample well to be tested: Add 40µl sample and then 10µl CAT antibodies, 50µl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37℃ for 60 minutes.

4. Preparation of washing solution: Dilute the washing concentration (30X) with distilled water for later use.

5. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain the liquid after 30 seconds' standing. Then repeat this procedure five times and blot the plate.

6. Color development: Add 50µl chromogen solution A firstly to each well and then add 50µl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for color development.

7. Stop: Add 50µl Stop Solution to each well to stop the reaction (the blue color changes into yellow immediately at that moment).

8. Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within the 10 minutes after having added the stop solution.

9. According to standards' concentrations and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding sample. Special software could be employed to calculate as well.

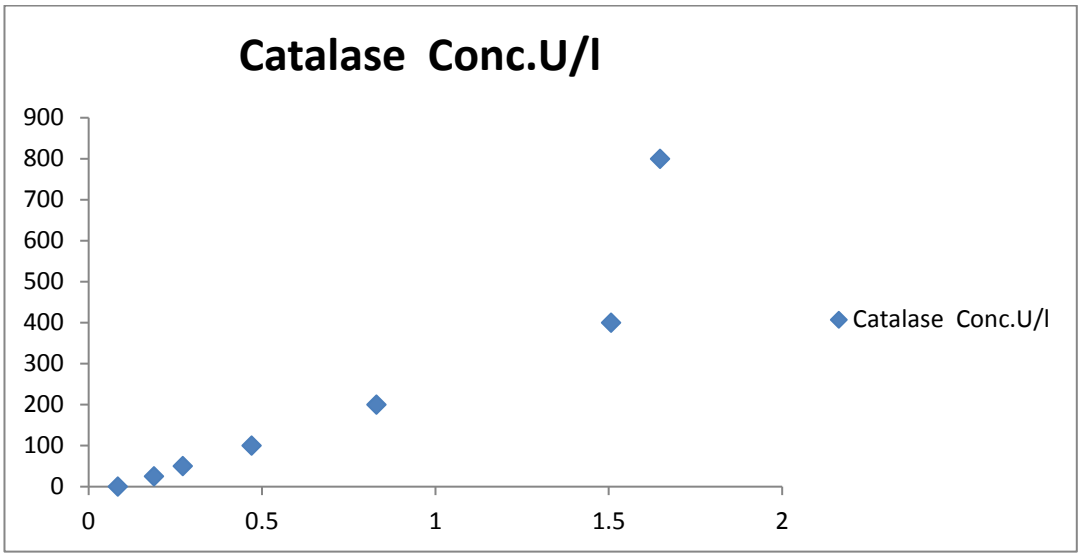

Figure 2.2 Standard curve of Catalase.

2.4.8 Creatinine, microalbumin and protein detection by urine test strip.

2.4.8.1 Test Principles.

2.4.8.1.1 Creatinine.

The test is based on a reaction of creatinine with 3,5-dinitrobenzoic acid in alkaline medium.

2.4.8.1.2 Microalbomin.

The test is based on the "protein error" principle of the indicator, which is caused by the presence of albumin.

2.4.8.1.3 Protein.

The test is based on the "protein error" principle of the indicator. The test pad is not specific for a particular protein and proteins other than albumin can cause a positive results.

2.4.8.2 Assay procedure.

1. Only well mixed, non-centrifuged urine was used which should not be older than two hours.
2. The test strip was immersed in the urine for approximately 2 sec, so that all reagent are covered. The excess urine was removed from the strip by wiping the edge of the strip on the urine container or on absorbent paper.
3. The urine strip was reflectometric read with Combilyzer.

2.4.9 Determenation of the C_3, C_4 protein, by radial immunodiffusion plate.

2.4.9.1 Test principle.

The examined protein, diffusing in agarose gel containing a specific antibody will form an immune-complex, visible as a ring around the well. The ring diameter is direct proportional to the concentration of the analysed protein. The proportion corresponde to the diffusion time. In fact, at the end (72), the square of diameter will be in linear proportion to the concentration of the sample.

With the plate is supplied a reference table in which each diameter of the halo is associated a concentration.

2.4.9.2 Assay procedure.

Remove the plate from its envelope and leave to stand at room temperature for few minutes so that any condensed water in the wells can evaporate. Fill the wells with 5 µl of sample and/or controls and wait it has been completely adsorbing before handing the plate. Close the plate and place it in a moist chamber for 72 hours.

2.5 Statistical analysis.

The Statistical Analysis System- SAS (2012) program was used analyzed of differente factors in study parameters . Least significant difference –LSD test was used to significant $P< 0.05$ compare between means in this study (SAS., 2012).

Chapter Three

Results and Discussion

3. Results and discussion.

3.1 Results.

3.1.1 Serum and urine biochemical parameters in healthy control subjects, diabetic and diabetic nephropathy patients at the age 45-56 years.

1. Random blood sugar (RBS) concentration.

Figures3.1 shows random blood sugar concentration in healthy control subjects, diabetic and diabetic nephropathy patients at the age 45-56. The Figure demonstrates that RBS increases significantly ($p<0.05$) in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

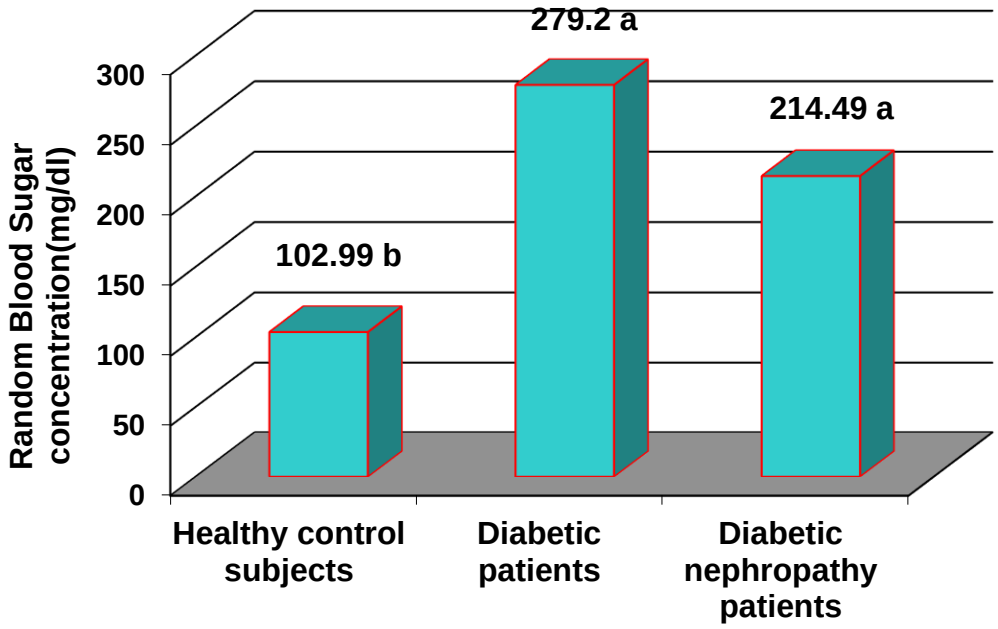

Figure 3.1:Random blood sugar concentration in healthy control subjects, diabetic and diabetic nephropathy patients (age 45-56 years). different letters mean significant differences at P< 0.05.

2. Serum urea concentration.

The results of serum urea concentration are shown in Figure 3.2. No change is observed in serum urea concentration in diabetic patients, whereas in diabetic nephropathy patients, serum urea concentration shows a significant ($p<0.05$) increase when compared with the healthy control subjects (Appendix 1).

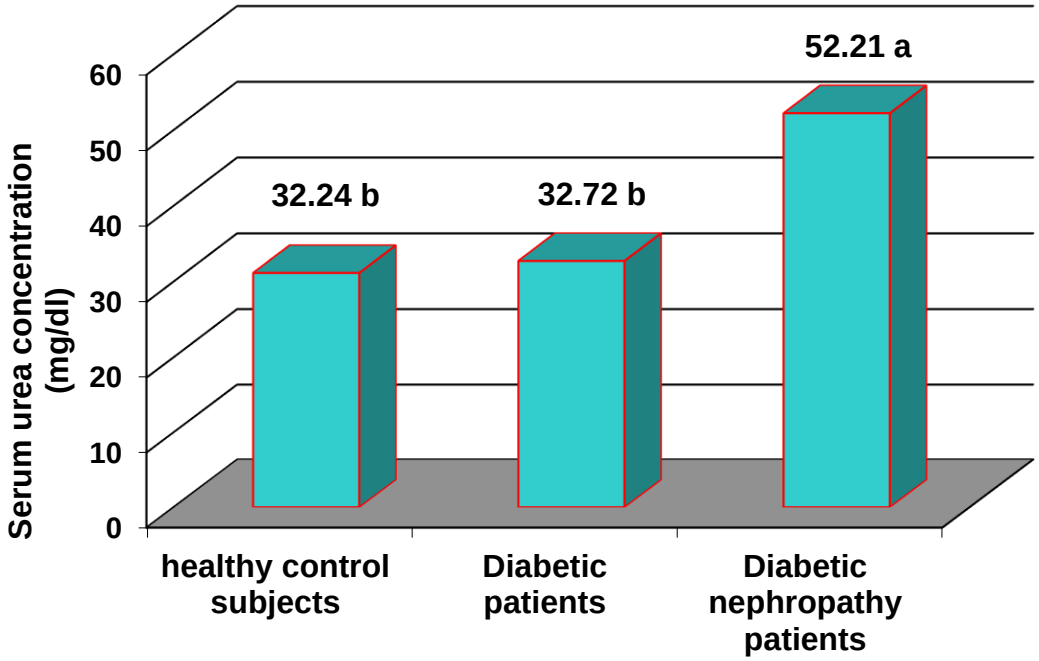

Figure 3.2: Serum urea concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 45-56 years). different letters mean significant differences at P< 0.05.

3. Serum creatinine concentration.

Figure3.3 shows nonsignificant ($p>0.05$) changes was observed in serum creatinine concentration in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

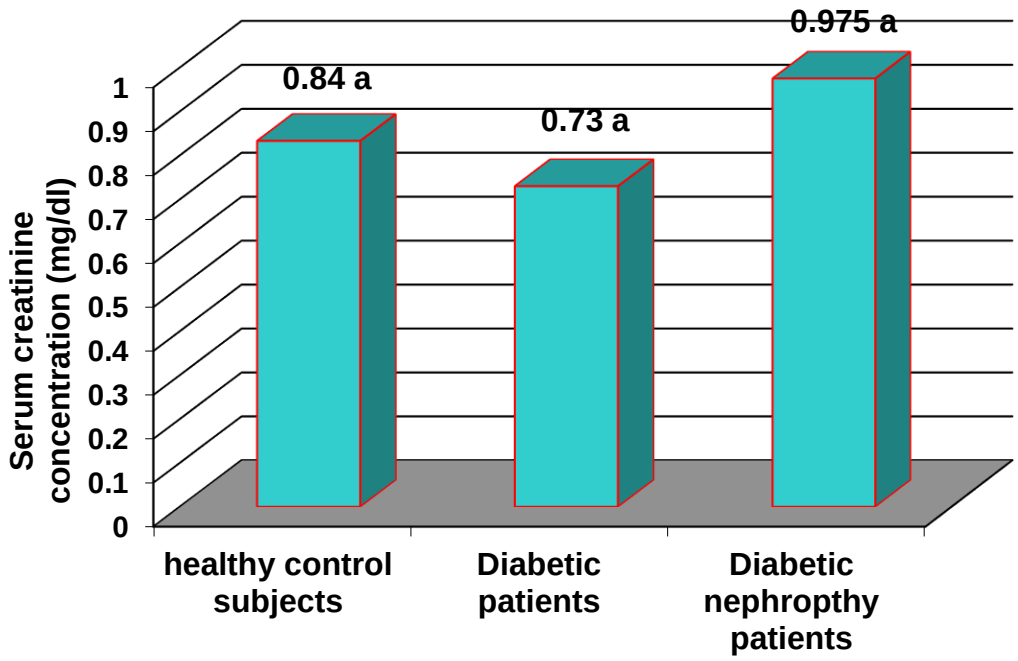

Figure 3.3: Serum creatinine concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age = 45-56 years). similar letters mean nonsignificant (P>0.05) differences.

4. Glycosylated hemoglobin (HbA1c).

The results of the percentages of HbA1c in healthy control subjects, diabetic and diabetic nephropathy patients are shown in Figure 3.4. The percentages of HbA1c increased significantly (P<0.05) in diabetic and diabetic nephropathy patients in comparison with the healthy control subjects (Appendix 1).

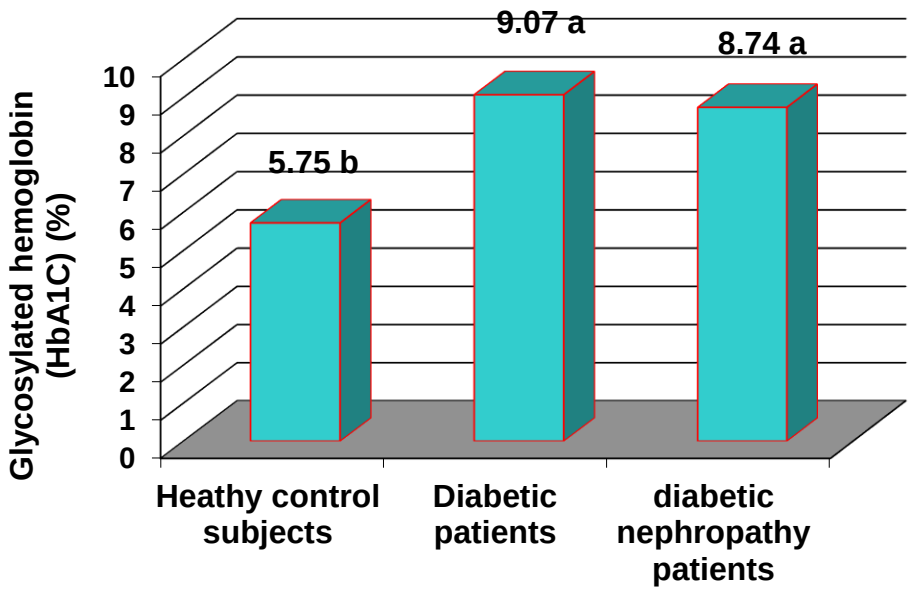

Figure 3.4: the percentages of glycosylated hemoglobin HbA1c in healthy control subjects, diabetic and diabetic nephropathy patients (age 45-56 years). different letters mean significant differences at P< 0.05.

