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Biochemical and Immunohistochemical Study of Sildenafil on Various Tissues of Male Rats

A thesis
Submitted to the Department of Pharmacology and Toxicology and the Committee of Graduate Studies of the College of Pharmacy/ the University of Mustansiriyah in Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacy (Pharmacology and Toxicology)

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We, the examining committee after reading this thesis, (**Biochemical and Immunohistochemical Study of Sildenafil on Various Tissues of Male Rats)**, in its contents, find it adequate as a partial fulfillment of the requirements for the degree of master of science in Pharmacy (Pharmacology and Toxicology).

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا
عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ)

صَدَقَ اللَّهُ العَلِيُّ العَظِيمُ

سورة البقره / ألآية 32

Dedication

The words at my command are inadequate both in form and spirit to express my deep felt appreciation and sincerity towards my family members for their forbearance of my long absence which come in the wake of long hours of working on research. They patiently provided all the support when I needed for renewal and encouragement.

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With full respect and love

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Ibrahim H. Nashmee Al-malki

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List of Abbreviations

ACs	Adenylyl cyclases.
ADP	Adenosine diphosphate
Akt	Serine/threonine-specific protein kinase.
p-Akt	Phospho-Serine/threonine-specific protein kinase.
AMPK	Adenosine monophosphate -activated protein kinase
ANP	Atrial natriuretic peptide.
AP-1	Activator Protein-1.
AR	Amphiregulin.
ATP	Adenosine triphosphate
BAY 60-7550	2-[(3,4-dimethoxyphenyl)methyl]-7-[(2R,3R)-2-hydroxy-6 phenylhexan-3-yl]-5-methyl-1H-imidazo[5,1f][1,2,4]triazin-4 one.
BAY 73-6691	1-(2-chlorophenyl)-6-[(2*R*)-3, 3, 3-trifluoro-2-methylpropyl]-1, 5-dihydro-4*H*-pyrazolo [3, 4-*d*] pyrimidine-4-one.
BH_4	Tetrahydrobiopterin
BK channels	Ca^{2+}-activated K^+ channels
BRL 50481	3-(N,N-dimethylsulfonamido)-4-methyl-nitrobenzene
BTC	Betacellulin
CaM	Calmodulin
CAMs	Cell adhesion molecules
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin

cDNA	Complementary DNA
C domain	C-terminal catalytic domain
c-Fos	Proto-oncogene
cGMP	Cyclic guanosine monophosphate
CNG	Cyclic nucleotide gated
cNT	Cyclic nucleotide
CN -V	Fifth cranial nerve
CREB	Cyclic Adenosine monophosphate -response element binding protein
Cripto-1	Teratocarcinoma-derived growth factor
CTGF	Connective tissue growth factor
DPX	Digital picture exchange
EBV	Epstein–Barr virus
ED	Erectile dysfunction
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHNA	Erythro-9-(2-**h**ydroxy-3-**n**onyl)**a**denine
ELK-1	E26 transformation-specific domain-containing protein
eNOS	Endothelial nitric oxide synthase
Epac	Cyclic adenosine monophosphate - responsive rap1 guanine nucleotide exchange factor
EPR	Epiregulin
ER	Endoplasmic reticulum
ErbB	Receptor tyrosine kinase family
ERK	Extracellular signal-regulated kinase
ETS	E-twenty six (family transcription factors)
FBG	Fasting blood glucose
Fig.	Figure
GAB-1	Growth factor receptor-bound protein 2 -associated-binding protein-1
GC	Guanylyl cyclase
GCT	Granular convoluted tubule
GEF	Guanine nucleotide exchange factor
GFR	Glomerular filtration rate
GP	Glycogen phosphorylase

GPCR	guanine nucleotide-binding protein coupled receptor
GS	Glycogen synthase
GTP	Guanosine triphosphate
GTPCH-I	Guanosine triphosphate cyclohydrolase I
5'-GMP	Guanosine 5'-monophosphate
G6P	Glucose-6-phosphate
HB-EGF	Heparin-binding epidermal growth factor
HCMV	Human cytomegalovirus
HCN	Hyperpolarization-activated cyclic nucleotide gated
HER	Human epidermal growth factor receptor
H&E	Hematoxylin and eosin stain
ICAM-1	Intercellular Adhesion Molecule 1
IHC	Immunohistochemistry
IHR	Intrahepatic resistance
IL-1β	Interleukin 1-beta
IL-18	Interleukin-18
Ins (1,4,5) P3	Inositol 1, 4, 5-trisphosphate
iNOS	Inducible nitric oxide synthase
I/R	Ischemic / reperfusion
IRS	Insulin receptor substrate
[Na+]i	Intracellular sodium
IP3R	Inositol trisphosphate receptor
JAK	Janus kinase
JNK	c-Jun NH2-terminal kinase
K_{ATP} channels	ATP - sensitive K^+ channels
kDa	Kilodalton
K_{ir} channels	Inward rectifier K^+ channels
K_v channels	Voltage-gated K^+ channels
LGC	Liver glycogen content
LMP1	Latent membrane protein 1
LPS	Lipopoly-saccharide
LTCC	L-type Ca^{2+} currents channel
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemoattractant protein-1
mitoKATP	Mitochondrial ATP-dependent K+ channel
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MLC20	Myosin light chain 20
µm	Micrometer
MPO	Myeloperoxidase
MPT	Mitochondrial permeability transition
MRP	Multidrug resistance proteins
MSNA	Muscle sympathetic nerve activity
Na+	Sodium
ANP	Atrial natriuretic peptide
NPRA	Natriuretic peptide receptor type A
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NCX	Na^{+}/Ca^{2+} exchanger
NHCT	$Na^{+}/HCO3^{-}$ cotransporter
NHE	Na^{+}/H^{+} exchanger
Neu	Neuregulins
NF-kB	Nuclear factor kappa B
NO	Nitric oxide
NO-cGMP	Nitric oxide- Cyclic guanosine monophosphate
NRG1-4	Neuregulins 1–4
NTP	Nucleotide triphosphate precursor
$O_2^{\bullet-}$	Superoxide anion
$ONOO^{\bullet-}$	Peroxynitrite
PAS	Periodic acid-schiff stain
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PKB	Protein kinase B
PKG	Protein kinases G
PLC	Phospholipase C

PLC yl	Phospholipase C-yl
PMCA	Plasma Membrane Ca^{2+}-ATPase
p38 MAPK	P38 mitogen-activated protein kinases
PP-1	Phosphoprotein phosphatase-1
PVP	Portal vein pressure
PYG	Phosphorylase glycogen gene
PYGB	Phosphorylase glycogen brain
PYGL	Phosphorylase glycogen liver
PYGM	Phosphorylase glycogen muscle
Raf-1	Proto-oncogene, serine / threonine kinase
R domain	N-terminal regulatory domain
RBF	Renal blood flow
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated coiled kinase
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
sGC	Soluble guanylyl cyclase
SMC	Smooth muscle cells
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
TAC	Total antioxidant capacity
S.c.	Subcutaneous
STAT	Signal transducers and activators of transcription family of transcription factors.
TGF	Transforming growth factor
TGF-*β*	Transforming growth factor - beta
TNFα	Tumor necrosis factor alpha
UDP	Uridine diphosphate
UTP	Uridine-5'-triphosphate
V-ATPase	Vacuolar H^+-ATPase
VASP	Vasoactivator stimulated phosphoprotein

Abstract

Background: Sildenafil a selective phosphodiesterase-5 (PDE5) inhibitor, elevates cyclic nucleotide intracellular second messengers which contribute in several intracellular signaling pathways involved in physiological and pathological situation. Epidermal growth factor is produced in rodents mainly by the submandibular glands and kidney, it is important in β-cell development. Glycogen phosphorylase is the key enzyme of glycogen breakdown that catalyzes the degradative phosphorolysis of glycogen. In the liver, glycogen phosphorylase has important role in blood glucose homeostasis. Interleukin 1-beta is a pro-inflammatory cytokine that plays important roles in inflammation. Interleukin 1-beta signal influence insulin action and insulin secretion.

Objective: To investigate the histological and immunochemical effects of sildenafil citrate on kidney, liver, pancreas and submandibular gland of adult male rats.

Materials and Methods: Forty eight adult male rats were divided into four equal groups. Group D: as control. Group A, B and C: rats were treated with (0.5, 1, and 2) mg/kg oral sildenafil citrate respectively (once daily by gastric tube). Animals were euthanized after 6 weeks of treatment. Kidney, liver, pancreas and submandibular glands were processed for histological and immunohistochemical examination. Blood was collected for serum examination of Interleukin 1-beta (IL-1β), total antioxidant capacity (TAC) and C-peptide. Body weight and blood glucose recorded before treatment and at last day of experiment.

Results: There was a high significant decrease in body weight after the oral dose of sildenafil (0.5, 1, 2 mg/kg) when compared with initial body weight for each animal in all groups. High significant elevation ($p<0.01$) was observed with liver

glycogen content in group A, B, C when compared to group D. Interestingly, sildenafil was reduced hepatic glycogen phosphorylase significantly in group B and high significantly in group C when compared with group D. After sildenafil treatment, immunohistochemical detection for epidermal growth factor (EGF) revealed increased number of immunopositive cells and strong immunoreactivity for epidermal growth factor (EGF) of kidney and submandibular compared with the control group. Strong immunoreactivity for the epidermal growth factor (EGF) expressed on its receptor was detected in the β-cell. Sildenafil markedly increased serum total antioxidant capacity (TAC) and reduced serum IL-1β. Serum C-peptide levels showed a high significant elevation ($p< 0.01$) in group C when compared with control and significant elevation when compared with other treatment group. In addition, blood glucose level was significantly reduced ($p<0.05$) in group A and high significant reduction was observed in group B and C when compared with initial glucose level related to each group.

Conclusion: Sildenafil has a potential role in elevating the epidermal growth factor (EGF) production which improve the synthesis of insulin in β-cells which subsequently lowers the blood glucose concentration and reduces hepatic glycogenolysis.

Chapter One

Introduction

1.1 Sildenafil:

Sildenafil citrate is an orally active selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific PDE5 (phosphodiesterase type 5) which is the predominant PDE isoenzyme in human corpora cavernosa. The empirical formula for sildenafil citrate is C28H38N6O11S with a molecular weight of 666.7 g/mol, white to off-white crystalline powder with a solubility of 3.5 mg/mL in water [(1)]. Sildenafil citrate has the following structural formula (fig. 1-1).

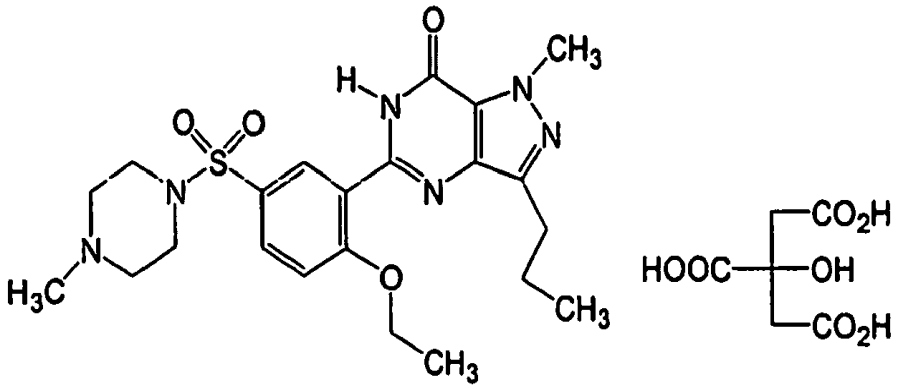

Figure (1-1) [(1)]: The structural formula of sildenafil citrate.

Sildenafil citrate, a vasoactive agent was approved as a drug for treating male erectile dysfunction (ED) by the US Food and Drug Administration on 27 March 1998 [(2)]. It restores impaired erectile function by increasing blood flow to the penis, resulting in a natural response to sexual stimulation. The erection physiological mechanism of the penis involves the discharge of nitric oxide (NO) in the corpus cavernosum through sexual stimulation. The activation of the guanylate cyclase by nitric oxide, which results in increased levels of cGMP, producing smooth muscle relaxation in the corpus cavernosum and allowing inflow of blood [(3)]. Sildenafil binds to the catalytic site of PDE5 and inhibits the degradation of intracellular cGMP in smooth muscle within the corpus cavernosum, resulting in increased levels of cGMP that causes smooth muscle cell relaxation, vasodilation, and improves

erectile function [4]. Cyclic GMP Intracellular receptors include cyclic nucleotide-gated channels, cGMP-dependent protein kinases (PKG), and cGMP-binding PDEs; cGMP may also cross-activate cAMP pathways by binding to cAMP-binding sites on cyclic adenosine monophosphate (cAMP) receptors such as cAMP-protein kinases A [5]. Cyclic GMP levels of tissue are determined by a balance between the activities of the guanylyl cyclases that responsible of formation of cGMP from guanosine triphosphate (GTP) and the cyclic nucleotide PDEs that catalyze the breakdown of cGMP (fig. 1-2). The mixture of a stimulator of guanylyl cyclase and a cGMP PDE inhibitor (such as sildenafil) produces synergistic enhancement of tissue cGMP levels [6].

1.1.1 Mechanisms of cyclic nucleotide-induced relaxation:

Cyclic adenosine monophosphate and cyclic guanosine monophosphate are main messengers that mediate relaxation under physiological conditions. Three distinct mechanisms are thought to mediate the dilator effect of cyclic nucleotides and their dependent protein kinases [7]:

(1) The decrease in the cytosolic Ca^{2+} concentration (fig. 1-3).

(2) The hyperpolarization of the smooth muscle cells **(**SMC) membrane potential (fig. 1-4).

(3) The reduction in the sensitivity of the contractile machinery by uncoupling contraction from myosin light chain (MLC) phosphorylation (fig. 1-5).

1- Cytosolic Ca^{2+} concentration modulation in the smooth muscle cells (SMC) [7]: a- Decreased Ca^{2+} release from the endoplasmic reticulum (ER). **b-** Increased Ca^{2+}-sequestration into the ER. **c-** Decreased influx of extracellular Ca^{2+}. **d-** Increased efflux of intracellular Ca^{2+} through the plasma membrane Ca^{2+}-ATPase (PMCA). **e-** Increased efflux of intracellular calcium through stimulation of the Na^{+}/Ca^{2+} -exchanger (NCX).

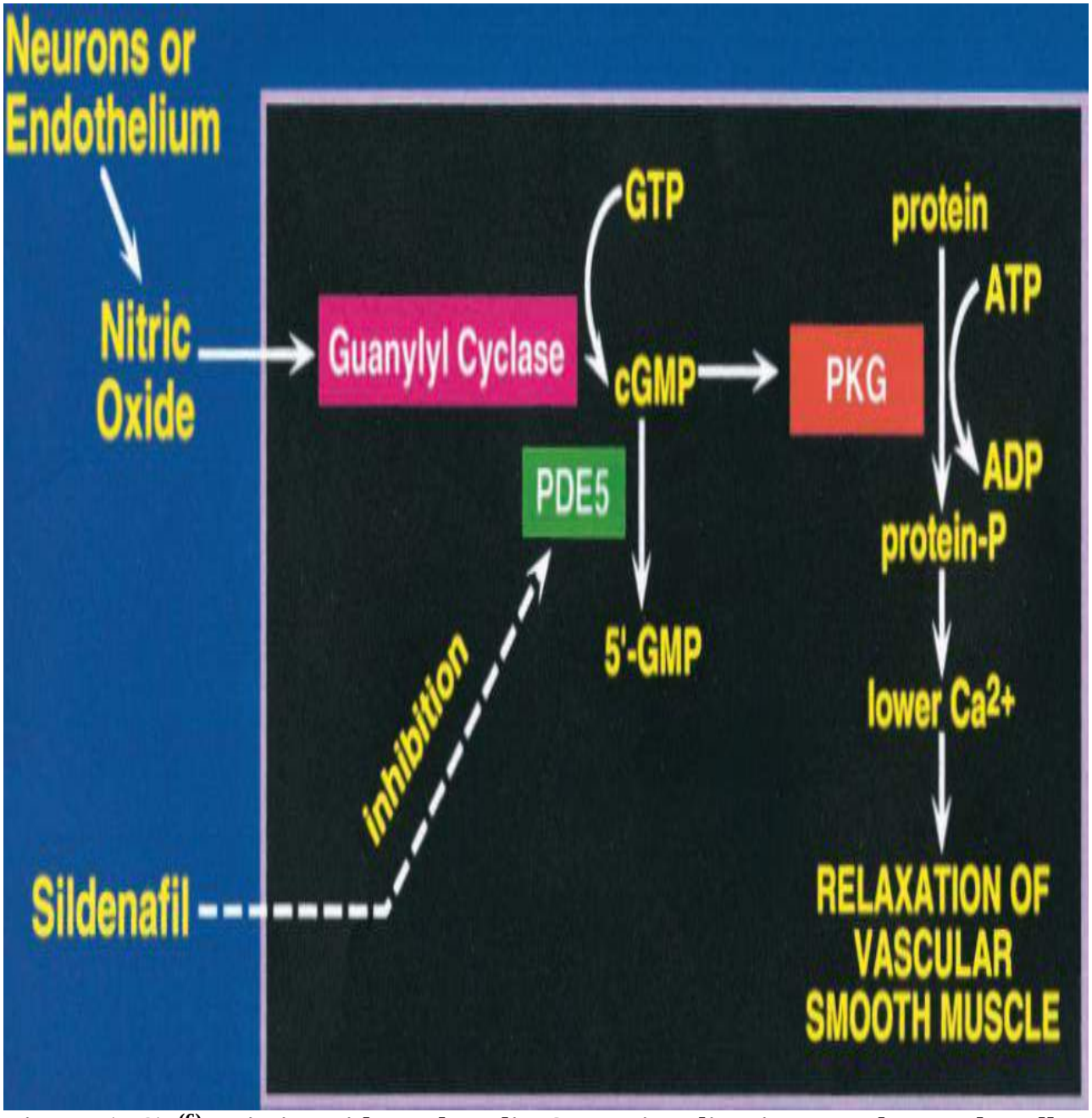

Figure (1-2) [6]: Nitric oxide and cyclic GMP signaling in smooth muscle cells. **cGMP:** Cyclic guanosine monophosphate**; ATP:** Adenosine triphosphate**; ADP:** Adenosine diphosphate**; GTP:** Guanosine triphosphate**; 5'-GMP:** Guanosine 5'-monophosphate.

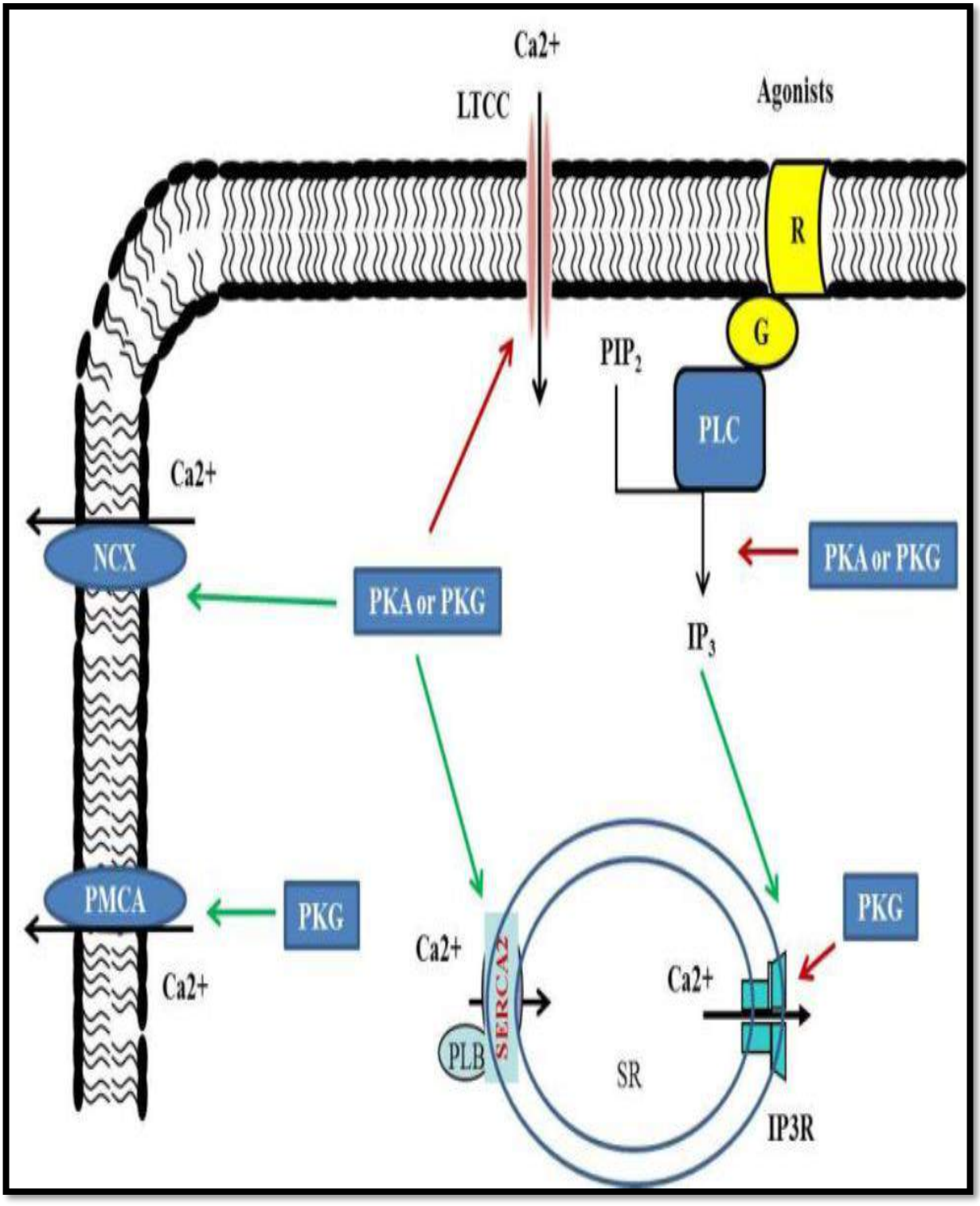

Figure (1-3) [7]: Mechanisms involved in the reduction of cytosolic Ca^{2+} concentration induced by cyclic nucleotides and their dependent protein kinases. Green arrows imply stimulation, and red arrows imply inhibition. IP3R: Inositol trisphosphate receptor; LTCC: L-type Ca^{2+} currents; NCX: Na^{+}/Ca^{2+} exchanger; PIP2: Phosphatidylinositol 4, 5-bisphosphate; PLC: Phospholipase C; PMCA: Plasma membrane Ca^{2+}-ATPase; SR: Sarcoplasmic reticulum.

2- Hyperpolarization of the smooth muscle cells (SMC) [7]:

a- Voltage-gated K^+ channels (K_v channels).

b- Large conductance Ca^{2+}-activated K^+ channels (BK channels).

c- ATP - sensitive K^+ channels (K_{ATP} channels).

d- Inward rectifier K^+ channels (K_{ir} channels).

3- Decrease in myosin light chain 20 (MLC20) phosphorylation:

Myosin light chain phosphorylation promotes the contraction of the SMC, and conversely a decrease in MLC20 phosphorylation promotes its relaxation, this occur through [8]:

a- Inhibition of MLCK activity:

The activity of myosin light chain kinase (MLCK) is primarily mediated by the Ca^{2+} / CaM complex. Elevation of cAMP levels inhibits phosphorylation of MLCK through a PKA-dependent pathway. This decreases the affinity of MLCK for the Ca^{2+}/CaM complex and thereby reduces myofilament Ca^{2+} sensitivity. There is no direct evidence for PKG-dependent phosphorylation of MLCK. However, cross-activation of PKA by cGMP is reported to participate in inhibition of MLCK activity, and subsequent vasodilation [8].

b- Increase in myosin light chain phosphatase (MLCP) activity:

Multiple of evidences show that PKG could activate MLCP, thereby decreasing MLC20 phosphorylation and SMC contraction [8]. It has also been reported that cGMP -induced inhibition of Ca^{2+} sensitization can be directly mediated by Ras homolog gene family, member A / Rho-associated coiled kinase (RhoA / ROCK) pathway. Cyclic adenosine monophosphate - responsive rap1 guanine nucleotide exchange factor (Epac) has been proposed to mediate the relaxation induced by cAMP in several smooth muscles through down-regulation of RhoA [9].

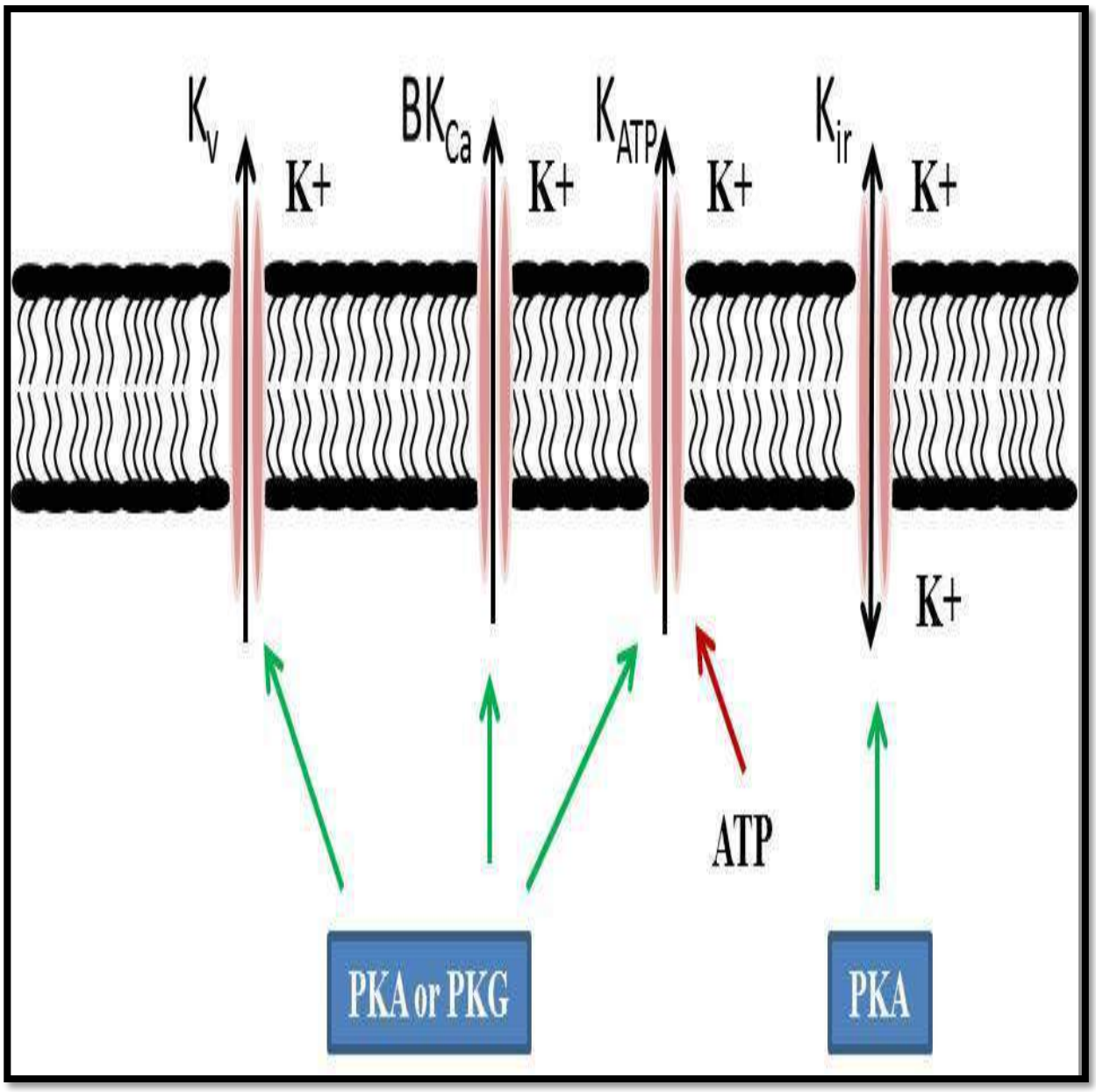

Figure (1-4) [7]: Modulation of K^+ channels by cyclic nucleotide-dependent protein kinases in smooth muscle (SM). Green arrows imply stimulation; red arrows imply inhibition. K_{ATP} channels: ATP - sensitive K+ channels; Kir channels: Inward rectifier K+ channels; Kv channels: Voltage-gated K+ channels; ATP: Adenosine triphosphate; PKG: Protein kinases G; BK_{Ca} channels: Ca2+-activated K+ channels.

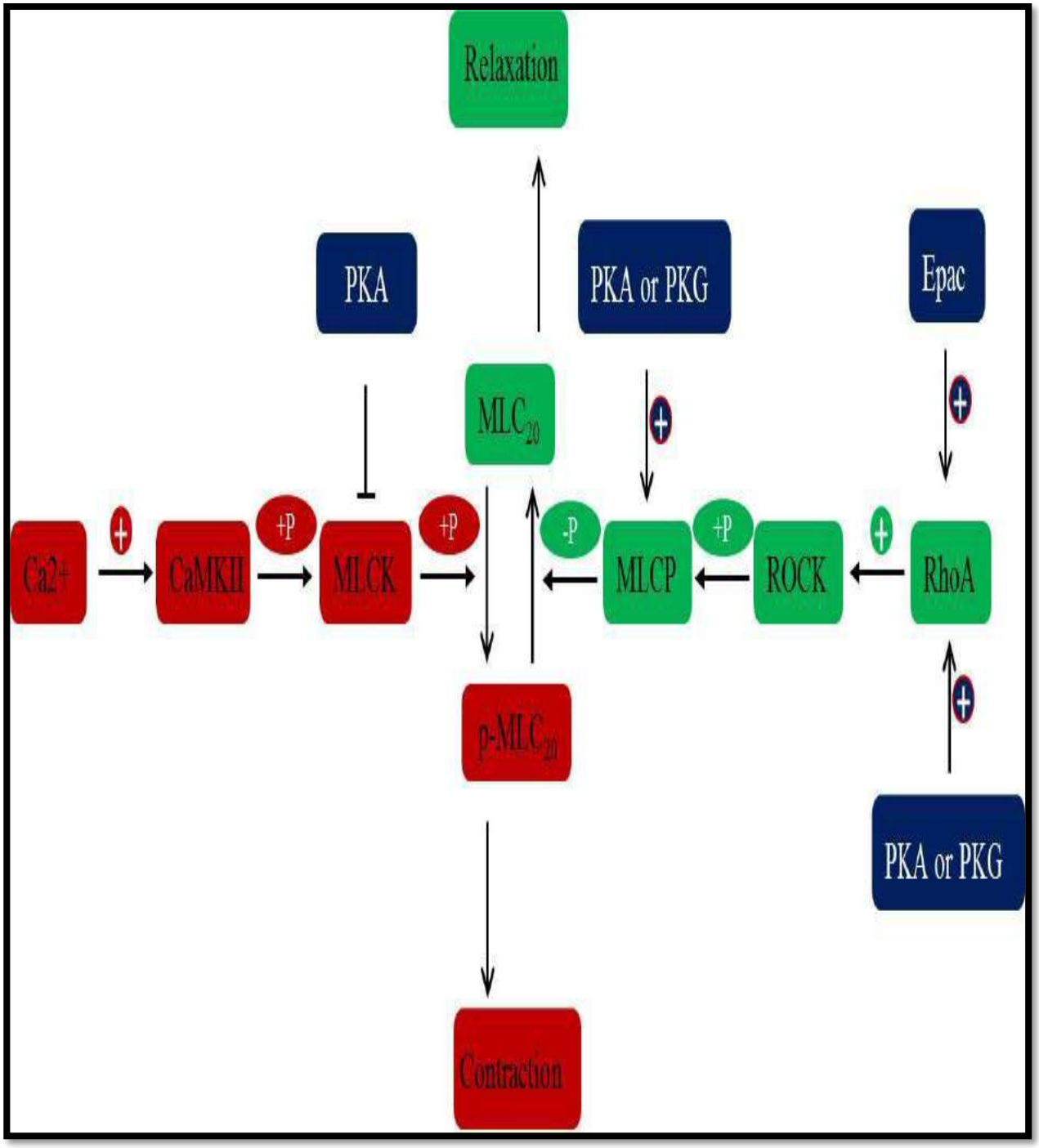

Figure (1-5) [7]: Role of myosin light chain twenty (MLC20) in the regulation of the contractile state in smooth muscle (SM). PKA: protein kinase-A; PKG: cGMP-dependent protein kinase; MLCP: myosin light chain phosphatase; MLCK: myosin light chain kinase; ROCK: Rho-associated protein kinase; P-MLC20: phosphorylated myosin regulatory light chain twenty; Epac: cAMP - responsive rap1 guanine nucleotide exchange factor; RhoA: Ras homolog gene family, member A; Ca^{2+}/Calmodulin protein kinase II; P refer to phosphorylation.

1.1.2 Pharmacokinetics:

Sildenafil is rapidly absorbed after oral administration. Maximum observed plasma concentrations are reached within 30 to 120 minutes (median 60 minutes) of oral dosing in the fasted state. The mean absolute oral bioavailability is 41% (range 25-63%). In clinical trials, single oral doses of sildenafil between 25 and 100 mg have been shown to be effective in the treatment of erectile dysfunction of organic, psychogenic or mixed etiologies. When sildenafil film-coated tablets are taken with a high fat meal, the rate of absorption of sildenafil is reduced with a mean delay in T_{max} of 60 minutes and a mean reduction in C_{max} of 29%. Patients may need to individualize their dosing relative to their food intake based on their own experienced clinical response **(10)**. Sildenafil is cleared predominantly by the cytochrome P450 3A4 (major route) and cytochrome P450 2C9 (minor route) hepatic microsomal isoenzymes. The major circulating metabolite results from N-demethylation of sildenafil. This metabolite has a PDE selectivity profile similar to sildenafil and an *in-vitro* potency for PDE5 approximately 50% of the parent drug. Plasma concentrations of this metabolite are approximately 40% of those seen for sildenafil. The N-desmethyl metabolite is further metabolised with a terminal half-life of approximately 4 hours **(11)**. After oral administration, sildenafil was excreted as metabolites predominantly in the faeces (approximately 80% of administered oral dose) to a lesser extent in the urine (approximately 13% of administered oral dose), where sildenafil half-life is 3-5 hours **(12)**.

1.1.3 Adverse effects of sildenafil:

The most common adverse effects of sildenafil use included headache, flushing, indigestion, nasal congestion, and impaired vision, including photophobia and blurred vision (mild and transient predominantly colour tinge to vision, but also increased perception to light or blurred vision), also there is evidence that sildenafil usage linked with nonarteritic anterior ischemic optic neuropathy **(13)**. Rare but

serious adverse effects found through post-marketing surveillance include prolonged erections, severe low blood pressure, myocardial infarction (heart attack), ventricular arrhythmias, stroke, increased intraocular pressure, and sudden hearing loss [14].

