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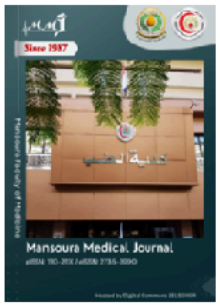

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RENOPROTECTIVE EFFECT OF LEFLUNOM1DE AGANIST ISCHEMIA-REPERFUSION INJURY IN RATS

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ABSTRACT

Renal ischemia is of great clinical interest because of its role in renal failure and renal graft rejection. The purpose of this study was to investigate the possible protective effect of leflunomide against ischemia/ reperfusion (I/R) injury in the rat. Methods: Three groups of Sprague-Dawley rats (10 rats each), the control group, I/R group and the leflunomide - treated I/R group. A renal I/R injury was induced by a left renal pedicle occlusion to induce ischemia for 45 min, followed by 60 mins of reperfusion with contralateral nephrectomy in rats. The rats in Leflunomide treated I/R group were pretreated intragastrically with a leflunomide suspension (10 mg/kg) 60 min before the ischemia induction. Thiobarbituric acid reactive substances (TBARS), nitric oxide (NO), tumor necrosis factor alpha (TNF-α), catalase (CAT) superoxide dismutase (SOD) activities were determined in renal tissue, while, creatinine, blood urea nitrogen (BUN) were measured in blood. Results: Our results indicate that TBARS, NO, TNF-α, BUN and creatinine levels, were significantly higher in the I/R group than those in the control group. Leflunomide administration significantly decreased these parameters. SOD and CAT activities significantly decreased after I/R injury when compared to the control group. Leflunomide treatment significantly increased activities of these enzymes when compared to the I/R group.

Conclusions : These results demonstrated that reactive oxygen species (ROS) and TNF-α play causal role in I/R induced renal injury and

leflunomide exerted renoprotective effects by anti-inflammatory effect with radical scavenging and antioxidant activities.

Key words : Ischemia-reperfusion, Oxidative stress, Leflunomide, TNF-α.

INTRODUCTION

Renal failure as a consequence of ischemia /reperfusion is of particular relevance to transplantion[1], coronary bypass surgery[2], aortic cross clamping[3], and sepsis[4] and remains a major cause of morbidity and mortality among patients in intensive care units.

I/R reperfusion injury leads to production of excessive amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing oxidative stress which results in alterations in mitochondrial oxidative phosphorylation, depletion of ATP, an increase in intracellular calcium and activation of protein kinases, phosphatases, proteases, lipases and nucleases leading to loss of cellular function/integrity.[5]

Many studies showed that an inflammatory response induced by ischemia followed by reperfusion is largely responsible for tissue damage [6, 7]. The acute inflammatory response initiated by I/R is characterized among others by the induction of a proinflammatory cytokine cascade [8], expression of adhesion molecules [9] and cellular infiltration [10]. Peripheral monocytes infiltrating the kidney have been considered the primary source of TNF-α.

TNF-α is capable of upregulating its own expression as well as the expression of other genes pivotal to the inflammatory response [11] ultimately leading to a reduction in the glomerular filtration rate (GFR). TNF- α also stimulates the production of reactive oxygen species from mesangial cells as well as the production of other inflammatory mediators [12].

The expression of proinflammatory cytokines during I/R injury results in the upregulation of inducible nitric oxide synthase (iNOS) thereby producing large amounts of nitric oxide (NO) for longer periods of time. Under oxidative stress conditions, NO reacts with superoxide to generate peroxynitrite, which is capable of nitrating tyrosine residues of proteins and enzymes leading to tissue injury [13-15].

Identification of drugs that down regulate the production of TNF-α and NO should provide the opportunity for therapeutic intervention against I/R injury

Leflunomide, an isoxazole derivative, is a unique immunomodulatory agent capable of treating rheumatoid arthritis, allograft and xenograft rejection, systemic lupus erythematosus, prostate carcinoma, and neuronal-glial tumours [16-27]. Recent study demonstrated the protective effect of leflunomide against hepatic ischemia/reperfusion injury in rats owing to its antioxidant and anti-inflammatory effects [28].