5. Serum complements 3 and 4(C3 and C4) concentrations.

The Figures 3.5 and 3.6 demonstrate that serum complements 3 and 4 concentrations show nonsignificant (P>0.05) changes in diabetic and diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

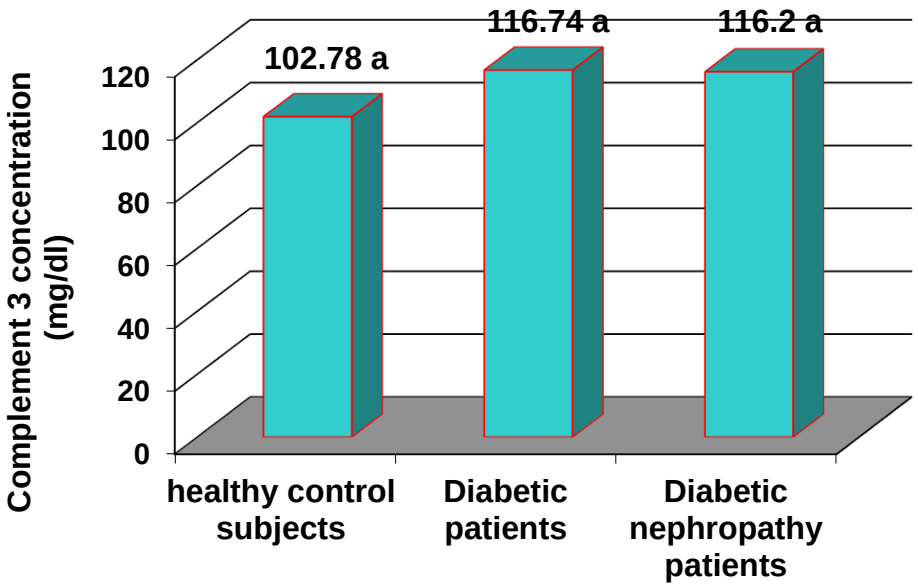

Figure 3.5: Serum complement3 concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 45-56 years). similare letters mean nonsignificant (P< 0.05) differences`.

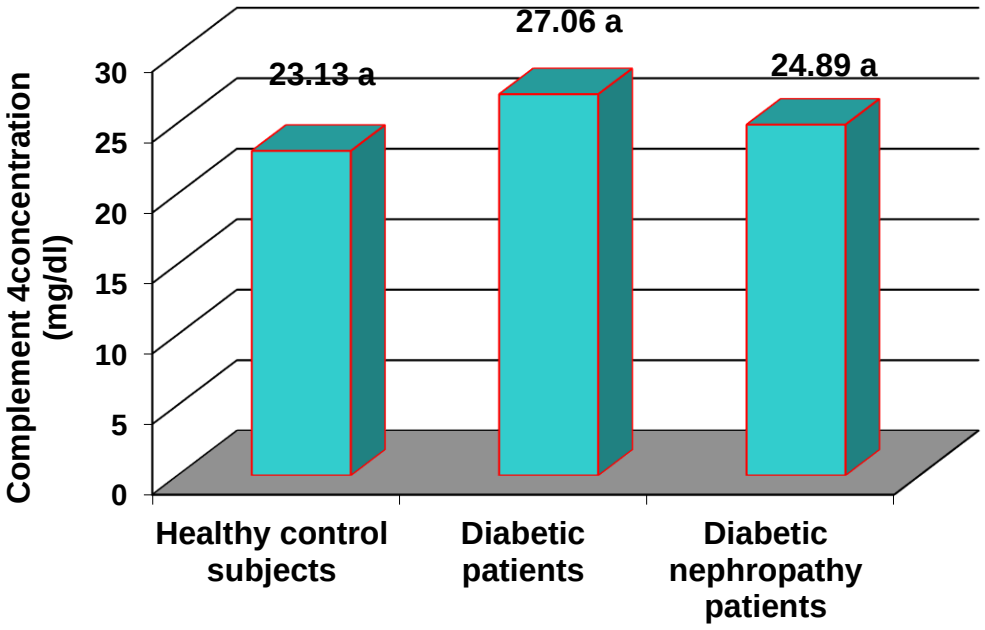

Figure 3.6: Serum complement 4 in healthy control subjects, diabetic and diabetic nephropathy patients (age 45-56 years). similar letters mean nonsignificant (P>0.05)differences.

6. Serum malondialdehyde (MDA) concentration.

Malondialdehyde concentrations in healthy control subjects, diabetic and diabetic nephropathy patients at the age 45-56 are shown in Figure 3.7. No significant (P>0.05) changes are seen in serum MDA concentrations in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

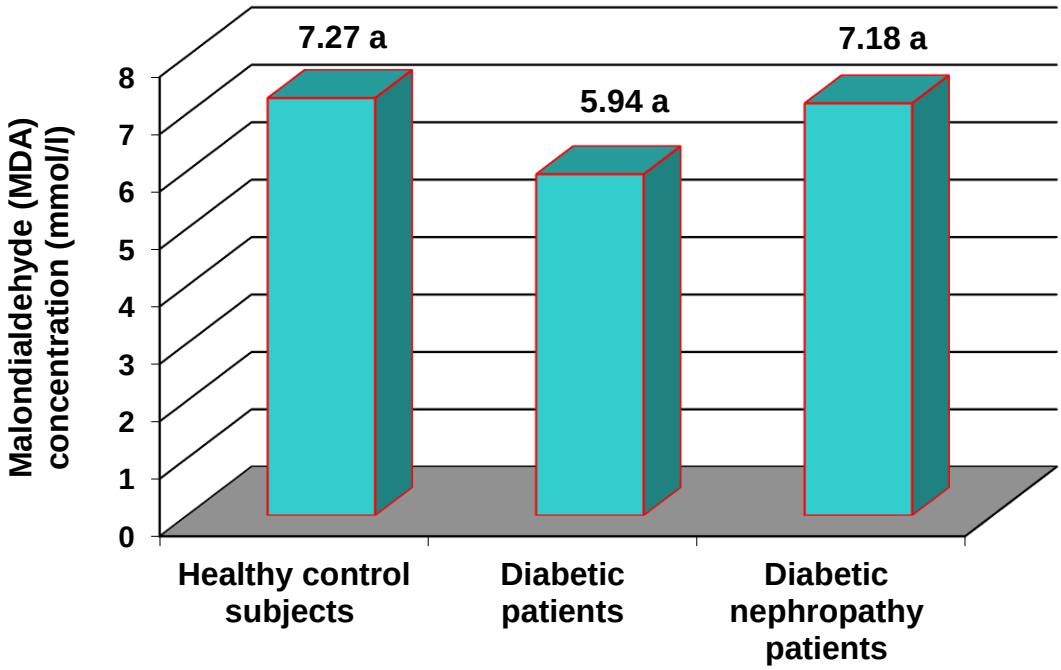

Figure 3.7: Malondialdehyde in healthy control subjects, diabetic and diabetic nephropathy patients(age 45-56 years). different letters mean significant differences at P< 0.05.

7. Serum glutathione (GSH) concentration.

The results of glutathione (GSH) concentrations in healthy control subjects, diabetic and diabetic nephropathy patients are shown in Figure 3.8. Serum glutathione (GSH) concentrations show nonsignificant (P>0.05) changes in diabetic and diabetic nephropathy patients in comparison with the healthy control subjects (Appendix 1).

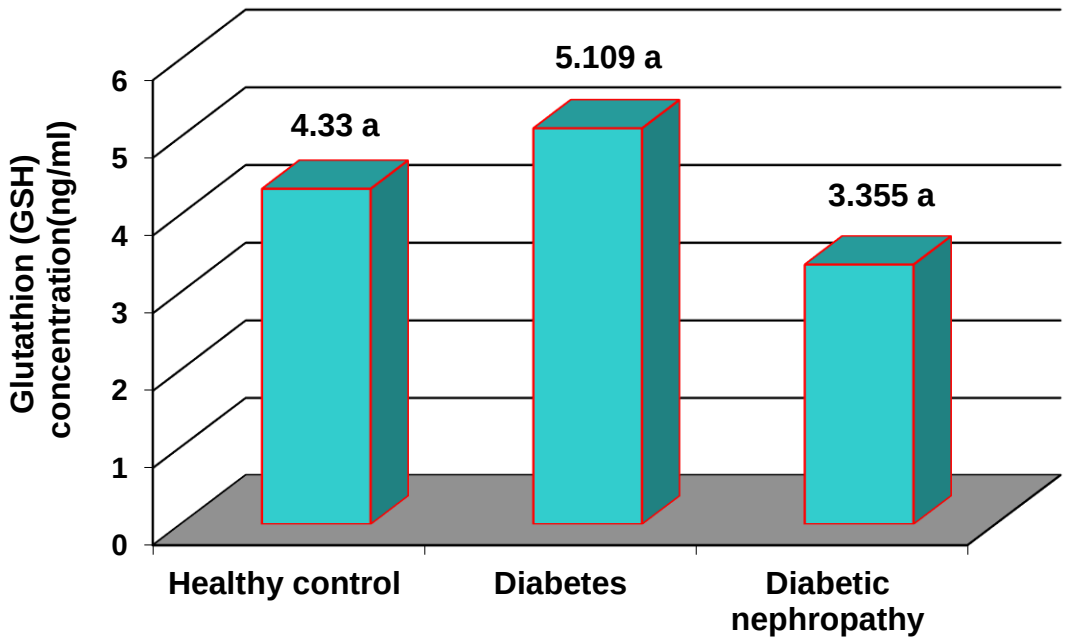

Figure 3.8: Glutathino (GSH)in healthy control, diabetic and diabetic nephropathy (age 45-56 years). different letter mean significant differences at P< 0.05* and a p< 0.01.**

8. Serum Catalase activity.

The activity of serum catalase in healthy control subjects, diabetic and diabetic nephropathy patients at the age 45-56 is shown in Figure 3.9. The activity of serum catalase increases significantly ($p<0.05$) in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

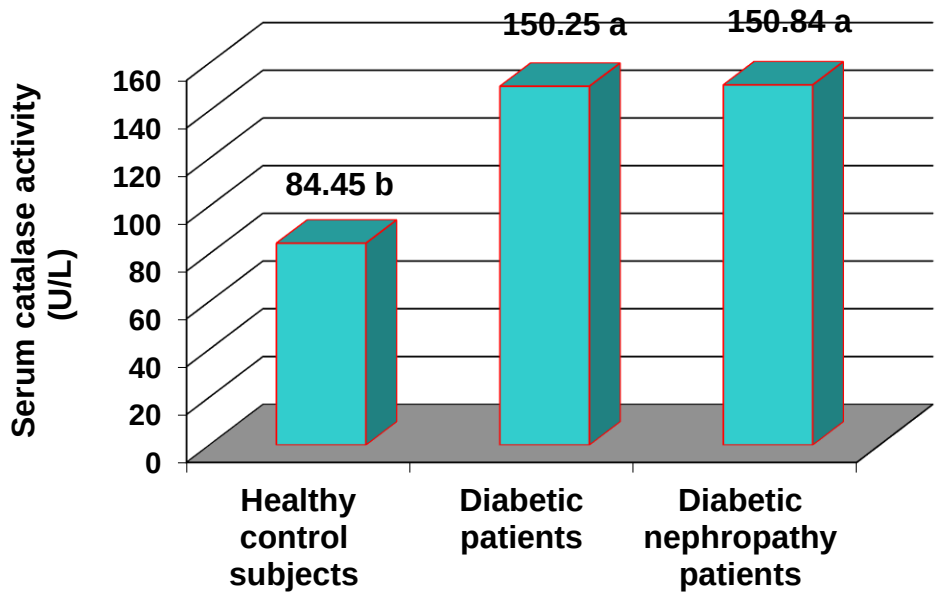

Figure 3.9: Serum catalase activity in healthy control subjects, diabetic and diabetic nephropathy patients (age 45-56 years). different letters mean significant differences at P< 0.05.

9.Urine microalbumin concentration.

Figure 3.10. shows urine microalbumin concentration in healthy control subjects, diabetic and diabetic nephropathy patients. No significant (P>0.05) change is observed in urine microalbumin concentration in diabetic patients, while a significant (P<0.05) increase is noticed in urine microalbomin concentration of diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

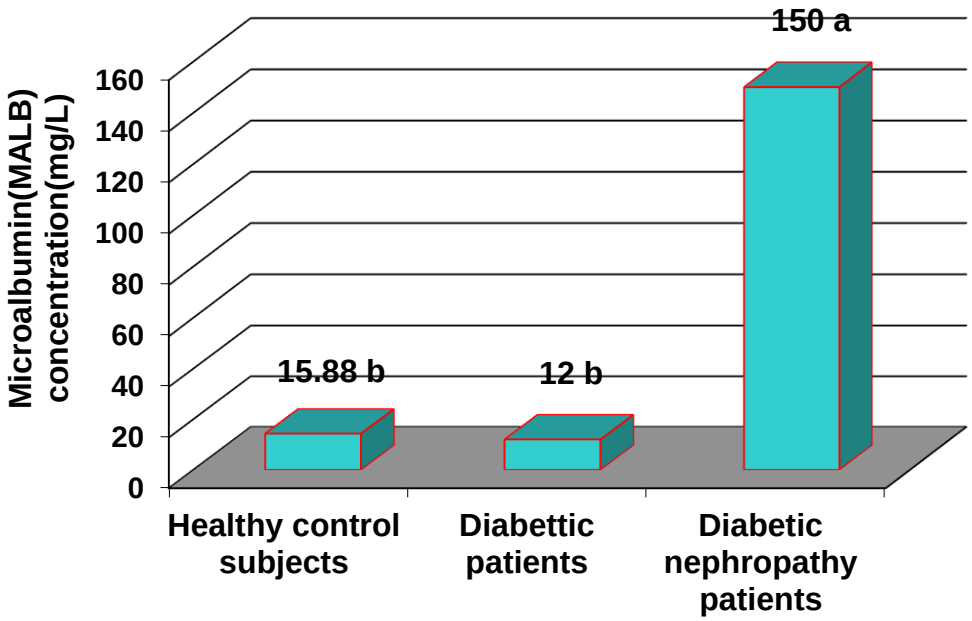

Figure 3.10: Urine microalbumin concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 45-56 years). different letters mean significant differences at P< 0.05.

