Effect of co-supplementation of vitamin C and E on diclofenac-induced renal injury in male albino mice

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Abstract
Drug-induced kidney disease constitutes an important cause of acute renal failure and chronic kidney disease in present day clinical practice. The present study was designed to investigate the nephroprotective effects of vitamin C and vitamin E both alone and in combination form on diclofenac-induced nephrotoxicity. The nephrototoxicity was induced by diclofenac at dose of 10 mg/kg in male albino mice. It was administered intraperitoneal once a day, every 24h at the same time for twenty one days. The treatments of vitamin C (200mg/kg, orally) and vitamin E (200mg/kg, orally) were given 1hour prior to the administration of diclofenac. The biochemical renal functional tests urea, creatinine as well as lipid peroxidation and antioxidant activities such as superoxide dismutase (SOD) and superoxide catalase (CAT) were assessed. Our results showed that levels of urea, creatinine and malondialdehyde (MDA) were significantly enhanced by administration of diclofenac while antioxidants SOD and CAT were decreased. However, the pretreatment with vitamin C and E alone significantly decreased the serum urea, creatinine and lipid peroxidation in kidney in comparasion with the diclofenac treated group. Furthermore the renal antioxidant status SOD

and CAT were enhanced in the vitamin C and E plus diclofenac treated group than the diclofenac alone treated group. On the other hands, the protection by the combination of vitamin C and vitamin E was additive when compared to each agent alone. The present study clearly demonstrate the marked nephroprotective effects using a combination of vitamin C and E through their antioxidant activity on diclofenac induced nephrotoxicity in mice.
Key Words: Diclofenac, Nephrotoxicity, Vitamin C, Vitamin E, Antioxidants, Nephroprotective.

Introduction:

Nephrotoxicity occurs when kidney-specific detoxification and excretion do not work properly due to the damage or destruction of kidney function by exogenous or endogenous toxicants.(1) Diclofenac is one of the world's most widely-prescribed non steroidal anti-inflammatory drug (NSAID) derived from phenylacetic acid,(2) it has analgesic, antipyretic and anti-inflammatory actions.(3) It acts by the inhibition of both cyclooxygenase 1 and 2 (COX1 and COX2) enzymes, resulting in decreased formation of prostaglandins from arachidonic acid.(4) COX1 is expressed in gastrointestinal tissue, in kidney and in platelets, and serves a protective and regulatory function. Whereas, The inflammatory process is influenced by COX 2 enzyme.(5) For this purpuse, diclofenac is used in the treatment of inflammatory and degenerative rheumatic diseases, gout attacks and pain management in cases of kidney and gall stones, an additional indication is the treatment of acute migraine and mild to moderate postoperative pain,(6-9) under such conditions if the use of diclofenac is prolonged, it produces sever renal injury due to reduction in synthesis of renal prostaglandins leading to an imbalance of vasodilation and vasoconstrictive forces.(10-13) Therefore, diclofenac alters renal hemodynamics resulting in inadequate renal perfusion and renal ischemia.(14) Diverse studies have shown that during the biotransformation of diclofenac, reactive metabolites and intra-renal

reactive oxygen species are produced which induce oxidative stress and damage to biomolecules contributing to development of renal papillary necrosis and acute interstitial nephritis.(15,16) Therefore, kidney is an important target site for untoward effect of diclofenac. The usage of this drug by large number of patients indicates its efficacy. However, it also indicates that a large population is at risk.