Thus based on this study and on the immunological dysfunction in renal ischemia/reperfusion and leflunomide's immunomodulatory feature with high efficacy and low toxicity, we assumed that leflunomide might have protective effect on renal ischemia reperfusion injury. In this study therefore we aimed to clarify the possible protective effect of leflunomide on renal ischemia/reperfusion injury in rat.

MATERIALS AND METHODS

Drugs :

Leflunomide (Arthfree tab. EVA pharma.Co.Egypt) is a prodrug that is rapidly converted in the gastrointestinal tract and plasma to its active metabolites A 77 1726. The A 77 1726 was highly bound to plasma protein (> 99%) and had a half life of between 15 and 18 days .The total plasma clearance was 0.3ml/Kg/hr.The majority of A 77 1726 (60-70%) is metabolized in liver and excreted in urine.

Chemicals :

Chemicals for TBARs, Catalase and superoxide dismutase were purchased from Sigma Chemical Co. (St Louis, MO). TNF-α detection kit was purchased from Genzyme, (Cambridge, MA, USA), NO detection chemicals from R&D system Inc. (U.S.A). other chemicals were from analytical grades.

Animals and experimental protocols :

Sprague-Dawley rats weighing 200-250 gm were used. The rats were fed with a standard rat chow and allowed to freely drink water. The rats were anaesthtized with thiopental (50mg/kg intraperitoneally) and the body temperature was kept at 36-38°C by placing the rats under light source. The abdominal region was

shaved with a safety razor and sterilized with povidine iodine solution. A midline incision was made and a right nephrectomy is done after ligating the pedicle. A non-traumatic vascular clamp was applied to the left renal pedicle. The control group (n=10) underwent identical surgical treatment, including isolation of the left renal pedicle but without pedicle occlusion. Ischemia was applied for 45 min to I/R group (n=10) followed by 60 min of reperfusion. The rats in I/R + leflunomide (I/R + lefl) group (n=10) were pretreated intragastric with leflunomide suspension [10 mg/kg] [28] in physiological saline 60 min before the ischemia induction. The control group and the I/R group received a comparable volume of vehicle physiological saline. At the end of each experimental procedure, the left kidneys were removed and kept frozen at 20°C until analyses. Blood samples were collected and the serum sample was stored at 20°C until detection of blood urea nitrogen (BUN) and serum creatinine as indices of renal injury

Tissue homogenization and Biochemical assay :

The left kidney of each rat was homogenized for all assays. The homogenization was performed in 1:10 (w:v) 0.1 M potassium phosphate buffer (pH 7.4) with tissue tearor homogenizer(cole parmer Chicago II,60698). Renal homogenates were centrifuged at 5000 rpm, and +4!C for 10 min, the supernatant was separated for analysis of the followings.

- Thiobarbituric acid reactive substances (TBARs) level was measured by the modified method of Ohkawa et al [29].
- Nitric oxide level was measured according to the method of Green etal [30].
- Catalase (CAT) activity was measured by Beutler's methods [31].
- Superoxide dismutase (SOD) activity was determined by the method of Winterbourn et al [32].
- TNF-α level was determined using a commercially available ELISA kit.

The result was expressed as nmol/mg tissue for TBARs and NO levels, as U/mg tissue for SOD and CAT activities and as pmol/mg tissue for TNF-α level. Serum samples were analyzed for creatinine level by the method of Jaffe [33] and blood urea nitrogen by the method of Patton and Crouch [34].

STATISTICAL ANALYSIS

Results are expressed as means ± standard deviation of mean. For statistical analysis, the non-parametrical Mann-Whitney U test was used. A p-value of less than 0.05 was considered statistically significant.

