Effect of Combination of L-Arginine and *N*-Acetylcysteine in Rat Model of Renal Ischemia-Reperfusion Injury

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Abstract:. **Background:** Ischemia, followed by reperfusion (I/R) is one of the major causes of acute renal failure (ARF). The pathogenesis of I/R is multifactorial .Nitric Oxide(NO) proved to be closely related to the pathogenesis of I/R-induced ARF .Therefore, the aim of the present study was to analyze the role of NO-dependent mechanisms in the renal response to I/R by providing a source of(NO) precursor (L-Arginine) in the presence or absence of *N*-acetylcysteine (NAC)which protect NO from interacting with oxygen free radicals that may contribute to the pathogenesis of ischemia reperfusion injury. **Materials and Methods:** Forty male Sprague-Dawley rats were submitted to right nepherectomy and divided into four groups: control, renal I/R, renal I/R withL-Arginine, renal I/R withL-Arginine and NAC, I/R injury was induced by 45 min of left renal artery ischemia followed by60 min of reperfusion. After reperfusion, 24 hours urine was collected to measure creatinine and para amino hippuric (PAH) clearance and urinary nitrites. Blood samples were collected to measure blood urea nitrogen (BUN) and serum creatinine. All animals are scarified, the left kidney are removed to measure protein content, superoxide dismutase, malondialdehyde and glutathione in the renal tissue, histopathological examination for the left kidneys were performed. **Results:** Renal I/R resulted in significant elevation in blood urea, serum creatinine and renal tissue malondialdehyde and significant lowering in superoxide dismutase, kidney glutathione, PAH clearance, creatinine clearance and urinary nitrites secretion. L-Arginine alone result in significant improvement in renal function parameters when compared to I/R group but this improvement is still insignificant when compared to control group also parameters of oxidative stress and urinary nitrites secretion does not significantly affected., but in the renal I/R+ L-Arginine and NAC, all biochemical results and histopathological parameters were significantly improved as compared to control group. **Conclusions:** Combined treatment with antioxidant (NAC) and nitric oxide precursors (L-Arginine)attenuates renal ischemia-reperfusion injury, by direct scavenging of reactive oxygen species (ROS), thus protecting NO from reaction with it.

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Key words: Ischemia reperfusion (I/R), L-Arginine, Nitric Oxide (NO), *N*-acetylcysteine and experimental rats

1. Introduction

Renal Ischemia/reperfusion (I/R) injury is a complex pathophysiologic process that occurs in cases of heminephrectomy, kidney transplantation, cardiac arrest with recovery, and vascular surgery**.** It represents a common cause of renal failure and renal graft rejection[(1)]**.** The mechanisms that explains I/R damage to kidneys are likely multifactorial and interdependent, involving anoxia, release of oxygen-derived free radicals, neutrophil accumulation, and inflammatory responses .[(2)] There is accumulating evidence that, in the kidney, various cells, including vascular endothelial and tubular epithelial cells, can generate nitric oxide (NO), nitric oxide (NO) incriminated in renal physiology and pathophysiology[(3)] .

The role of nitric oxide in the pathogenesis of renal failure is still controversial **,** where NO may be harmful due to either its direct cytotoxic effect and /or its reaction with superoxide generating peroxynitrate which has harmful effect on renal tissue[(4),] in contrast several studies demonstrate the beneficial effect of NO in different ARF module such as cyclosporine nephrotoxicity ,radiocontrast media ARF and ischemic models in regulation of vasomotor tone, immune defense modulation and neurotransmission[(5).] Therefore the dual opposite effect of NO has demonstrated , so we hypothesized that the net effect of NO in I/R may result from the balance between beneficial homodynamic effect and cytotoxicity.