10. Urine creatinine concentration.

The results concerning urine creatinine concentrations in Figure 3.11. reveals a significant (P< 0.05) decrease in diabetic patients as compared with healthy control subjects. In diabetic nephropathy patients, urine creatinine concentration demonstrates nonsignificant (P>0.05) change in comparison with the healthy control subjects (Appendix 1).

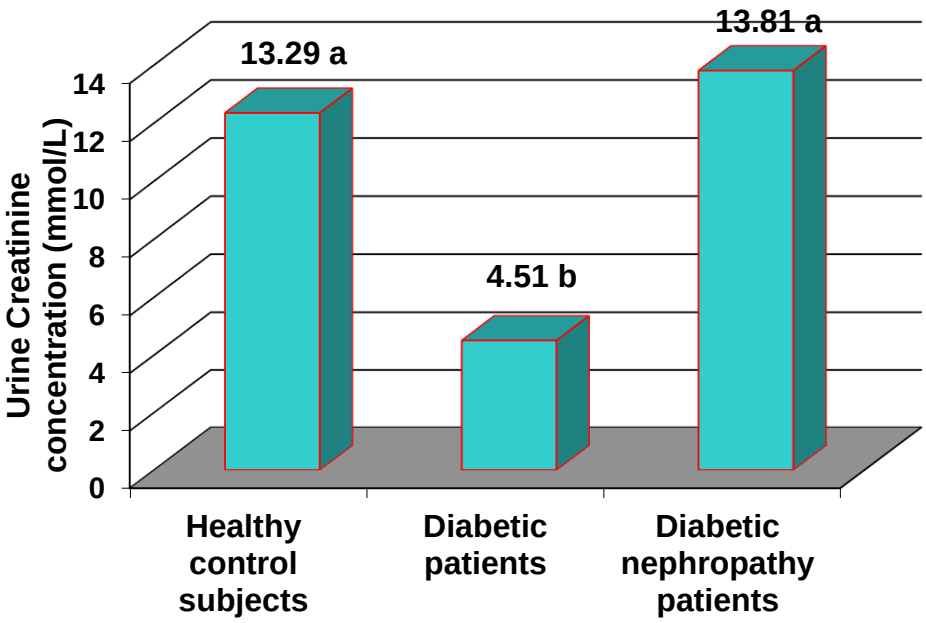

Figure 3.11: Urine creatinine concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 45-56 years). different letters mean significant differences at P< 0.05.

11. Albumin/creatinine ratio.

The results of albumin/creatinine(A/C) ratio are shown in Figure 3.12. A/C ratio shows a significant ($P<0.05$) increase in both diabetic and diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

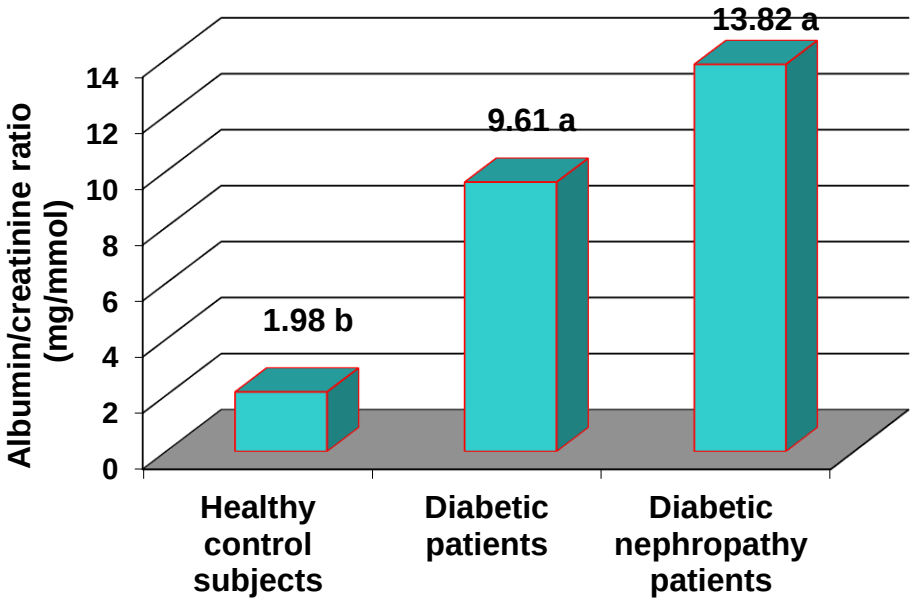

Figure 3.12: Albumin/creatinine ratio in healthy control subjects, diabetic and diabetic nephropathy patients(age 45-56 years). different letters mean significant differences at P< 0.05.

3.1.2 Serum and urine Biochemical parameters in healthy control subjects, diabetic and diabetic nephropathy patients at the of age 57-68 years.

1. Random blood sugar (RBS) concentration.

Figures3.13. shows random blood sugar concentration in healthy control subjects, diabetic and diabetic nephropathy patients at the age 45-56. The Figure demonstrates that RBS increases significantly ($p<0.05$) in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

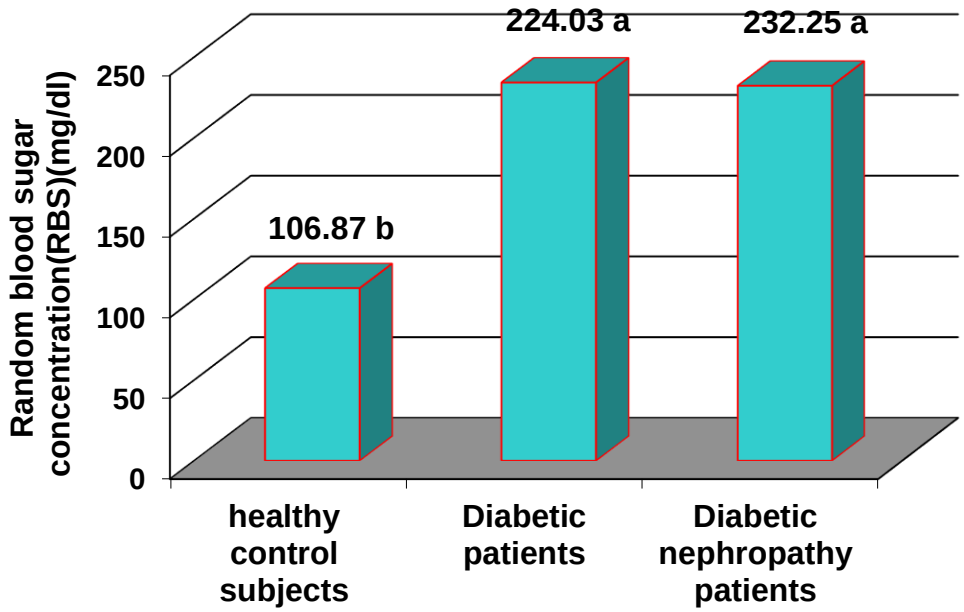

Figure 3.13: Random blood sugar concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). different letters mean significant differences at P< 0.05.

2. Serum urea concentration.

Figure 3.14. shows blood urea concentration in healthy control subjects, diabetic and diabetic nephropathy patients. No significant ($P>0.05$) change is observed in serum urea concentration in diabetic patients, while a significant ($P<0.05$) increase is noticed in serum urea concentration in diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

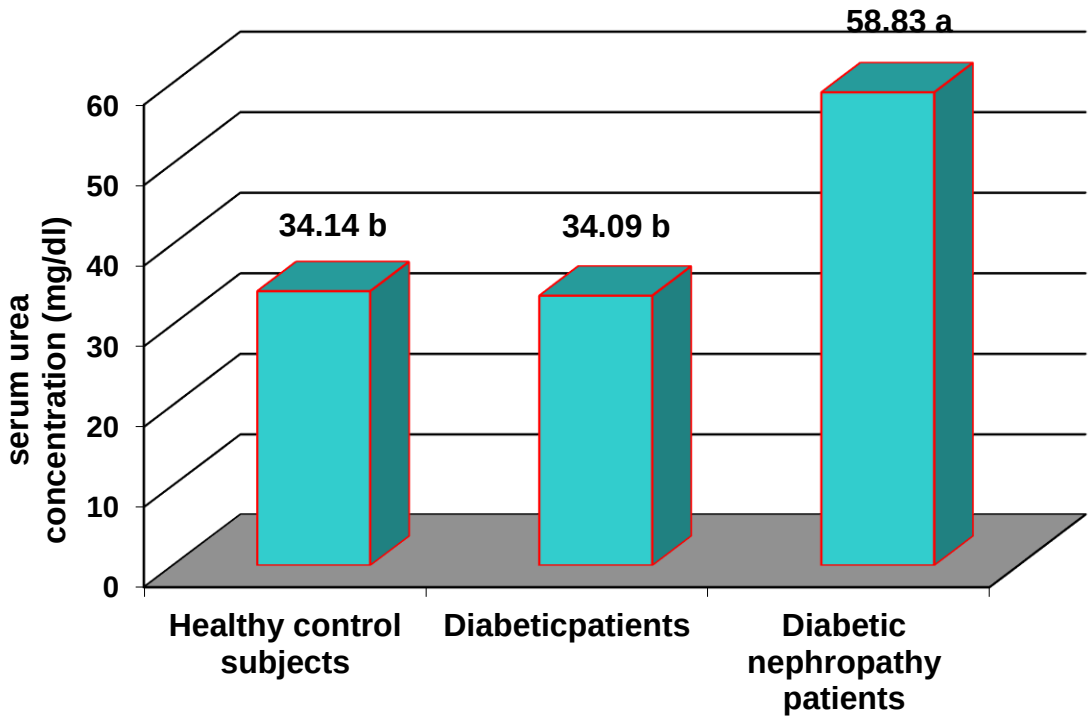

Figure 3.14: serum urea concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). different letters mean significant differences at P< 0.05.

3. Serum creatinine concentration.

The results of serum creatinine concentrations in healthy control subjects, diabetic and diabetic nephropathy patients are shown in Figure 3.15. No change is observed in serum creatinine concentration in diabetic patients, where as in diabetic nephropathy patients, serum creatinine concentration shows a significant ($p<0.05$) increase when compared with the healthy control subjects (Appendix 1).

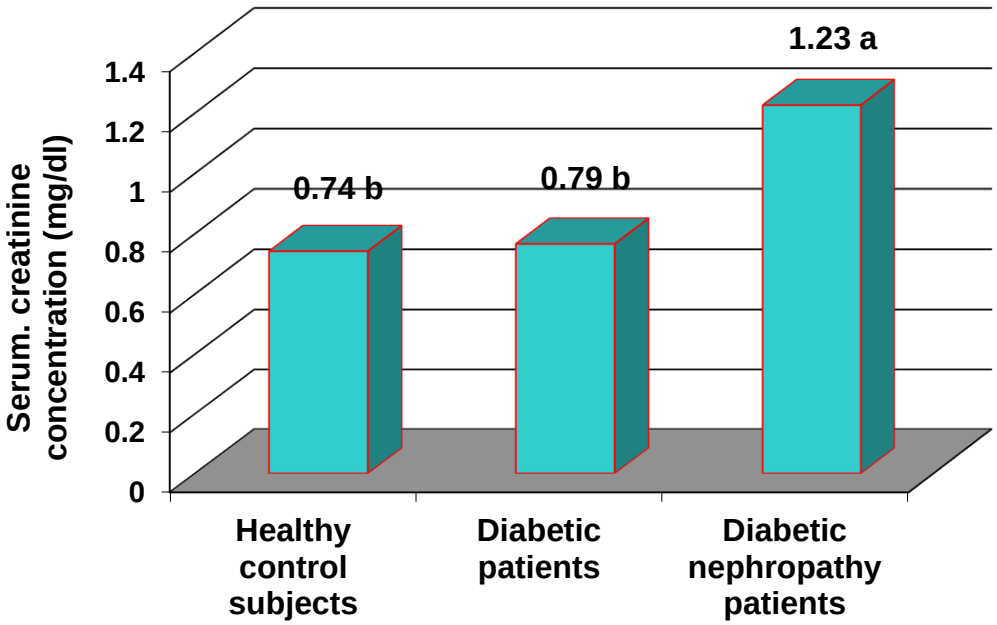

Figure 3.15: Serum creatinine concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). different letters mean significant differences at P< 0.05.

4. Glycosylated hemoglobin HbA1c.

The results of the percentages of HbA1c in healthy control subjects, diabetic and diabetic nephropathy patients are shown in Figure 3.15. The percentages of HbA1c increase significantly ($P<0.05$) in diabetic and diabetic nephropathy patients in comparison with the healthy control subjects (Appendix 1).

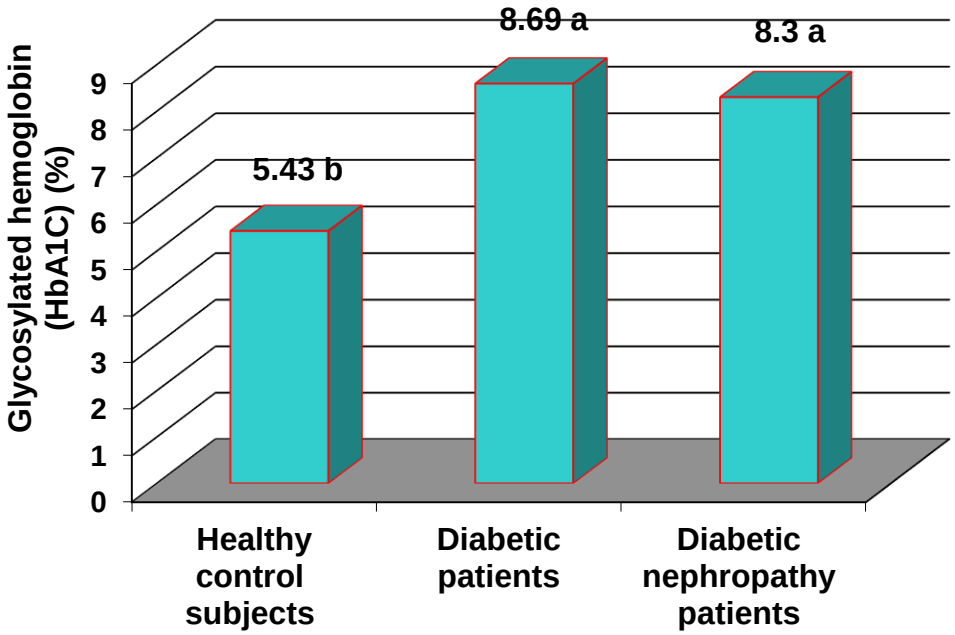

Figure 3.16: The percentage of glycosylated hemoglobin HbA1c in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). different letters mean significant differences at P< 0.05.

