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Department of Chemistry

Biochemical studies and partially purification of GPCR from iraqi patients with diabetic type 2 and diabetic nephropathy

A Thesis

Submitted to College of Education for Pure Science (Ibn Al-Haitham), University of Baghdad in Partial fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry

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We, the examining, committee, after reading this thesis (**Biochemical studies and partially purification of GPCR from Iraqi patients with diabetic type 2 and diabetic nephropathy**) and examining the student Sulaiman Mahmod Hasan in its content, find that it is qualified for pursuing the degree of Doctor of Philosophy in Clinical Biochemistry with grade on / /2018.

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Dedication

To my parents who have always supporting me

To my wife who supported me along the time

To my sisters and brothers

To my children

To all researchers who are working to improve the quality of life

To all of them I dedicate this work.

Sulaíman . M . Hasan

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Sulaiman M. Hasan

Summary 1

The study aimed to the determination of glucagon Like Peptide-1 (GLP-1) and G-Protein Coupled Receptors (GPCR) levels in diabetic and diabetic nephropathy patients and compare results with control group. In addition to study the relationship of GLP-1 and GPCR with HbA1c%,FBG,urea,createnine and lipid profile , that may be useful in marker combat diabetes and its complication

One hundred fifty samples from individuals were used in this study with ages ranged between (40-65) years that were divided into three groups as follows: first group (G1) consists of 50 healthy individuals as a control group, second group (G2) consists of 50 patients with diabetic and third group (G3) consists of 50 patients with diabetic nephropathy.

Results reveled a significant elevation in FBG , HbA1c , urea , creatinine in G2 and G3 comparing to G1 and in the G3 comparing to G2 . Results display a nonsignificant decrease in albumin levels in G2 comparing to G1 however, there is a significant decrease in G3 comparing to G2 and G1.

Results revealed a significant elevation in levels of (TC, TG, LDL-c, VLDL-c) in G2 and G3 comparing to G1 .While a significant decrease was found in HDL levels in G2 and G3 comparing to G1.Also, there is a significant elevation in (TC, TG, LDL-c, VLDL-c) in G3 comparing to G2 , while there is a nonsignificant decrease was found in HDL in G3 comparing to G2 .

Results showed a significant elevation in the insulin and IR levels in G2 and G3 comparing to G1 .Also ,a significant increase was noticed in G3 comparing to G2 .

A significant elevation in GLP-1 and GPCR levels was observed in G2 and G3 comparing to G1. Also, a significant differences was observed in G3 comparing to G2 .

Results showed a nonsignificant negative correlation between GLP-1 and urea in G1 (r_1=-0.146,p>0.05), however , a significant correlation was found in G2 (r_2=0.435, P<0.05) . A significant negative correlation was seen in G3 (r_3=-0.0729, P>0.05) . Study also showed a nonsignificant correlation between GLP-1 and creatinine for G1 (r_1=0.453, P<0.05) .While a significant positive correlation was found in G2 (r_2=0.210, P<0.05) was found . A significant negative correlation for G3 (r_3= -0.439, P>0.05) was observed . Correlation relation between GLP-1 and FBG, results illustrated a nonsignificant negative correlation in G1 (r_1= -0.120, P>0.05) , while a significant positive correlation was found in G2 and G3 (r_2=0.523, r_3= 0.323, P<0.05) . Study revealed a nonsignificant positive correlation between GLP-1 and insulin in G1 (r_1=0.070, P>0.05) .while there are a significant negative correlation in G2 and G3 (r_2= -0.113, r_3= -0.309, P<0.05) was detected .

Results, also , revealed a nonsignificant positive correlation between GPCR and HbA1c in G1 (r_1=0.172) , a significant positive correlation in G2 (r_2=0.427) and a significant negative correlation in G3 (r_3=-0.042) in these parameters . Results , also, indicated a nonsignificant positive correlation in G1 between GPCR and cholesterol (r_1=0.306 , P>0.05) . while there are a significant positive correlation in G2 (r_2=0.514, P<0.05) . and a significant negative correlation between GPCR and cholesterol in G3 (r_3= -0.288, P<0.05) were found . In this study a nonsignificant negative correlation between GPCR and TG in G1 (r_1=-0.0009, P>0.05) and a significant negative correlation in G2 was found

(r_2=-0.292, P<0.05) . finally, results revealed a significant positive correlation between GPCR and triglyceride in G3 (r_3= 0.276, P<0.05) .

Conclusion could be drawn from this study that the differences in GPCR and GLP-1 levels among groups indicate that GPCR may be used as a marker in development of diabetic nephropathy depending on a significant relation with HbA1c% ,urea , creatinine and insulin .

To the best of our knowledge this is the first study in determination and comparison of GPCR in diabetic and diabetic nephropathy patients. In addition to found relationship for GPCR with HbA1c% ,urea , creatinine and insulin .

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List of Abbreviations

Abbreviations	Name
ACE	Angiotensin-converting enzyme
ADA	American Diabetes Association
AGEs	Advanced Glycation End products
T2DM	Type 2 diabetic mellitus
GFR	Glomerular filtration rate
cAMP	cyclic Adenosine Monophosphate
DAG	Diacylglycerol
DM	Diabetes mellitus
DN	Diabetic Nephropathy
DPN	Diabetic Peripheral Neuropathy
DPP-4	dipeptidyl peptidase-IV
DRGs	Dorsal Root Ganglia Neurons
DW	Distal Water
FFAR1	Free Fatty Acid Receptor-1
GLUT1	Glucose Transporters 1
GLUT2	Glucose Transporters 2
GPCR	G-Protein Coupled Receptor
GPLR	G Protein–Linked Receptors
GPR146	G Protein-Coupled Receptor 146
GRPP	Glicentin-Related Pancreatic polypeptide
Gs protein	Gs protein (stimulating)
GTP	Guanosine-5-Triphosphate
HbA_0	Non-Glycosylated Hemoglobin

HbA_{1c}	Hemoglobin A1c
HDL-c	High Density Lipoproteins- Cholesterol
HOMA-IR	Homeostatic Model Assessment- Insulin Resistance
HRP	Horseradish Peroxidase
IGR	insulin glucagon ratio
IkB	inhibitor of kappa B
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical Density
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
POD	Peroxidase
ROS	Reactive Oxygen Species
TMB	Tetra Methyl Benzidine

Introduction (1) :

1.1 Diabetes Mellitus (1):

Diabetes is a multi-factorial disease process with a complex etiology. This involving insulin resistance, aberrations in glucose homeostasis, lipid metabolism, and inflammation . Further pancreatic beta cell toxicity [1,2] are linked to the microvascular complications in type 1 and type 2 diabetes such as retinopathies, nephropathies, and neuropathies, which can lead to blindness and kidney disease [3] .

Genetic background is an important issue in determining susceptibility to diabetic nephropathy, but exposure of tissues to chronic hyperglycemia is the main initiating factor [4] .

Early detection and active treatment can help in preventing the progression of complications and are therefore, considered to be very important for controlling diabetes mellitus. Oral glucose tolerance tests (O-GTT) also, have been used to measure fasting or postprandial blood glucose in the diagnosis of diabetes [5,6] .

1.2 Diabetic Nephropathy

Diabetic nephropathy is a microvascular complication of diabetes ,which is due to end-stage renal disease . It is linked to elevation in cardiovascular disease[7] . In nephropathy complicating Type 2 DM, chronic hyperglycaemia is central to the development of renal pathology [8]. Although glomerular lesions are characteristic, it is increasingly recognized that the pathology within the tubulointerstitium, for example fibrosis, tubular atrophy and ischaemic damage, are ultimately more predictive of the renal outcome [9] .

The renin–angiotensin system has an important role in the determination of renal disease, by inducing changes in arterial pressure , glomerular hemodynamics [10] . Also by increasing oxidative stress and promoting expression of proinflammatory genes and pro-fibrotic factors in mesangial cells and vascular smooth muscle [11] . Additionally, angiotensin II induced mesangial cell and vascular smooth muscle proliferation, therefore this will lead to the enlargement of the arteriolar wall. Activated inflammatory cells, (T cells) producing angiotensin, this will produce reinforcing of the intrarenal angiotensin [12] . Prospective controlled clinical studies show that ,angiotensin convert enzyme inhibitors , as well as angiotensin receptor blockers , which are able to stop or slow the progression of DN [13] .

In subjects with T2DM, the Glomerular filtration rate decreases by approximately 6 mL/min per year , however, it has been observed that the addition of Angiotensin-converting enzyme inhibitors or ARBs to patients' treatment regimens, while not hypertensive, achieved a reduction in the rate of progression of the fall in Glomerular filtration rate, thereby stabilizing renal function for a prolonged period [14] . Moreover, metabolic control reduces catabolism and improves nutritional status, decreases or delays the onset of DN, and reduces glomerular hyperfiltration [15] .

Continued investigations into mechanisms of kidney failure in patients with DM will lead to the finding of novel strategies to limit cardiovascular and renal disease are imperative. Hence , investigating the effects of incretins pathology is an important goal for glucose , cardiovascular disease and renal pathology which are inherently attractive in the treatment of patients with DM [16] .

More recently, there has been increasing interest in alternative mechanisms of glucose toxicity that resulting in nephropathy, including lipotoxicity, activation of inflammatory pathways and disruption of mitochondrial DNA bioenergetics among others. Among the[17] cytokines, most prominent in the development of diabetic nephropathy are the pro-fibrotic cytokines (transforming growth factor) β1 and connective tissue growth factor [18] .

Stimuli inherent in the diabetic milieu that activate these pathways and hence are considered to be integral to the development of diabetic nephropathy include hyperglycaemia [19] , AGE formation [20], glucosamine overproduction [21] , Ang II (angiotensin II) [22]. Progressive proteinuria coupled with extracellular matrix (fibronectin and collagen) expansion and inflammation lead to scarring that ultimately compromises renal function [23] .

1.3 Insulin

Insulin (from the Latin, insula meaning island) is a anabolic peptide hormone produced by beta cells in the pancreas which regulates the metabolism of carbohydrates and fats by promoting the absorption of glucose from the blood to skeletal muscles and fat tissue and by storing fat as well as using it for energy [24].

The human insulin consists of 51 amino acids, with a molecular weight of 5808 Dalton. It is a dimer of an A and B-chain, which are linked together by disulfide bridge as shown in figure (1.1 A) [25].

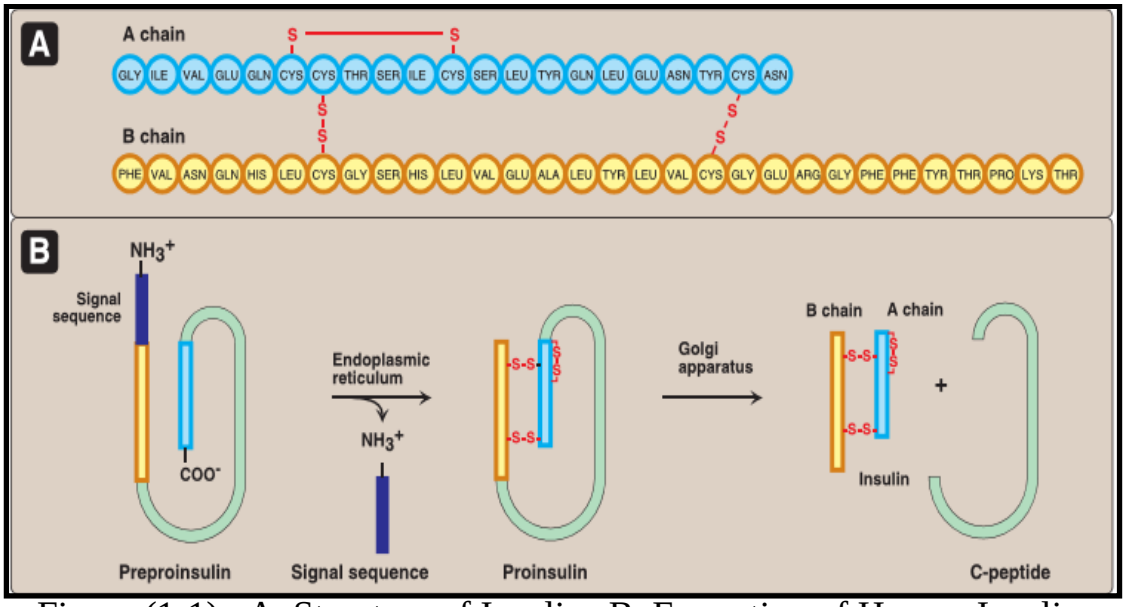

Figure (1-1): A. Structure of Insulin. B. Formation of Human Insulin from Preproinsulin[25] .

Insulin produced by the norepinephrine which leads to an increase in the blood glucose levels during stress. Release of catecholamines by the sympathetic nervous system led to release of insulin by beta cells, that inhibited by α_2-adrenergic receptors [26] and stimulated by β_2-adrenergic receptors [27]. The net effect of norepinephrine from sympathetic nerves and epinephrine from adrenal glands on insulin release is inhibition due to dominance of the α-adrenergic receptors [28].

The net effect of insulin is to enhance storage and block mobilization and oxidation of fatty acids. Insulin exerts its effect by formation of stimulating lipoprotienlipase, thus circulating triglycerides are hydrolyzed and free fatty acids can enter the adipocyte. Insulin is also required for the transport of glucose, which is needed for re-esterification of the triglycerides once inside the adipocyte. The conversion of glucose to fatty acids is accomplished by insulin's activation of several enzymes[29] .

1.4 Glucagon-Like Peptide-1

Glucagon-like peptide 1 (GLP-1) is traditionally recognised as a peripheral incretin hormone. Released postprandially from intestinal L-cells, it binds to GLP-1 receptors (GLP-1R) on pancreatic β-cells to increase insulin secretion. Additionally, GLP-1 considered as a neuropeptide produced by preproglucagon neurons found in the brainstem, (lower) in the caudal nucleus tractus solitarii and the intermediate reticular nucleus intermediate [30] .

Pro-glucagon is secreted by the intestinal mucosa which then cleaved to GLP-1, that acts on the GLP-1 receptor in the pancreas in a glucose-dependent manner and function to stimulate pancreatic insulin release and suppress glucagon secretion, with the net effect of regulating postprandial glucose excursions. These hormones have a very short half-life that degraded rapidly by dipeptidyl peptidase-4, DPP-4 to which cleaves two amino acids at the N-terminal end of peptides (proline or less commonly an alanine) residue at the penultimate position. Hence GLP-1-(7–36)amide is cleaved to GLP-1-(9–36) amide, (the major form) [31,32] . As shown in figure (1-2)

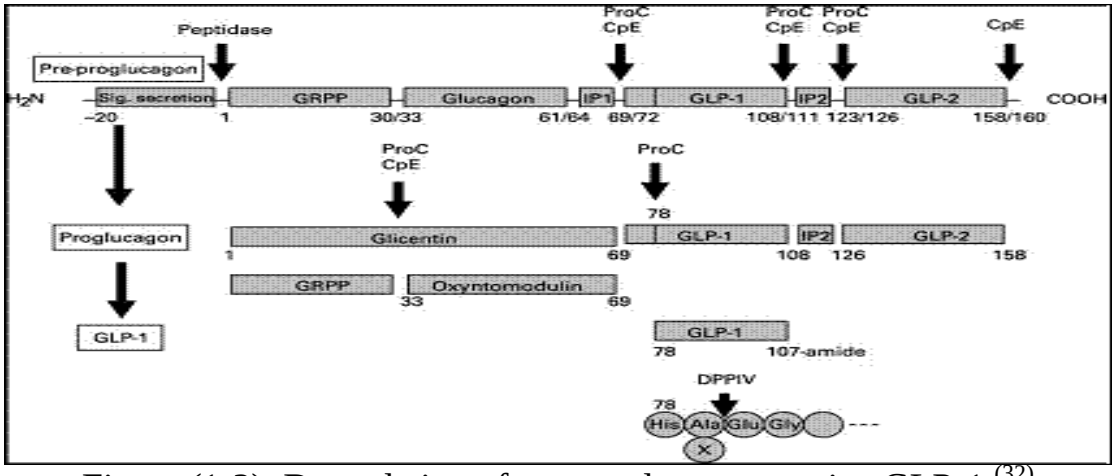

Figure (1-2): Degradation of pre proglucagon to give GLP-1 [32]

The GLP-1 receptor agonist class has important actions for type 2 diabetes patients which increase in glucose-mediated insulin production by pancreatic β-cells[33,34] leading to hyporglycemia . Other actions include a decrease or no change in fasting endogenous glucose release via a reduction in glycogenolysis but not gluconeogenesis and a reduction in glucagon secretion [35] . The GLP-1 receptor agonists also act via the central nervous system, and lowered intake food [36,37] .

The GLP-1 analogue drug class offers a drug regimen for people with type 2 diabetes. It is well tolerated by users, weight neutral and with a low risk of hypoglycaemia [38] .

1.4.1 GLP-1 and diabetic nephropathy

Researchers suggested that a long- term treatment with the GLP-1R agonist ameliorates diabetic nephropathy in DM animal models, has an effect on the glomerular endothelial and inflammatory cells infiltrating[39].Effects of the GLP-1 on the kidney include modulation of sodium homoeostasis in the kidney via its action on proximal tubular [40] . This has been with intravenous infusions of GLP-1 which enhanced sodium excretion, reduced H+ secretion and reduced glomerular hyperfiltration in obese improving its effect on proximal renal tubule level [41] .

1.5 G-Protein Coupled Receptors

G protein coupled receptors (GPCRs) are the most abundant receptor family encoded by the human genome. Therefore , they are the target of a large percentage of drugs currently prescribed in the United States. Recently, several GPCRs have been identified as potential therapeutic targets for the treatment of diabetes and diabetes-associated

complications, including retinopathy, nephropathy, and neuropathy [42]. All GPCRs have the same structural features comprise seven transmembrane domains, three extracellular and three intracellular loops, and contain cysteine residues in the second extracellular loop that could be have a role in the formation of the ligand binding pocket . The N-terminal region of GPCRs are extracellular and action in ligand specificity, while the C-terminal cytoplasmic tail may has an effect in signaling of GPCR comprising linked with G protein Receptor Kinases (GRKs) [43].

GPCRs classified into five classes; however, most small peptide hormones that signal via a GPCR, The rhodopsin family (701 members) , the adhesion family (24 members) , the frizzled/taste family (24members), the glutamate family (15 members) , and the secretin family (15 members) [43] .

The “classical” signalling pathways initiated by the activation of these Class A and B receptors comprise a complex series of steps, containing dissociation of G proteins, GTP hydrolysis, and re-association of the G protein trimer . The GPCR signalling comprise induction or inhibition of adenylate cyclase by Gαs or Gαi, respectively . Adenylate cyclase activation triggers cAMP formation, cAMP linked to the regulatory subunits of PKA and a consequent allosteric change leads to release of the PKA catalytic subunits. then PKAc phosphorylates downstream targets, that have a role in the regulation of metabolic enzymes and activation of transcription factors [44]. As shown in Figure (1-3).

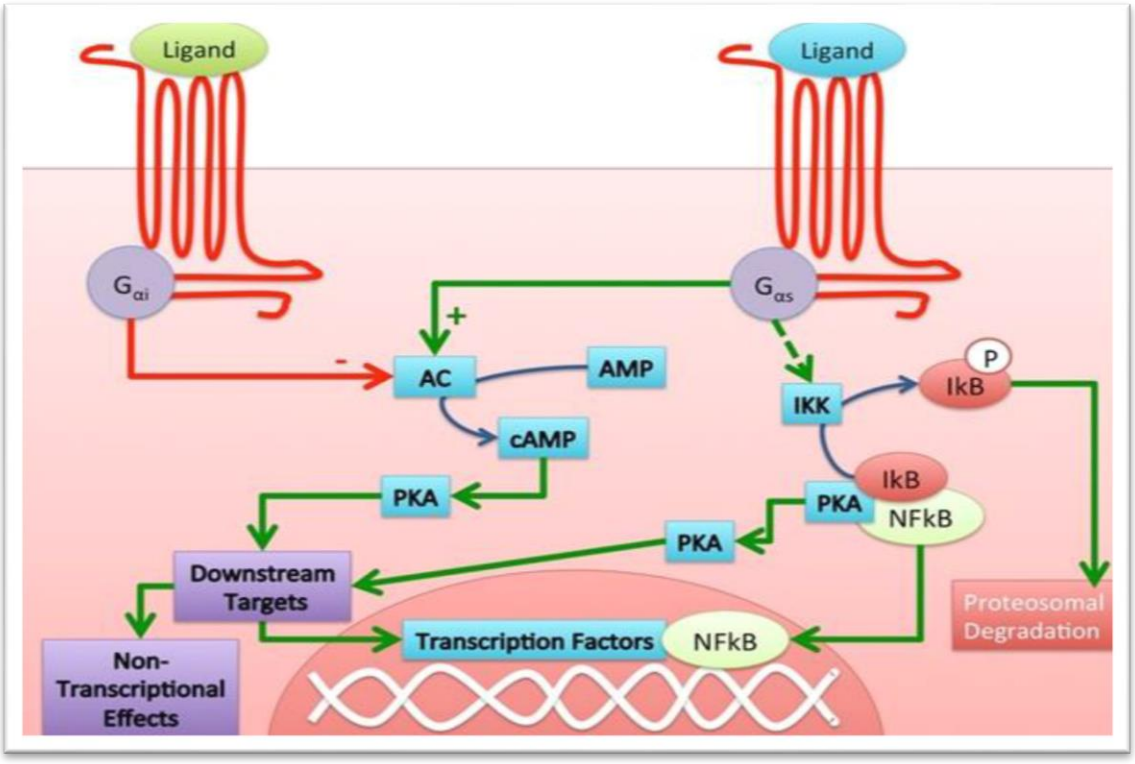

Figure (1-3): Classical and Non-Traditional GPCR-Mediated Signaling Cascades [44] .