Oxidative stress appears to be the main mechanism causing tissue ischemia-reperfusion damage[(6)] N-acetylcysteine (NAC), a precursor of l-cysteine and reduced glutathione (GSH) affects a variety of biological and pathological processes, as it is a source of sulfhydryl groups in cells and acts as reactive oxygen species (ROS) scavenger [(7, 8).]NAC has frequently been used in experimental studies to interfere in mechanisms of oxidative injury [(9)] In addition, NAC has consistently proved to be a potent antioxidant in certain experimental and clinical conditions [(10).] Therefore, the aim of the present study

was to analyzed the effect of the NO precursor L-Arginine in animals submitted to renal I/R, both in the presence or in the absence of (NAC)

2. Materials and Methods

This study was conducted on forty male Sprague-Dawley rats of normal house strain weighing between 200-250gm. purchased from the faculty of science Tanta University , The rats were housed in a temperature controlled room and on a 12-h light/dark cycle. The rats were fed a standard Purina rat chow diet and allowed water. All protocols were approved by Tanta Faculty of medicine ethical Committee .
They were classified into four groups' ten rats each.

Group I: (control group.). The rats of this group submitted to the right-sided nepherectomy and the left renal artery was dissected and manipulated but no clip applied, and it is considered as control group [11].

Group II: Ischemia/reperfusion (I/R) group. Renal I/R was carried out as described by ***(Basireddy et al., 2006)*** [11] animals anaesthetized with xylazine (10mg/kg, i.p.) and ketamine (75mg/kg, i.p.) Through flank incisions, a right-sided nepherectomy was performed then the left renal artery was clamped for 45min by a non-traumatic microvascular clip, then the clamp was removed following the ischemic period for 60 minutes to re-establish blood flow to the ischemic kidney (reperfusion).

Group III: I/R with L-Arginine group. After the basal period ischemia induced by clamping of left renal artery (45 min). Thereafter, in the reperfusion period, we intravenously infused L-Arginine (Arg, 300 mg/kg/60 min) [12].

Group IV: I/R with L-Arginine and NAC group .The rats of this group received L-Arginine as in group III. *N*-acetylcysteine (Acetylcysteine NM Pharma, Sweden) was administered in a dose of dose of 150 mg/kg i.v. 15 minutes before reperfusion and maintained at 50 mg/kg/h during reperfusion;[7]

Rectal temperature was kept at 37–38°C. After surgery, fluid losses were replaced by administration of 5ml of warm (37°C) isotonic saline i.p., and rats were returned to their cages 24h after renal I/R:

The rats had free access to normal rat chow and tap water. All these animals were kept in individual metabolic cages **for** 24 hours urine collection for Renal clearance experiments:

1- Creatinine clearance (ml/min/day) [13]
2- Para amino hippuric clearance (ml/min) [14]
3- Measurement of Urinary Nitrites (NO_2s); Total nitrate and nitrite concentrations were estimated by the conversion of nitrate into nitrite. Total nitrite content was then measured by the Greiss reaction using $NaNO_2$ as standard with detection limit of 1 mM [15]. Briefly, 30 µl of each sample was incubated for 1 h at 37°C with 5 µl Aspergillus nitrate reductase (5 U/ml; Sigma) and 15 µl of NADPH (1.25 mg/ml; Sigma). After incubation, 100 µl of Greiss reagent (1% sulfanilamide/ 0.1% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4) and 100 µl trichloroacetic acid (10% aqueous solution) were added and incubated at room temperature for 10 min. Protein precipitates were removed by centrifugation at 13,000 3 g for 5 min. the Absorbance of Supernatants was measured at 570 nm, and NO concentrations were estimated against a sodium nitrate standard curve.

Blood samples were collected from the orbital sinus under ether anesthesia and analyzed for Blood urea nitrogen (mg/dl) [16], and Serum creatinine (mg/dl) [17]

Then all the animals were scarified and left kidney were removed. Then after weighing the tissues from injured left kidneys 24h after IR., homogenized in volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.4), homogenization (homogenizer: IKA Ultra-Turrax t 25 Basic, Germany) was carried out for 2 min at 13 000 rpm. All procedures were performed at 4°C. Homogenate, supernatant and extracted samples were prepared and the following determinations were made on the samples using commercial chemicals supplied by Sigma.