5. Serum Complement 3 concentration.

Figure 3.17. shows serum complement 3 in healthy control subjects, diabetic and diabetic nephropathy patients at the age 57-68. The Figure demonstrates a nonsignificant (P>0.05) differentes in diabetic and diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

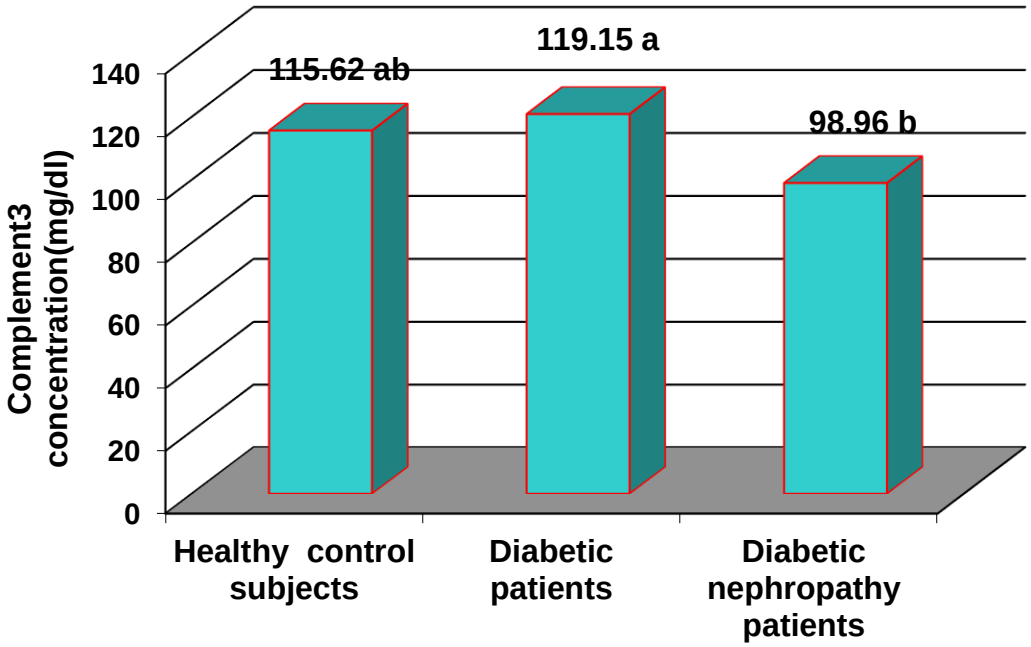

Figure 3.17: Serum complement3 concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). different letters mean significant differences at P< 0.05.

6. Serum Complement4 concentration.

The Figure 3.18. Demonstrates that serum complement 4 concentration show nonsignificant (P>0.05) changes in diabetic and diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

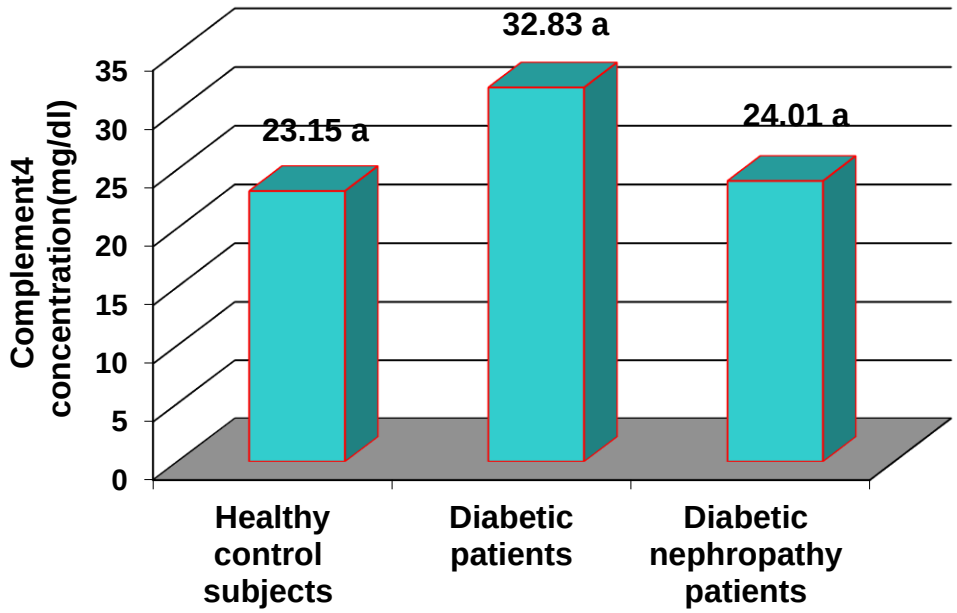

Figure 3.18. Serum complement 4 concentration in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). similar letters mean nonsignificant(P>0.05) differences.

7. Serum malondialdehyde (MDA) concentration.

Figure 3.19. shows nonsignificant (p>0.05) changes in serum malondialdehyde concentration in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

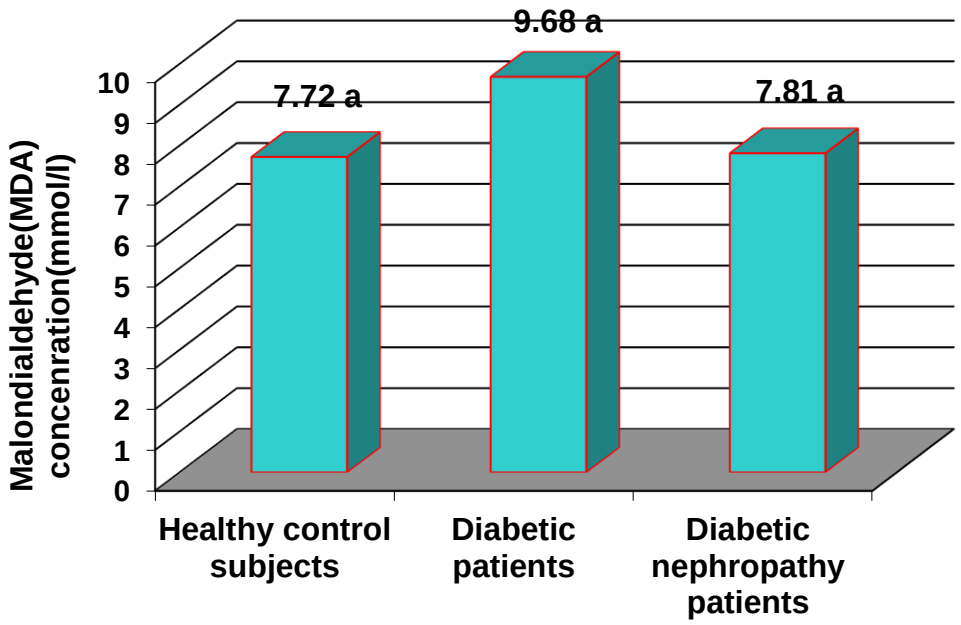

Figure3.19:Serrum malondialdehyde(MDA) concentration in healthy control subjects, diabetic and diabetic nephropathy patients(age 57-68 years). similar letters mean nonsignificant (P>0.05)differences.

8. Serum glutathione (GSH) concentration.

Serum glutathione (GSH) concentrations in healthy control subjects, diabetic and diabetic nephropathy patients at the age 57-68 are shown in Figure 3.20. no significant (P>0.05) changes are seen in GSH concentration in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

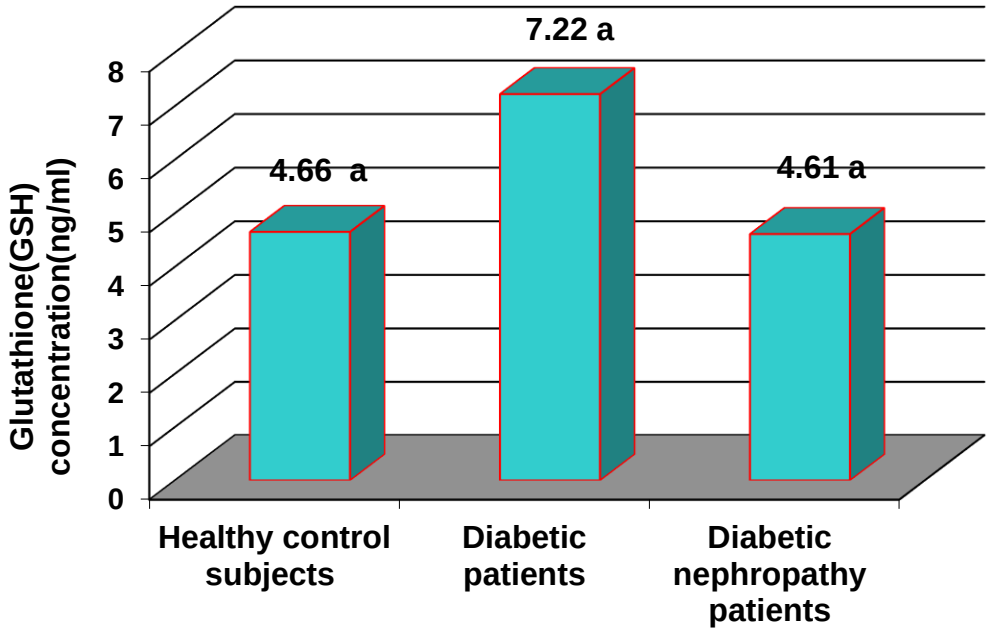

Figure 3.20: Serum glutathione(GSH) concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years).similar letters mean nonsignificant(P>0.05) differences.

9. Serum catalase activity.

Figures3.21. shows the activity of serum catalase in healthy control subjects, diabetic and diabetic nephropathy patients at the age57-68. The Figure demonstrates a significant ($P<0.05$) increase in serum catalase activity in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

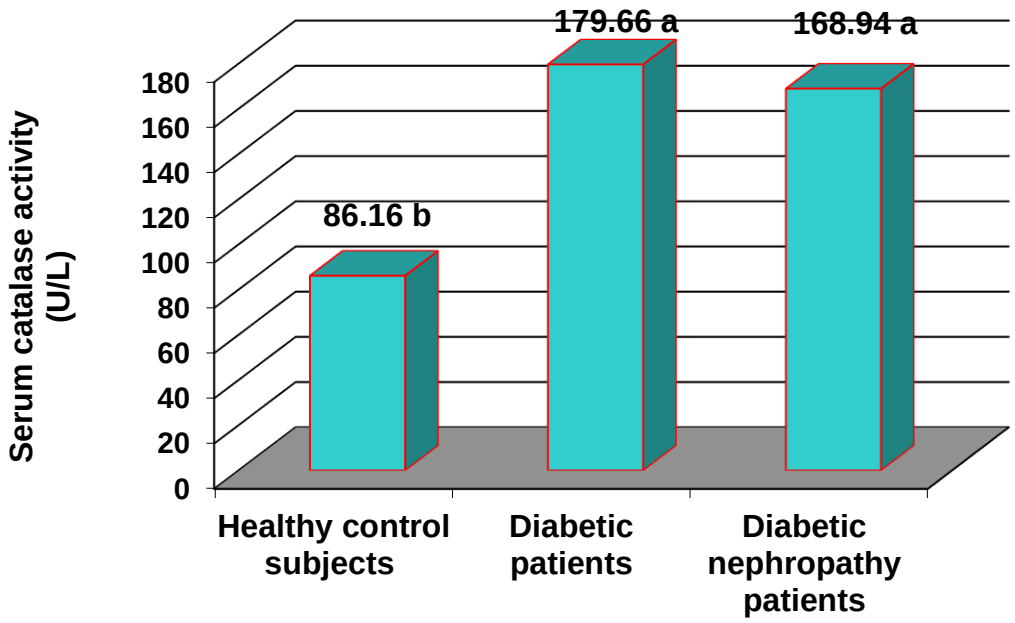

Figure 3.21: Serum catalase activity in healthy control subjects, diabetic and diabetic nephropathy patients (age 57- 68years). different letters mean significant differences at P< 0.05.

10. Urine microalbumin concentration.

Figure 3.22. shows urine microalbumin concentration in healthy control subjects, diabetic and diabetic nephropathy patients. No significant (P>0.05) change is observed in urine microalbumin concentration in diabetic patients, while a significant (P<0.05) increase is noticed in urine microalbumin concentration in diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

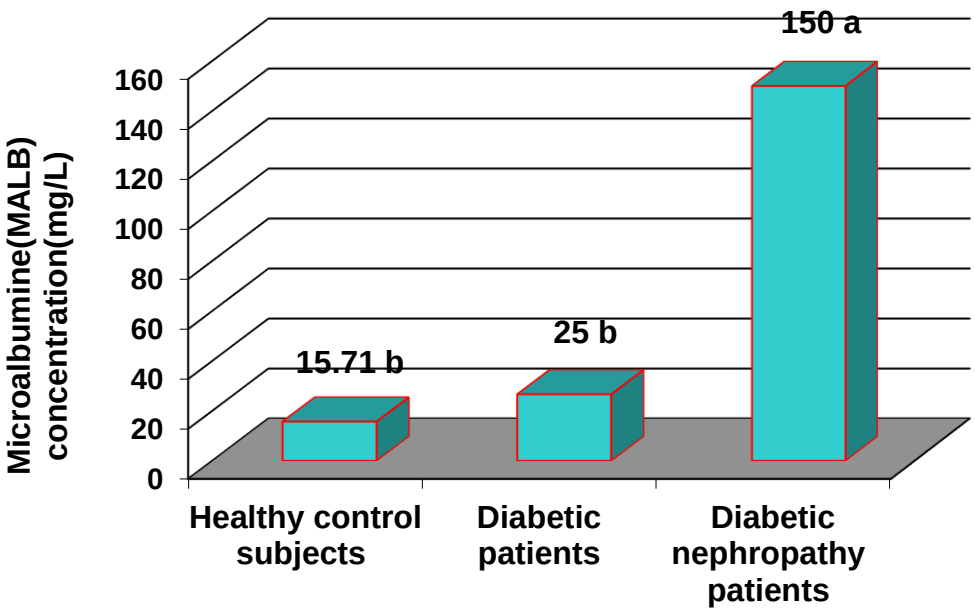

Figure 3.22: Urine microalbumine(MALB) concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). different letters mean significant differences at P< 0.05.

