A STUDY OF THE PROTECTIVE EFFECTS OF MONTELUKAST AGAINST DICLOFENAC INDUCE NEPHROTOXICITY

A project

SUBMITTED TO THE COLLEGE OF PHARMACY UNIVERSITY OF AL-QADISIYAH IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF B.Sc. pharmacy

By

Faiik H. Jowerah

Zainb .N Shanbooj

Supervised by

Assistant teacher

AHMED M. SULTAN

M Sc Pharmacology and Therapeutics

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Dedication

To whom Allah sent as mercy to the worlds

▫▫▫

To the prophet Mohammed.....

TO my parents

To my family

To everyone I love.....

Acknowledgement

This thesis would not have been possible without the blessing of God

I am heartily thankful to my supervisor, assistant teacher Ahmed M sultan, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

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List of abbreviations:

AIN	Acute interstitial nephritis
NSAIDs	Non-steroidal anti-inflammatory drugs
AKI	Acute kidney injury
GFR	Glomerular filtration rate
OTC	Over-the-counter
ATN	Acute tubular necrosis
TINU	Tubule-interstitial nephritis and uveitis
ATBM	Anti-tubular basement membrane
COX	cyclooxygenase
CysLTs	Cysteinyl leukotrienes
5-LO	5-lipoxygenase
LTs	Leukotrienes
PG	Prostaglandin
I.P.	Intraperitoneally
BUN	blood urea nitrogen
S. CR.	serum creatinine
MMPT	Mitochondrial Membrane Permeability Transition
SOD	superoxide dismutase
CAT	catalase
GPx	glutathione peroxidase

Summary

Background: Acute kidney injury (AKI) is characterized by a rapid, potentially reversible, decline in renal function including rapid fall in glomerular filtration rate (GFR) and retention of nitrogenous waste products over a period of hours or day. AIN is a common cause of acute renal dysfunction.The main causes of AIN can be grouped as drug induced, infection related, idiopathic forms (which would include tubule-interstitial nephritis and uveitis syndrome (TINU) and anti-tubular basement membrane (anti-TBM) disease), and AIN associated with sarcoidosis and other systemic diseases (systemic lupus erythematosus, Sjo ¨gren, malignancies).

Therefore this study was undertaken to evaluate the protective effect of montelukast against acute interstitial nephritis (ain) induced by diclofenac.

Materials and Methods: A total of Thirty-six young male Wister albino rats weighing 130-150 g, were used in this study. After acclimatization, rats were divided into 4 groups (5 rats each): Negative control group: (Rats were received nothing).Positive control group: (Rats were i.p. injected with diclofenac sodium at dose 100mg/kg body weight with water deprivation). Vehicle group: (Rats were i.p. injected with normal saline 2 ml/ kg body weight).Treatment group: (Rats were i.p. injected with montelukast 7 mg/kg i.p. injection) then after 30 minute inject diclofenac sodium at dose 100mg/kg body weight.

Five rats from each group were killed after 8 hours from beginning of diclofenac sodium and montelukast administration.At the end of the experiment, animals were anesthetized by thiopental sodium. The chest was opened by thoracotomy; blood sample was collected directly from the heart for measurement of blood urea nitrogen (BUN) and serum creatinine were measured by alkaline picrate method.

Results: The mean BUN was normal in negative control group (16.12± 0.6 mg/dl).Positive control group (diclofenac treated group) associated with higher

BUN levels (42.61± 6.3 mg/dl) when compared with negative control group.The effect of normal saline (0.9% Nacl) solvent used in the preparation of tested drug was evaluated next. There was statistically insignificant difference in mean BUN between positive control group and vehicle control group, and the effect of the solvent used in this study was almost negligibl**e.**Treatment group (Montelukast and diclofenac) group associated with statistically significant (P< 0.05 lower mean BUN (18.86± 1.5 mg/dl) when compared with the positive control group.