1.1.4 Therapeutic effect of sildenafil:

Sildenafil is a selective inhibitor of phosphodiesterase type 5 (PDE5). Inhibition of PDE5 enhances the vasodilator effects of nitric oxide in pulmonary hypertension by preventing the degradation of cyclic guanosine monophosphate (cGMP), which promotes relaxation of vascular smooth muscle and increases blood flow. It has been found to produce selective reduction in pulmonary artery pressure without adverse systemic hemodynamic effects [15]. In addition, cyclic nucleotides play a prominent role in the regulation of cellular functions and PDE inhibition can therefore elicit a variety of effects [16]. The pharmacologic actions of PDE inhibitors (PDEIs) are due to their potential to increase intracellular cAMP and cGMP as intracellular signal messengers that modulate both the intensity and the nature of immediate and delayed cellular responses. Cyclic GMP has emerged recently as a principal focus in signal transduction. Additionally, the conventional regulatory roles ascribed to cGMP such as stimulation of smooth muscle relaxation, inhibition of platelet aggregation, neutrophil degranulation, and initiation of visual signal transduction, numerous other physiological roles have recently been uncovered [17]. Sildenafil has been reported to provide cardioprotection against ischemic injury when infused at the onset of reperfusion in rabbits. Recently, another study have shown that, in addition to treating erectile dysfunction, sildenafil can prevent or decrease tissue injury. *In vivo* and *in vitro*, early treatment with sildenafil ameliorated the progression of renal damage in the “5/6 nephrectomy” model, and sildenafil was found to reduce cisplatin-induced nephrotoxicity in rats [18]. *In vitro* studies sildenafil was shown to potentiate the antiaggregatory effect of the nitric

oxide donor, sodium nitroprusside [19]. Studies in animals and humans have demonstrated an impact of PDE5 inhibitors upon glucose metabolism but the reports are controversial. There is evidence that liver is one of the organs that well responds to PDE inhibitors by influencing glucose output [20].

1.2 Phosphodiesterases:

Phosphodiesterases are a large family of enzymes that specifically hydrolyse the second messengers (cAMP and / or cGMP) into their inactive forms, the non-cyclic nucleotides 5'-AMP and 5'GMP, respectively as shown in (Fig.1-6) [21]. There are 11 families of PDE enzymes (PDE1-PDE11) which are recognized by substrate specificity, kinetic characteristics and amino acid sequence, each with a different tissue distribution as shown in table (1-1). All of them have an N-terminal regulatory domain (R domain) and a C-terminal catalytic domain (C domain). Some PDEs are highly specific for hydrolysis of cAMP (PDEs 4, 7, and 8) or cGMP (PDEs 5, 6 and 9), while others hydrolyze both cAMP and cGMP [22].

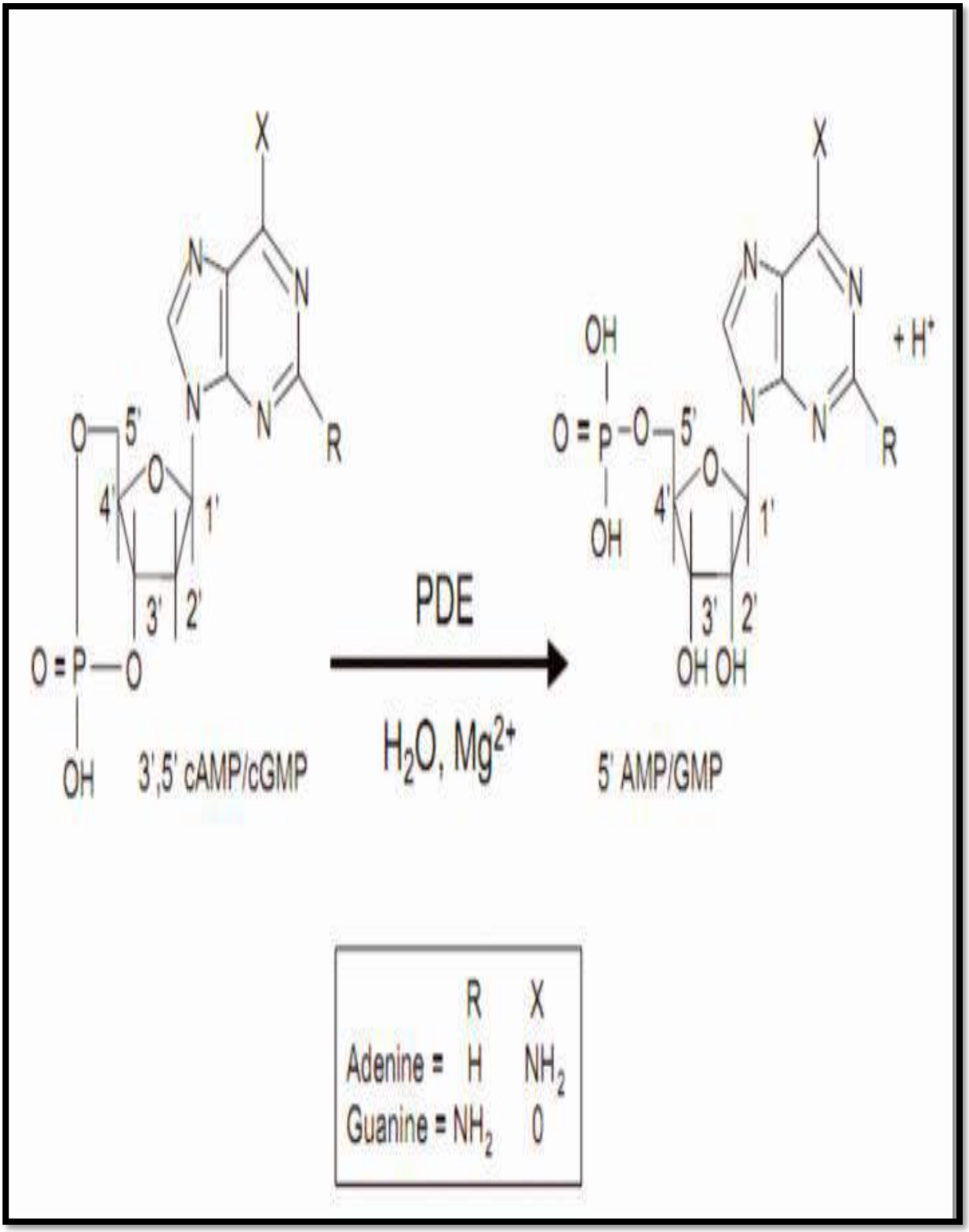

Figure (1-6) [21]: Degradation of cyclic nucleotides by PDEs.

Table (1-1): Characteristics of PDE isozymes [23, 24].

PDEs	Substrate	Property	Specific inhibitors	Tissue expression
PDE1	cAMP/cGMP	Ca^{2+}/calmodulin-stimulated	KS-505a IC 86340	Heart, brain, lung, smooth muscle
PDE2	cAMP/cGMP	cGMP-stimulated	EHNA BAY 60-7550	Adrenal gland, heart, lung, liver, platelets
PDE3	cAMP/cGMP	cGMP-inhibited, cAMP-selective	Cilostamide, enoxamone, milrinone	Heart, lung, liver, platelets, adipose tissue, inflammatory cells
PDE4	cAMP	cAMP-specific cGMP-insensitive	Rolipram, roflumilast, cilomilast	Sertoli cells, kidney, brain, liver, lung, inflammatory cells
PDE5	cGMP	cGMP-specific PKA/PKG-Phosphorylated	Sildenafil, tadalafil, vardenafil	Lung, platelets, vascular smooth muscle, heart, teste brain
PDE6	cGMP	cGMP-specific	Dipyridamole	Photoreceptor
PDE7	cAMP	cAMP-specific, high-affinity	BRL-50481	Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes
PDE8	cAMP	cAMP-selective	None	Testes, eye, liver, skeletal muscle, heart, kidney, ovary brain, T lymphocytes
PDE9	cGMP	cGMP-specific	BAY 73-6691	Kidney, liver, lung, brain, heart
PDE10	cAMP/cGMP	cAMP-selective	None	Testes, brain
PDE11	cAMP/cGMP	cGMP-sensitive, dual specificity	None	Skeletal muscle, prostate, kidney, liver, pituitary and salivary glands, testes

1.3 Cyclic nucleotides and their targets:

The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), have long been recognized as important intracellular signal transduction molecules, acting as second messengers between an extracellular signal such as a hormone, neurotransmitter, or cytokine and the elicited intracellular response. While the specific function of a given signal varies according to the cell type, extracellular environment, stimulus activating the signal, localization of the signal, and the type of cyclic nucleotide formed, an extracellular signal will generally activate a cyclase enzyme, which catalyzes the formation of the cyclic nucleotide from its nucleotide triphosphate precursor (NTP) [(25)]. Once formed, the cyclic nucleotide will affect the activity of downstream effector molecules including kinases, ion channels, transcription factors, and scaffolding proteins. Both the amplitude and duration of a cyclic nucleotide signal also varies and is largely dependent on the expression and activity levels of cyclic nucleotide phospho-diesterase (PDE) enzymes, which catalyze the hydrolytic breakdown of cyclic nucleotides [(25)].

1.3.1 Cyclic adenosine monophosphate Signaling:

The balance of cAMP signaling is essential to multiple cellular processes, including immune function, growth, differentiation, gene expression and metabolism. The intracellular level of the second messenger, cAMP, is regulated by [(26)]:

1- Opposite activities of two enzymes, adenylyl cyclase (ACs) and PDEs. Both ACs and PDEs are regulated by numerous signaling pathways including, calcium signaling through calmodulin (CaM) and calcineurin, G-proteins, inositol lipids (e.g., PKC) and receptor tyrosine kinases [(26)]. Concentrations of cAMP can be regulated by processes throughout the whole cell and within membrane regions by multidrug resistance proteins (MRP4, MRP5) which have demonstrated an energy

dependent export of cyclic nucleotide (cNT), MRP4 promotes the modulation of localized membrane cyclic adenosine monophosphate (cAMP) concentrations which are coupled to G protein coupled receptor (GPCR)-mediated events, MRP4 forms macromolecular complexes in specialized subcellular domains; while, MRP5 can efflux both cGMP and cAMP, thus reducing their intracellular availability; therefore both MRP4 and MRP5 regulating cNT levels when there is an overproduction of cNTs or inhibited PDE activity (Fig.1-7) [(26)].

2- The regulation of cAMP levels can be inhibited by GPCR subunits which modulate the activity of adenylyl cyclase (ACs) in some cell types and cAMP-dependent serine/threonine protein kinase (PKA) activity can act as a negative regulator of cAMP signaling by phosphorylating and inactivating ACs [(27)], the amplitude and duration of a cAMP signal within most cells is largely dependent on phosphodiesterase (PDE) enzyme activity, which is responsible for hydrolyzing cAMP to 5'AMP in order to terminate its signal [(28)]. Despite relatively equal levels of AC, cAMP and PDE expression in most cell types, the rate of cAMP hydrolysis in virtually all human tissues far exceeds the rate of synthesis, making PDE enzymes an important determinant of intracellular cAMP levels, which, under basal conditions, are typically less than 5 picomol/mg of protein [(29)]. Intracellular receptors for cAMP include the guanine nucleotide exchange factor (GEF) exchange protein activated by cAMP-responsive rap1 guanine nucleotide exchange factor (Epac), ion channels such as the cyclic nucleotide gated (CNG) channels and the hyperpolarization-activated cyclic nucleotide gated (HCN) channels, and PKA, Epac, a GEF, that activates the small G protein Rap1 in order to mediate cell adhesion, junction formation, and secretion, Epac can mediate cAMP anti-apoptotic and pro-apoptotic signaling through a PKA-independent mechanism in a cell-type specific manner [(30)]. Protein kinase A phosphorylates metabolic enzymes to mediate glucose metabolism and the transcription factor cAMP-response element binding

protein (CREB) to promote gene transcription. The transcription factor cAMP-response element binding protein is a 43 kDa basic / leucine zipper transcription factor that is highly conserved and expressed in most tissue types. The activation of CREB is not PKA specific, CREB phosphorylation can be stimulated by multiple kinases including Akt, pp90 ribosomal S6 kinase (p90Rsk), and calcium/calmodulin dependent kinases. The transcription factor cAMP-response element binding protein regulates a multitude of genes and can be overexpressed and constitutively activated in many cancer types. The transcription factor cAMP-response element binding protein regulates the expression of several genes involved in metabolism, signaling, proliferation, differentiation and survival [(31)].

1.3.2 Cyclic guanosine monophosphate Signaling:

Similar to cAMP, cGMP levels are predominantly controlled by degradation via PDE enzymes. Phosphodiesterase type 5 is the isoform predominantly responsible for cGMP hydrolysis and subsequent termination of a cGMP signal. The regulation of PDE activity is unique to cGMP signaling and serves multiple functions within cells such as acting as negative feedback for cGMP signaling by activating cGMP specific PDE5 or acting as crosstalk between cyclic nucleotide pathways by increasing or decreasing the activity of non-selective PDE isozymes such as PDE2 or PDE3, respectively [(32)]. Some guanylyl cyclase (GC) isoforms serve as substrates for phosphorylation by PKG resulting in decreased catalysis and, therefore, decreased formation of cGMP. However, similar to cAMP signaling, the specific effect of a cGMP signal depends largely on the receptors that is activated. Under standard physiological concentrations, which for cGMP are typically tenfold less than those observed for cAMP, cGMP preferentially activates receptors distinctly different than those activated by cAMP, including certain PDE isozymes, PKG, and cyclic nucleotide gated (CNG) ion channels [(33)].

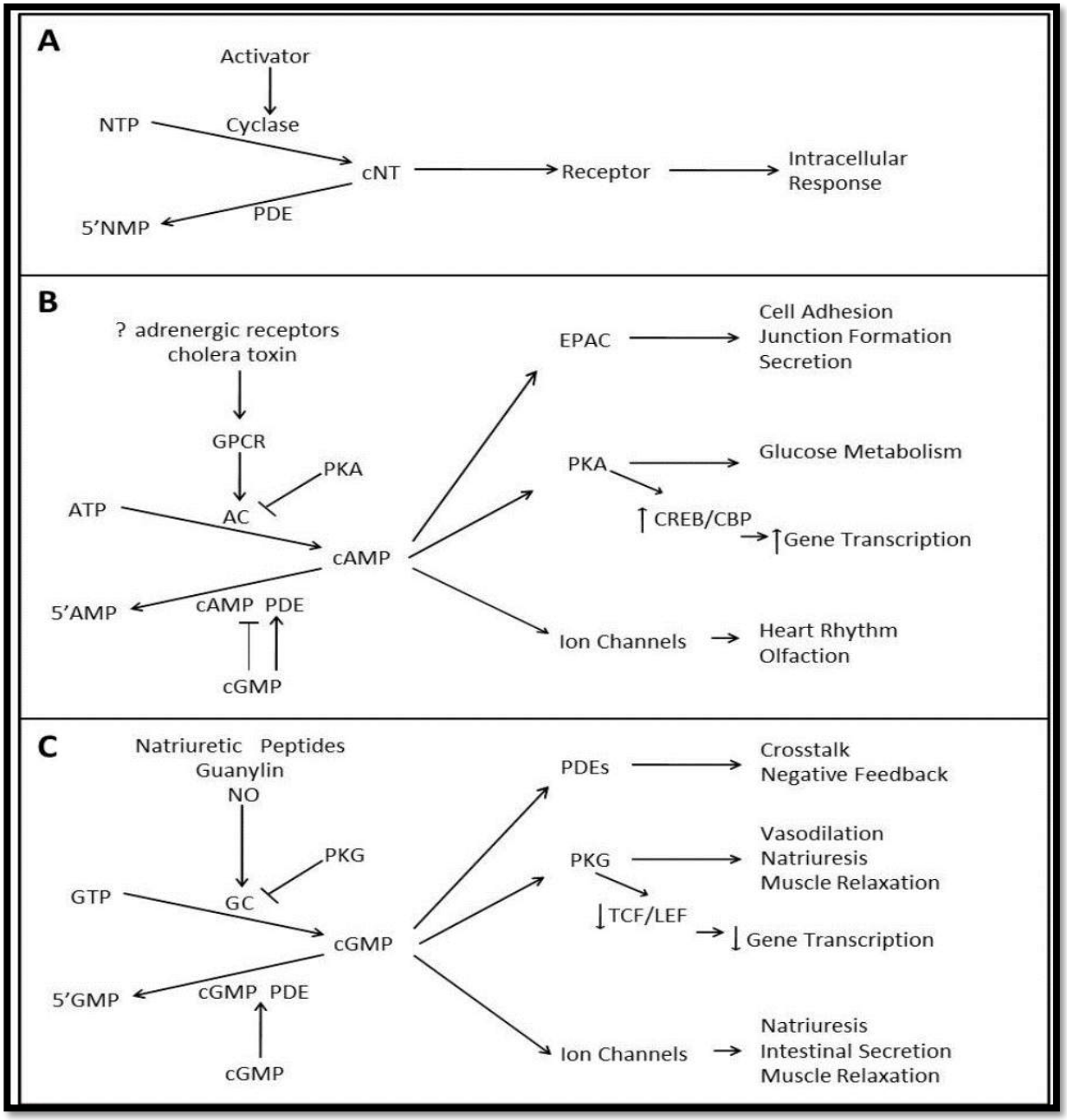

Figure (1-7) [26]: Generalized illustrations of cyclic nucleotide (cNT) signaling (A); cyclic adenosine monophosphate (cAMP) signaling (B); and cyclic guanosine monophosphate (cGMP) (C). A, demonstrates cyclic nucleotide (cNT) production through nucleotide precursor (NTP) and activation of selective cyclase and activation receptor and intracellular response or degradation of cNT through phosphodiesterase (PDE) activity. B, production of cAMP by adenylyl cyclase (AC) can be regulated by GPCR and PKA. cAMP signaling can lead to the activation of PKA, Epac and ion channels. Cyclic AMP can also be degraded by PDEs (PDE1, 2, 3, 4, 7, 8, 10, or 11) and cGMP can inhibit selective PDE cAMP degrading activity. C, production of cGMP by guanylyl cyclase (GC) can be regulated by nitric oxide (NO) and PKG. cGMP signaling can lead to the regulation of other PDEs, PKG and ion channels. Cyclic GMP can also be degraded by PDEs (PDE1, 2, 3, 5, 6, 9, 10, or 11).

Modulation of cyclic nucleotide gated channel activity is a more common event in cGMP signaling compared to cAMP signaling and serves as an important step for mediating the effects of cGMP on phototransduction, natriuresis, and intestinal fluid and electrolyte secretion. Similar to PKA and cAMP signaling, PKG mediates cGMP signaling by modulating down-stream signaling effects. For example, PKG can phosphorylate and activate myosin phosphatase to promote vasodilation and muscle relaxation. A central mediator of cGMP signaling is PKG, which activated by cGMP binding and phosphorylates down-stream substrates. PKG is a serine/threonine protein kinase which is highly versatile and plays a diverse role in regulating multiple cellular processes (i.e., vasodilation, cell differentiation, cell proliferation and apoptosis) [(34)].

1.3.3 Crosstalk between cyclic nucleotide signaling pathways:

An additional level of complexity arises when considering the crosstalk between cAMP and cGMP signaling pathways. The most notable source of crosstalk between the pathways is found in the ability of cGMP to modulate the activity of various PDEs, particularly PDE2 and PDE3, which can hydrolyze both cAMP and cGMP, but have higher affinity for cAMP [(35)]. For example, low nanomolar concentrations of cGMP are sufficient to produce more than a tenfold increase in cAMP hydrolysis by PDE2 and all but complete inhibition of cAMP hydrolysis by PDE3. In fact, it is thought that the effects of atrial natriuretic peptide (ANP) on aldosterone secretion are at least partially due to a drop in cAMP levels that is mediated by cGMP signaling following the activation of PDE2 [(35)].

Phosphorylation events mediated by PKA and PKG serve as another source of crosstalk between the pathways. Both kinases share a number of substrates allowing for specific downstream events to be mediated by both cAMP and cGMP signaling. For example, vasoactivator stimulated phosphoprotein (VASP) is preferentially phosphorylated at its serine 157 residue by PKA and its serine 239 residue by PKG

in order to modulate focal adhesions, cell shape, or platelet aggregation in response to a number of stimuli. PKA and PKG predominantly activated by their respective cyclic nucleotides, while in some instances, be activated by the alternate cyclic nucleotide. For instance, high levels of cAMP have been found to activate PKG *in vitro* in the absence of cGMP, but the validity of this effect is questionable as it has not been observed in intact cells or under physiological conditions [36].

1.4 Effect of sildenafil on various tissues:

1.4.1 Effect on kidney tissue:

The kidney is a complex organ with myriad functions, including filtration of the blood; preservation of fluid, electrolyte, and acid-base balance, and regulation of blood pressure. Every day, the two kidneys filter about 120 to 150 quarts of blood to produce about 1 to 2 quarts of urine, composed of wastes and extra fluid that make kidney more susceptible to injury by various insults and pathological condition [37, 38]. Sildenafil is a selective inhibitor of phosphodiesterase-5 (PDE5), which degrades cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (AMP) to lesser extended, then has a relaxant effect on the smooth muscle cells of the arterioles [39]. Several studies have shown that, in addition to treating erectile dysfunction, sildenafil has an ameliorative effect on tissue injury including renal ablation model of kidney damage [40]. These ameliorative effects indicate that sildenafil has improved both renal blood flow (RBF) and glomerular filtration rate (GFR) which may be attributed to its vasodilation effect of sildenafil. Moreover, sildenafil may preserve tubular integrity through vasodilation effects as well as, via attenuation of inflammation and pro-inflammatory mediators including TNF-α, which explain tubular function maintenance observed upon co-treatment with cisplatin [41]. Furthermore, because of the inflammation is known to induce renal vasoconstriction and to reduce renal blood flow and glomerular filtration rate, prevention of inflammation should result in a better filtration [42, 43].

Additional to sildenafil anti-inflammatory effect, it reduces oxidative stress through NO/cGMP pathway [44]. Previous study, found that sildenafil has markedly renoprotective effects, attenuating kidney tissue injury, especially in the vascular bed, and decreasing oxidative stress, as confirmed by biochemical assays and histopathological study. This protection is due primarily to the inhibition of oxidative stress and decrease in the level of TNF-α, which is one of the important mechanisms of organ injury. Principal among the reactive oxygen species (ROS) generated by oxidative stress is superoxide anion ($O_2^{\cdot -}$), which reacts with NO to produce peroxynitrite ($ONOO^{\cdot -}$), thereby reducing bioavailable NO. A reduction of NO availability promotes not only vasoconstriction but also the adhesion of leukocytes and platelets, which in turn release a vasoconstrictors and cytokines, thereby exacerbating ongoing inflammatory cascades. Adherent neutrophils and monocytes release tumor necrosis factor-alpha (TNF-α) and interleukins (ILs), the blood levels of which are markedly elevated [45]. In turn, TNF-α and ILs and lipopolysaccharide (LPS) upregulate enzymes that generate $O_2^{\cdot -}$ in cultured vascular tissues, in particular, NADPH oxidase. A self-perpetuating inflammatory cascade ensues. sildenafil is a potent inhibitor of $O_2^{\cdot -}$ formation in endothelial cells, both acutely and in the longer term. Acutely, this effect is mediated by the direct inhibition of NADPH oxidase activity and in the longer term by suppression of NADPH oxidase expression. This effect is mediated by the cGMP-PKG system and not by the cAMP-PKA axis. So, the NO-cGMP axis plays a role in blocking not only the expression of NADPH oxidase in response to inflammation but also the intrinsic acute activity of the enzyme [45]. Sildenafil exerts ameliorating effects by decreasing lipid peroxidase and myeloperoxidase activities as markers of lipid peroxidation [45]. Inflammatory cells, cytokines, and profibrotic growth factors including transforming growth factor-β (TGF-β), monocyte chemoattractant protein-1 (MCP-1), connective tissue growth factor (CTGF), tumor necrosis factor-α (TNF-

α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-18 (IL-18), and cell adhesion molecules (CAMs) have all been implicated in the pathogenesis of diabetic nephropathy via increased vascular inflammation and fibrosis [(46)]. Little attention has been paid to the effect of sildenafil on EGF production by the kidney. The high concentrations of epidermal growth factor (EGF) found in urine in the face of non-detectable levels of EGF in plasma support the hypothesis that EGF in urine was derived from kidney synthesis and secretion. The significant positive correlation between urine creatinine and urine EGF suggests a functional correlation between glomerular filtration and the tubular EGF [(47)].

1.4.2 Effect on liver tissue:

Experimental evidence using various animal models and experimental techniques indicated that NO derived from L-arginine by eNOS activity through cGMP pathway protected hepatocytes and liver endothelial cells from the deleterious effects of ischemia/reperfusion (I/R) injury [(48)], by the following mechanisms shown in (fig. 1-8) [(49)]:

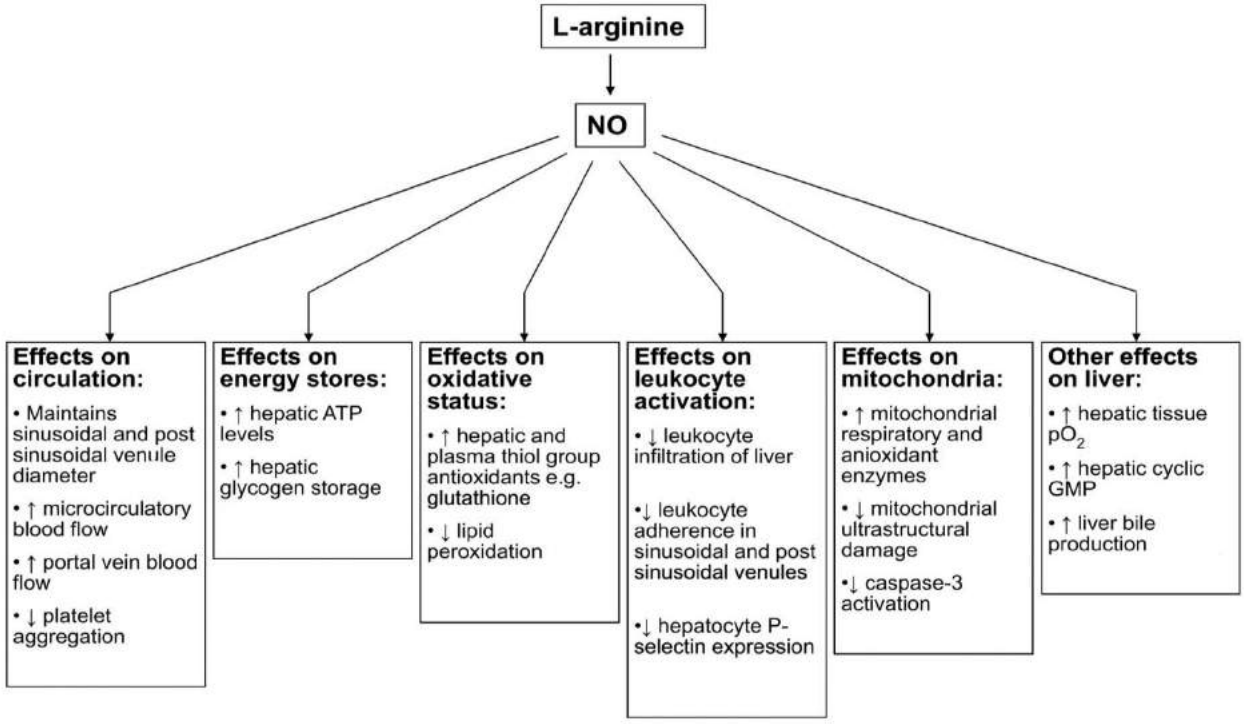

Figure (1-8) [(49)]: The protective effects of NO in liver I/R injury.

Previous study has been shown that administration of the phosphodiesterase inhibitor sildenafil, raises cGMP levels, in mice subjected to liver ischemic reperfusion (I/R) and has also been shown to significantly attenuate the I/R injury [(50)]. Therefore, attenuating the manifestations of I/R injury by inhibition of cGMP degradation by PDE5 inhibitors that might preserve the cGMP pool, thus promoting the favorable action of NO generated by eNOS that engage the soluble guanylate cyclase/cGMP/PKG pathway, rather than iNOS derived NO which is cytotoxic and exerts PKG-independent proinflammatory effects [(51)]. cGMP-dependent protein kinase or PKG plays an important role in the downstream pathway of cell protection elicited by NO. Moreover, the protection offered by PKG resulted in the phosphorylation, and activation of p38 mitogen-activated protein kinases (p38 MAPK) and phosphatidylinositol-3-kinases (PI3K) leading to a significant reduction in intracellular [Na^+] in hypoxic hepatocytes [(52)]. Phosphorylation of p38 MAPK in hepatocytes results in activation of vacuolar H^+-ATPase (V-ATPase) [(53)], V-ATPase are proton pumps spanning the membranes of various intracellular compartments as well as the plasma membranes of hepatocytes and play an important role in maintaining intracellular pH homeostatsis and correction of intracellular acidosis results from hepatic ischemia [(54)]. The activation of PI3K in hepatocytes, result in the exocytosis of lysosomes and consequent insertion of lysosomal V-ATPases into the plasma membrane [(55)]. Therefore, through activation of two separate signalling molecules (p38 MAPK and PI3K); the NO-PKG cascade results in not only the activation of V-ATPase pumps but also in an increase in, the number of plasma membrane V-ATPase pumps. Activated PKG has one other consequence, which pertains to the prevention of onset of the mitochondrial permeability transition (MPT) in hepatocytes, an effect that averts hepatocellular death [(56)]. A summary of the mechanisms through which the NO-PKG pathway exerts its protective actions in liver I/R injury is provided in (fig. 1-9).

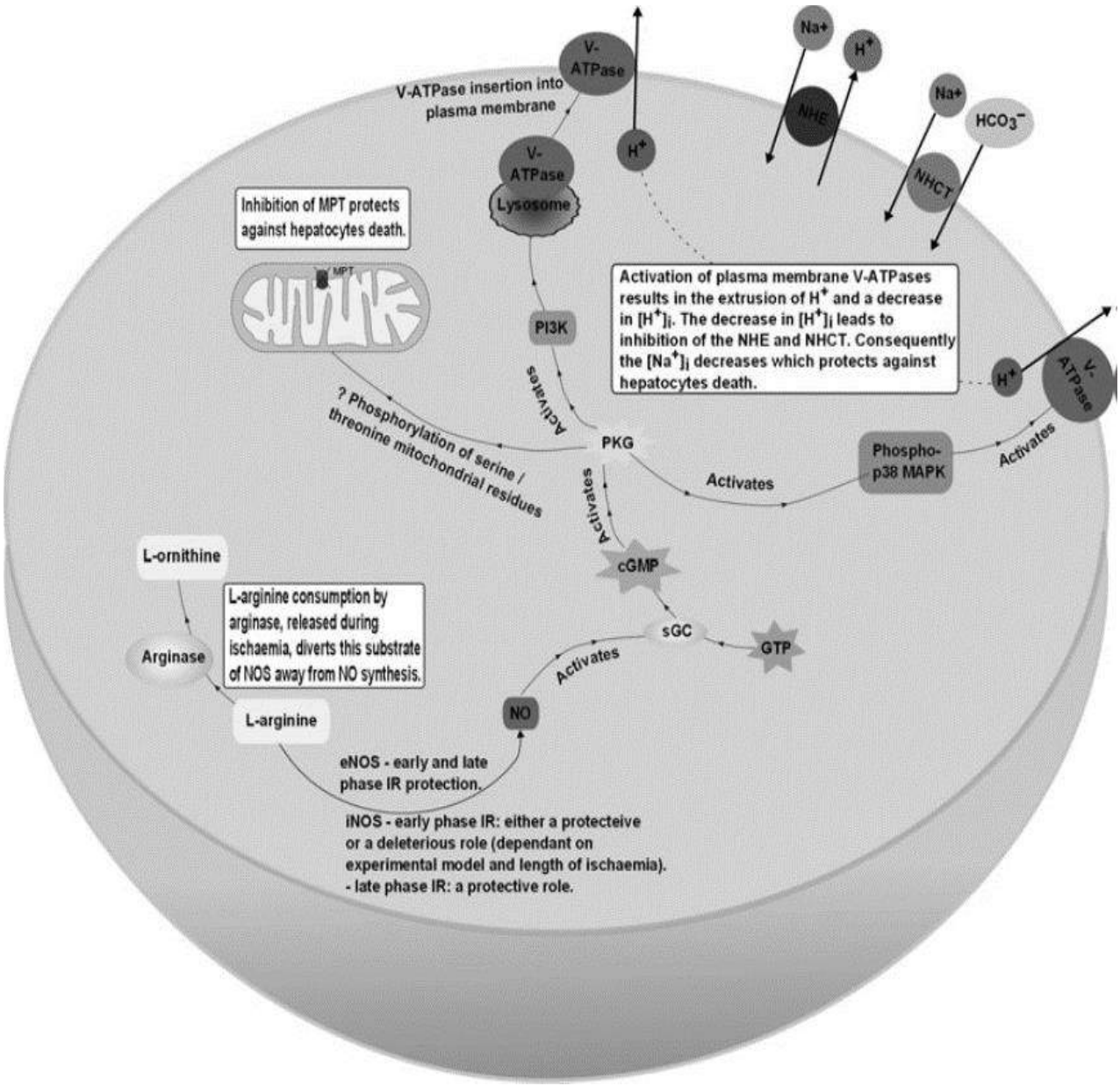

Figure (1-9) [49]: A summary of the main mechanisms involved in NO-mediated protection against liver I/R injury. A schematic representation of a liver cell with NO-mediated protective pathways shown. cGMP: cyclic 3′ ,5 guanosine monophosphate ; eNOS: endothelial nitric oxide synthase; GTP: guanosine triphosphate; H^+: hydrogen ions; $HCO3^-$: bicarbonate; iNOS: inducible nitric oxide synthase; MPT: mitochondrial permeability transition; Na^+: sodium ions; NHCT: $Na^+/HCO3^-$ cotransporter; NHE: Na^+/H^+ exchanger; NO: nitric oxide; PI3K: phosphoinositide 3-kinase; PKG: protein kinase G (cGMP-dependent protein kinase); sGC: soluble guanylyl cyclase; V-ATPase: vacuolar H^+-ATPase.

High-fat feeding–dependent hepatic inflammation and insulin resistance were prevented by daily oral dosing of sildenafil [57], also improves hepatic steatosis and decrease Kupffer cell inflammation by reduces NF-κB signaling in hepatocytes, macrophage and endothelial cells [58]. Another authors reported that sildenafil treatment also blocks M1 activation of Kupffer cells induced by high-fat feeding [59]. Sildenafil decreased expression of intercellular Adhesion Molecule 1 (ICAM-1)-mRNA [ICAM-1 is one of the adhesion molecules that are known to play an important role in the process of leukocyte-endothelial cell interaction] and reduction of leukocyte-endothelial interaction. Additionally, sildenafil may attenuate oxidative aggression-induced tissue injury through hepatocyte and sinusoidal endothelial cell membrane stabilization, also by minimization of intracellular calcium overload and by attenuated myeloperoxidase (MPO) and through improvement of sinusoidal blood flow [51]. In an experimental study, acute incubation of sildenafil increased the vasodilator response to NO in cirrhotic rat livers [60]. In a human study, acute administration of sildenafil increased hepatic production of NO and cGMP and decreased the hepatic sinusoid resistance in cirrhotic patients [61]. Sildenafil up regulated the hepatic protein expression of eNOS and increased intrahepatic NO production. Additionally, PDE-5 levels of sildenafil treated cirrhotic livers were significantly reduced [62].

