University of Al Qadisiya

College of Pharmacy

Renal protective effect of cilostazol against diclofenac induced nephrotoxicity

Graduation research submitted to College of Pharmacy

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2017 A.D

1438 A.H

بسم الله الرَّحْمَنِ الرَّحِيمِ

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

صدَقَ آلله العليٌ آلعظيْم

سورة البقرة – الاية ٣٢

DEDICATION

I dedicate this project to God Almighty
my creator, my strong pillar
to my family , my friends , my
professors , and to all Iraqis who
sacrifice to make us live in peace ..

Mohammad A. Rahman Mohammad K. Lahmud Ali T. Saeed

ACKNOWLEDGEMENTS

We are greatful to Allah for giving us the strength to successfully conduct this project and for sustaining our efforts which many time did ascillate .

We are deeply indebted to ou supervisor pharmacist **Hussam H. Sahib** without whose valuable advices and suggestions for the correction , modification and improvement this project would not have been success .

Abstract :

Diclofenac : a non- selective NSAID with greater selectivity to COX-2 , used to treat mild to moderate pain . The primary mechanism responsible for its anti-inflammatory, antipyretic, and analgesic action is thought to be inhibition of prostaglandin synthesis by inhibition of cyclooxygenase (COX) enzyme . Cilostazol, a selective phosphodiesterase III inhibitor, has potent anti platelet and vasodilator effects . Cilostazol has inhibitory effect on reactive oxygen species and superoxide generation as well as hydroxyl radicals scavenging action .

This work aimed to investigate the possible protective effect of cilostazol on diclofenac –induced nephrotoxicity and the possible underlying mechanisms.

Materials and methods: 24 male albino rats were divided into 4 equal groups:

1- Control .
2- diclofenac100mg/kg i.p once daily for one day .
3- (Diclofenac + cilostazol) diclofenac 100mg/kg i.p once daily and one hour after that the administration of cilostazol intraperitoneally at a dose of 20 mg/kg for one day .
4- (Vehicle group) : the rats in the DMSO group received 1.5 ml of DMSO was administered intra-peritoneally to rat . On second day blood samples were collected for the estimation of creatinine, urea in serum .

Results : Diclofenac elevated the serum levels of creatinine, urea Administration of cilostazol decreased urea, creatinine .

Conclusion : Cilostazol protected rats from diclofenac-induced nephrotoxicity possibly, in part through its antioxidant and anti-apoptotic activity .

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List of abbreviations

AKI	Acute Kidney Injury
GFR	Glomerular Filtration Rate
PG	Prostaglandin
DCLO	Diclofenac
S.Cr	Serum Creatinin
BUN	Blood Urea Nitrogen
TMA	Thrombotic Microangiopathy
DMSO	DiMethylSulfoxide

Chapter One

Introduction

Chapter One

Introduction

- AKI can be defined as an abrupt (1 to 7 days) and sustained (more than 24 hours) decrease in kidney function.
- Over 30 definitions of acute renal failure/AKI have been used in the literature.
- The ADQI formulated the RIFLE criteria in 2004 to allow for AKI to be objectively and uniformly defined (1) .

