Histopathological study of diclofenac induced acute renal failure under lipoic acid and bosentan therapy in male albino rats

Lina Bahjat Qasim*, Ghaith A. Jasim*, Ihsan S. Rabeea**
***Pharmacology and Toxicology Department, College of Pharmacy, Mustansiriyah University**
****Pharmacology and Toxicology Department, College of Pharmacy, Kufa University**

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Corresponding Author email:
ihsans.mohammed@uokufa.edu.iq.
orcid: https://orcid.org/ 0000-0001-5153-4094

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Abstract:

Acute kidney injury (AKI), formly known as acute renal failure (ARF), is an abrupt and reversible decrease in kidney function as indicated by the glomerular filtration rate (GFR). Diclofenac-induced AKI is due to toxic effect of it on renal glomeruli, resulting in glomerular lesions. Furthermore, diclofenac causes autolysis, which increase renal intracellular osmolarity that leads to proximal renal tubular dilatations. Lipoic acid (LA) has antioxidant and anti-inflammatory activities. Bosentan is a competitive endothelin A (ETA) and endothelin B (ETB) receptors antagonist. In this study, the evaluation of effectiveness of lipoic acid and bosentan against diclofenac-induced AKI was done by histopathological examination. The results showed that diclofenac caused histopathological changes include; retracted glomerulus, tubular cast, tubule-interstitial inflammation and tubular necrosis. Lipoic acid or bosentan alone could not reduce the histopathological alterations caused by diclofenac. Meanwhile, the combination therapy was able to reduce the histopathological changes significantly ($p<0.05$). Therefore, the combination therapy of lipoic acid and bosentan showed promising ameliorative effect against diclofenac-induced AKI.

Key words: acute kidney injury, diclofenac, lipoic acid, bosentan.

دراسة التشريح المرضي للديكلوفيناك المحدث للفشل الكلوي الحاد تحت تأثير حمض الليبويك وعلاج البوسنتان في ذكور الجرذان البيضاء

لينا بهجت قاسم *, غيث علي جاسم*, احسان صلاح ربيع**
***فرع الادوية والسموم/ كلية الصيدلة/ الجامعة المستنصرية*
****فرع الادوية والسموم / كلية الصيدلة/ جامعة الكوفة*

الخلاصة:

إصابة الكلى الحادة (AKI) ، والمعروفة رسميًا بالفشل الكلوي الحاد (ARF) ، هي انخفاض مفاجئ وقابل للعكس في وظائف الكلى كما يتضح من معدل الترشيح الكبيبي (GFR). اصابة الكلى الحادة الناجمة عن الديكلوفيناك تحدث نتيجة التأثير السام له على الكبيبات الكلوية ، مما يؤدي إلى حدوث آفات كبيبية. علاوة على ذلك ، يتسبب الديكلوفيناك في حدوث انحلال ذاتي ، مما يزيد من التناضح الكلوي داخل الخلايا والذي يؤدي إلى توسع الانبوب الكلوي القريب. حمض ليبويك (LA) يعمل كمضاد للأكسدة ومضاد للالتهابات. البوسنتان هو أحد مضادات مستقبلات endothelin A (ETA) و endothelin B (ETB) التنافسية. في هذه الدراسة ، قمنا بتقييم فعالية حمض ليبويك والبوسنتان ضد اصابة الكلى الحادة الناجمة عن الديكلوفيناك من خلال فحص الانسجة المرضية. لاحظنا أن الديكلوفيناك يسبب تغيرات نسيجية تشمل: تراجع الكبيبات ، الزهر الأنبوبي ، الالتهاب الخلالي النبيبي والنخر الأنبوبي. لا يمكن لحمض ليبويك أو

بوسنتان كلا على حدة تقليل التغيرات النسيجية المرضية التي يسببها الديكلوفيناك. وفي الوقت نفسه ، كان العلاج المركب قادرًا على تقليل التغيرات النسيجية المرضية بشكل ملحوظ (P> 0.05). نستنتج أن العلاج المركب لحمض ليبويك والبوسنتان أظهر تأثيرًا محسنًا واعدًا ضد اصابة الكلى الحادة الناجمة عن الديكلوفيناك.