RESULTS

BUN and creatinine levels in the I/R group were significantly higher than in control rats ($p<0.001$) table (1). When leflunomide was administered before I/R, BUN levels were still significantly higher than control group, but the elevation in both BUN and creatinine were significantly lower in comparison to I/R group alone ($p<0.001$).

TBARS levels and TNF-α levels were significantly higher in the I/R group than those of the control group [Table 2, Fig 1]. Pretreatment with leflunomide in the I/R + leflunomide group, significantly lower than these levels in comparison to I/R group alone, ($p<0.001$, $p<0.001$) respectively. Nitric oxide level was significantly increased in I/R group in comparison to control group .Pretreatment with leflunomide decreased the level of NO in comparison to I/R group ($p<0.01$) [Table 2]. CAT and SOD activities significantly decrease in I/R group when compared to the control group [Table 3 ,Fig 2,3]. Leflunomide treatment increased the CAT and SOD enzyme activities in comparison to the I/R group ($p<0.001$, $p<0.05$) respectively.

Table 1: ***Serum creatinine and blood urea nitrogen(BUN) levels in control and studied groups .(Mean ±SD).***

Groups	Creatinine (mg/dl)	BUN (mg/dl)
Control	0.85±0.03	14.27±0.24
I/R group	1.61±0.03	27.60±0.62
P_1	<0.001	<0.001
I/R +leflunomide	0.93±0.15	18.05±0.91
P_2	<0.05	<0.01
P_3	<0.001	<0.001

P_1: statistical significance between control group and saline treated ischemia-reperfusion(I/R) group.
P_2: statistical significance between control group and Leflunomide- treated ischemia-reperfusion(I/R) group.
P_3: statistical significance between saline treated ischemia- reperfusion(I/R) group. and Leflunomide- treated ischemia- reperfusion(I/R) group.

Table 2: ***Thiobarbituric acid reactive substance(TBARs),Tumour necrosis factor alpha(TNF-α) and Nitric oxide (NOx) levels in control and studied groups .(Mean ±SD).***

Groups	TBARs (nmol/mg tissue)	TNF-α (Pmol/mg tissue)	NOx (nmol/mg tissue)
Control	0.459±0.111	39±13.88	2.53±0.928
I/R group	0.647±0.117	53.8±6.03	4.49±0.989
P_1	<0.001	<0.001	<0.001
I/R +leflunomide	0.490±0.120	17.5±6.04	3.53±0.948
P_2	<0.05	<0.001	<0.001
P_3	<0.001	<0.001	<0. 01

P_1: statistical significance between control group and saline treated ischemia-reperfusion(I/R) group.P_2: statistical significance between control group and Leflunomide- treated ischemia- reperfusion(I/R) group.
P_3: statistical significance between saline treated ischemia- reperfusion(I/R) group. and Leflunomide- treated ischemia- reperfusion(I/R) group.

Table 3: ***Catalase and superoxide dismutase(SOD) levels in control and studied groups .(Mean ±SD).***

Groups	Catalase (U/mg tissue)	SOD (U/mg tissue)
Control	2276±286.9	8.84±0.736
I/R group	1800±150.92	7.14±0.59
P_1	<0.001	<0.05
I/R +leflunomide	2190±96.69	8.82±0.749
P_2	NS	NS
P_3	<0.001	<0.01

P_1: statistical significance between control group and saline treated ischemia-reperfusion(I/R) group.

P_2: statistical significance between control group and Leflunomide- treated ischemia-reperfusion(I/R) group.

P_3: statistical significance between saline treated ischemia- reperfusion(I/R) group and Leflunomide- treated ischemia- reperfusion(I/R) group.