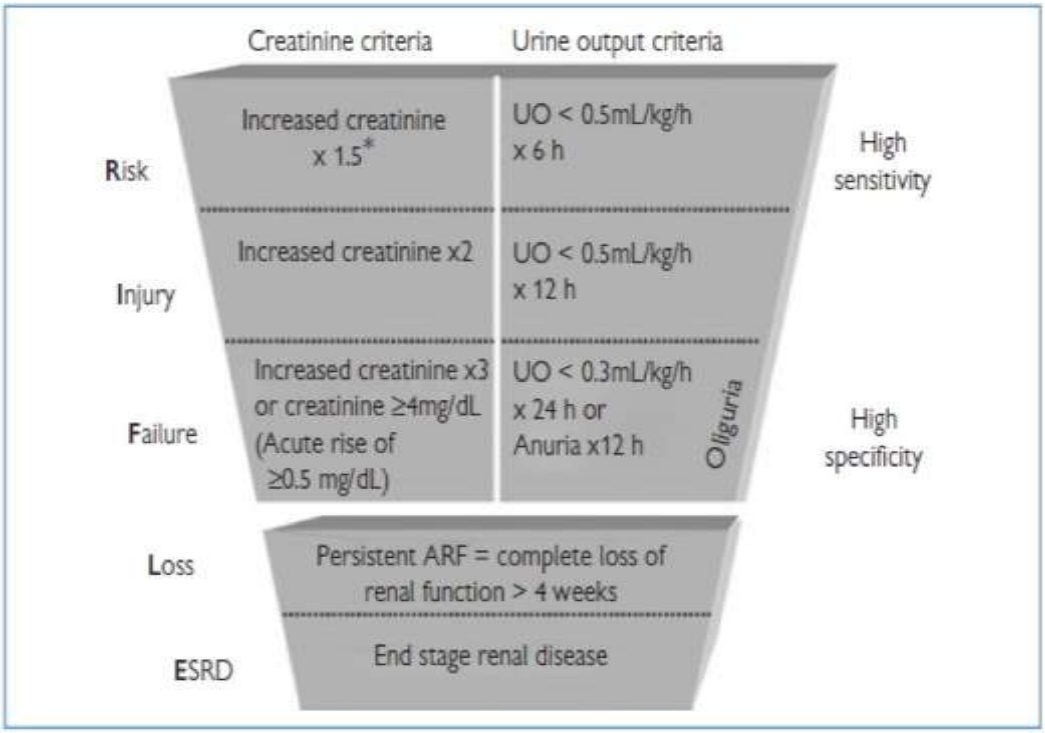

Figure 1 : RIFLE CRITERIA Of AKI (1)

Based on the amount of urine that is excreted over a 24-hour period, patients with ARF are separated into two groups :

- Oliguric: patients who excrete less than 500 milliliters per day(< 16 oz/day)
- Nonoliguric: patients who excrete more than 500 milliliters per day (> 16 oz/day) ,Approximately 50-60% of all causes of AKI are nonoliguric. (2)

In nonoliguric patients, the urine is of poor quality (i.e., contains little waste) because the blood is not well filtered, despite the fact that an adequate volume of urine is excreted.

Both kidneys are failing when ARF occurs. One normally functioning kidney can maintain adequate blood filtering.

Signs and symptoms of acute kidney injury differ depending on the cause and may include :

- Too little urine leaving the body
- Swelling in legs, ankles, and around the eyes
- Fatigue or tiredness
- Shortness of breath
- Confusion
- Nausea
- Seizures or coma in severe cases
- Chest pain or pressure

In some cases, AKI causes no symptoms and is only found through other tests done by your health care provider.(2)

Types of Acute Renal Failure

The three types of AKI are named for their location within the renal (kidney) system :

- Prerenal AKI : As an adaptive response to severe volume depletion and hypotension, with structurally intact nephrons .
- Intrinsic renal AKI: In response to cytotoxic, ischemic, or inflammatory insults to the kidney, with structural and functional damage .
- Postrenal ARF: From obstruction to the passage of urine .(3)

Risk Factors for drug induced AKI

A simplified approach to understanding the renal vulnerability to nephrotoxins entails the classification of risk factors into three major categories . Each category or specific risk factor contributes to the enhanced development of kidney injury. In general, more than one of the risk factors is acting to promote renal injury. Most often, at least two or all three conspire to cause various forms of clinical kidney disease. It is these factors that explain the variability and heterogeneity seen with drug or toxin-induced kidney disease (4).

1- Patient-Specific characteristics

The patient exposed to drugs and other substances is predisposed to develop nephrotoxicity when certain underlying risk factors are present. Many of these factors are non modifiable, such as older age and female gender. Risk in the elderly and females occurs through the following :

1) Changes in total body water, which is reduced in setting of decreased lean body mass and leads to drug overdose .

2) Unrecognized lower GFR despite normal serum creatinine concentration .

3) Reduced drug binding to proteins due to hypoalbuminemia, which results in increased free drug concentrations (5).

The elderly also have increased propensity to vasoconstriction from excessive angiotensin II and endothelin and have higher levels of oxidatively modified biomarkers (4). These factors combine to expose the patient to excess drug concentration and risk of nephrotoxicity.