The mean serum creatinine was normal in negative control group (0.75 ± 0.25 mg/dl). Positive control group (diclofenac treated group) associated with higher but insignificant serum creatinine levels (1.15 ± 0.05 mg/dl) when compared with negative control group.The effect of normal saline (0.9% Nacl) solvent used in the preparation of tested drug was evaluated next. There was statistically insignificant difference in mean serum creatinine between positive control group and vehicle control group, and the effect of the solvent used in this study was almost negligible. Treatment group (montelukast and diclofenac)associated with lower but statistically insignificant lower mean serum creatinine (0.8 ± 0.01mg/dl) when compared with positive control group.

Conclusion: Montelukast has a nephroprotective effect against diclofenac induces acute interstitial nephritis (that investigated by higher levels of BUN and S creatinine) through its antioxidant effect on free oxygen radicals or directly increase the antioxidant enzymes activity (superoxide dismutase and catalase enzymes) and prevent the inhibition of these enzymes.

Chapetr one:

Introduction

1.1. Acute interstitial nephritis

The kidneys receive approximately 25% of the cardiac output and are the major organ for drug excretion and due to this function, the renal arterioles and glomerular capillaries are especially vulnerable to the effects of drugs (1).

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly used over-the-counter (OTC) medications and are known to have adverse effects on kidney function (2). OTC NSAIDs, including diclofenac, are routinely taken by teenagers for pain and fever (3). Because of their frequent and accepted use, NSAIDs are widely considered safe, but in reality, even therapeutic doses carry a risk of loss of renal function (2).

Acute kidney injury (AKI) is characterized by a rapid, potentially reversible, decline in renal function including rapid fall in glomerular filtration rate (GFR) and retention of nitrogenous waste products over a period of hours or day (4)**.** AKI increases the risk of death in patients after thoraco-abdominal aortic surgery, bone marrow transplantation, amphotericin B therapy, in patients with liver cirrhosis and in cardiac surgery (5) **.** AKI is classically divided into pre-renal, renal (intrinsic) and post-renal failure. Pre-renal ARF is a consequence of decreased renal perfusion (due to hypovolemia/shock or ischemia), which leads to a reduction in GFR. Intrinsic renal failure occurs when there is damage to the structures of the nephron such as the glomeruli, tubules, vessels, or interstitium(4).

The major cause of intrinsic ARF is acute tubular necrosis (ATN) that results from ischemic or nephrotoxic injury. Pre-renal ARF and ischemic ATN may occur as a

continuum of the same pathophysiological process, and together account for 75% of the causes of ARF(4).

Post-renal ARF follows obstruction of the urinary collection system with an increase in pressure within the renal collecting systems resulting in reduced GFR and renal failure (4). The various factors that predispose to ARF are hemodynamic instability, major vascular surgery, hypovolemia, atherosclerosis, diuretic therapy, preoperative starvation, congestive cardiac failure, peritonitis, ileus obstruction, biliary surgery, jaundice, diabetes mellitus, hypoxia, ischemia and reperfusion (I/R), pre-eclampsia/eclampsia, sepsis, major burns and pancreatitis (6).

AIN is a common cause of acute renal dysfunction (7,8).The main causes of AIN can be grouped as drug induced, infection related, idiopathic forms (which would include tubule-interstitial nephritis and uveitis syndrome (TINU) and anti-tubular basement membrane (anti-TBM) disease), and AIN associated with sarcoidosis and other systemic diseases (systemic lupus erythematosus, Sjo ¨gren, malignancies) (9,10) .

A large and expanding number of drugs have been implicated in causing AIN and it can be stated that any drug can theoretically induce an episode of AIN. However, the majority of cases have been caused by antimicrobial agents and NSAIDs (11, 12). AIN is based on an immunologic reaction against endogenous nephrogenic antigens or exogenous antigens processed by tubular cells, with cell-mediated immunity having a major pathogenic role after NSAID exposure of about a week (13, 14).

The inflammatory cellular infiltrates that characterize AIN, mainly composed of T lymphocytes and macrophages, are a powerful source of cytokines that increase the production of extracellular matrix and the number of interstitial fibroblasts, and

induce an amplification process recruiting more inflammatory cells and eosinophils into the interstitium (15, 16). AIN is now recognized as a major cause of drug induced AKI and accounts for about 15% of all patients with unexplained AKI (17).

