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Evaluation of Renoprotective Effect of Lipoic Acid and Bosentan Against Diclofenac-Induced Acute Renal Failure

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Abstract

Acute renal failure is also known as acute kidney injury (AKI) is a complex health condition related to significant morbidity and mortality. Non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac have potential risk of renal injury. The direct effect of diclofenac-induced renal injury depends on the formation of reactive oxygen species (ROS) resulting in oxidative stress. Secondly, diclofenac inhibit renal prostaglandin production, limiting renal afferent arteriole vasodilation; thus glomerular filtration rate will decrease resulting in acute kidney injury. Alpha-Lipoic acid (ALA) acts as an antioxidant and anti-inflammatory micronutrient. Bosentan is a competitive antagonist with dual endothelin-1 (ET-1) receptors. In present study, we investigated the effect of α-lipoic acid and bosentan in diclofenac-induced acute renal failure in male rats. Measurement of serum levels of urea, creatinine, were done by colorimetric technique. On the other hand, serum levels of malondialdehyde, superoxide dismutase-1, kidney injury molecules-1, transforming growth factor β1, fibronectin and collagen type I were measured by enzyme linked immunosorbent assay technique. The receiver operating characteristic (ROC) curve analysis was used for evaluating accuracy of biomarkers in diagnosis AKI. Area under the curve (AUC) is a metric that summarize the diagnostic accuracy of the test. We observed that diclofenac increased serum levels of urea, creatinine, malondialdehyde, KIM-1, TGFβ1 and fibronectin significantly ($p<0.05$) in the induction group compared to control group. While, SOD_1 significantly ($p<0.05$) reduced in the induction group compared to control group. Both of α-lipoic acid and bosentan alone did not significantly protect against diclofenac induced AKI. However, the combination group showed a significant protection against AKI. Pearson correlation analysis showed a significant positive correlation between (urea and KIM-1) and between (creatinine and KIM-1) (r^2=0.792 and r^2=0.677 respectively). Furthermore, there was a significant positive correlation between fibronectin and urea (r^2= 0.498, $p<0.01$) and fibronectin and creatinine (r^2=0.356, $p<0.05$). Interestingly, KIM-1 showed a significant positive correlation with fibronectin (r^2=0.536, $p<0.01$). The AUC for KIM-1 was 0.986 and for fibronectin was 0.829. We concluded that combination therapy of α-lipoic acid and bosentan showed a significant protective effect against diclofenac-induced AKI. In addition, fibronectin could be a promising biomarker for detection and diagnosis of acute kidney injury.

Keywords: Diclofenac, Oxidative stress, Alpha- lipoic acid, Endothelin-1, Bosentan.

تقييم التأثير الوقائي لحمض الليبويك والبوسنتان ضد الفشل الكلوي الحاد الناجم عن الديكلوفيناك

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الخلاصة

يُعد الفشل الكلوي الحاد المعروف أيضًا باسم إصابة الكلى الحادة (AKI) حالة صحية معقدة تتعلق بالمراضة والوفيات الكبيرة. ان مضادات الالتهاب غير الستيروئيدية مثل الديكلوفيناك لها مخاطر محتملة من اصابة الكلى. يعتمد التأثير المباشر للإصابة الكلوية التي يسببها ديكلوفيناك على تشكيل أنواع الأكسجين التفاعلية (ROS) مما يؤدي إلى الإجهاد التأكسدي. ثانيًا، يمنع الديكلوفيناك إنتاج البروستاغلاندين الكلوي، مما يحد من توسع الأوعية الشرياني الكلوي الوارد، وبالتالي سينخفض معدل الترشيح الكبيبي مما يؤدي إلى الإصابة الحادة للكلى (AKI). يعمل حمض ألفا ليبويك (ALA) كمضاد للأكسدة ومضاد للالتهابات. يعتبر البوسنتان مضادًا تنافسيًا مع مستقبلات endothelin-1 المزدوجة. في الدراسة الحالية، درسنا تأثير حمض ألفا ليبويك والبوسنتان في الفشل الكلوي الحاد الناجم عن الديكلوفيناك في ذكور الجرذان. تم قياس مستويات اليوريا والكرياتينين في الدم بتقنية قياس الألوان. من ناحية أخرى، تم قياس مستويات مصل الدم من malondialdehyde، superoxide disutase-1 ، جزيئات إصابة الكلى -١ ، عامل النمو المحول β1 ، الفبرونكتين والكولاجين من النوع الأول بتقنية الفحص المناعي المرتبط بالإنزيم.