11. Urine creatinine concentration.

Urine creatinine concentration in healthy control subjects, diabetic and diabetic nephropathy patients at the age 57-68 is shown in Figure 3.23. No significant (P>0.05) changes are seen in urine creatinine concentrations of diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

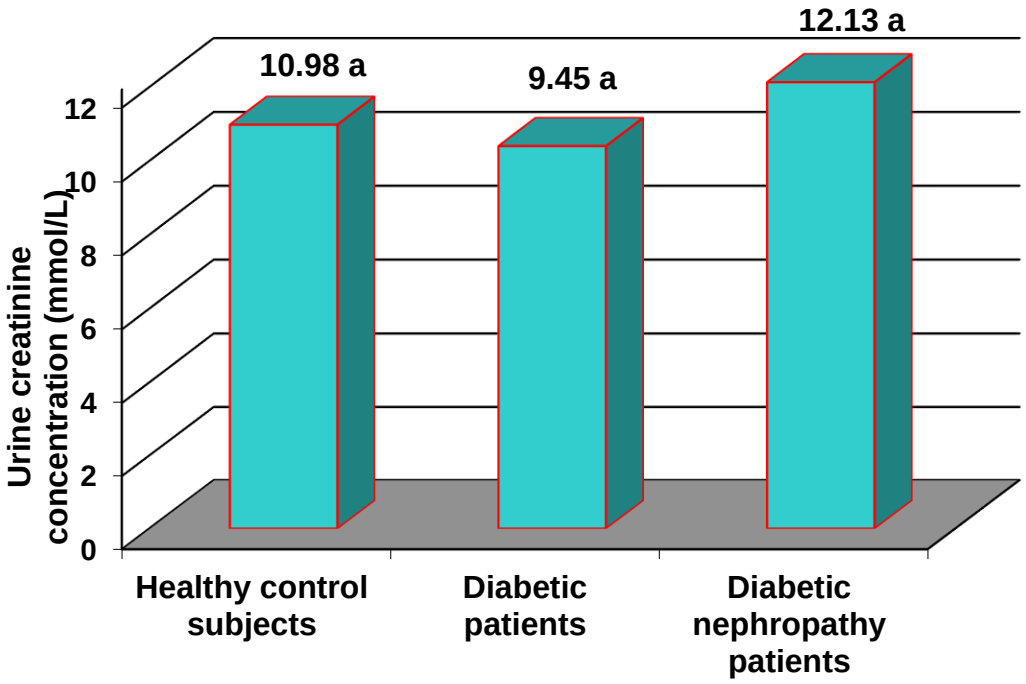

Figure 3.23: Urine creatinine concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). similar letters mean nonsignificant (P>0.05) differences.

12. Albumin/creatinine ratio.

The results of albumin/creatinine ratio are shown in Figure 3.24. No change is observed in albumin/creatinine ratio in diabetic patients, whereas in diabetic nephropathy patients, albumin/creatinine ratio shows a significant ($p<0.05$) increase when compared with the healthy control subjects (Appendix 1).

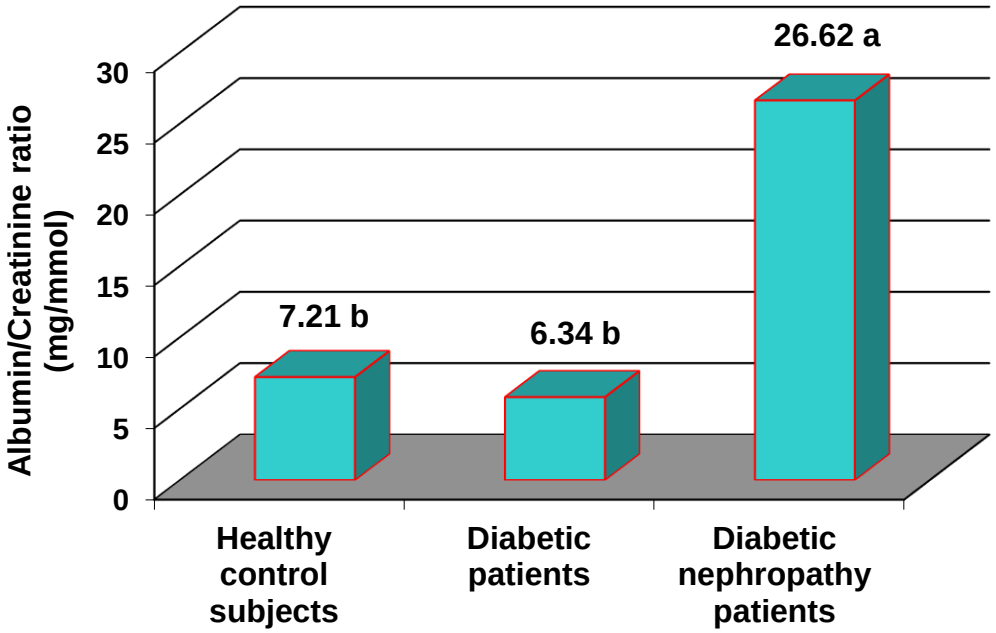

Figure 3.24: Albumin/Creatinine ratio in healthy control subjects, diabetic and diabetic nephropathy patients (age 57-68 years). different letters mean significant differences at P< 0.05.

3.1.3 Serum and urine Biochemical parameters in healthy control subjects, diabetic and diabetic nephropathy patients at the age 69-80 years.

1. **Random blood sugar concentration.**

Figures3.25 shows random blood sugar concentration in healthy control subjects, diabetic and diabetic nephropathy patients at the age 69-80. The Figure demonstrates that RBS increases significantly ($p<0.05$) in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

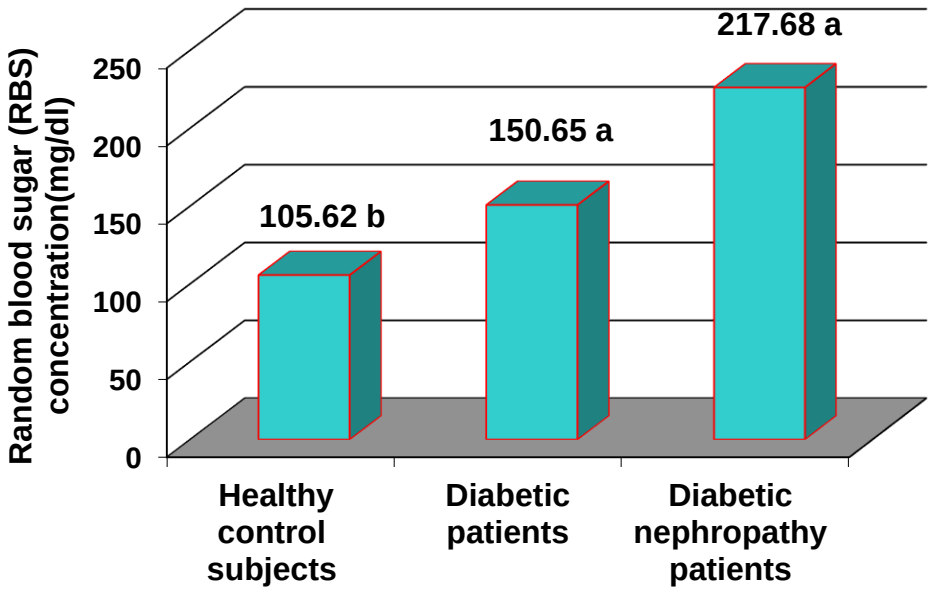

Figure 3.25: Random blood sugar (RBS) concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 69-80 years). different letter mean significant differences at P< 0.05.

2. Serum urea concentration.

The results of serum urea concentrations are shown in Figure 3.26. No change is observed in serum urea concentration in diabetic patients, whereas in diabetic nephropathy patients, serum urea concentration shows a significant ($p<0.05$) increase when compared with the healthy control subjects (Appendix 1).

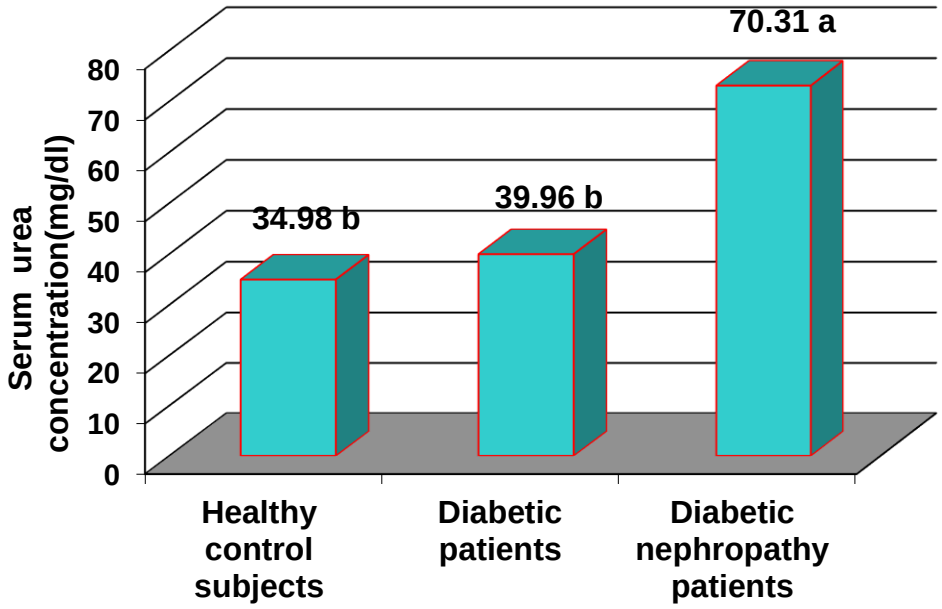

Figure 3.26: Serum urea concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 69-80 years). different letters mean significant differences at P< 0.05.

3. **Serum creatinine concentration.**

Figure 3.27. shows serum creatinine concentrations in healthy control subjects, diabetic and diabetic nephropathy patients. No significant ($P>0.05$) change is observed in serum creatinine concentration of diabetic patients, while a significant ($P<0.05$) increase is noticed in serum creatinine concentration in diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

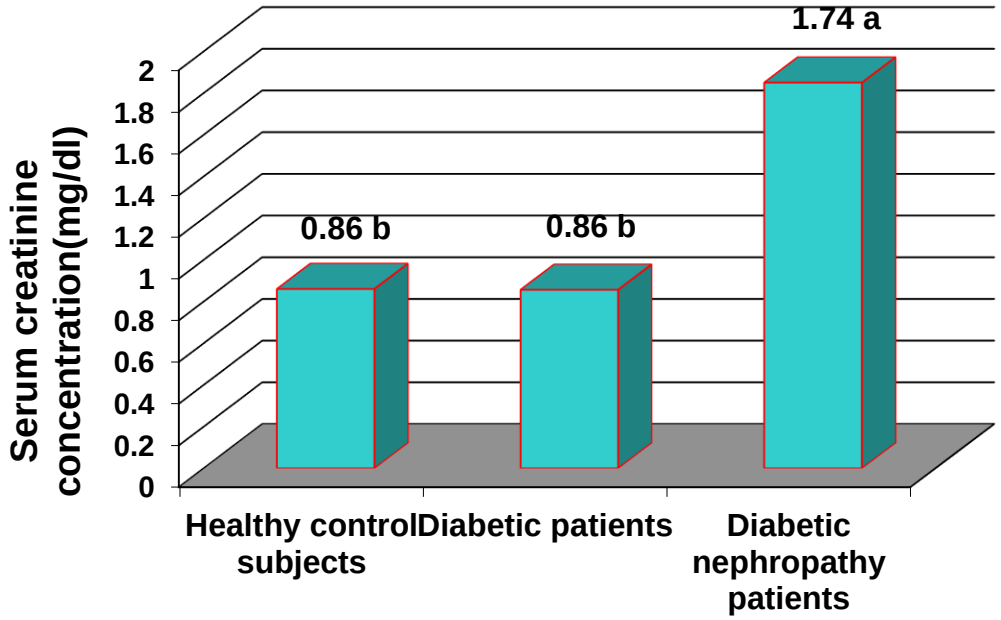

Figure 3.27: Serum creatinine concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age = 68-80 years). different letters mean significant differences at P< 0.05.

4. Glycosylated hemoglobin HbA1c.

The results of the percentages of HbA1c in healthy control subjects, diabetic and diabetic nephropathy patients are shown in Figure 3.28. The percentages of HbA1c increase significantly ($P<0.05$) in diabetic and diabetic nephropathy patients in comparison with the healthy control subjects (Appendix 1).

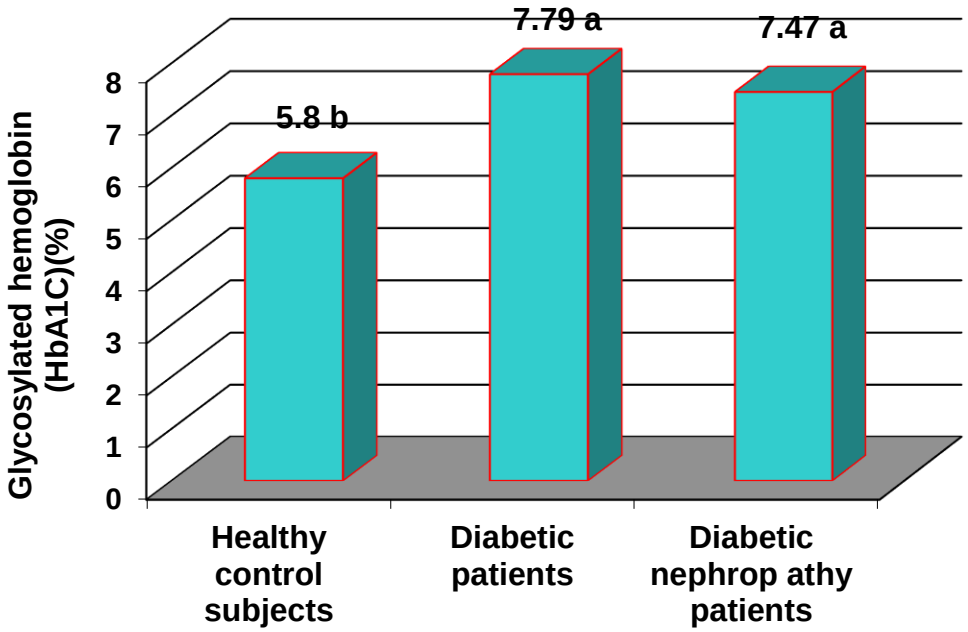

Figure3.28. The percentage of glycosylated hemoglobin(HbA1C)in healthy control subjects, diabetic and diabetic nephropathy patients (age 69-80 yrars). different letters mean significant differences at P< 0.05.