L-ascorbic acid (vitamin C) is a water-soluble antioxidant that performs numerous physiological functions in the human body,(17) it is a cofactor for the enzymes involved in biosynthesis of carnitine,(18,19) norepinephrine, dopamine and collagen hydroxylation,(20-22) collagen is important for bone and cartilage formation.(23,24) It also works along with antioxidant enzymes such as glutathione peroxidase and superoxide dismutase. It is responsible for regenerating oxidized vitamin E in the body and increases the antioxidant benefits o vitamin E.(25) Moreover, literature has shown the most abundant and effective antioxidant in the human body to be vitamin C,(26) which exhibits a powerful scavenging effects against various free radicals by neutralizing reactive oxygen species (ROS) and decreasing oxidative damage to cell membranes.(27-30)

α-tocopherol (vitamin E) is the major lipid-soluble antioxidant and is known to protect cellular membranes and lipoproteins from peroxidation.(31) Previous studies have shown that vitamin E inhibits free radical formation and may augment the activity of antioxidant enzymes in biological system.(32,33) In addition vitamin E is involved in immune function, neurological functions, cell signaling, enzymatic activities, regulation of gene expression, and other metabolic processes.(34)

Although, previous studies authors reported that, vitamin C and vitamin E have a nephroprotective effect,(35-39) but to the best of our knowledge, there is no enough data available in which vitamin C together with vitamin E have been studied to investigate their antinephrotoxic effects in diclofenac-induced renal injury. So, the aim of this study was to determine

the effect of subacute diclofenac exposure on kidney tissues of male mice and assess whether these effects can be ameliorated by co-treatment with vitamins C and E. These findings may lead to further potentiate the research to evaluate the nephroprotective effects of this combination therapy.

Materials and Methods:

Experimental animals

thirty sexually mature male albino mice weighing approximately between 25-40g were purchased from Omar Al-Mukhtar University Animal House and were housed 6 per cages (320×180×160cm), placed in well ventilated house with optimum condition (temperature 20 ± 2°C; photoperiod: 12 h natural light and 12 h Dark). They were also allowed free access to water and standard food pellets. The cages were cleaned regularly to avoid any chance of infection, experiments were carried out between 9:00a.m and 2 p.m.

Drugs and chemicals

The drugs used in the experiment include diclofenac injection ampoule (diclofenac sodium 75mg/3ml purchased from ITALFARMACO, Itay), vitamin C tablet (vitamin C 500mg manufactured in the UK, Vitameen Ltd. London), Vitamin E soft gelatin capsules (vitamin E 400mg, was supplied by PHARCO Pharmaceuticals, Alexandria-Egypt), commercial kits to estimate oxidant and antioxidant parameters were from Biodignostic company, and renal function tests were carried out in Al-Saleem Laboratory.

Animal treatment schedule

Animals were adapted to laboratory conditions for seven days before commencement of the experiment. Before the experiment began, the mice were fasted over night but tap water was made available ad libitum. The animals in each group were kept in a separate cage and labeled. Each animal was weighed prior to the treatment, mice were randomly divided into 5 groups of 6 mice per group and treated as below:

Group I: Served as control and received 0.2 ml/day of corn oil via the oral route.
Groups II: This group received intraperitoneal injections (i.p) of diclofenac sodium 10mg/kg/day for 3 weeks without any other treatment.
Group III: Mice were administered single, daily, oral 200mg/kg of vitamin C dissolved in water 1 hour before 10 mg/kg/day of i.p diclofenac sodium for 3weeks.
Group IV: Once a day, mice were treated with vitamin E dissolved in water and corn oil (200 mg/kg/day, orally) 1 hour before 10 mg/kg/day of i.p diclofenac sodium for 3 weeks.
Group V: Once a day, mice were treated with vitamin C dissolved in water (200 mg/kg per day, orally) and vitamin E dissolved in corn oil (200 mg/kg per day, orally), and 1 hour later diclofenac (10 mg/kg/day) was administered via i.p rout for 3 weeks.
Blood collection and measurement of serum urea and creatinine in mice
Following termination of the experiment on the day 21, mice were fasted overnight for 14 hours and were sacrified after 22 days from beginning of drugs and vehicle administration, they were killed by cervical dislocation followed by decapitation to obtain blood samples, blood was collected into plain sample plastic tubes and sent to Al-Saleem Medical Laboratory for the determination of the biochemical parameters. Blood samples were centrifuged at 5000 rpm/min for 3 minutes to separate serum. Serum was preserved in eppendrof tubes and serum urea and creatinine were all assayed using Roche diagnostic kits (USA). The absorbance of the reaction was determined at 340-409nm for urea and 512-583nm for creatinine by COBAS INTEGRA 400 PLUS.
Assessment of antioxidant parameters of the kidney
The abdomen was opened and the liver tissue was removed, washed with ice-cold saline to remove the blood, blotted on filter paper and weighed, renal tissue supernatants obtained from the kidney tissue homogenization were used to determine the superoxide dismutase (SOD), superoxide

catalase (CAT) and lipid peroxidation measured as malondialdehyde (MDA) activities.