In 1997, Zhong et al. [45] were the first to identify a new signalling pathway with cAMP-independent activation of PKA in rabbit lung cytosol extract .

Suggested that there are two separate vasoactive peptides, endothelin-1 and angiotensin II, via GPCR binding, induced the cAMP-independent PKAc activation. A major cells of the cortical collecting duct in the mammalian kidney, when induced , display elevation in ouabain binding sites and an increase in the PKA that prevented by PKA inhibitors. No elevation in the cAMP was observed . Further, PKA activation was prevented by inhibiting the proteasome [46] .

Activation of GPCRs coupled to Gαs leads to stimulation of AC activity, led to the elevation at protein kinase A (PKA) activity, and downstream signaling events, including alterations in gene transcription.

Alternatively, dissociation of Gαs can cause activation of the IKK (IkB kinase) . In turn IKK phosphorylates IkB (inhibitor of kappa B), leading to the proteosomal degradation of IkB and dissociation of the IkB-NFkB-PKA complex. This will led to activation of B cells, then translocates to the nucleus to act as a transcription factor [47] .

One such receptor, GPR40 (free fatty acid receptor 1 or FFAR1), is expressed in pancreatic beta cells as well as in brain, omental adipocytes, and endocrine cells of GI mucosa. Activation by medium- or long-chained free fatty acids leads to insulin secretion in a glucose-dependent manner [48] .

Recent study ,revealed that GPCRs are abundantly expressed in the pancreatic islets and may play an important role in the normal glucose homeostasis and microvascular function [49].

Aim of the study (1)

Aim of the present study is :

1- to determined GLP-1 levels in diabetic and diabetic nephropathy and compare the results with control individuals .

2- to determination of GPCR levels in diabetic and diabetic nephropathy patients and compare the results with control group .

3- to find relationship for GLP-1with urea, creatinine , insulin and HbA1c in these patient , in order to be used as a marker in monitoring the development of diabetic to its complication in these patients **.**

4- to find relationship of GPCR with HbA1c, TC and TG in these patients ,in order to that GPCR could be used as a marker combat diabetes and its complication .

Chemicals and Methods (1):

2.1 Chemicals:

Chemicals used in the current study were obtained from various companies, see table (2-1).

Table (2-1):- Chemicals and Suppliers.

Chemicals	Company, Origin
Glucose (GLUC-PAP) Kit	Randox Laboratories Limited, United Kingdom.
HbA1c Kit	Stanbio Laboratory An EKF Diagnostics Co., USA.
Insulin kit	Elabscience Biotechnology Co. Ltd, China
GLP-1 kit	
Lipid Profile	Human Gesellschaft for biochemical and Diagnostica mbH, Germany.
Urea kit	Biosystem S.A. (spain)
Creatinine kit	Biosystem S.A. (spain)
Albumin kit	Biosystem S.A. (spain)
GPCR kit	BlueGene Biotechnology Co. Ltd, China

2.2 Instruments:

Instruments-company and their origin- which are used in this study are listed in table (2-2).

Table (2-2):- Instruments, company and their origin.

Instruments	Company, origin
ELISA human reader and washer	Bio Tek, USA
Analytical balance	Ohans (France)

Spectrophotometer UV-Visible beam	Labomed (England)
Deep freeze	FROILABO, France
Water bath	Gemmy , YCW-01
Centrifuge	Hettich, Japan
pH meter	Genway (UK)
Shaker incubator	Gallenkamp (UK)
Micropipettes	Oxford (USA)

2.3 Subjects:

One hundred fifty individuals with age ranged between (40-65) years were enrolled in this study. They divided into three groups as follows:-

1. Group (G1) that consists of 50 healthy individuals as control group .

2. Group (G2) that consists of 50 diabetic patients .

3. Group (G3) that consists of 50 diabetic nephropathy patients.

The patients attended the diabetic & endocrinology center in Al-Yarmouk Teaching Hospital between June 2017 to September 2017. Patients with smoking and kidney disease were excluded

2.4 Blood Sample Collection:

Ten milliliters of venous blood was drawn from the study cases and control sample placed in a plane tube and left for (15 min) at room temperature, and then centrifuged at 3500 rpm for 10 min. Serum that obtained was stored at (-20^{o}C) unless used immediately. whole blood was used in the determination of HbA1c .

2.5 Analytical Methods and Procedures:

2.5.1 Determination of Fasting Blood Glucose:

Principle:

Glucose was estimated by enzymatic reaction in the presence of glucose oxidase (GOD) and peroxidase (POD), A red-violet quinoneimine dye was produce according to the following equation[50].

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminophenazone} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4\ H_2O$$

Reagents	**Concentration**
1) Phosphate buffer pH 7.0 2) Phenol 3) GOD-PAP Reagent which involved: - 4-Aminophenazone - Glucose oxidase - Peroxidase	0.1mmol/L 11mmol/L 0.77mmol/L ≥1.5kU/L ≥1.5kU/L
4) Standard glucose	100mg/dL

Procedure:

Working solution: the content of a one vial of GOD-PAP reagent was mixed with the amount of buffer 1(phosphate buffer and phenol). The solution transferred to bottle 1, with rinsing several times. The series of tubes were prepared as follows:

	Blank	Standard	Sample
Standard	----	10µL	-----
Serum	----	-----	10µL
Distilled water	10µL	-----	------
Working solution	1000µL	1000µL	1000µL
Tubes were mixed , and incubated for 5 minutes at 37° C . The absorbance was recorded for the sample (A_{sample}) and standard ($A_{Standard}$) via blank at (λ_{max}=500nm).			

Calculation:

$$\text{Glucose conc.}\,(\text{mg}/\text{dL}) = \frac{\text{A sample}}{\text{A standard}} \times 100\ \text{mg/dl}$$

2.5.2 Determination of Glycated Hemoglobin (HbA_{1C}):

Principle:

A hemolyzed whole blood was mixed with a weakly binding cation-exchange resin. The non-glycosylated hemoglobin (HbA_0) was linked to the resin, leaving (HbA_1) free to be removed by a resin separator. The percent of HbA_1 was measured by evaluation of the absorbance values at 415nm of the HbA_1 fraction and of the total Hb fraction,by calculating the ratio of absorbances (R).This ratio comparing was compared to the ratio of a glycohemoglobin standard that obtained from the same procedure[51].

Reagents:

1. Glycohemoglobin Ion-Exchange Resin (Tubes) in which tube includes: 3.0 mL cation-exchange resin and 8 mg/dL buffer at pH 6.9.

2. Glycohemoglobin Lysing Reagent: which consists of potassium cyanide (10 mmol/L) and surfactants.

3. Glycohemoglobin Standard (Lyophilized): that prepared from packed human erythrocytes.

Reagent Preparation:

A liquate of 1.0 mL DW was added to the vial of standard glycohemoglobin. Rubber stopper was replaced and allowed to stand for 10min at room temperature. The mixture was swirled gently until the solution was completed.

Procedure:

Hemolysate Preparation:

1. A liquate of 0.5 mL Lysing reagent was pipetted into Standard (S), Unknown (U) and Control (C) tubes.

2. A liquate of 0.1 mL of each blood sample was added into the appropriately labeled tubes, mixed and allowed to stand for 5min at room temperature (15-30^{0}C) to complete hemolysis.

Glycohemoglobin Separation and Assay:

1. Pre-Fill resin tube Standard (S), Unknown (U) and Control (C) were labeled.

2. A liquate of 0.1 mL of the prepared hemolysate was added into the appropriately labeled resin tube.

3. A resin separator was put in the Pre-Fill tube.

4. Tubes were mixed on a hematology rocker for 5min.

5. At the end of the 5min mixing, resin eluent was introduced into tube until resin was firmly packed in the bottom of the 13min tube.

6. Each supernatant was poured directly into separate cuvettes for absorbance that recorded against D.W at 415nm, within 60 minutes.

Total Hemoglobin Assay:

1. A liquate of 5.0 mL DW was pipetted into the labeled tubes; Standard (S), Unknown (U) and Control (C).

2. Hemolysate (0.02 mL) was pipetted into , mixed , and then transferred to cuvettes to measure absorbance .

3. Absorbance (A_{tot}) of Control, Standard and Unknown were recorded against D.W at 415nm, within 60 minutes.

Results:

The HbA_{1C} percentage was calculated as follows :

$$\mathrm{R}(unknown) = \frac{\mathrm{Agly}(unknown)}{\mathrm{Atot}(unknown)}$$

$$\mathrm{R}((\mathrm{standard})) = \frac{\mathrm{Agly(standard)}}{\mathrm{Atot(standard)}}$$

$$\text{Glycohemoglobin (\%)} = \frac{\text{R (unknown)}}{\text{R (standard)}} \times \text{Conc. of Glycohemoglobin standard (\%)}$$

2.5.3 Determination of blood urea :

Principle:

The urea level was estimated after enzymatic hydrolysis by urease enzyme an Indophenol compound was generated from salicylate and

Hypochlorite as shown below .The intensity of the green complex is equal to the amount of urea found in the sample[52] .

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_4^+ + CO_2$$

$$NH_4^+ + \text{Salicylate} + NaClO \xrightarrow{\text{Nitroprusside}} \text{Indophenol}$$

Reagents:

Reagents	Concentration
R1:	
Sodium salicylate	62 mmol/L
Sodium nitroprusside	3.4 mmol/L
Phosphate buffer pH(6.9)	20 mmol/L
R2 : Urease	>500U/mL
Sodium hypochlorite	7 mmol/L
Sodium hydroxide	150 mmol/L
Standard / Urea	50mg/dL

Procedure:

	Reagent blank	Standard or Sample
Standard / Sample	------	10µL
Distilled water	10µL	----
Reagent R1	1000µL	1000µL
Tubes were mixed and incubated for 5 minutes at 37° C after that R2 was added to the mixture ,which Incubated for 5 minutes at 37° C . The absorbance of the sample / standard was recorded versus the blank reagent (ΔA) during 60 minute at (λ_{max}=600nm).		

Calculation:

$$\text{Urea Concentration } \left(\frac{\text{mg}}{\text{dL}}\right) = \frac{\Delta\text{A sample}}{\Delta\text{A standard}} \times 50 \text{ mg/dl}$$

2.5.4 Determination of Creatinine :

Principle:

Creatinine in the sample was reacted with picrate in alkaline medium forming a coloured complex (J affe method)[53] .

Reagents:

Reagents	Concentration
R1:Sodium hydroxide **R**2 : Picric acid	0.4 mol/L 25 mmol/L
Standard / Creatinine	2 mg/dL

Procedure:

	Reagent blank	Standard or Sample
Standard / Sample	------	100µL
Distilled water	100µL	----
Reagent R1	500 µL	500 µL
Reagent R1	500 µL	500 µL
Tubes were mixed and absorbance was recorded at 500 nm after 30 second and 90 second for (A_1) and (A_2),respectively .		

Calculation:

$$\text{Creatinine Con.}\left(\frac{\text{mg}}{\text{dL}}\right) = \frac{(A2-A1)\text{sample}}{(A2-A1)\text{standard}} \times 2 \text{ mg/dl}$$

2.5.5 Determination of Albumin :

Principle:

The method of determination of albumin is depending on the specific binding of bromocresol green (anionic dye) , and the protein at acidic pH. This resulting a shift in the absorption wavelength of the complex . The concentration of albumin is equal to the intensity of color [54].

$$\text{BCG} + \text{Albumin} \xrightarrow{\text{pH 4.3}} \text{BCG-albumin complex}$$

Reagents:

Reagents	Concentration
Bromocresol reagent :	
Succinate buffer pH 4.3	75 mmol/L
BCG	0.12 mmol/L
Standard / Albumin	5 mg/dL

Procedure:

	Reagent blank	Standard or Sample
Standard / Sample	------	10µL
Distilled water	10µL	----
Reagent	2000µL	2000µL
Tube were mixed and let to stand 1 minute at 25° C . The absorbance were read for (A) samples , standard at 630 nm via blank .		

Calculation:

$$\text{Albumin Concentration (g/dL)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Conc. of Standard}$$

2.5.6 Determination of Total Cholesterol:

Principle:

Cholesterol was evaluated using spectrophotometeric method after enzymatic hydrolysis of cholesterol ester and oxidation of the free cholesterol. The marker (quinoneimine) was generated from 4-aminophenazone and phenol in the presence of hydrogen peroxide and peroxidase .The intensity of the pink complex is proportional to the total cholesterol found in the sample [55].

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine}_{(\text{pink complex})} + 4H_2O$$

Reagents:

Reagents	Concentration
Phosphate buffer pH(6.5)	100 mmol/L
4-aminophenazone	0.3 mmol/L
Phenol	5 mmol/L
Cholesterolesterase	> 150 U/L
Cholesteroloxidase	> 100U/L
Peroxidase	> 5 KU/L
Sodium azide	0.05%
Standard / Cholesterol	200mg/dL or 5.17mmol/L

Procedure:

	Reagent blank	Standard or Sample
Standard / Sample	------	10µL
Distilled water	10µL	----
Reagent	1000µL	1000µL
Tubes were mixed and incubated for 5 minutes at 37° C. The absorbance of the sample / standard was recorded versus the blank reagent (ΔA) during 60 minutes at (λ_{max}=500nm).		

Calculation:

$$\text{Cholesterol Concentration } \left(\frac{\text{mg}}{\text{dL}}\right) = \frac{\Delta\text{A sample}}{\Delta\text{A standard}} \times 200\text{mg/dl}$$

2.5.7 Determination of Triacylglycerol:

Principle:

The triacylglycerol (TG) level was estimated after enzymatic hydrolysis by lipoprotein lipase. Quinoneimine that formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxide as an indicator, is shown in the following equation [56].

$$\text{Triacylglycerols} + H_2O \xrightarrow{\text{LPL}} \text{glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate + ADP}$$

$$\text{Glycerol-3-phosphate} \xrightarrow{\text{GPO}} \text{Dihydroxyaceton phosphate} + H_2O_2$$

$$2\ H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine}$$

Reagents:

Reagents	Concentration
PIPES buffer (pH 7.5)	50 mmol/L
4-chlorophenol	5 mmol/L
4-aminophenazone	0.25 mmol/L
Magnesium Chloride	4.5 mmol/L
ATP	2 mmol/L
lipoprotein lipase	≥1300 U/L
glycerol kinase	≥400 U/L
glycerol-3-phosphate oxidase	≥1500 U/L
Peroxide	≥500 U/L
Sodium azide	0.05%
Standard Triacylglycerol	200mg/dL

Procedure:

	Reagent blank	Standard or Sample
Standard / Sample	------	10µL
Distilled water	10µL	----
Reagent	1000µL	1000µL
Solutions were mixed, and then incubated for 5 minutes at 37° C. The absorbance of the sample (ΔA_{sample}) and standard (ΔA_{blank}) was recorded versus the blank reagent during 60 minutes at (λ_{max}=500nm).		

Calculation:

$$\text{Triacylglycerol Concentration (mg/dL)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 200 \text{ mg/dl}$$

2.5.8 Determination of High Density Lipoproteins-Cholesterol (HDL-c):

Principle:

Phosphotungstic acid was added magnesium ions presence led to LDL, VLDL and chylomicron fractions precipitation quantitatively. The cholesterol concentration in the HDL fraction, which remains in the supernatant, after centrifugation was determined [57].

Reagents	**Concentration**
- Precipitant: Phosphotungstic Acid Magnesium Chloride	 0.55 mmol/L 25 mmol/L
Standard Cholesterol	55 mg/dL

Procedure:

1. Precipitation

	Semi- micro
Sample/Standard	200 µL
Precipitant (R1)	-----
Diluted Precipitant (R1)	500µL
The above reagents were piptted into a centrifuge tube, mixed and then incubated 10 minutes at room temperature. The sample was centrifuged for 10 minutes, for at 40,000×g. The clear supernatant was separated from the precipitate within 1 hour after centrifugation and cholesterol concentration was estimated by using cholesterol liquicolor reagent.	

2. Cholesterol CHOD- PAP Assay:

	Blank	Standard	Sample
DW	100 µL	-----	------
Supernatant	------	------	100 µL
Standard Supernatant	------	100 µL	------
Reagent	1000 µL	1000 µL	1000 µL
The mixture was incubated for10 minutes at 25° C. The absorbance of the sample and standard was recorded, respectively, versus the blank reagent through 60 minutes at (λ_{max}=500nm).			

Calculation:

$$\text{HDL Concentration } \left(\frac{\text{mg}}{\text{dl}}\right) = \frac{\Delta\text{A sample}}{\Delta\text{A standard}} \times 55 \text{ mg/dl}$$

2.5.9Estimation of LDL-c and VLDL-c:

The LDL-c and VLDL-c were calculated according to Friedewald formula [58], as displayed in below:

$$\text{LDL} - \text{c (mg/dL)} = \text{TC} - [\text{HDL} - \text{c} + \tfrac{\text{TG}}{5}]$$

$$\text{VLDL} - \text{c(mg/dL)} = \frac{\text{TG}}{5}$$

2.5.10 Determination of Insulin Levels:

Principle:

Competitive (ELISA) technique was used in the estimation of insulin levels which the microtiter plate provided in this kit was pre-coated with insulin . During the reaction, insulin in the sample or standard competes with a fixed amount of INS on the solid phase supporter for sites on the biotinylated detection Ab specific to INS. Excess conjugate and unbound sample or standard were washed from the plate. Avidin was added to conjugate Horseradish Peroxidase in well microplate and then incubated. Then a Tetra Methyl Benzidine substrate solution was added per well. The enzyme-substrate reaction was stopped by adding sulphuric acid and changing in the color was measured spectrophotometrically at 450 nm. The concentration of insulin in the samples was obtained from the optical density of the samples that compared to a standard curve[(59)].

Materials:

1) Micro ELISA Plate (96 well)

2) Reference Standard

3) Reference Standard & Sample Diluent (20mL)

4) Biotinylated Detection Ab (120µL) (Concentrated)

5) Biotinylated Detection Ab (Diluent 10mL)

6) Concentrated HRP Conjugate (120µL)

7) HRP Conjugate Diluent (10mL)

8) Concentrated Wash Buffer (25x) (30mL)

9) Substrate Reagent (10mL)

10) Stop Solution (10mL)

11) Plate Sealer

Reagent Preparation:

1) Wash Buffer: A liquate of 30 mL of concentrated wash buffer was diluted into 750 mL of wash buffer with DW. Unused solution was stored at 4℃. The solution was warmed to 40℃ in a water bath (heating temperature should not exceed 50℃), if crystals formed, in a tubes were mixed gently until the crystals have completely dissolved. The solution was cooled to room temperature before use.

2) Standard: A standard was prepared 15 minutes before use, centrifuged at 10,000×g for 1 minute, and then was mixed with 1.0mL of reference standard and sample diluent. The lid was tightened, stands for 10 minutes and turned it upside down for several times. After it dissolves fully, the lid mixed thoroughly with a pipette. This mixture produced a stock solution of 2500pg/mL. A series of dilutions were prepared as follows: 2500, 1250, 625, 312.5, 156.25, 78.125, 39.063 and 0 pg/mL. Standard and sample diluents were refereed and mixed. The undiluted standard was served as the highest standard (2500pg/mL) while the reference standard &sample diluents were served as the zero (0 pg/mL).

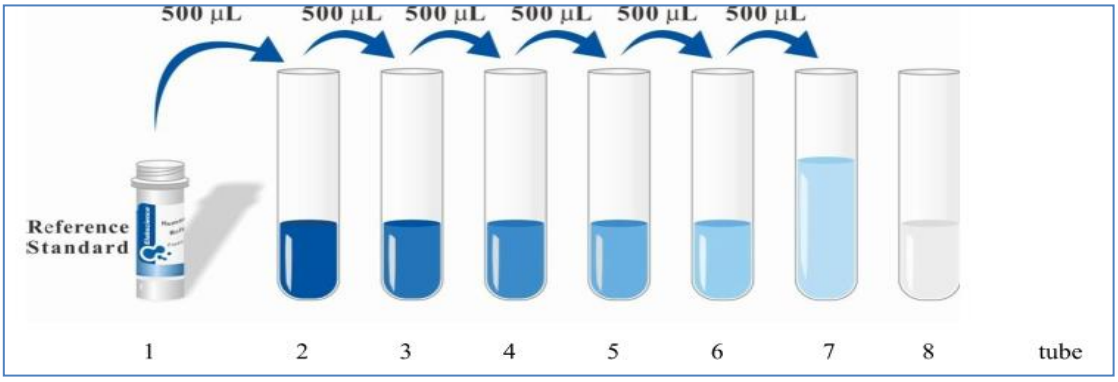

Figure (2.1): A standard sequence for Preparation of Insulin.

3) Biotinylated Detection Ab: The required amount was calculated before experiment (50μL/well). The stock tube was centrifuged before use; the concentrated biotinylated detection Ab was diluted to the working concentration using biotinylated detection Ab diluent (1:100).

4) Concentrated HRP Conjugate: Amount that required was competed before experiment (100μL/well). The HRP (concentrated) conjugate was diluted by using HRP conjugate diluent (1:100).

5) Substrate Reagent: The needed dosage of the reagent can be aspirated with sterilized tips.