Collectively, phosphodiesterase-5 inhibitor increases portal flow and lowers portal pressure by a decrease in sinusoidal resistance in healthy individuals and patients with liver cirrhosis [63]. The hepatoprotection afforded by sildenafil to a certain extent could be related to hepatoproliferative properties that have been attributed to sildenafil and its downstream signaling pathway. In particular, sildenafil promoted hepatocellular regeneration in a rat model of liver injury caused by chronic ethanol feeding, manifested by a greater mitotic index of liver cells in sildenafil treated rats [64]. Finally, nitric oxide-Cyclic guanosine monophosphate

(NO-cGMP) signaling pathway could underlie the protective effect of sildenafil against hepatic apoptosis through its mitochondrial ATP-dependent K+ channel (mito-KATP) channel opening properties, as this has already been demonstrated in a rabbit heart model [65]. The studies in animals and human have demonstrated an impact of PDE5 inhibitors upon glucose metabolism but the reports were controversial. In some studies, cGMP accumulation in the liver cells has been shown to have little effect on glycogenolysis while on the other hand, the inhibition of hepatocyte protein synthesis and gluconeogenesis caused by cytokines and nitric oxide (NO) has been attributed to an increase in cGMP level. There is evidence that liver is one of the organs that well responds to PDE5 inhibitors by influencing glucose output [20].

1.4.3 Effect on pancreatic tissue:

Increased oxidative stress plays a role in the pathogenesis of islets damage and β-cell failure by lipid peroxidation and damages cellular organelles. It results from imbalance between production and scavenging of reactive oxygen species (ROS) [66, 67]. Many evidences indicate that any increase in intracellular cAMP and cGMP may prevent induction of oxidative stress [68-72]. Specifically, there was good evidence on reduction of diabetes-induced oxidative stress by use of PDEIs in rats [6].

Additionally, several agents from herbal sources with marked PDE inhibitor and antioxidant activities have been tested and showed improvement of islets function in diabetes and islet transplantation [73]. Therefore, by increasing cyclic nucleotides, PDEIs can overcome oxidative stress [67]. After previous study has been shown that sildenafil restored diazinon-induced increase in plasma glucose concentration, islets TNF-α and lipid peroxidation on the other hand islets total antioxidant power and plasma insulin would enhanced [74]. Therefore, phoso-diesterase inhibitors increase viability of islets and insulin secretion and decrease formation of ROS [6]. Additional to direct relation between production of free

radicals and damage to islets, there is a relationship between nitric oxide (NO), epidermal growth factor (EGF), oxidative stress and phoso-diesterase inhibitors with β-cell and islets of Langerhans [(75)]. Where, there are studies of adult human islets have reported their ability to undergo dedifferentiation, proliferation, and redifferentiation [(76)]. Accordingly, interest has been directed toward factors identified as critical to pancreatic organogenesis. Likewise, epidermal growth factor (EGF) ligands contribute to islet development and therefore may represent candidates for modulating the differentiated state of adult human islet cells. In addition fetal pancreatic explants respond to EGF while pharmacologic inhibition of the extracellular signal-regulated kinase (ERK) pathway, a known mechanism of EGF signaling, elicits an opposite effect [(77)]. Individual EGF ligands have differential effects on specified pancreatic endocrine progenitors [(78)]. New approaches have used EGF for stimulating β-cell regeneration directly or by EGF stimulated mesenchymal cells. Therefore, it was reasonable for β-cells to recover after increased EGF secretion due to sildenafil antioxidative actions [(79, 80)].

1.4.4 Effect on Submandibular Gland:

The submandibular is the second largest major salivary gland and weighs about 7 grams which is important parts of the gastrointestinal tract with a high implication in health of the whole body [(81)]. The submandibular polypeptides promote health and well-being in wound healing, mucosal protection, tissue and organ regeneration. Moreover, submandibular gland produces EGF and releases it into the saliva, where it is involved in mucosal repair in the mouth and in the gastro-oesophageal tract [(82)]. Epidermal growth factor (EGF) is synthesized by the granular convoluted tubule (GCT) of rodent submandibular glands [(83)]. Previous study has been observed that reduction of submandibular saliva components such as protein and calcium in rat treated with lead acetate, which induces oxidative stress [(84)]. It was proved that in diabetic mice, the levels of EGF, its messenger RNA in the

submandibular glands and the circulating level were greatly reduced [83]. The use of cGMP and cAMP phosodiesterase inhibitors, sildenafil, prevented lead induced increased lipid peroxidation and also protected from decreased thiol groups content and total antioxidant power of the gland. Therefore, sildenafil had a profound effect on rat submandibular gland by its antioxidant activity [85]. The exocrine function of salivary glands was entirely dependent on autonomic nerves, the question had arisen whether sildenafil might affect the neural regulation of salivary secretion. Indeed the secretion of polypeptides from the granular ducts is predominantly an adrenoreceptor mediated Ca^{+2}-dependant event. Whereas sildenafil will augmenting the receptor stimulation by beta-adrenergic agonists, which activates adenylate cyclase via heterotrimeric GTP-binding protein (G protein), which leads to an increase in intracellular cAMP levels. The increased cAMP subsequently activates cAMP-dependent protein kinase, which has been well recognized to be essential for consequent exocytosis. Sildenafil stimulate submandibular secretion of protein, EGF and flow rate of saliva in normal rats [86].

1.5 Epidermal growth factor (EGF)

Epidermal growth factor is an acid- and heat-stable 53 amino acid protein originally found in rodents and humans. Epidermal growth factor is a potent mitogenic peptide of molecular weight 4800 that was first isolated from mouse salivary glands by Cohen in 1962 [87]. When the EGF-complementary DNA (cDNA) was cloned, *in situ* hybridization studies showed that the gene was expressed at high levels in kidney as well as in salivary glands. Besides the salivary gland, detectable levels of EGF were also found in various tissue extracts and body fluids, including amniotic fluid, milk, saliva, gastric and duodenal contents, pancreatic juice, bile, urine and platelet-rich plasma. The discovery of EGF led to its characterization as an embryotrophic factor, it enhances mitogenesis, development, and implantation in different mammalian species. This growth factor has been shown to have various

effects on numerous cellular systems [88]. The biological and physiological role of EGF during development and in adult animals have opposing actions on processes such as proliferation and apoptosis in many different cell types. These effects have been postulated to be a feature of receptor expression levels, cell surface receptor density, and ligand concentration, all of which can lead to biphasic responses [89].

1.5.1 Epidermal growth factor (EGF)-Related Factors:

At least 11 different EGF-like polypeptides have been reported, which together comprise the EGF family of growth factors. Each of these peptide shares at least one EGF-like domain comprising disulfide linkages. The EGF family includes transforming growth factor (TGF)-α, amphiregulin (AR), heparin-binding EGF (HB-EGF), epiregulin (EPR), betacellulin (BTC), neuregulins 1–4 (NRG1-4), and teratocarcinoma-derived growth factor (Cripto-1) [88].

1.5.2 Epidermal growth factor receptor (EGFR):

Four family members are human epidermal growth factor receptor-1 (Her1) or receptor tyrosine kinase family (ErbB1), Her2 (ErbB2), Her3 (ErbB3), and Her4 (ErbB4). Each of these receptors exhibits structural similarities in its extracellular, transmembrane, and cytosolic domains. The EGFR signaling pathway is one of the most important pathways that regulate growth, survival, proliferation, and differentiation in mammalian cells. Binding of EGF results in EGF receptor dimerization, autophosphorylation of the receptor, and tyrosine phosphorylation of other proteins. The EGF receptor activates ras and the MAP kinase pathway. Phosphatidylinositol signaling and calcium release induced by EGF activate protein kinase C is another component of EGF signalling pathway. Crosstalk of EGF signaling with other pathways make the EGF receptor a junction point between signaling systems therefore EGF have several physiological functions [90].

1.5.3 Common cited effects of epidermal growth factor (EGF):

1- Its ability to induce mitogenesis in various cells types of ectodermal and mesodermal origin, but EGF has also been shown to promote secretion of hormones such as prolactin and human chorionic gonadotropin as well as pituitary hormones and steroids. EGF also induces the differentiation of specific cell types including the differentiation of human trophoblasts to syncytiotrophoblasts. Additionally, EGF has been shown to influence glucose metabolism [(91)].

2- Epidermal growth factor and transforming growth factor increase the tyrosine phosphorylation and p85 binding to several intracellular docking proteins; insulin receptor substrate 1 (IRS-1), IRS-2, growth factor receptor-bound protein 2 -associated-binding protein 1 (GAB-1) as well as EGFR, leading to the activation of both the metabolic and mitogenic pathways. Furthermore, by activating PI3-kinase pools that are additional to those of insulin, EGF can also augment the down-stream signaling of insulin in insulin-resistant states like Type 2 diabetes [(92)].

3- Epidermal growth factor receptor activation leads to a cascade of biochemical reactions, which include the phosphorylation of phospholipase C -yl (PLC yl). The last factor induces the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2), and the generation of diacylglycerol and inositol 1, 4, 5-trisphosphate (Ins (1,4,5) P3). Diacylglycerol activates protein kinase C, whereas Ins (1,4,5) P3 releases calcium from intracellular stores. It is conceivable that excessive activation of these critical signal transduction pathways may interfere with important cellular functions, like promote secretion of hormones [(93)].

4- In streptozotocin-induced diabetic rats**,** pancreatic β-cell mass was increased threefold, insulin content was increased eightfold and hyperglycemia would reduce in mice treated with EGF plus gastrin compared with pretreatment values. As well as, EGF induce islet regeneration from exocrine pancreatic duct cells .Combination therapy of EGF with gastrin significantly increased cell mass in adult human

pancreatic islets *in vitro* and *in vivo*, an increase that appeared to result from the induction of cell neogenesis from pancreatic duct cells [(94)].

5- Epidermal growth factor receptor ligands are expressed in the developing pancreas, and EGF receptor signaling stimulates proliferation and branching morphogenesis of fetal pancreatic ducts. This process is impaired and islet cell differentiation is delayed in mice lacking EGF receptors [(95)].

6- Epidermal growth factor (EGF) and PDGF stimulated MAPK and Akt (PKB) phosphorylation in a time-dependent manner in freshly isolated cells from the adult ductal network of regenerating adult pancreas [(95)].

7- Epidermal growth factor has been implicated in epithelial cytoprotection, cell survival regulation, cardiac function, and accelerated wound healing in experimental animals. EGF has also been reported to play an essential role in normal epithelial regeneration, which occurs regularly in vital organs such as the gastrointestinal, genitourinary, respiratory, and corneal epithelia. These diverse actions are consistent with the identification of EGFR on cells of different origin, including fibroblast, corneal cells, and breast cells [(96)].

1.6 Glycogen:

Glycogen is a very large, branched polymer of glucose residues that can be broken down to yield glucose molecules when energy is needed. Most of the glucose residues in glycogen are linked by α-1,4-glycosidic bonds. Branches at about every tenth residue are created by α-1,6-glycosidic bonds. It is found in the form of granules in the cytosol in many cell types [(97)]. Hepatocytes (liver cells) have the highest concentration of it-up to 8% (100-120g) of the fresh weight in well fed state in an adult. In the muscles, glycogen is found in a much lower concentration (1% of the muscle mass), but the total amount exceeds that in liver. Small amounts of glycogen found in the kidneys, and even smaller amounts in certain glial cells in the brain and white blood cells. Glycogen plays an important role in the glucose

cycle [97]. The most common disease in which glycogen metabolism becomes abnormal is diabetes, in which, because of abnormal amounts of insulin, liver glycogen can be abnormally accumulated or depleted. In the liver, glycogen synthesis and degradation regulated to maintain blood-glucose levels as required to meet the needs of the organism as a whole [98].

The regulation of these processes is quite complex. Several enzymes taking part in glycogen metabolism mainly glycogen phosphorylase and glycogen synthase which allosterically respond to metabolites that signal the energy needs of the cell. Hormonally stimulated cascades that lead to the reversible phosphorylation of enzymes also regulate glycogen metabolism. By both these mechanisms, glycogen degradation is integrated with glycogen synthesis where phosphorylation activate glycogen phosphorylase enzyme while deactivate glycogen synthase enzyme [99]. Glycogen Phosphorylase is one of the phosphorylase enzymes that represent the rate-limiting step in glycogenolysis, it is a dimer of two identical 94 kDa subunits and has three isozymes: Phosphorylase glycogen muscle (PYGM), Phosphorylase glycogen liver (PYGL), and Phosphorylase glycogen brain (PYGB). A separate isozyme of phosphorylase expressed in liver is less sensitive to these allosteric controls [100].

- AMP (present significantly, when ATP is depleted) activates phosphorylase, promoting the relaxed (active) conformation.
- ATP and glucose-6-phosphate inhibit phosphorylase, promoting the tense (inhibited) conformation.
- Thus, glycogen breakdown inhibited when ATP and glucose-6-phosphate are plentiful.

Glycogen synthase is allosterically activated by glucose-6-phosphate (G6P) (opposite of the effect on phosphorylase), thus glycogen synthase is active when high blood glucose leads to elevated intracellular G6P as shown in table (1-2) [100].

Table (1-2): Allosteric activators and inhibitors of glycogen phosphorylase and glycogen synthase [100].

Enzyme	Activators	Inhibitors
Glycogen phosphorylase	AMP	ATP, G6P
Glycogen synthase	G6P	—

In response to low blood sugar, glucagon which is synthesized by alpha-cells of the pancreas, activates G-protein coupled receptors to trigger cAMP cascades in liver. The cAMP cascade results in phosphorylation and activates glycogen phosphorylase enzyme, so the needs of the organism take precedence over the needs of the cell [101]. The cAMP cascade has the opposite effect on glycogen synthesis. The regulation of Glycogen synthase (GS) by covalent modifications is highly complex and consists of sequences of hierarchal phosphorylations on at least nine different sites catalyzed by a range of kinases including PKA, phosphorylase kinase and glycogen synthase kinase 3. AMP-activated protein kinase (AMPK) has also been shown to directly phosphorylate GS. Phosphorylation of glycogen synthase promotes the less active conformation [102].

In response to high blood sugar (immediate after meal), glucagon level will decrease and insulin is secreted from pancreatic β cells. Insulin stimulates insulin-stimulated protein kinase which phosphorylates G-subunit of phosphoprotein phosphatase-1 (PP-1). This phosphatase catalyzes removal of regulatory phosphate residues from phosphorylase, phosphorylase kinase, and glycogen synthase enzymes, thus insulin antagonizes effects of the cAMP cascade induced by glucagon and epinephrine [103]. In liver, glucose in blood enters freely into the hepatocyte when the glucose is high in bloodstream. Glucose itself inhibits glycogen phosphorylase. Phosphoprotein phosphatase-1 (PP-1) activates glycogen synthase and deactivates glycogen phosphorylase. Therefore, the liver can store the excess of glucose as glycogen. The

two kinds of GP regulation can be viewed as reflecting temporal and spatial differences in energy demanding states: As opposed to the rising AMP concentration that is mainly a result of declining energy levels inside the cell, calcium and cAMP are second messengers triggered by signals from outside the cell, mediating in this case a future energy need as a consequence of cell activation as revealed in (fig. 1-10) [104]. As an allosteric modulator AMP is a direct activator of GP, while the phosphorylation cascade consists of a sequence of reactions. Finally, decrease in AMP could be viewed as a fast route to a slower activation by the cAMP-triggered phosphorylation cascade [105].

1.7 Interleukin-1β (IL-1β)

Interleukin 1-beta (IL-1β) is a pro-inflammatory cytokine that plays important roles in inflammation. It is produced after infection and it is a key regulator of the body's inflammatory response injury, and an antigenic challenge. Numerous biological effects has been shown by the single polypeptide IL-1β, cloned in 1984 [106]. It shows a role in several diseases, involving autoimmune diseases such as rheumatoid arthritis, inflammatory bowel diseases, and type 1 diabetes, also in diseases associated with metabolic syndrome such as atherosclerosis, chronic heart failure, and type 2 diabetes. The primary source of IL-1β is the macrophage, but epidermal, epithelial, lymphoid, and vascular tissues also synthesize IL-1β. Recently, IL-1β production and secretion have also been informed from pancreatic islets, IL-1β signal effects influencing insulin action and insulin secretion [106]. Another study showing that genetic variation in IL1 gene family is associated with hyperglycemia and insulin resistance provides another proof for the involvement of IL-1β in the pathogenesis of diabetes [107].

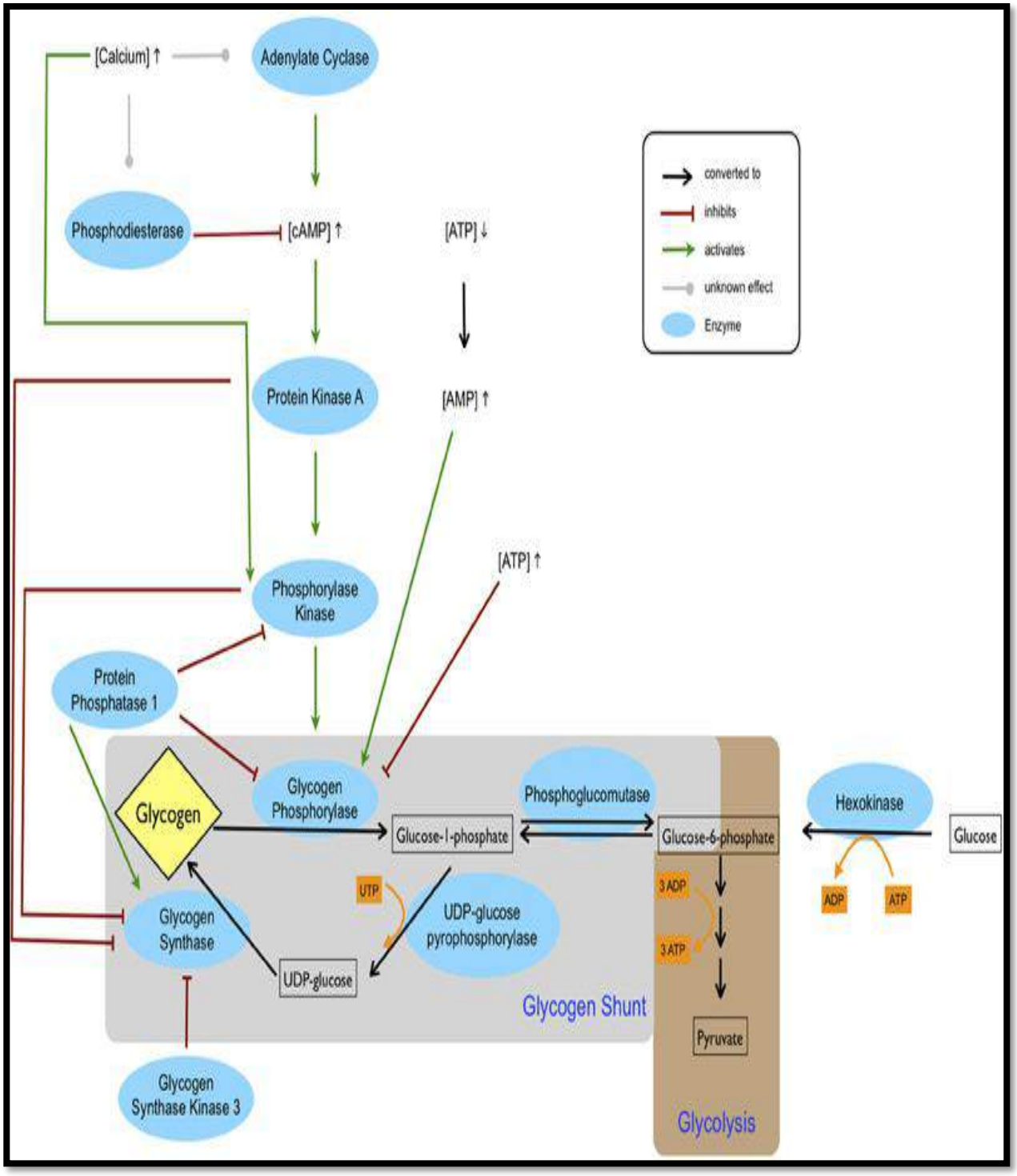

Figure (1-10) [104]: Regulation of glycogen metabolism and role of PDE enzymes in controlling this processes. **cAMP:** Cyclic adenosine monophosphate**; ATP:** Adenosine triphosphate**; ADP:** Adenosine diphosphate; **UTP**: Uridine-5'-triphosphate; **UDP:** Uridine diphosphate.

The central involvement of IL-1β iAn a wide range of inflammatory and autoimmune diseases makes it an attractive target for therapeutic interventions. IL-1β expression is believed to be regulated largely at the level of transcription and by its processing and release. Pro–IL-1β synthesis is induced by lipopoly-saccharide (LPS) through activation of the nuclear factor kappa B (NF-kB) [108]. Pro–IL-1β can be cleaved into the biologically active cytokine by several proteases in the extracellular space but caspase 1 is the main protease responsible for cleavage within macrophages [109]. Cyclic guanosine monophosphate (cGMP) influences the signal transduction that leads to the transcription of TNFα and IL-1β. The major cGMP target in many cell types is protein kinase G, and cGMP-dependent inhibition of proto-oncogene, serine / threonine kinase (Raf-1) by phosphorylation on serine 43 by protein kinase G has been reported. High cGMP concentrations can cross-activate protein kinase A and lead to extracellular signal-regulated kinases (ERK) as well as Raf-1 inhibition followed by decrease activation of nuclear transcription factor of its target genes encoding cytokines including IL1 beta [110].

IL-1β has been proposed to mediate both impaired function and destruction of pancreatic β cells during the development of autoimmune type 1 diabetes. In keeping with this, treatment of rodent islets with IL-1β results in a potent inhibition of insulin secretion followed by islet destruction. In human islets, IL-1β has been shown to impair insulin release and to induce Fas expression, enabling Fas-triggered apoptosis. Finally, activation of the nuclear transcription factor NF-κB is required for IL-1β–induced Fas expression. Rat β-cells also produce IL-1β–converting enzyme, the enzyme required for activation of IL-1β. Human β cells themselves are capable of producing IL-1β independently of any viral infection or immune mediated process, in response to glucose [111]. Antagonizing IL-1 receptor-mediated activity reduces hyperglycemia and tissue inflammation in diabetic mice and humans and favors the possibility of β-cell regeneration in addition to normalizing

insulin secretion. Endoplasmic reticulum (ER) stress may provide the link between IL-1β and β-cell dysfunction, reduce ER calcium storage, and induce ER stress, suggesting a possible mechanism of islet dysfunction and apoptosis [112]. Cytokines induces excessive production of reactive oxygen species (ROS) and activation of caspases, which inhibit insulin secretion and promote apoptosis of pancreatic β cells. Several studies have found an overexpression of superoxide dismutase (SOD) also protects β-cells from IL-1β or other cytokine induced apoptosis by repressing NF-κB activation. It has been demonstrated that pioglitazone also protects human islet β-cells from IL-1β-induced apoptosis by blocking NF-κB activation [113]. Collectively, IL-1β inhibits β-cell function and promotes Fas-triggered apoptosis in part by activating the transcription factor NF-κB. Increased production and release of IL-1β, followed by NF-κB activation, Fas upregulation, DNA fragmentation, and impaired β-cell function [111].

1.8 Aims of the study

This study was designed:

- To investigate the biochemical and immunohistochemical effects of sildenafil on kidney, liver, pancreas and submandibular gland of adult male rats.
- To study the effect of sildenafil on EGF production by the submandibular glands and kidneys in male rats.
- To study the effects of sildenafil on glycogen phosphorylase, hepatic glycogenolysis and hepatic glycogen content.
- To study antioxidant and anti-inflammatory effect of sildenafil, and the relation with blood glucose level, C-peptide and body weight.

Chapter Two

Materials and Methods

2.1 Chemicals

All chemicals and reagents were of the highest available purity and need no more purification (table 2-1). Specific kit, chemicals and reagents used in this study are shown in tables (2-2, 2-3, 2-4, 2-5, 2-6 and 2-7).

Table (2-1): Chemical and solvent.

Chemical	Suppliers
Diethyl Ether	QualiKems-India
Dimethyl sulfoxide (DMSO)	SIGMA-ALDRICH
DPX mounting medium	SyrBio-Switzerland
Eosin solution (water-based)	SyrBio-Switzerland
Ethanol (99%)	Scharlau-Spain
Formalin (37%-40%)	SIGMA CHEMICAL co.-USA
Hematoxylin solution	SyrBio-Switzerland
NaH_2PO sodium dihydrogen phosphate	fluka germany
Na_2HPO_4 sodium phosphate dibasic	fluka germany
Paraffin wax (5 kilos)	Medite-USA
Phosphate buffer solution (PBS)	DAKO, Denmark
Positively charged microscope slides	Fisherbrand Superfrost- USA
Sildenafil citrate powder	SDI
Tween 20	SCRC-China
Xylene	Scharlau-Spain

Table (2-2): Periodic Acid Schiff staining (PAS) Kit (500 ml) (Atom Scientific, UK).

Contains	size
Haemalum Mayer	500 ml
Feulgen Stain (Schiff)	500 ml
Periodic Acid 0.5%	500 ml

Table (2-3): The primary immunohistochemical kits.

Name	Cat no.	Company
Rabbit anti epidermal growth factor polyclonal antibody.	**bs-2010R**	**Bioss**
Rabbit polyclonal antibody to glycogen phosphorylase , Liver (PYGL).	**PAA849Ra02**	**Cloud-Clone Corp.**

Table (2-4): The Secondary immuno-histochemical detection system.

Materials	Quantity	Part Number
HRP-anti-Mouse, Rat & Rabbit Polymer	5ml	K405-50-4
Peroxidase Block (H_2O_2)	5 ml	K405-50-1
Primary Ab dilution buffer	8ml	K405-50-3
Protein Blocking solution	5ml	K405-50-2
Reagent BS (buffer & substrate)	5ml	K405-50-5
Reagent C (conc. DAB chromogen)	1ml	K405-50-6

Table (2-5): Interleukin-1 beta (IL-1β) Rat ELISA Kit.

Materials	Quantity	Cat no.
Assay Diluent A	30mL	ab100767
Biotinylated anti-rat IL-1β	2 vials	
Recombinant Rat IL-1β Standard	2 vials	
IL-1β Microplate (12 x 8 wells)	96 wells	
Stop Solution	8 mL	
20X Wash Buffer Concentrate	25mL	
200X HRP-Streptavidin Concentrate	200 µL	
TMB One-Step Substrate Reagent	12 mL	

Table (2-6): Total Antioxidant Capacity (TAC) Assay kit.

Materials	Quantity	Cat no.
Assay Diluent	10 mL	ab65329
Cu2+ Reagent	200 µL	
Protein Mask	10 mL	
Trolox Standard (1 µmol) Lyophilized	1 vial	

Table (2-7): C-peptide roche kit.

Materials	suppliers
Cobas C–peptide kit	Roche-germany

2.2 Instruments:

Instruments, equipment and software used in this study are summarized in tables (2-8, 2-9) correspondingly.

Table (2-8): Instruments with their suppliers.

Instruments	suppliers
ACCU-CHECK Glucometer	Roche Diagnostics (Switzerland)
Automated Upright Microscope System with LED Illumination for Life Sciences Leica DM4000 B LED	Leica-microsystems
Centrifuge	Hettich Universal (Germany)
Cold Plate for Modular Tissue Embedding System Leica EG1150 C	Leica-biosystems
Electrical oven	Memmert(Germany)
Heated Paraffin Embedding Module Leica EG1150 H	Leica-biosystems
Mettler H54 A.R. Microbalance	Karl Kolb (Germany)
Microplate reader	Biotec ELx 800 (Germany)
Microplate washer ELx50	Biotec ELx50 (Germany)
Multistainer Leica ST5020	Leica-biosystems
Orbital Shaker	GFL (Germany)
pH meter	Inolab (Germany)
Semi-automated Rotary Microtome Leica RM2245	Leica-biosystems
Semi-enclosed Benchtop Tissue Processor Leica TP1020	Leica-biosystems
Thermostatic waterbath	Gemmy (Tiawan)
Vortex Mixer	Cleaver(Germany)

Table (2-9): Equipment and Software.

Equipment			
1-	Cotton swabs	**14-**	Micropipette (volac. U.K) 100 - 1000 µL
2-	Eppendorf tubes	**15-**	Pap pen
3-	Forceps	**16-**	Plates for colorimetric assay
4-	Flasks	**17-**	Pipette tips
5-	Filter paper	**18-**	Positively charged microscope slides (superfrost plus, thermo - scientific, USA)
6-	Glass cover slips	**19-**	Rat gavage needle
7-	Glass staining jar	**20-**	Rat surgical kit
8-	Gloves	**21-**	Slide holders
9-	Graduated cylinder	**22-**	Syringes 10 cc + 5cc + 1cc
10-	Humid champer	**23-**	Timer
11-	Leica microtome disposable blades	**24-**	Tissue Cassettes
12-	Micropipette (capp. Denmark) 0.5-10 µL	**25-**	Tubes
13-	Micropipette (volac. U.K) 10-100 µL		

Software	
1.	Adobe Photoshop CC (version 14.2.1, 2104)
2.	Image J (version 1.48, NIH, USA, 2014)
3.	SPSS statistics (version 22, 2014)

2.3 Animals and study design:

Forty eight adult Wistar male rats (weighing 200–250 gm) were used in this study. They were obtained from the Animal House of Department of Pharmacology & Toxicology, College of Pharmacy/Al Mustansiriyah University. Animals were randomly divided into 4 groups each group contains 12 animals as shown in table (2-10):

Table (2-10): The rats in the control group D and treatment groups (A, B, C).

Group	No.	Treatment	Duration
A	12	administered 0.5 mg/kg/day sildenafil oral solution by using oral gavage tube	Six weeks
B	12	administered 1 mg/kg/day sildenafil oral solution by using oral gavage tube	Six weeks
C	12	administered 2 mg/kg/day sildenafil oral solution by using oral gavage tube	Six weeks
D (control)	12	administered saline by using oral gavage tube	Six weeks

Plastic cages of (20x25x35 cm) dimension used to keep three animals per cage. Animals within the same cage were differentiated by tail markings using a waterproof marker. Before starting study protocol the animals were kept for 2 weeks under controlled conditions of temperature of (22 ± 1^0C) with light schedule of 12-12 hour's light/dark cycles and the animal house was provided with an air vacuum to be adapted with the environment of the animal house. Foods (commercial pellets from a local) and water were accessible freely to the animals. Animals care started in 23/11/2014 and ended in 26/3/2015. The study was initiated after seeking approval from the ethics committee of the college of Pharmacy/AL-Mustansiriyah University.

2.4 Body weight measurements:

Baseline body weight measurements were taken on the first day of the study immediately following the acclimatization period and at the end of 6th week of treatment period.

2.5 Blood glucose measurements:

All the animals were bled on the first day of the experiment immediately following the acclimatization period and at the end of 6th week of treatment period, by sequential snipping of the tip of the tail as described by Fluttert *et al*. 2000 **(114)**. A glucometer (Accu-Chek Go, Roche) was used to measure the fasting blood glucose (FBG) levels **(115)**.

2.6 Treatment administration:

Each day of the study sildenafil citrate oral stock solution (0.5mg/ml) is prepared freshly by dissolving (10mg) white to off-white sildenafil citrate crystalline powder in 20 ml distilled water. A calculated dose of sildenafil for each rat (in treatment group) was drawn in an insulin syringe then delivered to the animal via direct instillation into the stomach or lower esophagus (gavage) of a conscious rat is by far the most accurate method to administer drugs into the gastrointestinal tract. However, because this technique is performed in a conscious rat, iatrogenic injury to the esophagus and pulmonary aspiration are possible sequelae. Using adequate manual restraint, the rat was held in a vertical position with its nose aimed toward the ceiling so as to form a straight line between the rat's mouth and stomach. Next, the gavage needle was gently inserted into the oral cavity through the left diastema and passed along the roof of the oral cavity toward the ramus of the right mandible. As the animal swallows, the instrument was advanced down into the esophagus. Finally, once the needle was advanced to the appropriate depth, the solution slowly infused by depressing the plunger of the syringe **(116)**.

2.7 Animal sacrifice:

At the end of 6th week, blood collected from animals (3-4 ml) by cardiac puncture then rats euthanized by decapitation [117].

2.7.1 Serum sample preparation:

Collected blood was allowed to clot for 30 min in plane tube without an anticoagulant at 25°C. Then, blood samples were centrifuged at 2,000 x g for 15 min, and serum layers were pipetted off without disturbing the white buffy layers. Subsequently, serums were stored in frozen state for detection of interleukin 1-beta (IL-1β), total antioxidant capacity (TAC) and C-peptide.

2.7.2 Organ harvest:

After opening the abdominothoracic cavity, the liver was easily identified, and all four lobes of liver were transferred to the fixative after washing with normal slaine. The pancreas was identified by pushing the stomach upwards to reveal the spleen that was then held by a forceps with a little traction. The pancreas extended from the spleen to the duodenum along the greater curvature of the stomach. It was carefully dissected away from these organs and isolated [118]. The kidney located toward the back of the abdominal cavity on either side of the spine, dissect them by cutting longitudinally. The submandibular a lobular structure located on the sides of the neck, between muscles ventral to the parotid gland. Carefully remove the skin of the neck and face to reveal these glands.

2.8 Tissue preparation and staining

2.8.1 Tissue preparation:

After fixation with 10% buffered formalin, tissue processing will be started according to Bancroft and Stevens the tissues were processed as follows [119]: Fixation, dehydration, clearing, impregnation, embedding, sectioning, using semi-enclosed Benchtop tissue processor Leica TP1020, heated paraffin embedding

module Leica EG1150 H, cold Plate for modular tissue embedding system Leica EG1150 C, after programing according to type of staining (fig. 2-1).

Figure (2-1): Tissue processing machines.

1- Fixation:

Immediately after organs separation, organs were cut to two or three part to ensure proper infusion of fixative , prolong fixation usually yields less accurate results (false negative) and affect the tissue binding sites of the immunohisto-chemical markers used in the study.

2. Dehydration:

Tissue processor basket was transferred automatically from the fixative to the graded concentration alcohol jars at room temperature as follows:

a- Two changes of 70% ethanol, each change for 2 hours.

b- Ethanol 90% for 2 hours.

c- Two changes of absolute ethanol, each change for 2 hours.

3. Clearing:

Leica basket was transferred automatically in two change of xylene jar, 1 hour for each to remove alcohol and give the tissues some degree of transparency.

4. Wax impregnation:

Leica basket was transferred automatically in two change of molten paraffin wax (56^0C melting point), 3 hours for each to remove the clearing agent.

5. Embedding:

Embedding was done by leica paraffin embedding module, the specimens cassette were transferred from the paraffin jar of leica processor to warming tray of leica paraffin embedding module, then working area to embedding the mold, finally mold putted on cold spot and labeled. When the wax surface (in the mold) was solidified, the mold was transferred to lieca cold plat, after being solid, the blocks were kept in refrigerator at 4 c° until used.