1- Protein measurements were made according to the method explained by **Lowry et al.** [18]
2- Estimation of Total super oxide dismutase (SOD) activity was determined according to the method of **Sun et al.**[19].
3- The malondialdehyde (MDA) levels were determined by the method [20]. based on the coupling of MDA with thiobarbituric acid briefly, samples were diluted in phosphate buffer (20mM, pH7.4) and heated together with a thiobarbituric acid TBA solution (375 mg/ml) in a boiling water bath for 15 min. The tubes were cooled, and the absorbance was measured by spectrophotometry at 535nm. 1,1,3,3- Tetraethoxypropane. Sigma, St. Louis, MO was used as a stander **(Bird and Draper,1984)**[21]. Results were expressed as nmol/g wet tissue
4- Total kidney glutathione concentrations were measured with an assay based on a reaction using Ellman's reagent, the method is based on the reduction of 5.5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by GSH to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance was measured at 405 nm [22] according to the

manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI, USA).

Histopathological examination of the left kidneys were performed in all these animals using a standard protocol and paraffin sections were stained with hematoxylin and eosin and examined by light microscope [23].

Statistical analysis:

Data are expressed as means±SD. The statistical significance of differences between experimental groups was calculated using a one-way ANOVA .We considered statistically significant a *P* value <0.05

3. Results

The results of clearance studies and renal function tests were presented in table (1) figure (1, 2,3, and 4) this show that I/R results in significant elevation in BUN and serum creatinine and significant lowering in creatinine clearance and PAH clearance if compared to control group, treatment with L-arginine results in significant lowering in BUN and serum creatinine if compared to I/R group but the level of both parameters is still significantly high if compared to control group ,regarding creatinine clearance and PAH clearance in L- arginine group there is significant elevation in both clearance parameters if compared to I/R group but it is still significantly low if compared to control group .Combined treatment by L-arginine and NAC in group IV result in significant lowering in BUN and serum creatinine if compared to I/R group and L-arginine treated group and significant elevation in creatinine clearance and PAH clearance if compared to I/R group and L-arginine treated group.

Table 1: Effect of L-Arginine alone or with combination of NAC on BUN, serum creatinine, creatinine clearance and PAH clearance in rats 24 hours after IR injury.

Parameters	Control (I)	I/R (II)	I/Rwith L-arginine (III)	I/RwithL-arginine and NAC (1V)
BUN (mg/dl)	22.2±4.32	90.3±3.43[a]	42±5.44[b,c]	28±4.47[d]
Serum creatinine (mg/dl)	0.73±0.07	1.53±0.14[a]	0.98±0.13[b,c]	0.85±0.06[d]
Creatinine clearance (ml/min.)	1.16±0.06	0.60±0.10[a]	1.04±0.11[b,c]	1.22±0.06[d]
PAH clearance (ml/min.)	8.44±0.74	5.72±0.67[a]	7.20±0.93[b,c]	8.45±0.99[d]

a denotes II vs I b denotes III vs I c denotes III vs II d denotes IV vs II & III.

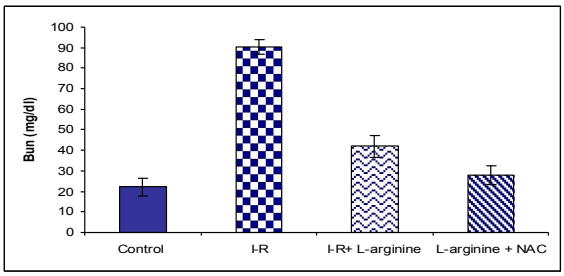


Figure 1: Effect of L-arginine alone or with combination of NAC on BUN, in rats 24 hours after IR injury.

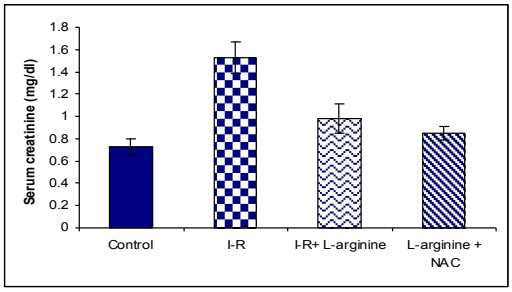


Figure 2: Effect of L-arginine alone or with combination of NAC on serum creatinine, in rats 24 hours after IR injury.