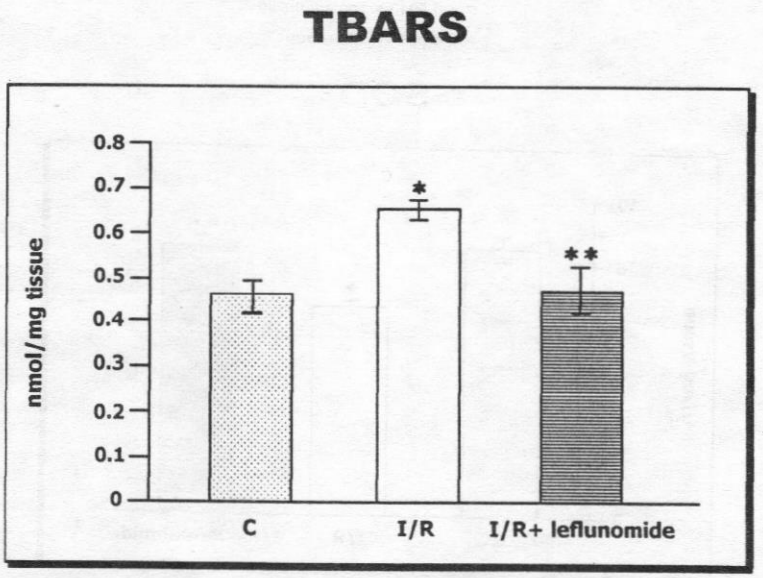

Fig. 1: Renal tissue TBARS levels in control rats, in rats after 45 min ischemia and 60 min reperfusion (I/R) and in I/R rats pretreated with leflunomide (10 mg/kg) (I/R+ leflunomide)

*** P < 0.001 vs. control group**

**** P < 0.001 vs. I/R group.**

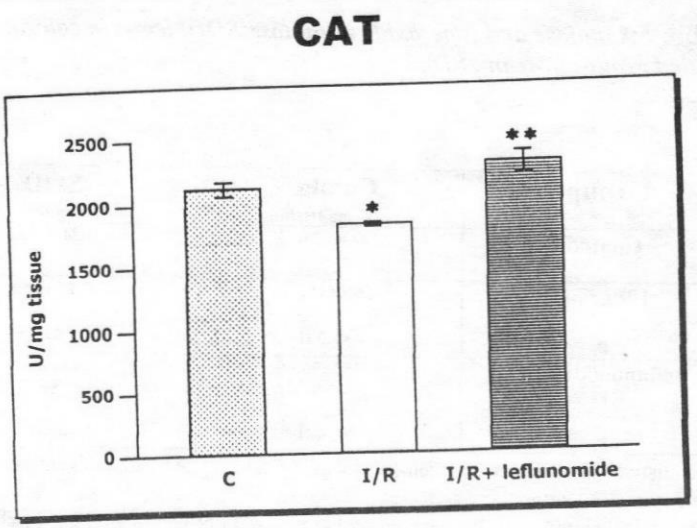

Fig. 2: Renal tissue CAT activities in control rats, in rats after 45 min ischemia and 60 min reperfusion (I/R) and in I/R rats pretreated with leflunomide (10 mg/kg) (I/R+ leflunomide)

* P < 0.001 vs. control group

** P < 0.001 vs. I/R group.

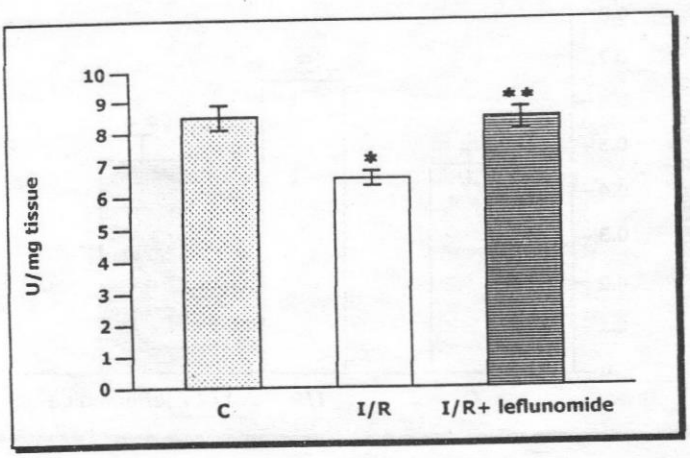

Fig. 3: Renal tissue SOD activities in control rats, in rats after 45 min ischemia and 60 min reperfusion (I/R) and in I/R rats pretreated with leflunomide (10 mg/kg) (I/R+ leflunomide)