SOD activity was determined according to the method described by (Nishikimi et al., 1972).(40) The reduction of nitro blue tetrazolium (NitroBT) with NADH mediated by phenazine methosulfate (PMS) was inhibited upon addition of SOD. This observation indicated the involvement of superoxide aninon radical (O2−) in the reduction of NitroBT, the radical being generated in the reoxidation of reduced PMS. Similarly, the reduction of NitroBT coupled to D-amino acid oxidase-PMS system was also inhibited by SOD, the absorbance was read at 560 nm.

On the other hands, CAT was determined using the method (Aebi, 1984).(41) The principle of the method is based on the measured of a decrease in absorbance of the test sample by the induced decomposition of H2O2 in the presence of analyte enzyme. This rate is recorded by measuring the reduction in absorbance during 3 minutes at 240 nm in 1.5 ml of reaction mixture consisting of: 13.2 mM H2O2 in 50 mM phosphate buffer (pH 7.0) and 0.1 ml of the homogenate. As a control a mixture containing: 50 mM phosphate buffer (pH 7.0) and 0.1 ml of the tissue homogenate is used.

Determination of lipid peroxidation

MDA was determined using the method (Ohkawa et al., 1979),(42) as it reacts with thiobarbituric acid (TBA), the reaction yields a pink MDA-TBA adduct the product of two moles of TBA plus 1 mole of MDA. The colored complex can be assayed by measuring absorption at 532nm,

Statistical analysis:

Multiple group comparasions were interpreted by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test to determine the specific pairs of groups that were statistically different. Calculations were done using statistical software Graph Pad Prism (Version 6.01) for Windows. Data were presented as means plus or minus

the standard error. The minimum level of significance was set at P values of less than 0.05.

Results:

Effect of treatments on serum levels of urea and creatinine

Table 1 shows the effect of repeated, intraperitoneal diclofenac administration and pretreatment with oral 200 mg/kg of vitamin C and 200 mg/kg of vitamin E on the serum urea, creatinine for 21 days. Serum levels of urea and creatinine were determined as measures of renal function. The administration of diclofenac induced sever renal injury, as shown by marked increase serum levels of these parameters in comparison with the control group ($P<0.001$). Whereas, the pretreatment of mice with vitamin C and E separatedly demonstrated a significant decrease in serum levels of urea and creatinine when compared to those of diclofenac treated group ($P<0.001$). Although the levels of urea and creatinine were significantly lower in the vitamin C and E plus diclofenac-treated group compared with the diclofenac group, these levels were still higher than that of the control group. Here in our present findings, we found that the combined effects of vitamin C and vitamin E were more significant as compared to the individual effects of vitamin C and vitamin E alone (see Figures 1 and 2), this interesting interaction indicates that vitamin C can work synergistically with vitamin E in protecting against renal injury induced by diclofenac resulting in the decreased levels of serum creatinine and blood urea.

Determination of oxidant and antioxidant parameters of the kidney

The SOD and CAT activities were significantly reduced in the diclofenac treated group compared with the control group ($P<0.001$). These antioxidant activities were significantly ($P<0.001$) increased in the vitamin C and E groups when compared with the diclofenac treated group (Table 2). On the other hands, MDA was significantly ($P<0.001$) enhanced in animals exposed to diclofenac compared to the normal control group, and this value was significantly ($P<0.001$) decreased with the combinations of diclofenac plus vitamin C and E. Also favourable

results were obtained with combined antioxidant therapy of vitamin C and E compared with monotherapy (see Figures 3,4 and 5).