Other risk factors include:

- Pre-existent renal disease
- Specific disease (diabetes mellitus, multiple myeloma, proteinuric patients)
- Sodium-retaining states (cirrhosis, heart failure, nephrosis)
- Dehydration and volume depletion
- Acidosis, potassium and magnesium depletion
- Hyperuricemia, hyperuricosuria
- Sepsis, shock
- Renal transplantation(6)

The underlying genetic makeup of the host can also enhance renal vulnerability to potential nephrotoxins (7,8). The drug or its metabolite form adducts that modify their physical structure, making them more immunogenic. There is, however, significant heterogeneity in the response of patients to drugs and exogenous exposures. One obvious example is the heightened allergic response of some individuals as compared with others. Differences in innate host immune response genes can predispose certain patients to develop an allergic response to a substance. The variability of immune responses has been demonstrated in patients who develop drug-induced interstitial nephritis, which appears to be a T cell driven process (9). This translates into enhanced vulnerability to an allergic response in the kidney and development of an acute interstitial nephritis.

Polymorphisms of genes encoding proteins involved in the metabolism and subsequent renal elimination of drugs have been described and are correlated with various levels of drug sensitivity. Specific to the discussion of nephrotoxicity, mutations may cause loss-of-function in apical secretory transporters (reduce cell drug efflux into the urine), and mutations in kinases that regulate drug carrier proteins can impair drug elimination and promote toxicity by elevating intracellular toxin concentrations (8).

2- Kidney-Specific Factors

The next category of risk relates to the mechanism by which the kidney metabolizes and excretes various drugs and toxins .(Significant renal exposure to potential nephrotoxins occurs due to the high rate of drug and toxin delivery to the kidney, a result of the high blood flow to the kidney, which approaches 25% of cardiac output. Many renal cells, particularly those in the loop of Henle, exist in a relatively hypoxic environment due to the high metabolic rates required to actively transport many solutes via Na^{+}-K^{+}-ATPase driven transport. This excess cellular workload and hypoxic environment promotes increased sensitivity to injury when exposure to potentially nephrotoxic substances occurs (10,11). Another factor that enhances renal nephrotoxicity is the high concentration of parent compounds and their metabolites that develop in the renal medulla and interstitium from the enormous concentrating ability of the kidney (10,11). Elevated tissue concentration of these toxins promotes injury through both direct toxicity and ischemic damage (reduced prostaglandins, increased thromboxane).

Biotransformation of drugs, xenobiotics, and other substances by multiple renal enzyme systems, including CYP450 and flavin-containing monooxygenases, favors the formation of toxic metabolites

and reactive oxygen species (10,12). The presence of these byproducts of metabolism tilts the balance in favor of oxidative stress, which outs trips natural antioxidants and increases renal injury via nucleic acid alkylation or oxidation, protein damage, lipid peroxidation, and DNA strand breaks (10,12).

Enhanced toxicity in proximal tubular cells occurs due to the extensive cellular uptake of potential toxins and drugs by both apical (aminoglycosides, sucrose) and basolateral transport systems (tenofovir) .(12)

3- Drug-Specific Factors , include :

- Inherent nephrotoxic potential
- Dose
- Duration , frequency and form of administration
- Repeated exposure
- Drug interactions
- Combined or closely associated use of diagnostic or therapeutic agents with added or synergistic nephrotoxic potential

- Most drugs found to cause nephrotoxicity exert toxic effects by one or more of the following pathogenic mechanisms :

ALTERED INTRAGLOMERULAR HEMODYNAMICS

In an otherwise healthy young adult, approximately 120 mL of plasma is filtered under pressure through the glomerulus per minute, which corresponds to the glomerular filtration rate (GFR). The kidney maintains or auto regulates intraglomerular pressure by modulating the afferent and efferent arterial tone to preserve GFR and urine output.

For instance, in patients with volume depletion, renal perfusion depends on circulating prostaglandins to vasodilate the afferent arterioles, allowing more blood flow through the glomerulus.