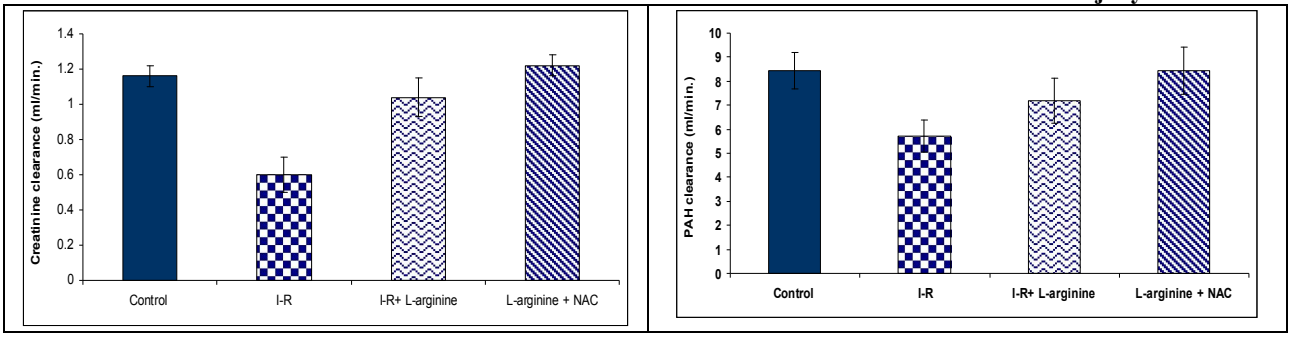


Figure 3 and 4: Effect of L-arginine alone or with combination of NAC on creatinine clearance and PAH clearance in rats 24 hours after IR injury.

Table (2) and figure (5, 6, 7) show that in I/R injury in group (II) and after treatment by L-Arginine in group III there is significant lowering in urinary excretion of nitrites, kidney glutathione and renal tissue SOD if compared to control group but combined treatment by L-Arginine and NAC result in significant elevation in urinary nitrites secretion, kidney glutathione and renal tissue SOD if compared to I/R group and L-Arginine group. As regard renal tissue MDA, renal I/R injury and after treatment with L-Arginine result in significant elevation in its level if compared to control group but after treatment by both L-Arginine and NAC there is significant lowering in its level if compared to I/R group and L-Arginine group.

Table 2: Effect of L-arginine alone or with combination of NAC on renal tissue MAD, SOD, glutathione and urinary nitrites in rats 24 hours after IR injury

Parameters	Control (I)	I/R (II)	I/Rwith L-Arginine (III)	I/RwithL-Arginine and NAC(1V)
MAD kidney (nmol/g protein)	2.73±.18	4.88±0.25[a]	4.60±0.33[b]	2.85±0.13[c]
SOD u/mg protein	0.17±0.04	0.09±0.03[a]	0.11±0.02[b]	0.18±0.05[c]
Kidney glutathione (µmol/g protein)	0.37±0.10	0.15±0.06[a]	0.17±0.05[b]	0.28±0.16[c]
Urinary Nitrites (nmol/h)	223.9±30.8	126.1±15.88[a]	148.29±10.94[b]	199.3±11.02[c]

a denotes significant differences group II vs I b denotes significant differences group III vs I
c denotes significant differences group IV vs II & III.

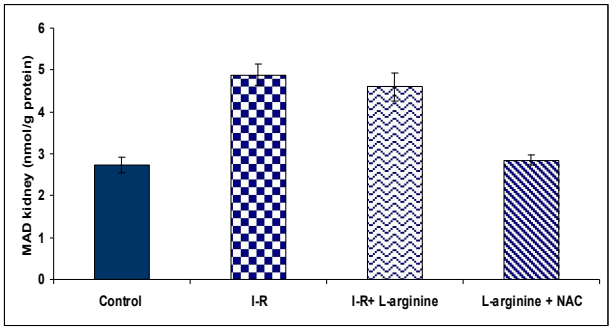


Figure 5: Effect of L-arginine alone or with combination of NAC on renal tissue MAD, in rats 24 hours after IR injury.