الكلمات المفتاحية: اصابة الكلى الحادة , الديكلوفيناك, حامض الليبويك, البوسنتان

Introduction

Acute renal failure (ARF), also known as acute kidney injury (AKI) is a complex health condition related to significant mortality and morbidity[1]. In hospitalized patients, around 19-33 percent of AKI episodes are linked to nephrotoxic drugs. Diclofenac (2-[(2,6-diclorophenyl) amino] phenyl acetate) is phenyl acetic acid derivate that inhibits prostaglandin biosynthesis. It has antipyretic, pain-relieving, anti-rheumatic and anti-inflammatory activities[2]. It also has many uses include: gout, ureteric colic, as well as rheumatoid arthritis, and osteoarthritis [3].

Diclofenac-induced AKI is due to toxic effect of it on renal glomeruli, resulting in glomerular lesions. Furthermore, diclofenac causes autolysis, which increase renal intracellular osmolarity that leads to proximal renal tubule dilatations [4].

Diclofenac causes ischemia by inhibiting renal prostaglandin production, limiting renal afferent arteriole vasodilation, increasing afferent resistance; thus decreasing the glomerular capillary pressure below normal values and GFR will decrease resulting in AKI[5]. In the last few decades, many animal studies and molecular experiments have been done and revealed that endothelial injury, leukocyte-mediated inflammation and decrease microvascular blood flow are central mechanisms for AKI induced by ischemia [6]. Lipoic acid (LA) is a naturally occurring micronutrient, synthesized in small amounts by plants and animals [7]. LA can act as antioxidant in lipophilic and hydrophilic environments[8]. In addition, LA has anti-inflammatory action by inhibiting nuclear factor kappa beta (NF-kappaβ), which responsible for regulation of gene expression of many pro-inflammatory cytokines e.g. tumor necrosis factor alpha (TNF- α), interlukine-1 (IL-1), interlukine-6 (IL-6)[9]. Bosentan is a derivative of non-peptide pyrimidine that acts on both endothelin A (ETA) and endothelin B (ETB) receptors as a competitive, unique antagonist[10]. It was approved in patients for treatment of pulmonary arterial hypertension (PAH)[11]. Endothelin-1 (ET-1) is a 21 amino acid peptide[12]. It has two structurally related G protein-coupled receptors, endothelin type A receptor (ETAR) and endothelin type B receptor (ETBR)[13]. ET-1 is involved in many transcription factors activation such as NF-κB and expression of pro-inflammatory cytokines including TNF-α, IL-1, and IL-6 [14]. ET-1 is also a potent proliferative and mitogenic peptide[15].

Materials and methods

Animals

In this study, thirty mature male albino rat weighing between 200 and 250g were included. The animals were provided by the Iraqi center for cancer research and medical inheritance/Mustansiriyah University. The study was approved by the ethical committee for animal experimentation of college of pharmacy / Mustansiriyah University, where the work was done. Each six animals were placed in disinfected cage, with artificial light cycle of 12/12 and a temperature of (22±2°C). They were provided unrestricted access to water and normal chow pellets and left for 14 days to acclimate.

Chemicals

The chemical substances with their sources are clarified in the table (1):

Table (1): Chemicals and their origins

Chemicals	Source
Sodium salts of diclofenac (Olfen ®)	Acino pharma company, Liesberg, Switzerland
Lipoic acid (Lipoic forte®)	America Medic & Science company, USA
Bosentan	Cipla Ltd, India

Experimental design

Thirty adults male wistar rats were divided into five groups of six rats in each.

1. Control group: rats received distilled water (5 ml/kg, p.o.) for 11 days, on the 5th day an intraperitoneal injection of normal saline (5 ml/kg) was adminstrated.
2. Induction group: rats were administrated distilled water (5ml/kg, p.o.) for 11 days, on the 5th day they received an intraperitoneal injection of diclofenac sodium (100mg/kg).
3. Lipoic acid group: rats received lipoic acid (200mg/kg p.o.) for 11 days, on day five they received diclofenac sodium (100mg/kg, i.p.).
4. Bosentan group: rats received bosentan (100 mg/kg p.o.) for 11 days, and on the day five they received diclofenac sodium (100 mg/kg, i.p.).
5. Combination group: rats received a combination of lipoic acid (100mg/kg p.o.) and bosentan (100mg/kg p.o.) for 11 days, and on the 5th day they were given an intraperitoneal injection of diclofenac sodium (100mg/kg).

On 12th day, the rats were anesthetized with ketamine (alfasan Woerden-Holland) (90mg/kg) and xylazine (Kepro-Holland) (10mg/kg). The rat's abdominal cavity was opened using forceps and scissors in order to harvest and preserve the kidneys in 10% formalin for histopathological examinations.