At the same time, intraglomerular pressure is sustained by the action of angiotensin-II–mediated vasoconstriction of the efferent arteriole. Drugs with antiprostaglandin activity (e.g., nonsteroidal anti-inflammatory drugs [NSAIDs]) or those with antiangiotensin-II activity (e.g., angiotensin-converting enzyme [ACE] inhibitors, angiotensin receptor blockers [ARBs]) can interfere with the kidneys' ability to auto regulate glomerular pressure and decrease GFR (13,14) . Other drugs, such as calcineurin inhibitors (e.g., cyclosporine, tacrolimus), cause dose-dependent vasoconstriction of the afferent arterioles, leading to renal impairment in at-risk patients (15).

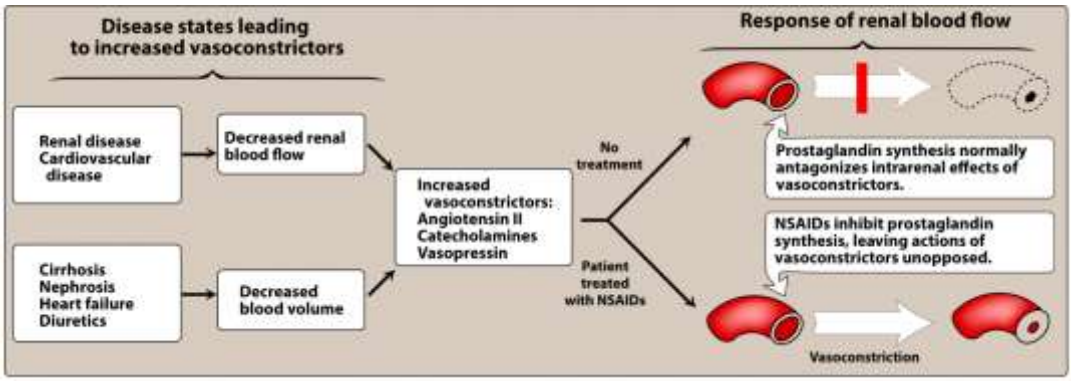

Figure 2: Renal effect of NSAIDs inhibition of prostaglandin synthesis (14)

TUBULAR CELL TOXICITY

Renal tubular cells, in particular proximal tubule cells, are vulnerable to the toxic effects of drugs because their role in concentrating and reabsorbing glomerular filtrate exposes them to high levels of circulating toxins (16). Drugs that cause tubular cell toxicity do so by impairing mitochondrial function, interfering with tubular transport, increasing oxidative stress,or forming free radicals(17,18). Drugs

associated with this pathogenic mechanism of injury include aminoglycosides,amphotericin B, antiretrovirals (adefovir, cidofovir, tenofovir) , cisplatin , contrast dye, foscarnet, and zoledronate (12,19)

INFLAMMATION

Drugs can cause inflammatory changes in the glomerulus, renal tubular cells, and the surrounding interstitium, leading to fibrosis and renal scarring. Glomerulonephritis is an inflammatory condition caused primarily by immune mechanisms and is often associated with proteinuria in the nephrotic range. Medications such as gold therapy, hydralazine, interferon-alfa, lithium, NSAIDs, propylthiouracil (16,18) .

Acute interstitial nephritis, which can result from an allergic response to a suspected drug, develops in an idiosyncratic, non–dose-dependent fashion(20) . Medications that cause acute interstitial nephritis are thought to bind to antigens in the kidney or act as antigens that are then deposited into the interstitium, inducing an immune reaction(20). However, classic symptoms of a hypersensitivity reaction (i.e., fever, rash, and eosinophilia) are not always observed (18,20) . Numerous drugs have been implicated, including allopurinol , antibiotics (especially beta lactams, quinolones, rifampin , sulfonamides, and vancomycin) , antivirals (especially acyclovir and indinavir), diuretics (loops, thiazides), NSAIDs, phenytoin, proton pump inhibitors, and ranitidine(21) .

Chronic interstitial nephritis is less likely than acute interstitial nephritis to be drug induced; it is also insidious in onset, and signs of hypersensitivity are often lacking(22) . Drugs associated with this mechanism of nephrotoxicity include calcineurin inhibitors (e.g. cyclosporine, tacrolimus), certain chemotherapy agents, Chinese herbals containing aristocholic acid, and lithium. Chronic interstitial nephritis has been reported with analgesics such as acetaminophen, aspirin, and NSAIDs when used chronically in high dosages (i.e., more

than 1 gram daily for more than two years) or in patients with preexisting kidney disease (23,24) . Early recognition is important because chronic interstitial nephritis has been known to progress to end-stage renal disease. Diagnosis may be difficult because most patients do not consider over-the-counter preparations to be medications and tend to under report frequency of use (22).