6. Sectioning:

Leica semi-automated rotary microtome used for sectioning, after trimming, the section thickness was set to 5 µm for (H&E, PAS) and 4 µm for immunohisto-chemistry (IHC). Serial sections were taken from each block then placed into water bath, preheated to 40-44 Cº to relax the sections from compression due to sectioning then the sections were placed onto slides (positive charged for IHC).

7. De waxing and hydration:

Loading slides to holder then leave it in 70 ºC oven for 2 hour then immersed in Pre-warmed (55 ^{0}C) xylene for 5 minutes and xylene (at room temperature 20-25 ^{0}C) for 2 minutes followed by passing the slides through 1: 1 xylene to absolute

ethanol for 3 minutes then descending concentration of ethyl alcohol baths (99%, 90%, 70% and 50%) three minute for each and then washed with distilled water.

2.8.2 Tissue staining:

2.8.2.1 Hematoxylin and eosin staining:

A- Staining: Loading slides in (Leica Multistainer ST5020) after programing as follow [119]:

a- Harri's Hematoxylin solution 3 minutes.

b- Running tap water for 5 minutes.

c- Acid alcohol 5 seconds.

d- Running tap water 1minute.

e- Eosin 1% solution for 2 minutes.

f- Running tap water for 5 minutes.

g- Dehydrated in graded alcohol (70%, 80%, 90% and 99%) 1minute for each, cleared in xylene (2-3 minutes).

Dislodging slides from Leica multistainer and mounted by DPX mounting medium.

B- Microscopical study:

A digital microscope System with Leica DM4000 B LED was used to capture five zones of a slide (corners and the center) which randomly at X400 magnification were selected.

2.8.2.2 Liver periodic acid schiff (PAS) stain:

Liver sections were stained with periodic acid schiff (PAS) for histochemical detection of glycogen [120].

A- Principle:

The PAS stain is a histochemical reaction in that the periodic acid oxidizes the carbon to carbon bond forming aldehydes which react to the fuchsin-sulfurous acid which form the magenta color. PAS Stain Kit (Atom Scientific, UK) was used and the manufacturer's protocol was a modified McManus protocol as follows:

a- De waxing and rehydration of slides section.

b- Sections were oxidized in 0.5% periodic acid solution for 5 minutes and then washed in several changes of distilled water.

c- The slides were placed in Schiff reagent solution for 20 minutes at room temperature (sections became light pink color during this step) and then washed in lukewarm tap water for 5-10 minutes (Immediately sections turned dark pink/magenta in color).

d- Counterstaining in haemalum Mayer's haematoxylin was then performed for 5 minutes. Slides were washed in tap water for 5 minutes, differentiated in 0.5% acid alcohol and then blued in tap water for two minutes.

e- Slides were finally dehydrated in graded alcohol (70%, 80%, 90% and 99%) one minute for each, cleared in xylene (2-3 minutes) and mounted.

B- Assessment of hepatic glycogen content:

A digital microscope system with Leica DM4000 B LED was used to capture five zones of a slide (corners and the center) which randomly at X400 magnification were selected PAS stained liver sections were examined for glycogen content by Adobe Photoshop CC14 **(121)**.

Adobe Photoshop digital subtraction techniques were utilized to facilitate assessment of hepatic glycogen content. First, all central veins and interlobular vascular spaces were selected and subtracted from the digital image using the commands (magic wand, feather, threshold and cut). Then, the area of interest (PAS positive color) was selected using the magic wand tool and the whole “of interest” surface area was calculated using the record measurement command (fig.2-2).

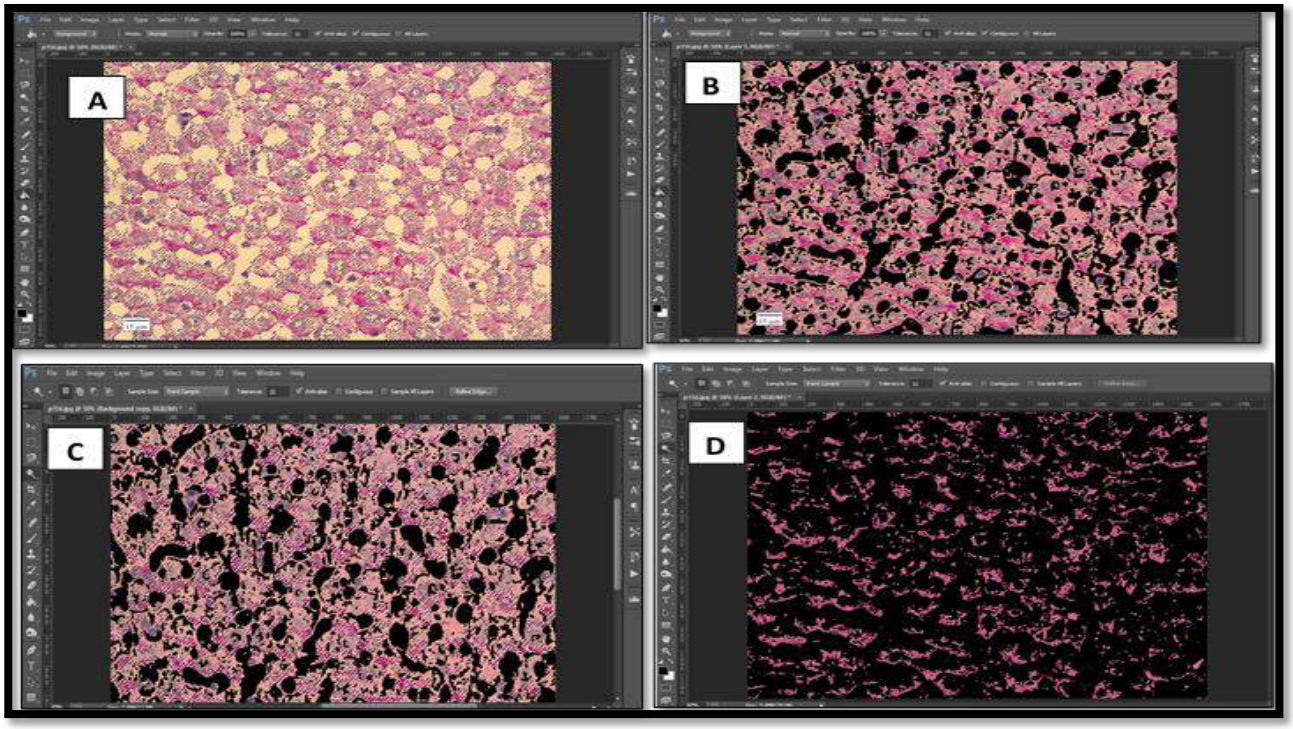

Figure (2-2): Selection (A) & subtraction (B) of central vein and interlobular vascular spaces followed by selection (C) and calculation of surface area (D) of area of interest (PAS positive signal of hepatic glycogen) using adobe Photoshop software.

2.8.2.3 Immunohistochemistry staining:

A- Principle of the test:

A biotin free-horseradish peroxidase conjugate, 3-3'diaminobenzidine HRP/ DAB system is intended for use with primary antibody from rabbit or mouse for the qualitative identification of antigens by light microscopy and immunohistochemistry in formalin fixed and paraffin embedded tissues. After application of primary antibody which bind to specific antigens present in the specimen, any excess antibody is removed by washing, followed by sequential incubation with a horseradish peroxidase conjugate to the primary anti body and unbound enzyme is removed by washing. Staining is completed and the brown end product is formed after incubation with the substrate-chromogen (3-3'diaminobenzidine) (DAB) and it is oxidized when it donates electrons to activate the HRP/H_2O_2 reaction [(119)].

B- Immunohistochemistry procedure: involve the following steps [119]:

1- After de waxing and rehydration slides section, slides holder changed in the water until ready to perform antigen retrieval. After this point the slides section should not allowed to dry.

2- Retrieval jar filled with antigen retrieval solution (Sodium Citrate buffer (PH 6)).

Sodium Citrate Buffer (for antigen retrieval):
Tri-sodium citrate (dihydrate) (2.94 g) dissolve in 1000 ml of distilled water.
1NHCl added to adjust pH to (6), finally 0.5 ml of Tween 20 mixed.

3- Retrieval jar kept in preheated water path (100^0 C) for 30 min.

4- Loading slides in retrieval jar, preserved 20 min. Less than 20 minutes may leave the antigens under retrieved, leading to weak staining. More than 20 minutes may leave them over-retrieved, leading to nonspecific background staining and also increasing the chances of sections dissociating from the slides. Then slides removed to holder in distal water.

5- Hydrophobic barrier circle drawn surrounded tissue sections by pap pen hold aqueous solutions within a defined area, reducing the use of excess reagents.

6- In the humid chamber slides stuffed then enough drops of peroxidase blocking reagent was placed onto the sections and incubated for 15 minutes.

7- Phosphate buffered saline used twice for wishing the slides then then drained and wipe around the sections with tissue paper gently, recollected slides to humid chamber.

8- Tow drops of protein-blocking reagent was placed onto the section and incubated for 10 minutes in a humid chamber. Then slides were drained and blotted gently.

9- One drops of diluted primary antibody was placed onto the section and incubated for 1 hour at 37 ^{0}C in a humid chamber. After incubation, the slides were drained and blotted gently.

10- Slides were rinsed twice with PBS, then drained and wipe around the sections with tissue paper gently.

11- Tow drops of secondary antibody, HRP (horseradish peroxidase), was placed on to the section and incubated for 10 minutes at 37^0C in a humid chamber. Slides were drained and blotted gently.

12- Slides were rinsed twice with PBS, then drained and wipe around the sections with tissue paper gently.

13- Tow drops of freshly prepared substrate – chromogen solution was placed on to sections and incubates for 10 minutes at 37 Cº in humid chamber.

14- Loading slides in slides holder, kept holder for 4 mints in slowly running tab water.

15- Slides stuffed in humid chamber.

16- Tow drops of Hematoxylin was placed onto the section an incubated for three minutes.

17- Slides reloaded in holder then rinse in slowly running tab water.

18- Slides holder changed into distilled water jar then dehydrated by placing them in ethanol and xylene in the following order:

a- 70% ethanol for 3 minutes.

b- 95% ethanol for 3 minutes.

c- Absolute ethanol for 3 minutes.

d- 1:1 Xylene to absolute ethanol for 3 minutes.

e- Xylene for 3 minutes.

f- Fresh xylene for 3 minutes.

19- Two drops of mounting medium distyrene (a polystyrene), a plasticiser (tricresyl phosphate), and xylene (DPX) was placed onto the xylene-wet section then covered with a cover slip.

C- Quality control:

1- Positive control: The same sample of normal tissue was included for each antibody for all immunohistochemical staining as a positive control.

2- Negative control: negative control slides used to check specificity of staining by omitting primary antibody and applying antibody diluents alone. This was taken under identical test condition (in the same slide).

D- Assessment of immunohsitochemical stain uptake:

1- A digital microscope System with Leica DM4000 B LED was used to capture five zones of a slide (corners and the center) which randomly at X400 magnification were selected.

2- Glycogen phosphorylase and EGF immunohistochemical stain uptake by tissues was assessd using Image J software [(122)]. All scaled images were stacked in RGB mode (Red, Green, and Blue). The stain of interest was selected using the hue, saturation threshold. The selected area was then inverted to black and the number and cumulative surface areas of interest were calculated using the "record measurement" command (fig.2-3).

3- For EGF, membranous and cytoplasmic brown staining was notable. Qualitative estimation of immunostaining of EGF positive cells was graded as follow:

a- Undetectable = - ve;

b- Weak = +ve;

c- Strong = 2+ve;

d- Very strong or intense = 3+ve.

This classification according to positive cell number: (-), 0-5% of the positive cells; (+), 5-50% of positive; (2+), 50-75% of positive; (3+), 75-100% of positive [(123)]. The final IHC score value was obtained by multiplying the number of extent and intensity [(124)].

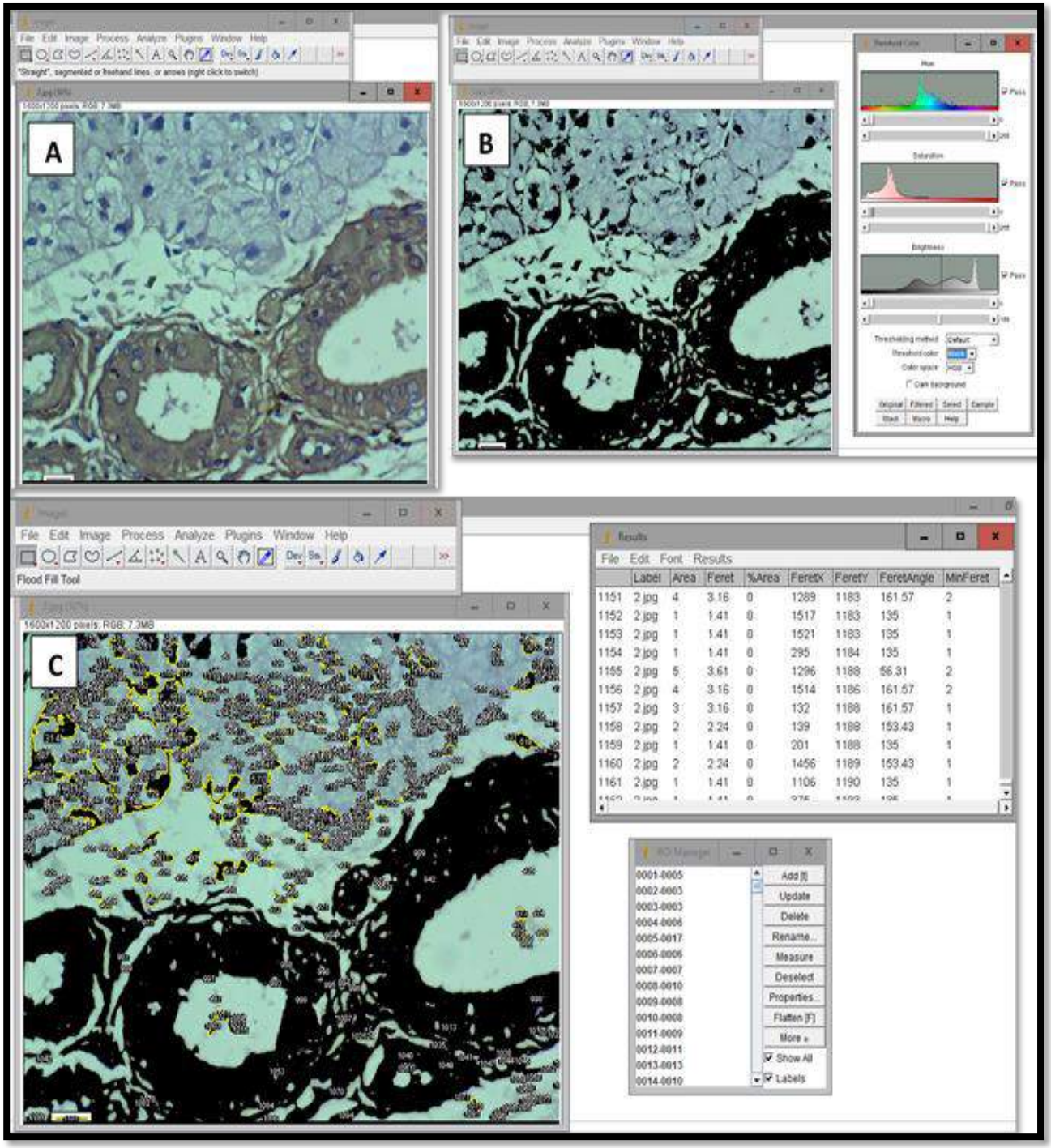

Figure (2-3): Scaling and RGB stacking of images (A) followed by selection & inversion of areas of interest (B) and finally calculating the numbers & surface area (C) using Image J software.

4- For Liver glycogen phosphorylase assessment surface areas of interest were calculated and used for comparison.

2.9 Detection of serum rat Interleukin-1 beta (IL-1β):

Detection of rat IL-1β performed according to manufacturer's protocol use enzyme-linked immunosorbent assay technique (sandwich ELISA) [125]. Preparing 96-well plate coated with an antibody specific for IL-1β. Pipetting samples and standards into the wells and IL-1β present in a sample is bound to the wells by the immobilized antibody. After washing the wells, biotinylated anti-rat IL-1β antibody is added. The wells are washed away unbound biotinylated antibody, then HRP-conjugated streptavidin is added to the wells. Again the wells are washed, a TMB substrate (3,3',5,5'-tetramethylbenzidine) solution is pipetted to the wells and color develops in proportion to the amount of IL-1β bound. After stop solution the color changes from blue to yellow (fig. 2-4), and the intensity of the color is measured at 450 nm.

2.10 Detection of serum total anti-oxidant capacity (TAC):

According to manufacturer's protocol, total antioxidant capacity can measure both small molecule antioxidants (ascorbate, uric acid, GSH, vitamin E, etc.) and proteins (GSH reductase, catalase, peroxidase albumin, transferrin, etc.) [126]. Both small molecule and protein convert copper ion (Cu^{2+}) to Cu^{+} by. Chelating Cu^{+} ion with a colorimetric probe giving a broad absorbance peak around 570 nm, proportional to the total antioxidant capacity. Trolox is used as standard to antioxidants, so antioxidants capability of biological fluids being measured in Trolox equivalents.

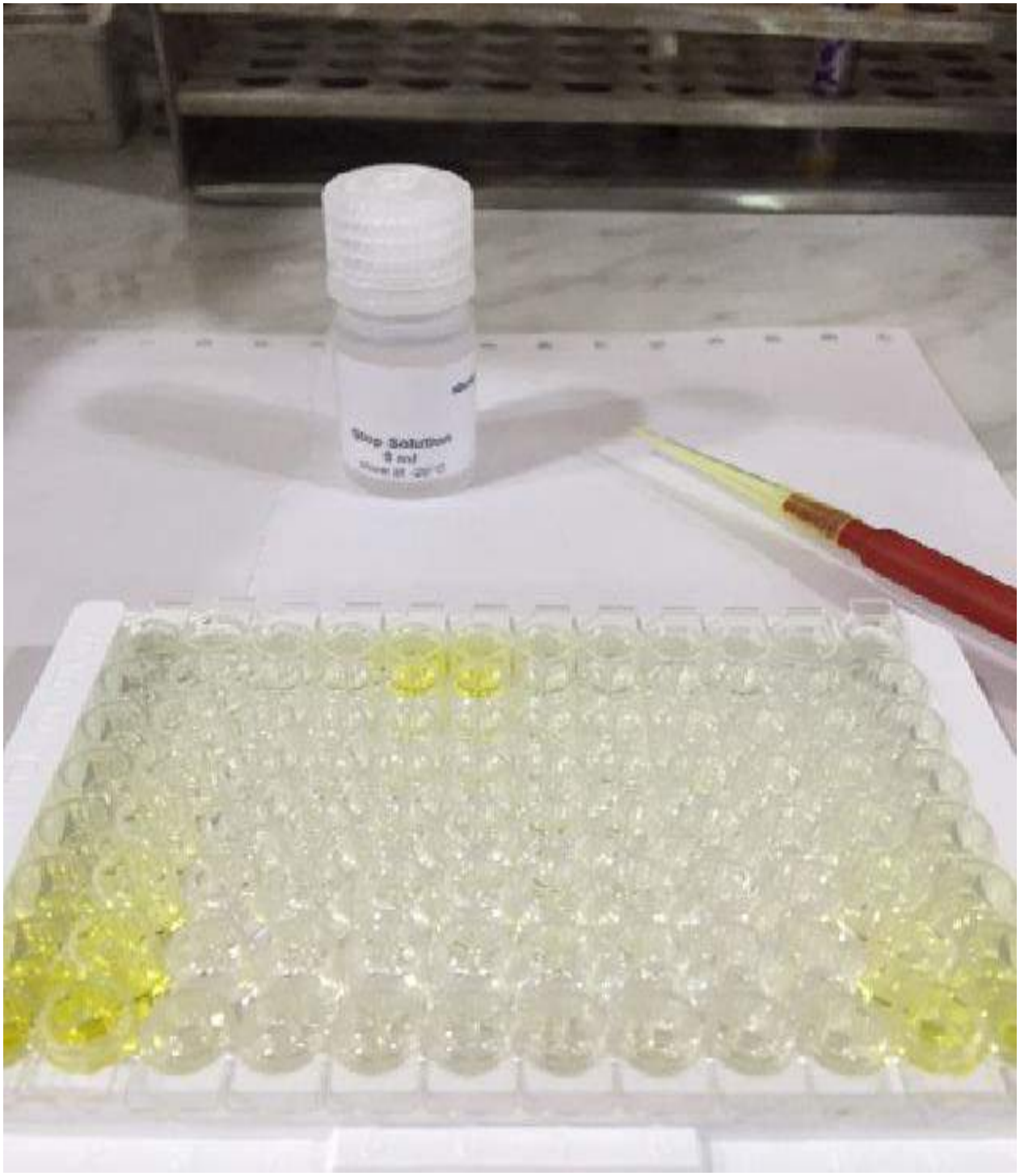

Figure (2-4): Micro wells plate after adding stop solution.

2.11 Detection of serum rat C-peptide level:

C-peptide level was measured by using electro-chemi-luminescence (ECL) immunoassay detection technology based on applying a voltage to the sample solution resulting in a precisely controlled reaction, using Cobas analyzer from Roche [127, 128]. This test applied by Al nadaer clinical laboratory.

2.12 Statistical analysis

The statistical package of SPSS-22 (Statistical Packages for Social Sciences-version 22) used for analysis of data. Data were presented as mean ± standard deviation. The significance of difference of different means were tested using **independent -t-test** for difference between two independent means , **paired-t-test** for difference of paired observations (or two dependent means), and **ANOVA** test for difference among more than two independent means. **Pearson correlation** was calculated for the correlation between two quantitative variables with its t-test for testing the significance of correlation. The correlation coefficient value (**r**) either positive (direct correlation) or negative (inverse correlation) with value < 0.3 represent no correlation, 0.3 ─ 0.5 represent weak correlation, 0.5 ─ 0.7 moderate strength, > 0.7 strong correlation. Statistical significance was considered whenever the P value was less than 0.05 and highly significant if it was less than 0.01 [129].

Chapter Three

Results

3.1 Effect of various doses of sildenafil citrate on body weight:

The statistical values of rats body weight represented as mean ± SD in control and treated groups with sildenafil are summarized in table (3-1).

The mean body weight of rats in group A was initially (226.3±12.9), but after six weeks of treatment with an oral dose of sildenafil (0.5 mg/kg/day), they show a high significant decrease (P= 0.0002), where their mean weights were (202±20.7) (table 3-1).

A high significant reduction (P= 0.00003) in body weight was also observed in group B which treated with 1 mg / kg / day oral sildenafil in which their initial mean weight was (230 gm ±13.1) and after six weeks of treatment the mean weight became (197.4 gm ±20.6) (table 3-1).

In addition, rats of group C with initial mean body weight (230.4 gm ±10.9) showed a high significant reduction (P= 0.001) when treated with 2 mg / kg / day oral sildenafil where the mean weight became (209.2 gm ±12.6) (table 3-1).

Meanwhile, those rats in group D showed a mean weight of (220.9 gm ± 12.1), in which after six weeks of oral saline treatment their mean weight was (233.6 gm ±13.8) which highly significant increase (P= 0.000) when compared with baseline (table 3-1).

In comparison of all groups to control group (group D) there was highly statistical difference using ANOVA test where P = (0.0007).

Table (3-1): Changes in body weight (gm). Data are expressed as mean±S.D.

Group	Initial weight (gm)	Final weight (gm)	% changes in weight (gm)
Group A	226.3±12.9	202±20.7 **	-10.9±6 [b, **]
Group B	230±13.1	197.4±20.6 **	-14.5±5.3 [a, c, **]
Group C	230.4±10.9	209.2±12.6 **	-9.7±3.5 **
Group D	220.9±12.1	233.6±13.8 **	5.7±2.3
P-value (ANOVA)			**0.0007**

** High significant difference of paired T-test at P<0.01when compared final weight with initial weight.
** High significant difference of independent T- test when compared with control at P<0.01.
[a] Significantly different compared with group A at P <0.05.
[b] Significantly different compared with group B at P <0.05.
[c] Significantly different compared with group C at P <0.05.

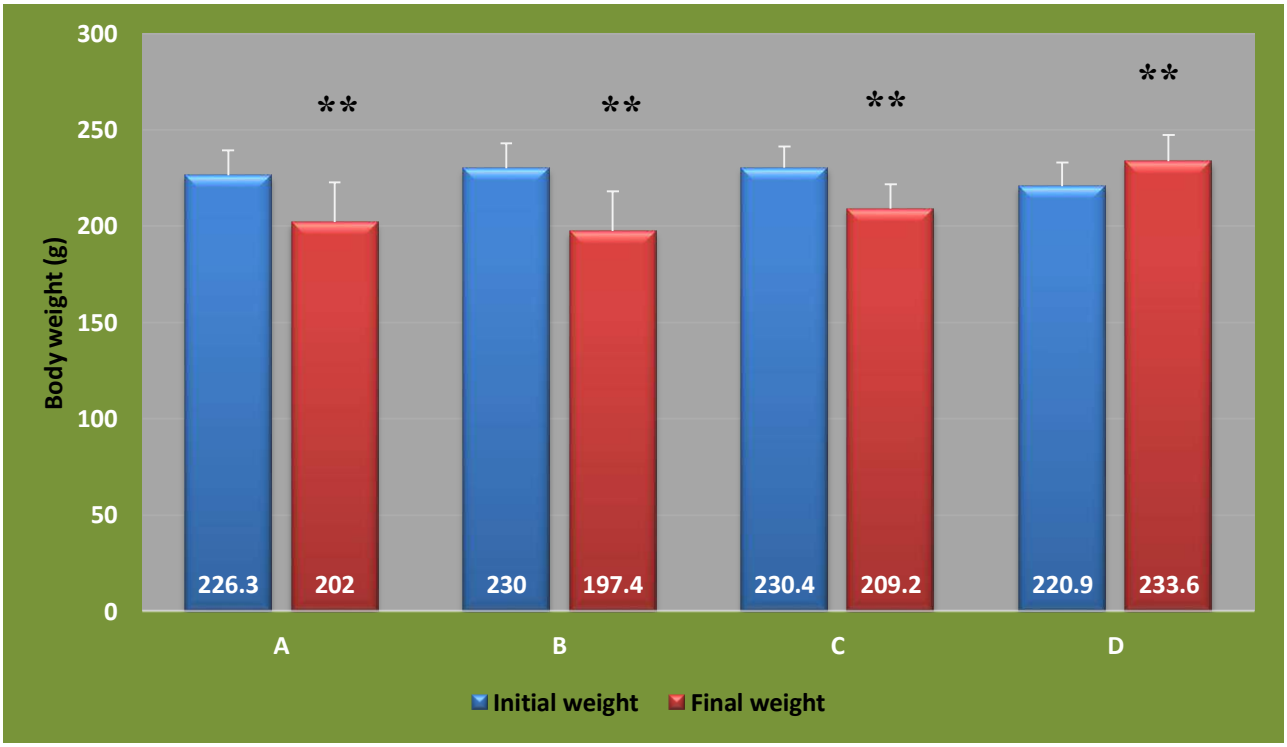

Figure (3-1): The difference in the body weight before and after sildenafil treatment in all groups. ** = P < 0.01 refer to highly significant compared with initial weight.

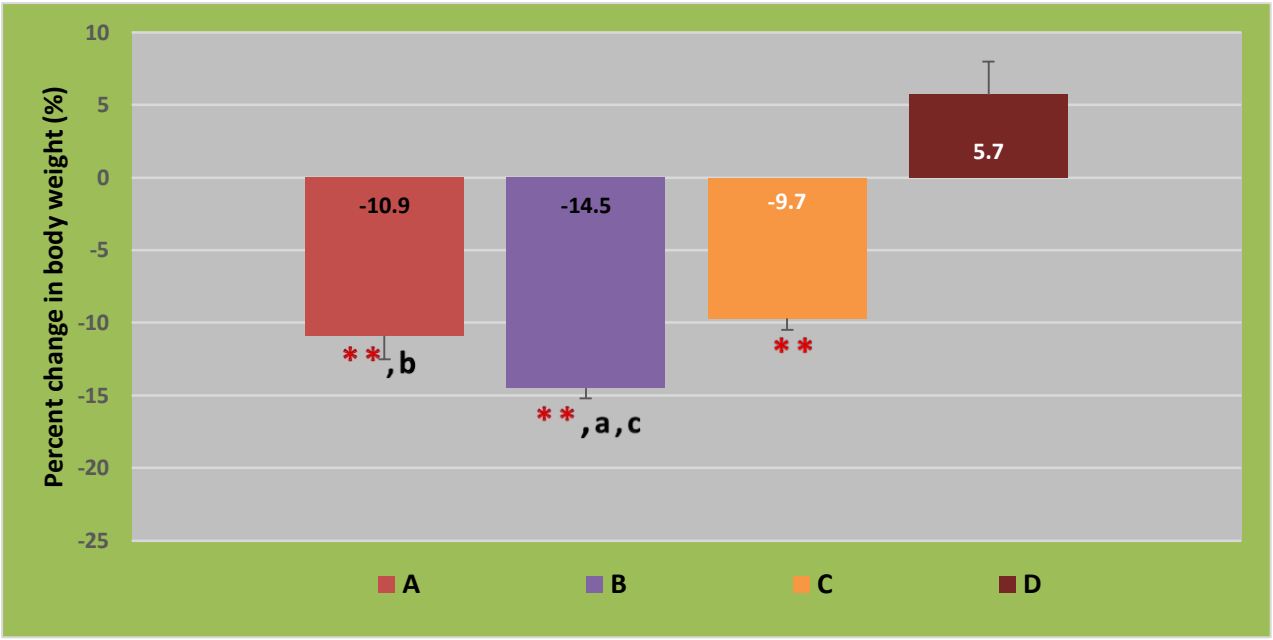

Figure (3-2): The percent of changes (increase and decrease) in body weight among all groups. ** High significant difference of independent T- test when compared with control at P<0.01; [a] significantly different compared with group A at P<0.05; [b] significantly different compared with group B at P<0.05; [c] significantly different compared with group C at P<0.05.

3.2 Effect of various doses of sildenafil citrate on liver:

3.2.1 Effect on hepatic glycogen content:

The descriptive statistics, which represent the mean ± SD value for the score of hepatic glycogen content (area %) expression in control and treated with sildenafil groups are displayed in table (3-2).

Table (3-2): Changes in liver glycogen content (area %). Data are expressed as mean ± SD.

Group	Liver glycogen content (%) after sildenafil treatment
A	23.1±1.8 [b, c, **]
B	34.5±2.1 [a, c, **]
C	65.8±2.7 [a, b, **]
D	20.5±1.6
P-value (ANOVA)	**0.0001**

** High significant difference of independent T- test when compared with control at P<0.01.
[a] Significantly different compared with group A at P<0.05.
[b] Significantly different compared with group B at P<0.05.
[c] Significantly different compared with group C at P<0.05.

Analysis of data statistically by independent T-test revealed a significant difference ($P< 0.05$) between each two treated groups.

Independent T-test from control for each treated group shows that the group A glycogen content (fig. 3-4) after six weeks treatment of orally 0.5 mg/kg/day sildenafil was (23.1±1.8%) which is highly statistical different ($P< 0.01$) than glycogen content (20.5±1.6%) of control group D (fig. 3-3).

While the mean of glycogen content of group B (fig. 3-5) after oral sildenafil treatment 1 mg/kg/day for six weeks was (34.5±2.1%), that highly significant increase in total surface area of glycogen content than control group D ($P< 0.01$) (fig. 3-3).

In addition, after six weeks of orally sildenafil treatment 2 mg/kg/day, highly significant increase in total surface area of glycogen content (65.8±2.7%) of group C (fig.3-6) detected when compared to group D at ($P< 0.01$) (fig. 3-3).

In comparison of all groups to control group there was highly statistical significant using ANOVA test where $P= 0.0001$ (table 3-2).

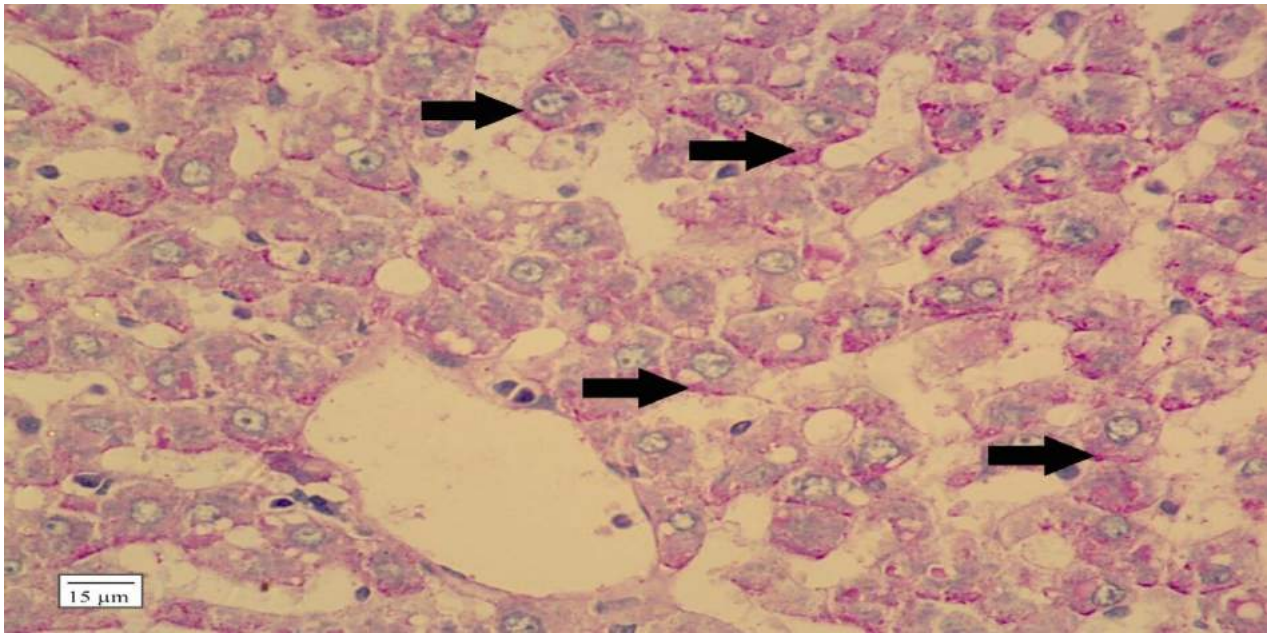

Figure (3-3): Microphotograph of periodic acid–schiff (PAS) staining of rat liver of control group D showing glycogen content of hepatocyte. X400. Black arrow refer to glycogen distribution in hepatocyte.