Kidney tissues processing for histopathological examination

For light microscopy, there are many techniques can be used such as paraffin sections, semithin sections, and frozen sections techniques. The paraffin technique has been used in present study. It can be summarized in the following steps [16]:

1. **Fixation:** The whole kidney of the male rat was fixed by adding chemical fixative agent (formaldehyde 10%). **2. Dehydration and clearing**: The tissue was embedded in paraffin wax and then cut into sections. Because the wax is not soluble in alcohol or water and soluble in xylene (paraffin wax solvent). Xylene was used to replace the water in the sample with alcohol. This was accomplished by exposing the tissue to increasing concentrations of ethanol (from 0 to 100%).Finally, alcohol was replaced with xylene, which is miscible with alcohol. The final stage is known as clearing. **3. Embedding:** The tissue was dipped in paraffin wax, which fills the spaces that usually have water in them. The tissue hardens after cooling. **4. Sectioning:** The cooled tissue was trimmed and placed on a microtome. Thin pieces of tissue are sliced into 4 µm and stained before being placed on microscope slide. **5. Staining and mounting:** The wax has to be dissolved and replaced with water (rehydration) since most staining solutions are aqueous. The sections are processed through xylene, and then decreasing strengths of ethanol (100% to 0%) and finally water. The staining reagents that used in this study are hematoxylin and eosin (H&E). The section is dehydrated and placed in xylene after it has been stained. It is then placed on

microscope slide in mounting medium dissolved in xylene. To keep the sample secure, a coverslip put on top. Evaporation of xylene that surrounding the edges of the coverslip dries the mounting medium and securely adheres the coverslip to the slide. The histological changes in current study were scored by EGTI (endothelial, glomerular, tubular, and interstitial) scoring system as clarified in table (2)[17].

Table (2): EGTI histology scoring system (17)

Tissue type	Histopathological changes	score
Endothelial	Endothelial loss	**3**
	Endothelial disruption	**2**
	Endothelial swelling	**1**
	No change	**0**
Tubular	(Plus) necrosis in more than sixty percent of tubular cells	**4**
	(Plus) inflammation, necrosis up to sixty percent of tubular cells, and cast formation	**3**
	Loss of brush border (BB) in less than twenty-five percent of tubular cells. Thickened basal membrane	**2**
	Loss of BB in less than twenty-five percent of tubular cells. Integrity of basal membrane	**1**
	No change	**0**
Glomerular	Glomerular fibrosis	**3**
	Retraction of glomerular tuft	**2**
	Thickening of bowman capsule	**1**
	No change	**0**
Tubule-interstitial	Necrosis > sixty percent	**4**
	Necrosis up to sixty percent	**3**
	(Plus) necrosis < twenty-five percent	**2**
	Inflammation, hemorrhage in <twenty-five percent	**1**
	No change	**0**

Statistical analysis

A non-parametric kruskal wallis H test, and Mann whitney U test were used for comparison of histopathological changes among all groups.

Results

The kidney tissue sections that were stained with hematoxylin and eosin as previously described were placed under light microscope using x400 magnification for histopathological evaluation. Histopathological changes were assessed by experienced pathologist using the EGTI scoring system as mentioned in table (1). Figure (1) showed kidney section of control rat with apparently normal renal tissues (glomerulus, tubules) and EGTI scores: tubular: 0, endothelial: 0, glomerular: 0, tubule-interstitial: 0

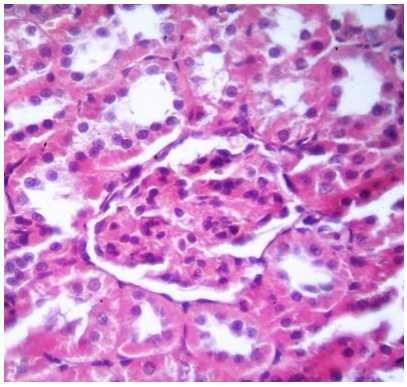


Figure (1): Control group section showed apparently normal renal tissues {glomerulus (black arrow), tubules (blue arrow)}, H&E, X400.

On other hand, the induction group which received diclofenac 100mg/kg showed histopathological changes characterized by retracted glomerulus, tubule-interstitial inflammation, tubular cast and necrosis as shown in figure (2 A-D) with EGTI scores: tubular:3, endothelial :1, glomerular:2, tubule-interstitial: 2.