Table 1: Effects of intraperitoneal administration of diclofenac alone and in combination with vitamin C and E on serum renal functions.

Parameter	Control	Diclofenac	VitaminC+ diclofenac	VitaminE+ diclofenac	Vitamin C + vitamin E+diclofenac
Urea mg/dl	66±.3.33	160±3.75***	86±1.78# # #	88±2.62 # # #	71±3.03 # # #
Creatinine mg/dl	0.4±0.02	1.9±0.05***	0.7±0.02# # #	0.8±0.04# # #	0.5±0.02 # # #

Values are expressed as mean±SEM.
*** (P<0.001) denotes significant difference vs. control values.
# # # (P<0.001) denotes significant difference vs. diclofenac values.

Table 2: Shows the levels of MDA, SOD and CAT in the kidney tissue of normal and experimental groups of mice.

Parameter	Control	Diclofenac	VitaminC+ diclofenac	VitaminE+ diclofenac	Vitamin C +vitaminE+ diclofenac
MDA (nmol/g protein)	99±2.26	228±10.38***	135±2.39# # #	126±3.14# # #	105±5.09 # # #
SOD (Unit/mg protein)	20 ±0.99	51 ±1.78***	27 ±1.22 # # #	29 ±1.25# # #	22 ±1.25 # # #
CAT (Unit/mg protein)	54±1.61	27±1.38***	46±1.54 # # #	44±2.06 # # #	51±2.70 # # #

Values are expressed as mean±SEM.
*** (P<0.001) denotes significant difference vs. control values.
# # # (P<0.001) denotes significant difference vs. diclofenac values.

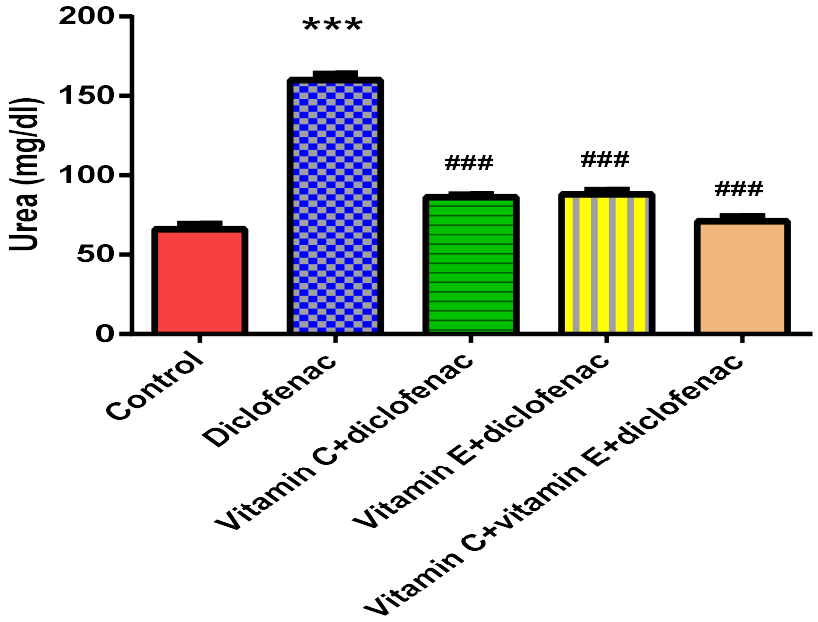

Figure 1. Effects of diclofenac, vitamin C and vitamin E on the kidney urea level (mg/dl).Values are expressed as mean±SEM. ***(P<0.001) denotes significant difference vs. control values and # # #(P<0.001)denotes significant difference vs. diclofenac values.