تم استخدام تحليل منحنى خاصية تشغيل المستقبل (ROC) لتقييم دقة المؤشرات الحيوية في تشخيص AKI. المنطقة الواقعة تحت المنحنى (AUC) هي مقياس يلخص الدقة التشخيصية للاختبار. لاحظنا أن ديكلوفيناك زاد من مستويات المصل من اليوريا والكرياتينين والمالونديالديهايد و KIM-1 وTGFβ1 والفيبرونكتين بشكل ملحوظ (p <٠,٠٥) في مجموعة الحث مقارنة بمجموعة التحكم. بينما انخفض معدل SOD في الدم بشكل كبير (p <٠,٠٥) في مجموعة الحث مقارنة بمجموعة التحكم. كلا من حمض الليبويك والبوسنتان على حده لم يحميا بشكل كبير من اصابة الكلى الحادة الناجم عن الديكلوفيناك. ومع ذلك ، أظهرت المجموعة المركبة حماية كبيرة ضد القصور الكلوي الحاد.

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أظهر تحليل ارتباط بيرسون علاقة ارتباط موجبة معنوية بين (اليوريا و KIM-1) وبينcreatinine (و KIM-1) (r2 = 0.792) و(r2 = 0.677) على التوالي. علاوة على ذلك، كان هناك ارتباط إيجابي معنوي بين الفبرونيكتين واليوريا (r = 0.498 ،p< ٠,٠١) والفيبرونيكتين والكرياتينين (r = 0.356 ،p< ٠,٠٥).
ومن المثير للاهتمام، أظهر KIM-1 ارتباطًا إيجابيًا معنويًا مع الفبرونيكتين (r = 0.536 ،p< ٠,٠١). استنتجنا إلى أن العلاج المركب لحمض ليبويك والبوسنتان أظهر تأثيرًا وقائيًا كبيرًا ضد اصابة الكلى الحادة الناجم عن ديكلوفيناك. بالإضافة إلى ذلك، يمكن أن يكون الفبرونيكتين مؤشر حيوي واعد للكشف عن إصابة الكلى الحادة وتشخيصها.

الكلمات الأساسية: ديكلوفيناك، الإجهاد التأكسدي، حمض ألفا ليبويك، إندوثيلين -١، بوسنتان

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are considered one of the most commonly prescribed medicines in the world, with over 30 million people taking them every day[1]. Diclofenac as a NSAIDs has gained special attention over the past few years due to the potential risk of renal injury [2]. NSAIDS can cause two different types of acute renal failure (ARF) which also known as acute kidney injury (AKI); Hemodynamically mediated (pre-renal injury and/or acute tubular necrosis) and Immune mediated (acute interstitial nephritis (AIN)) [3]. The direct effect of diclofenac-induced renal injury depends on targeting the mitochondria of the kidney, and formation of reactive oxygen species (ROS) leading to oxidative stress [4]. In addition, diclofenac inhibits renal prostaglandin production, limiting renal afferent arteriole vasodilation, increase afferent resistance; consequently the glomerular capillary pressure will drop below normal values with diminished GFR resulting in AKI [2]. Diclofenac (2-[(2,6-diclorophenyl) amino] phenyl acetate) is a derivative of phenyl acetic acid that exhibits antipyretic, pain-relieving, anti-rheumatic and anti-inflammatory activities with inhibitory effect on prostaglandin biosynthesis [5]. It is used to treat gout and ureteric colic, as well as rheumatoid arthritis, osteoarthritis and prescribed postoperatively[6].