Washing Procedure:

1. Automated Washer: A liquate of 350μL wash buffer was added for each well. The interval between injection and suction should be set about 60s.

2. Manual Wash: A liquate of 350μL wash buffer was added per well, soaked for 1~2minutes. After the last wash, any remaining wash buffer was decanted by inverting the plate and blotting it dry by rapping it firmly against clean and absorbent paper , which was toweled on a hard surface.

Procedure:

After thawing, the sample was centrifuged again before the assay. All the reagents were mixed thoroughly by gently swirling before pipetting.

1. A liquate of 50μL of standard, blank, or sample was added into each well. The blank was added with reference standard and sample diluents. Immediately, 50μL of biotinylated detection Ab working solution was added per well, covered with the plate sealer. The plate was tapped to ensure thorough mixing and incubated for 45minutes at 37^0C.

2. Every well was aspirated and washed. The process was repeated 3 times by filling every well with wash buffer (approximately 350µL). Any remaining wash buffer was removed after the last wash by aspirating or decanting, invert the plate which was pated via thick clean absorbent paper.

3. A 100µL of HRP conjugate was added per well, covered , incubated for 30 minutes at 37^0C.

4. The aspiration/wash process was repeated for five times as conducted in step2.

5. A liquate of 90µL of substrate solution was added per well, covered with a new plate sealer and incubated for about 15 minutes at 37^0C. The reaction time could be reduced or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in the standard wells, the reaction was stopped by the addition of liquate of (50µL) of stop solution for every well which the color was turned to yellow immediately.

6. The optical density of each well was evaluated at once at 450 nm.

Calculation:

The average of the two recording data for every standard and samples were calculated then substrate the average zero standard optical density. A standard curve was created by plotting the mean of the OD value for every standard on the y-axis against the concentration on the x-axis and curve was drawn through the points on the graph.

2.5.11 Estimation of Homeostatic Model Assessment-Insulin Resistance:

Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) was calculated according to Matthews *et al.* equation in 1985 as follows:

$$\text{HOMA} - \text{IR} = \frac{\text{Glucose} \times \text{Insulin}}{405}$$

This equation is used when glucose concentration is in mg/dL [60].

2.5.12 Determination of Glucagon Like Peptide -1:

Principle:

ELISA kit was used in the estimation of GLP-1,in which the micro plate was pre-coated with antibody specific to GLP-1. Samples, standards were added to the plate wells and a biotinylated detection antibody specific for GLP-1 . Avidin-HRP conjugate was added to every micro plate well and incubated. The substrate solution was added to every well. Wells colored with blue that contain GLP-1, biotinylated detection antibody and Avidin-HRP conjugate . The enzyme-substrate reaction was stopped by sulphuric acid addition then , the color become yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm . The OD value was proportional to the concentration of GLP-1. The concentration of GLP-1 in the samples was calculated by comparing the OD of the samples to the standard curve.

Materials:

1) Micro ELISA Plate (96 well)

2) Reference Standard

3) Reference Standard & Sample Diluent (20mL)

4) Biotinylated Detection Ab (120µL) (Concentrated)

5) Biotinylated Detection Ab Diluent (10mL)

6) Concentrated HRP Conjugate (120µL)

7) HRP Conjugate Diluent (10mL)

8) Concentrated Wash Buffer (25x) (30mL)

9) Substrate Reagent (10mL)

10) Stop Solution (10mL)

11) Plate Sealer

Reagent Preparation:

1) Wash Buffer: A liquate of 30 mL of concentrated wash buffer was diluted into 750 mL with DW. Unused solution was stored at 4℃. The solution was warmed to 40℃ in water bath (heating temperature should not exceed 50℃). If crystals were formed, the solution was mixed gently until crystals have completely dissolved. The solution was cooled to room temperature before use.

2) Standard: The standard was prepared within 15 minutes prior to use, centrifuged at 10,000×g for 1 minute, and the standard was mixed with 1.0mL of reference standard &sample diluent. The lid was tightened, standed for 10 minutes and turned it upside down for several times. After it dissolved fully, the lid mixed thoroughly with a pipette. This mixture produced a stock solution of 20ng/mL. Serial dilutions were prepared as follows: 20, 10, 5, 2.5, 1.25, 0.625, 0.313 and 0 ng/mL. Standard &sample diluents were refereed and mixed. The undiluted standard was served as the highest standard (20ng/mL) while the reference standard &sample diluents were served as the zero (0 ng/mL).

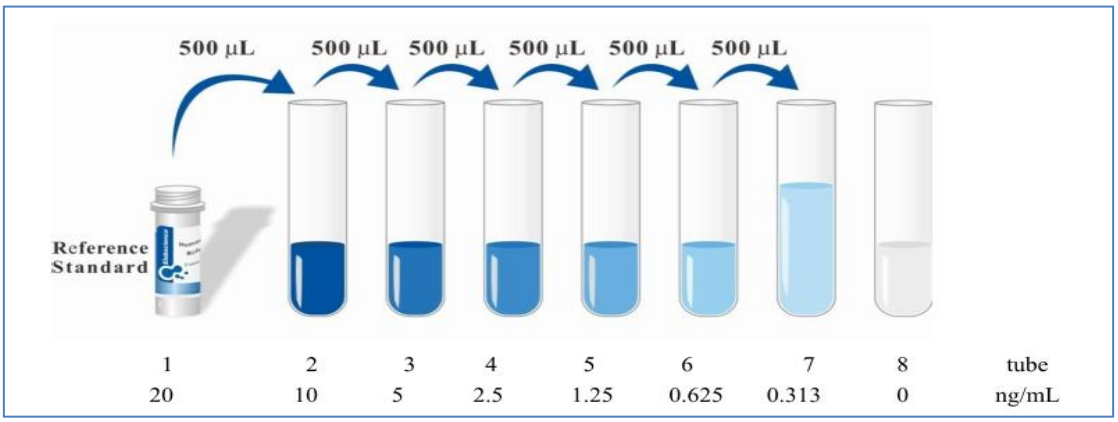

Figure (2.2): Standard Preparation of Glucagon Like Peptide-1.

3) Biotinylated Detection Ab: The required amount was calculated before experiment (50µL/well). The stock tube was centrifuged before use; the concentrated biotinylated detection Ab was diluted to the working concentration using biotinylated detection Ab (diluent 1:100).

4) Concentrated HRP Conjugate: The required amount was calculated before experiment (100µL/well). The concentrated HRP conjugate was diluted to the working concentration using HRP conjugate diluent (1:100).

5) Substrate Reagent: As it is sensitive to light and contaminants, so the vial was opened until need it. The needed dosage of the reagent can be aspirated with sterilized tips and the unused residual reagent shouldn't be dumped back into the vial again.

Washing Procedure:

1. Automated Washer: A liquate of 350µL wash buffer was added for every well, the interval between injection and suction should be set about 60s.

2. Manual Wash: A liquate of 350µLwash buffer was added per well, soaked for 1~2minutes. After the last wash, any remaining wash buffer was

decanted by inverting the plate and blotting it dry by rapping it firmly against clean and absorbent paper was toweled on a hard surface.

Procedure:

After thawing the sample was centrifuged again before the assay. All the reagents were mixed thoroughly by gently swirling before pipetting.

1. A liquate of 100µL of standard, blank, or sample was added into each well. The blank was added with reference standard &sample diluents. Immediately 50 µL of biotinylated detection Ab working solution was added per well, covered with the plate sealer. The plate was tapped to ensure thorough mixing incubated for 90 minutes at 37^0C.

2. Every well was aspirated and washed. The process was repeated three times by filling every well with wash buffer (approximately 350µL). Any remaining wash buffer was removed after the last wash by aspirating or decanting, inverted the plate which was pated via thick clean absorbent paper.

3. A liquate of 100µL of HRP conjugate working solution was added per well, covered with a new plate sealer, incubated for 30 minutes at 37^0C. 4. The aspiration/wash process was repeated for five times as conducted in step2.

5. A liquate of 90µL of substrate solution was added per well, covered with a new plate sealer, incubated for about (15 minutes at 37^0C). The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, the reaction was stopped by addition of liquate of 50µL of stop solution for every well where the color turn to yellow immediately.

6. The optical density of each well was estimated at once, using a microplate reader set to 450 nm.

Calculation:

The average of the duplicate reading of each standard and samples were calculated, and then subtract the average zero standard optical density. A standard curve was created by plotting the mean of OD value of each standard on the y-axis against the concentration on the x-axis and a curve was drawn through the points on the graph.

2.5.13 Determination of G- Protein Coupled Receptor:

Principle:

Sandwich ELISA technique was used in the determination of GPCR. The micro titer plate was pre-coated with a monoclonal antibody specific for GPCR. Standards or samples were added to the microtiter plate wells and GPCR if present will link to the antibody pre-coated wells. In order to determine the amount of GPCR in the sample, a standardized preparation of HRP-conjugated polyclonal antibody, specific for GPCR, were added to each well to "sandwich" the GPCR immobilized on the plate. The microtiter plate undergoes incubation, and then the wells were thoroughly washed to remove all unbound components. Next, substrate solutions were added to every well. The enzyme HRP and substrate were allowed to react over a short incubation period. Only those wells that contain GPCR and enzyme-conjugated antibody will exhibit a change in color. The enzyme-substrate reaction was stopped by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. A standard curve was plotted relating the intensity of the color OD to the concentration of standards. The GPCR concentration in every sample was interpolated from this standard curve.

Materials:

All reagents provided in the kit which stored at 2-8° C are mentioned below:

1) Microtiter Plate (96 wells)

2) Enzyme Conjugate (10 mL)

3) Standard A (0 ng/mL)

4) Standard B (1.0 ng/mL)

5) Standard C (2.5 ng/mL)

6) Standard D (5.0 ng/mL)

7) Standard E (10 ng/mL)

8) Standard F (25 ng/mL)

9) Substrate A (6 mL)

10) Substrate B (6 mL)

11) Stop Solution (6 mL)

12) Wash Solution (100 x) (10 mL)

13) Balance Solution (6 mL)

Reagents Preparation:

Wash solution was prepared by diluting of 10 mL of wash solution concentrate (100×) with 990 mL of DW. The solution was stable (2 weeks at 2-8°C).

Procedure:

1) The desired numbers of coated wells were secured in the holder then 50 µL of standards or samples were added to the appropriate well in the antibody pre-coated microtiter plate. A liquate of 50 µL of PBS (pH 7.0-7.2) was added in the blank control well.

2) A liquate of 5 µL of balance solution was dispensed into 50 µL specimens and mixed well.

3) A liquate of 100 µL of conjugate was added to every well (not blank control well), them mixed well. Incubated for 1 hour at 37°C.

4) The microtiter plate was washed using one of the specified methods indicated below:

a) Manual Washing: The incubated mixture was removed by aspirating contents of the plate into a sink or a proper waste container. Every well was filled completely with 1× wash solution, and then aspirate contents of the plate into a sink or proper waste container. Procedure was repeated (5 times) for a total of five washes. After washing, the plate was inverted, and blotted dry by hitting the plate onto absorbent paper or paper towels until no moisture appears.

b) Automated Washing: The plate was washed five times with diluted wash solution (350-400 µL/well/wash) using an auto washer. Then the plate is dried as above. It was recommended that the washer should be set for a soaking time of 10 seconds and shaking time of 5 seconds between every wash.

5) A liquate of 50 µL of substrate A and 50 µL of substrate B were added to every well including theblank control well, than covered and incubated for 10-15 minutes at 20-25°C. (Avoid sunlight).

6) A liquate of 50 µL of Stop Solution was added to every well including blank control well, mixed well.

7) The OD was recorded at 450 nm using a microplate reader immediately.

Calculation:

1) Tthe estimation level of GPCR in samples was found by using standard curve .

2) The average of the duplicate was made for every standard and sample. All OD values were subtracted by the mean value of blank control before the interpretation of result .

3) A standard curve was performed by ploted the average OD for every standard on the vertical (Y) axis versus the concentration on the horizontal (X) axis. A curve was drawn using graph paper or statistical software to generate a linear regression, four parameter logistic (4-PL) curve-fit or curvilinear regression of second degree. An x-axis for the optical density and a y-axis for the concentration was also a choice. The data may be linearized by plotting the log of the concentrations versus the log of the OD and the best fit line can be determined by regression analysis.

4) The concentration of samples was calculated corresponding to the mean absorbance from the standard curve.

2.6 Statistical analysis:

Results expressed as mean ± SEM. Students t-test was applied to compare the significance of the difference between DN, Diabetic patient`s and control groups. p- Value ($P>0.05$), ($P<0.05$) considered statistically no significant and significant respectively. The correlation coefficient (r) test is used for describing the association between the different studied parameters.

Results & Discussion (1):

3.1- Analytical Parameters:

Results in table (3-1) illustrated levels of (FBS , HbA_{1C} , Urea , Creatinine , Albumin) in G1, G2 and G3.

Table (3-1):- Descriptive Parameters for G1,G2,G3

Parameters	(G1)	(G2)	(G3)	T-Test G1 vs G2	T-Test G1 vs G3	T-Test G2 vs G3
FBG (mg/dL)	90.62±7.223	177.62±25.742	230.67±50.974	S	S	S
HbA_{1C} (%)	5.32±0.475	8.42±1.068	11.77±1.47	S	S	S
Urea (mg/dL)	20.31±3.4	33.67±4.62	107.37±57.2	S	S	S
Creatinine (mg/dL)	0.47±0.11	0.92±0.164	3.94±2.42	S	S	S
Albumin (mg/dL)	4.029±0.3	3.97±0.215	2.4±0.836	NS	S	S

*G1: control group. G2: diabetic group. G3: diabetic nephropathy group. S: significant NS: nonsignificant

Results in table (3-1) and figures (3-1,3-2,3-3 and 3-4) reveled a significant elevation in F.B.S , HbA1c , urea , creatinine in G2 and G1 comparing to G1 and in the G3 comparing to G2 . Results display a nonsignificant decrease in albumin levels in G2 comparing to G1 however, there is a significant decrease in G3 comparing to G2 and G1.

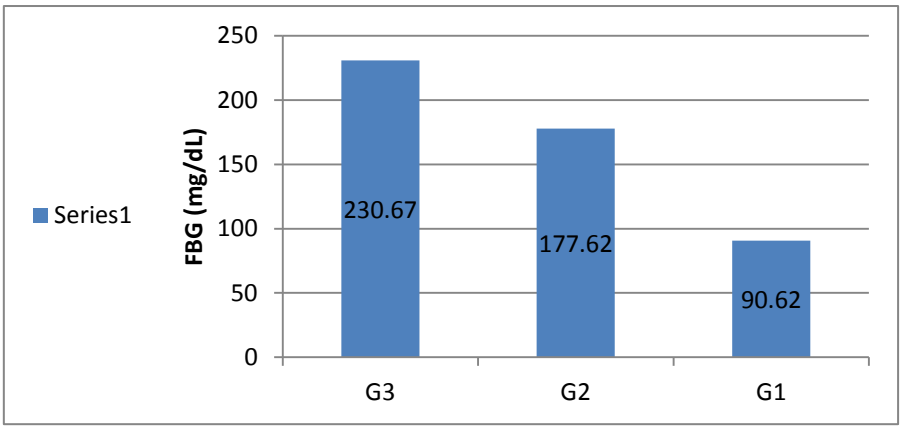

Figure (3.1): Fasting Blood glucose Levels in Sera of Three Studied Groups.

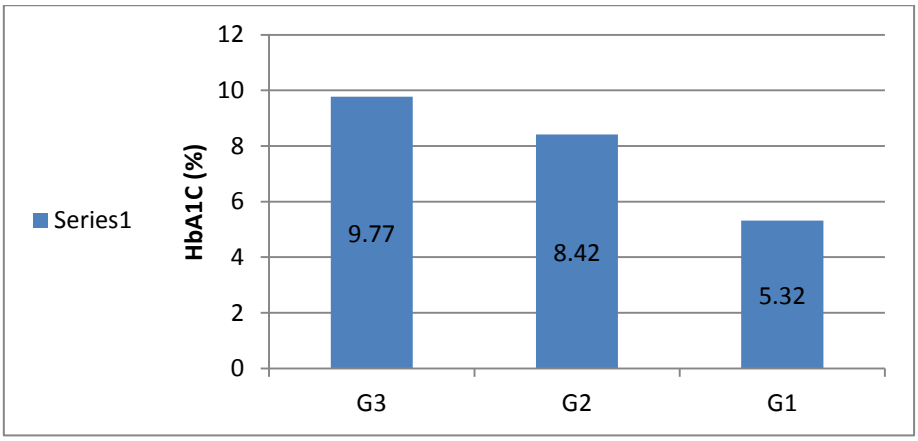

Figure (3.2): HbA_{1C} Levels in Blood of Three Studied Groups.

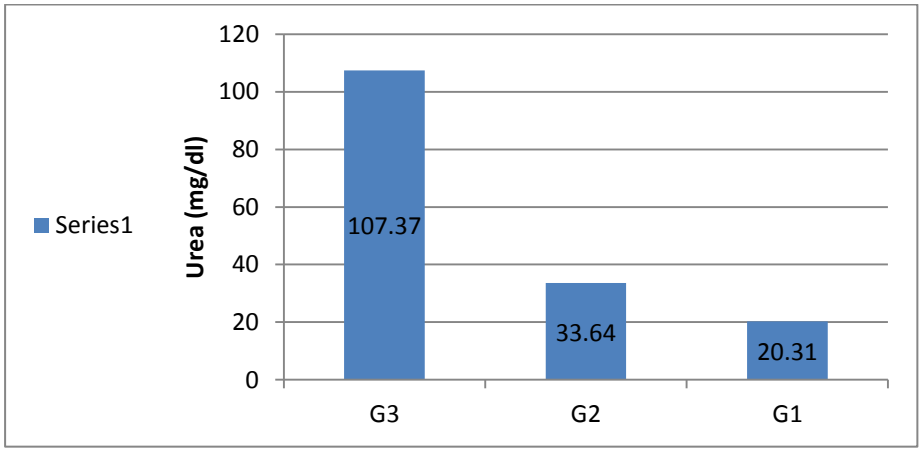

Figure (3.3): Urea Levels in Blood of Three Studied Groups.

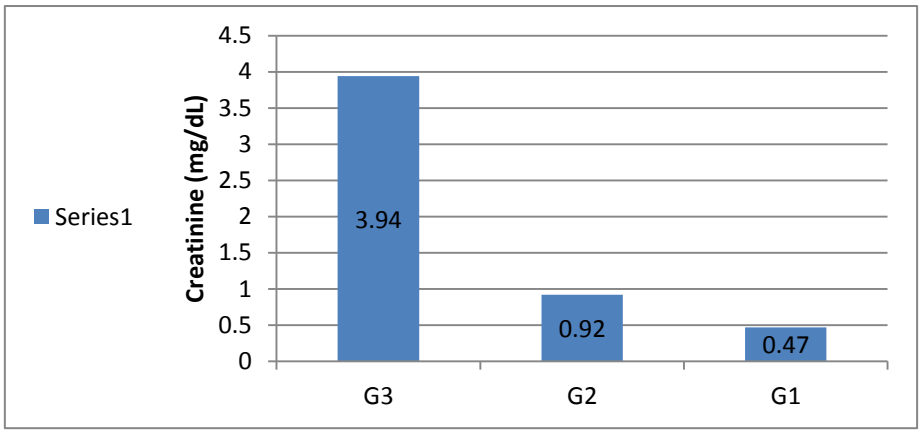

Figure (3.4): Creatinine Levels in Blood of Three Studied Groups.

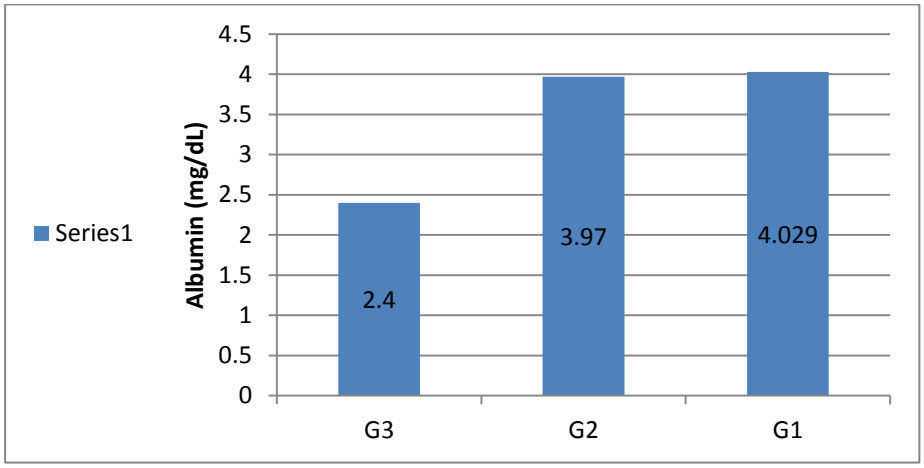

Figure (3.5): Albumin Levels in Blood of Three Studied Groups.

Diabetic nephropathy (DN) pathogenesis is very complex and multifactorial . Many mechanisms explained that hyperglycemia causes kidney injuries through many which mediators . Common between the pathways such as reactive oxygen species (ROS) and TGF-β and there are many overlaps and interference between the pathways [61] .

Table (3-2) and figures (3-6,3-7,3-8,3-9 and 3-10) display levels of lipid profile (TC, TG, HDL-c, LDL-c, VLDL-c) in all studied groups . Results revealed a significant elevation in levels of (TC, TG, LDL-c, VLDL-c) in G2 and G3 comparing to G1 .While a significant decrease was found in HDL levels in G2 and G3 comparing to G1.Also, there is a

significant elevation in (TC, TG, LDL-c, VLDL-c) in G3 comparing to G2 were seen , while there is a nonsignificant decrease was found in HDL level in G3 comparing to G2 .