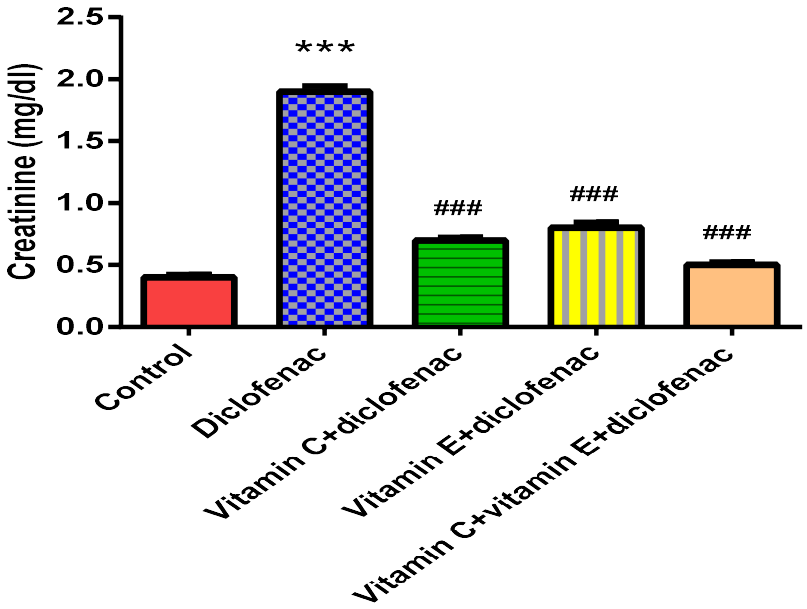

Figure 2. Effects of diclofenac, vitamin C and vitamin E on the kidney creatinine level (mg/dl).Values are expressed as mean±SEM. ***(P<0.001) denotes significant difference vs. control values and # # #(P<0.001)denotes significant difference vs. diclofenac values.

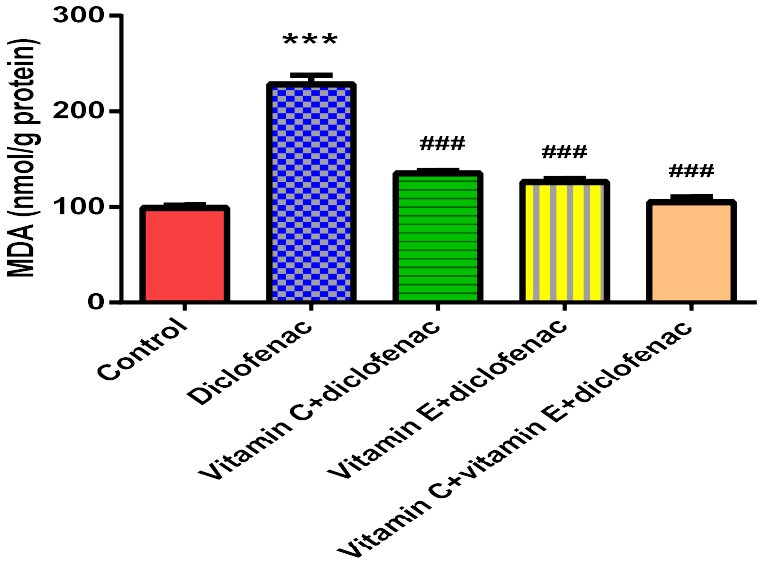

Figure 3. Showed the effects of diclofenac, vitamin C and vitamin E on the kidney MDA level (nmol/g protein). Values are expressed as mean±SEM. ***(P<0.001) denotes significant difference vs. control values. # # # (P<0.001) denotes significant difference vs. diclofenac values.