* P < 0.05 vs. control group

** P < 0.01 vs. I/R group.

DISCUSSION

Lipid peroxidation, as a free radical generating system, may be closely related to I/R induced tissue damage, and TBARs is a good indicator of the degree of lipid peroxidation[35]. In the present study, the levels of TBARs were significantly increased by I/R. This observation is in agreement with previous studies, in which levels of lipid peroxidation products were increased from 40 to 100% from baseline [36- 37]. Our results show that leflunomide causes significant inhibition of TBARs production probably in part by scavenging the very reactive hydroxyl($OH^{\cdot}$) and superoxide anion ($ROO^{\cdot}$) radicals indicating a reduction in lipid peroxidation and cellular injury.

Superoxide radicals formed by I/R injury are converted into H_2O_2, either spontaneously (in pH 4.8) or by dismutation with the SOD enzyme (especially, in neutral and alkaline pH). H_2O_2 is then converted to H_2O by either CAT or glutathione peroxidase. It has been reported that SOD activity was reduced after I/R injury [38, 39]. Dobashi et al [40] also demonstrated mRNA levels of CAT significantly decreased after I/R. In our study SOD and CAT activities were found to be significantly decreased in the I/R group when compared to the control group. The decrease in renal SOD and CAT activities is probably the result of the inactivation by ROS produced by I/R. leflunomide treatment increased levels of these enzymes in comparison with the I/R group. The increase in the SOD and CAT activities is possibly due to the scavenging of ROS, i.e. $\cdot O2$ and $\cdot OH$ by leflunomide.

Induction of iNOS under inflammatory conditions leads to the production of large amounts of NO for longer periods of time. The toxic effects of NO may be attributed to peroxynitrite (ONOO-) which is a reaction product of NO with $\cdot O_2$. NO has also been seen to inactivate the antioxidant enzymes glutathione peroxidase [41] and catalase [42]. Alterations in NO synthesis have been implicated in pathophysiological changes of ischemia/reperfusion injury in several key organs [43, 44]. For example, nephrotoxicity [45, 46] or neurotoxicity [47]. An animal model of kidney ischemia and brain focal ischemia is mediated at least in part by NO, since this toxicity is blocked by antisense to iNOS and inhibitors of NOS respectively. Several groups have reported that

iNOS is the injurious isoform involved in ischemic and endotoxin-induced acute renal failure, based on in vivo studies in both rats and mice [48, 49]. In a recent study, kidneys of iNOS knockout mice have been protected against ischemic acute renal failure [50], where treatment with the iNOS inhibitor has improved renal function and has decreased apparent OONO- formation. OONO- is believed to be responsible for the shedding of proximal tubule cells seen in ischemic sections [51].

Previous studies have shown that proinflammatory cytokines play a key role in ischemia/reperfusion injury. Ischemia/reperfusion-induced renal TNF-α expression may result in renal cell injury by at least two distinct mechanisms: (i) direct cytotoxicity (induction of dysfunction and/or apoptosis) [52] and (ii) neutrophil mediated tissue injury [53]. In a recent study, ischemia/reperfusion-induced renal TNF-α production has been found to be associated with impaired renal function and anti- TNF-α treatment results in diminished histologically evident damage and improved renal function [54].