1.2 NSAIDS

They are among the most widely used medications in the world because of their demonstrated efficacy in reducing pain and inflammation(18).Their efficacy has been documented in a number of clinical disorders, including osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, gout, dysmenorrhea, dental pain and headache(19,20).

The basic mode of action is inhibition of the pro-inflammatory enzyme cyclooxygenase (COX). NSAIDs as a class comprise both traditional nonselective NSAIDs (tNSAIDs) that nonspecifically inhibit both COX-1 and COX-2, and selective COX-2 inhibitors. Although effective at relieving pain and inflammation, tNSAIDs are associated with a significant risk of serious gastrointestinal adverse events with chronic use (21). Therefore, specific inhibitors of the COX-2 isoenzyme were developed, thus opening the possibility to provide anti-inflammatory and analgesic benefits, while theoretically leaving the gastroprotective activity of the COX-1 isoenzyme intact. However, important concerns have recently been raised regarding the potential cardiovascular toxicity of COX-2 inhibitors(22) .

1.2.1. NSAIDs mechanism of action

NSAIDs exert their actions by inhibiting enzymatic activity of the COX enzymes. These enzymes are the first committed step in the synthesis of PG from arachidonic acid (Figure 1). Arachidonic acid is an omega-6 poly-unsaturated fatty acid commonly found at the *sn*-2 position of cell membrane glycerophospholipids and cleaved from cell membranes by one of several different phospholipase A_2 enzymes (23).

COX-1 and COX-2 are bifunctional enzymes that mediate a COX reaction whereby arachidonate plus two molecules of oxygen are converted to the cyclic endoperoxide PGG_2, followed by a hydroperoxidase reaction in which PGG_2undergoes a two-electron reduction to PGH_2 (23).

The unstable inter-mediate PGH_2 spontaneously rearranges or is enzymatically converted by specific synthases to biologically active PG, of which there are many isoforms (24) . The overall regulation of the type and amount of PG produced in a given cell or tissue is determined by the expression levels of COX-1, COX-2, and terminal synthase enzymes.(25)