Table (3-2):- Lipid Profile Levels for G1,G2,G3.

Parameters	(G1)	(G2)	(G3)	T-Test G1 vs G2	T-Test G2 vsG3	T-Test G1 vsG3
TC (mg/dL)	90.79±4.01	145.91±29.1	249.62±48.38	S	S	S
TG (mg/dL)	105.04±20.111	177.01±52.8	268.62±80.97	S	S	S
HDL-c (mg/dL)	48.166±4.444	35.167±4.32	35.2±4.211	S	NS	S
LDL-c (mg/dL)	82.2±14.37	110.1±32.47	138.04±18.51	S	S	S
VLDL-c (mg/dL)	22.475±6.34	34.42±9.21	53.62±12.41	S	S	S

*G1: control group. G2: diabetic group. G3: diabetic nephropathy group. S: significant NS: nonsignificant

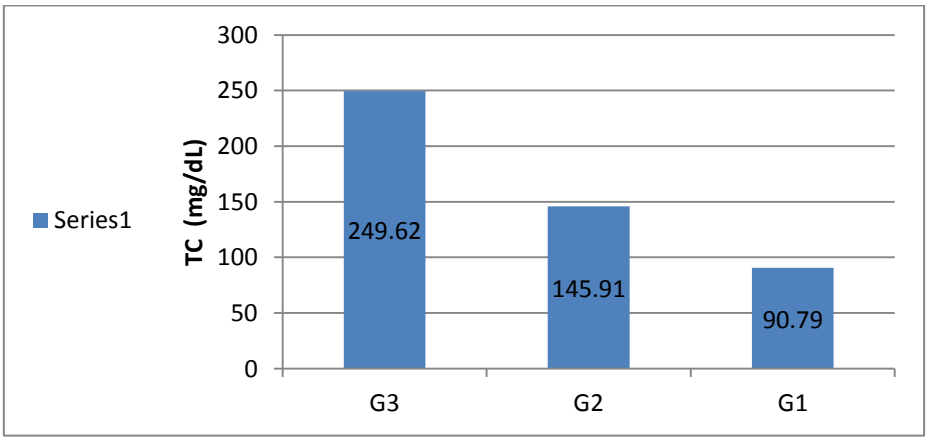

Figure (3.6): Total Cholesterol Levels in Sera of Three Studied Groups.

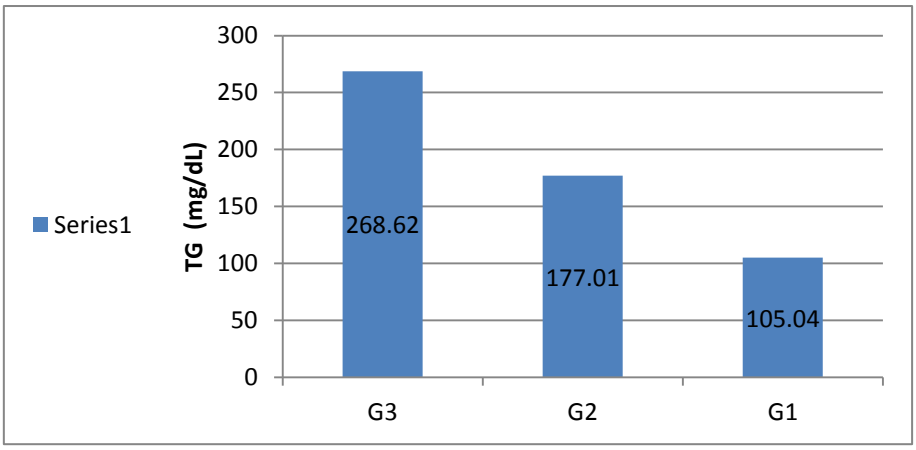

Figure (3.7): Triglyceride Levels in Sera of Three Studied Groups.

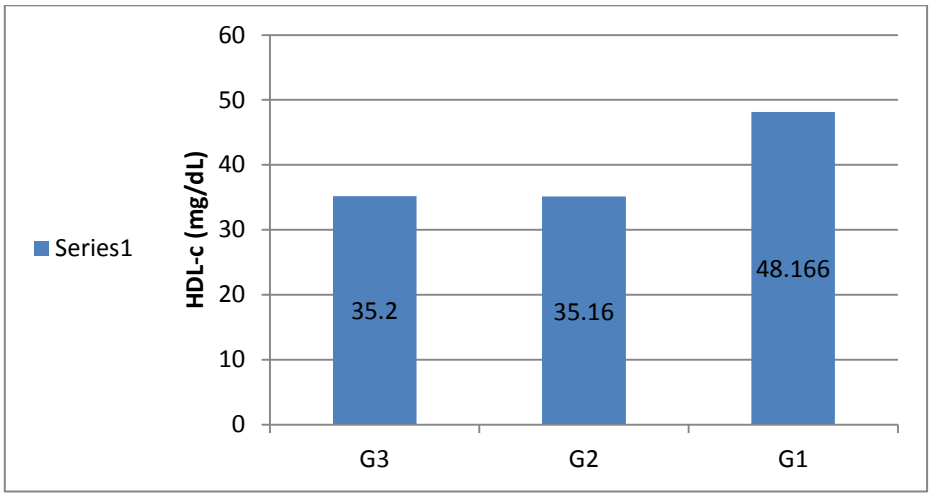

Figure (3.8): HDL-c Levels in Sera of Three Studied Groups.

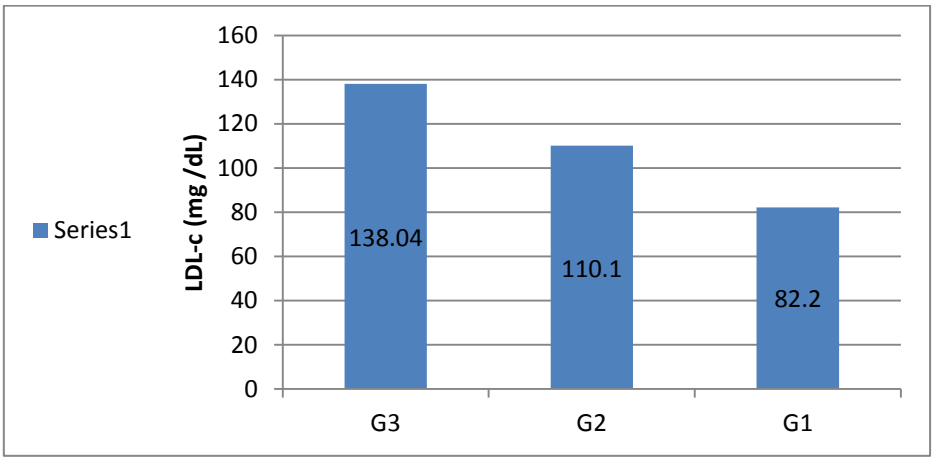

Figure (3.9): LDL-c Levels in Sera of Three Studied Groups.

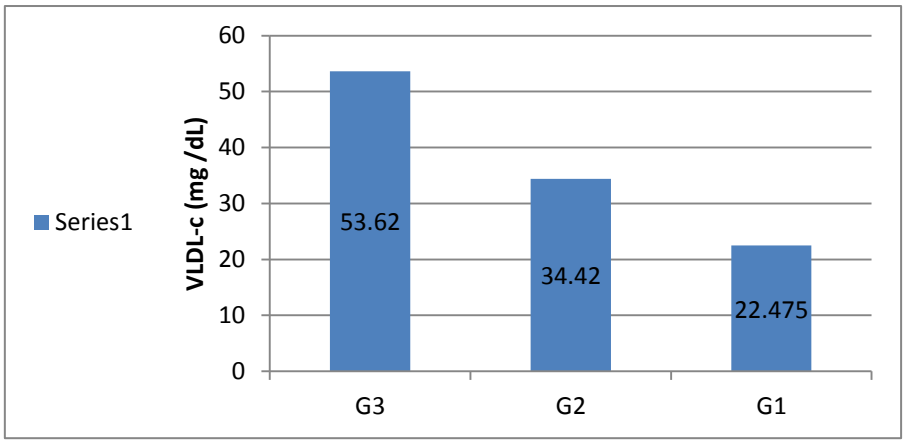

Figure (3.10): VLDL-c Levels in Sera of Three Studied Groups.

Diabetic dyslipidemia comprises a triad of raised triglycerides, reduced HDL-c and excess of LDL particles, which is inagreement with the present study .The dyslipidemia are found in diabetes patients due to IR or deficiency that affect the key enzymes and pathways in lipid metabolism[62].

Study reported that elevation in fasting glucose led to elevation in the transfer of cholesterol esters from HDL-C to VLDL-C particles; while denser LDL particles acquire a large percentage of these HDL esters and more diminishing the HDL-C level .The same study showed that elevation in postprandial lipid excursion in T2D patients was found which change due to the same mechanisms in chylomicron setting of and secretion by the intestine [63].

Table (3-3) and figures (3-11,3-12,3-13 and 3-14) illustrated levels of insulin , IR , GLP-1 and GPCR in G1 , G2 and G3 . Results showed a significant elevation in the insulin and IR levels in G2 and G3 comparing to G1 .Also ,a significant increased was noticed in G3 comparing to G2 .

Table (3-3): Insulin , IR , GLP-1and GPCR Levels for G1 , G2 and G3.

Parameters	(G1)	(G2)	(G3)	T-Test G1 vs G2	T-Test G1 vs G3	T-Test G2 vsG3
Insulin (IU/mL)	4.27±3.19	13.26±8.64	22.6±6.831	S	S	S
IR	2.068±0.631	6.314±1.505	12.634±3.616	S	S	S
GLP-1 (ng/mL)	0.529±0.132	1.207±0.255	1.880±0.355	S	S	S
GPCR (ng/mL)	0.54±0.158	1.307±0.299	2.10±0.59	S	S	S

A significant elevation in GLP-1 and GPCR levels was observed in G2 and G3 comparing to G1. Also, a significant differences was observed in G3 comparing G2 .

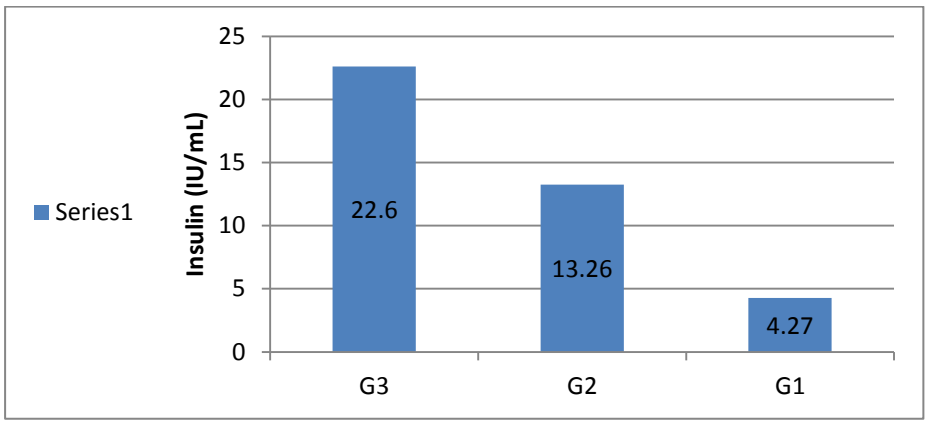

Figure (3.11): Insulin Levels in Sera of Three Studied Groups.

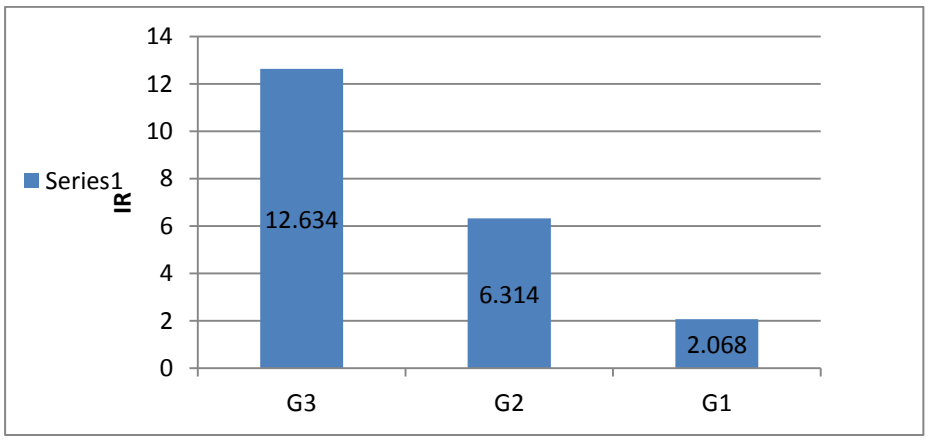

Figure (3.12): IR Levels in Sera of Three Studied Groups.

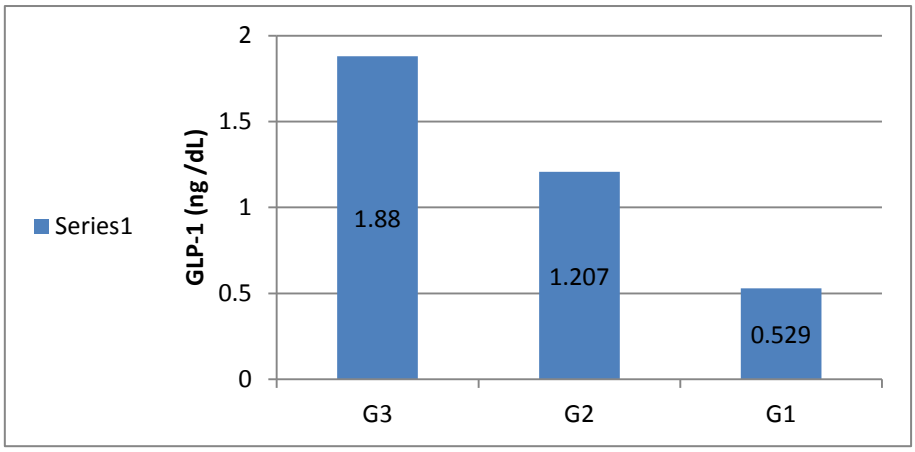

Figure (3.13): GLP-1 Levels in Sera of Three Studied Groups.

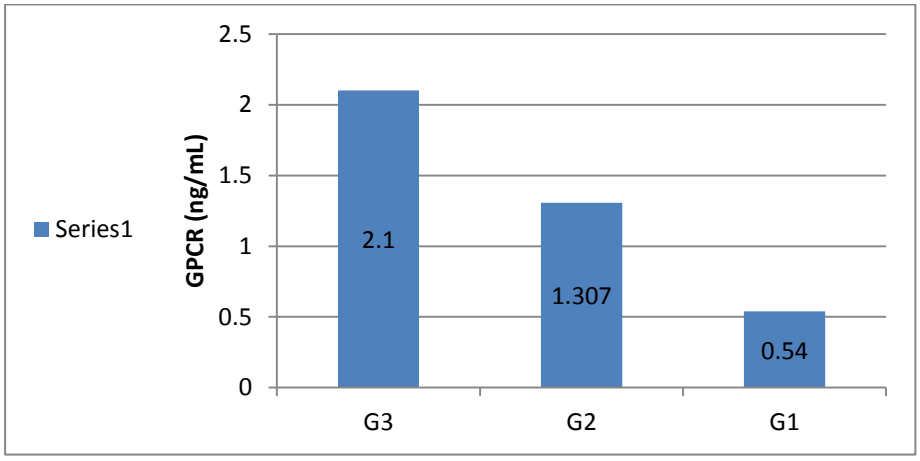

Figure (3.14): GPCR Levels in Sera of Three Studied Groups.

Study revealed that insulin affects the liver apolipoprotein secretion and control the lipoprotein lipase activity and cholesterol ester transport protein .This will led to dyslipidemia in diabetes mellitus[63]. Results in current study are agreement with this study .

Other study demonstrated that to display the loss of tonic restraint normally exerted by elevation in concentration of insulin on alpha-cells. Both of beta-cell destruction and beta-cell failure to liberate first phase of insulin due to alpha-cells insulin resistance. In addition to the lack of inhibitory tone exerted by insulin on glucagon release, other mechanisms have been investigated to illustrate the inappropriate elevate in alpha-cell [64].

The progressive impairment of β cell function and increased insulin demand as tissue becomes insulin resistance have a role development of the increase of FBS in type 2 diabetes. One of the major mechanisms of the genesis and progression of type 2 diabetes is progressive ectopic lipid deposition (e.g., in myocytes and hepatocytes, rather than in adipocytes), which stimulate insulin resistance, cell lipotoxicity, and diminished cell function, causing metabolically inadequate insulin secretion [(65)] . Obesity, insulin resistance is a prerequisite condition in diabetes type 2, that chronically increased insulin levels [(66)]. Result of this study are inagreement with present study .

Other study demonstrated that the lipoprotein lipase activity and VLDL-C clearance in uncontrolled DM were diminished causes insulin resistance[(67)], which agree with current study .

Induction of insulin liberate by glucagon was observed in experiments on human subjects nearly 50 years ago - glucagon's insulinogenic effect. In addition the insulin receptor is required in alpha-cells for glucagon secretion stimulate by reduced glucose levels due to siRNA-mediated "knockdown" of the insulin receptor in a pancreatic alpha-cell line (alpha-TC6), abolishes this glucagon secretion [(68)].

The GLP-1 receptors are found in the pancreas, brain, heart, vasculature, lung, kidney, and gastrointestinal tract, so may affect systemic metabolism in multiple organs involved CV systems as a multifunctional hormone. The metabolic Syndrome patients tend to have a binge-eating disorder and it may be one of the causes of elevated levels of GLP-1. The same study suggested that metabolic Syndrome including dyslipidemia patients, have increased of GLP -1 levels are high-risk

patients for CVD, independent with the presence of diabetes[69-71]. Conclusion of this study are in agreement with present study .

The study showed that hyperinsulinemic-insulin resistance *invitro* and in*vivo* is associated with impaired GLP-1 release in response to insulin and heterologous GLP-1 secretagogues response . The same study demonstrated that insulin was induced GLP-1 secretion, but it was only under high glucose conditions[72]. This results are inagreement with our study .

More recent study demonstrated that three classes of diabetes medications - GLP-1agonists , DPP-4 inhibitors are also thought to slow down the progression of diabetic nephropathy [73].

Treatment of T2D patients with GLP-1R agonists leading to lower in fasting TG, that pronounced with GLP-1R agonists than DPP-4 inhibitors in postprandial TG excursion [74,75] .

A research reported that GLP-1 has a role in the hypertensive heart failure, and myocardial infarction. Also, preliminary clinical studies suggested that GLP-1 infusion may improve cardiac contractile function in chronic heart failure patients with and without diabetes, and in myocardial infarction patients after successful angioplasty [76].

Study hypothesized that circulating of GLP-1 was linked with insulin resistance/hyperinsulinemia and metabolic syndrome [77].

Recent study demonstrated that GLP-1 may inhibit lipogenesis in mature adipocytes by down –regulating FASN expression , which is at least partially mediated by PKA and MAPK pathways [78,79] .

The ability of GLP-1 to reduced postprandial hyperglycemia by elevation in insulin secretion and lowering glucose secretion makes this

peptide an ideal candidate for the treatment of type 2 diabetes. Additionally, as GLP-1 is able to retain its glucose lowering activity in patients with type 2 diabetes it is also of significant clinical relevance. The main limitation of GLP-1 is a very short half-life and as a result therapeutic strategies that activate the GLP-1R and improve GLP-1 actions have been extensively studied and developed. [79]

Most small peptide hormones that signal via a GPCR, such as glucagon and GLP-1, interact with either Class A (Rhodopsin-Like) or Class B (Secretin-Like) GPCRs . Members of the class B family of GPCRs bind peptide hormones and play important roles in many diseases, like diabetes, osteoporosis and anxiety[80].

In β-cells, the main action of GLP-1 through the GPCR is the formation of cAMP and its insulinotropic activity[81,82]. Upon agonist binding, the Gαs subunit dissociates from the receptor, couples to AC and generates cAMP [83]. When blood glucose levels rise, it enters the β-cell through GLUT-1 and GLUT-2 transporters. Glucose is phosphorylated by glucokinase to glucose-6-phosphate, and led to elevation in the ATP/ ADP ratio in the cytosol and the plasma membrane depolarizing by closing Na^+/K^+ channels. The closure of Na^+/K^+ channels, in turn opens calcium channels, releasing intracellular stores of calcium. The elevation of the cytosolic calcium led to secretory granules containing insulin to fuse to the plasma membrane and insulin is exocytosed [84-86]. It is also likely that human glucokinase activity is more important in glucose-stimulate insulin secretion than the rate at which glucose enters the β-cell [87,88].

Despite the disorder in insulin function signaling it is often implicated in the etiology of diabetes-associated complications, many of these cellular activities may be modulated by GPCR signaling [89,90].

Study suggested that fatty acid-induced GLP-1 secretion is based on GPCR[91,92] by fatty acid receptor (GPR40) which is expressed in enteroendocrine L cells, it is mainly coupled to the Gq protein, which activates phospholipase C (PLC) upon fatty acid binding to the receptor[93].

Resent study revealed that the GPCR expression across many tissues in mice and humans will aid and may help in identify novel therapeutic targets and predict on-target side effects [94].