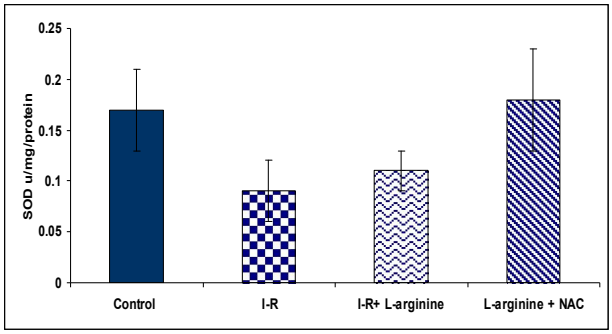
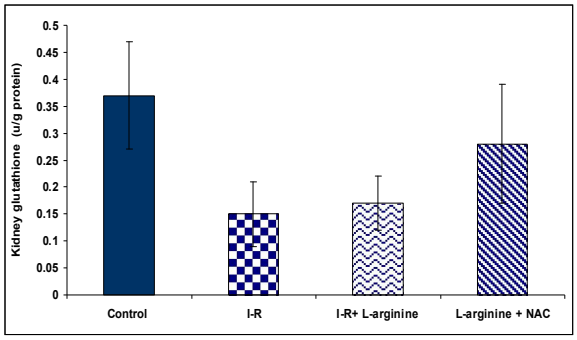


Figure 6: Effect of L-arginine alone or with combination of NAC on renal tissue SOD in rats 24hours after IR injury



Nitrites (nmol/h)

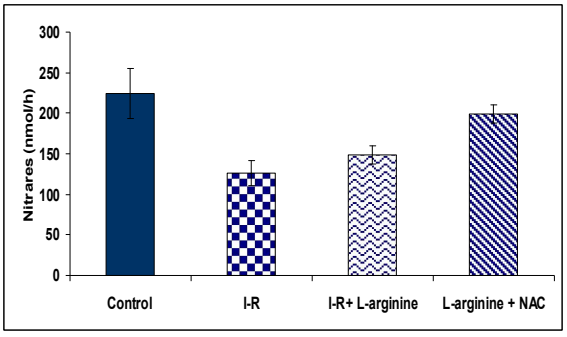


Figure 7 and 8: Effect of L-arginine alone or with combination of NAC on kidney glutathione and urinary nitrites in rats 24 hours after IR injury.

RENAL HISTOPATHOLOGY

The histopathological findings of this study revealed the following:

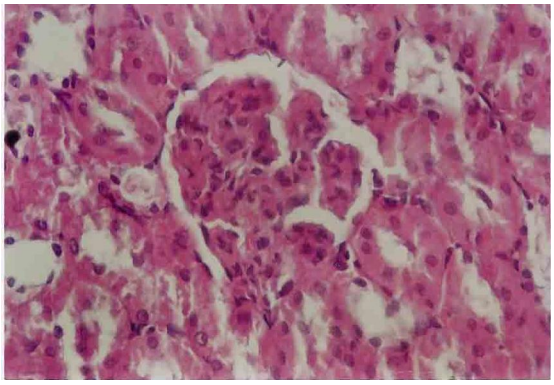


Figure 9: Section from the kidney of the control group revealed apparently normal kidney architecture with slight glomerular hypertrophy

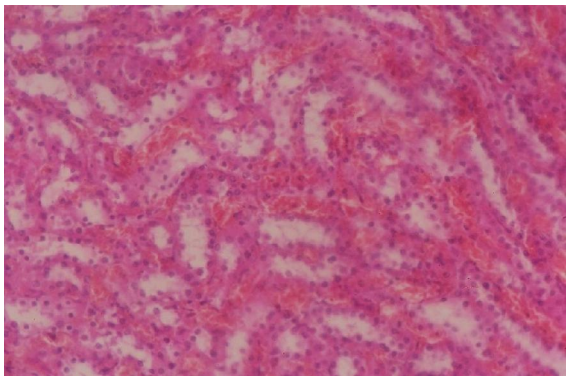


Figure 10: Section from the kidney of ischemia/reperfusion group revealed severe tubular necrosis, proteinaceous casts and severe medullary congestion and haemorrhage.