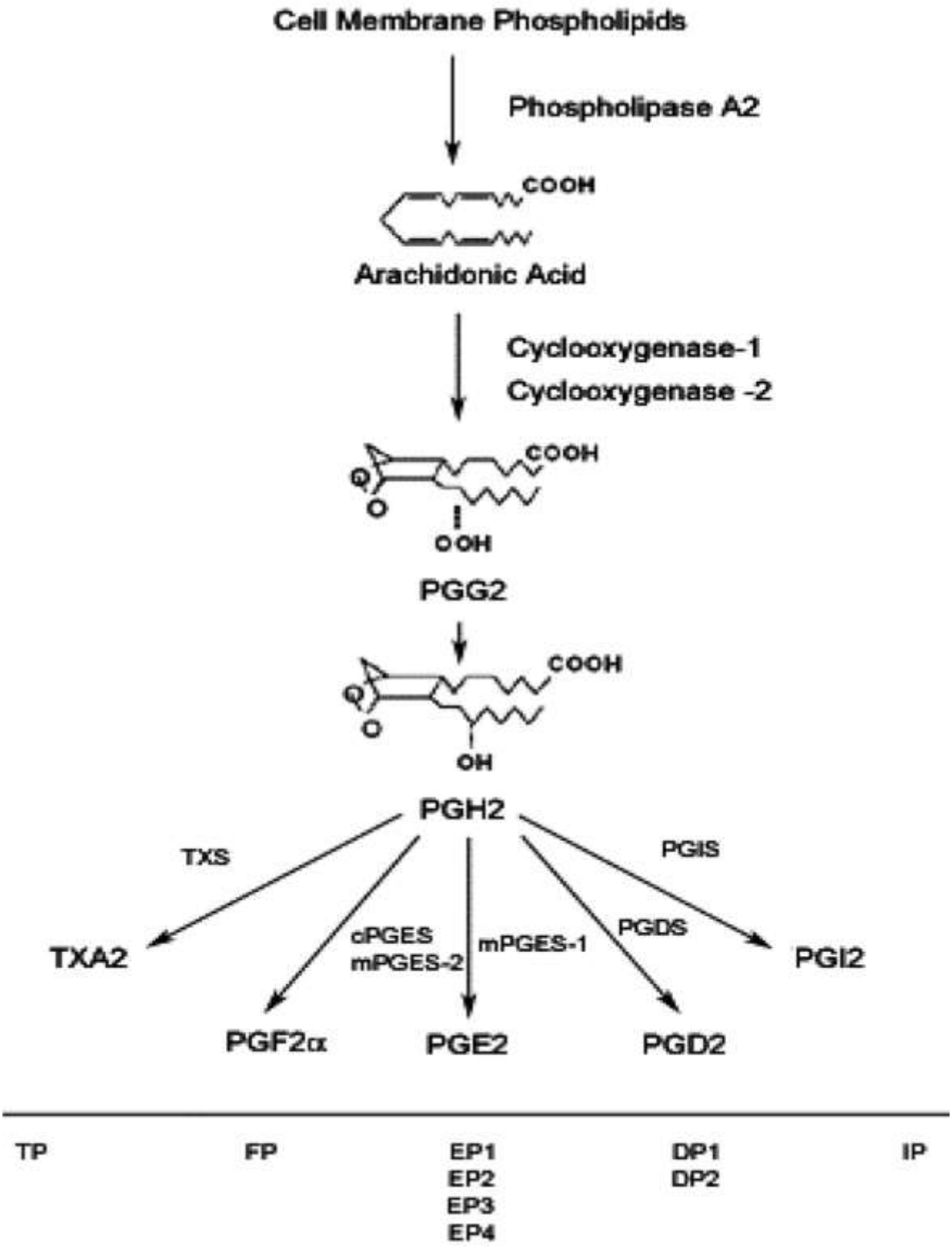
Cell Membrane Phospholipids
Phospholipase A2
COOH
Arachidonic Acid
Cyclooxygenase-1
Cyclooxygenase -2
COOH
OOH
PGG2
COOH
OH
PGH2
TXS
PGIS
TXA2
cPGES
mPGES-2
mPGES-1
PGDS
PGI2
PGF2α
PGE2
PGD2
TP
FP
EP1
EP2
EP3
EP4
DP1
DP2
IP

Figure 1(15)

Prostaglandin biosynthetic pathway. Prostaglandins (PGs) are produced from cell membrane phospholipids from the precursor omega-6 polyunsaturated fatty acid, arachidonic acid. The cyclooxygenase enzymes are bifunctional enzymes that generate PGG_2 and then the unstable intermediate PGH_2. This intermediate is converted by tissue-specific synthases to PG that act on their respective receptors. cPGES, cytosolic prostaglandin E synthase; DP, prostaglandin E receptor; EP, prostaglandin E receptor; FP, prostaglandin F receptor; IP, prostaglandin I receptor; mPGES, microsomal prostaglandin E synthase; PGDS, prostaglandin D synthase; PGIS, prostaglandin I synthase; TP, thromboxane A receptor; TXS, thromboxane synthase; TXA2, thromboxane A_2.(25)

1.2.2 NSAIDS INDUCE ACUTE RENAL FAILUER

The kidneys receive approximately 25% of the cardiac output and are the major organ for drug excretion , Due to this function, the renal arterioles and glomerular capillaries are especially vulnerable to the effects of drugs (26).

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly used over-the-counter (OTC) medications in the United States and are known to have adverse effects on kidney function (27). OTC NSAIDs, including ibuprofen, are routinely administered to children or taken by teenagers for pain and fever (28).

Use of NSAIDs has increased dramatically during recent years, especially in children in the United States (29). Because of their frequent and accepted use, NSAIDs are widely considered safe, but in reality, even therapeutic doses carry a risk of loss of renal function (27). Adverse renal effects from these drugs are caused by two distinct pathological entities. The first mechanism of acute kidney injury (AKI) from NSAIDs is due to reduced renal plasma flow caused by a decrease in prostaglandins, which regulate vasodilation at the glomerular level.

NSAIDs disrupt the compensatory vasodilation response of renal prostaglandins to vasoconstrictor hormones released by the body (30).