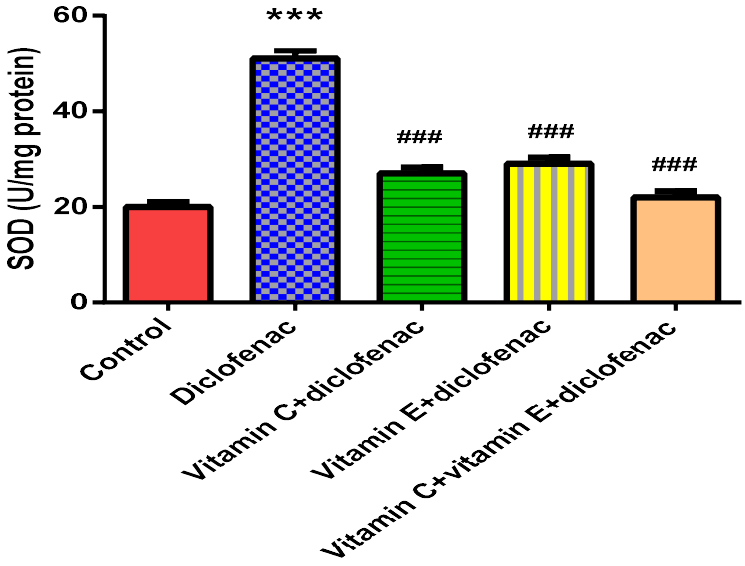

Figure 4. Showed the effects of diclofenac, vitamin C and vitamin E on the kidney SOD level (U/mg protein).Values are expressed as mean±SEM. ***(P<0.001) denotes significant difference vs. control values. # # #(P<0.001) denotes significant difference vs. diclofenac values.

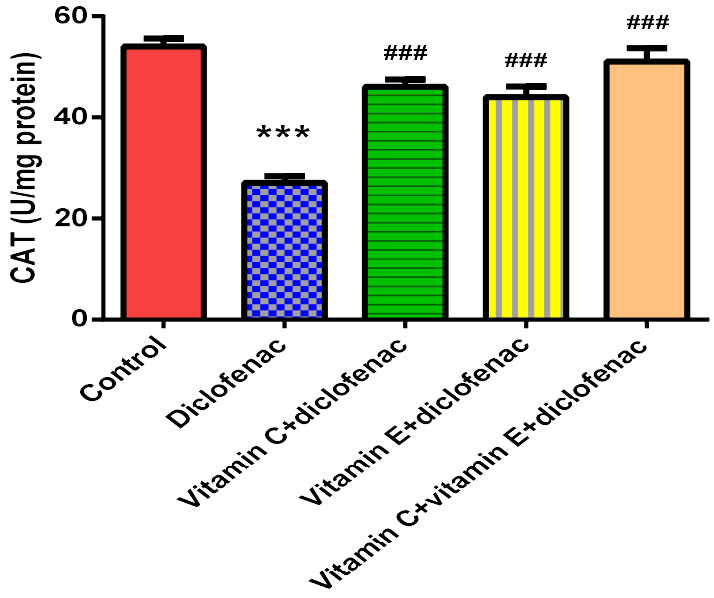

Figure 5. Showed the effects of diclofenac, vitamin C and vitamin E on the kidney CAT level (U/mg protein). Values are expressed as mean±SEM. ***(P<0.001) denotes significant difference vs. control values. # # #(P<0.001) denotes significant difference vs. diclofenac values.

Discussion:

Diclofenac is one of the most frequently prescribed therapeutic agent, used for treatment of considerable painful conditions because it has analgesic, antipyretic and anti-inflammatory actions. The damaging effects of diclofenac on kidney tissue in human and animals is well-documented in the literature.(43,44) However, different treatment strategies have been proposed to prevent diclofenac-induced nephrotoxicity. For this purpose, adult male albino mice were divided into five groups and were treated with a high dose of diclofenac in the absence or presence of vitamin C and E. The kidney damage was reflected by an

increase in levels of serum urea and creatinine. The present study revealed a significant ($P<0.001$) rise in levels of serum urea and creatinine on exposure to a high dose of diclofenac, indicating considerable renal injury. However, these elevations were significantly ($P<0.001$) attenuated by vitamin C and E pretreatment, this effect clearly indicated that vitamin C and E may offer protection in renal damage induced by diclofenac.