Alpha-Lipoic acid (ALA) is also called thiotic acid [7] , is chemically known as 1,2-dithiolane-3-pentanoic acid (C8H14O2S2)[8]. ALA has beneficial effects on prevention or relief of symptoms of oxidative stress-related diseases. In which, a recent study reported by Oktan M et al. (2020) evaluated that ALA could be an effective strategy for the management of nephrotoxicity induced by Colistin [9]. It acts as antioxidant and dehydrogenase enzymes cofactor, which is involved in metabolism of mitochondrial macronutrients and energy production [10]. ALA can act as antioxidant in lipophilic and hydrophilic environments [11], it also acts as a free radical scavenger of ROS such as (hydrogen peroxide, hydroxyl radical) and reactive nitrogen species (RNS) such as (peroxynitrite, nitroxyl and nitrogen dioxide)[7]. In addition, ALA has metal chelating activity and its able to regenerate endogenous antioxidants such as vitamin E, vitamin C [12]. ALA has anti-inflammatory activity through inhibiting nuclear factor kappa beta (NF-κB), a transcription factor involve in regulation of gene expression of many pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interlukine-1 (IL-1) & interlukine-6 (IL-6) [13]. In addition, ALA has anti-inflammatory effect through inhibition of intercellular adhesion molecule-1 (ICAM-1) and vascular cells adhesion molecule-1 (VCAM-1) expression by central nervous system endothelial cells [14].

Bosentan is a competitive an antagonist of endothelin-1 receptors, it acts as antagonist of both endothelin-A receptor (ETAR) and endothelin-B receptor (ETBR)[15]. Bosentan was the first endothelin receptor antagonist (ERA) approved for treatment of patients with pulmonary arterial hypertension (PAH) [16]. It is chemically designed as monohydrate of 4- tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxy-phenoxy)-[2,2´]-bipyrimidin-4-yl]-benzenesulfonamide [17].

Endothelin-1 (ET-1) is a 21 amino acid peptide [18] , involved in activation of many transcription factors particularly NF-κB and expression of pro-inflammatory cytokines including TNF-α, IL-1, and IL-6 [19]. In addition, ET-1 up-regulates expression of adhesion molecules on vascular endothelial cells and induces the aggregation of polymorphonuclear neutrophils (PMNs) contributing to inflammation and endothelial dysfunction [20]. Renal vascular ET-1 system is upregulated under many pathophysiological situations, therefore using of bosentan as competitive an antagonist of endothelin-1 receptors could decrease the inflammation that involved in AKI. The recent study reported by Caires et al (2017) showed that bosentan could reverse cyclosporine-induced changes in renal function [21]. Therefore, AKI induced by diclofenac could be avoided while using ALA and/or bosentan as renal protective agents.

Although , a rise in serum creatinine and urea are current hallmarks for diagnosing AKI [22], they are insensitive, nonspecific, biomarkers[23]. Many biomarkers have been shown to indicate the onset of AKI before serum creatinine and urea rise such as kidney injury molecule-1 (KIM-1)[24]. After ischemia or nephrotoxic injury, the transmembrane glycoprotein KIM-1 is upregulated in proximal tubular cells [25]. Renal ischemia represents the main trigger of ROS resulting in oxidative stress. Oxidative stress can be assessed by indirect methods which can measure the stable

by-products of ROS activity on biomolecules (lipid, protein, DNA) like malondialdehyde (MDA). Many human and animals models are focusing on modifying AKI with the help of antioxidants [26]. The main antioxidants fighting oxidative stress and can be used as biomarkers in AKI are superoxide dismutase (SOD), catalase, and glutathione peroxidase[27]. Acute kidney injury may also involve glomerular injury, which can detect by measuring mesangial extracellular matrix (ECM) secretions such as fibronectin, and collagen type I that undergo upregulation upon mesangial cells proliferation [28].

Materials and methods

Animals

Thirty adult male albino rats weighing between 200 and 250g were used in the present study. These animals were supplied by the animal house of Iraqi center for cancer research and medical inheritance / Mustansiriyah University and was accepted by the ethics committee for animal experimentation of college of pharmacy / Mustansiriya University, where the study took place. The animals were separated into six rats in each sterilized cage which was installed with an artificial light/dark 12/12 cycle at an appropriate temperature (22±2°C). They left for 14 days for acclimation with free access to water and normal chow pellets.

Drugs

Materials were collected from following sources: diclofenac sodium (Olfen ®) was purchased from Acino pharma AG, Liesberg, Switzerland. Alpha Lipoic acid (Lipoic forte®) was purchased from America Medic & Science, USA. Bosentan was purchased from Cipla Ltd, India. Lipoic acid and bosentan were suspended in distilled water immediately before oral administration.