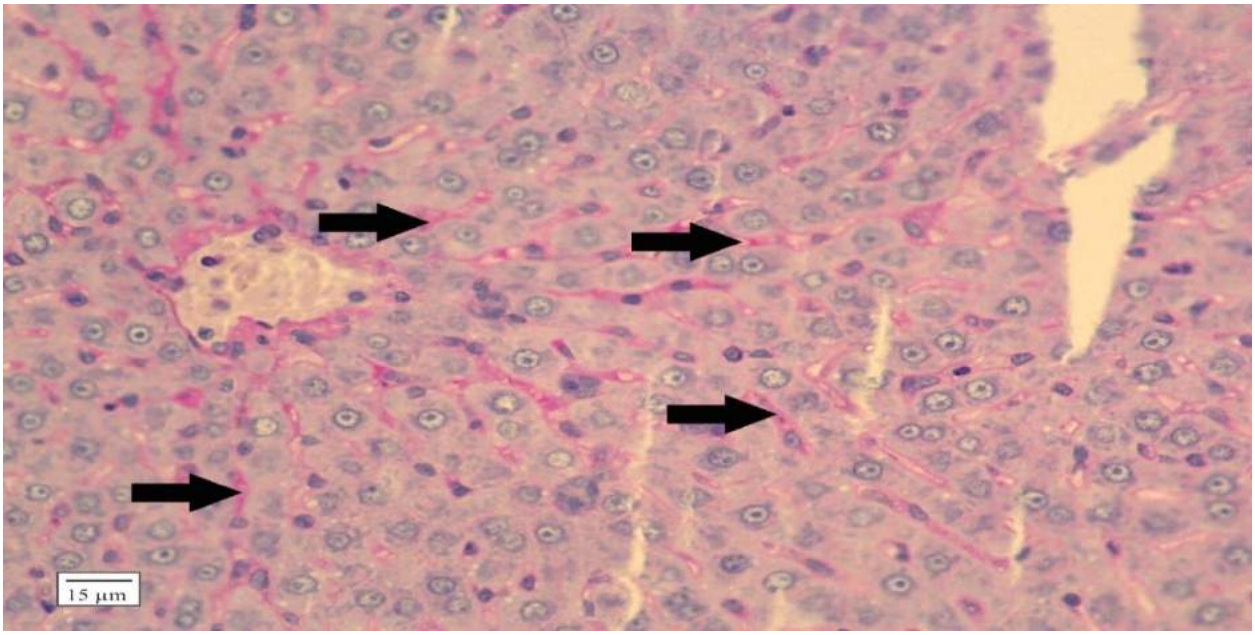

Figure (3-4): Microphotograph of periodic acid–schiff (PAS) staining of rat liver of group A after 0.5 mg/kg/day sildenafil six weeks treatment showing increasing in glycogen content of hepatocyte. X400. Black arrow refer to glycogen distribution in hepatocyte.

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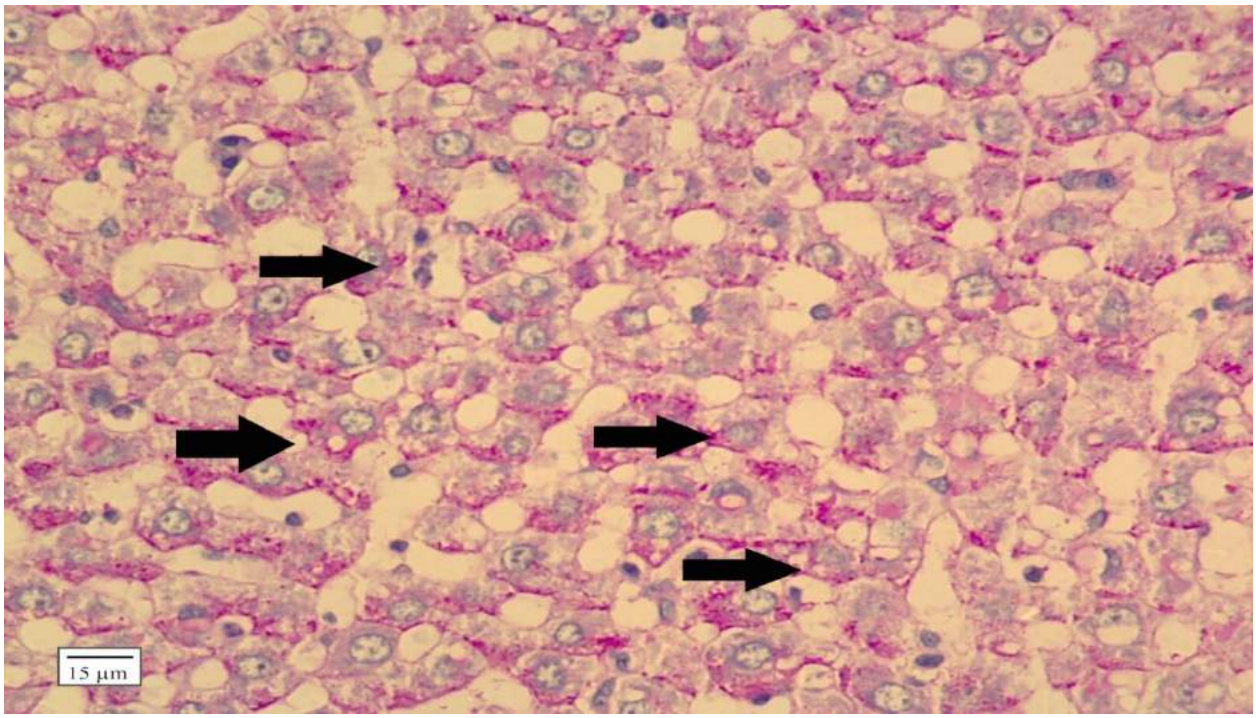

Figure (3-5): Microphotograph of periodic acid–schiff (PAS) staining of rat liver of group **B** after 1 mg/kg/day sildenafil six weeks treatment showing increasing in glycogen content of hepatocyte. X400. Black arrow refer to glycogen distribution in hepatocyte.

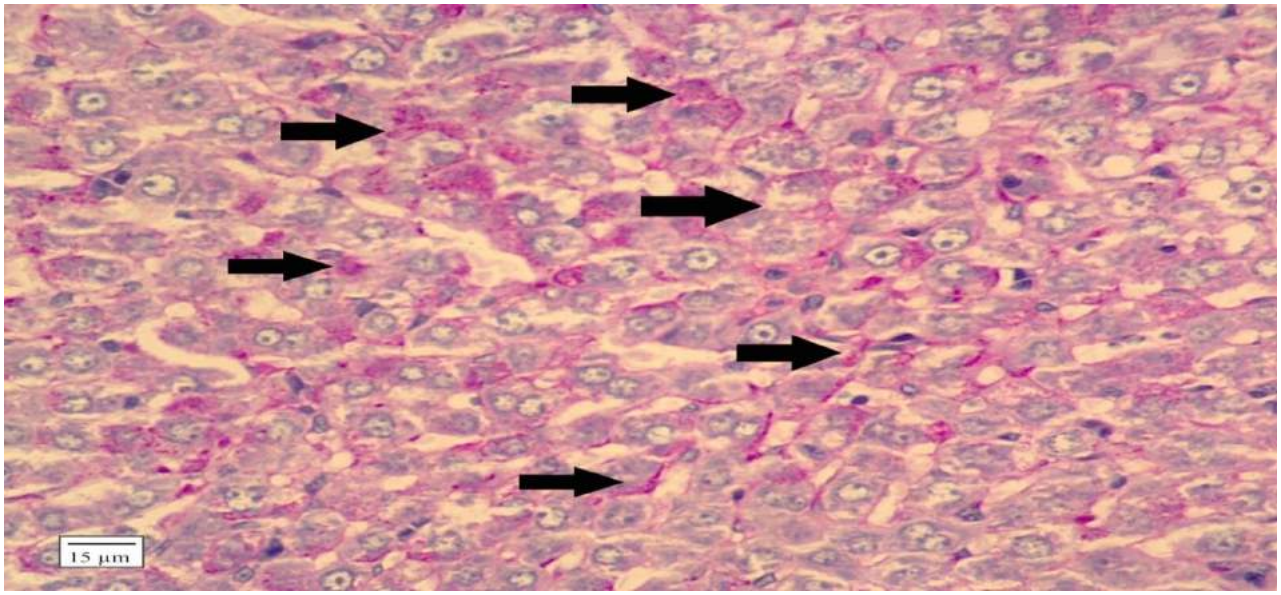

Figure (3-6): Microphotograph of periodic acid–schiff (PAS) staining of rat liver of group **C** after 2 mg/kg/day sildenafil six weeks treatment showing increasing in glycogen content of hepatocyte. X400. Black arrow refer to glycogen distribution in hepatocyte.

3.2.2 Effect on liver glycogen phosphorylase (GP) activity:

Glycogen phosphorylase data expressed as mean ± SD of (area %) staining score in rats livers. Independent T-test between each treated groups showed a significant difference at ($P< 0.05$), which summarized in table (3-3).

Table (3-3): Changes in liver GP content (area %). Data are expressed as mean ± SD.

Group	Liver glycogen phosphorylase (%) after sildenafil treatment
A	86.6±1.5 [b, c]
B	76.9±1.8 [*, a, c]
C	63±1.7 [**, a, b]
D	89.3±19.1
P-value (ANOVA)	**0.00005**

* Significant difference of independent T- test when compared with control at P<0.05.
** High significant difference of independent T- test when compared with control at P<0.01.
[a] Significantly different compared with group A at P<0.05.
[b] Significantly different compared with group B at P<0.05.
[c] Significantly different compared with group C at P<0.05.

Statistical analysis by independent T-test from control group D (89.3±19.1%) show the following:

- No statistical significant changing of liver glycogen phosphorylase expression in group A (86.6±1.5%) after six weeks (0.5mg / kg / day) oral sildenafil treatment (fig.3-8) when compared to control group D (fig.3-7) where P= 0.64.
- While the mean of GP expression in group B (76.9±1.8%) revealed significant decrease after six weeks of oral sildenafil treatment (1 mg/kg/day) (fig.3-9) when compared to control group D (fig.3-7) where P = 0.03.
- There was highly significant decrease (P< 0.01) when the control group D (fig.3-7) was compared with group C after six weeks of 2 mg/kg/day orally sildenafil treatment (fig.3-10) where GP content in hepatic tissue was (63±1.7%).

All groups when compared to control group (group D) using ANOVA test, had highly statistical significant where P= 0.00005.

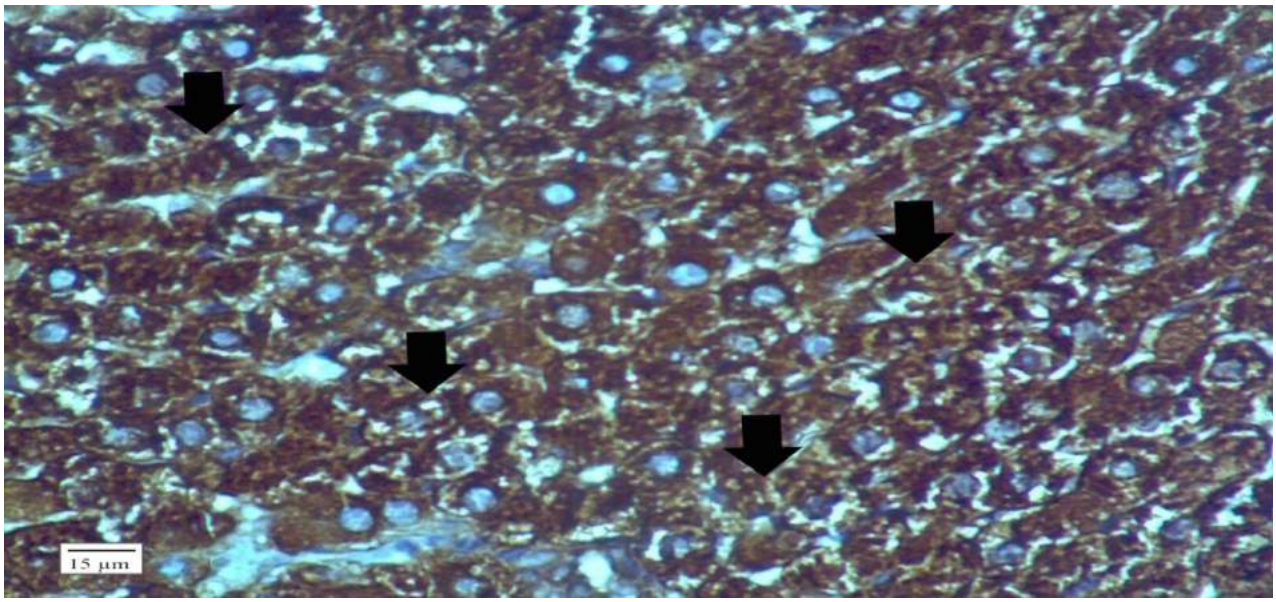

Figure (3-7): Microphotograph of Immunohistochemical (IHC) staining of Glycogen phosphorylase in liver of control **D** group show diffuse reactivity of hepatocyte cytoplasm against GP. X400. Black arrow refer to GP distribution in hepatocyte.

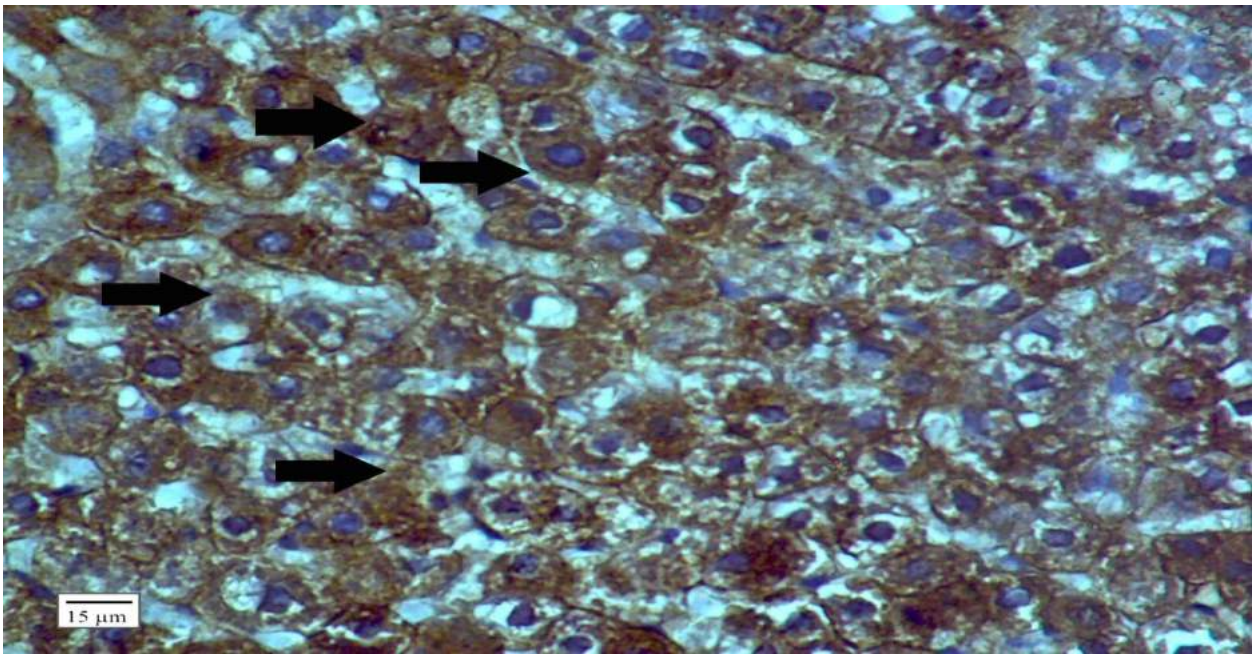

Figure (3-8): Microphotograph of Immunohistochemical (IHC) staining of liver GP in group **A** after 0.5 mg/kg/day of sildenafil six weeks treatment, showing cytoplasmic reaction of hepatocyte against GP less than control group. X400. Black arrow refer to GP distribution in hepatocyte.

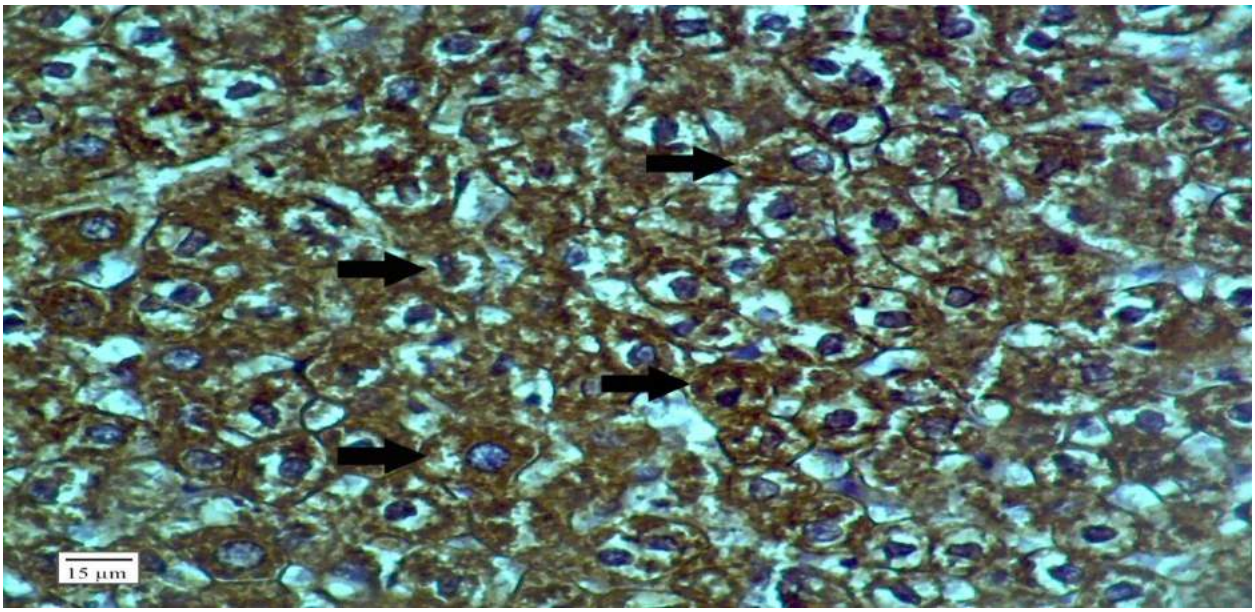

Figure (3-9): Microphotograph of Immunohistochemical (IHC) staining of liver GP in group **B** after 1 mg/kg/day of sildenafil six weeks treatment, showing cytoplasmic reaction of hepatocyte against GP less than control group. X400. Black arrow refer to GP distribution in hepatocyte.

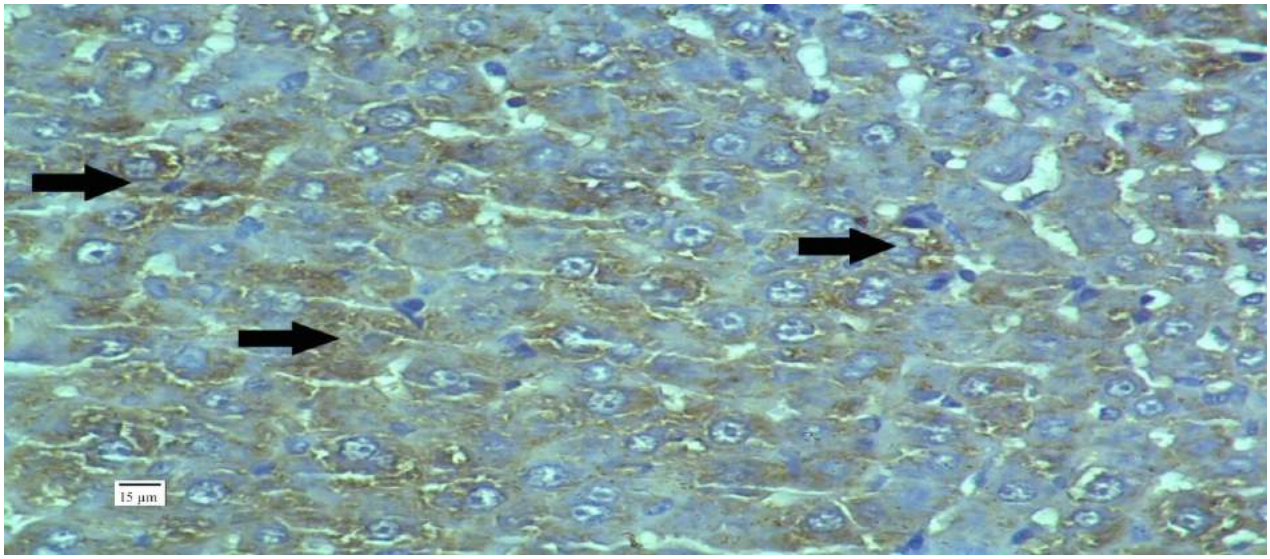

Figure (3-10): Microphotograph of Immunohistochemical (IHC) staining of liver GP in group **C** after 2 mg/kg/day of sildenafil six weeks treatment, showing cytoplasmic reaction of hepatocyte against GP less than group **A** and **B**. X400. Black arrow refer to GP distribution in hepatocyte.

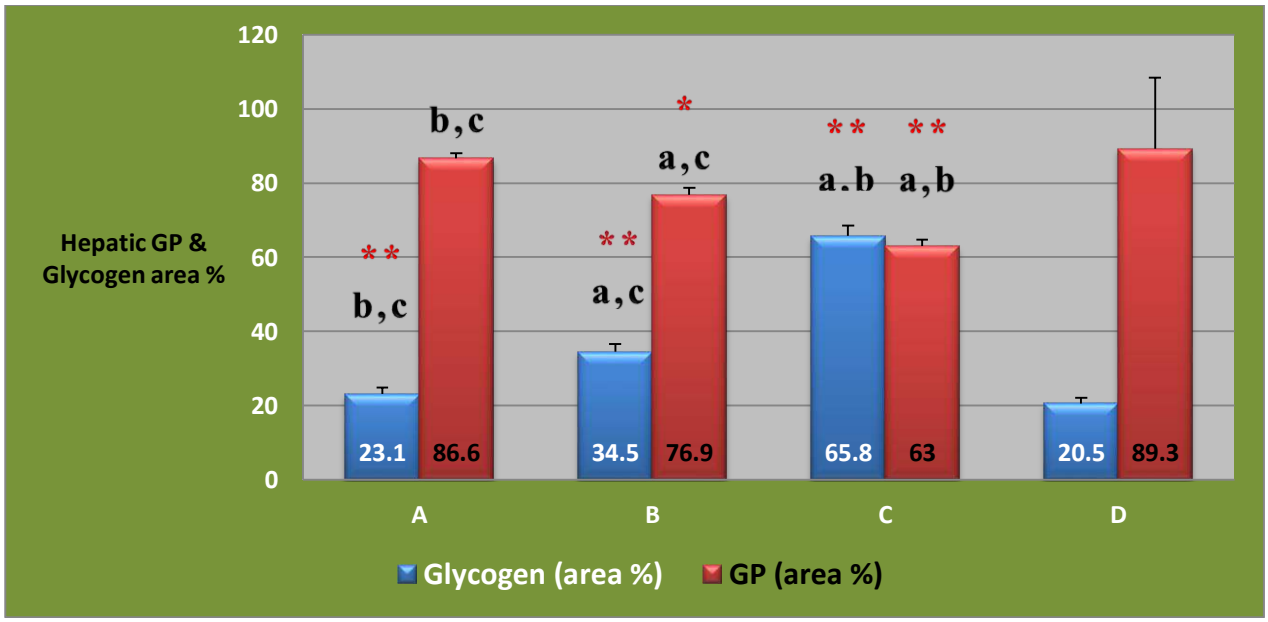

Figure (3-11): The changes in glycogen content and glycogen phosphorylase of the liver after sildenafil treatment in all groups. * = P < 0.05 refer to statistically significant when compared with control; ** = P < 0.001 refer to highly significant when compared with control; [a] Significantly different when compared with group A at P < 0.05. [b] Significantly different when compared with group B at P<0.05; [c] Significantly different when compared with group C at P<0.05.

3.3 Effect of various doses of sildenafil citrate on epidermal growth factor (EGF):

The descriptive statistics, which represent as mean ± standard deviation for the score of EGF in kidneys, pancreas and submandibular gland of sildenafil treated groups of rats and control group, are expressed in table (3-4).

Table (3-4): Changes in epidermal growth factor (EGF) scores. Data are expressed as mean ± S.D.

Group	Kidney	Pancreas	Submandibular gland
A	0.88 ± 0.29 **, b, c	0.75 ± 0.48 **, b, c	0.93 ± 0.27 **, b, c
B	1.3 ± 0.17 **, a, c	1.52 ± 0.67 **, a, c	1.32 ± 0.30 **, a, c
C	2.04 ± 0.35 **, a, b	2.12 ± 0.51 **, a, b	1.74 ± 0.31 **, a, b
D	0.73 ± 0.12	0.26 ± 0.28	0.51 ± 0.07
P-value (ANOVA)	**0.000**	**0.000**	**0.000**

** High significant difference of independent T- test when compared with control at P<0.01;
[a] Significantly different when compared with group A at P<0.05;
[b] Significantly different when compared with group B at P<0.05;
[c] Significantly different when compared with group C at P<0.05;

3.3.1 The effect on EGF score in the kidney:

The mean EGF score of group A after six weeks of 0.5 mg/kg/day oral sildenafil treatment (fig.3-14) was (0.88±0.29), therefore high significant increase (P<0.01) in EGF in group A when compared to control group which administered saline orally for same duration was detected.

When comparing EGF score of group B (1.3±0.17) with control group, a high significant increase (P<0.01) noticed after six weeks of oral sildenafil treatment 1mg /kg/day (fig.3-15). Treatment of rats in group C (fig.3-16) with orally sildenafil 2mg

/kg/day for six weeks revealed highly significant increment (P<0.01) of EGF score when compared to control group (fig.3-13).

When comparing all groups score to control group, there were highly statistical significant where P = 0.000.

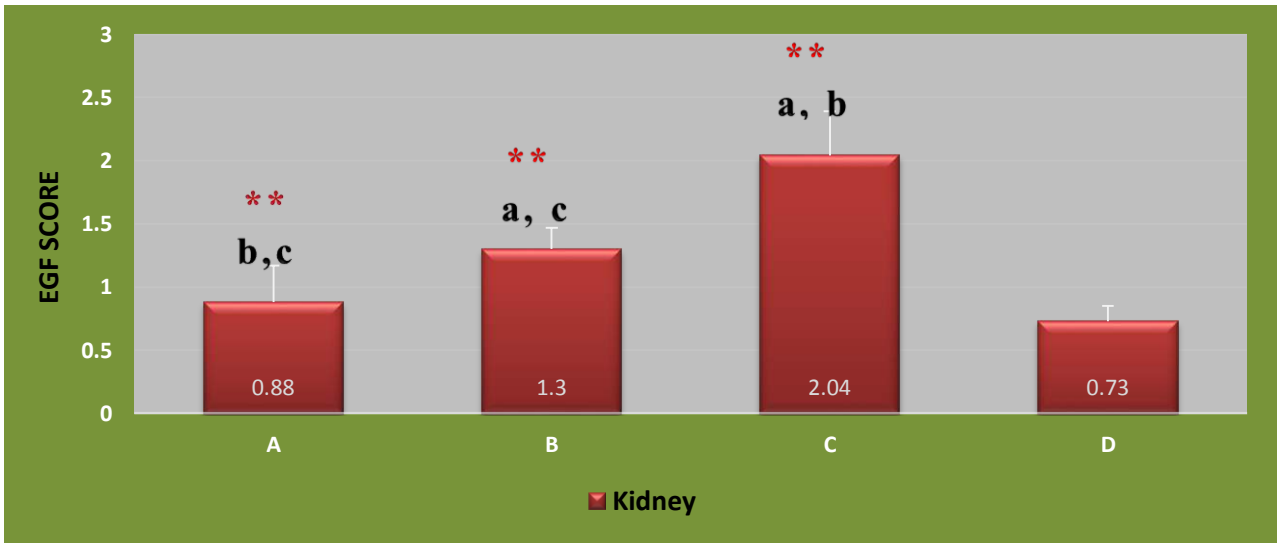

Figure (3-12): EGF changes in kidney after sildenafil treatment in all groups. * = P < 0.05 refer to statistically significant when compared with control; ** = P < 0.01 refer to highly significant when compared with control; [a] Significantly different when compared with group A at P < 0.05. [b] Significantly different when compared with group B at P < 0.05; [c] Significantly different when compared with group C at P < 0.05.

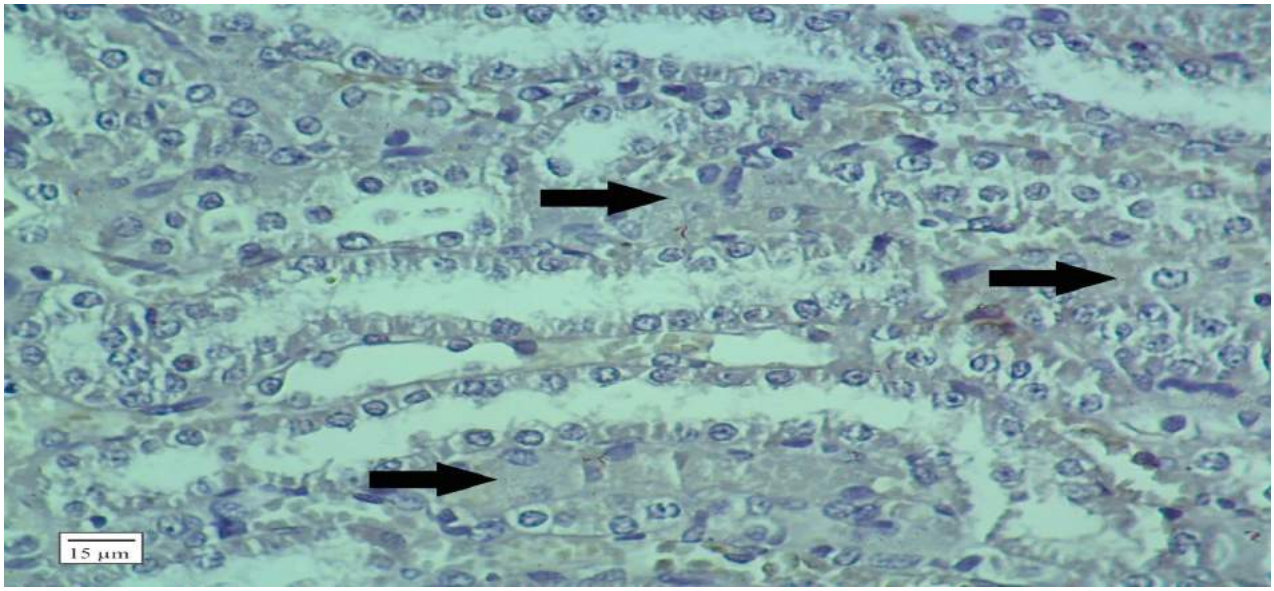

Figure (3-13): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in kidney (medulla) of control group **D** showing reactivity against EGF. X400. Black arrow refer to cytoplasmic EGF.

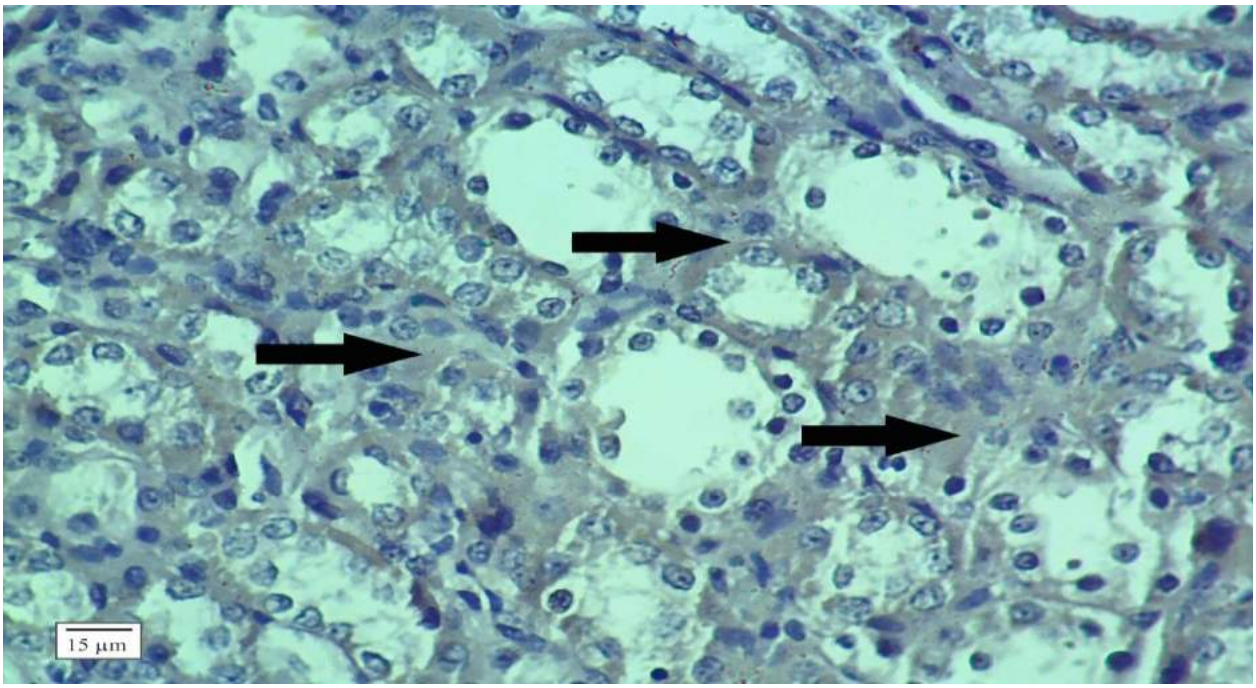

Figure (3-14): Microphotograph of immunohistochemical (IHC) staining of Epidermal growth factor (EGF) in kidney (medulla) of group **A** after 0.5 mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF.X400. Black arrow refer to cytoplasmic EGF.

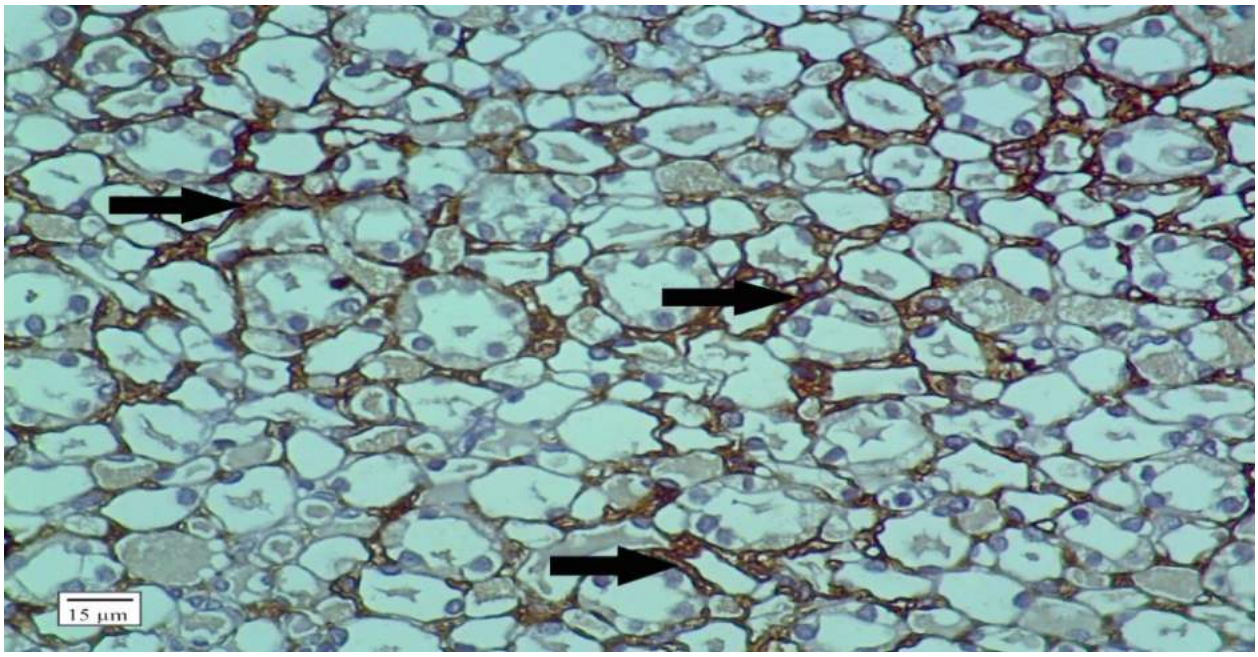

Figure (3-15): Microphotograph of immunohistochemical (IHC) staining of Epidermal growth factor (EGF) in kidney (medulla) of group **B** after mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF.X400. Black arrow refer to cytoplasmic EGF.