5. Serum complements 3 and 4(C3 and C4) concentration.

The Figures 3.29. and 3.30. demonstrate that serum complements 3 and 4 concentrations show no significant (P>0.05) changes in diabetic and diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

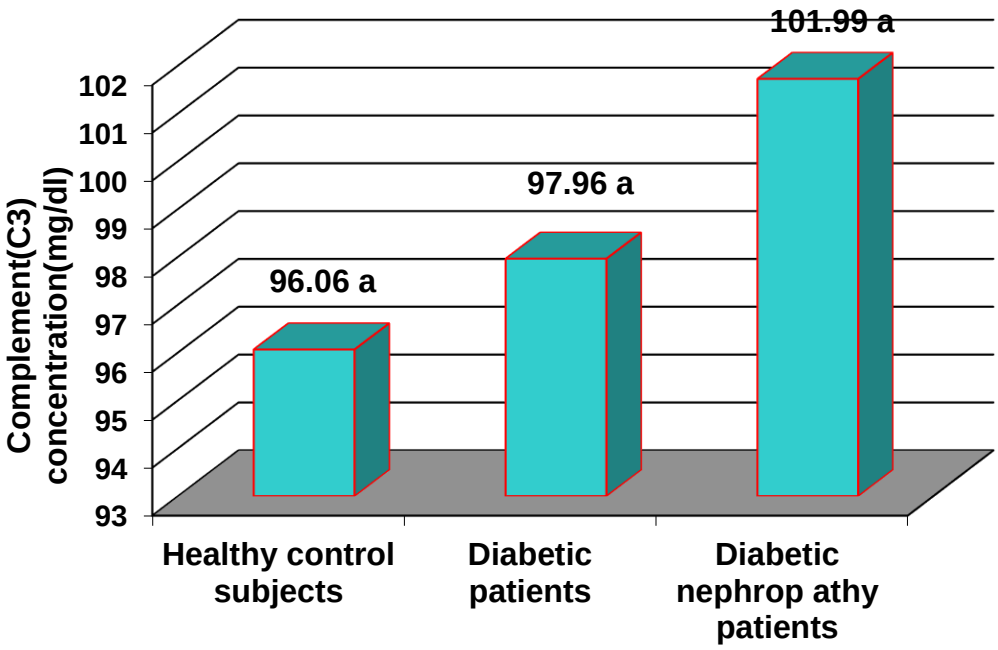

Figure 3.29: Serum complement3 concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 69-80 years). similare letters mean nonsignificant(P>0.05) differences.

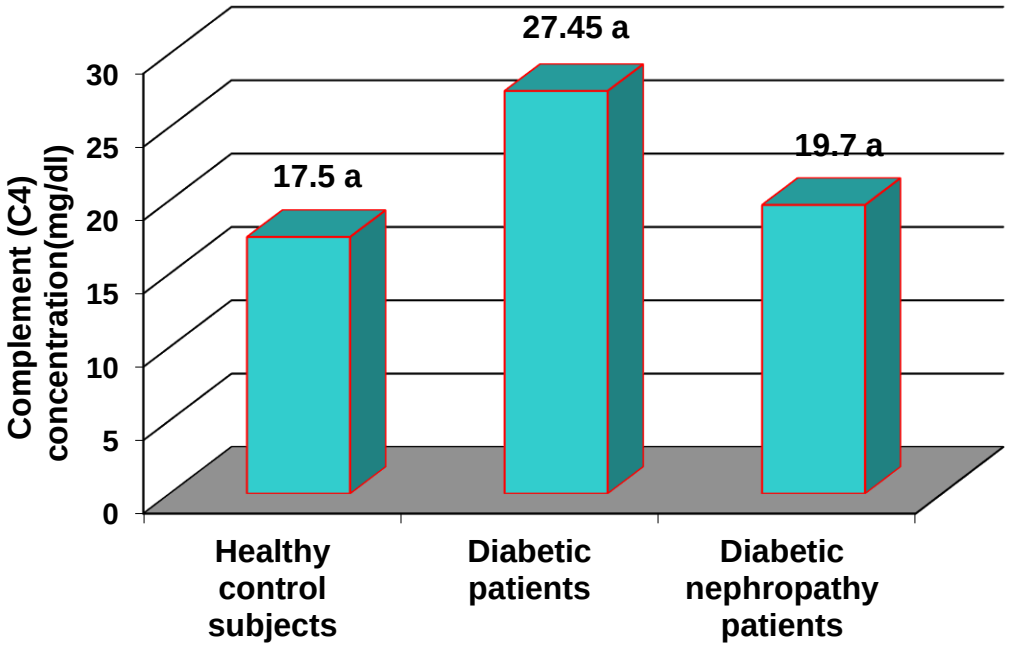

Figure 3.30: Serum complement 4 concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 69-80 years). similar letters mean nonsignificant(P>0.05) differences.

6. Serum malondialdehyde (MDA) concentration.

Figure 3.31. shows serum malondialdehyde concentrations in healthy control subjects, diabetic and diabetic nephropathy patients at the age 68-80. The Figure demonstrates that serum MDA concentration decrease significantly (P<0.05) in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

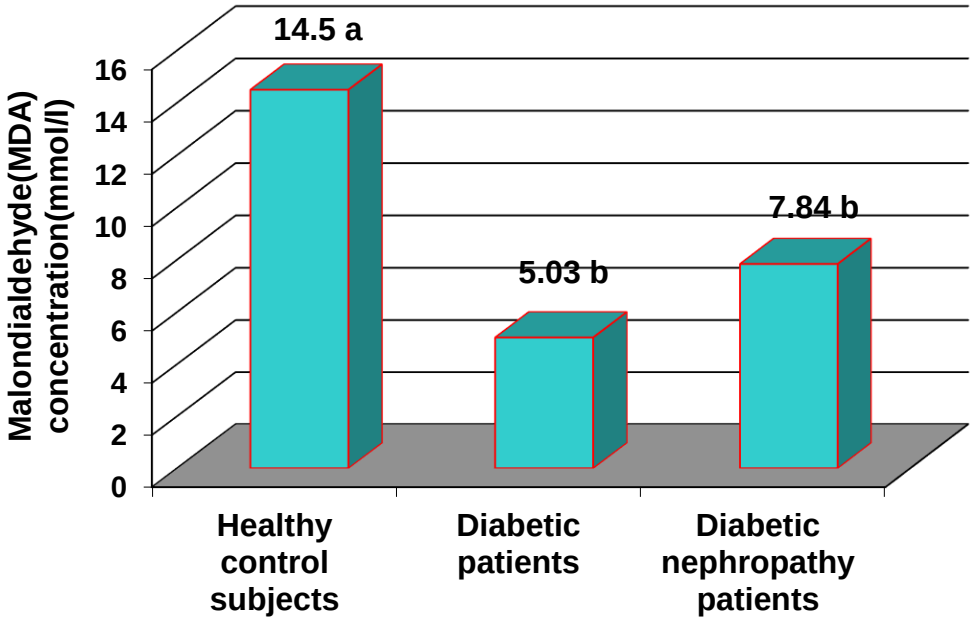

Figure3.31:Serummalondialdehyde(MDA) concentrations in healthy control, diabetic and diabetic nephropathy patients (age 69-80 years). different letters mean significant differences at P< 0.05.

7. Serum glutathione (GSH) concentration.

The results of serum glutathione (GSH) concentrations are shown in Figure 3.32. serum GSH concentrations decrease significantly (P<0.05) in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

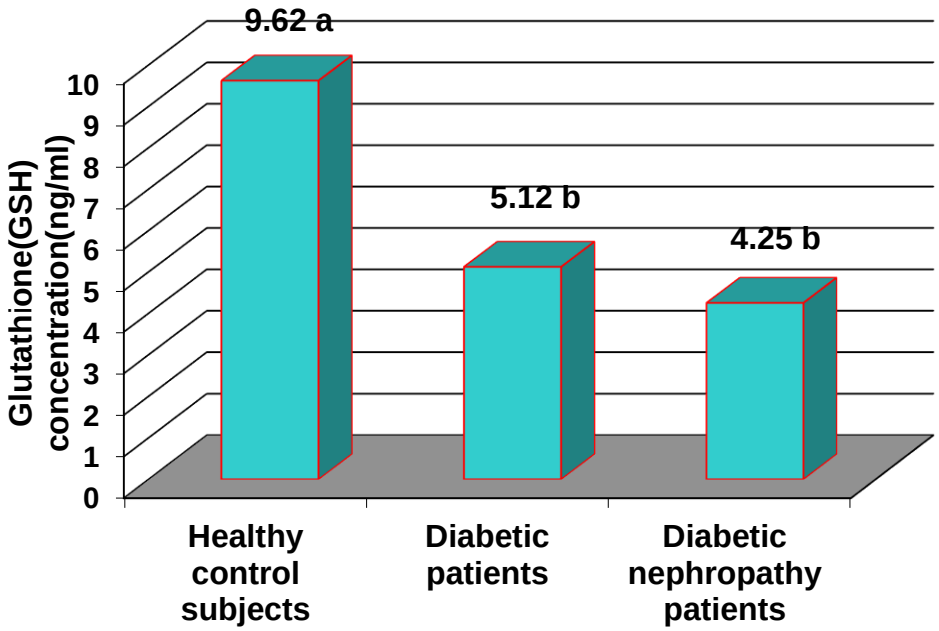

Figure 3.32: Serum glutathione(GSH) concentrations in healthy control subjects, diabetic and diabetic nephropathy patients (age 69-80 years). different letters mean significant differences at P< 0.05.

8. Serum catalase activity.

Figure 3.33 shows no significant ($p>0.05$) change in the activity of serum catalase in diabetic and diabetic nephropathy patients as compared with the healthy control subjects (Appendix 1).

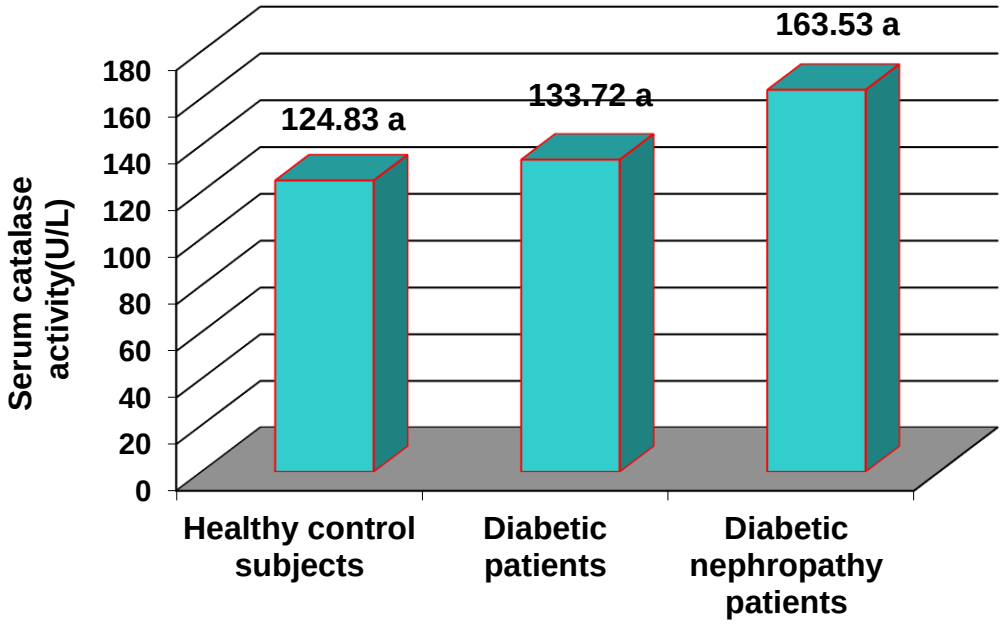

Figure 3.33.Serum catalase activityin healthy control subjects, diabetic and diabetic nephropathy patients (age 69-80 years). similar letters mean nonsignificant(P>0.05) differences.

9. Urine microalbumin (MALB) concentration.

Figure 3.34. shows urine microalbumin concentrations in healthy control subjects, diabetic and diabetic nephropathy patients. No significant (P>0.05) change is observed in urine microalbumin concentration in diabetic patients, while a significant (P<0.05) increase is noticed in urine microalbomin concentration in diabetic nephropathy patients when compared with the healthy control subjects (Appendix 1).

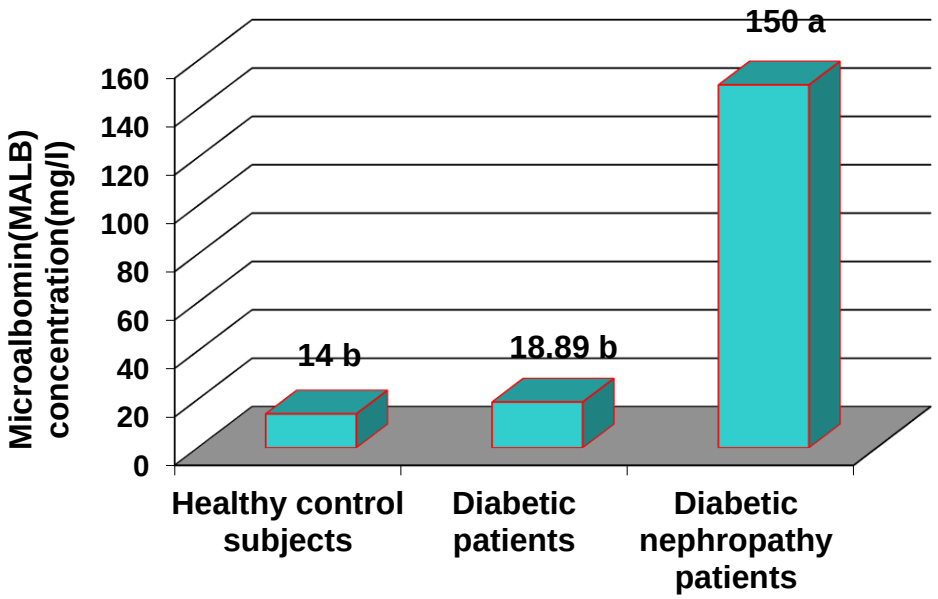

Figure3.34. Urine microalbomin(MALB) concentration in healthy control, diabetic and diabetic nephropathy patients(age 69-80 years). different letters mean significant differences at P< 0.05.