Nevertheless, multiple research groups have reported the role of prostaglandins in diclofenac-induced nephrotoxicity, as similar to other NSAIDs, diclofenac suppresses prostaglandins (PGE2 and PGI2) synthesis in glomeruli, convoluted tubules, medulla and renal arterioles decreasing renal perfusion (pressure natriuresis) and glomerular filtration rate (GFR),(45,46) thus causing renal ischemia which is induced by loss of vasodilatory effects of PGs and affecting the coagulation mechanism.(47) Moreover, PGs have also been shown to play a role in stimulating renin and angiotensin-mediated aldosterone release.(48) Thus, diclofenac can result in hyperkalemia and metabolic acidosis. diclofenac may cause hyponatremia which is possibly related to release of inhibitory effect of PGs on antidiuretic hormone (ADH)-facilitated water absorption at the distal collecting tubules.(49) Additionally, diclofenac triggers a delayed-type hypersensitivity response with shunting of arachidonic acid metabolites to lipoxygenase pathway increasing leukotrienes synthesis which mediate chemotaxis for WBCs leading to cellular infiltrates (T-cell and eosinophils) and tubulointerstitial nephritis.(50)

Although the underlying pathophysiologic mechanism of renal injury under the treatment of diclofenac is not clearly known, there are some hypotheses such as an elevation in ROS may play a key intermediary role in the pathophysiologic processes of renal injuries induced by diclofenac.(51,52) ROS have been demonstrated to be capable of degrading glomerular basement membrane and inducing glomerular injury, leading to impaired glomerular filtration and sieving function.(53,54) In order to eliminate toxic ROS, cells are equipped with

various antioxidant defense systems. Therefore, the development of tissue injury depends on the balance between ROS generation and tissue antioxidant defense mechanism.(55) The glomerular antioxidant enzymes are suggested to play an important role in the functional derangement induced by the ROS.(56) In the current study, diclofenac produced alterations in the levels of oxidative stress biomarkers in kidney of male mice, as marked by a significant decrease in SOD, CAT activities and increase in MDA level. In turn, the elevation of MDA level indicates lipid peroxidation which is known to cause cellular injury by inactivation of membrane enzymes and receptors, as well as protein cross-linking and fragmentation.(57)

SOD is a key defense enzyme that accelerates the dismutation of superoxide radicals ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2).(58)Whereas,CATcatalyzes thedecomposition
of H_2O_2 to water and oxygen.(59) However, our findings showed that vitamin C and E separatedly significantly increased the antioxidative activities of SOD, CAT and decreased MDA level in comparasion with diclofenac-treated mice. Additionally, we presume that the combined effects of vitamin C and E were also high as compared to the individual effects of vitamin C and E alone, the reason for these augmented additive effects of this combination may be due to the inhibition of ROS resulting in the decreased levels of serum creatinine and urea with increased antioxidant activity.

The current observations reported in this study suggest that another mechanism has been involved in the explanation of this kidney damage induced by diclofenac apart of the role of PGs, is its ability to produce free radicals inducing prooxidative damage in renal tissue.(60) Such observation is in accordance with the data reported by Hickey et al., who suggested that diclofenac induced renal injury may involve production of ROS leading to oxidative stress and massive genomic DNA fragmentation and these free radicals may ultimately translate into apoptotic cell death of

kidney cells. These phenomenons ultimately decrease the GFR along with renal dysfunctions.(61)

According to our results about the antioxidants "SOD and CAT" in addition to the lipid peroxidation indicator "MDA", we may suggest that the therapeutic potential of vitamin C and E is dependent on an antioxidant mechanism against diclofenac-induced nephrotoxicity and these findings could have considerable clinical implication.

Conclusion:

To the best of our knowledge, this is the first study, in which the nephroprotective effects of vitamin C and vitamin E were studies together in combination form for the prevention of diclofenac-induced nephrotoxicity. Our results demonstrate that diclofenac is capable of inducing marked alterations in biochemical parameters of the kidney in a mice model, this result is contributed to oxidative stress induced by the drug. Whereas, vitamin C and E can ameliorate diclofenac-induced renal damage due to their free radical-scavenging and antioxidant activities, the protection by the combination of vitamin C and E was additive when compared to each agent alone. Clearly, These interesting findings await for further study using other types of experimental animals. Furthermore, direct testing in human and histopathological study are needed in order to judgment if this combination could be used as safe agents in human therapy.

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