3.2 Correlation Relation of GLP-1 with the Studied Parameters:

Correlation relation for GLP-1 with FBG , urea , createnine , insulin and T-test were studied for all groups, see table (4) .

Table (3-4) : r- value and T-test for GLP-1 with urea , createnine ,FBG , insulin and HbAIc% for G1,G2 and G3

Parameters	GLP-1			T-test		
	r-value					
	r1	r2	r3	G1	G2	G3
Urea (mg/dl)	-0.146	0.435	-0.0729	NS	S	S
Createnine (mg/dl)	0.453	0.210	-0.439	NS	S	S
FBG (mg/dL)	-0.120	0.523	0.323	NS	S	S
Insulin (IU/mL)	0.070	-0.113	-0.309	NS	S	S
HbAIc%	-0.322	0.291	0.326	NS	S	S

Results showed a nonsignificant negative correlation between GLP-1 and urea for G1 (r_1=-0.146, P >0.05), while a significant positive correlation was found in G2 (r_2=0.435, P<0.05) . A significant negative correlation was observed in G3 (r_3=-0.0729, P<0.05), as shown in table (3-4) and figure (3-15).

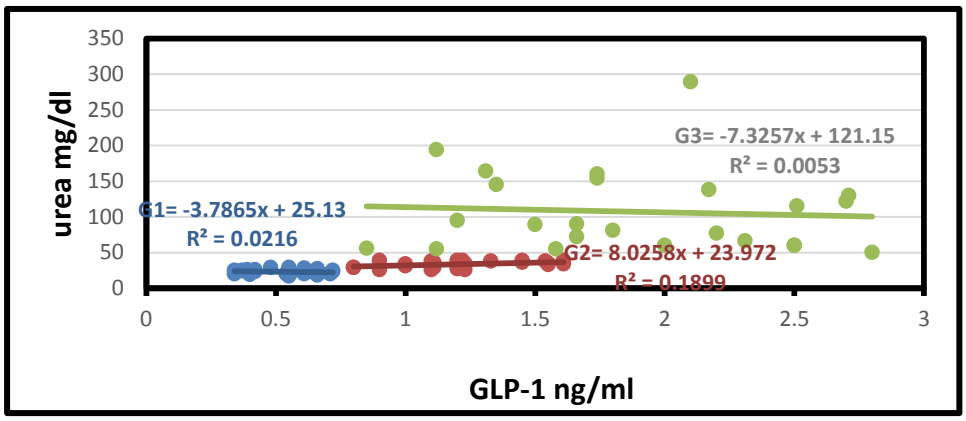

Figure (3.15): Correlation between GLP-1 and urea for G1,G2,G3

Study also showed a nonsignificant correlation between GLP-1 and creatnine for G1 (r_1=0.453, P>0.05) .while there are a significant positive correlation in G2 (r_2=0.210, P<0.05) was found . A significant negative correlation for G3 (r_3= -0.439, P<0.05) was observed as shown in table (3-4) and figure (3-16) .

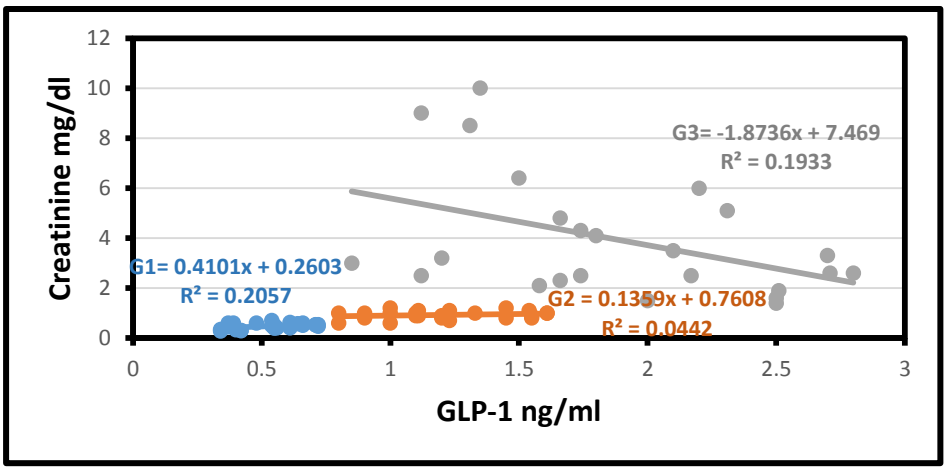

Figure (3.16): Correlation between GLP-1 and Creatinine for G1,G2,G3

Table (3-4) displays the correlation relation between GLP-1 and FBG. Results illustrated a nonsignificant negative correlation in G1 (r_1= -0.120, P>0.05) . While, a significant positive correlation was found in G2 and G3 (r_2=0.523, r_3= 0.323, P<0.05) , as shown in figure (3-17) .

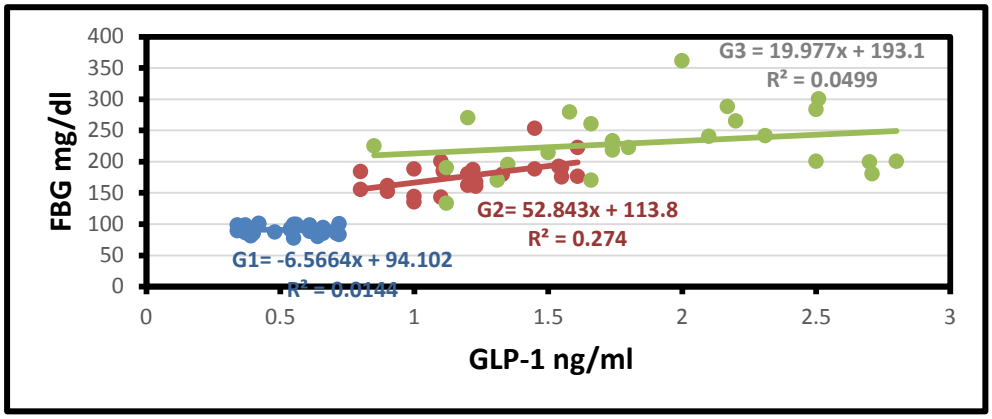

Figure (3.17): Correlation between GLP-1 and FBG for G1,G2,G3

This study revealed a nonsignificant positive correlation between GLP-1 and insulin in G1 (r_1=0.070, P<0.05) .while there are a significant negative correlation in G2 and G3 (r_2= -0.113, r_3= -0.309, P<0.05) , as presented in table (3-4) and figure (3-18).

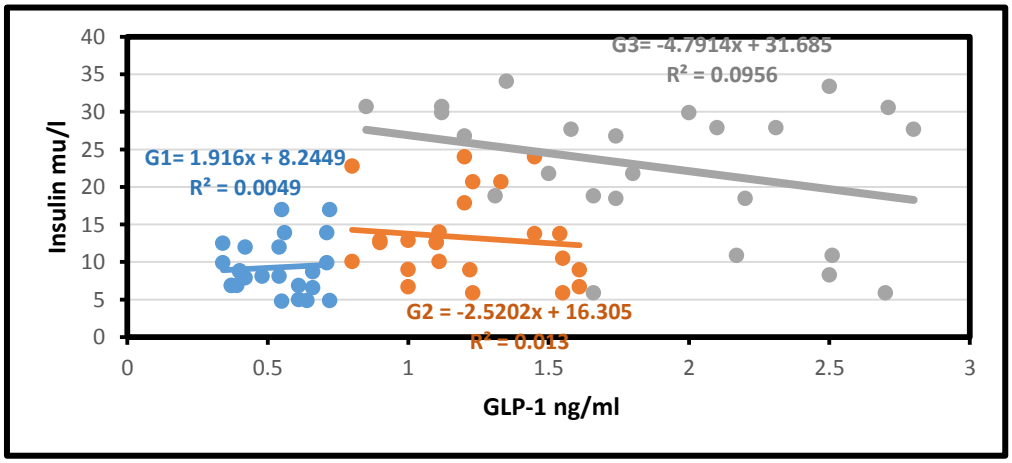

Figure (3.18): Correlation relation between GLP-1 and insulin for G1,G2,G3

Results display nonsignificant negative correlation in G1 between GLP-1 and HbAIc% (r_1=-0.322 , P>0.05) . while a significant positive correlation in G2 (r_2=0.291, P<0.05) , G3 (r_3= 0.326, P<0.05) , as shown in table (3-4) and figure (3-19).

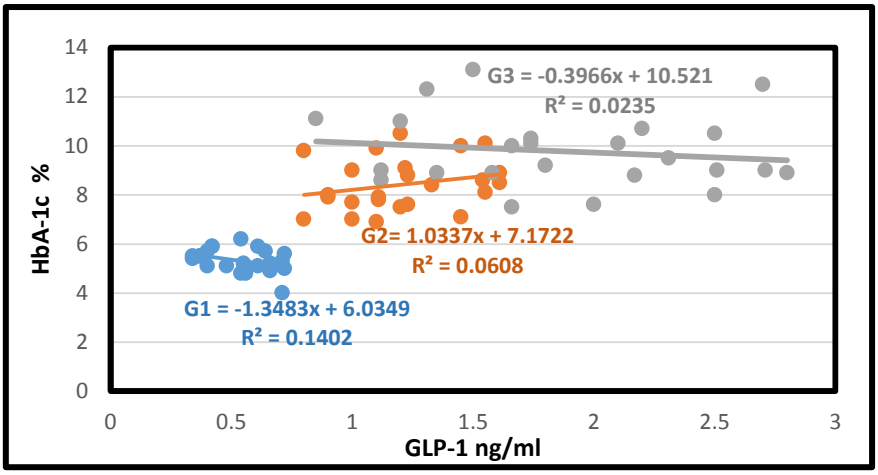

Figure (3.19): Correlation relation between GLP-1 and HbAIc% for G1,G2,G3

3.3 Correlation Relation of GPCR with Studied Parameters:

The correlation relation of GPCR with HbA1c , TC , TG and GLP-1were studied for all examined groups .The T-test and p- value are shown in table (3-5) .

Table (3-5): r- value and p- value for GPCR with HbA1c , TC and TG for G1,G2 and G3 .

Parameters	GPCR(ng/ml)			T-test		
	r-value					
	r1	r2	r3	G1	G2	G3
HbA1c %	0.172	0.427	-0.042	NS	S	S
TC (mg/dl)	0.306	0.514	-0.288	NS	S	S
TG (mg/dl)	-0.0009	-0.292	0.276	NS	S	S
GLP-1 (ng/ml)	0.167	-0.167	0.034	NS	S	S

Results in table (3-5) revealed a nonsignificant positive correlation between GPCR and HbA1c in G1 (r_1=0.172) while a significant positive correlation was found in G2 (r_2=0.427) and a significant negative correlation in G3 (r_3=-0.042) in these parameters , as shown in table (3-5) and figure (3-20).

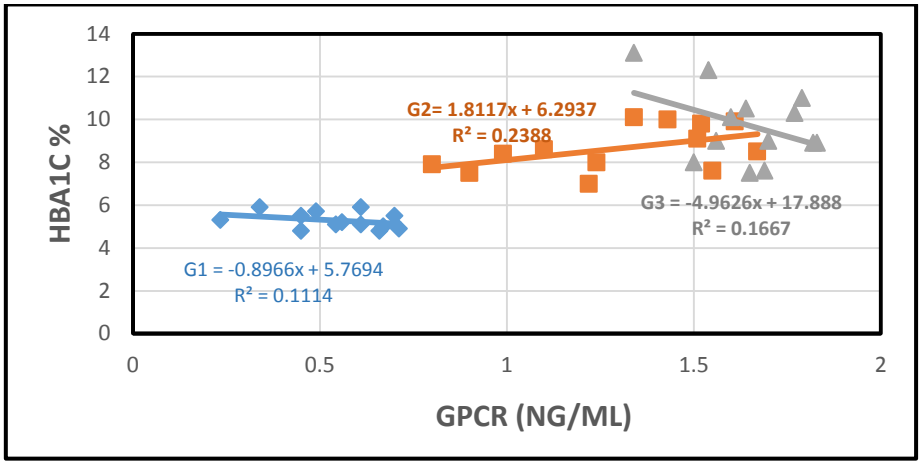

Figure (3.20): Correlation between GPCR and HbA1c % for G1,G2,G3

Results , also, showed a nonsignificant positive correlation in G1 between GPCR and cholesterol (r_1=0.306 , P>0.05) . However , there is a significant positive correlation in G2 (r_2=0.514, P<0.05) . and a significant negative correlation between GPCR and cholesterol in G3 (r_3= -0.288, P<0.05) , as shown in table (3-5) and figure (3-21).

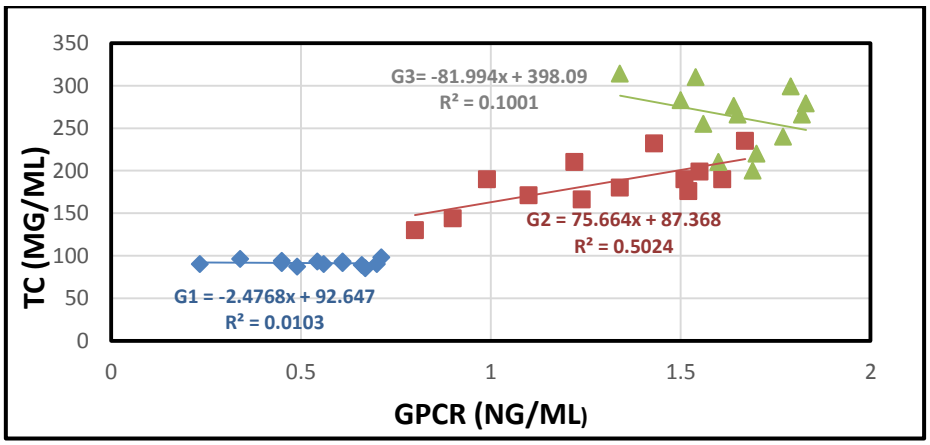

Figure (3.21): Correlation relation between GPCR and TC for G1,G2,G3

Table (3-5) showed a nonsignificant negative correlation between GPCR and TG in G1 (r_1=-0.0009, P>0.05) . while, there is a significant negative correlation in G2 (r_2=-0.292, P<0.05) . Also, results revealed a significant positive correlation between GPCR and triglyceride in G3 (r_3= 0.276, P<0.05) , are seen in table (3-5) and figure (3-22).

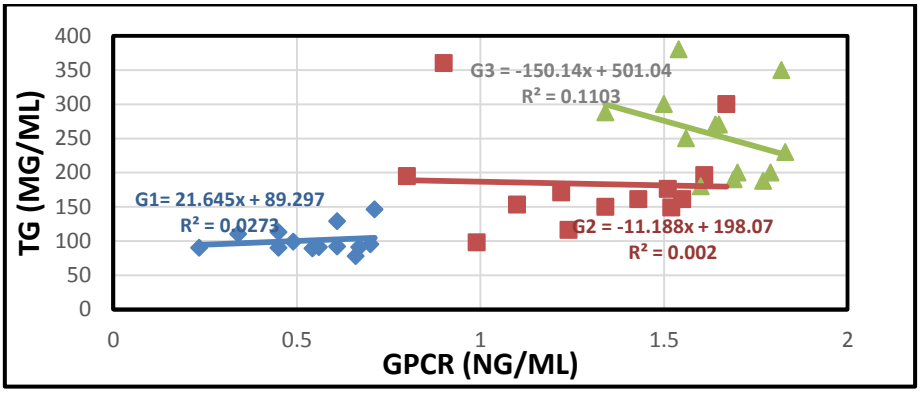

Figure (3.22): Correlation between GPCR and TG for G1,G2,G3

Results showed nonsignificant correlation between GPCR and GLP-1in G1 (r_1=0.167 , P>0.05) . while there are significant negative correlation was see in G2 (r_2=-0.167, P<0.05) and a significant positive correlation in G3 (r_3= 0.034, P<0.05) , as shown in table (3-5) and figure (3-23).

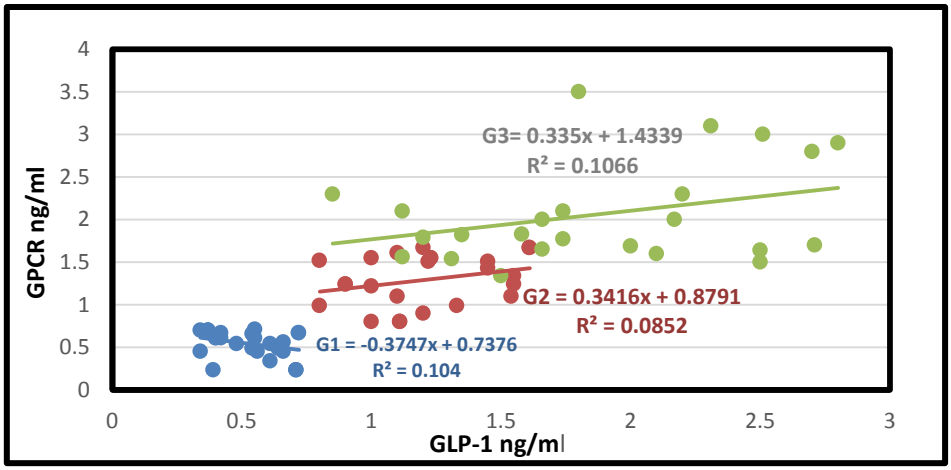

Figure (3.23): Correlation between GPCR and GLP-1 for G1,G2,G3

Conclusion could be drawn from this study that the differences in GPCR and GLP-1 levels among groups indicate that GPCR may be used as a marker in development of diabetic nephropathy depending on a significant relation with HbA1c% ,urea , creatinine and insulin .

To the best of our knowledge this is the first study in determination of GLP-1 and GPCR in diabetic and diabetic nephropathy patients and compare results with control group .In addition to study relation for GPCR and GLP-1with urea , cretinine and insulin .

Conclusions

1- Results showed a significant increase in the insulin and IR levels in G2 and G3 comparing to G1 .Also ,a significant increases was noticed in G1 comparing to G2 .

2- A significant elevation in GLP-1 and GPCR levels were observed in G2 and G3 comparing to G1. Also, a significant differences was observed in G3 comparing to G2 .

3- Results showed a significant correlation for GLP-1 and GPCR with TC,TG,VLDL and HDL in G2 and G3 .

4- Results showed a significant correlation for GLP-1 and GPCR with urea , creatinine ,HbA1c% , insulin and IR in G2 and G3 .

5- Conclusion could be drawn from this study that the differences in GPCR and GLP-1 levels among groups indicate that GPCR may be used as a marker in development of diabetic nephropathy depending on a significant relation with HbA1c% ,urea , creatinine and insulin .

Summary 2

The study aimed to purified G- Protein Coupled Receptor from the whole blood cell and membrane of control , diabetic and diabetic nephropathy patients by different chromatography techniques . The GPCR extracted from whole cell and membrane by precipitation of proteins with 50 – 75% ammonium sulfate , it was cooled and centrifuged at 3500g for 10 minutes . Finally , the pellet was dissolved in a minimum volume of (1.5 mM) of PBS at pH 7.3 .The Pellet cells were thawed and resuspended in the extraction buffer and then solubilized in phosphate buffer containing n-Dodecyl- β- D-Maltoside as detergents and protease inhibitor cocktail and glass bead. The extracted G Protein Coupled Receptor from whole cell was purified by ion exchange chromatography, as a first step using DEAE-Sepharose .A gel filtration chromatography was applied as a second step of purification using sepharose 6B.The molecular weight and purity of GPCR was determined by SDS.PAGE .

Results of ion exchange chromatography for control patients showed two proteins peaks that appeared after elution by the gradient concentration of sodium chloride , and there was no protein peaks appeared in the washing fractions . The GPCR concentration was measured in the fractions of these two protein peaks . Data indicated that the GPCR is located in the first protein peak (eluted at 0.1 M of NaCl) at fraction numbers between (6 and12) . The second peak (eluted at 0.2 M of NaCl) gave a peak between (18-20) fraction numbers .

Results for diabetic patient showed two proteins peaks that appeared after elution with the gradient concentration of sodium chloride , while no protein peak appeared in the washing fractions . The GPCR concentration was measured in the fractions of these two protein peaks . Data indicated

that GPCR located in the first protein peak (eluted at 0.1 M of NaCl) at fraction numbers between (7 and13) . The second peaks (eluted at 0.2 M of NaCl) gave a peak between (18-20) fraction numbers .

Results for diabetic nephropathy patient showed two proteins peaks that appeared after elution with the gradient concentration of sodium chloride . While no protein peaks appeared in the washing fractions . The GPCR concentration was measured in the fractions of these two protein peaks . Data indicated that GPCR located in the first protein peak (eluted at 0.1 M of NaCl) in fraction numbers between (7 and13) . The second peaks (eluted at 0.2 M of NaCl) gave a peak between (19-21) fraction numbers .

After purification by ion exchange chromatography fractions were collected pooled and concentrated to be applied in gel filtration chromatography by using sepharose 6B column . Aliquot of five mL of concentrated fraction was injected into column (65 x 1.5) cm which previously equilibrated with 50 Mm phosphate buffer saline (pH=7.4) , and eluted with elution buffer PBS pH =7.4 containing (0.5Mm) DDM . Flow rate was (1ml /min) with 5 ml for each fraction that monitored at 280 nm .

Results showed a single active protein peak after eluted with elution buffer that was identical with the peak that presented GPCR concentrations at fractions number (14) for control group and show of single active protein peak that was identical with the peak that presented GPCR concentrations at fractions number (12) for diabetic patients which show a single active protein peak that was identical with the peak that presented GPCR concentrations at fractions number (12) for diabetic nephropathy patients .