Our data demonstrated that leflunomide plays an important role in the attenuation of I/R-induced renal injury by decreasing TBARs, TNF-α levels and NO activities and by increasing SOD and CAT activities. These results are in agreement with others (28) who demonstrated the protective effects of leflunomide in hepatic I/R injury by decreasing levels of TBARs, nitric oxide and TNF-α and by increasing activity of catalase and SOD respectively. Also Jankovic reported that A771726, leflunomide's active metabolite, also had inhibitory effect on NO production and iNOS mRNA expression in interferon gamma (IFN-γ) + lipopolysaccharide (LPS)-activated murine and rat primary fibroblast [55-56]. Furthermore, it was found that leflunomide lower significantly the TBARs content as well as the generation of of NO and TNF-α in liver homogenate in mice[57]. Also others [58] demonstrated that leflunomide within the therapeutic range causes a dose-dependent reduction of production of IL-1, TNF-α and NO in synovial tissue culture media.

The mechanism of the immunomodulatory and anti-inflammatory actions of Leflunomide, is mainly due to inhibition of the activity of dihydroorotate dehydrogenase (DHODH) in-

volved in de novo pyrimidine biosynthesis. Also, at a higher concentration, it mainly inhibited protein tyrosine kinases initiating signaling [59-62], and therefore could reduce the cell response to mitogen and cytokine. Other investigators found that leflunomide exerts its action via inhibition of nuclear factor kappa (NF-KB) activation in T cell and other cells by suppression of the MAPK activated by TNF-α [63-64].

Finally, these results indicate that the renoprotective effects of leflunomide in the renal injury induced by I/R could be related to its antioxidant properties, which reduce the lipid peroxidation and increase SOD activity, CAT activity, and anti-inflammatory properties by reducing level of TNF-α Therefore, leflunomide can have a role as renoprotective against ischemia- reperfusion injury .

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التأثير الوقائى لعقار الليفلوناميد على القصور المؤقت فى الدورة الدموية الكلوية للفئران

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قصور الدورة الدموية الكلوية المؤقت له أهمية إكلينيكية لدوره فى احداث الفشل الكلوى ورفض أنسجة الكلى ولذلك هدفت هذه الدراسه إلى بحث التأثير الوقائى لعقار الليفلوناميد على حماية الأنسجه ضد قصور الدورة الدموية المؤقت بكلية الفئران. وقد أجريت هذه الدراسة على ثلاث مجموعات من الفئران، المجموعة الأولى، المجموعة الضابطه، والثانية مجموعة قصور الدوره الدموية المؤقت والأخيرة مجموعة قصور الدوره الدمويه + الليفلوناميد. أحدث قصور الدوره الدموية المؤقت بواسطة غلق العنق الكلوى الأيسر لمدة ٤٥ دقيقة ثم فكه لمدة ٦٠ دقيقه مع استئصال الكلية اليمنى. تم اعطاء فئران المجموعة الثالثة عقار الليفوناميد ٥٠مجم/كجم فى المعدة بواسطة أنبوبة معدية ٦٠ دقيقة قبل إحداث القصور المؤقت. تم قياس كل من معامل نخر الورم ألفا، الشقوق الحرة، أكسيد النيتريك، إنزيم الكاتاليز وانزيم السوبر أوكسيد الدسميوتيز فى أنسجة الكلى وكذلك نسبة الكرياتينين واليوريا فى الدم. وقد أظهرت النتائج زيادة ذات دلالة إحصائية فى مستوى معامل نخر الورم ألفا، الشقوق الحره وأكسيد النيتريك وكذلك نسبة الكرياتينين واليوريا فى الدم فى مجموعة قصور الدورة الدموية عن المجموعة الضابطة وإعطاء عقار الليفلوناميد يؤدى إلى إنخفاض مستوى هذه العناصر.

انخفض نشاط إنزيم الكاتاليز وانزيم السوبر أوكسيد الدسميوتيز بعد إحداث القصور المؤقت عن المجموعة الضابطة. العلاج بعقار الليفلوناميد أدى إلى زيادة نشاط هذه الإنزيمات. ولذلك فإن العلاج بعقار الليفلوناميد ذو فاعلية فى حماية الكلى من قصور الدورة الدموية المؤقت .