The kidneys of lipoic acid group also showed acute structural damages represented by retracted glomerulus, tubule-interstitial inflammation, tubular cast and necrosis as demonstrated in figure (3 A-D) with EGTI scores: tubular: 3, endothelial: 2, glomerular: 2, tubule-intersitial: 2.

In addition, bosentan group showed non histological protection against acute renal failure induced by diclofenac as demonstrated in figure (4 A-C) with EGTI scores: tubular: 3, endothelial: 1, glomerular: 2, tubule-interstitial: 2

The kidney tissue sections of combination group which received 100mg lipoic acid and 100 mg of bosentan showed apparently normal appearance as shown in figure (5) with EGTI scores: tubular: 0, endothelial: 0, glomerular: 0, tubule-intersitial: 0

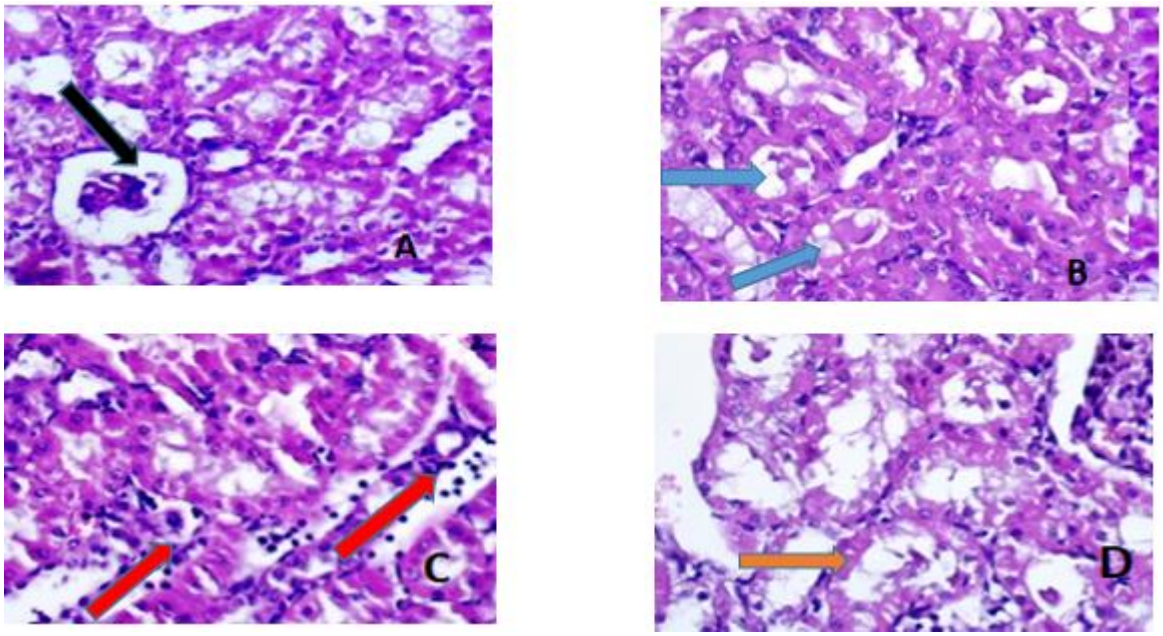
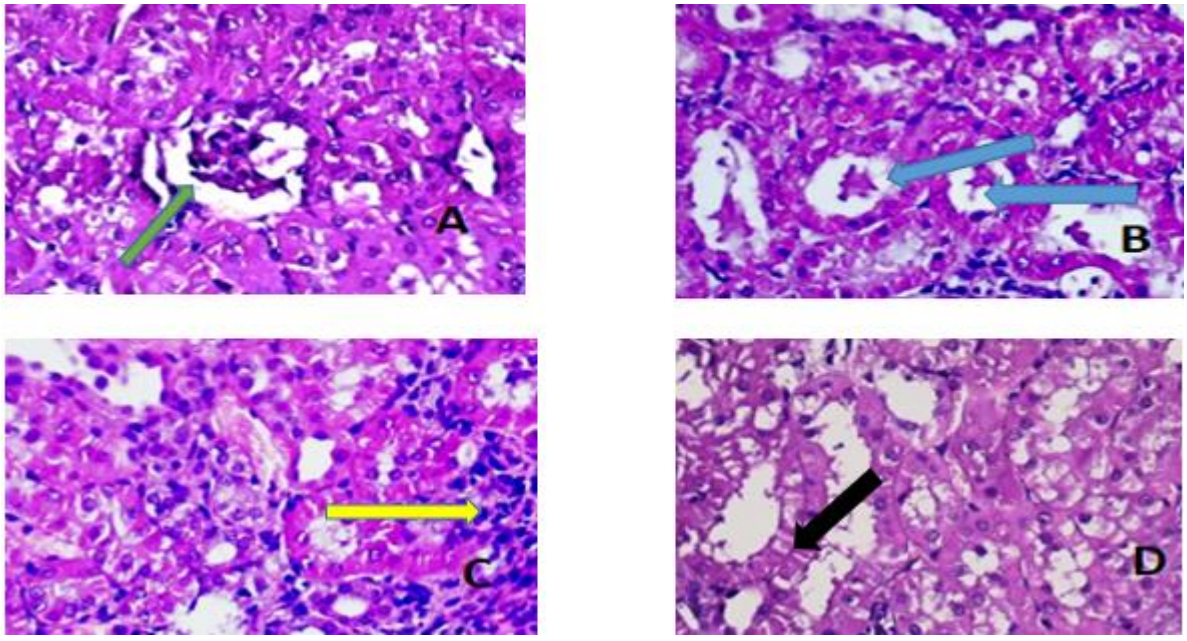