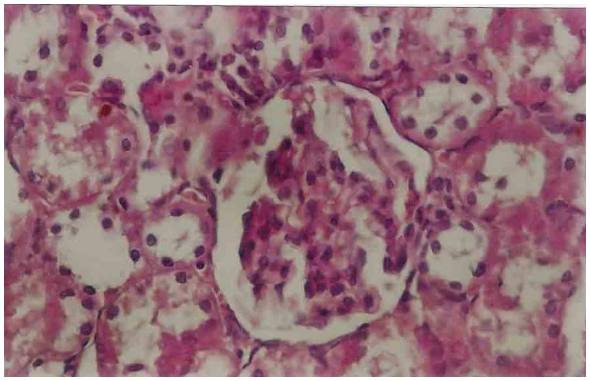


Figure 11:Section from the kidney of L-arginine group revealed morphological changes in the form of hypocellular glomerulus with widdened Bowman's space with degenerated tubules and hyaline casts

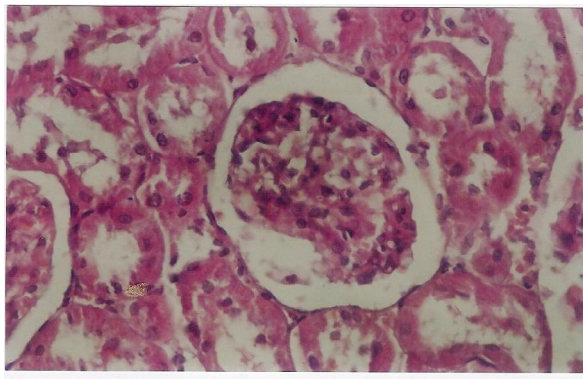


Figure 12: Section from the kidney In L-arginine +NAC treated group revealed mild morphological changes in the form of mild tubular necrosis and widening of Bowmsnn's space

4. Discussion

Renal ischemia reperfusion (I/R) injury remains one of the biggest obstacles in the field of renal transplantation causing acute renal failure.[24] Tissue ischemia with inadequate oxygen supply followed by successful reperfusion initiates a wide and complex array of inflammatory responses that may both aggrevate local injury as well as induce impairment of remote organ function. [25]

It is clear from the results of the present work that in ischemia reperfusion group there significant decrease in urinary nitrite, which indicate a decrease nitric oxide production as urinary nitrite is believed to be waist product of nitric oxide.[24]

Altered NO production and /or decreased bioavailability of NO in ischemia reperfusion injury may result in neutrophil adhesion and infiltration which may build obstruction of small blood capillaries leading to more ischemia [26]. with consequent significant decrease in glomerulr filtration rate (GFR) and renal plasma flow (RPF) which is observed in the present work after ischemia reperfusion. A possible mechanism of significant decrease in urinary nitrite in I/R group is a decrease L-Arginine production which contribute to decrease NO levels[27], in addition when the concentrations of L-Arginine are low, nitric oxide synthase (NOS) may generate superoxide instead of NO, a process called NOS uncoupling (i.e., uncoupling of NADPH oxidation and NO synthesis)[28]. Superoxide generation by endothelial nitric oxide synthase (eNOS) is dependent on the presence of its substrate, Arginine, and its cofactor tetrahydrobiopterin (BH4)[29]. When there is an abundance of both factors eNOS produces NO,