The molecular weight of GPCR that estimated by SDS-PAGE electrophoresis was approximately two bands of GPCR appear in control subject at approximady (~ 30 KD) and at (~ 51 KD) . This could be due to the isoform of GPCR that papered in these groups .

Results ,also , showed appearance of three bands in G2 related to GPCR in (~ 29 KD) and (~ 47,49 KD) . While it is not very clear related to its little concentrations . In diabetic nephropathy, bands appeared similar to the bands appeared in G2 but with highly clearance related to its highly concentration .This three bands related to the type of GPCR and its isoform in this disease .

The conclusion could be drawn from this study for the first time that 2 bands of GPCR appear in the control group while there are three bands appeared in G2 and G3.This finding could be useful in the early detection and active treatment for diabetic and diabetic nephropathy patients . Further study is important to find type of GPCR that purified by amino acid analyzer .

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List of Abbreviations

Abbreviations	**Name**
AEBSF	4-(-Amino Ethyl)-Benzene Sulfony Fluoride
DDM	n-Dodecyl -β-maltoside
ROS	Rod outer segments
Gβγ	Beta gamma Subunit G-protein
Gα	Alfa subunit G-protein

4. Introduction (2):

4.1 purification of GPCR from various sources by different methods .

Purification is a major challenge in the field of GPCR research because many GPCRs are denatured by detergents (due to the difficulty of purifying GPCRs) . Membrane mimics provide a range of choice for GPCR reconstitution and biophysical characterization that beginning to be applied to GPCR research , and detailed biophysical date remain scarce for receptors other than rhodopsin [95-97] .

Rhodopsin is the favorite type of GPCR that studied due to its stability in the dark state and to large quantities that purified from retinal extracts [98] .

Previous studies suggested that combination of purification and crystallization of rhodopsin of other membrane protein which the initial rhodopsin structure utilized the selective precipitation of opsins and other membrane proteins by $Zn(oAc)_2$ treatment of alkyl-glucose extraction of rod outer segments (ROS) that isolated from dark adapted biovine retina [99,100].

It was reported that to obtain new rhodopsin crystals which are stable to photo activation a combination of previously reported methods of purification was employed . The expression of ID4-tagged GPCRs in the retinas represents a promising a approach for the expression of recombinant GPCRs in rod cells , where they are homogeneously processed and transported to ROS and rhodopsin . These tagged GPCRs can be purified and concentrate using the immunoaffinity and crystallized by using $(NH_4)_2SO_4$ concentrations[101]. The human melatonin receptors

were purified by tandem affinity tag technique at their carboxyl – terminal tails and expressed in human embryonic kidney 293 cells [102] .

Human $_5$-HT_4 receptor was purified by one – step immunoaffinty chromatography and the purified receptor in detergent solution preserved its legend binding properties . This expression methods may prove generally useful for generation functional , high – quality GPCR protein [103] .

Immunoaffinity purification was used to purify rho ID4 monoclonal antibody (cell essentials) Linked to CNBr-actvated sepharose 4B beads then 17-4 protein was subjected to gel filtration chromatography using a HiLoad 16/60 superdex 200 column . This system is feasible for the production of large quantities of olfactory receptor with yields of 3mg/L of culture medium and > 90% purity which is important for structure and functional analysis [104] .

Another study, based on purified synthetic human GPCR in two steps from membrane fractions . Firstly, by Ni^{+2} affinity purification of His_6 – tagged protein using a Hitrap chelating HP 5ml column and Gel filtration purification was used to the second step by using superdex 200 10/300 GL. The protein purified from the first step include monomer , dimmer , and higher oligomer for receptor , these species could be further resolved in the gel filtration step. The yields of purified Trx-hcRs finally achieved in this system was ~ 1.3/L [105] .

A systematic detergent screen showed that foes – chalic -14 was the optimal detergent to solubilize and subsequently purify the GPCR receptors by using immunoaffuity chromatography followed by gel filtration . Circular dichroism showed that the purified receptors had helical secondary structures , indicating that they were properly folded

suitable for functional analysis and for subsequent crystallization trials[106].

Thromboxane A_2 receptor is a GPCR that mediate vasoconstrictin and promotes thrombosis in response to binding of thromboxane this receptor expressed and purified by using a single step affinity purification [107].

In order recent study Human Mu Oproid Receptor from *E.coli* was carried out with several purification steps as a affinity chromatography and ionic exchange chromatography [108].

4.2 GPCR in diabetes mellitus :

Islet function is regulated by a number of different signals. A main signal is generated by glucose, which stimulates insulin secretion and inhibits glucagon secretion. The glucose effects are modulated by many factors, including hormones, neurotransmitters and nutrients[109-116]. Receptors activation are linked with the postprandial phase that is the targets for nutrients, bile acids and gut hormones [117] .

Many GPCRs are directly involved in the development of insulin resistance and β-cell dysfunction , and in the etiology of inflammation that can lead to obesity – induced T2DM[118,119] . Although there is a well-recognised GPCR capable of detecting glucose—the sweet taste receptor TAS1R2/3 heterodimer non-GPCR mechanisms appear to dominate postprandial glucose-dependent insulin and GLP-1 release, which are instead achieved through metabolism-dependent closure of ATP-sensitive potassium (K_{ATP}) channels in beta cells or electrogenic sodium-dependent glucose transport in GLP-1-secreting L cells [120] .

Glucose-dependent stimulation of insulin secretion is not restricted to $G_{\alpha s}$-coupled receptors, and has also been observed with $G_{\alpha q}$-coupled receptors linked to protein kinase C activation and inositol 1,4,5-trisphosphate (IP_3)-dependent calcium release from intracellular stores. One of the classical $G_{\alpha q}$-coupled receptors linked to insulin secretion is the muscarinic acetylcholine receptor M_3, responsible for the vagal stimulation of insulin release in some species [121,122] . GPCRs that signal via the $G\alpha_q$ and $G\alpha_s$ pathways led to elevation insulin liberation , which GPCRs that signal via the $G\alpha_i$ pathway that inhibit insulin secretion. the GPCRs couple to a complex of $G\alpha\beta\gamma$, that activated GPCRs led to the secretion of $G\beta\gamma$ as well as $G\alpha$ subunits. The effects of any one type of GPCR on insulin liberation that quite complex [123] ,as show in figure (4.1).

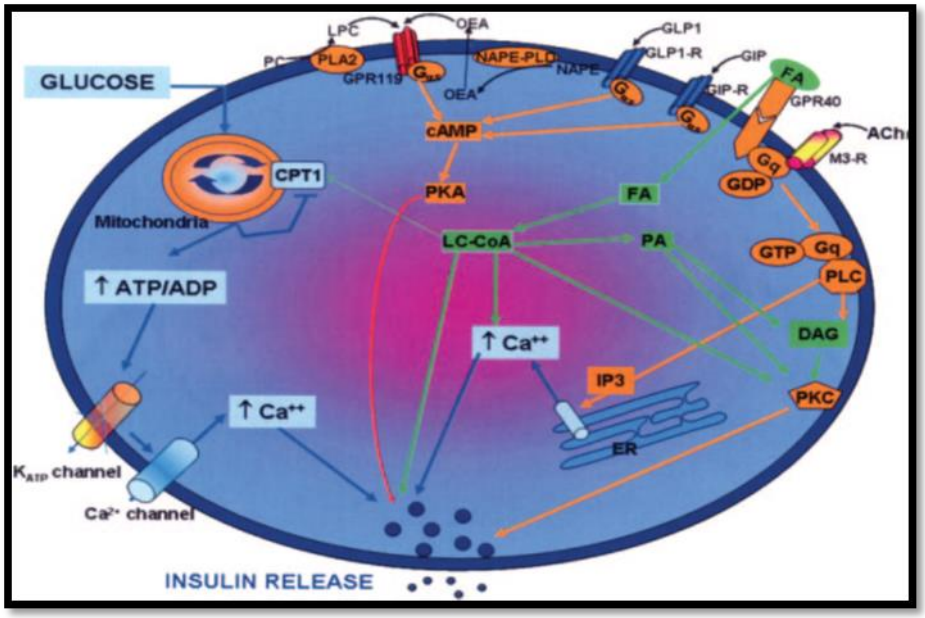


Figure (4.1): GPCRs –mediated amplification of insulin secretion [122] .

Table (4.1) display GPCRs that found in beta cells that affect insulin liberation and their ligands . levels of glucose are a primary regulator of insulin liberation , signaling via various GPCRs that positive

or negative effects on insulin secretion via their regulation of intracellular signaling pathways[123].

Table (4.1) type of GPCRs that found in beta cell that affect insulin secretion

GPCRs	Full name	Ligand	Alpha subunit	Cell type	Insulin secretion
ADRB2	Beta-2 adrenergic	Epinepherine	Gs	beta	+
ADRA2A	Alpha-2 adrenergic (A)	Norepinepherine	Gi	beta	-
MTNR1A	Melatonin 1A	Melatonin	Gq/Gi	beta	?
MTNR1B	Melatonin 1B	Melatonin	Gq/Gi	beta	?
HTR2B	Serotonin-2B	Serotonin	Gq	beta	+
HTR1D	Serotonin-1D	Serotonin	Gi	beta	-
M3	Muscarinic-3	Acetylcholine	Gq	beta	+
SSTR2	Somatostatin-2	Somatostatin	Gi	beta	-
GLP1R	Glucagon-like Peptide 1	Glucagon-like Peptide 1	Gs	beta/alpha?	+
GPR40	G-protein receptor40	Free fatty acids	Gq	beta	+
GPR119	G-protein receptor119	Free fatty acids	Gs	beta	+
GCGR	Glucagon	Glucagon	Gs/q	beta/alpa	+

These receptors have important effect in the regulation of blood pressure, study revealed that adrenergic family of GPCRs, (α2 and β2 receptors) regulate islet function. Which α2-adrenergic receptors inhibit insulin liberation and stimulate glucagon secretion. The β2-adrenergic receptors stimulate both insulin and glucagon secretion. Studies revealed that α2 receptor have role in type 2 diabetes. People who carry a specific mutation (single nucleotide polymorphism) in the gene encoding the α2 receptor (subtype 2A) have an increased risk of type 2

diabetes. This genetic variant is associated with overexpression of the α2 receptor and lowered insulin secretion [123].

In clinic, two strategies are widely applied to treat the diabetes, GLP-1 analogs and inhibitors of DPP-4 that degrades both GLP-1 and GIP. These strategies had limitations in clinical practice: GLP-1 analogs can only be administered by injection and the effectiveness of DPP4 inhibitors is mild. Therefore, development of agent(s) that may enhance GLP-1 pathway received increasing attentions at first. Basically, GLP-1 is released in response to activation of two GPCRs ; GPCR119 (GPR119) and GPCR 131 (GPR131). Adverse reaction due to activating GPRs has been conducted and limited it to develop in clinical application[124]. Interestingly, agonist for GLP-1 receptor from natural product has been developed and geniposide were introduced as the potential one. It will be the new target in the development of agent(s) for therapeutics of diabetes in the future. Also, we suggest the suitable target(s) to call the attention(s) for better treatment of diabetes [125-128]

The number of β-cell GPCRs involved in the control of insulin secretion has increased steadily and there are six to seven different known G protein-coupled mechanisms. Although these receptors are expressed in high levels in pancreatic β-cells [129] .

Glucose metabolism raises the ATP/ADP ratio, which closes ATP-sensitive potassium channels (KATP channels), depolarizes the cell membrane, activates voltage-gated calcium channels (Ca2_ channels), and results in calcium influx, which in turn triggers the release of insulin. Several extracellular signals can amplify this process through the activation of GPCRs.These receptors couple to various downstream signaling pathways in the cell leading to an increase in cAMP levels and/or intracellular calcium and converge to amplify GSIS.

Acetylcholine, carnitine-palmitoyl transferase 1, diacylglycerol , endoplasmic reticulum , fatty acid , inositol triphosphate , long-chain coenzyme-A, M3-muscarinic receptor , N acylphosphatidylethanolamine , phosphatidic acid , phosphatidylcholine , cAMP-dependent protein kinase , protein kinase C , phospholipase A2 , phospholipase C , phospholipase D , G protein subunit q. [130-132] .

Results for the previous study show for the first time the occurrence of potentially patho genic agAAB directed against GPCR in patients suffering from type 2 diabetes. The presence of agAAB that mainly interact with the a α_1-AR suggests an increased risk of hypertension and vascular complications for diabetic patients [133-135] .

The Gi-GPCRs, that signal through Gα proteins of the i/o class ($G\alpha_{i/o}$), control cellular behaviors in mammalian tissues, such as Gi-GPCRs control insulin release from pancreatic β cells, and variants in genes encoding several Gi-GPCRs—including the α-2a adrenergic receptor, ADRA2A—elevation the risk of type 2 diabetes mellitus. Increased Gi-GPCR activity in perinatal β cells lowered β-cell proliferation, lowered adult β-cell mass, and impaired glucose homeostasis. The Gi-GPCR inhibition enhanced perinatal β-cell proliferation, increased adult β-cell mass, and improved glucose homeostasis. Transcriptome analysis detected the expression of multiple Gi-GPCRs in developing and adult β cells, and gene-deletion experiments suggest that ADRA2A as a key Gi-GPCR regulator of β-cell replication[136] .

Insulin secretory capacity depends on both the secretory capacity of individual β cells and total β-cell mass, which is reduced in both type 1 and type 2 diabetes . Two sources contribute to the pool of β cells in the

pancreas: neogenesis from progenitor cells and proliferation of preexisting β cells. The β-cell population expands most dramatically during the perinatal and early postnatal period because of increased proliferation, which then falls markedly as adulthood approaches in both rodents and humans [137,138] .

Previous study aimed to synthesis many mini-G proteins that used in the structure estimation of GPCRs in their fully active state. Mini-G proteins was performed on G_s , which turned out to be one of the best expressed and most stable of the mini-G proteins. The structures of GPCRs and G proteins are highly conserved , and there are thought to be highly conserved networks of side chain interactions within the GPCR , and G protein , that are essential for receptor activation and G protein activation[139] .

Phylogenetic analyses revealed several kinds of evolutionary patterns that occurred during GPCR evolution including one-to-one orthologous relationships, species-specific gene expansion, and episodic duplication of the entire GPCR repertoire in certain species lineages. This study suggested dynamic process of birth and death of GPCR genes since hundreds of millions of years . A range of amino acid residues can be accommodated in this interface and therefore it plays a less important role in defining both specificity and the affinity of G protein binding [140]. Genetic drift and selective forces have shaped the individual structure of a given receptor gene but also of the species-specific receptor repertoire – a process that is still ongoing [141-143] .

Aim of the study

Researchers are seeking to understand which GPCRs involved in normal and diabetic islet function, as this knowledge has the potential to suggest new approaches to treat diabetes. Given the importance of GPCRs in transmitting signals from the extracellular environment and potential as drug targets, scientists have sought to understand their role in pancreatic islet cell biology. A first step in this process was to understand which GPCRs are expressed in islets.

Therefore , this study designed for the first time to improve the number and type of GPCR that found in control , type 2 diabetic and diabetic nephropathy patients in order to treat these patients successfully .

Chemicals and Methods (2):

5.1 Chemicals:

Chemicals used in the present study were obtained from various companies as displayed in table (5-1).

Table (5-1):- Chemicals and Suppliers.

Chemicals	Company, Origin
AEBSF	Sigma / USA
BSA	BHD/England
Blue dextrin	BHD/UK
Coomassie brilliant blue G-250	Sigma / USA
DEAE- Sepharoe	Sigma / USA
DDM	Sigma / USA
KH_2PO_4	BHD/England
KCL	Fluka/Switzerland
Na_2HPO_4	BHD/England
NaCL	BHD/England
Tris -HCL	BHD/UK
Sepharose 6B	Sigma / USA

5.2 Instruments:

Instruments-company and their origin that used in this study are summarized in table (5-2).

Table (5-2):- Instruments, company and their origin.

Instruments	Company, origin
Analytical balance	Ohans (France)
Cooling Centrifuge	Gallenkamp(UK)
Deep freeze	FROILABO, France
ELISA human reader and washer	Bio Tek, USA
Gel electrophoresis system	Bio- Red (USA)
Micropipettes	Oxford (USA)
pH meter	Genway (UK)
Spectrophotometer UV-Visible beam	Labomed (England)
Water bath	Gemmy , YCW-01, Taiwan

5.3 Solutions and Buffers

5.3.1 Solutions and Buffers that used for Extraction and solubilization of GPCR [144,145]:

A. Phosphate Buffer Saline PBS [146], (1.5mM), pH =7.3

According to Gruikshank et. al (1975) method. Na_2HPO_4 (2.1 g) was dissolved in a 1 L of distilled water. The pH was adjusted to 7.4, sterilized by autoclave and stored at 4 ^oC prior to use.

B. NaCl solution (100 mM).

C. 4- (2-amino ethyl)- benzene sulfonyl fluoride, (a Serine protease inhibitor), (1mM).

D. n-Dodecyl- B- D- Maltoside.

5.3.2 -Solutions for Protein purification :

1) Sodium Chloride Solution (0.1-0.5)M

2) (Wash Buffer) Phosphate Saline (1.5mM) pH =7.3 :

It was prepared as described in (5.3.1. A). The pH was adjusted to (7.4) then buffer sterilized by autoclave and stored at 4^{o}C prior to use.

3) Elution Buffer:

A) For Ion Exchange Chromatography:

This buffer was prepared by adding (50 mM of DDM to the PBS(1.5 mM, pH =7.3) containing step wise of NaCl (0.1-0.5)M.

B) For Gel Filtration Chromatography:

It was prepared by adding 1.5 mM of DDM to the phosphate buffer saline (1.5mM,pH =7.3).

4) Sodium hydroxide Solution (0.25M) .

5) Hydrochloric acid Solution (0.25M) .

5.3.3-Solutions and Reagents for Determination of Protein Concentration :

The solution and reagent for determination of protein concentration. were prepared according to Bradford [147] methods as follows:

A-Bovine Serum Albumin (BSA):

Stock Solution of BSA it was prepared by dissolving (0.01 g) of BSA in (10 mL) of distilled water .The solution was used to prepare (20,40,60,80,100 µg/mL) concentration of BSA. These concentrations were used to determine the standard curve of protein.

B- Coomassie Brilliant Blue G-250 Stain: It was prepared by dissolving(100 mg) of the dye in (50mL) ethanol (95%). Aliquot of 100 mL of 85% (w/v) phosphoric acid was added to this solution. The solution was completed to 1 liter with distilled water and it was filtered via whatman-1 filter papers . The reagent was stored in dark bottle at $4^{o}C$.

C-Tris -HCl Stock Solution (0.15M):

This solution was prepared by dissolving (0.3g) of Tris -HCl in (80ml) of distilled water. The pH was adjusted to (7.5) then its volume was completed to (100 mL) with distilled water.

D- Reagent Blank :

This reagent was prepared by mixing (0.1 mL)of 0.15 M (Tris HCl pH 7.5) and (5mL) of Coomassie Brilliant Blue G-250 reagent.

5.3.4- Solutions for Polyacrylamide Gel Electrophoresis (SDS-PAGE) [148].

A. Stock 30% Acrylamide -Bisacrylamide Gel Solution:

It was prepared by dissolving (3g) polyacrylamide and (0.08g) Bis-acrylamide in (10mL) of distilled water. The solution was filtered through whatman-1 filter paper , then stored in refrigerator at 4 ^{o}C in a dark bottle for no longer than one month.

B. Stock 4x Resolving Gel, Tris base (pH =8.8):

It was prepared by dissolving (3.64g) of Tris base and (0.8ml) of (10%SDS) in (11 mL) of distilled water, The pH was adjusted to (8.8) and then the volume was complete to (20 mL).

C. Stock 4x Stacking Gel ,Tris base (pH 6.8):

This solution was prepared by dissolving (1.2g) of (Tris -base), (0.8mL) of (10% SDS) in (11 mL) of distilled water, the pH was adjusted to (6.8) the volume was completed to(20 mL).

D. Stock (4x) Tris-glycine tank buffer –SDS:

Quantities of (3 g) Tris- base and (14.5 g) glycine were dissolved in (250mL) distilled water.

E. (1x) Tris- glycine tank buffer-SDS: Mixed (250mL) of 4x Tris- glycine reservoir buffer SDS and (10mL) of (10%) SDS solution then the volume was completed to I L.

F. Catalyst: 10% Ammonium per sulfate solution (A.P.S): It was prepared by dissolving (100 mg) of (A.P.S) in (1 ml) of distilled water.

G. Sodium Dodecyl Sulfate (SDS) 10% (w/v):

It was prepared by dissolving (1 g) in (6mL) distilled water .The volume was completed to (10mL) with distilled water.

H. TEMED (N,N,N,N-Tetra Methylene Diamine).

I. Coomassie Brilliant Blue R-250 .

J. Resolving Gel Solution .

K. Stacking Gel Solution .

L. Staining Solution: It was prepared by dissolving (0.6 mg) of comassie Brilliant Blue in a mixture of (23mL) of acetic acid and (112mL) of methanol. The volume was completed to (250mL) by distilled water **.**

M. De Staining Solution: It was prepared by mixing (50 mL) of methanol with (25 mL) acetic acid. The volume was completed to (500 mL) with distilled water.

N. loading Dye: It was prepared by dissolving (2 mg) of bromophenol blue in a mixture of (5ml)of C, (8 mL) of G,(4ml) of glycerol, (2mL) of 2- mercaptoethanol and (1 ml) distilled water.