CRYSTAL NEPHROPATHY

Renal impairment may result from the use of drugs that produce crystals that are insoluble in human urine. The crystals precipitate, usually within the distal tubular lumen, obstructing urine flow and eliciting an interstitial reaction(18) . Commonly prescribed drugs associated with production of crystals include :

-antibiotics (e.g., ampicillin, ciprofloxacin , sulfonamides)

-antivirals (e.g., acyclovir, foscarnet, ganciclovir)

- indinavir; methotrexate; and triamterene (17,19)

The likelihood of crystal precipitation depends on the concentration of the drug in the urine and the urinary pH(25) . Patients most at risk of crystal nephropathy are those with volume depletion and underlying renal insufficiency (25) .

AKI in this setting may be preventable if it is anticipated by appropriate drug dosing, volume expansion with high urinary flow, and alkalinization of the urine when appropriate .

RHABDOMYOLYSIS

Rhabdomyolysis is a syndrome in which skeletal muscle injury leads to lysis of the myocyte, releasing intracellular contents including myoglobin and creatine kinase into the plasma. Acute kidney injury (AKI) develops in this setting via the following 3 mechanisms :

- Renal vasoconstriction
- Heme-mediated proximal tubular cell toxicity
- Intratubular cast formation (26)

Heme proteins are believed to be involved in the generation of reactive oxygen species (ROS), which are known to cause tubular injury through peroxidation of membrane lipids and intracellular enzymes(26)

Clinical manifestations of rhabdomyolysis include weakness, myalgia, and tea-colored urine (27) .

- Drugs and alcohol are causative factors in up to 81 percent of cases of rhabdomyolysis, and up to 50 percent of patients subsequently develop acute renal failure (27) .
-
- Statins are the most recognizable agents associated with rhabdomyolysis, but more than 150 medications and toxins have been implicated .
- Many drugs of abuse, such as cocaine, heroin, ketamine , methadone, and methamphetamine, have been reported to cause rhabdomyolysis(26,27) .

THROMBOTIC MICROANGIOPATHY

In the normal kidney (as in the rest of the body), there are small blood vessels called capillaries. They are lined with a slippery coating of cells known as endothelial cells . When the endothelial cells of capillaries become damaged, blood flow through the kidney slows .

The liquid part of the blood, called plasma, helps waste flow to the kidney to be removed from body through urine . There are also solid particles in the blood, including red blood cells and platelets . Red blood cells carry oxygen from lungs to the rest of the body, including kidney cells . Platelets have the job of plugging up any damaged part of the blood vessel to keep it from leaking .

Defects in the blood vessel wall lining can produce rough patches that are like potholes on a road – they can slow traffic and cause a lot of damage (30) . Red blood cells can become deformed and then burst. Platelets can activate and cause clots to form . The wreckage from all these events can close off entire blood vessels. In the end, parts of your kidney can die from lack of blood flow, and body can run low on red blood cells and platelets .

Mechanisms of renal injury secondary to drug-induced thrombotic microangiopathy include an immune-mediated reaction or direct endothelial toxicity . Drugs most often associated with this pathogenic mechanism of nephrotoxicity include antiplatelet agents (e.g., clopidogrel, ticlopidine), cyclosporine, mitomycin-C , and quinine (31)

Diclofenac is a non- selective NSAID with greater selectivity to COX-2 , used to treat mild to moderate pain, or signs and symptoms of osteoarthritis or rheumatoid arthritis .The primary mechanism responsible for its anti-inflammatory, antipyretic, and analgesic action is thought to be inhibition of prostaglandin synthesis by inhibition of cyclooxygenase (COX) enzyme (29) .

Cilostazol, a selective phosphodiesterase III inhibitor, has potent antiplatelet and vasodilator effects. The drug is approved for treatment of intermittent claudication in patients with peripheral vascular diseases (32) . Cilostazol was generally well tolerated. Adverse events reported are headache, palpitation and tachycardia with mild to moderate intensity and rarely required treatment withdrawal (33) . Several investigation in different cells and tissues have indicated to inhibitory effect of cilostazol on reactive oxygen species and superoxide generation as well as hydroxyl radicals scavenging action (34) .