Figure (2 A-D): Induction group histopathological changes, diclofenac intraperitoneal injection 100mg/kg once on day 5th, H&E, X400. [A: Retracted glomerulus (black arrow), B: Tubular cast (blue arrow), C: Tubule-interstitial inflammation (red arrow), D: Tubular necrosis (orange arrow)]



(3 A-D): Histopathological sections for lipoic acid group (200mg/kg) oral administration, H&E, X400. [**A**: Retracted glomerulus (green arrow), **B**: Tubular cast (blue arrow), **C**: Tubule-interstitial inflammation (yellow arrow), **D**: Tubular necrosis (black arrow)]

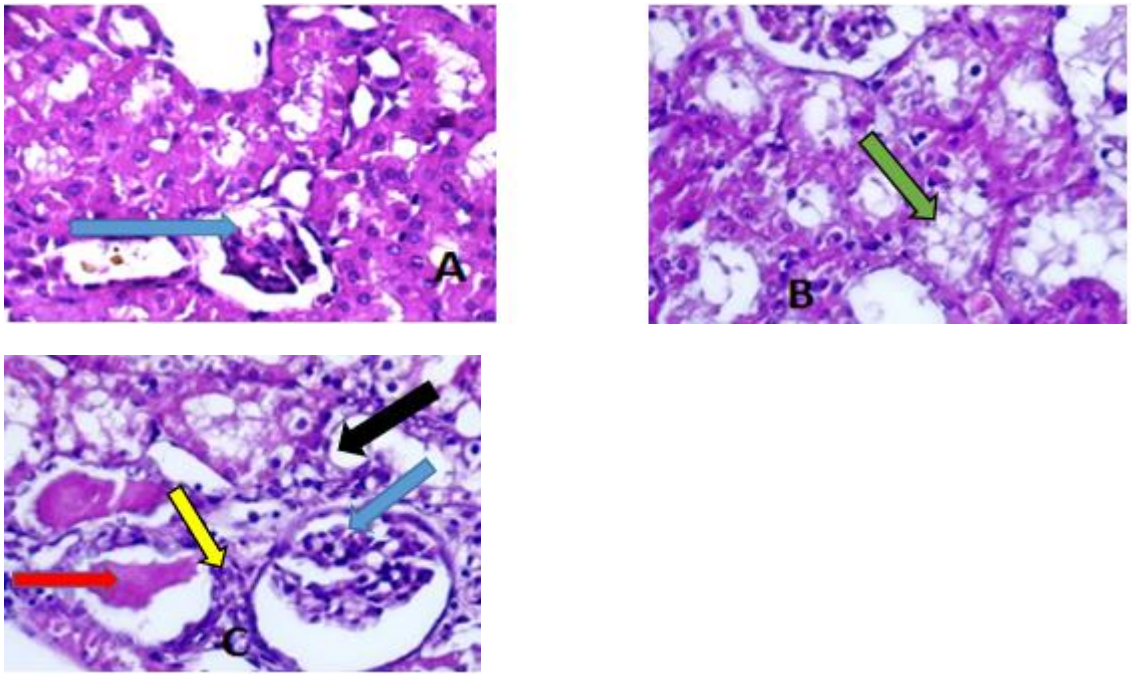


Figure (4 A-C): Histopathological sections for bosentan group (100mg/kg) oral administration, H&E, X400. [**A**: Retracted glomerulus (blue arrow), **B**: Tubular cast (green arrow), **C**: Tubule-interstitial inflammation (yellow arrow); tubular necrosis (black arrow); congested blood vessels (red arrow); retracted glomerulus (blue arrow)]