Inhibition of renal prostaglandins results in acute deterioration of renal function after ingestion of NSAIDs. The second mechanism of AKI is acute interstitial nephritis (AIN), which is characterized by the presence of an inflammatory cell infiltrate in the interstitium of the kidney. AIN is caused by an immunological reaction after NSAID exposure of about a week (31-23). AIN is now recognized as a major cause of drug induced AKI and accounts for about 15% of all patients with unexplained AKI (32).

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID) which belongs to the acetic acid group (33, 34), is frequently prescribed in human and veterinary medicine as an anti-inflammatory, antipyretic and analgesic agent. Diclofenac acts by inhibiting cycloxygenase-1 (COX-1) and cycloxygenase-2 (COX-2) enzymes, thus preventing prostaglandin synthesis from arachidonic acid (33). COX-1 and COX-2 are continuously expressed in the kidney and its vessels (33, 35, 36). Diclofenac may cause stomach-related side effects and nephrotoxicity, similar to other NSAIDs, by blocking prostaglandin synthesis (34). Liver synthesized fibrinogen which is converted into fibrin and participates in coagulation, while antithrombin acts as an anti-coagulant. NSAIDs inhibit thromboxane production and platelet aggregation, thus expressing anti-coagulation activity (37,38). In addition, NSAID administration depresses the excessive production of adenosine deaminase by immune system cells during infection (39,40).

Reactive oxygen species (singlet oxygen, superoxide anion, hydroxyl radical, hydrogen peroxide, nitric oxide, peroxynitrite, etc.) are continuously produced in the body(41,42) and are deactivated by enzymatic (superoxide dismutase,

glutathione peroxidase, catalase, etc.) or non-enzymatic (glutathione, vitamin A, vitamin C, etc.) substances (41,42). Normally, oxidants and antioxidants are in balance in the body. However, oxidative stress occurs when reactive oxygen radicals are produced excessively and/or antioxidants are insufficient. As a result, oxidative stress causes lipid peroxidation. Malondialdehyde (MDA) is the world-wide accepted biological marker of lipid peroxidation (41,42,43). NSAIDs can affect oxidative balance in the body, and some of them have antioxidant activity while others exhibit oxidant activity (44, 45).

Similar to other NSAIDs, diclofenac inhibits prostaglandin thus causing kidney ischemia and affects the coagulation mechanism; hence it has been hypothesized that it may have dose-dependent effects on kidney-linked stereological parameters such as renal damage, oxidative stress, plasma coagulation parameters and adenosine deaminase activity.

1.3 montelukast

Its ameliorating effect on oxidative damage and myeloperoxidase (MPO) activity (46,47). Montelukast ameliorates burn- and sepsis-induced multiorgan damage by a neutrophil-dependent mechanism (48, 49). Furthermore, montelukast has been shown to reduce I/R-induced oxidative damage in the liver, intestine, kidney, testes and bladder, through its anti-inflammatory and antioxidant properties (50, 51, 52, and 53). However, to our knowledge, the effect of montelukast on ALI caused by HSR has not been reported.

Leukotrienes (LTs) are synthesized from membrane phospholipids in response to cell activation. Cysteinylleukotrienes (CysLTs) are produced from arachidonic acid through 5-lipoxygenase (5-LO) pathway and act on the CysLT1 and CysLT2 receptors (54). In fact, several pathways are involved in production of reactive

oxygen species (ROS), it has been reported that bioactive metabolites of LTs have a pivotal role in oxidative stress (55).

In another study, Beytur et al. (56) reported that the selective reversible CysLT1 receptor antagonist, montelukast (ML) (MK-0476), has significant antioxidant properties against CP-induced testicular damage. Also, the protective effects of ML have previously been addressed in other models of cell damage induced by several drugs (57). The beneficial effects of ML in various experimental models of inflammation have also been reported (58, 59).

1.4 Aim of study

Study the protecting effect of montelukast for treating acute renal failure induced by non steroidal anti inflammatory drugs .

Chapter two

MATERIALS AND METHODS.