The aim of the present study is to assess the possible protective effect of cilostazol on DCLO -induced nephrotoxicity and the possible underlying mechanisms .

Chapter two

Materials and Methods

Chapter two

Materials and method

- Materials

Twenty four male Wistar albino rats over 16 weeks old (body weight 200-250 g) were fed a standard laboratory diet, given water ad libitum, and housed at a controlled room temperature (24.5-25 °C) with a 12-hour light-dark cycle before and during the experiment .

All experiments conducted in the college of pharmacy, Al-Qadisiyah University, according to the guidelines for the care and use of laboratory animals in scientific research.Tablets of CIL (Pletal ® 100 mg tb.Abdi İbrahim İlaçSanayi, Istanbul, Turkey) were crushed in sterile containers, and each tablet was dissolved in 1.5 ml of dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and centrifuged using an IKA Yellow Line TTS2 low profile vortex mixer (IKA -Werke GmbH & Co. KG, Staufen, Germany) at 2500 rpm. for five minutes to obtain a homogenous mixture . Diclofenac sodium (Voltaren) 75mg ampoules each one contain 75 mg diclofenac sodium is produced by Novartis Pharma Co.

Experimental protocol:

Twenty four female albino rats were used divided to Four groups each group contains 6 albino rats :

Group I: **(non-treated) control group**: The rats in the control group received no treatment .

Group II: **Diclofenac group (induce group)**: Diclofenac was administered intra-peritoneally at a dose of 100mg/kg once daily for one dayfor induction of experimental nephrotoxicity .

Group III: (Diclofenac + cilostazol) group: Diclofenac was administered intraperitoneally at a dose of 100 mg/kg once daily and one hour after that the administration of cilostazol intraperitoneally at a dose of 20 mg/kg for one day . (30)

Group IIII: (vehicle group): The rats in the DMSO group received 1.5 ml of DMSO was administered intra-peritoneal to rat (the rats administered DMSO without CIL at a volume which was similar to that used in the CIL group over the same period of time) . (30)

Blood samples were collected from the heart under thiopental sodium anaesthesia (70 mg/kg, intraperitoneal route, Pental sodium®1 g inj., I.E. UlagayIlac Sanayi, Istanbul, Turkey) 8 hours afterthe administration of diclofenac, cilostazol and dimethyl sulfoxide respectively. Serum was separated by centrifugationat 3000 _g for 10 min for estimation of creatinine and urea levels . (31)

Statistical analysis:

Results were expressed as the means + S.E.M. Statistical significant difference was determined by one-way analysis of variance(ANOVA) Probability values (P) less than 0.05 were considered to be statistically significant .

Chapter three

Results

Chapter three

Results:

Serum urea and creatinine levels : diclofenac significantly increased (P <0.05) urea and creatinine levels in relation to control groups. The previous parameters were significantly decreased (P <0.05) by diclofenac– cilostazol administration in relation to diclofenac -treated group , the use of vehicle was associated with a slightly increase on Sr.Cr and Sr.Urea levels , so the vehicle statistically insignificant increase the levels of Sr.Cr and Sr.Urea as shown in (Table 1) , figures (3 and 4) .

Group	Serum Creatinine mg/dL	Serum Urea mg/dL
Control	0.62± 0.06	32±2.33
Diclofenac	1.85± 0.13	75± 4.3
Cilostazol + Diclofenac	0.75± 0. 21	42± 3.5
DMSO	0.7± 0.02	35± 1.2

(Table 1) : Biochemical parameters of renal function evaluation at the end of the one day study protocol .