5.4 - subjects : Blood were collected from control , diabetic and diabetic nephropathy patient . That aged in the range (40-65) years .

5.5 - Methods :

5.5.1- Extraction of GPCR from blood :

1- Blood that obtained from subjects were centrifuged at 2500g for 15 minutes to separate pack cell volumes . Supernatant plasma and buffy coat were discarded . RBCs were washed with (3) volume of normal saline and hemolysed by adding (5)

volumes of water . After 20 minutes , hemolysed was centrifuged at 3500g for 20 minutes . Transparent supernatant was collected and filtered through qualitative filter paper (whatmen-1) to remove left out cell debris . It was followed by precipitation of proteins with 50 – 75% ammonium sulfate . For precipitation an equal volume of saturated solution of $(NH_4)_2SO_4$ was mixed with filtrate and after 15 minutes , it was cooled and centrifuged at 3500g for 10 minutes . Finally , the pellet was dissolved in a minimum volume of (1.5 mM) of Phosphate Buffer Saline PBS pH 7.3 .

2- The Pellet cells were thawed and resuspended in extraction buffer (1.5 mM PBS buffer, pH =7.3, 1 mM AEBSF) and then protease inhibitor cocktail (up 10 ml for 1ml) was added with gentle swirling on ice.

3- DDM (1%) was added to the lysed sample and stirred on ice for 1 h.

4-The dissolved pellet was dialyzed for 72 hrs against 1500 ml of dialysis buffer (1.5 mM PB, pH 7.3) in a cold room . buffer was changed three times during 72 hrs.

5.5.2-Determination of Protein Concentration:

Protein concentration was estimated according to the Bradford method (1976), (0.1ml) of each concentration of BSA that prepared in (5.3.3.A) was added and mixed with five mL, of Coomassie Brilliant Blue G-250(5.3.3.B), after 3 minutes the absorbance measured at 595 nm against a reagent blank(5.3.3.D).

5.5.3 Determination of GPCR Concentration by ELISA:

A-The desired numbers of coated wells in the holder were secured then (50ml) of standards or samples were added to the appropriate well in the antibody

pre-coated micro titer plate. Aliquot of (50 ml) of PBS pH (7.0-7.2) was added in the blank control well.

B- Five ml of balance solution was dispensed into (50 ml) of specimens, mixed well.

C-Aliquots of (100 ml) of conjugate solution was added to each well, except blank control well and mixed well.

D-The microtiter plate was washed by manual washing.

E- Add (50ml) of substrate A and(50ml) of substrate B to each well including blank control well, and the covered and incubated for 10-15 minutes at 20-25 °C.

F- Aliquots of (50 ml) of stop solution was added to each well including blank control and mixed well.

G- The absorbance was determined for samples at 450 nm by using a microplate reader immediately.

5.5.4 - G-protein Coupled Receptor Purification:

5.5.4.1- GPCR Extraction from blood Cell :

GPCR that extracted from blood was purified by using ion exchange chromatography as a first step of purification, and gel filtration chromatography as a second step of purification.

5.5.4.1.1- Ion Exchange Chromatography:

The GPCR solution that obtained from step (5.4.1.2) was passed through DEAE-Sephorose anion column (2.5 x8cm) after equilibration of column and washing with 1.5 mM phosphate buffer saline at pH =7.3. Then GPCR crude was

eluted with (5.3.2. 3.A) buffer; Aliquot of 5ml fraction was collected in each tube with flow rate of 0.5 ml/min. Protein concentration was estimated for each fraction which measured at 595 nm using UV- VIS spectrophotometer . The GPCR concentration was determined by ELISA Kit from (BlueGene). The fractions that gave the highest absorbance were collected. Protein and GPCR concentrations were measured for these fractions.

5.5.4.1.2- Gel filtration chromatography [149] :

***Column Preparation (Sepharose 6B):**

Column was prepared according to bioworld company procedure in which the suspension gel was degassed by vacuum pump to remove the air bubbles and poured into column (96 x 1.6 cm) after packaging, the gel was equilibrated with(5.3.1.A) buffer at pH 7.3.

*** Loading of Sample**

The concentrated sample was applied to the sepharose 6B column that pre equilibrated with (1.5 mM PBS pH 7.4), then eluted with (5.3.2.3.B) buffer. Aliquot of 5ml fractions were collected in each tube with flow rate of 0.5 ml/min . Protein concentration was estimated for each fraction was measured at 595 nm using UV- VIS spectrophotometer . The GPCR concentration was determined and fractions which gave the highest absorbance and concentration of GPCR were collected.

5.6 - Estimation of GPCR Molecular Weight [150] :

Molecular weight of GPCR was determined by SDS-PAGE Electrophoresis

5.7- SDS-PAGE Electrophoresis [151-153]

Sodium dodecal sulfate (SDS) -gel electrophoresis was used to improve the purity of the purified GPCR,SDS anionic detergent, is very effective in solubilizing most membrane proteins. .However, it unravels protein structures and cover the polypeptide in negative charge The molecular weight and purity of individual polypeptide chain can be determined by polyacrylamide gel electrophoresis in the presence of SDS .Since protein solubilized in SDS are all negatively charged and bind SDS such that the charge to molecular weight ratio is constant ,their subsequent migration in an electric field is dependent under ideal conditions on the size of the protein and the sieving effect of the polyacrylamide gel electrophoresis support.

Stock solution	**Stacking gel solution**	**Resolving gel solution**
30% Stock acrylamide acrylamide	1.3 ml	5 ml
Stacking gel buffer	1.9 ml	-
Resolving gel buffer	-	3.7 ml
Resolving buffer	-	-
10% ammonium Persulfate	75 µL	150 µL
Distilled Water	4.4 ml	6.3 ml
TEMED	3.5µL	15µL

The gel was run after mixing immediately between two securely sealed glass plates.The bottom of the glass plates was sealed with sealant (30% polyacrylamide plug). When the gel was set, a stacking gel was formed over the resolving gel and formed the sample wells for the protein samples. Samples were injected into the sample wells and a running buffer was laid over the top .The plates were placed in position and a 150 voltage was applied across the gel at room temperature. The gel was removed, stained in a staining solution to visualise the protein bands, destained in a destaining solution for a few hours until a colourless background was obtained.

Results & Discussion (2):

6.1- Extraction and solubilization of GPCR from control ,diabetic and diabetic nephropathy patients .

Phosphate buffer saline is often used as a buffer in biochemistry to maintain the pH of protein , neutral or charges on certain amino acids which allow the structure of protein to be in its native form [154] .

Detergents break the lipid barrier surrounding cells by proteins solubilization and disrupting lipid-lipid , and protein-lipid interaction, self associate and bind to hydrophobic surfaces . A suitable detergent for solubilization an individual membrane of proteins were determined , for successfully purification , conjunction with homogenization and mechanical grinding [155] .

Many detergents have been used for solubilzation of different GPCRs but the one of chaise was , non –ionic detergent , dodecal-β-D-maltoside (DDM) was used in the extraction and solubilization of GPCR from different membrane tissues [156-160].

Table (6-1) shows protein concentration which was determined by Bradford test and GPCR concentration for extraction and solubilization steps of lysate . Results show an decrease in protein and GPCR concentration after extraction and solubilization steps , control which were (0.89) mg/ml and (6.61) ng/ml respectively in the crude lysate in control group and (0.718) mg/ml , (3.038) ng/ml after precipitate by $(NH_4)_2SO_4$ in (50 -75%) . In diabetic patients the concentration of protein and GPCR were (0.92) mg/ml and (6.19) ng/ml in the crude lysate respectively and (0.799) mg/ml , (3.052) ng/ml after precipitation by $(NH_4)_2SO_4$ in (50 -75%) . diabetic nephropathy which protein and

GPCR concentration were (0.94) mg/ml and (5.322) ng/ml respectively in the crude lysate and (0.801) mg/ml , (3.088) ng/ml after precipitation by $(NH_4)_2SO_4$ in (50 -75%) .

Table (6.1): protein and GPCR concentrations for extraction and solubilization and precipitation steps of lysate .

Control

Sample	Volume (ml)	Protein con.(mg/ml)	GPCR con.(ng/ml)
Crude	30	0.89	6.61
precipitate by $(NH_4)_2SO_4$ (50-75%)	15	0.718	5.34
Diabetic			
Sample	Volume (ml)	Protein con.(mg/ml)	GPCR con.(ng/ml)
Crude	30	0.92	6.19
Precipitate by $(NH_4)_2SO_4$ (50-75%)	15	0.799	6.052
Diabetic nephropathy			
Sample	Volume (ml)	Protein con.(mg/ml)	GPCR con.(ng/ml)
Crude	30	0.94	5.322
precipitate by $(NH_4)_2SO_4$ (50-75%)	14	0.801	4.95

6.2- purification of GPCR Extracted from cell membrane

Ion Exchange and gel filtration chromatography were applied , respectively in the purification of GPCR that extracted from the whole cell .

6.2.1 Ion Exchange chromatography :

The GPCR that extracted from blood was purified by ion exchange chromatography using DEAE-Sepharose (an anionic ion exchanger) which has multiple uses led to its high capacity for bio separation , easy preparation and simplicity to separate biomolecules [161-163] .

Five ml of crude of the extract was applied on the DEAE-sepharose column (2.5 x 8) cm . The column was equilibrate and washed with an equal volume of 50 mM of phosphate buffer saline contained DDM (pH=7.4) to wash uncharged and positive charged proteins in protein mixture of crude GPCR . The bound proteins (negatively charged) were then eluted using gradient concentrations of NaCl ranged (0.1- 0.5) M . Fraction were collected in 5 ml tube at a flow rate of 0.5 ml/min and eluted with gradient (0.1 – 0.5) of sodium chloride solution .

Results of control group showed two proteins peaks ,figure (6.1) that appeared after elution by the gradient concentration of sodium chloride , while no protein peaks appeared in the washing fractions . The GPCR concentration were measured in the fractions of these two protein peaks . Data indicated that GPCR located in the first protein peak (eluted at 0.1 M of NaCl) at fraction numbers between (6 and12) . The second peaks (eluted at 0.2 M of NaCl) gave a peak between (18-20), thus its neglected . Fractions represent GPCR were pooled and concentrated to (5ml) by sucrose .

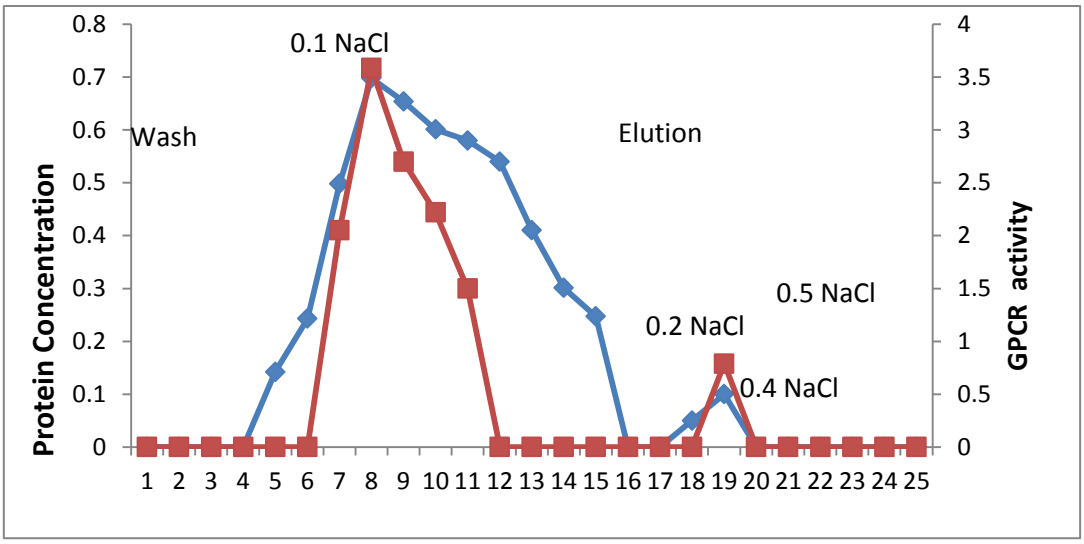
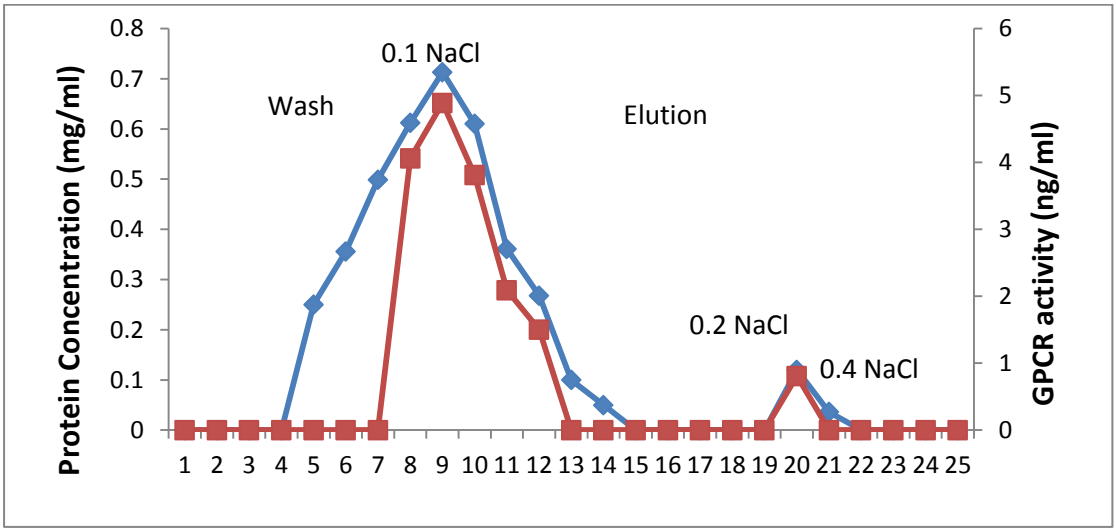


Figure (6.1) : Ion Exchange for Control group

Results for diabetic patient , figure (6.2), showed two proteins peaks that appeared after elution with the gradient concentration of sodium chloride , while no protein peaks appeared in the washing fractions . The GPCR concentration were measured in the fractions of these two protein peaks . Data indicated that GPCR located in the first protein peak (eluted at 0.1 M of NaCl) at fraction numbers between (7 and13) . The second peaks (eluted at 0.2 M of NaCl) give peak between (18-20) .



Figure(6.2) : Ion Exchange for Diabetic patient

Results for diabetic nephropathy patient in figure (6.3) showed two proteins peaks that appeared after elution by the gradient concentration of sodium chloride , while no protein peaks appeared in the washing fractions . The GPCR concentration were measured in the fractions of these two protein peaks . Data indicated that GPCR located in the first protein peak (eluted at 0.1 M of NaCl) in fraction numbers between (7 and13) . The second peaks (eluted at 0.2 M of NaCl) give peak between (19-21) **.**

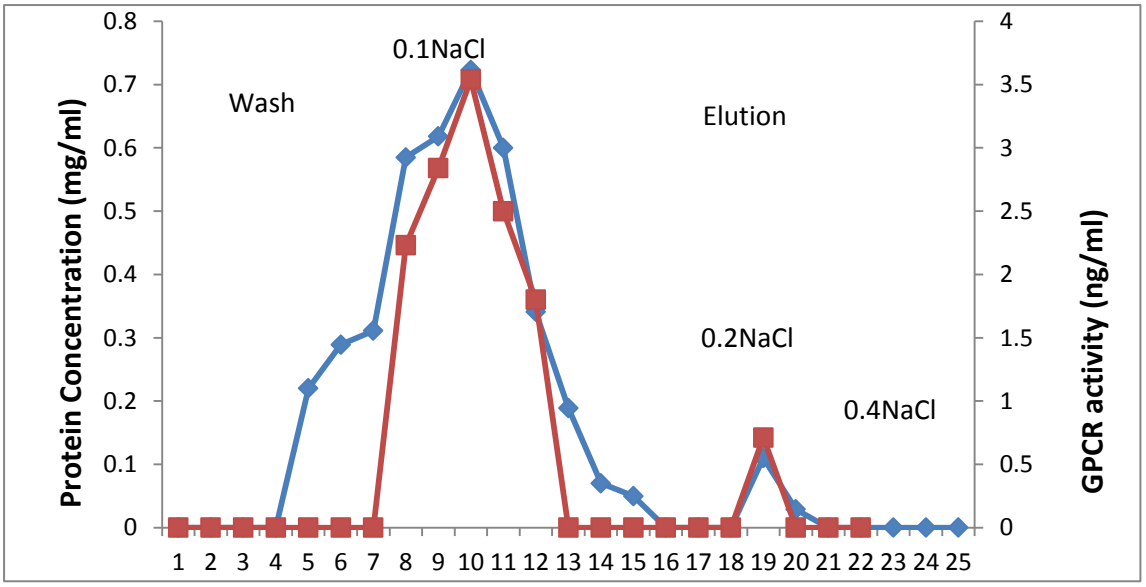


Figure (6.3) : Ion Exchange for Diabetic Nephropathy patient

6.2.2 – Gel filtration chromatography :

After purification by ion exchange chromatography fractions were collected pooled and concentrated to be applied in gel filtration chromatography by using sepharose 6B column . Aliquot of five mL of concentrated fraction was injected into column (65 x 1.5) cm which previously equilibrated with 50 Mm phosphate buffer saline (pH=7.4) , and eluted with elution buffer PBS pH =7.4 containing (0.5Mm) DDM . Flow rate was (1ml /min) with 5 ml for each fraction that monitored at 280 nm .

Results displayed in figures (6.4-6.5-6.6) showed a single active protein peak after eluted with elution buffer that was identical with the peak that presented GPCR concentrations at fractions number (14) for control patients and show of single active protein peak that was identical with the peak that presented GPCR concentrations at fractions number (12) for diabetic patients which show a single active protein peak that was identical with the peak that presented GPCR concentrations at fractions number (12) for diabetic nephropathy patients .

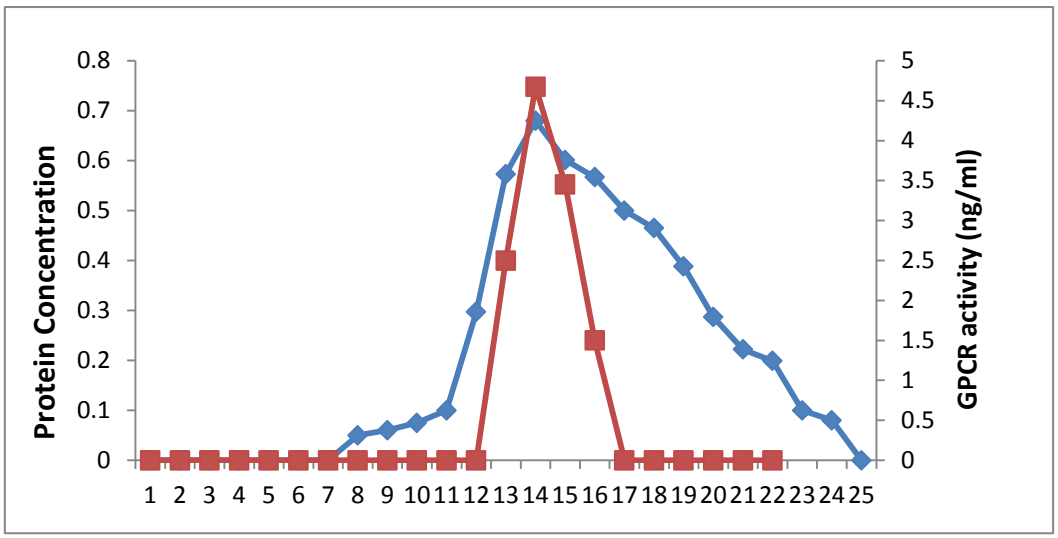


Figure (6.4) : Gel -filtration for Control patient

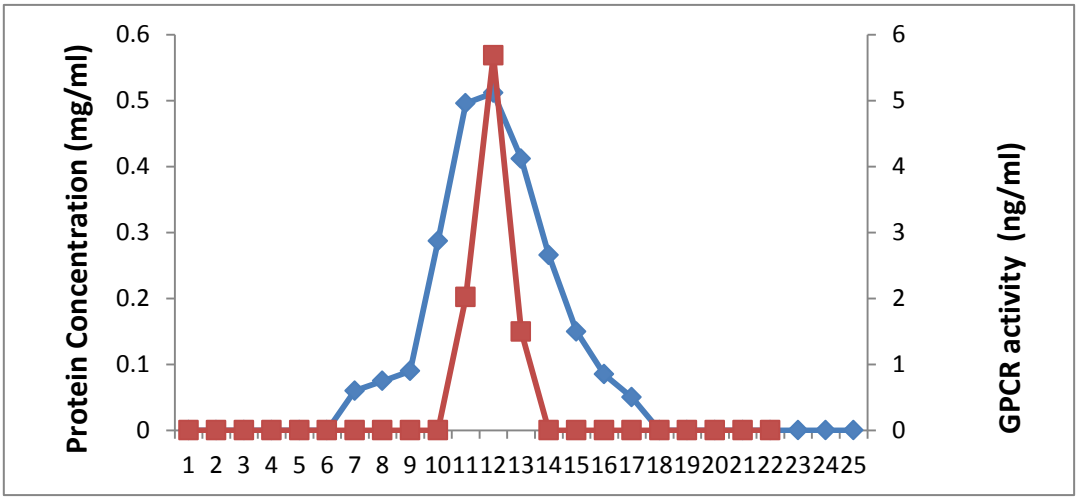


Figure (6.5): Gel -ffiltration for Diabetic patient

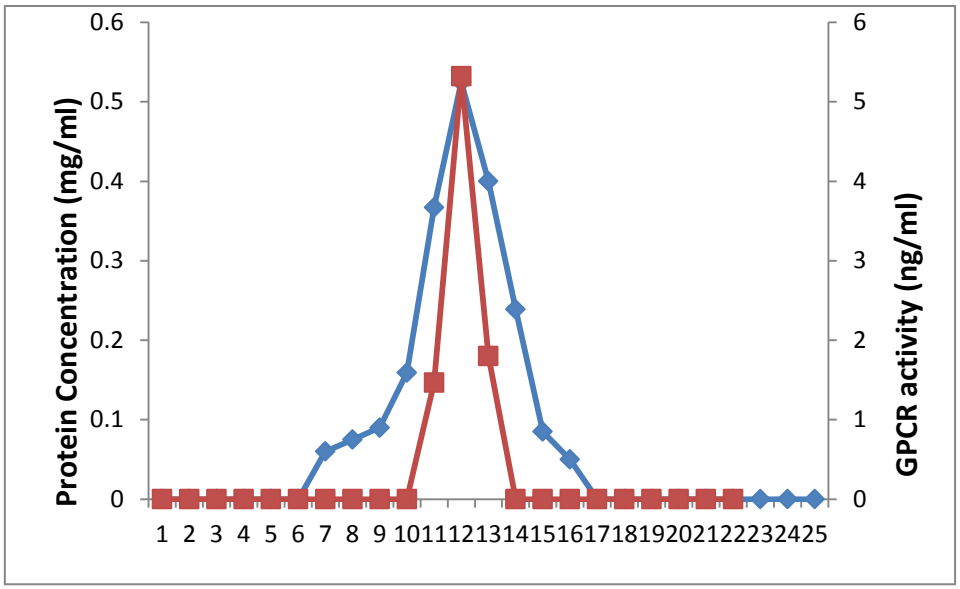


Figure (6.6) : Gel -ffiltration for Diabetic Nephropathy patient

Volume , protein concentration , GPCR concentration , specific activity , purification fold for all purification steps of GPCR from control , diabetic and diabetic nephropathy are shown in table (6.2).