Animals

A total of Thirty-six young male Wister albino rats weighing 130-150 g, were used in this study. All experiments were conducted in the College of pharmacy, Department of Pharmacology, Al-Qadisiyah University, according to the guidelines for the Care and Use of Laboratory Animals in scientific research. The animals were placed in an animal house, in a group caging system, at controlled temperature (25±2◦C) and ambient humidity. Lights were maintained on a 12-h light/dark cycle. The animals had free access to water *ad libitum*.

Drugs

- Diclofenac sodium (Hemofarm, Serbia)was used and administered in a single dose of 100 mg/kg to the animal according to body weight by intraperitoneally (i.p.).(60)
- Montelukast (TAD Pharma GmbH, Germany)was dissolved in 0.9% sodium chloride solution(63)and administered in a dose of(7 mg/kg i.p. injection 30 min before administration of diclofenac.(61)

Animal model of acute interstitial nephritis

Induction of acute interstitial nephritis and subsequent development of acute kidney damage were carried out by injection rats with single diclofenac sodium intraperitoneally.(60)

Experimental Protocol

After acclimatization, rats were divided into 4 groups (5 rats each):

Negative control group: Rats were received nothing.

Positive control group: Rats were i.p. injected with diclofenac sodium at dose 100mg/kg body weight with water deprivation.

Vehicle group: Rats were i.p. injected with normal saline 2 ml/ kg body weight.

Treatment group: Rats were i.p. injected with montelukast (7 mg/kg i.p. injection) then after 30 minute inject diclofenac sodium (63) at dose 100mg/kg body weight.

Five rats from each group were killed after 8 hours from beginning of diclofenac sodium and montelukast administration.

At the end of the experiment, animals were anesthetized by thiopental sodium (I.E. Ulagay Ilac Sanayi, Istanbul, Turkey) in a dose of (70 mg/kg, by intraperitoneal route) (60). The chest was opened by thoracotomy; blood sample was collected directly from the heart for measurement of blood urea nitrogen (BUN) and serum creatinine were measured by alkaline picrate method (Bartels et al., 1972) (62).

Statistical analysis

Statistical analyses were performed using SPSS 12.0 version. Data were expressed as mean ± SEM. Analysis of Variance (ANOVA) was used for the multiple comparisons among all groups. In all tests, $P< 0.05$ was considered to be statistically significant.

Chapter three

3.1 Effect of diclofenac and montelukast on BUN

As shown in table 1 and figure 1, the mean BUN was normal in negative control group (16.12± 0.6 mg/dl).

Positive control group (diclofenac treated group) associated with higher BUN levels (**42.61± 6.3** mg/dl)when compared with negative control group.

The effect of normal saline (0.9% Nacl) solvent used in the preparation of tested drug was evaluated next. There was statistically insignificant difference in mean BUN between positive control group and vehicle control group, and the effect of the solvent used in this study was almost negligible.

Treatment group (Montelukast and diclofenac) group associated with statistically significant(P< 0.05 lower mean BUN (**18.86± 1.5** mg/dl) when compared with the positive control group.

Table 2: The difference in mean BUN between the 4 study groups.

Animal group \	negative control group	Positive control group	vehicle group	treatment group
BUN (mg/dL)	16.12± 0.6	42.61± 6.3	40.8± 0.69	18.86± 1.5