10. Urine creatinine concentration.

The results of urine creatinine concentrations are shown in Figure 3.35. No change is observed in urine creatinine concentration in diabetic patients, whereas in diabetic nephropathy patients, urine creatinine concentration shows a significant ($p<0.05$) increase when compared with the healthy control subjects (Appendix 1).

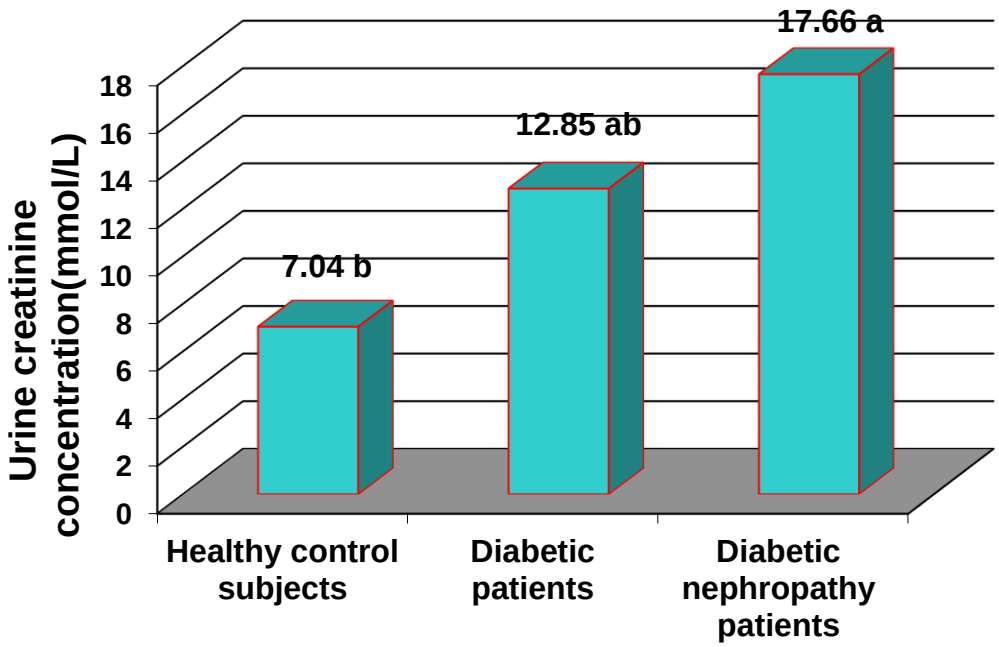

Figure 3.35: Urine creatinine concentrations in healthy control subjects, diabetic and diabetic nephropathy patient(age 69-80 years). different letters mean significant differences at P< 0.05.

11. Albumin/creatinine ratio.

The results concerning albomin/creatinine ratio in Figure 3.36. reveals a significant (P< 0.05)increase in diabetic nephropathy patients as compared with healthy control subjects. In diabetic patients, Albumin/Creatinine ratio demonstrates no significant (P>0.05) change in comparison with the healthy control subjects (Appendix 1).

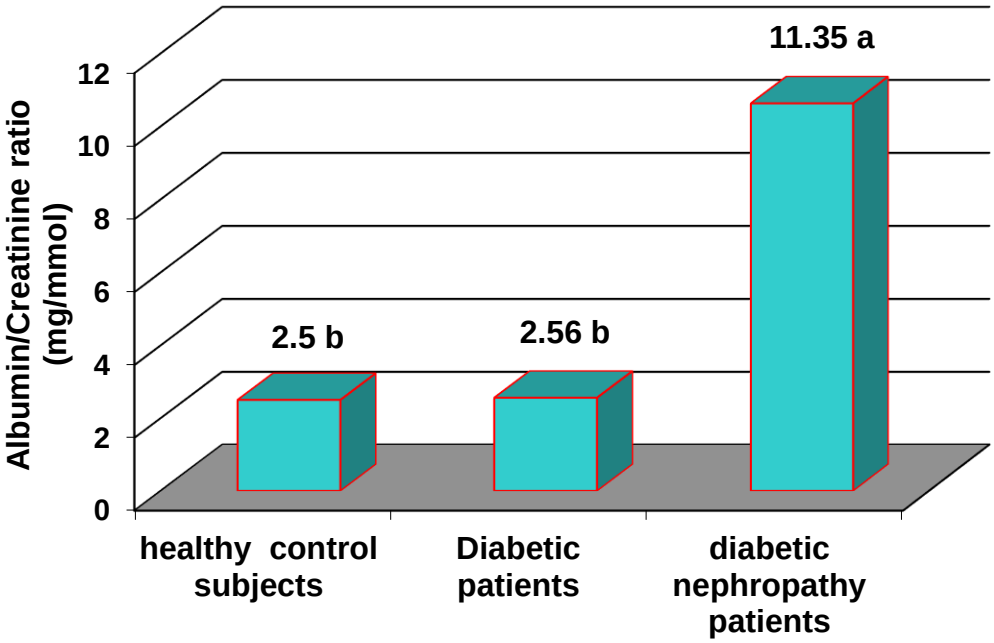

Figure 3.36: Albumin/Creatinine ratio in healthy control, diabetic and diabetic nephropathy patients(age 69-80 years). different letters mean significant differences at P< 0.05.

3.2 Discussion.

3.2.1Random blood sugar.

The results of the present study showed an in increase in random blood sugar in diabetic and diabetic nephropathy patients at the ages of 45-80 years. Similar results were obtained in diabetic (Deepa *et al*., 2011; Singh *et al*., 2014; Kanwar *et al*., 2015; Bamanikar *et al*., 2016) and diabetic nephropathy patients (Ahmed *et al*., 2010; Usman *et al*., 2012). Hyperglycemia results from defects in insulin secretion and insulin action or both. Type 2 diabetes mellitus is characterized by insulin resistance in peripheral tissue and a defect in insulin secretion from the beta cell of the pancreas (Garber, 2000). The chronic hyperglycemia associated with diabetes mellitus is due to derangement in carbohydrate, fat, and protein metabolism (Kanwar *et al*., 2015). It can increase oxidative stress which has been considered to be a pathogenic factor of diabetic complications including nephropathy (Kumawat *et al*., 2013).

3.2.2 Serum urea:

No change was observed in serum urea concentration in diabetic patients, whereas an increase was demonstrated in diabetic nephropathy patients at the ages of 45-80 years. Bamanikar *et al*. (2016) demonstrated that out of 100 diabetic samples, 18 had high urea level. They showed that poorly controlled blood sugar levels would cause increase in serum urea levels thus increasing the chances of the patient suffering from diabetic nephropathy. The duration and severity of diabetes strongly correlate with serum urea levels (Bamanikar *et al*., 2016). The increase in serum urea concentration in diabetic nephropathy patients in the current study is in accordance with other studies (Ahmed *et al*., 2010; Singh *et al*., 2014; Kanwar *et al*.,2015). Bamanikar *et al*.(2016) reported that an

increase in urea level is observed when there is damage to the kidney, and the increase in blood urea level in the presence of hyperglycemia in diabetic patients indicates damage to the kidney.

3.2.3Serum creatinine:

The findings of the current study indicated no change in serum creatinine concentrations in diabetic patients at the ages of 45-80 years and in diabetic nephropathy patients at the age of 45-56 years. Serum creatinine concentrations increased significantly in diabetic nephropathy patients at the ages of 57-80 years. Creatinine is a waste product in the blood formed by the normal breakdown of muscles cells during activity, and the healthy kidneys filters creatinine out of the blood into the urine (Usman *et al*., 2012). When kidneys are not functioning well, creatinine levels build up in the blood, since creatinine is an indicator of normal functioning of the kidney and its increase in the blood indicates kidney dysfunction (Kamal, 2014). Anjaneyulu and Chopra (2004) reported that the increase in serum creatinine in diabetic rats indicated progressive renal damage.

3.2.4 Glycosylated hemoglobin:

The results obtained from this study revealed that the percentages of glycosylated hemoglobin increased in diabetic and diabetic nephropathy patients at the ages of 45-80 years. These findings seem to agree with Rani *et al*. (2016) who observed a significant increase in glycosylated hemoglobin (%) in complicated and non-complicated patients with nephropathy. Carbohydrates such as glucose is known to bind non-enzymatically to proteins such as hemoglobin in a process called glycosylation (glycation) (Varashree and Gopalakrishna, 2011). Glycosylated hemoglobin is formed by non-enzymatic, substrate-

concentration dependent irreversible process of combination of aldehyde group of glucose with the amino-terminal valine of the β- chain of hemoglobin (Chandalia and Krishnaswamy, 2002). The formation of glycosylated hemoglobin is dependent on glucose concentration, and the individuals with hyperglycemia will have higher levels of glycosylated hemoglobin (Gabby *et al*., 1979).

3.2.5 Serum complements 3 and 4:

No changes were seen in serum complements 3 and 4 concentrations in diabetic and diabetic nephropathy patients of all age groups with the exception of serum complement 3 concentration at the age of 57-68 years which showed a decrease. Conflicting results were reported in the literature concerning the role of complement in diabetes mellitus. Nikolova *et al*. (2004) found that serum complements 3 and 4 levels were within the normal range in diabetic nephropathy patients. On the contrary, Dezayee and Alnakshabandi (2011) demonstrated an increase in serum complement 3 in type 2 diabetic patients with a decrease in serum complement 4 as compared with the healthy subjects. On the other hand, there is evidence that complement may be involved in susceptibility to and progression of diabetic nephropathy (Fearn and sheerin, 2015).

3.2.6 Serum malondialdehyde:

In the present study, no changes were observed in serum malondialdehyde concentrations in diabetic and diabetic nephropathy patients at the age of 45-68 years, whereas a decrease in serum malondialdehyde concentration was noticed in diabetic and diabetic nephropathy patients at the ages of 69-80 years. Human body possesses natural antioxidants to control the level of reactive oxygen species by scavenging them both enzymatically and non-enzymetically (Matough *et*

al., 2012). In addition to the naturally occurring antioxidants, the antioxidant activity of drugs may play a role in ameliorating the oxidative stress induced renal damage during diabetes (Agarwal *et al*; 2015) and good glycemic control could be supportive and beneficial in reducing effects caused by oxidative stress (Jabeen *et al*., 2013).

3.2.7 Serum glutathione:

Serum glutathione concentrations show nonsignificant changes in diabetic and diabetic nephropathy patients at the ages of 57-68 years. At the age of 69-80 years, serum glutathione concentration decreased in diabetic and diabetic nephropathy patients. Glutathione level has been shown to decrease in type 2 diabetes mellitus (Dongre and Meshram, 2015) with and without nephropathy (Kumawat *et al*., 2013). Glutathione can maintain SH groups of protein, detoxify foreign radicals and also act as coenzyme in several enzymatic reactions (Tsai *et al*., 2012). The decrease in the glutathione level and glutathione peroxidase activity was correlated with glutathione reductase activity in diabetic nephropathy (Kumawat *et al*., 2013). Glutathione peroxidase catalyzes the reduction of lipid peroxides at the expense of glutathione. H_2O_2 is inactivated by glutathione peroxidase resulting in consumption of glutathione (Waggiallah and Alzohairy, 2011). The oxidized glutathione is reduced to two molecules of glutathione by glutathione reductase.

3.2.8 Serum catalase activity:

The results of the current study demonstrated an increase in serum catalase activity in diabetic and diabetic nephropathy patients at the ages of 45-80 years. These results are in agreement with those obtained by others in type 2 diabetes mellitus with or without nephropathy (Kumawat *et al*., 2013 ; Jabeen *et al*., 2013; Goicheva *et al*., 2014). On the contrary,

Ezeiruaku *et al*. (2016) reported that the mean plasma catalase activity decreased in type 2 diabetics. Kedziora-Kornatowska *et al*. (1998) found lower catalase activities in erythrocytes of non-insulin dependent diabetes mellitus patients with or without nephropathy. Catalase acts as main regulator of hydrogen peroxide metabolism. It catalyzes the decomposition of hydrogen peroxide to water and oxygen (Jabeen *et al*., 2013). The reason for increased catalase activity can be linked to a secondary compensatory activation of the enzyme (Goicheva *et al*., 2014).

3.2.9 Urine microalbumin:

The present study revealed no change in urine microalbumin concentration in diabetic patients, while an increase was seen in diabetic nephropathy patients. The microalbuminuria levels were shown to be higher in complicated cases (Rani *et al*., 2016). The development of vascular complications in diabetes correlates with the intensity of hyperglycemia, and the high intracellular glucose concentration has been suggested to be a prerequisite for the development of structural and functional changes in the kidney (Kumawat *et al*., 2013). Diabetic nephropathy is characterized by proteinuria, reduced glomerular filtration rate, podocyte injury, mesangial matrix accumulation and tubulointerstitial damage (Wolf, 2004). Therefore, The cause of increased in microalbuminuria may be attributed to a defect in the glomerulare membrane filtration.

3.2.10 Urine creatinine:

The results of the present study showed no changes in urine creatinine concentrations in diabetic and diabetic nephropathy patients, with the exception of diabetic patients at the age of 45-56 years which

demonstrated a decrease, and diabetic nephropathy patients at the age of 69-80 years which showed an increase. It is assumed that creatinine excretion rates are fairly constant during the day, as long as the glomerular filtration rate (GFR) remains constant (Price *et al*., 2005). Differences in creatinine excretion are likely in part due to differences in muscle mass (Mattix *et al*., 2002). Diabetes affects the kidney in stages, and at the onset of diabetes, the GFR becomes disturbed (Dabla, 2010). Creatinine is used for the determination of GFR. It is filtered by the glomerulus , and a small amount is secreted into the glomerular filtrate by the proximal tubule. Creatinine clearance is the rate at which creatinine is cleared from the blood (i.e. excreted into the urine) and normal kidneys have higher filtration rate than the affected ones in terms of kidney disease (Bazari, 2007). Diabetic nephropathy occurs in conjunction with hyperfiltrative period in which creatinine clearance and GFR are high (USRDS, 2002)followed by a gradual decrease in GFR that leads to kidney failure (Gal *et al*., 1997).