Table (6.2) : Volume , protein concentration , GPCR concentration , specific activity , purification fold for all purification steps of GPCR from control , diabetic and diabetic nephropathy

Control

Steps	Volume (ml)	Protein con.(mg/ml)	GPCR con.(ng/ml)	Specific activity (ng/mg)	Purification fold
Crude	30	0.89	6.61	7.42	1.0
Ion exchange	12	0.718	5.32	7.49	1.1
Gel filtration	8	0.32	3.26	10.18	1.37
Diabetic					
Crude	30	0.92	6.19	6.72	1.0
Ion exchange	10	0.713	5.688	7.9	1.17
Gel filtration	8	0.512	4.89	9.55	1.42
Diabetic nephropathy					
Crude GPCR	30	0.94	5.322	5.66	1.0
Ion exchange	9	0.723	4.49	6.21	1.1
Gel filtration	7	0.525	3.52	6.7	1.18

6.3- Determination of purity and molecular weight of GPCR by SDS-PAGE:

The molecular weight and purity of GPCR that purified from the G1,G2 and G3 were determined by sodium dodecyl sulfate –polyacrylamide gel electrophoresis (SDS-PAGE) ,as shown in figure (6.7) **.**

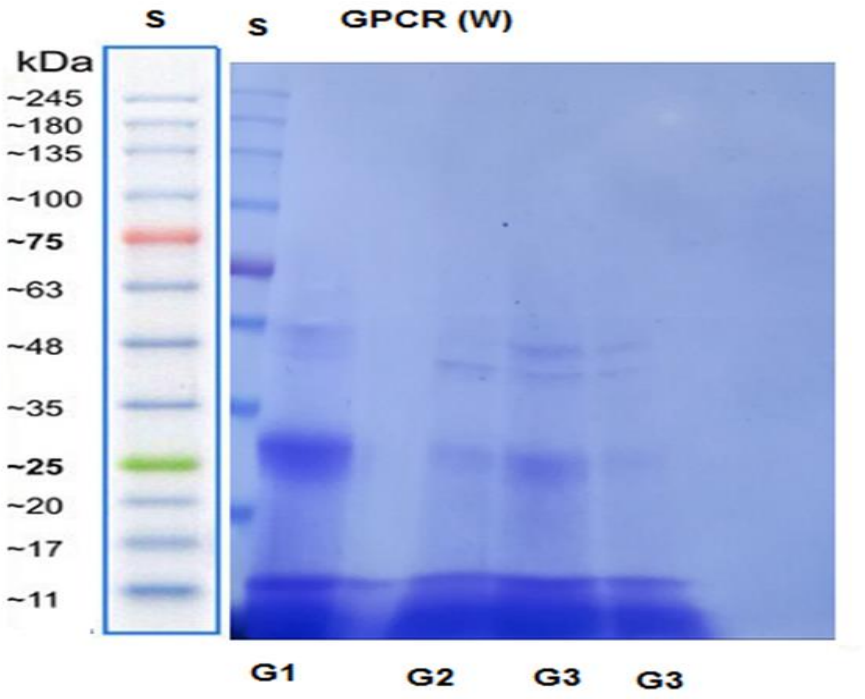


Figure (6.7) SDS-PAGE of purified GPCR G1,G2 and G3

Results indicated the appearance of two bands of GPCR in the control subject at approximady (~30 KD) and at (~51 KD) . This could be due to the isoform of GPCR that papered in these groups . As it was reported that the monomeric form is important to activate G-protein for binding , and they stated that further studies are required to understand the function of the dimerization of such receptor for its biological function [(164,165)].

Results ,also , showed the appearance of three bands in G2 that related to GPCR in (~ 29 KD) and (~ 47,49 KD) . While, it is not very clear related to its little concentrations . In diabetic nephropathy G3 bands ,also , appeared similar to the bands appeared in G2 but with highly clearance related to its highly concentration as show in table (6-2) . This three bands related to the type of GPCR and its isoform in this disease .

Study revealed that expression does not always lead to successful functional membrane purification which do not retain their activity of post – purification [166] . Engineering a robust microbial host for GPCR expression , investigated the expression of 12 GPCRs in the diabetic disease . All receptors were expressed at the mg/L scale , however only the human adenosine A2a receptor with M.wt of (47.05 KD) is active for ligand – binding and located primarily at the plasma membrane , whereas other tested GPCRs mainly retained within the cell which suggests that a pool of receptors may be folded incorrectly [166] .

superimposed NMR-derived structure to the homologous regions in the crystal structure of the β_2 receptor and , revealed a sensible conformation and orientation for the TM2 and TM3 mimetics . [167]

Previous study for 5-hydroxytryptamine$_{1B}$ revealed bands with apparent molecular weights of ~43 and ~86 kDa, corresponding to 5-HT_{1B} receptor monomers and dimers . In cells expressing the 5-HT_{1D} receptor, bands with apparent molecular weights of ~38 and ~76 kDa representing 5-HT_{1D} receptor monomers and dimers were immunodetected . 5-HT_{1B} and 5-HT_{1D} receptor monomers are predicted by sequence analysis to be ~43 and ~38 kDa, respectively . [168]

GPR40 is a membrane-bound receptor paired with medium and long-chain fatty acids (FFA) as endogenous ligands. Its acute activation potentiates insulin secretion from beta cells, whereas prolonged binding might contribute to the deleterious effects of chronic exposure to FFA. Little information is available on the expression of GPR40 and its regulation in human islets (HI)[169] .

G-protein coupled receptor 40 (GPR40) has drawn a considerable attention as a potential therapeutic target for type 2 diabetes. As GPR40

agonist may offer advantages to commonly used agents, by acting ambient glucose dependent manner which mechanistically leads to reduced risk of developing hypoglycemia [169] .

The glucagon – like peptide -1 receptor (GLP1R)is a class B GPCR with M.wt 53 KD, that found in type two diabetes in previous study [170] .

Study found that a short form of dopamine receptor D2 (DRD2S) is efficiently transported to the primary cilia, while a long form of dopamine receptor D2 (DRD2L) is rarely transported to the primary cilia. Using an anti-Prlhr antibody, we found that Prlhr localized to the cilia on the surface of the third ventricle in the vicinity of the hypothalamic periventricular nucleus. Predicted molecular seizes of GPCRs are as follows: DRD2S, 47.3kDa; DRD2L, 50.6kDa; NPFFR1, 47.8kDa NMUR1, 47.4kDa; NPFFR2, 47.4kDa; NMUR2, 47.8kDa; PRLHR, 41.1kDa. [171]

G-protein-coupled receptors (GPCRs) modulate cytoplasmic signalling in response to extracellular stimuli, and are important therapeutic targets in a wide range of diseases. Structure determination of GPCRs in all activation states is important to elucidate the precise mechanism of signal transduction and to facilitate optimal drug design. However, due to their inherent instability, crystallisation of GPCRs in complex with cytoplasmic signalling proteins, such as heterotrimeric G proteins and β-arrestins, has proved challenging. Study engineered mini-Gs, using rational design mutagenesis, to form a stable complex with detergent-solubilised β1-adrenergic receptor (β1AR). Mini G proteins induce similar pharmacological and structural changes in GPCRs as heterotrimeric G proteins, but eliminate many of the problems associated with crystallisation of these complexes, specifically their large size,

conformational dynamics and instability in detergent. They are therefore novel tools, which will facilitate the biochemical and structural characterisation of GPCRs in their active conformation. [172]

According to the previous researches that improved molecular weight of GPCRs about ~30 and ~50 KD , such as GLP-1R (~53 KD), GPCR 40 (31,438), α_2- Adregeric receptor (~50 KD) ,β_2- Adregeric receptor (47.058KD) ,so , in this study one or more of the above receptors were purified in diabetic and diabetic nephropathy . further study is important to know amino acid sequences in the purified receptors to know the type of GPCR that purified in these patients exactly .

Conclusion

1- Results showed the appearance of two bands of GPCR in the control subject at approximady (~ 30 KD) and at (~51 KD) .

2-Results ,also , showed appearance of three bands related to GPCR in (~ 29 KD) and (~ 47,49 KD) . While it is not very clear related to its little concentrations . In diabetic nephropathy bands ,also , appeared similar to the bands appeared in G2 but with highly clearance related to its highly concentration .

3-The conclusion could be drawn from this study that 2 bands of GPCR appear in control group while there are three bands appeared in G2 and G3 with different concentrations which may be useful in early detection and active treatment to controlling diabetic and diabetic nephropathy patients .

As far as to our knowledge this is the first study reported the purification of GPCR from Iraqi control subjects , diabetic patients and diabetic nephropathy patients by using DEAE-sepharose in ion exchange chromatography and sepharose 6B in gel filtration chromatography . In order to compare the levels and types of GPCR that appear in these groups which may be useful in diagnosis the defect in GPCR type to predict treatment for these patient's to improve disease in these patient's and to reduce the development of the disease .

Recommendation :

Study recommended to :

1- Study make on large scale of GPCR .

2- Number of patients increased to increase GPCR concentration in diabetic and diabetic nephropathy patients .

3- Amino acid analyzer must be doing to the purified GPCR from all groups to improve the type of GPCR that appear in this disease .

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الخلاصة

هدفت الدراسة إلى تحديد مستويات الجلوكاجون مثل الببتيد -1 و المراسل المزدوج للبروتين ج في مرضى السكري ومرضى الاعتلال السكري الكلوي ومقارنة النتائج مع مجموعة السيطرة. بالإضافة إلى دراسة العلاقة بين الجلوكاجون مثل الببتيد -1 و المراسل المزدوج للبروتين ج مع نسبة السكر التراكمي و السكر و اليوريا والكرياتينين والدهون ، والتي قد تكون مفيدة في مكافحة داء السكري ومضاعفاته .

تم اخذ مائة وخمسون عينة في هذه الدراسة حيث تراوحت الأعمار (40-65) سنة والتي تم تقسيمها إلى ثلاث مجموعات على النحو التالي: المجموعة الأولى (G1) تتكون من 50 فردًا أصحاء كمجموعة تحكم ، المجموعة الثانية (G2) تتكون من 50 مريضًا مع مرضى السكري ومجموعة ثالثة (G3) يتكون من 50 مريضا يعانون من اعتلال الكلية السكري.

أظهرت النتائج ارتفاعًا ملحوظًا في السكر ونسبه السكر التراكمي و اليوريا و الكرياتينين في G2 و G3 مقارنة بـ G1 و G3 مقارنة بـ G2. تظهر النتائج انخفاضا غير ملحوظ في مستويات الألبومين في G2 مقارنة مع G1 ، ومع ذلك ، هناك انخفاض كبير في G3 مقارنة G2 و G1.

أظهرت النتائج ارتفاعًا ملحوظًا في مستويات (TC، TG، LDL-c، VLDL-c) في G2 و G3 مقارنة بـ G1. في حين وجد انخفاض ملحوظ في مستويات HDL في G2 و G3 مقارنة بـ G1.، ارتفاع ملحوظ في (TC، TG، LDL-c، VLDL-c) في G3 مقارنة G2 ، في حين أن هناك انخفاض غير ملحوظ في HDL في G3 مقارنة G2.

أظهرت النتائج ارتفاعًا ملحوظًا في مستويات الأنسولين في G2 و G3 مقارنةً بـ G1. كما لوحظت زيادة ملحوظة في G3 مقارنةً بـ G2.

لوحظ ارتفاع ملحوظ في مستويات GLP-1 و GPCR في G2 و G3 مقارنة مع G1. أيضا لوحظت فروق ذات دلالة إحصائية في G3 مقارنة G2

أظهرت النتائج وجود علاقة سلبية غير معنوية بين GLP-1 واليوريا لـ G1 (r1 = -0.146)، ($p<0.05$) ، بينما توجد علاقة ارتباط ذات دلالة في G2 (r2 = 0.435)، ($P< 0.05$). شوهد ارتباط سلبي كبير في G3 (R3 = -0.0729)،($P <0.05$). وأظهرت الدراسة أيضا وجود علاقة غير ذات دلالة بين GLP-1 و كرياتنين لـ G1 (r1 = 0.453)،($P >0.05$). في الوقت

نفسه توجد علاقة ارتباط موجبة معنوية في (r2 = 0.210) G2، (P <0.05). ولوحظ ارتباط سلبي كبير لـ (R3 = -0.439) G3، (P <0.05). علاقة الارتباط بين GLP-1 و السكر. أوضحت النتائج وجود علاقة سلبية غير محددة في (r1 = -0.120) G1، (P <0.05). في حين تم العثور على ارتباط إيجابي كبير في G2 و (R2 = 0.523) G3، (R3 = 0.323)، (P <0.05) . كشفت الدراسة عن وجود علاقة إيجابية غير واضحة بين GLP-1 والأنسولين في (r1 = 0.070) G1، (P>0.05). في الوقت نفسه هناك ارتباط سلبي كبير في G2 وG3 (r2 = -0.113)، (r3 = -0.309، P<0.05).

كشفت النتائج أيضًا عن وجود علاقة موجبة غير معنوية بين GPCR و HbA1c في G1 (r1=0.172) بينما وجد ارتباط إيجابي موجب في (r2 = 0.427) G2 وارتباط سلبي كبير في (r3 = -0.042) G3. أظهرت النتائج أيضًا وجود ارتباط إيجابي غير معنوي في G1 بين GPCR والكولسترول (r1 = 0.306، P>0.05). في حين أن هناك علاقة إيجابية كبيرة فيG2 (R2 = 0.514، P>0.05). وعلاقة سلبية كبيرة بين GPCR والكوليسترول في G3 (r3=-0.288، P>0.05). علاقة سلبية غير معنوية بين GPCR و TG في G1 (r1 = -0.0009، P>0.05). في حين أن هناك علاقة سلبية كبيرة في G2 تم العثور عليها (r2= -0.292، P>0.05). أيضا ، كشفت النتائج وجود علاقة إيجابية كبيرة بين GPCR و الدهون الثلاثية في G3 (R3 = 0.276)، P>0.05).

الجزاء الثاني من البحث تضمن:

هدفت الدراسة إلى تنقية مستقبل البروتين G من الدم لمرضى اعتلال الكلية السكري ومرضى السكري عن طريق تقنيات الكروماتوغرافيا المختلفة ، والتي تعتبر أبسط نموذج للتنقية ودراسة بنية ووظيفة مستقبل البروتين في خلية حقيقية النواة . تم استخلاص مستقبل Gالبروتين من خلية كاملة وغشاء بواسطة ترسيب البروتينات مع 50 - 75٪ من كبريتات الأمونيوم ، وتم تبريده بالطرد المركزي عند 3500 جم لمدة 10 دقائق. وأخيرا ، تم حل الكرية في الحد الأدنى من حجم (1.5 ملم) محلول منظم PBS (Ph 7.3) . تم إذابة خلايا المترسبه وعلق في عازلة استخراج ثم تذوب في عازلة الفوسفات التي تحتوي على DDM كمنظفات. تم تنقيته المستقبلة G البروتين المتلقية من خلية كاملة بواسطة كروماتوجراف التبادل الأيوني كخطوة أولى باستخدام -DEAE Sepharose. تطبيق كروماتوجرافيا الجل الترشيح كخطوة ثانية للتنقية.

تم تحديد الوزن الجزيئي لـ مستقبل Gالبروتين بواسطة SDS.PAGE.

أظهرت نتائج التحليل الكروماتوجرافي التبادلي الأيوني لمريض السيطرة ذروتين بروتينتين ظهرتا بعد الشطف بواسطة تركيز متدرج من كلوريد الصوديوم ، في حين لم تظهر أي قمم للبروتين في أجزاء الغسل. تم قياس تركيز GPCR في كسور هذين القمتين البروتين. أشارت البيانات إلى أن GPCR تقع في أول ذروة البروتين (المصفوفة في 0.1 M من كلوريد الصوديوم) بأرقام الكسور بين (6 و 12). القمم الثانية (المصفوفة في 0.2 M من NaCl) تعطي الذروة بين (18-20).

أظهرت النتائج لمريض المصاب بالسكري اثنين من قمم البروتينات التي ظهرت بعد شطف تركيز متدرج من كلوريد الصوديوم ، في حين لم تظهر أي قمم للبروتين في أجزاء الغسيل. تم قياس تركيز GPCR في كسور هذين القمتين البروتين. أشارت البيانات إلى أن GPCR تقع في أول قمة بروتين (تمت التصفية في 0.1 M من NaCl) بأرقام الكسور بين (7 و 13). القمم الثانية (المصفوفة في 0.2 M من NaCl) تعطي الذروة بين (18-20).

أظهرت النتائج لمريض اعتلال الكلية السكري اثنين من قمم البروتينات التي ظهرت بعد شطف بواسطة تركيز التدرج من كلوريد الصوديوم ، في حين لم تظهر قمم البروتين في الكسور الغسيل. تم قياس تركيز GPCR في كسور هذين القمتين البروتين. أشارت البيانات إلى أن GPCR تقع في أول ذروة البروتين (المصفوفة في 0.1 M من كلوريد الصوديوم) في أعداد الكسور بين (7 و 13). القمم الثانية (المصفوفة في 0.2 M من NaCl) تعطي الذروة بين (19-21).

كان الوزن الجزيئي لـ GPCR الذي يقدره الترحيل الكهربائي SDS-PAGE هو ما يقرب من شريطين من GPCR يظهران في عينه الاصحاء (~ 30 د.ك) و عند (~51 د.ك). GPCR. كما ذكر أن الشكل الأحادي مهم لتنشيط ج بروتين للارتباط

كما أظهرت النتائج ظهور ثلاثة نطاقات متعلقة بـ GPCR في (~ 29 د.ك) و (47،49 د.ك). في حين أنه ليس واضحا جدا لتركيزاته الصغيرة. في مجموعه اعتلال الكلية السكري أيضا بدا مشابها للأشرطةالتي ظهرت في G2.

الإهداء

الى كل من احب العراق ....

ارضاً وانساناً

الى الطيبينِ الخيرينِ اللذينِ انجبتهما ارض الرافدين ...

أمي وأبي

الى الذين طاب بهم الثرى....

شهداء وطني

الى الامير الحر الكريم ذخرنا في حياتنا وخلودها...

اخي الشهيد (شاكر)

الى سندي ورفيقة دربي ....

زوجتي العزيزه

الى النور الذي ينير دربي ...

اولادي

الى من زرع الامل والابتسامه في حياتي....

اخوتي وخواتي

الى الشموع التي أضاءت لي طريق العلم ... اساتذتي الافاضل

أهدي جهدي المتواضع

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بغداد
كلية التربية للعلوم الصرفة
(إبن الهيثم)/قسم الكيمياء

دراسات كيموحيوية وتنقية جزئية للبروتين المراسل ج المزدوج من مرضى عراقين مصابين بمرض السكري من النوع الثاني و الاعتلال السكري الكلوي

أطروحة مقدمة إلى
كلية التربية للعلوم الصرفة (إبن الهيثم)/ جامعة بغداد
كجزء من متطلبات نيل درجة دكتوراه فلسفة في الكيمياء الحياتية

من قبل
سليمان محمود حسن

بكالوريوس علوم في الكيمياء (2012) / كلية التربية للعلوم الصرفة (إبن الهيثم)/ جامعة بغداد.
ماجستير علوم في الكيمياء العضوية (2015) / كلية التربية للعلوم الصرفة (إبن الهيثم)/ جامعة بغداد.

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