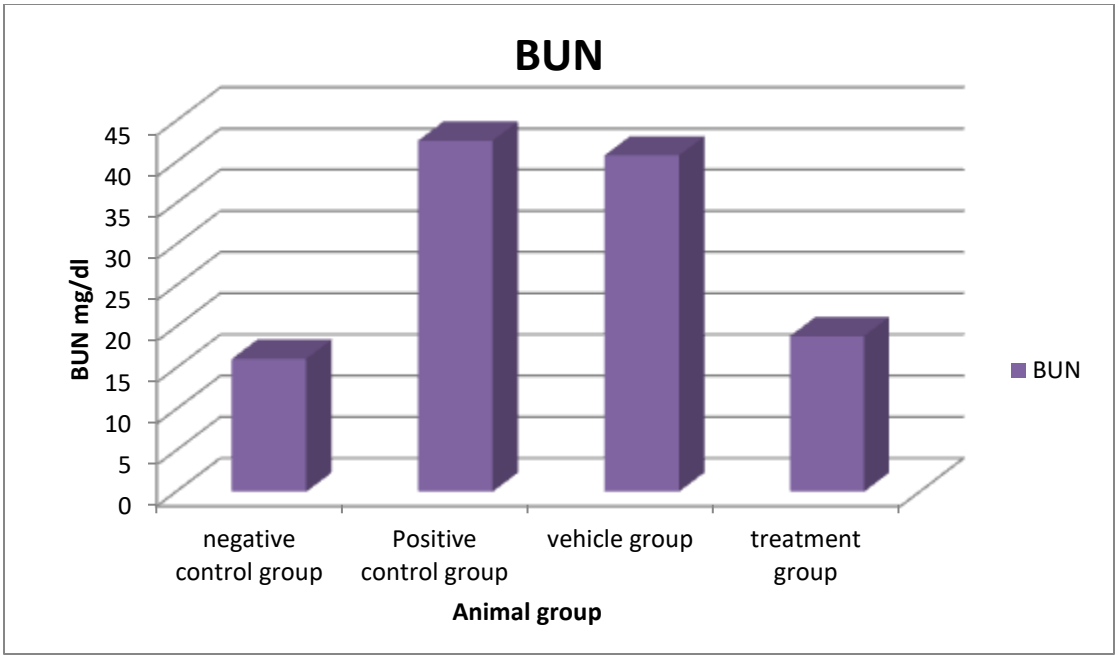

Fig. 1: Fig. 2: effect of diclofenac on serum level of blood urea nitrogen (BUN). Results are mean ± SE for each group of rat. The value are significantly differ from negative-control group ($p<0.05$). where $p<0.05$ was considered to be statistically significant.

3.2 Effect of diclofenac and montelukast on serum creatinine

As shown in table 2 and figure 2, the mean serum creatinine was normal in negative control group (0.75 ± 0.25 mg/dl).

Positive control group (diclofenac treated group) associated with higher but insignificant serum creatinine levels (1.15 ± 0.05 mg/dl) when compared with negative control group.

The effect of normal saline (0.9% Nacl) solvent used in the preparation of tested drug was evaluated next. There was statistically insignificant difference in mean

serum creatinine between positive control group and vehicle control group, and the effect of the solvent used in this study was almost negligible.

Treatment group (montelukast and diclofenac)associated with lower but statistically insignificant lower mean serum creatinine (0.8 ± 0.01mg/dl) when compared with positive control group.

Table 2: The difference in mean serum creatinine between the 4 study groups.

Animal group \	negative control group	Positive control group	vehicle group	treatment group
s.creatinine (mg/dL)	0.75 ±0.25	1.15 ± 0.05	1.08 ± 0.02	0.8 ± 0.01