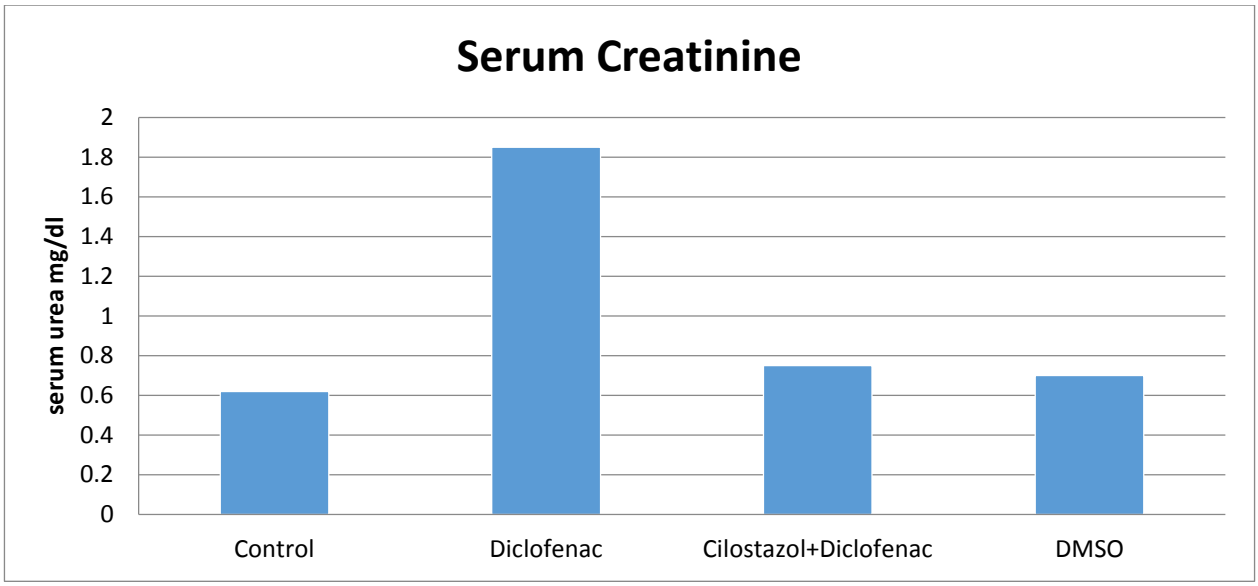

Figure (3): Shows S.Cr level for each group

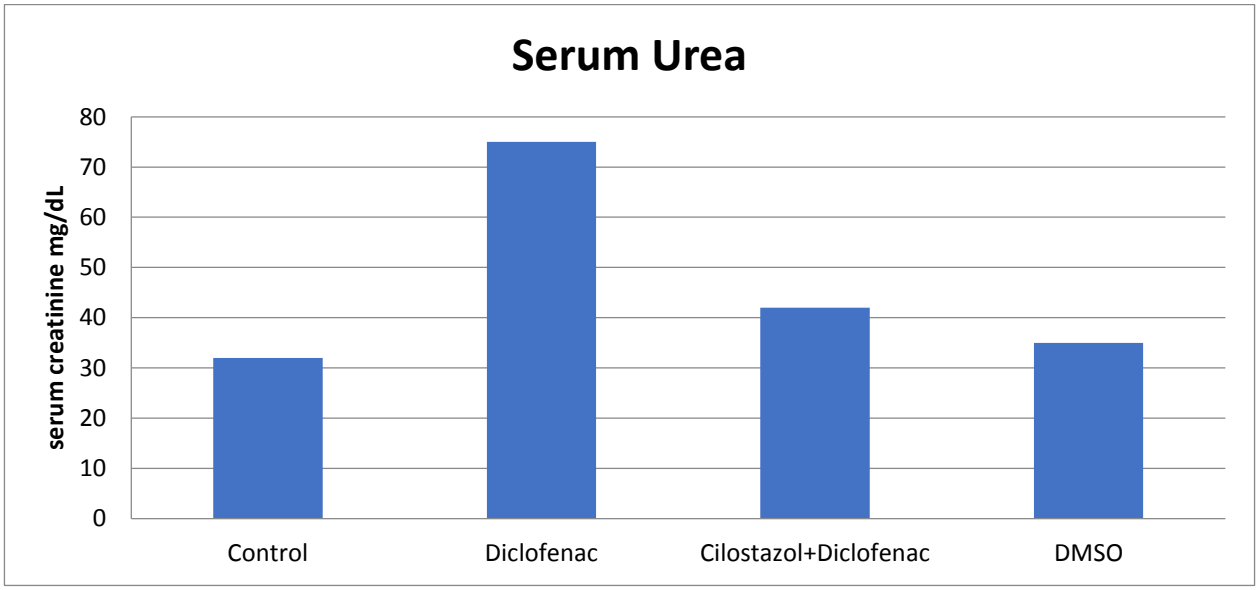

Figure (4): Shows S.urea level for each group

Chapter four

Discussion

Chapter four

Discussion

Diclofenac is one of the Non-steroidal anti-inflammatory drugs alter renal functions through their effects on renal prostaglandins leading to reversible renal ischemia . Although Prostaglandins do not play a physiologic role in maintaining renal blood flow in normal subjects , it plays a role in maintaining glomerular filtration rate (GFR) in intravascular depleted states, renal plasma flow is maintained by a balanced between the vasoconstrictor influence of the renin–angiotensin system and the vasodilatory effects of prostaglandins (35). In fluid depleted states, prostacyclin (PGI2) mostly affects renal homeostatic mechanisms. PGE2 and PGD2 cause dilatation of the renal vascular bed along with the lowering of renal vascularresistance. Thus, it enhances renal perfusion with redistribution of blood flow from the renal cortex to nephrons in the juxta-medullary region (36) . So, Prostaglandins become critical in maintaining GFR in volume depleted states. Hence, when the production of prostaglandins is blocked due to NSAIDs, it may lead to hyperkalemia, peripheral edema, increased blood pressure, weight gain and acute renal failure (37) .

In this study, uraemia, as an indicator of kidney damage, was significantly increased depending on dose. Diclofenac may cause kidney damage depending on dose and this effect may also be observed in rodents (38, 39). NSAIDs induced nephrotoxicity maybe due to the inhibitory effect of these drugs on prostaglandin synthesis, thus causing kidney ischemia (40).

cilostazol was effective to mitigate renal ischemia-reperfusion injury in rats It is known that oxidative stress plays an essential role in the development of Diclofenac nephrotoxicity .GSH has a very important

role in protecting against oxygen free radical detrimental effect through providing reducing equivalents for several enzymes act as a Scavenger to hydroxyl radicals and singlet oxygen. The results of the present study showed that administration of Diclofenac increased MDA level while decreased GSH in the renal tissue. Administration of cilostazol with Diclofenac decreased MDA level while increased GSH in relation to Diclofenac-treated group(40) .