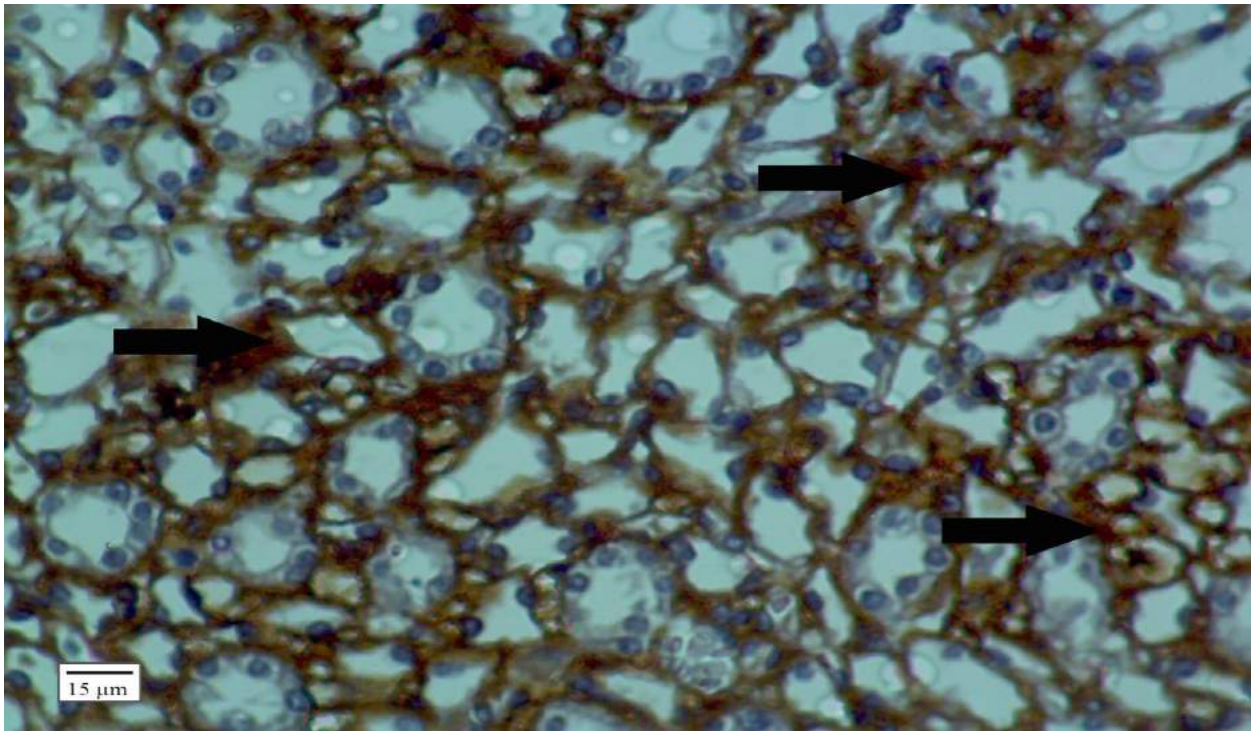

Figure (3.16): Microphotograph of immunohistochemical (IHC) staining of Epidermal growth factor (EGF) in kidney (medulla) of group C after 2 mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF. X400. Black arrow refer to cytoplasmic EGF.

3.3.2 The effect on EGF score in the pancreas:

The mean score of EGF in group D after six weeks of oral saline (fig.3-18) was (0.26±0.28), while the mean score of EGF in group A (fig.3-19) after six weeks of oral sildenafil treatment 0.5mg / kg / day was (0.75±0.48), consequently there was highly significant statistical increment ($P<0.01$) in EGF score when compared to group D.

Meanwhile the mean score of EGF in group B (fig.3-20) after orally sildenafil for six weeks 1mg/ kg/ day was (1.52±0.67) with highly significant increase when compared to group D.

Additionally high statistical significant increment ($P<0.01$) in score of EGF in group C (fig.3-21) when compared to group D after 2mg / kg / day orally sildenafil treatment.

All three groups had significant difference in EGF score when compared to control group where P = 0.000.

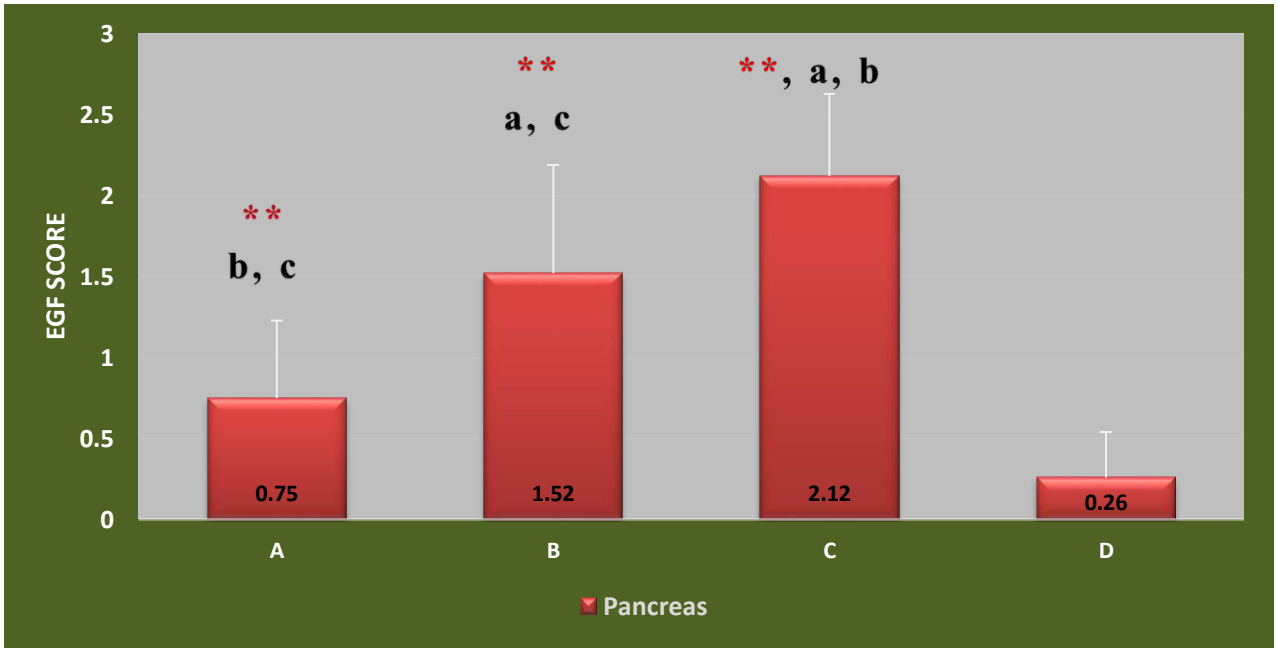

Figure (3-17): Changes of epidermal growth factor (EGF) in pancreas after sildenafil treatment in all groups. * P < 0.05 refer to statistically significant when compared with control; ** P < 0.01 refer to highly significant when compared with control; [a] Significantly different when compared with group A at P < 0.05. [b] Significantly different when compared with group B at P<0.05; [c] Significantly different when compared with group C at P < 0.05.

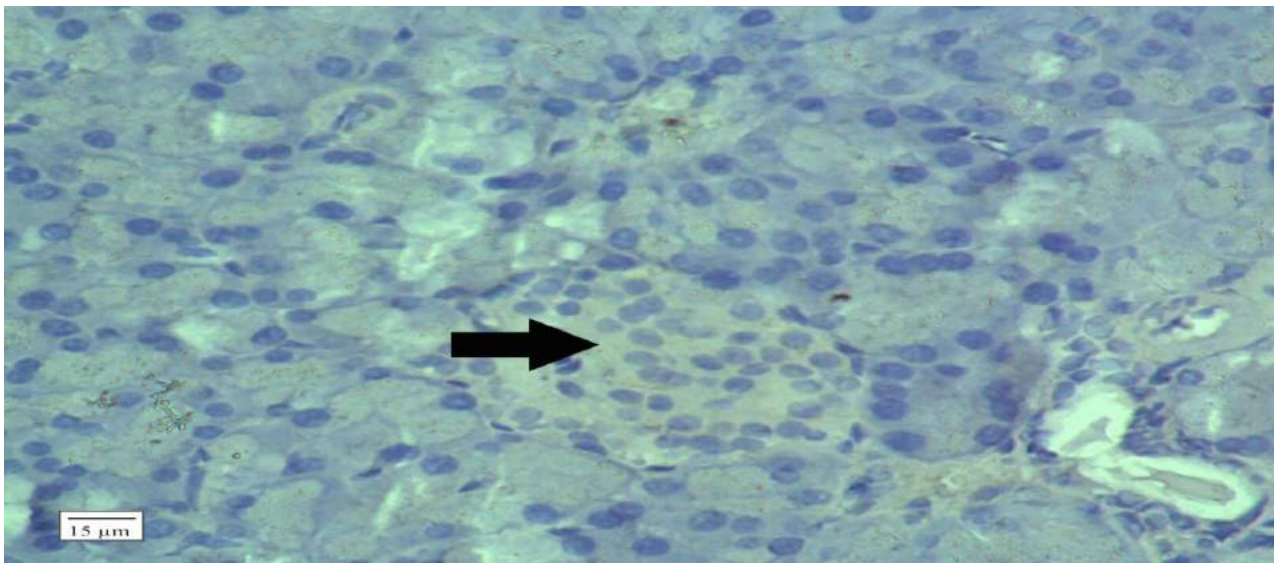

Figure (3-18): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in pancreas (islet of Langerhans) of group **D**: showing few reactivity against EGF.X400. Black arrow refer to EGF expression in islet of Langerhans.

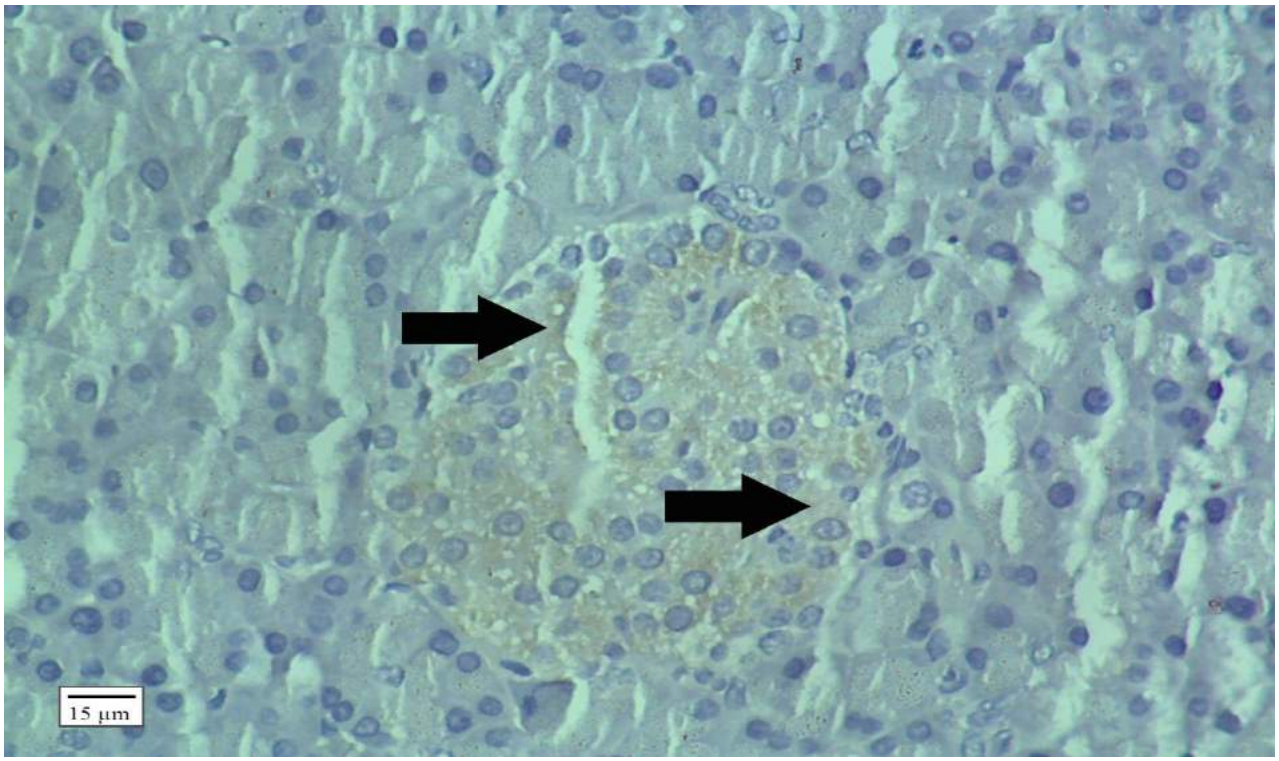

Figure (3-19): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in pancreas (islet of Langerhans) of group **A** after 0.5 mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF.X400. Black arrow refer to EGF expression in islet of Langerhans.

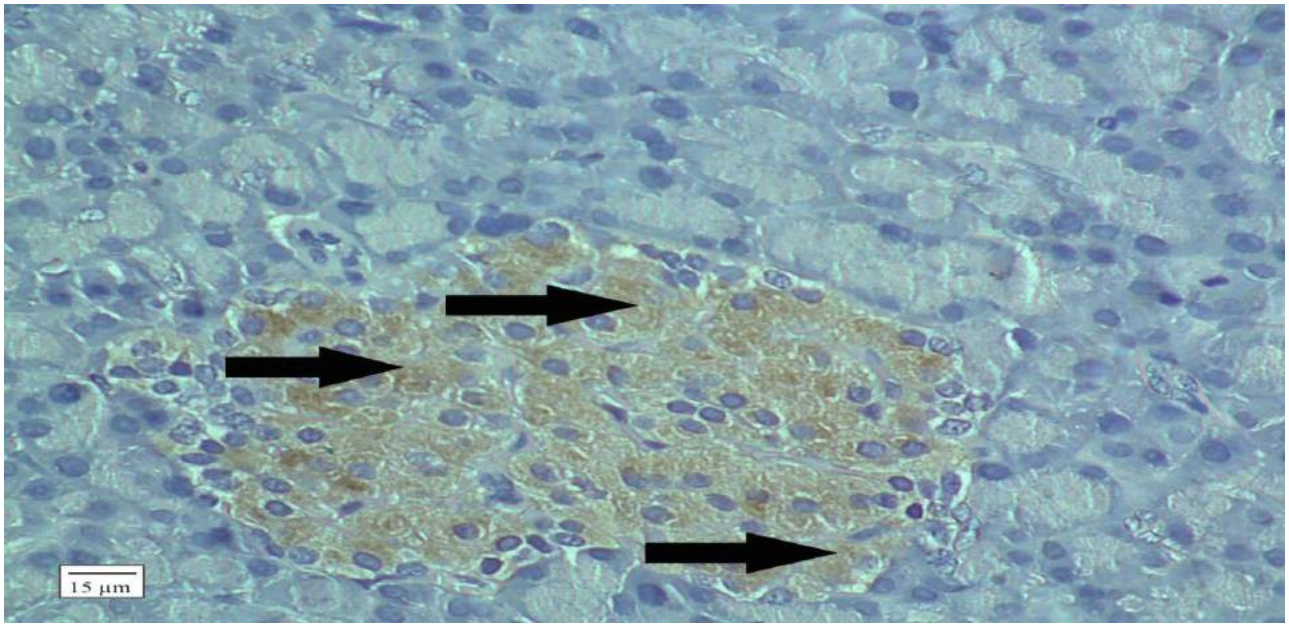

Figure (3-20): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in pancreas (islet of Langerhans) of group **B** after 1 mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF.X400. Black arrow refer to EGF expression in islet of Langerhans.

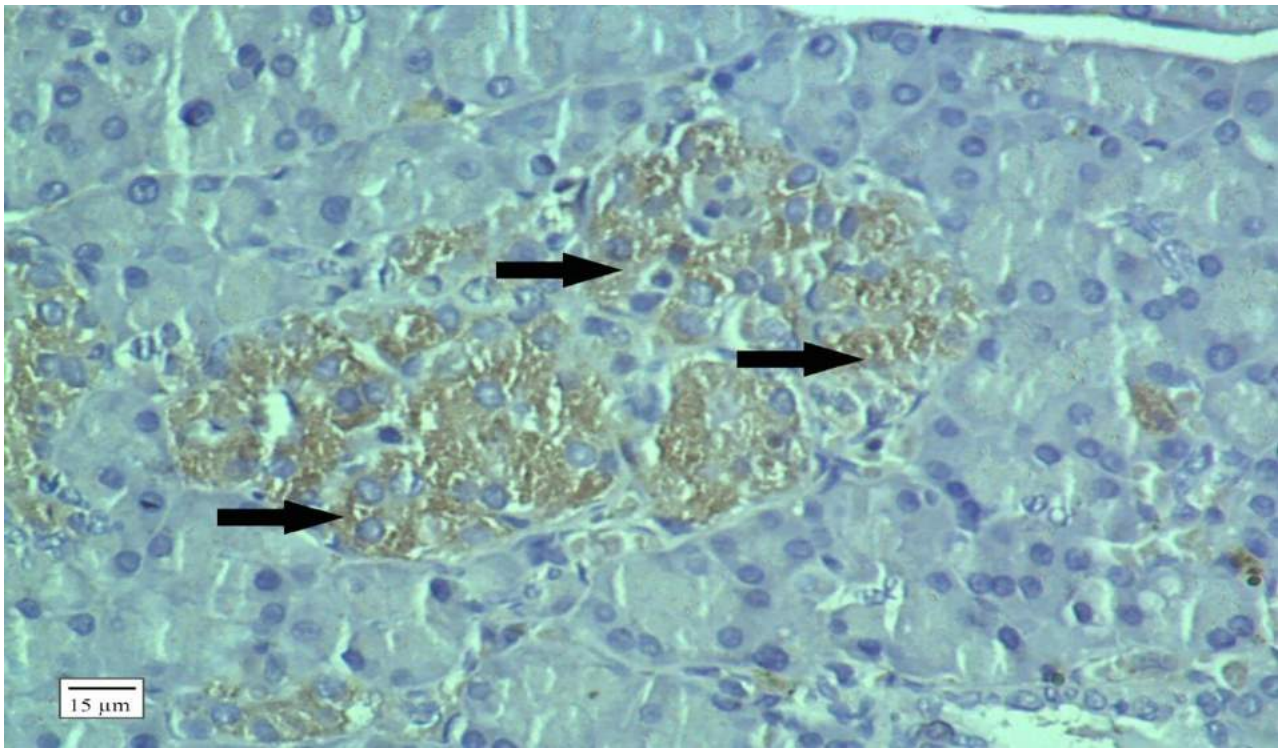

Figure (3-21): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in pancreas (islet of Langerhans) of group **C** after 2 mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF.X400. Black arrow refer to EGF expression in islet of Langerhans.

3.3.3 The effect on EGF score in the submandibular gland:

Sildenafil markedly effect EGF expression in the submandibular gland of group A (fig.3-24) after (0.5 mg / kg / day) orally dose treatment for six weeks was with highly statistical difference ($P<0.01$) when compared to control group D (fig.3-23).While the mean score of EGF in submandibular gland of group B (fig.3-25) after six weeks of 1mg / kg / day orally sildenafil treatment was (1.32±0.30) with highly significant increase ($P < 0.01$) score when compared to control group D. Finally, EGF score in group C (fig.3-26) after six weeks treatment with orally sildenafil (2mg / kg / day) was (1.74±0.31) with highly significant increase ($P < 0.01$) when compared to control group D.

In ANOVA test, All three group had significant difference in EGF score when compared to control group where $P = 0.000$.

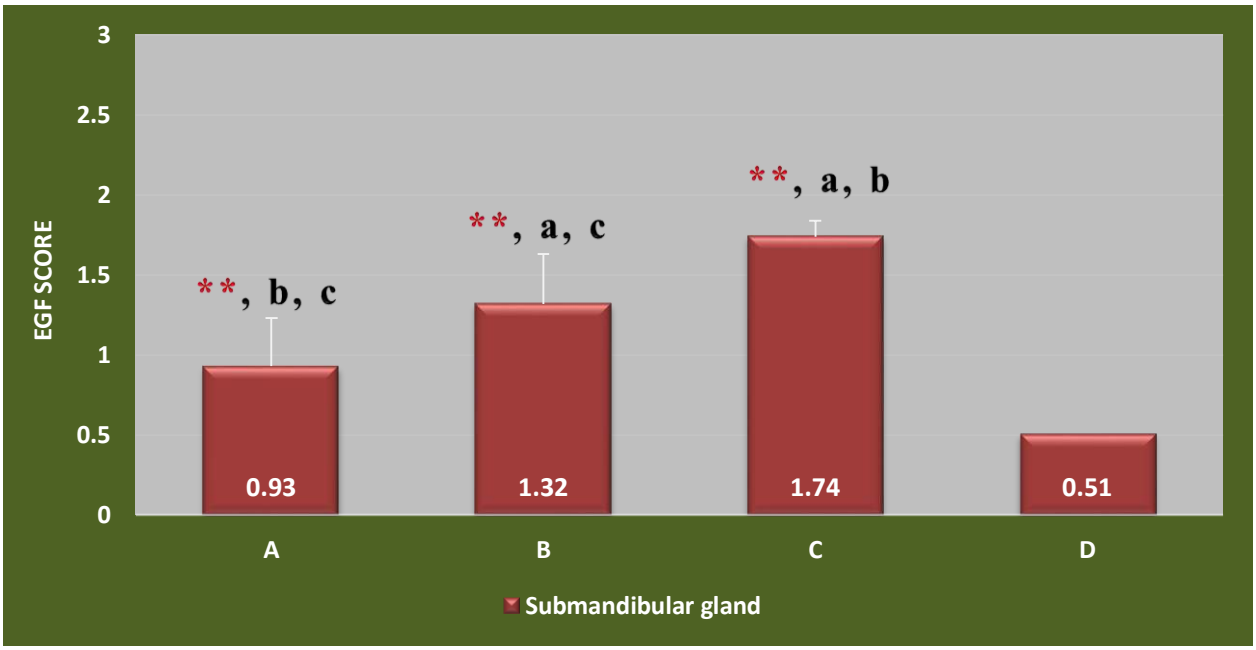

Figure (3-22): Changes of EGF in submandibular after sildenafil treatment in all groups. * = P < 0.05 refer to statistically significant when compared with control; ** = P < 0.01 refer to highly significant when compared with control; [a] Significantly different when compared with group A at P < 0.05. [b] Significantly different when compared with group B at P<0.05; [c] Significantly different when compared with group C at P < 0.05.

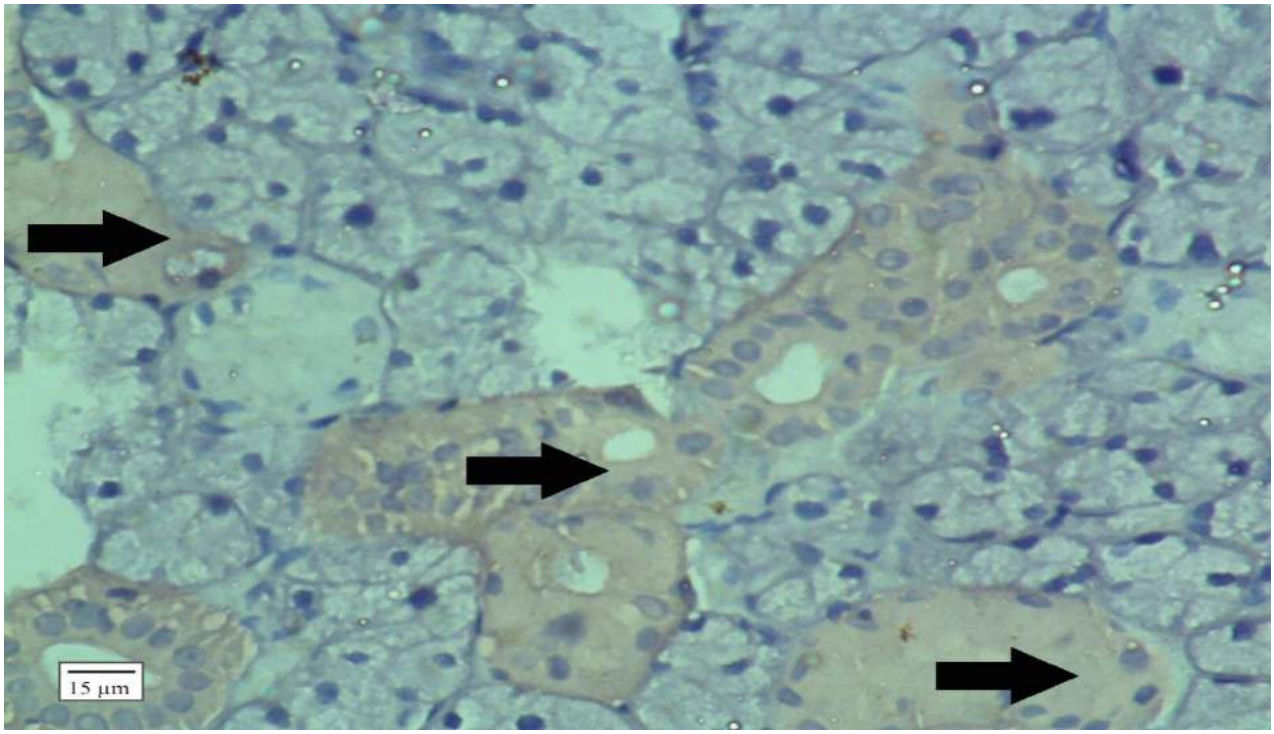

Figure (3-23): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in submandibular gland of group **D**: showing weak reactivity against EGF.X400. Black arrow refer to cytoplasmic EGF.

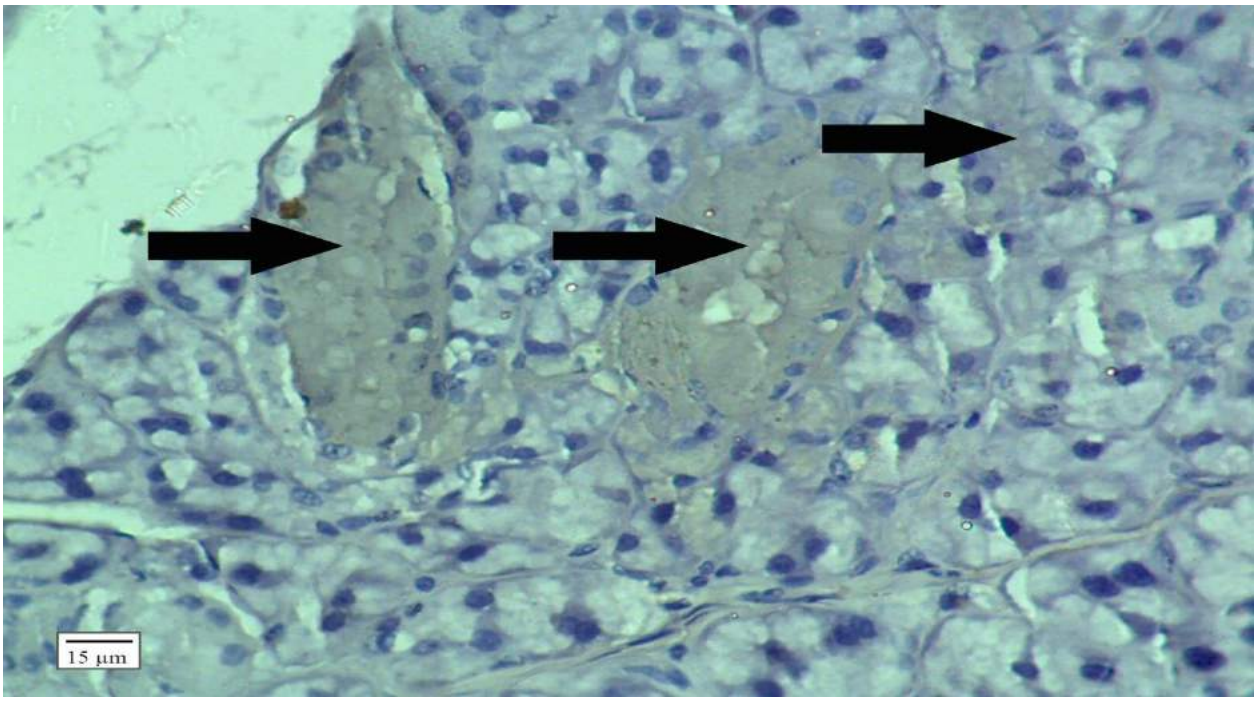

Figure (3-24): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in submandibular gland of group **A** after 0.5 mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF.X400. Black arrow refer to cytoplasmic EGF.

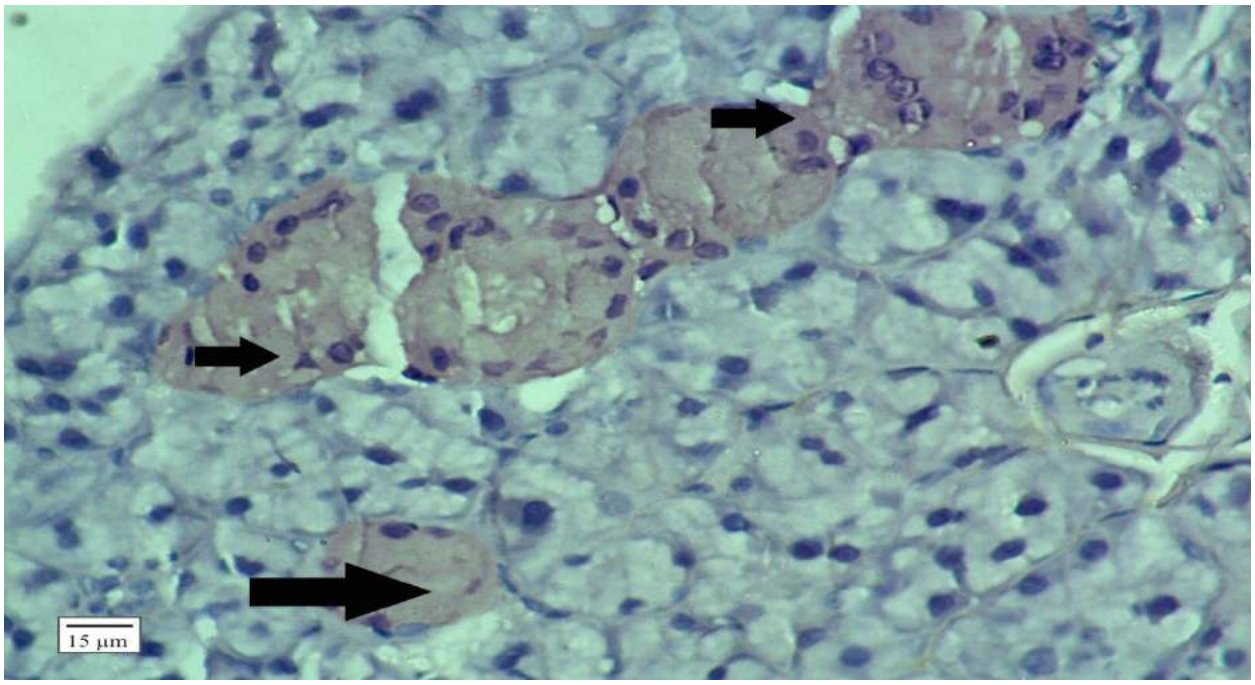

Figure (3-25): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in submandibular gland of group **B** after 1 mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF.X400. Black arrow refer to cytoplasmic EGF.

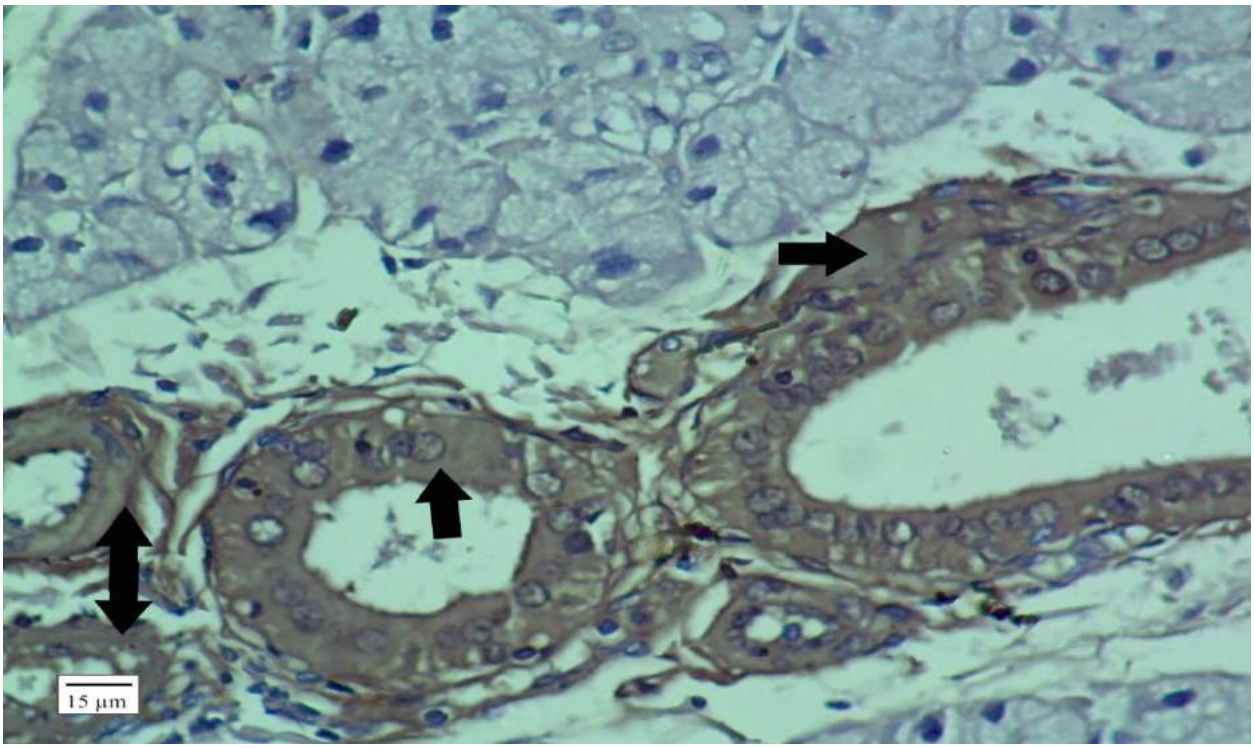

Figure (3-26): Microphotograph of immunohistochemical (IHC) staining of epidermal growth factor (EGF) in submandibular gland of group **C** after 2 mg/kg/day of sildenafil six weeks treatment: showing increase reactivity against EGF. X400. Black arrow refer to cytoplasmic EGF.