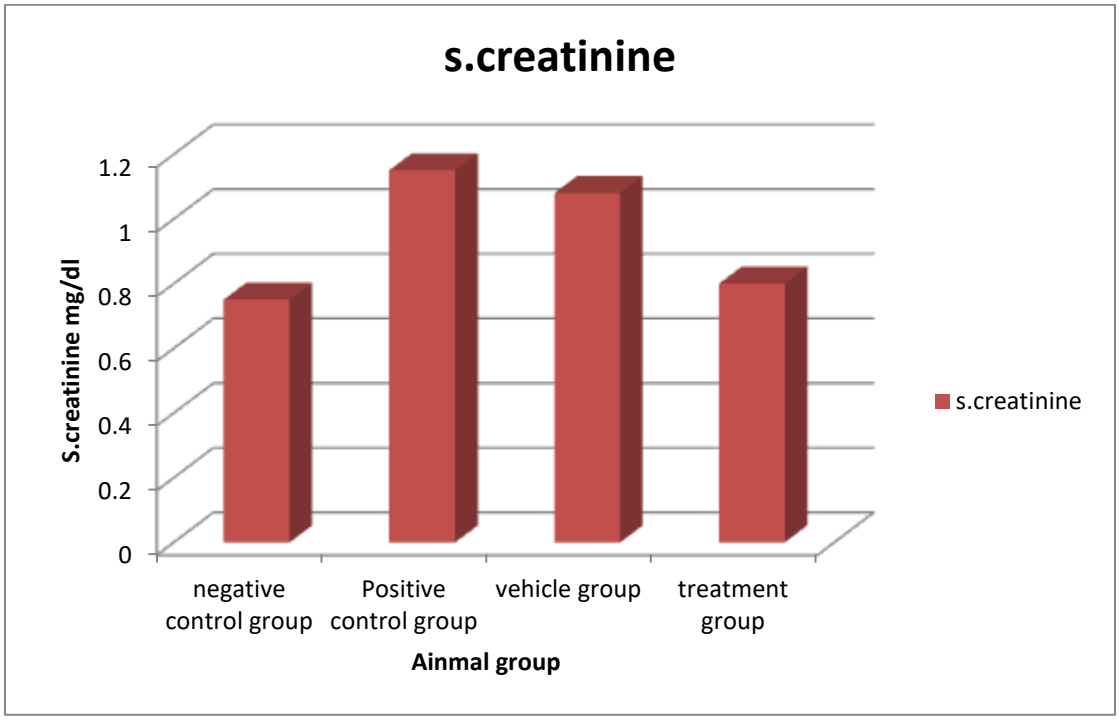

Fig. 2: effect of diclofenac on serum level of creatinine (s.cr). Results are mean ± SE for each group of rat. The value are significantly differ from negative-control group ($p<0.05$). where $p<0.05$ was considered to be statistically significant.

Chapter four

Discussion

4.1The effect of diclofenac on study parameters (blood urea nitrogen and serum creatinine)

In this study intraperitoneal administration of diclofenac to rats in a dose of (100 mg\kg) resulted in marked increment in BUN and serum creatinine. Serum creatinine and BUN are important indicators of kidney damage (64). Similar findings were reported by Hickey et al. (2001)(65),

Diclofenac has been shown to target renal tissue and is well known to be nephrotoxic (66, 67). Their nephrotoxic effects are due to the induction of the Mitochondrial Membrane Permeability Transition (MMPT), a phenomenon where mitochondrial degeneration is initiated by calcium ion flow into mitochondria due to peroxidants, inorganic phosphate inducer or reactive oxygen species (68, 69).

4.2 The effect of montelukast on the study parameters

In comparison to positive control (diclofenac treated group), the present study demonstrated that montelukast treatment group produced significant decrease in blood urea with little or insignificant decrease in serum creatinine. These findings were in agreement with (70). Montelukast, one of the selective reversible CysLT1 receptor antagonists, is used for the maintenance treatment of asthma and to relieve symptoms of wound healing(71,72) Recently, Sener et al.16(73) have reported that montelukast has protective effects on chronic renal failure-induced multiple organ

injury, they attributed this to montelukast's ability to inhibit neutrophil infiltration and apoptosis, they also suggested that montelukast balances the oxidant–antioxidant status and regulates the generation of proinflammatory mediators(74).In a different study it has been shown that montelukast reversed ischemia reperfusion induced oxidant responses and improved microscopic damage and renal functions(75). MDA is a stable metabolite of the free radical-mediated lipid peroxidation cascade and it is widely used as a marker of oxidative stress and lipid layer destruction(76). Montelukast may directly eliminate free oxygen radicals or directly increase the antioxidant enzyme activity and prevent the inhibition of these enzymes. Moreover, when the other antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) are insufficient to protect the cell against free radical attacks, glutathione peroxidase (GPx) enzyme expression is stimulated for further defense systems (77).

Taking into consideration the reduced oxidative damage caused by montelukast treatment, all investigators attributed the beneficial actions of montelukast to its anti-oxidative and anti-inflammatory activity (70). These data are consistent with our results that montelukast reduced BUN and Cr levels in the treatment group.

Conclusions:

- Montelukast has a nephroprotective effect against diclofenac induces acute interstitial nephritis (that investigated by higher levels of BUN and S creatinine) through its antioxidant effect on free oxygen radicals or directly increase the antioxidant enzymes activity (superoxide dismutase and catalase enzymes) and prevent the inhibition of these enzymes .

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