3.2.11 Albumin / creatinine ratio:

In the current study, an increase in albumin / creatinine ratios was seen in diabetic patients at the age of 45-56 years, and diabetic nephropathy patients at the age of 45-80 years. No change was observed in albumin / creatinine ratio in diabetic patients at the ages of 57-80 years. Manjunatha Goud *et al*. (2010)showed a significant increase in urine albumin / creatinine ratio in diabetic cataract patients without nephropathy and in diabetic cataract patients with nephropathy. The urine albumin / creatinine ratio is commonly used as an index of albuminuria (Wang *et al*., 2013). The presence of microalbuminuria in type 2 diabetic patients is an important predictor of progressive renal failure (Manjunatha Goud *et al*., 2010).

Conclusions and Recommendatios

4. Conclusions and Recommendations.

4.1 Conclusions.

In general, diabetic patients demonstrated hyperglycemia, an increase in the percentages of glycosylated hemoglobin and catalase activity with an associated no changes in serum complements 3 and 4, MDA. GSH at the different age groups. With the exception of GSH, MDA at the age group of 69-80 years which showed a decrease.

Diabetic nephropathy patients showed more profound biochemical changes than diabetic patients as reflected by hyperglycemia, an increase in serum urea, creatinine, the percentages of glycosylated hemoglobin, catalase activity, urine creatinine and A/C ratio with an associated microalbuminuria at the different age groups. Serum complements 3 and 4, MDA and GSH (with the exception of GSH, MDA at the age of 69-80 years which showed a decrease) remained unchanged.

4.2 Recommendations.

1. Carrying out a molecular study for diabetic nephropathy.
2. Evaluation of inflammatory markers such as IL-10, IL-17, IL18, in diabetic nephropathy patients.
3. Study the formation of advanced glycation end products.
4. Study the immunoglobulin secretion in urine.
5. Measurement of interferon gama (INF-δ).

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Appendices

Appendix-1-

Group: 45-56 years of age:

Table1. Compare between difference groups in RBS, Urea, Creat. And HbA1C (age = 45-56 years).

Group	Mean ± SD			
	RBS ()	Urea	Creatinine	HbA1C
Normal	103.70 ± 9.82 b	31.13 ± 7.18 b	0.833 ± 0.19 a	5.72 ± 0.44 b
Diabetic nephropathy	214.49 ± 63.03 a	52.21 ± 30.87 a	0.975 ± 0.528 a	8.74 ± 2.35 a
Diabetes	279.20 ± 141.94 a	32.72 ± 6.98 b	0.73 ± 0.11 a	9.07 ± 1.97 a
LSD value	77.444 **	14.892 **	0.269 NS	1.474 **
P-value	0.0001	0.0133	0.236	0.0001
** (P<0.01), NS: Non-significant.				

Means having with the different letters in same column differed significantly.

Table 2 . Compare between difference groups in C_3 , C_4, MDA and GSH (age = 45-56 years).

Group	Mean ± SD			
	C_3	C_4	MDA	GSH
Normal	101.91 ± 33.02 a	23.03 ± 7.67 a	7.270 ± 3.28 a	4.33 ± 1.08 a
Diabetic nephropathy	116.20 ± 17.38 a	24.89 ± 6.03 a	7.178 ± 2.84 a	3.355 ± 1.69 a
Diabetes	116.74 ± 25.40 a	27.05 ± 7.27 a	5.943 ± 4.62 a	5.109 ± 2.97 a
LSD value	25.678 NS	6.660 NS	3.332 NS	1.812 NS
P-value	0.358	0.432	0.653	0.196
NS: Non-significant.				

Means having with the different letters in same column differed significantly.

Table 3. Compare between difference groups in Catalase, MALB, CRE in urine and A/C ratio (age = 45-56 years).

Group	Mean ± SD			
	Catalase	MALB	CRE in urine	A/C ratio
Diabetic nephropathy	150.84 ± 30.99 a	150.00 ± 0.00 a	13.81 ± 5.53 a	13.82 ± 9.05 a
Diabetes	150.25 ± 46.18 a	12.00 ± 6.32 b	4.51 ± 3.90 b	9.61 ± 5.54 a
Normal	84.45 ± 23.03 b	14.28 ± 8.51 b	12.35 ± 8.95 a	2.12 ± 2.72 b
LSD value	30.585 **	6.094 **	7.198 *	6.521 **
P-value	0.0001	0.0001	0.031	0.0024
* (P<0.05), ** (P<0.01).				

Means having with the different letters in same column differed significantly.

Group: 57-68 years of age:

Table4. Compare between difference groups in RBS, Urea, Creat. and HbA1C (age =57-68 years).

Group	Mean ± SD			
	RBS	Urea	Creatinine	HbA1C
Diabetic nephropathy	232.25 ± 60.82 a	58.83 ± 32.14 a	1.226 ± 0.62 a	8.30 ± 1.54 a
Diabetes	234.25 ± 125.97 a	33.58 ± 6.99 b	0.766 ± 0.11 b	8.59 ± 1.93 a
Normal	106.87 ± 6.07 b	34.14 ± 2.91 b	0.742 ± 0.07 b	5.43 ± 0.19 b
LSD value	75.713 **	21.852 *	0.419 *	1.469 **
P-value	0.0041	0.0295	0.0222	0.0005
* (P<0.05), ** (P<0.01).				

Means having with the different letters in same column differed significantly.

Table 5. Compare between difference groups in C_3 , C_4, MDA and GSH (age =57-68 years).

Group	Mean ± SD			
	C_3	C_4	MDA	GSH
Diabetic nephropathy	98.96 ± 25.39 b	24.01 ± `4.57 a	7.81 ± 4.33 a	4.61 ± 2.06 a
Diabetes	120.85 ± 21.72 a	31.97 ± 10.15 a	9.68 ± 5.62 a	7.22 ± 5.24 a
Normal	115.61 ± 14.57 ab	23.15 ± 11.82 a	7.72 ± 3.61 a	4.65 ± 1.38 a
LSD value	21.59 *	12.321 NS	6.594 NS	5.191 NS
P-value	0.0479	0.291	0.789	0.497
* (P<0.05), NS: Non-significant.				

Means having with the different letters in same column differed significantly.

Table 6. Compare between difference groups in Catalase, MALB, CRE in urine and A/C ratio (age = 57-68 years).

Group	Mean ± SD			
	Catalase	MALB	CRE in urine	A/C ratio
Diabetic nephropathy	168.94 ± 34.63 a	150.00 ± 0.00 a	12.13 ± 8.01 a	26.62 ± 14.03 a
Diabetes	179.66 ± 129.13 a	26.67 ± 21.08 b	10.40 ± 7.95 a	5.81 ± 4.76 b
Normal	86.16 ± 28.74 b	15.71 ± 9.75 b	10.98 ± 9.85 a	7.21 ± 5.11 b
LSD value	74.189 *	12.101 **	8.102 NS	17.827 *
P-value	0.0484	0.0001	0.8842	0.0483
* (P<0.05), ** (P<0.01), NS: Non-significant.				

Means having with the different letters in same column differed significantly.

Group: 69-80 years of age:

Table 7. Compare between difference groups in RBS, Urea, Creat. and HbA1C (age = 69-80 years).

Group	Mean ± SD			
	RBS	Urea	Creatinine	HbA1C
Diabetic nephropathy	226.14 ± 85.12 a	73.09 ± 34.76 a	1.850 ± 0.72 a	7.45 ± 1.01 a
Diabetes	150.65 ± 21.01 a	39.96 ± 9.15 b	0.856 ± 0.18 b	7.79 ± 0.67 a
Normal	105.62 ± 11.50 b	34.98 ± 5.72 b	0.860 ± 0.18 b	5.80 ± 0.27 b
LSD value	60.601 **	24.915 **	0.521 **	0.864 **
P-value	0.0023	0.0073	0.0005	0.0005
** (P<0.01).				

Means having with the different letters in same column differed significantly.

Table 8. Compare between difference groups in C_3 , C_4, MDA and GSH (age = 69-80 years).

Group	Mean ± SD			
	C_3	C_4	MDA	GSH
Diabetic nephropathy	101.72 ± 32.32	19.68 ± 8.76 a	7.84 ± 3.46 b	4.25 ± 2.82 b
Diabetes	97.96 ± 24.17	27.45 ± 12.00 a	5.03 ± 3.41 b	5.12 ± 2.09 b
Normal	96.06 ± 26.33	17.50 ± 5.13 a	14.50 ± 8.92 a	9.62 ± 4.07 a
LSD value	32.752 NS	11.343 NS	5.763 **	4.421 *
P-value	0.935	0.162	0.0126	0.055
* (P<0.05), ** (P<0.01), NS: Non-significant.				

Means having with the different letters in same column differed significantly.

Table 9. Compare between difference groups in Catalase, MALB, CRE in urine and A/C ratio (age = 69-80 years).

Group	Mean ± SD			
	Catalase	MALB	CRE in urine	A/C ratio
Diabetic nephropathy	163.53 ± 61.66 a	150.00 ± 0.00 a	17.66 ± 8.19 a	10.63 ± 5.43 a
Diabetes	133.72 ± 33.21 a	18.89 ± 10.54 b	12.85 ± 7.62 ab	2.56 ± 3.30 b
Normal	124.83 ± 106.29 a	14.00 ± 8.94 b	7.04 ± 2.41 b	2.50 ± 2.26 b
LSD value	73.523 NS	9.002 **	8.015 *	4.621 **
P-value	0.5173	0.0001	0.0520	0.0011
* (P<0.05), ** (P<0.01), NS: Non-significant.				

Means having with the different letters in same column differed significantly.

بينما ظهرت زياده في مرضى اعتلال الكليه لداء السكر. وقد اظهرت نتائج تركيز الكرياتنين في البول عدم حدوث تغيرات في مرضى داء السكر ومرضى اعتلال الكليه لداء السكر باستثناء مرضى داء السكر لفئات الاعمار ٤٥-٥٦ سنه. والذين اظهروا انخفاض في تركيز الكرياتنين في البول وكذلك مرضى اعتلال الكليه لداء السكر لفئات الاعمار ٦٩-٨٠ سنه. والذين اظهروا حدوث زياده في تركيز الكرياتنين في البول. وقد ازدادت نسبه الالبومين/الكرياتنين في مرضى داء السكر لفئات الاعمار ٤٥-٥٦ سنه وكذلك لمرضى اعتلال الكليه لداء السكر لفئات الاعمار ٤٥-٨٠ سنه. ولم تتغير هذه النسبه في مرضى داء السكر لفئات الاعمار ٥٧-٨٠ سنه.

أجريت هذه الدراسه للتعرف على دور الاجهاد التأكسدي وبعض الجوانب الكيمياويه الحياتيه في المرضى المصابين باعتلال الكليه لداء السكر النوع الثاني.

تضمنت الدراسه واحد و ثلاثون مريضاً مصاباً باعتلال الكليه لداء السكر النوع الثاني. وثلاثون مريضاً مصاباً بداء السكر النوع الثاني الذي تمت مراجعتهم لمستشفى الدكتور عبدالمجيد الخاص للفتره من كانون الاول /٢٠١٥ و لغايه تموز /٢٠١٦. كما شملت الدراسه لاغراض المقارنه بجمع عينه من الاصحاء (عدد٢٨) بنفس اعمار المرضى.

وقد تم قياس تراكيز السكر العشوائي واليوريا والكرياتنين والهيموكلوبين الكلايكوسيليتي والمتممين ٣ و٤ والمالوندايالديهايد والكلوتاثايون وفعاليه انزيم الكاتليز في الدم. فضلاً عن ذلك فقد تم قياس تراكيز المايكروالبومين والكرياتنين في البول مع قياس نسبة الالبومين/الكرياتنين.

واظهرت نتائج الدراسه وجود زياده في تركيز الكلوكوز العشوائي في المرضى المصابين بداء السكر والمرضى المصابين باعتلال الكليه لداء السكر لفئة الاعمار ٤٥-٨٠ سنه. ولم يلاحظ وجود تغير في تركيز اليوريا في مصل الدم في مرض داء السكر، في حين لوحظ وجود زياده في تركيز اليوريا في مصل دم المرضى المصابين باعتلال الكليه لداء السكر لفئة الاعمار ٤٥-٨٠ سنه. كما اشارت النتائج الى عدم حدوث تغير في تركيز الكرياتنين في مصل الدم في مرضى داء السكر لفئة الاعمار ٤٥-٥٦ سنه. وازداد تركيز الكرياتنين في مصل الدم بشكل معنوي في مرض اعتلال الكليه لداء السكر لفئة الاعمار ٥٧-٨٠ سنه. واوضحت النتائج بان هناك زياده في النسب المئويه للهيموكلوبين الكلايكوسيليت في مرضى داء السكر ومرضى اعتلال الكليه لداء السكر لفئة الاعمار ٤٥-٨٠ سنه. ولم تظهر تراكيز المتممين ٣ و٤ اي تغيرات في المرضى المصابين بداء السكر ومرضى اعتلال الكليه لداء السكر لكل فئات الاعمار. ولم تحدث تغيرات في تركيز المالوندايالديهايد في مرضى داء السكر ومرضى اعتلال الكليه لداء السكر لفئات الاعمار ٤٥-٦٨ سنه. بينما لوحظ وجود انخفاض في تركيز المالوندايالديهايد في مرضى داء السكر ومرضى اعتلال الكليه لداء السكر لفئات الاعمار ٦٩-٨٠ سنه. اما بخصوص تركيز الكلوتاثايون في مصل الدم فقد بينت النتائج عدم حدوث تغيرات معنويه في مرض داء السكر ومرض اعتلال الكليه لداء السكر للفئه العمريه ٤٥-٦٨ سنه. بينما لوحظ وجود انخفاض في تركيزه في مرض داء السكر لفئات الاعمار ٦٩-٨٠ سنه. وقد اوضحت النتائج زياده في فعالية انزيم الكاتاليز في مرضى داء السكر ومرضى اعتلال الكليه لداء السكر لفئات الاعمار ٤٥-٨٠ سنه. ولم يسجل اي تغير في تركيز المايكروالبومين في البول لمرضى داء السكر،

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جامعة النهرين
كلية العلوم

دور الاجهاد التأكسدي وبعض الجوانب الكيمياويه الحياتيه في المرضى المصابين باعتلال الكليه لداء السكر النوع الثاني

رسالة

مقدمة الى مجلس كلية العلوم/جامعة النهرين

كجزء من متطلبات نيل درجة الماجستير في كلية العلوم/التقانة الاحيائية

من قبل

آلاء وليد عباس

بكلوريوس علوم/تقانه احيائيه/كلية العلوم/ جامعة النهرين(٢٠١٤)

بأشراف

أ.د. وليد حميد يوسف

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