3.4 Serum marker:

3.4.1 Effect of various doses of sildenafil citrate on Interleukin-1 beta (IL-1β):

Table (3-5) demonstrates that serum IL-1β was insignificantly decrease in group A after 0.5mg / kg / day sildenafil oral dose when compared to serum IL-1β of control group D where P = 0.512.

However the mean IL-1β of group B which treated with 1mg / kg / day orally sildenafil was (245.1±10.4) where there was significant decrease in group B IL-1β when compared to control group D IL-1β where P= 0.04.

Whereas highly significant decrease ($P < 0.01$) in group C IL-1β (193±13.5) after treatment with sildenafil by oral gavage in dose of 2mg / kg / day when compared to control group D IL-1β.

All group when compared to control group (group D) using ANOVA test, had highly statistical significant where P= 0.000.

Table (3-5): Changes in IL-1β. Data are expressed as mean±S.D.

Group	Interleukin-1 beta (IL-1β) (pg / ml)
A	263±5.9 [c]
B	245.1±10.4 *, [c]
C	193±13.5 **, [a, b]
D	265.5±11.6
P-value (ANOVA)	**0.000**

* Significant difference of independent T- test when compared with control at P<0.05.
** High significant difference of independent T- test when compared with control at P<0.01.
[a] Significantly different when compared with group A at P<0.05.
[b] Significantly different when compared with group B at P<0.05.
[c] Significantly different when compared with group C at P<0.05.

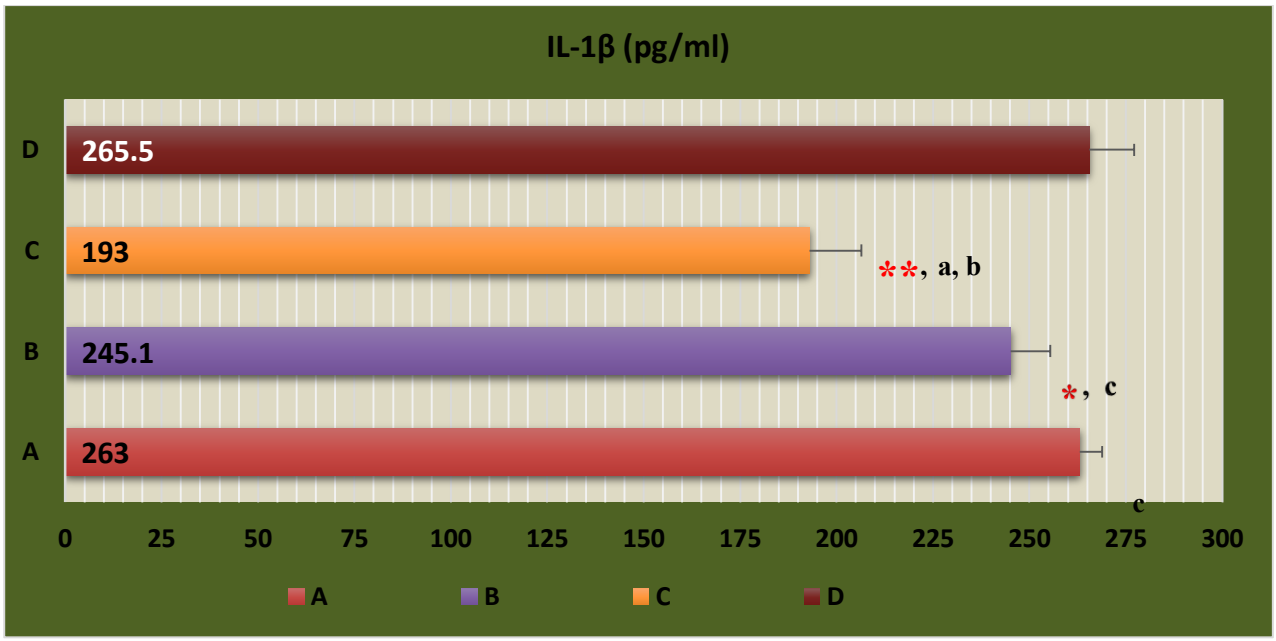

Figure (3-27): The amount of IL-1β in all groups after sildenafil treatment. * = P < 0.05 refer to statistically significant when compared with control; ** = P < 0.01 refer to highly significant when compared with control; [a] Significantly different from group A at P < 0.05. [b] Significantly different from group B at P<0.05; [c] Significantly different from group C at P < 0.05.

3.4.2 Effect of various doses of sildenafil citrate on total antioxidant capacity (TAC):

When rats treated with sildenafil for six weeks in orally dose 0.5 mg / kg / day in group A, the mean of serum total antioxidant capacity (TAC) in group A was (1.41±0.10), with significant increment ($P < 0.05$) than mean of serum total antioxidant capacity in control group D.

Also there was high significant elevation ($P < 0.01$) in serum TAC of group B and C which treated with 1mg/kg/day and 2mg/kg/day sildenafil respectively, when compared to control group D as shown in table (3-5).

All group when compared to control group (group D) using ANOVA test, had highly statistical significant where P= 0.000.

Table (3-6): Changes in TAC. Data are expressed as mean±S.D.

Group	total antioxidant capacity (TAC) (mmol trolox equivalents / L)
A	1.41±0.10 *, b, c
B	2.08±0.15 **, a
C	1.93±0.09 **, a
D	1.28±0.12
P-value (ANOVA)	**0.000**

* Significant difference of independent T- test when compared with control at P<0.05.
** High significant difference of independent T- test when compared with control at P<0.01.
[a] Significantly different when compared with group A at P<0.05.
[b] Significantly different when compared with group B at P<0.05.
[c] Significantly different when compared with group C at P<0.05.

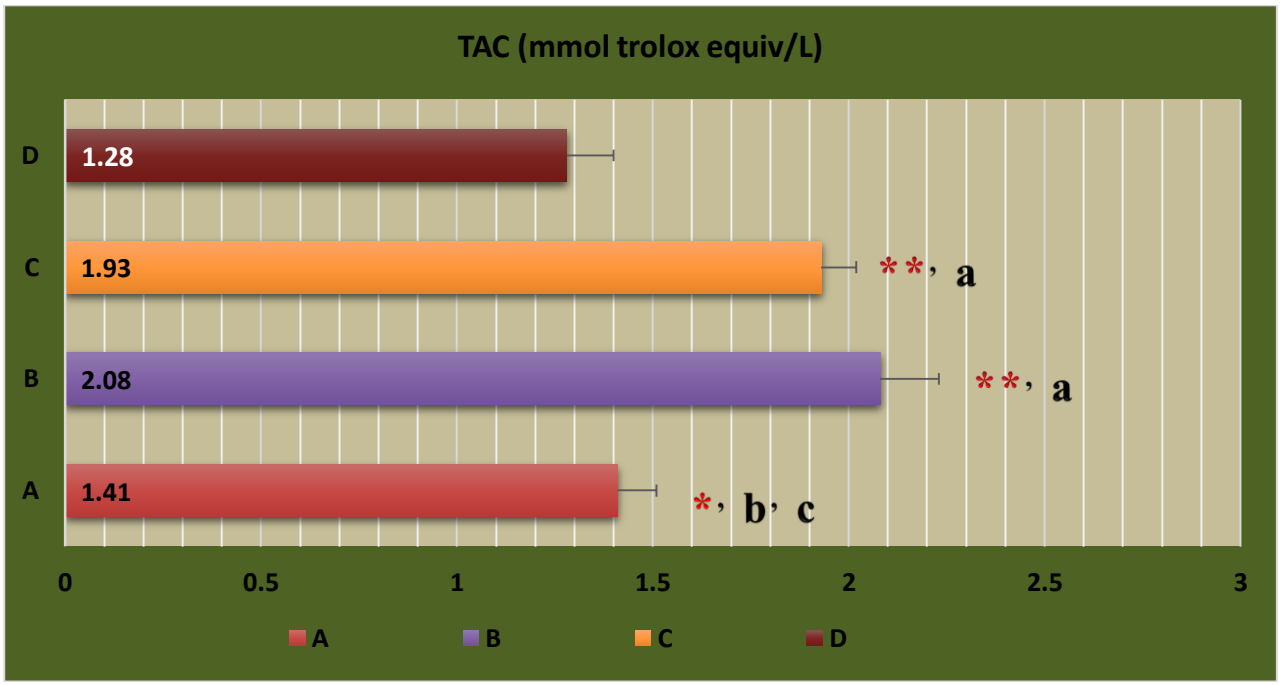

Figure (3-28): The amount of Total antioxidant capacity (TAC) in all groups after sildenafil treatment. * = P < 0.05 refer to statistically significant different when compared with group D; ** = P < 0.01 refer to highly significant different when compared with group D; [a] Significantly different when compared with group A at P < 0.05. [b] Significantly different when compared with group B at P<0.05; [c] Significantly different when compared with group C at P < 0.05.

3.4.3 Effect of various doses of sildenafil citrate on fasting blood glucose (FBG) and C-peptide:

The mean of initial FBG of group (A) was (87.8±2.4mg/dl) and after o.5 mg/kg /day of oral sildenafil treatment, the mean of final FBG was (77.8±1.4 mg/dl) with significant decrease in total percent of FBG (-11.3±0.9 %) where P= 0.014 and when compared with control group there was a highly significant difference where P = 0.008, this associate with insignificant increase in mean of C-peptide (1.7±0.03 ng/ml) in comparing to mean of C-peptide of control group D where P= 0.53.

Meanwhile the mean of initial FBG of group (B) was (88.2 ±1.4 mg/dl) and after six weeks of sildenafil treatment in oral dose of 1mg / kg / day, the mean of final FBG was (70.2 ±3.4 mg/dl) with highly significant decrease in total percent of FBG (-20.4±1.2%) where P= 0.000 and when compared with control group there

was a highly significant difference where P= 0.000, this associate with insignificant increase in mean of C-peptide (1.8±0.06 ng/ml) in comparing to mean of C-peptide of control group D where P= 0.08.

Finally the mean of initial FBG of group (C) was (85.2±1.6 mg/dl) and after 2mg/kg/ day oral sildenafil for six weeks treatment, the mean of final FBG was (62.3±1 mg/dl) with highly significant decrease in total percent of FBG (-26.9±2.5%) where P= 0.000 and when compared with control group there was highly significant difference where P= 0.000, this associate with highly significant increase in mean of C-peptide (2.6±0.15 ng/ml) in comparing to mean of C-peptide of control group D where P= 0.002.

The mean of initial FBG of control group (D) was (82.2±3.2 mg/dl) and after oral administration of saline for six weeks, final FBG was (89.3±2.1 mg/dl) with insignificant increase in total percent of FBG (8.6±1.3%) where P = 0.268, and the mean of C-peptide was (1.6±0.03 ng/ml).

Table (3-7): Changes in FBG & C-peptide. Data are expressed as mean ± S.D.

Group	Initial FBG (mg/dl)	Final FBG (mg/dl)	% changes in FBG	C-peptide (ng/ml)
A	87.8±2.4	77.8±1.4 *	-11.3±0.9 **, b, c	1.7±0.03 c
B	88.2 ±1.4	70.2 ±3.4 **	-20.4±1.2 **, a	1.8±0.06 c
C	85.2±1.6	62.3±1 **	-26.9±2.5 **, a	2.6±0.15 **, a, b
D	82.2±3.2	89.3±2.1 NS	8.6±1.3	1.6±0.03
P-value (ANOVA)			**0.000**	**0.000**

* Significant difference of paired t-test at P<0.050 when compared final FBG with initial FBG.
** High significance difference of paired t-test at P<0.01 when compared final FBG with initial FBG.
NS No significant difference of paired t-test when compared final FBG with initial FBG.
* Significant difference of independent T- test when compared with control at P<0.05.
** High significant difference of independent T- test when compared with control at P<0.01.
a Significantly different when compared with group A at P<0.05;
b Significantly different when compared with group B at P<0.05;
c Significantly different when compared with group C at P<0.05;

Figure (3-29): Changes in FBG before and after sildenafil treatment in all groups. * Significant difference of paired t-test at P<0.05 when compared final FBG with initial FBG.** High significance difference of paired t-test at P<0.01 when compared final FBG with initial FBG.

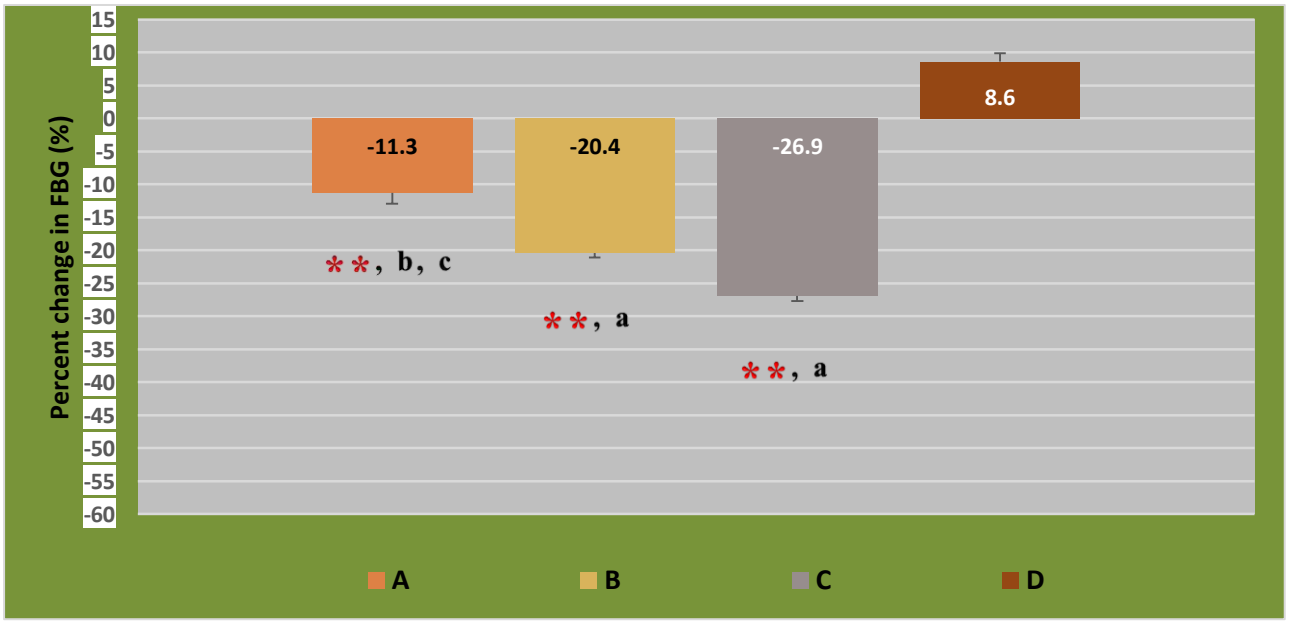

Figure (3-30): Changes in the level of FBG in all group before and after sildenafil treatment. * Significant difference of independent t-test at P<0.05 when compared with control. ** High significance difference of independent t-test at P<0.01 when compared with control. [a] Significantly different when compared with group A at P<0.05. [b] Significantly different when compared with group B at P<0.05. [c] Significantly different when compared with group C at P<0.05.

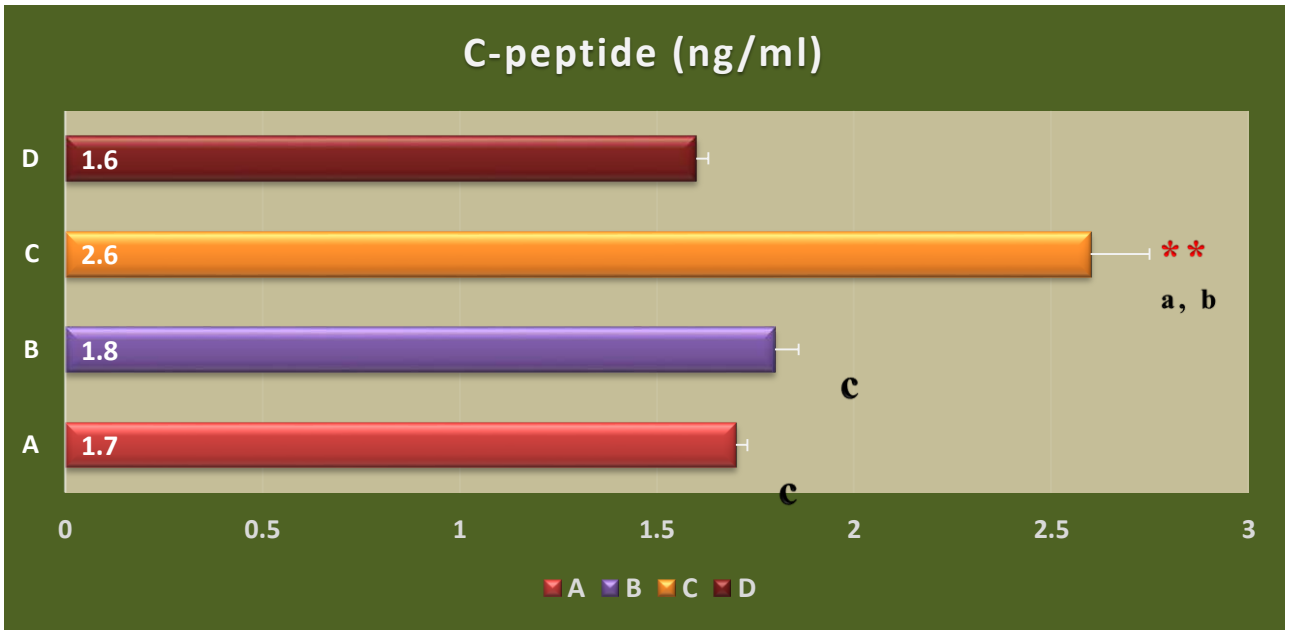

Figure (3-31): The level of C- peptide after sildenafil treatment in all groups. * Significant difference of paired t-test at P<0.05 when compared with control. ** High significance difference of paired t-test at P<0.01 when compared with control. [a] Significantly different when compared with group A at P<0.05. [b] Significantly different when compared with group B at P<0.05. [c] Significantly different when compared with group C at P<0.05.

3.5 Correlations:

3.5.1 Percent of hepatic glycogen content versus percent of hepatic glycogen phosphorylase (GP):

There was highly significant and strong inverse correlation between hepatic glycogen content and hepatic GP (-.962) with P= 0.000, when there is increasing in hepatic glycogen content there was decrease in hepatic GP.

Table (3-8): Correlation of percent of hepatic glycogen content versus percent of hepatic glycogen phosphorylase (GP).

		Hepatic glycogen area(%)	**Hepatic GP area (%)**
Pearson Correlation	Hepatic glycogen area%	1.000	-.962
	Liver GP area%	-.962	1.000
Sig. (1-tailed)	Hepatic glycogen area%	.	.000
	Liver GP area%	.000	.

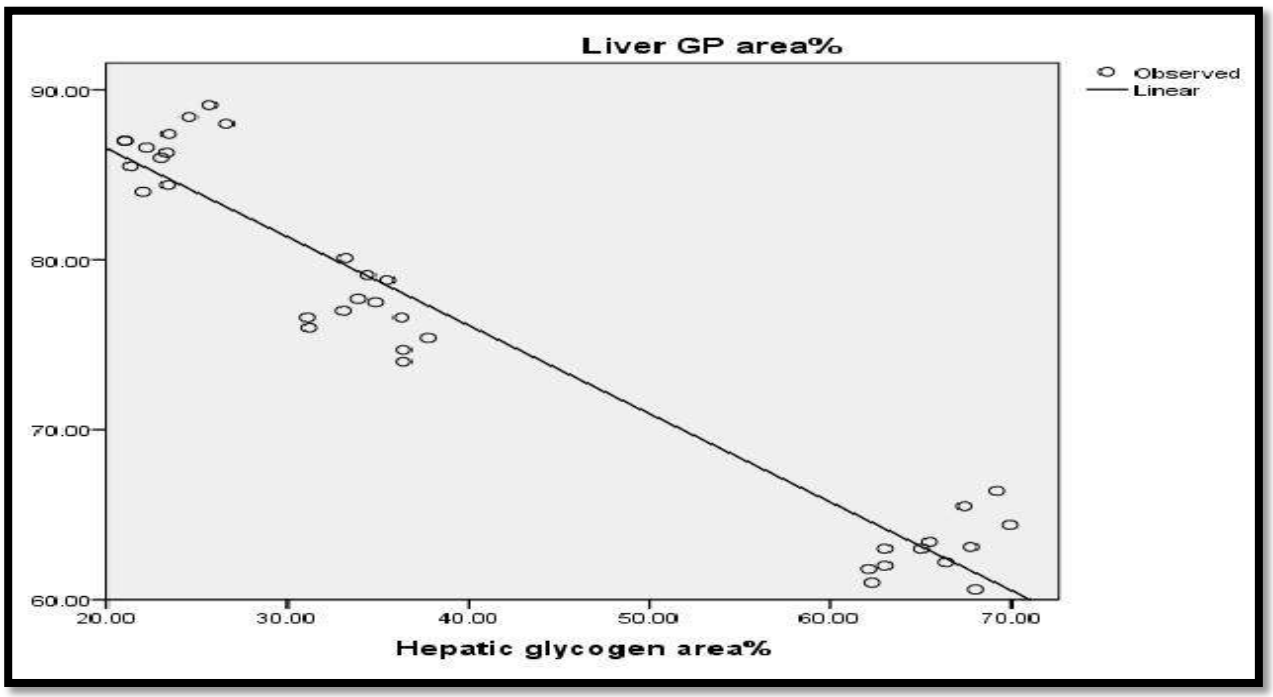

Figure (3-32): Linear graph shows inverse correlation between hepatic glycogen content and hepatic GP.

3.5.2 Percent of hepatic glycogen content versus serum C-peptide:

There was highly significant and strong direct correlation between hepatic glycogen content and serum C-peptide (0.947) with P= 0.000, when there is increasing in hepatic glycogen content there was increase in C- peptide.

Table (3-9): Correlation of hepatic glycogen content versus serum C-peptide.

		Hepatic glycogen area (%)	**C-peptide (ng/ml)**
Pearson Correlation	Hepatic glycogen area%	1.000	.947
	C-peptide /(ng/ml)	.947	1.000
Sig. (1-tailed)	Hepatic glycogen area%	.	.000
	C-peptide /(ng/ml)	.000	.

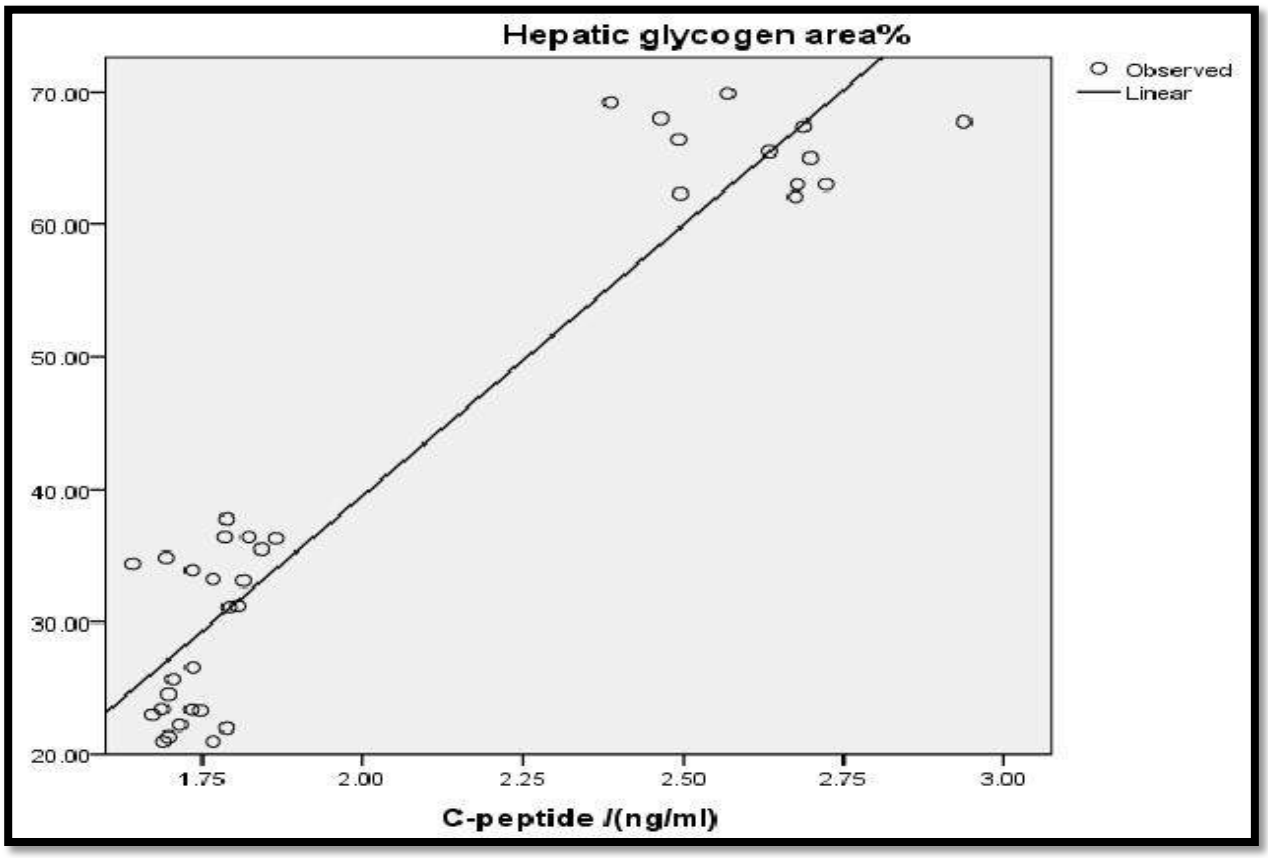

Figure (3-33): Linear graph shows direct correlation between hepatic glycogen content and serum C-peptide.

3.5.3 Serum C-peptide versus interlukin-1beta (IL-1β):

There was highly significant and strong inverse correlation between serum C-peptide and interlukin-1beta (IL-1β) (0.906) with P= 0.000, when there is increasing in serum C-peptide there was decrease serum IL-1β and vice versa.

Table (3-10): Correlation of serum C-peptide versus interlukin-1beta.

		C-peptide (ng/ml)	**Serum interlukin-1beta (pg/ml)**
Pearson Correlation	C-peptide (ng/ml)	1.000	-.906
	Serum interlukin-1beta (pg/ml)	-.906	1.000
Sig. (1-tailed)	C-peptide /(ng/ml)	.	.000
	Serum interlukin-1beta (pg/ml)	.000	.

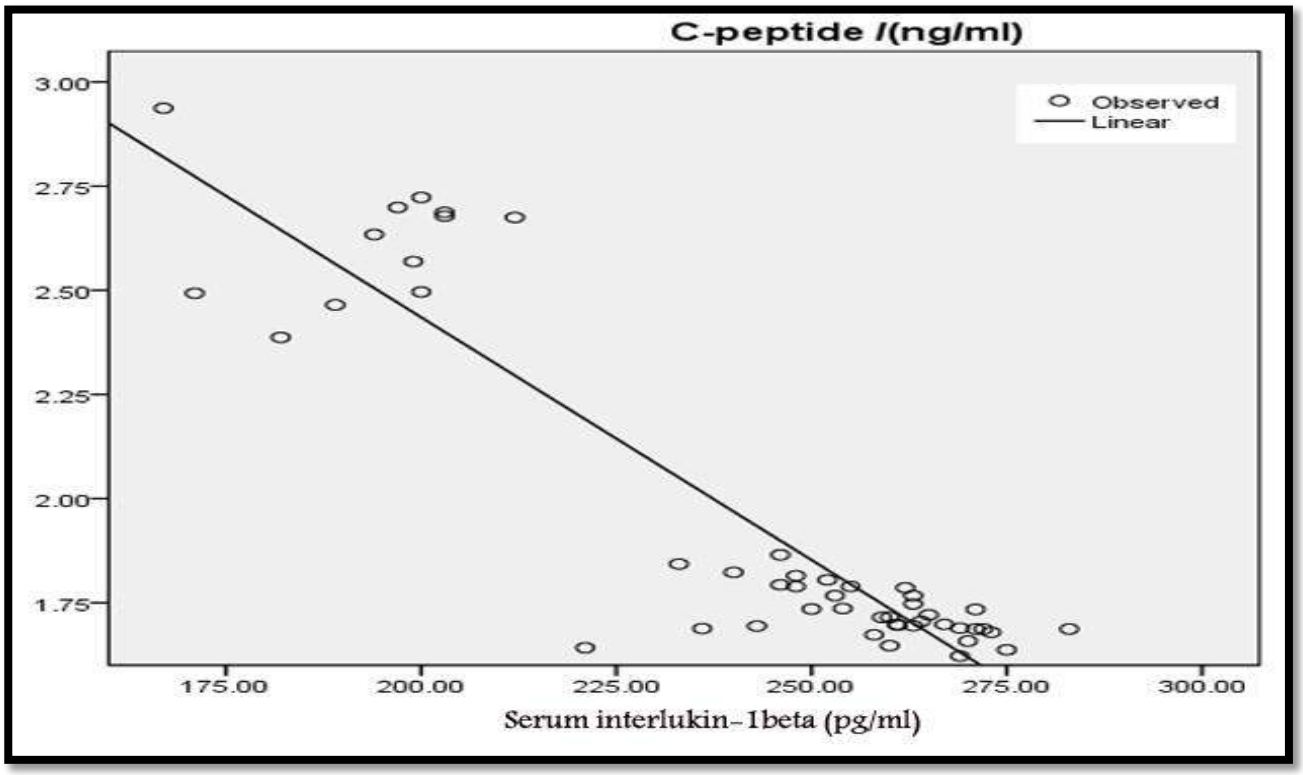

Figure (3-34): Linear graph shows inverse correlation between serum C-peptide versus interlukin-1beta.

3.5.4 Serum interlukin-1beta (IL-1β) versus final fasting blood glucose (FBG):

There was highly significant and moderate direct correlation between serum IL-1β and final FBG (0.697) with P= 0.000, when there is increasing in level of IL-1β there was increase in final FBG.

Table (3- 11): Correlation of serum interlukin-1beta (IL-1β) versus final fasting blood glucose (FBG).

		IL-1β (pg/ml)	**Final FBG (mgL/dl)**
Pearson Correlation	Serum IL-1β (pg/ml)	1.000	.697
	FBG (mgL/dl)	.697	1.000
Sig. (1-tailed)	Serum IL-1β (pg/ml)	.	.000
	FBG (mgL/dl)	.000	.

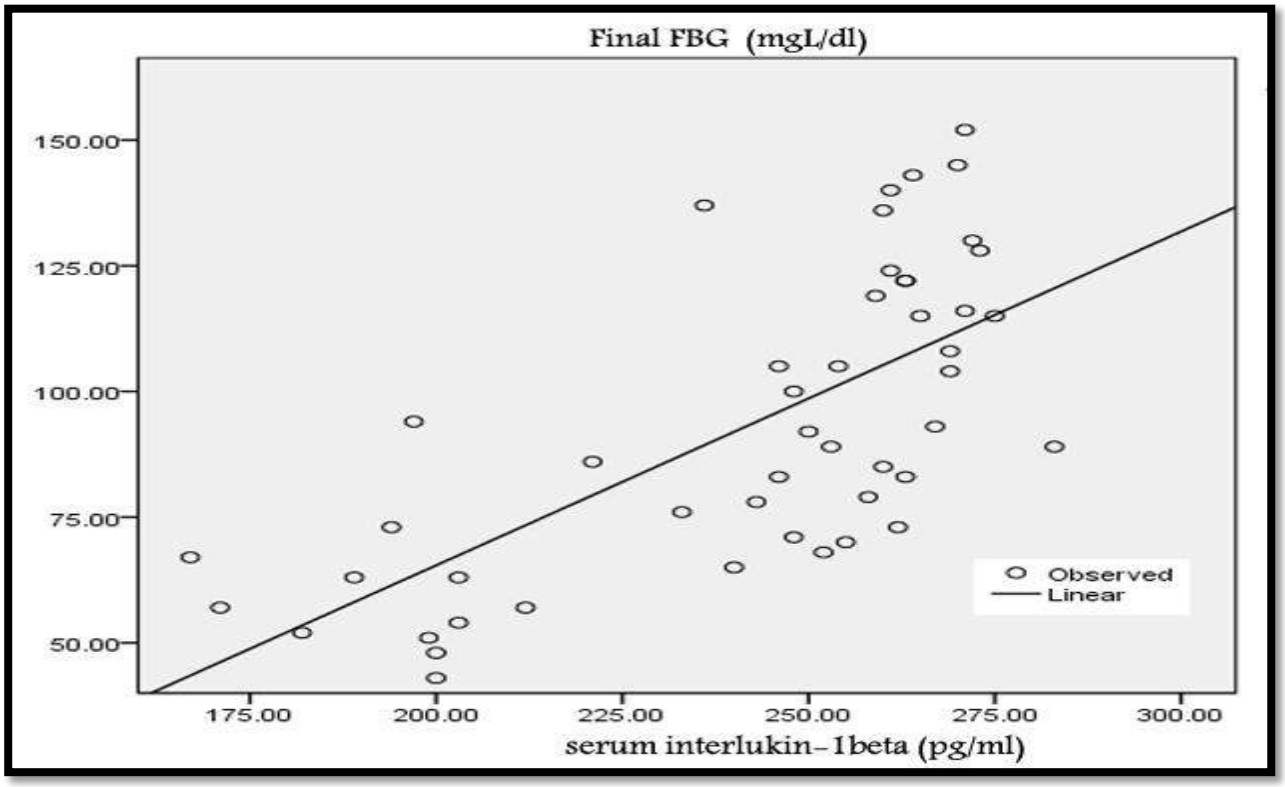

Figure (3-35): Linear graph shows moderate direct correlation between FBG and C-peptide.

Chapter Four

Discussion

4. Discussion

Sildenafil is a selective inhibitor of PDE5, decrease cGMP hydrolysis and is also characterized by antioxidant activity [130]. These studies given the security record of this drug, and reveal that phosphodiesterase-5 inhibition sildenafil is possibly a viable approach for the prevention of imbalance in carbohydrate metabolism, insulin resistance and energy expenditure. It is of importance to screen the effects of sildenafil on blood glucose level which is determined in the current study. The level of blood glucose is correlated primarily to insulin level and hepatic glucose metabolizing enzymes. So blood glucose, C-peptide levels, IL1beta, total anti-oxidant and liver glycogen phosphorylase activity and glycogen content, pancreatic changed and body weight are determined for 6 weeks [131, 58].