Experimental design

Thirty male wistar albino rats were randomly divided into five groups (n=6 for each group). Control group, rats received distilled water (5 ml/kg, p.o.) for 11 days, on 5th day they received an intraperitoneal injection of normal saline (5 ml/kg). Induction group, rats received distilled water (5ml/kg, p.o.) for 11 days, on the 5th day they received an intraperitoneal injection of diclofenac sodium (100mg/kg). Lipoic acid group, rats received lipoic acid 200mg/kg by preparing a stock solution of 600mg of α-lipoic acid in 10ml distilled water (each 1ml contains 60mg of α-lipoic acid as suspension) delivered orally by oral gavage for 11 days, on the 5th day they received an intraperitoneal injection of diclofenac sodium (100mg/kg). Bosentan group: rats received bosentan 100 mg/kg by preparing a stock solution of 125mg of bosentan in 10ml distilled water (each 1ml contains 12.5mg of bosentan as suspension) delivered orally by oral gavage for 11 days, on the 5th day they received an intraperitoneal injection of diclofenac sodium (100 mg/kg). Combination group: rats are treated with a combination of lipoic acid (100mg/kg p.o.) and bosentan (100mg/kg p.o.) for 11 days, on the 5th day they received an intraperitoneal injection of diclofenac sodium (100mg/kg). On 12th day ketamine (alfasan Woerden-Holland) (90mg/kg) and xylazine (Kepro-Holland) (10mg/kg) were used to anesthetize the laboratory animals. Blood samples were collected by cardiac puncture and were allowed to drain in sterile serum separatory tubes (CMC Medical Devices & Drugs S.L, Malaga-Spain), and then centrifuged for 7 min at 1500rpm at room temperature. Finally the supernatant layer was isolated in Eppendorf tubes (2ml) and kept at −20°C to be evaluated later.

Determination of serum creatinine and serum urea by Semi- automated biochemistry analyzer

Serum samples were processed according to the manufacturer's instructions (Spinreact, S.A.U. Ctra Santa Coloma, spain) and (Linear Chemicals, S.L.U. Joaquim Costa 18 2ª planta, Spain) to evaluate serum urea and creatinine levels respectively as indicators of acute renal failure [29].

Determination of serum biomarkers by sandwich Enzyme-linked immunosorbent assay (ELISA) technique

Determination of serum oxidative stress biomarkers (superoxide dismutase, malondialdehyde), selective acute kidney injury biomarker (kidney injury molecule-1), and fibrosis biomarkers (transforming growth factor-β, collagen-1, fibronectin) were performed according to manufacturer's protocols (mybiosource company, USA) [30].

Statistical analysis

The data were provided in the form of means ± standard error (M±SEM) by SPSS-16.0 statistical program. The analysis of variance (ANOVA) was used to determine the significance of various means. When *P-value* was less than 0.05, a statistically significant difference was reported. Pearson correlation analysis also was used in present study to evaluate whether there is an association between the serum biomarkers levels in AKI cases [31]. The receiver operating characteristic curve (ROC) analysis was also used for evaluating accuracy of biomarkers in diagnosis of AKI. Area under the curve (AUC) is a metric that summarizes the diagnostic accuracy of the test [32].

Results

The effect of lipoic acid, bosentan, and the combination of both on serum levels of kidney function biomarkers (urea and creatinine) in rats induced acute renal failure by diclofenac

Following diclofenac-induced acute renal failure, mean serum creatinine and urea levels in the induction group were significantly elevated ($p< 0.05$) compared to control group as shown in Table

(1). In addition, mean serum creatinine and urea levels in both lipoic acid and bosentan groups showed a non-significant difference in comparison to the induction group. Finally, mean serum creatinine and urea levels in the combination group were significantly reduced ($p<0.05$) when being compared to the induction group to achieve similar levels to control group as illustrated in the Table (1).

Table 1. The effect of lipoic acid, bosentan, and the combination of both on mean serum levels of creatinine and urea in rats induced acute renal failure by diclofenac.

