Protective Effect of Etanercept in Experimentally Generated Acute Kidney Failure

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ABSTRACT

Objective: Acute renal failure (ARF) is a clinical syndrome that causes a decrease in the glomerular filtration rate and leads to an accumulation of nitrogenous waste products and uremic toxins. This study investigated the protective effect of Etanercept on ARF.

Materials and Methods: Experiments were done on Balb-C female mice. Acute renal failure was created with an intraperitoneal folic acid injection. Etanercept was injected with folic acid in doses of 0.7 mg/kg, 3.5 mg/kg, and 7 mg/kg in treatment groups. Blood urea nitrogen levels, creatinine and cystatin-c were measured on the 7th day of the study. The kidneys were examined histologically with hematoxylin-eosin staining and Tunel assay.

Results: Etanercept improved kidney morphology damaged by folic acid. The apoptotic index was low, and there was less dilation in the renal tubules in the groups treated with Etanercept. Folic acid increased blood urea nitrogen levels. This increase was decreased gradually with increased doses of Etanercept. There were no differences in creatinine levels in all groups.

Conclusion: This study investigated the protective effects of Etanercept in the context of Acute Renal Failure (ARF). The results demonstrated that Etanercept improved kidney morphology damaged by folic acid, reduced the apoptotic index, and gradually decreased elevated blood urea nitrogen levels. Although creatinine levels showed no significant differences among the treatment groups, the findings suggest that Etanercept may hold promise as a potential agent for the treatment of ARF. Further research and clinical studies are warranted to validate its effectiveness and safety in human subjects.

Keywords: Acute renal failure; Etanercept; Folic acid; TNF-α; TNFR1; Nephropathy

INTRODUCTION

Acute renal failure (ARF) occurs when kidneys suddenly become unable to filter waste products from blood. ARF is a syndrome characterized by an acute decrease of glomerular filtration rate (GFR) in days and often in hours. Glomerular filtration is the process by which the kidneys filter the blood, removing excess wastes and fluids. GFR is a calculation that determines how well the blood is filtered by the kidneys, which is one way to measure remaining kidney function. Rapid decrease of GFR causes accumulation of nitrogen breakdown products in the blood and disturbances in fluid-electrolytes and acid-base balance [1, 2]. Acute kidney injury includes diseases, such as interstitial nephritis, which decreases kidney function by accumulating immune complexes in the interstitium, and acute tubular damage due to renal ischemia [3].

ARF has high morbidity and mortality rates. It is thought to develop in 20-25% of patients in intensive care units and 5% of all hospitalized patients [4]. Beside the morbidity associated with ARF, heavy financial burden is added.
Inflammatory events begin as a response to acute kidney damage. Cytokines and chemokines are secreted and restorative processes and profibritic cells are activated. Tumor Necrosis Factor-α (TNF-α) is one of these cytokines. Etanercept is a recombinant human receptor fusion protein that competitively suppresses the interaction of TNF-α with cell surface receptors. It blocks TNF-α mediated cellular response and regulates the activities of other proinflammatory cytokines affected by TNF-α [5]. Etanercept has been used in inflammatory diseases such as rheumatoid arthritis, ankylosing spondylitis, juvenile idiopathic arthritis, psoriasis and psoriatic arthritis [6].

In an experimental study, TNF-α inhibition was shown to have a protective effect against ARF. An experimental TNF-α antibody was used in that study [7]. The fact that Etanercept is a clinically used drug increases its importance. Therefore, in this present study, the protective effect of Etanercept against ARF was investigated.

MATERIAL and METHODS

91 Balb-C adult female mice were used. The animals were divided into 7 groups, randomly (Table 1).

Creation of Acute Renal Failure

The groups are characterized in Table 1. Folic acid (with 0.3 mM NaHCO3, in the volume of 0.2 mL) was administered intraperitoneally to the mice in Group 1, 2, 3, 4, and 5. After thirty minutes following folic acid injection, Etanercept was administered in the doses of 0.7 mg / kg, 3.5 mg / kg, and 7 mg / kg doses to Group 2, 3, and 4, respectively. A dissolved solution of Etanercept (Enbrel®, Pfizer Medicines) was administered intraperitoneally to mice in Group 5 thirty minutes after the folic acid injection.