4.1 Effect of various doses of sildenafil citrate on body weight:

Nitric oxide (NO) is one of the signaling molecules synthesized from l-arginine by NO synthase in animals. Cumulative evidence displays that NO regulates the mammalian metabolism of energy substrates. In intact cells, through cGMP and AMP-activated protein kinase signaling, nitric oxide regulates the metabolism of glucose, fatty acids and amino acids in mammals. Augmenting NO-cGMP pathway stimulate glucose uptake as well as glucose and fatty acid oxidation in skeletal muscle, heart, liver and adipose tissue; inhibit the synthesis of glucose, and fat in target tissues (e.g., liver and adipose tissue); enhance lipolysis in adipocytes; and in reducing unfavorable fat mass in animal [132, 133]. Cyclic GMP (cGMP) are largely mediated natriuretic peptide–mediated (NP-mediated) biological responses through the guanylyl cyclase domain of NP receptor type A (NPRA). NP stimulate a quick and continuous rise of intracellular cGMP that triggers a cGMP-dependent protein kinase, PRKG1, which consequently phosphorylates perilipin 1 and hormone-sensitive lipase, essential steps to initiate lipolysis [134]. Natriuretic peptide show a potent lipolytic effect in human adipocytes in a cyclic GMP–

dependent manner. So the expected from previews findings, revealed that any agent increase cGMP like selective PDE5 inhibitor sildenafil will improve muscle fat oxidative capacity and show increased energy expenditure [134, 135]. The results of this study shows there was decrease in rat body weight of group A about (-10.9) g after oral administration of 0.5mg of sildenafil from initial body weight, in group B there was more decrease in body weight about (-14.5) g with administration of 1mg of sildenafil from initial body weight, while in group C after 2mg of oral sildenafil the loss in body weight mimic the group A; about (-9.7) g so the group B shows more decrease in body weight after 1mg of sildenafil injection. So, data suggest that sildenafil will decrease rat's body weight but not in a dose dependent manner, this action is not certain, especially in the absence of data on feed intake. In summary treatment of rats with sildenafil significantly decrease body weight comprising with control. This results in concordance with Ayala J. 2007, where Ayala *et al.* show that in a mouse model of diet-induced insulin resistance, chronically inhibiting cGMP hydrolysis with sildenafil increases energy balance and enhances *in vivo* insulin action. Treatment with Sildenafil plus L-arginine or sildenafil alone for 12 weeks, had reduced weight and fat mass due to improved energy expenditure in High-fat–fed mice [58].

Phosphodiesterase-5 expression has been detected in the brain, and Sildenafil has been shown to cross the blood-brain barrier. Thus, cyclic GMP signaling in the central nervous system may have a role in the regulation of insulin action, energy homeostasis and improve energy expenditure [136, 137]. The drop in weight gain and fat mass was related to an increase in energy expenditure. This is reveal with other studies which demonstrating decrease oxygen consumption and increased weight gain in eNOS knockout mice [138]. Thus, NO-cGMP signaling impairment triggered by eNOS deletion results in reduced energy expenditure and improved weight gain, whereas preserving cGMP signaling, as done in the current studies, increases energy

expenditure and decreases weight gain. Signaling of NO through cGMP has been exposed to induce mitochondrial biogenesis *in vitro* and *in vivo* [138, 139]. It is probable that chronic sildenafil treatment increases in cGMP subsequently may encouragement mitochondrial function because NO-cGMP signaling has been exhibit to play a role in cellular energy sensing [140,141]. Dai Z *et al.* 2013 demonstrate that administration of sildenafil which acts by reducing cGMP hydrolysis through inhibition of PDE5, lead to increase blood flow, increases energy expenditure and reduces adiposity in diet-induced rats, genetically obese rats, growing-finishing pigs, and obese humans with type-II diabetes [133].

Either increased in energy expenditure, weight loss can result from reduced energy intake. Reduced energy intake can result from either decreased food intake or impaired food absorption. Dyspepsia (due to relaxation of the smooth muscle of the gastroesophageal sphincter with reflux) is one of secondary effects of sildenafil, a phosphodiesterase blocker that prolongs the relaxant properties of NO-mediated smooth muscle relaxation, which may be also explain the association of weight loss with sildenafil secondary effect on gastrointestinal [142]. It was suggested that the administration of rat sildenafil (10 mg/kg/day s.c.) for 5 days, there was statistically insignificant decrease in body-weight of treated rats. Wang L *et al.* 2015 report that there was no significantly change in mice body weight after 10 mg/kg of sildenafil (orally administered) for 8 weeks. The reason for this action is not certain which may be related to the difference in dose and duration of treatment [143].

4.2 Effect of various doses of sildenafil citrate on liver

4.2.1 Effect on hepatic glycogen content

Under normal conditions, the liver plays a critical role in disposing orally or internally delivered carbohydrate; therefore, limiting postprandial hyperglycemia. This response involves both a decrease in hepatic glucose production and a stimulation of hepatic glucose uptake. The latter is dependent on a number of inputs:

circulating concentrations of glucose, nonesterified fatty acids, amino acids, hormones (insulin and glucagon) and neural mediators (NO and norepinephrine) [144]. The data from the current study reveal that *in vivo* treatment of rats with sildenafil, dose-dependently increase hepatic glycogen content. These data are in accordance and support the results of Hoseini *et al.* 2006 and Abdollahi *et al.* 2004, they were demonstrated that the hypoglycemic effect of sildenafil is related to increase in liver glycogen content (LGC) where blood glucose is converted to hepatic glycogen and sildenafil administration markedly reduces liver glycogenolysis [20, 145]. There is also evidence that sildenafil elevate liver glycogen content (LGC) and decreased blood glucose level of diabetic control rats in dose dependent manner [146]. Abbott *et al.* 2004, reported that the main effects of sildenafil in rats, in the one and six month safety studies were dose related increases in liver weight [147]. The putative underlying mechanisms of sildenafil involve augmenting NO/ sGC/ cGMP pathway and activation multiple cyclic GMP pathways. Where NO stimulate glucose uptake, glucose and fatty acid oxidation in skeletal muscle, heart, liver and adipose tissue, also NO inhibit the liver synthesis of glucose [133]. Remarkably, NO is shown to stimulate the increase of glycogen possibly through inhibition of glycogenolysis [148]. Moreover glycogen phosphorylase inhibitors, might be anticipated to increase hepatic glycogen content [149]. There are studies showing conversely relationship between daily exposed to sildenafil and liver glycogen content, Abdel-Hafez AM and Othman MA. 2013 suggested that rats treated with 10 mg/kg/day sildenafil for 8 weeks, reduced glycogen staining in hepatocytes [150]. Also Jarrar BM, Almansour MI. 2015 report that male rabbit treated with sildenafil 9mg/kg for 5 days per week for 7 weeks provoked partial depletion of glycogen content [151]. To discuss this inconsistency in researchers findings, the exact molecular mechanism by which NO / sGC/ cGMP brings about this hepatic effect must be established, actually controversial opinion in

these aspects, where Moore *et al*. 2012, suggested that the role of NO / sGC/ cGMP pathway in the regulation of net hepatic glucose uptake and glycogen storage in conversely relationship [(152)]. Also An Z *et al*. 2012, reported that increase in cGMP in the liver after treatment of dogs with cGMP analog 8-Bromo-cGMP decreased net hepatic glucose uptake through activation of hepatic adenosine monophosphate - activated protein kinase (AMPK), but has no effect on hepatic glycogenolysis and gluconeogenesis [(153)]. While Choudhury J *et al*. 2004 data propose that phosphodiesterase inhibitor prolonged atrial natriuretic peptide (ANP) effects on insulin-stimulated glucose uptake in targeted organ by potentiate sGC-cGMP-PKG pathway also potentiate insulin promotes hepatic glycogen production through the activation of glycogen synthase [(154)]. That is in agreement and support the current study data, sildenafil effect on liver glycogen content mainly came from sildenafil increase insulin secretion which approved in the current study. There was highly significant and strong direct correlation between hepatic glycogen content and C-peptide and strong inverse correlation between hepatic glycogen content and liver glycogen phosphorylase (GP), where insulin promotes hepatic glycogen production through the activation of glycogen synthase and inhibiting glycogen phosphorylase.

4.2.2 Effect on liver glycogen phosphorylase (GP) activity:

There is an evidence that liver is one of the organs that well responds to PDE inhibitors by influencing glucose output. The liver plays a major role in blood glucose homeostasis by maintaining a balance between the uptake and storage of glucose via glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis. The regulation of these metabolic pathways involves the rapid modulation of the activity of specific enzymes including hepatic key enzymes phosphoenolpyruvate carboxykinase (PEPCK) and GP [(155)]. Glycogen phosphorylase is the key enzyme of glycogen breakdown that catalyzes the degradative phosphorolysis of glycogen to glucose-1-phosphate [(156)]. This enzyme is

promoted by substrate as well as allosteric effectors, including adenosine monophosphate (AMP).

There is evidence that glucagon and other cAMP-increasing agents exert a glycogenolytic effect by maintaining GP in a phosphorylated active state. Cyclic adenosine monophosphate or Ca^{2+} dependent glycogenolytic agents cause glycogen degradation through specific protein kinases which activate the GP **(157)**. The studies in animals and human have demonstrated an impact of PDE5 inhibitors upon glucose metabolism but the reports were controversial. The results of this study showed that there was insignificant decrease of GP expression in rat liver of group A in comparison to control group after 0.5mg sildenafil, while there was (-12.4) % decrease of GP in rat liver of group B after 1mg sildenafil in comparison to control group, also great decrease in GP of group C rat liver (-26.3%) after 2mg of sildenafil in comparison to control group, so the results of this study indicated that treatment of rats with sildenafil is dose-dependently reduces hepatic GP activity. Moreover, the hypoglycemic effect of sildenafil is related to increase in liver glycogen content (LGC) where blood glucose is converted to hepatic glycogen. Effects of sildenafil on LGC were in agreement with those reported by Abdollahi M *et al.* 2003 who found that sildenafil administration markedly reduces liver glycogenolysis **(158)**. Similarly, Hoseini S *et al* 2006 concluded that administration of sildenafil markedly reduces liver glycogenolysis which in turn lowers blood glucose concentration at higher doses. This effect of sildenafil seems to be in relation with its NO mimicking potential and antioxidant properties **(20)**. Sildenafil, through the inhibition of PDE5 and augmentation of NO-cGMP axis shows similar effects to NO in many organs. NO has been suggested as a second messenger molecule for the stimulatory effect of insulin in carbohydrate metabolism. Tissues Incubation with physiologic concentration of insulin of heart, liver, kidney, muscle and intestine from mice and erythrocytes or their membrane fractions from humans, results in the stimulation of

a membrane-bound NOS and NO synthesis. Furthermore, NO has shown an insulin-like effect in stimulating glucose transport and glucose oxidation in muscle, a major site for insulin action that is completely blocked in the presence of NOS inhibitor [(20)].

In addition, it has been reported that culturing hepatic parenchymal cells in the presence of insulin results in an NO-dependent inhibition of the glucagon stimulated glucose production via gluconeogenesis plus glycogenolysis. Where NO can inhibit both the basal and the glucagon-cyclic AMP-mediated mobilization of glycogen pathway. Also NO is shown to stimulate increase of glycogen possibly through inhibition of glycogenolysis [(148)]. Considering the ability of sildenafil to reduce hepatic GP activity, one mechanism would be that sildenafil inhibits cAMP-dependent processes. To explain this, it should be noted that increasing cGMP is a fundamental mechanism of action of sildenafil. Study on rat hepatocytes *in vitro* indicated that sildenafil increases hepatocytes cGMP levels but does not significantly alter glycogenolysis and gluconeogenesis [(159, 160, 86)]. That is partly in agreement with current findings when considering glycogen phosphorylase (GP) does not significantly change with low doses of sildenafil (0.5mg/kg). In the case of PDE2 (highly specific for hydrolysis of cAMP and stimulated by cGMP), this regulation primarily involves the allosteric stimulation of cAMP hydrolysis by cGMP. So, cGMP can inhibit both the basal and the glucagon- cyclic AMP-mediated mobilization of glycogen pathway [(161)]. These mentioned mechanisms all explain the inhibitory effect of sildenafil on glycogenolysis and also its blood glucose lowering effect.

4.3 Effect of various doses of sildenafil citrate on epidermal growth factor (EGF):

The granular convoluted tubule (GCT) of rodent submandibular glands synthesized epidermal growth factor (EGF) which is a potent mitogen in various

tissues. In man, EGF has been localized either in the duct cells or in serous cells. It is important in many physiological processes as completion of normal pregnancy, spermatogenesis, wound healing and mammary gland development [(83)]. Kidney is another site of the EGF-family ligands synthesis. Endogenous renal EGF production serves as an autocrine stimulus to renal growth, where the EGF associated with physiologic events in the luminal membrane of the thick ascending limb of the loop of Hanley [(162)]. The present study demonstrate the marked influence of sildenafil on EGF synthesis by the submandibular glands and kidney (the second site of EGF synthesis) in male rats, and the expression of EGF on its receptors on target organs, beta cells of islets of Langerhans.

The combination of increased submandibular and kidney EGF expression after sildenafil treatment with strongly positive immunoexpression of EGF on its receptors in beta cells, together with β-cell ultrastructure of islets of Langerhans which observed in previous study [(83)], strongly suggests that sildenafil has stimulate submandibular secretion of EGF. Likewise, epidermal growth factor (EGF) ligands contribute to islet development and therefore may represent candidates for modulating the differentiated state of adult human islet cells. These data agreed with earlier study [(86)], which proposed that sildenafil increased the secretion of EGF and total protein except amylase from the submandibular gland. Sildenafil antioxidant activities might postulate the exact mechanism of sildenafil's stimulatory effect, which might be indorsed to its augmenting effect on cellular cyclic GMP [(74)]. Another study by El-Gamal DA *et al* 2011, in agreement with the present data where cGMP have stimulatory effects on salivary functions. Regarding beneficiary effects of increased salivary flow rate and secretion of EGF in recover β-cells due to sildenafil antioxidative actions. Activation of the nitric oxide (NO) system against oxidative stress by overexpression of NO synthase could be another mechanism for the action of sildenafil [(83)]. Interestingly, β-cell enhancement detected in current

study was upgraded after sildenafil treatment, which could be also attributed to its antioxidative actions. New approaches have used EGF for enhancement β-cell regeneration directly or by EGF stimulated mesenchymal cells [82, 81]. So, it was reasonable for β-cells to recover and developed after increased EGF secretion. Other findings suggest that PDEIs may improve secretion of insulin significantly in response to glucose by the lower doses of tested PDE inhibitors. The level of ROS at the lower doses of PDEIs decreased and the viability of islets were increased [163]. Deletion or over-expression experiments in mice involving components of the cGMP signaling pathway have contributed to the understanding of long-term effects of altered cGMP signaling, including the regulation of gene expression , may explain modulatory effect of sildenafil on EGF synthesis but further studies are in proposal to investigate this possibility. Some of the effects of cGMP on gene expression are indirect, through cGMP modulation of other signaling pathways, e.g. mitogen activated protein kinase pathways [164].

However, some effects of cGMP can be directly attributed to cGMP regulation of specific transcription factors such as Cyclic AMP-response element binding protein (CREB) and Proto-oncogene (c-Fos), and are mediated by cGMP-dependent protein kinases [164, 165]. The secretion of EGF from the granular ducts of submandibular gland and released into plasma is predominantly an adreno-receptor mediated ca^{2+}-dependant event by cervical sympathetic nerve stimulation, suggesting that its release is regulated by the sympathetic nervous system [166]. It is conceivable that sildenafil may have direct central effects on sympathetic outflow. This potential mechanism is supported, in part, by evidence that sildenafil crosses the blood-brain barrier and that PDE5 is present in the brain [167]. The important findings of this possibility, that sildenafil elicits a marked increase in sympathetic nerve activity, as measured by intra neural recordings of muscle sympathetic nerve activity (MSNA) and by plasma catecholamine levels [136]. Later studies may have

important implications for understanding of sildenafil effect on submandibular sympathetic innervation. Considerably, raise in EGF expression can participate in explanation of liver changes after sildenafil treatment, where EGF receptor and insulin receptor networks share many downstream components. So, both insulin and EGF increase the tyrosine phosphorylation and activation of IRS-1 and IRS-2 while EGF is also capable of activating additional PI3-kinase pools and, thus, epidermal growth factor can evoke metabolic responses and augment the downstream signaling of insulin [(168)].

4.4 Serum marker

4.4.1 Effect of various doses of sildenafil citrate on Interleukin 1-beta (IL-1β)

In various signaling pathways, cyclic guanosine monophosphate serves as a second messenger participating and regulating many aspects of cell function. In addition the traditional adjusting role of cGMP in relaxation of smooth muscle and vascular tone regulation, several other physiological roles have lately been revealed. As example, cGMP signaling reported that it has been involved in the downregulation of P-selectin expression and leukocyte recruitment in mice. In both human monocytes and murine macrophages, cGMP analogues and cGMP elevating agent also have displayed some anti-inflammatory activity by inhibiting TNF-a and IL-1β secretion [(169)]. In the present study, *in vivo* effects of once daily oral administration of the PDE5 inhibitor sildenafil for six weeks in normal rats, led to a reduced concentration of serum IL-1β dose-dependently. Many studies on the synergistic of the current result, where sildenafil down regulate inflammatory cytokines [(170, 171)]. In alternative human study, sildenafil has no significant effect on serum IL1beta in low doses less than 0.3mg/kg. This completely in accordance with present data in serum IL-1β in group A compared to control group after 0.5mg/kg sildenafil [(172)]. Previous studies address that increasing the bioavailability of cGMP

might be beneficial in ameliorating the inflammation, elevates intracellular cGMP, but not cAMP suppresses the LPS-induced production of TNF- and IL-1β. Also cGMP analogues 8-bromo-cGMP and dibutyryl cGMP as well as the NO donor S-nitrosocysteine were used to inhibit TNF- and IL-1 release after stimulation with LPS. Inhibition of PDE by zinc abrogates the LPS-induced release of TNF- and IL-1 by increasing intracellular cGMP levels. Interleukin 1-beta processing and activation involves the IL-1-converting enzyme, a caspase family cysteine protease **(110)**.

An inhibition of caspase activity through cGMP-dependent pathways which has been demonstrated previously, may explain these findings. When the NO donor S-nitrosocysteine was used to activate sGC, LPS-induced TNF- and IL-1release was suppressed. Cyclic guanosine monophosphate (cGMP) influences the signal transduction that leads to the transcription of TNFα and IL-1β. The major cGMP target in many cell types is protein kinase G, and cGMP-dependent inhibition of Raf-1 by phosphorylation on serine 43 by protein kinase G has been reported. High cGMP concentrations can cross-activate protein kinase A and lead to extracellular signal-regulated kinases (ERK) as well as Raf-1 inhibition followed by decrease activation of nuclear transcription factor of its target genes encoding cytokines including IL1 beta **(110)**. This study has important findings, that serum IL-1β was negatively correlated with insulin so β-cell function and positively related with fasting glucose. Based on this data, it was concluded that IL-1β as an inflammation cytokine play important role insulin secretion of pancreatic beta cell. These data support the recent hypothesis that IL-1β involved with the demise of the β-cell **(112)**. Therefore blocking IL-1β, improve the function of the β-cells or possibly allows for partial regeneration of β-cells **(173)**.

4.4.2 Effect of various doses of sildenafil citrate on total antioxidant capacity (TAC):

The human body is equipped with a complete arsenal of defenses against external and internal aggression. Those protecting against the so called reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide are crucial in inflammatory and antibacterial responses [(174)]. The concentrations of these metabolic intermediates are kept under strict control by the activity of a complex defence system including enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) and molecule with thiol groups such as glutathione. However, an uncontrolled production of ROS is liable to occur in several conditions leading to a situation known as (oxidative stress) where high levels of ROS can attack many essential biomolecules (haemoglobin (Hb) protein, DNA, RNA, lipids) and various cell structures, causing oxidative damage. In fact, many pathological processes are initiated or aggravated by such processes [(175)], many clinical and experimental previous studies recognize the potent antioxidant effect of sildenafil. Sildenafil prevents induction of oxidative stress in salivary glands. There is also good evidence of lipid peroxidation protection properties of cAMP or cGMP analogues in plasma of diabetic rats, and rat neural and renal cells, where the current results for the oxidative status of sildenafil support these findings [(176, 177)].

On the other hand, a role for reactive oxygen intermediates in the regulation of hepatic glucose production also has been reported, indicated a protective role for cGMP from induction of oxidative stress inside cells. It has been reported that sildenafil, through the inhibition of PDE5 and augmentation of inhibitory action of the NO-cGMP axis on NADPH oxidase expression and activity inhibits superoxide formation. Thus antioxidant potential of sildenafil might be another mechanism for its glucose lowering effect and inhibition of glycogenolysis [(73)], the hypothesis that

was also established in another work that proposed the protective effects of cAMP and cGMP phosphodiesterase inhibitors in restoration of hyperglycemia stress back to their antioxidant potentials in diabetic rat β-cell [(74)]. Thus, reducing superoxide levels may be an important method to lowering glucose level. Sildenafil citrate by enhancing cGMP levels, inhibit the activity and expression of NADPH oxidase in mitochondria. If the activity and expression of NADPH oxidase are inhibited, then the formation of ROS will decrease and antioxidant enzymes will increase. Therefore, elevated cGMP levels and decreased superoxide formation would augment antioxidant enzyme activities. The resultant decrease in endogenous superoxide would in turn increase the bioavailability of NO. Enhanced NO availability would further inhibit NAD[P]H oxidase activity and expression. This would create a 'self-augmenting' therapeutic loop (fig. 4-1) [(178)].

Authors in previous studies also propose that PDE5 inhibiter might have potent antioxidant effect, either through upregulation of SOD and catalase, although MDA levels decreased slightly. The increase in red blood cell CAT and SOD values in subjects during sildenafil treatments has been attributed to the inhibition of free radicals and lipid peroxidation [(176, 179)]. Sildenafil citrate has been shown to have protective effects against oxidative stress by inhibiting free radical formation and supporting antioxidant redox systems, and it was suggested that this reduction in oxidative stress results in improvement of endothelial function [(180)].

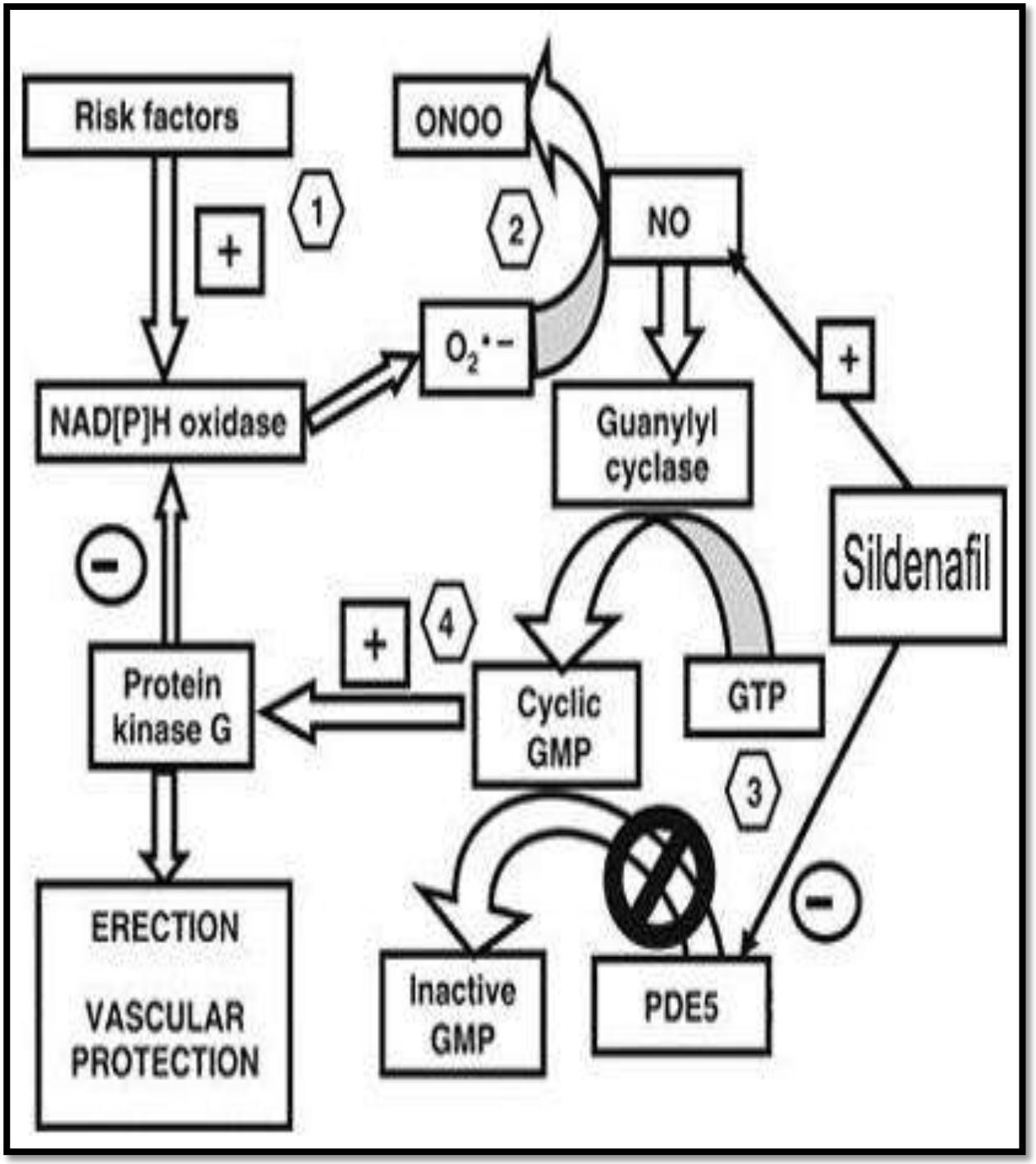

Fig. (4-1) [178]: Proposed mechanism underlying the antioxidant effect of sildenafil. **PDE5:** Phosphodiesterase-5; **NO:** Nitric oxide; **GMP**: Guanosine monophosphate; **GTP**: Guanosine triphosphate; **NADPH**: Reduced Nicotinamide adenine dinucleotide phosphate; **$O_2^{\bullet -}$**: Superoxide anion; **ONOO:** Peroxynitrite.

4.4.3 Effect of various doses of sildenafil citrate on serum fasting blood glucose (FBS) and C- peptide:

After all revealed explanation discussed previously, suppose that sildenafil after daily oral dose for six weeks lowered blood glucose and markedly increase C-peptide level in higher dose. Additionally, these results agreed with previous studies; where, nitric oxide (NO) may play a key role in mediating the metabolic effects of insulin, including stimulation of muscle glucose uptake in a mouse model of insulin resistance [(181)]. Given the role of NO-cGMP signaling in muscle metabolism, the current studies addressed the hypothesis that augmenting cGMP signaling by preventing cGMP hydrolysis would enhance *in vivo* insulin action. Because an increase in blood flow may enhance muscle glucose uptake by increasing delivery of substrates to skeletal muscles, inhibition of PDE5 in the arterial vasculature would be predicted as another mechanism in decrease fasting blood glucose [(182)]. As previously mentioned, a role for central inhibition of phosphodiesterase-5 on energy and glucose metabolism is also a possibility that cannot be ruled out [(57)]. Sildenafil, through the inhibition of PDE5 and augmentation on the action of NO-cGMP axis shows similar effects to NO in many organs. Also, injection of NO in alloxan-induced diabetic mice mimics the effect of insulin in the control of hyperglycemia by lowering plasma glucose concentration. Moreover, the hypoglycemic effect of sildenafil is related to increase in liver glycogen content (LGC) where blood glucose is converted to hepatic glycogen. Effects of sildenafil on LGC were in agreement with those reported by Hoseini S *et al* 2006 who found that sildenafil administration markedly reduces liver glycogenolysis [(73)].

Low doses of phosphodiesterase-5 inhibitors have distinct effects on liver, other insulin-sensitive tissues additional and main site of glucose utilization for storage as glycogen by promoting substrate uptake and glucose removal via NO-cGMP pathway increases blood flow and enhance the circulation. Further putative

underlying mechanisms of blood glucose lowering effect of sildenafil, NO-cGMP pathway activates expression of peroxisome proliferator-activated receptor-γ coactivator-1α, thereby enhancing mitochondrial biogenesis and oxidative phosphorylation [183, 133]. Interestingly, only high dose of sildenafil affected C-peptide level and increased its level, also this findings with agreement of other study where sildenafil elevated serum insulin, C-peptide levels, LGC and decreased blood glucose level in dose dependent manner [146]. Inhibition of cGMP hydrolysis activates cGMP-dependent protein kinase G, which phosphorylates and activates hormone-sensitive lipase then increases free fatty acid (FFA) availability and energy expenditure. Pancreatic β-cells are affected by FFAs depending on the duration of exposure. Acutely, FFAs exaggerated insulin secretion [184].

4.5 Conclusion

- Administration of sildenafil markedly lowers blood glucose concentration which in turn related to increase liver glycogen content, reduction in liver glycogenolysis and glycogen phosphorylase activity.
- Sildenafil increase EGF production which improve synthesis of insulin from the β cells of islets of Langerhans.
- Sildenafil improves the function of the β-cells or possibly allows for partial regeneration of β-cells by possessing the anti-oxidant properties and anti-inflammatory effect through down regulation of IL-1β.

4.6 Recommendations

- Longer duration of treatment with large scale of rats.
- Using of larger doses of sildenafil with different rout of administration.
- Study the effect of sildenafil on other markers such as eNOS, specific protein kinase, Natriuretic peptide (NP), glucagon, TNFα and IL-6.
- Investigate the effect of sildenafil on EGF and IL-1β gene expression.
- Study the effect of sildenafil on experimental diabetic animal model.
- Study the effect of sildenafil in treatment of obesity.

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الخلاصة

المقدمة:

السيلدينافيل هو مثبط انتقائي لأنزيم ثنائي الأستر الفسفوري النوع الخامس، مما يؤدي الى رفع النوكليوتيدات الدورية داخل الخلايا كمرسلات ثانوية تساهم في مسارات الإشارات بين الخلايا المشاركة في الاوضاع الفسيولوجية و المرضية. عامل نمو البشرة (EGF) يتم إنتاجه في القوارض بشكل رئيسي من قبل الغدد تحت الفكية والكلى ، وهو مهم في تطوير خلايا-بيتا . الجليكوجين فسفو-ريليز (GP) هو الأنزيم الرئيسي في هدم الجليكوجين الذي يحفز عملية هدم الجليكوجين . في الكبد، الجليكوجين فسفو-ريليز (GP) له دور مهم في توازن السكر في الدم. انترلوكين-1 بيتا (IL- 1β) هو بروتين خلوى مؤيد للالتهابات يقوم بأدوار هامة في الالتهاب . IL- 1β يرسل محفزات لنشاط الأنسولين و إفرازه.

الهدف:

التحقيق في التأثيرات النسيجية و الكيمياء المناعية للسيلدينافيل على الكلى والكبد والبنكرياس و الغدة تحت الفكية لذكور الجرذان البالغين.

المواد وطرق العمل:

تم تقسيم ثمان وأربعين من الجرذان الذكور البالغين إلى أربع مجموعات متساوية :

المجموعة 1: بمثابة المجموعة الضابطة . المجموعة 2 و 3 و 4 : أعطيت الجرذان فيها 0.5، 1، 2 ملغ / كغ من السيلدينافيل عن طريق الفم على التوالي (مرة واحدة يوميا عن طريق أنبوب المعدة) لمدة 6 اسابيع . تحت التخدير، تم تشريح جميع الحيوانات في هذه الدراسة بعد 6 أسابيع من العلاج . تم تجهيز الكلى والكبد والبنكرياس و الغدد تحت الفكية للفحص النسيجي و النسيجي الكيمياء المناعي . الدم تم جمعه لفحص انترلوكين-1 بيتا (IL- 1β) ، وإجمالي القدرة المضادة للأكسدة (TAC) وسي- الببتايد في المصل. وزن الجسم و نسبة الجلوكوز في الدم سجلت قبل العلاج و في اليوم الأخير من التجربة.

النتائج:

كان هناك انخفاض ذو دلالة معنوية عالية ($p<0.01$) في وزن الجسم بعد الجرعة الفموية من السيلدينافيل (0.5، 1، 2 ملغ / كلغ) بالمقارنة مع وزن الجسم الأولي لكل حيوان في جميع الفئات. وقد لوحظ ارتفاع ذو دلالة معنوية عالية ($p<0.01$) مع محتوى جليكوجين الكبد في مجموعة A، B، C بالمقارنة مع المجموعة الضابطة (D). ومن المثير للاهتمام، أن السيلدينافيل قام بتخفيض فسفوريليز جليكوجين الكبد (GP) بشكل ذو

دلالـة معنويـة ($p<0.05$) فـي المجموعـة (B) و بشـكل ذو دلالـة معنويـة عاليـة ($p<0.01$) فـي المجموعـة C مقارنـة مـع المجموعـة D. بعـد العـلاج بالسـيلدينافيل، الكشـف النسـيجي الكيميـاء المنـاعي لعامـل نمـو البشـرة (EGF) أظهر زيادة عدد الخلايا موجبة المناعة و تفاعل منـاعي قويـة لعامل نمو البشرة (EGF) فـي الكلـى و الغدد تحت الفك السفلي بالمقارنـة مـع المجموعـة الضـابطة. تفاعليـة مناعيـة قويـة لمستقبلات عامل نمو البشرة (EGF) تم تعيينها في الخلايا-بيتا. السلدينافيل و بشكل ملحوظ يزيد إجمـالي القدرة المضـادة للأكسدة (TAC) فـي المصـل ويقلل مستوى IL-1β في المصـل. مستويات السـي الببتايد فـي المصـل أظهـرت ارتفـاع ذو دلالـة معنويـة عاليـة ($p<0.01$) فـي المجموعـة C بالمقارنـة مـع المجموعـة الضـابطة وارتفـاع ذو دلالـة معنويـة ($p<0.05$) بالمقارنة مع مجموعة العلاج الأخرى. وبالإضـافة إلـى ذلك، تم تخفيض مستوى السكر فـي الـدم بشكل ذو دلالة معنوية ($p<0.05$) في المجموعة (A) ولوحظ انخفاض ذو دلالة معنوية عالية ($p<0.01$) في المجموعة B و C بالمقارنة مع مستوى السكر الأولي المتعلق بكل مجموعة.

الاستنتاج:

السيلدينافيل يمتلك فغالية عالية في رفع إنتاج عامل نمو البشرة (EGF) والذي من شأنه تحسين تصنيع الأنسولين في خلايا-بيتا و بالتالي يقلل من تركيز السكر في الدم ويقلل من تهدم الجليكوجين الكبدي الى كلوكوز.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
الجامعة المستنصرية
كلية الصيدلة

دراسة كيمياء حيوية و كيمياء مناعية نسيجية للسيلدنافيل على الأنسجة المختلفة في ذكور الجرذان

رسالة مقدمة الى فرع الأدوية والسموم والى لجنة الدراسات العليا في
كلية الصيدلة /الجامعة المستنصرية كجزء من متطلبات الحصول على
شهادة الماجستير في علوم الصيدلة (الأدوية والسموم)

من قبل

الصيدلاني

أبراهيم حسن نشمي

(بكالوريوس صيدلة 2002)

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