Groups	Number of rats per group (N=6)	Mean serum creatinine level (mg/dl)	Mean serum urea level (mg/dl)
control	6	0.6333±0.04216 $_a$	40.33±1.85$_a$
induction	6	1.0167±0.0872 $_b$	127±5.955 $_b$
lipoic acid	6	0.9±0.08165 $_b$	121±6.255 $_b$
bosentan	6	0.933±0.088433 $_b$	123.5±9.59 $_b$
combination	6	0.65±0.06191 $_a$	52.67±4.01 $_a$

Data are mentioned as means± SEM (SEM: standard error of mean)
Different small letters (a, b) indicate significant difference between groups ($p<0.05$)

The effect of lipoic acid, bosentan, and the combination of both on serum levels of oxidative stress biomarkers (MDA and SOD_1) & KIM-1 in rats induced acute renal failure by diclofenac

In the present study the mean serum levels of MDA and KIM-1 in the induction group were significantly rise ($p <0.05$) compared to control group. However, the mean serum MDA and KIM-1 levels in lipoic acid and bosentan groups showed a non -significant difference when being compared to the induction group. The combination group showed a significant decrease ($p<0.05$) in mean serum MDA and KIM-1 levels when compared to the induction group as clarified in Table (2).

On other hand, the mean serum SOD_1 levels in the induction group were significantly decreased ($p< 0.05$) compared to control group. Both lipoic acid and bosentan groups showed a non -significant difference in mean serum SOD_1 levels when being compared to the induction group. Meanwhile, the mean serum SOD_1 levels in combination group were markedly elevated ($p<0.05$) when compared to all other groups as illustrated in Table (2).

Table 2. The effect of lipoic acid, bosentan, and the combination of both on mean serum levels of MDA and SOD_1 in rats induced acute renal failure by diclofenac

Groups	Number of rats per group (N=6)	Mean serum MDA level (nmol/ml)	Mean serum SOD_1 level (U/ml)	Mean serum KIM-1 level (pg/ml)
control	6	0.2168±0.0165 $_a$	35.0868±3.5 $_a$	122±6.09 $_a$
induction	6	0.493±0.0617 $_b$	20.7957±2.15 $_b$	229±6.21 $_b$
lipoic acid	6	0.446±0.0764 $_b$	24.4232±1.835 $_b$	200±11.94 $_b$
bosentan	6	0.417±0.0651 $_b$	29.0912±2.78 $_b$	198±12.6 $_b$
combination	6	0.221±0.031 $_a$	48.5875±4.21 $_c$	118±9.03 $_a$

Data are mentioned as means± SEM (SEM: standard error of mean)
Different small letters (a,b) indicate significant difference between groups ($p<0.05$)

The effect of lipoic acid, bosentan and the combination of both on serum levels of fibrosis biomarkers (TGF-beta$_1$, fibronectin, and collagen type I) in rats induced acute renal failure by diclofenac

The mean serum TGF-beta$_1$ and fibronectin levels in the induction group were significantly rise ($p <0.05$) when compared to control group. On other hand, the lipoic acid group and bosentan group showed a non-significant difference in mean serum TGF-beta$_1$ and fibronectin levels among all other groups. The combination group showed a significant decrease ($p<0.05$) in mean serum TGF-beta[1] and fibronectin levels compared to the induction group as illustrated in Table (3). Finally, the mean serum collagen type I levels in the induction group did not differ significantly among all other groups as showed in Table (3).

Table 3. The effect of lipoic acid, bosentan and the combination of both on mean serum levels of TGF-beta, fibronectin, and collagen type I in rats induced acute renal failure by diclofenac

groups	Number of rats per group (N=6)	mean serum TGF beta level (pg/ml)	mean serum fibronectin level (ng/ml)	mean serum collagen level (ng/ml)
control	6	71.14±3.312 $_{a}$	72.5768±2.322 $_{a}$	0.0885±0.0334 $_{a}$
induction	6	86.626±3.054 $_{b}$	84.2857±2.45 $_{b}$	0.0933±0.049 $_{a}$
lipoic acid	6	78.48±2.34 $_{a\,b}$	75.4767±2.25 $_{ab}$	0.1090±0.0424 $_{a}$
bosentan	6	80.429±2.07$_{a\,b}$	78.303±2.12 $_{ab}$	0.1322±0.0541$_{a}$
combination	6	72.47±2.166 $_{a}$	70.1548±1.67 $_{a}$	0.0751±0.0378 $_{a}$

Data are mentioned as means± SEM (SEM: standard error of mean)
Different small letters (a, b) indicate significant difference between groups ($p< 0.05$)