but when the concentration of one of these factors is relatively low, eNOS generates superoxide. NOS-mediated superoxide generation has been suggested to occur in reperfusion injury after ischemia [30], but significant amount of free oxygen radicals generates due to reperfusion injury[31] These free radicals produced due to reintroduction of oxygen within the renal cells damaging cellular protein and plasma membrane, which in turn causes release of more free radicals [32] this is proved by the significant increase tissue oxidant MDA and decrease of renal tissue glutathione and SOD which was observed in this study in I/R group . The increase oxidative stress and eroding the antioxidant defense with lack of NO aggravate most alteration elicited in these animals[33,34] Generation of superoxide in ischemic reperfusion injury results in the formation of peroxynitrate, which is potent cytotoxic oxidant causing lipid peroxidation, injuring DNA, and nitrotyrosination of proteins[35]and this may explain the present finding of significant increase in BUN, serum creatinine and histopathological changes observed in the form of severe tubular necrosis,medullary congestion, hemorrhage and the presence of proteinurious cast . Oxygen-derived free radicals during reperfusion, leads to more endothelial cell dysfunction with subsequent decreased endothelial dependent vasorelaxation with decreased NO release and leukocyte-endothelial adhesion and activation[36] also the formation of peroxynitrate, produce further vasoconstriction contributing to more decrease in GFR and RPF[37].

Treatment with L-Arginine which is NO precursor result in significant improvement of renal functions as BUN ,Serum creatinine ,GFR,RPF and oxidative stress markers when these results are compared with those of I/R group. However the improvement of the renal function , oxidative stress and structural changes in the kidney with L-Arginine are insignificant when compared to control group this finding indicates that the pathogenesis of ischemia/reperfusion induced acute renal failure is still not completely corrected by L-Arginine in I/R rats.

The beneficial effect of L-Arginine on ischemia/ reperfusion renal injury secondary to hemodynamic vasodilator effect of nitric oxide [33] L-Arginine reduce 02- generation and improve nitric oxide signaling protein expression[38]

NO has important effect on renal hemodynamic ,renal nerves and direct tubular transport properties[39]. In the kidney various cells including vascular endothelium and tubular epithelium can generate NO which interact with vascular smooth muscles ,mesengeal and tubular cells to control renal blood flow and glomerulo tubular functions[40] Another mechanism by which L- Arginine partially protect the kidney through release of NO which is depressor to endothelin-1 a potent vasoconstrictors [41].

In group of I/R treated withL-arginine and NAC, there is significant increase in urinary nitrite this finding suggesting that the supplementation of L-Arginine with NAC represent an additional factors increased nitric oxide production ,this greater NO viability may result from increased eNOS protein expression ,previous study demonstrate that NAC upregulating the protein expression of (eNOS) and is capable of tripling endothelial NOS expression as well as increasing NO bioavailability. [7,42,43] Or may be attributed to the protection of released nitric oxide from being reacting with superoxide radicals, in the other meaning NAC increase NO formation and simultaneously eliminating superoxide as it acts as superoxide scavenger [44]. This could be proved by significant increase in GFR and RPF observed in this group of animals.

NAC is a thiol containing antioxidant and animal studies showed that NAC treatment had a protective effect in model involving increase oxidative stress such as I/R injury.[45,46] Part of the renoprotective effect of NAC in this group is due to ameliorating effect of reactive oxygen species[47] as judged by the significant increased in renal tissue glutathione, SOD and significant decrease of MDA in this group compared to I/R group and in group of I/R treated withL-arginine group. NAC interfere with (ROS) production by inhibiting active granulocyte and induce glutathione production. [48] also It has anti inflammatory effect by inhibiting cytokine-stimulated necrosis factor κB (NF-κB) activation, and by down-regulating the expression of vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1).[49]

Pretreatment of animals with NAC combine with L-Arginine markedly ameliorate renal dysfunction and histopathological alteration, it causes significant decrease in BUN Serum creatinine and significant increase in creatinine clearance and PAH clearance. The kidney shows normal morphology with mild

tubular necrosis in this group compared to either I/R or control group. This indicate complete correction of the changes observed in all parameters studied in I/R animals when treated with L-Arginine and NAC.

Conclusions

The Local effect of increased production of NO by L-Arginine may be partly lost depending on the balance between NO production and oxidative stress .so L-arginine /No pathway seems to have slightly protective effect of the kidney after I/R injury in Rats

The renoprotective effect of L-Arginine and NAC may be dependent not only on free radical scavenging but also on NO potentiating and increasing its production.

These results need to be confirmed by studies in human being

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