Agrawal et al. (2007) reported that cilostazol inhibited lipid peroxidation and reduced oxidative stress through decrease the MDA level and improved glutathione level in blood of diabetic patients . The scavenging of super oxide radicals is achieved through SOD, which catalyses the dismutation of superoxide to hydrogen peroxide.(41)

The anti-apoptotic role of cilostazol was confirmed in endothelial cells via counteract tumor necrosis factor-a (TNF- a)-induced cell death(39) and by suppressing mitochondria dependent apoptotic signaling with decreased DNA fragmentation and subsequent stimulation of Bcl2 expression and down-regulation of Bax protein and cytochrome c release from mitochondria in different tissues (42).Similar to that used by other studies to demonstrate the beneficial role of cilostazol (43).

DiaaRagab et al alevealed that I/R-induced renal injury can be protected by cilostazol, via modulating the I/R-effect on oxidative stress, iNOS, NF-kB, IL-18and PPAR-c in renal tissues (44) .

Ahmed A. Abdelsameea etal demonstrated that Cilostazol attenuates gentamicin-induced nephrotoxicity in rats through its antioxidant and antiapoptotic activity (45) .

The present findings indicated that, cilostazol prevented lipid peroxidation and opposed the Diclofenac-induced redox tissue imbalance, possibly via its free radical scavenging property and/or by increasing the activity of the endogenous antioxidants (SOD and CAT).

Chapter five

Conclusion

Chapter five

Conclusion :

Cilostazol protected rats from **Diclofenac** induced nephrotoxicity . The nephroprotective effect of cilostazol could be meditated through its antioxidant and antiapoptotic activity.

Recommendation :

Further studies are recommended :

1- To find highest doses of cilostazol at renoprotective effect .
2- To find the effect of cilostazol on hepatotoxicity .

Chapter Six

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Chapter Six

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