Correlations between the biomarkers

In Pearson correlation analysis, data showed a significant ($p<0.01$) positive linear relationship between urea and fibronectin as demonstrated in Figure (1). Also, urea has a significant ($p<0.01$) moderate positive linear relationship with KIM-1 as clarified in Figure (2) .On the other hand, creatinine has a significant ($p<0.01$) positive linear
relationship with KIM-1 biomarker as illustrated in Figure (3).
Figure (4) showed a significant ($p<0.05$) positive linear correlation between fibronectin and creatinine biomarker. Finally, a significant ($p<0.01$) positive linear relationship appeared between fibronectin and KIM-1 biomarkers as shown in Figure (5)

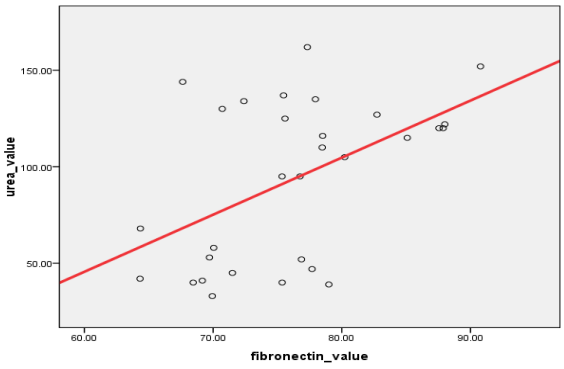

Figure 1. weak positive relationship between urea and fibronectin, pearson correlation coefficient (r) = 0.498.

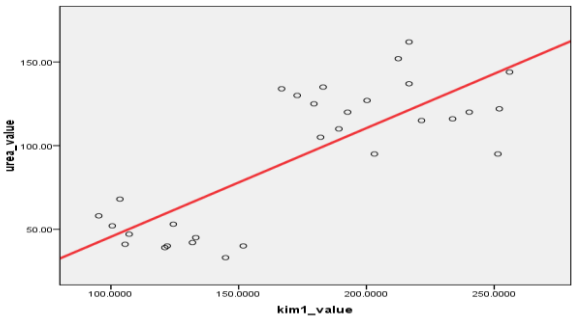

Figure 2. Moderate positive relationship between urea and KIM-1, pearson correlation coefficient (r) = 0.792.

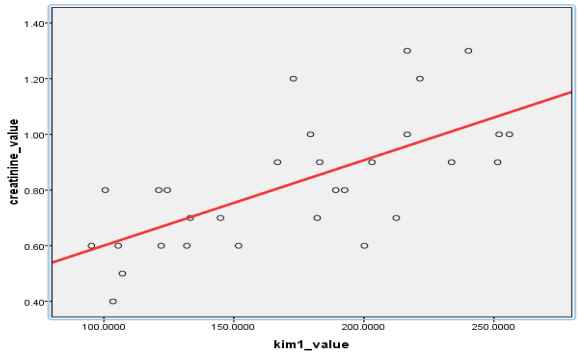

Figure 3. Moderate positive relationship between creatinine and KIM-1, pearson correlation coefficient (r) =0.677.

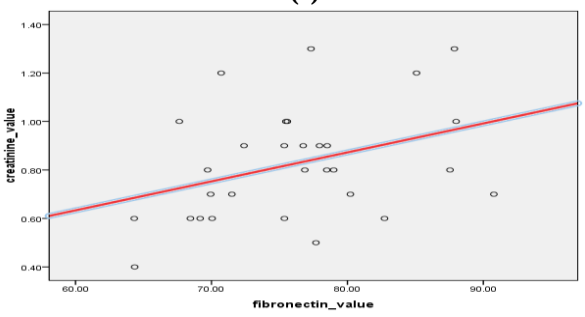

Figure 4. weak positive relationship between creatinine and fibronectin, pearson correlation coefficient (r) = 0.365.

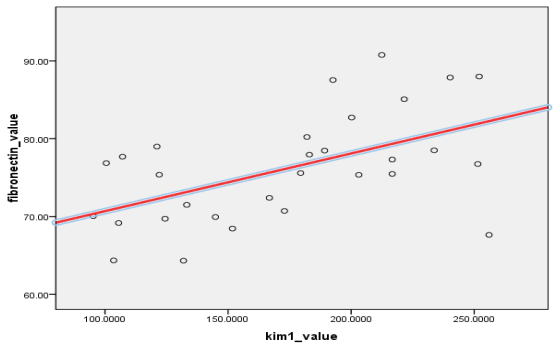

Figure 5. Moderate positive relationship between fibronectin and KIM-1, pearson correlation coefficient (r) =0.536.