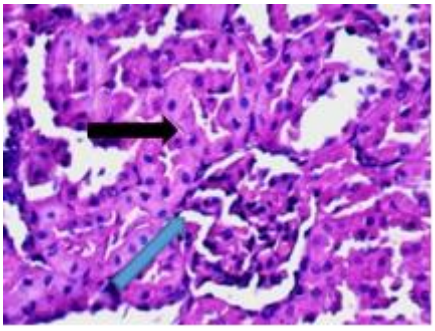


Figure (5): Histopathological changes in combined lipoic acid (100mg/kg) & bosentan (100mg/kg) oral administration, [glomerulus (black arrow), tubule (blue arrow)], H&E, X400

In present study, Kruskal-Wallis H test was used for general comparison among the groups for significant histopathological changes and Mann-whitney U test was also applied to determine which group exactly has a significant difference with other as shown in table (3).

Table (3): Global comparison among the groups for histopathological changes of kidney tissues.

histopathological changes	Kruskal-Wallis H test					
	Mean rank					
	Asymptote sig <0.05	control	Induction	Lipoic acid	Bosentan	Combination
Endothelial damage	0.001	4.5 a	12.5 b	18.5 c	12.5 b	4.5 a
Glomerular damage	0.001	4.5 a	14.5 b	14.5 b	14.5 b	4.5 a
Tubular damage	0.001	4.5 a	14.5 b	17.5 b	11.5 c	4.5 a
Tubule-interstitial damage	0.001	4.5 a	14.5 b	14.5 b	14.5 b	4.5 a

* Different lower-case letters indicate significant difference between groups ($p<0.01$) which was done by Mann-Whitney U test

Histopathological changes including endothelial, glomerular, and tubule-interstitial damages in the induction group showed a significant damage (p>0.01) when compared with the control and combination groups. On the other hand, these changes in lipoic acid group showed non-significant difference (p=1) when compared with that in the induction and bosentan groups. Finally, the combination group showed a non-significant difference (p=1) in morphological appearance when compared to the control group.

For tubular damages, the induction group showed a significant damage (p>0.01) in comparison to control and combination groups. Lipoic acid group showed a non-significant difference (p=0.127) when compared with the induction group. Meanwhile, the bosentan group showed a significant damage (p>0.01) in comparison to all other groups. Finally, the combination group has a non-significant difference (p=1) when compared to control group.

Discussion

The histopathological results in present study showed significant detrimental effects of diclofenac on renal tissue architecture, where retracted glomeruli, tubular cast, tubule-interstitial inflammation, and tubular necrosis

appeared in the induction group compared to control group. These results resembled two previous studies that showed diclofenac induced renal glomerular proliferation, significant interstitial inflammation and cast formation (18) (19). Histopathological results in lipoic acid group showed no protective effect aganist diclofenac induced acute kidney injury. In fact, there is more damage occur in this group compared to the induction group, this finding is in line with research done by Grdović N et al (2021), that found ALA contribute to the profibrotic processes and collagen formation. In which, treatment with ALA was linked with development of glomerulosclerosis, tubulointerstitial fibrosis and decrease in renal function (20).

On the other hand, the present study results partially agreed with previous study mentioning that lipoic acid attenuate histopathological changes significantly following methotrexate administration. This could be due to different induction mechanism that caused by methotrexate (21).

Bosentan group showed a non-significant reduction in renal injury following diclofenac administration when compared to the induction group, this result partially agreed with a previous study that found bosentan could attenuate significantly renal injury induced by arsenic, in which a positive correlation between high level of endothelin and renal dysfunction was noticed (22). The disagreement might be due to different induction models used and longer treatment duration. Finally, the rat's kidney in the combination group appeared to be almost normal, this finding suggests that lipoic acid and bosentan have additive effects giving promising role in protecting renal tissues against detrimental effect of diclofenac.

Conclusion

The study showed a promising protective effect of combination therapy of lipoic acid and bosentan against diclofenac-induced AKI.

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