RENOPROTECTIVE EFFECT OF LEFLUNOMIDE 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
Identification of drugs that downregulate 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 anaesthetized 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
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 3: Catalase and superoxide dismutase (SOD) levels in control and studied groups. (Mean ±SD).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase (U/mg tissue)</th>
<th>SOD (U/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2276±286.9</td>
<td>8.84±0.736</td>
</tr>
<tr>
<td>I/R group</td>
<td>1800±150.92</td>
<td>7.14±0.59</td>
</tr>
<tr>
<td>P1</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>I/R +leflunomide</td>
<td>2190±96.69</td>
<td>8.82±0.749</td>
</tr>
<tr>
<td>P2</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P3</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

P1: statistical significance between control group and saline treated ischemia-reperfusion (I/R) group.
P2: statistical significance between control group and Leflunomide-treated ischemia-reperfusion (I/R) group.
P3: statistical significance between saline treated ischemia-reperfusion (I/R) group and Leflunomide-treated ischemia-reperfusion (I/R) group.

**TBARS**

![TBARS Graph](image)

*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.**

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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(\( \cdot \)OH) and superoxide anion (\( \cdot \)O\(_2^\cdot \)) radicals indicating a reduction in lipid peroxidation and cellular injury.

Superoxide radicals formed by I/R injury are converted into \( \text{H}_2\text{O}_2 \), either spontaneously (in pH 4.8) or by dismutation with the SOD enzyme (especially, in neutral and alkaline pH). \( \text{H}_2\text{O}_2 \) is then converted to \( \text{H}_2\text{O} \) 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. \( \cdot \)O\(_2\) 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
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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