Receiver operating characteristic curve (ROC) analysis

In the present study, Receiver operating characteristic curve analysis was performed for KIM-1 biomarker as clarified in Figure (6). In which the sensitivity (true positive rate) was (94.4%) and specificity (False positive rate) was 91.7%. In addition, ROC for fibronectin biomarker also was done as demonstrated in Figure (7). In which, the sensitivity (true positive rate) was 83.3% and specificity (false positive rate) was 66.7%.

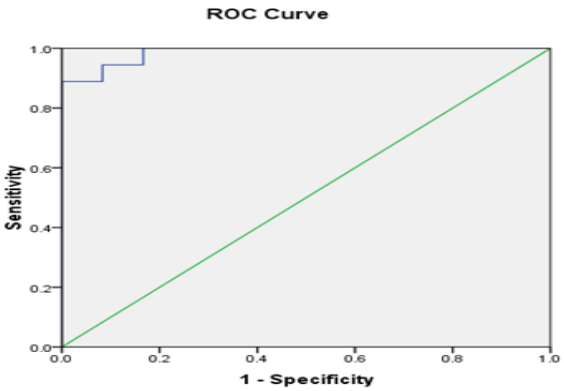

Figure 6. Roc of KIM-1 biomarker, area under the curve (AUC) =0.986.

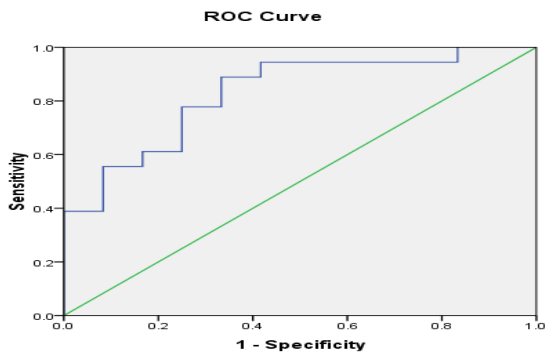

Figure 7. Roc curve of fibronectin biomarker, area under the curve (AUC) =0.829

Discussion

The present study emphasized that diclofenac induced AKI, as observed by elevated biomarkers of kidney injury, oxidative stress, and fibrosis[4]. The first effect of diclofenac in AKI is to reduce prostaglandins synthesis, which leads to afferent arteriole vasoconstriction, ischemia and decreased GFR [33].As a result, serum creatinine and serum urea levels elevated significantly ($p < 0.05$) in the induction group when compared to control group. Lipoic acid group showed non-significant reduction in both serum urea and creatinine levels when compared to induction group. In addition, serum urea and creatinine levels in bosentan group did not differ significantly ($p<0.05$) in comparison to induction group. This results disagreed with previous study reported by sharma et al (2020) that induced AKI by arsenic, which decrease endothelium nitric oxide synthase that was attenuated by bosentan[34]. The combination group showed a significant ($p<0.05$) reduction in serum creatinine and urea levels when compared to induction group. This could be related to additive effect of lipoic acid and bosentan.

After ischemia, transmembrane glycoprotein KIM-1 is up-regulated in proximal tubular cells [25] which was consistent with our findings that showed increased serum levels of KIM-1 significantly ($p<0.05$) in the induction group compared to the control group. Lipoic acid group showed a slight decrease in serum levels of KIM-1 compared to the induction group. This outcome disagreed with a recent study that stated ALA alleviate kidney damage by decrease KIM-1 serum levels significantly ($p<0.05$) in the induction group compared to control. This could be due to ALA ameliorate folic acid-induced AKI by increase iron storage and decrease fenton reaction resulting in decrease oxidative stress[35]. Meanwhile, present study showed a non-significant difference in serum KIM-1 levels in bosentan group when compared to the induction group. On the other hand, the combination group showed a non- significant difference in KIM-1 serum levels when compared to the control group. As a result, this lead us to believe that there could be an additive effect of lipoic acid and bsentan. The second cause of AKI caused by diclofenac is a mitochondrial dysfunction that leads to oxidative stress. The main biomarkers measured in the present study that indicated occurrence of oxidative stress are malondialdehyde (MDA) and superoxide dismutase (SOD), serum MDA levels were significantly elevated in the induction group when compared to control levels. On the other hand, serum SOD levels were significantly reduced. Meanwhile, serum levels of MDA in lipoic acid group were slightly decrease compared to the induction group whereas, SOD serum levels slightly increase. These findings were consistent

with previous study that stated ALA didn't significantly affect serum levels of MDA and SOD [(9)]. Similar results were obtained from the bosentan group. The MDA serum levels in combination group were significantly reduced compared to the induction group. Meanwhile SOD serum levels were significantly elevated. These findings might be explained by the fact that α-lipoic acid decrease NADPH oxidase 2 (NOX2) and NADPH oxidase 4 (NOX4) as major sources of free radicals[(36)].In addition, bosentan increase endothelial nitric oxide synthase (eNOS) through phosphatidylinositol 3 (PI3) kinase /AKI activation[(32)]. As a result, combination therapy can outperform each separate agent.

Many fibrotic mediators can be activated in AKI such as TGFβ1 [(37)], the serum levels of TGFβ1 in induction group significantly elevated when compared to control group. Whereas, lipoic acid group showed a slight reduction in TGFβ1 levels compared to the induction group. The TGFβ1 serum levels in bosentan group didn't differ significantly when compared to lipoic acid group. Finally, significant reduction in serum TGFβ1 levels were observed in combination group when compared to the induction group. High levels of circulating active TGFβ1 produce progressive renal illness such as glomerulosclerosis, and tubule-interstitial fibrosis which is characterized by mesangial enlargement and extracellular matrix protein like fibronectin build up[(38)]. This fact was consistent with another present study that indicated a significant elevation of serum levels of fibronectin in the induction group when compared to control group. On the other hand, lipoic acid showed a non-significant difference in serum levels of fibronectin when compared to the induction group. These results disagreed with a previous study that stated ALA alleviate mesangial cells damage significantly by inhibiting MAP-kinase phosphorylation resulting in attenuating TGFβ1 and fibronectin levels[(39)]. Meanwhile, fibronectin serum levels in bosentan group didn't differ significantly in comparison to induction and lipoic acid groups. In addition, combination group showed a significant decrease in fibronectin serum levels when compared to induction group. Finally, the serum levels of collagen type I in the present study didn't show significant differences between the all studied groups and further studies needed in concern to these results.

Pearson correlation test showed a significant positive correlation between fibronectin and urea (r^2= 0.498, $p<0.01$) and between fibronectin and creatinine (r^2=0.356, $p<0.05$). These results agreed with previous study that found prefibrogenic markers associated positively with creatinine and urea levels in short term induction of renal failure[(40)]. Interestingly, KIM-1 showed a significant positive correlation with fibronectin (r^2=0.536, $p<0.01$). These findings highlighted future perspective on the possibility of using these biomarkers for diagnosis of AKI.

In the present study, ROC curve analysis showed that KIM-1 was with high sensitivity and specificity and AUC of 0.986 and cut off value of 163.65 pg/ml. This result resemble a recent study that indicated an AUC for KIM-1 was 0.968 [(41)]. In addition, fibronectin ROC analysis recorded an AUC of 0.829 and cut off value of 73.87 ng/ml suggesting a promising predictable biomarker for AKI diagnosis. This data came in line with another study that indicated the prefibrogenic biomarkers can be utilized to predict AKI, increasing the opportunities of more interventions in treatment of renal conditions [(40)]. It should be mentioned that our study has some limitations. As with most experimental studies the research was limited to the measures used. Moreover, experimental animal sample size was small.

Conclusion

Alpha-Lipoic acid and bosentan as combinative therapy have a role in improving kidney functions by decreasing serum urea and creatinine levels significantly compared to using each treatment alone. Oxidative stress that occurred in diclofenac induced AKI was prevented by reducing serum MDA levels and increasing serum SOD-1 levels significantly in combinative treatment. Ischemic tubular injury was reversed in combinative treatment through reducing serum KIM-1 levels significantly compared to the induction group. The combinative treatment showed a significant decrease in fibrosis events through decreasing serum TGFβ1 and fibronectin levels significantly when compared to the induction group. Therefore, Alpha-Lipoic acid and bosentan as combinative therapy could have an ameliorate role against diclofenac induced AKI. Fibronectin could be a promising biomarker for detection and diagnosis of AKI.

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