The animals were followed for 7 days and given free water and food. Weight measurements were taken on the first two days and the last three days. On the 7th day of the study, 50 mg / kg Ketamine and 5mg / kg Xylazine intraperitoneal were administered. Afterwards, the blood was removed intracardially for sacrifice.

Blood Urea Nitrogen Measurement in Serum Sample

Blood urea nitrogen (BUN) measurement was performed on the first day after sacrifice. BUN Enzymatic Kit Manual (Catalog Number: 5602-01) from Bio Scientific Corporation (3913 Todd Lane Suite 312 Austin, TX 78744 United States) was used for BUN measurement.

Creatinine Measurement in Serum Sample

Creatinine measurement was done on the 3rd day after sacrifice using the Creatinine Assay Kit (Catalog Number: ab65340) supplied by Abcam.

Cystatin-c Measurement in Serum Sample

Cystatin-c measurement was performed on the 2nd day after sacrficence. Cystatin-c measurements were made using the Cystatin C Mouse ELISA Kit (Catalog Number: ab119590) obtained from Abcam.

Histological Follow up

At the end of the experiment, the mice were sacrificed with intraperitoneal ketamine anesthesia and the right kidney tissues were removed. Tissues were fixed in 10% neutral formalin. Tissues were then dehydrated by passing them through a graded alcohol series. Tissues that were made transparent with xylol were embedded in paraffin. 5-6 µm sections were stained with Hematoxylin-Eosin (HE) to evaluate the structures and photos were obtained with an Olympus BX51 photomicroscope.

Tunel Assay

The Tunel assay was used to show apoptosis in the kidney tissue. An in situ Cell Death Detection kit from Roche was used. After the 5-6 µm thick slides of kidney tissues were deparaffinized and rehydrated, they were washed with phosphate buffered saline (PBS). For antigen recovery, they were kept in a microwave oven in 0.01 M sodium citrate buffer at 350 W for 5 minutes and then left to cool at room temperature for 20 minutes. Tissues were washed with PBS and incubated with the Tunel reaction mixture of the kit for 60 minutes at 37°C in a wet and dark environment. 4’, 6-diamidino-2-phenylindole was used for the contrasting dye.

An apoptotic index was created by counting the apoptotic cells in the tissues. For the apoptotic index, images were taken from 10 different areas at 40X magnification from each section.

Statistical Analysis: Data are expressed as mean ± standard error (x ± SH). The comparison between the groups was done by one way ANOVA. The Tukey procedure was used for multiple comparison (Post-hoc) test. Significant value was determined as p <0.05.

<table>
<thead>
<tr>
<th>Groups</th>
<th># of animals</th>
<th>Characteristics of groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>ARF group: 250 mg/kg dose of folic acid</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>ARF+ 0.7 mg etanercept group: 250 mg/kg folic acid and 0.7 mg/kg etanercept</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>ARF+3.5 mg etanercept group: 250 mg/kg folic acid and 3.5mg/kg etanercept</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>ARF+7 mg etanercept group: 250mg/kg folic acid and 7 mg/kg etanercept</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>ARF + adjuvant group: 250 mg / kg folic acid and mannitol (e-421) 40 mg/ml, sucrose 10 mg/ml, trometamol 1.2 mg/ml, tromethamine hcl 0.2 ml (to adjust pH).</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Control group</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>Etanercept at a dose of 7 mg / kg</td>
</tr>
</tbody>
</table>
RESULTS

An experimental ARF model was created in seventy-five mice, in Groups 1-5. Before the 7th day, thirty-four mice died because of complications. The number of deaths in each group is shown in Table 2.

The weight difference between the 7th and 0th days of the study was compared between the groups (Table 3). Statistical comparison of BUN, creatinine and cystatin-c values is shown in Table 4.

Table 2: Mortality Rates

<table>
<thead>
<tr>
<th>Groups</th>
<th># of mice before folic acid administration</th>
<th>Deaths</th>
<th>% Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>7</td>
<td>46.6</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>8</td>
<td>53.3</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>8</td>
<td>53.3</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>8</td>
<td>53.3</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3: Weight Differences

<table>
<thead>
<tr>
<th>Groups</th>
<th>Difference between the 7th and 0th days (in grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-4.36 ± 0.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>-2.61 ± 1.26&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>-2.79 ± 0.99&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>-0.43 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>-4.31 ± 1.14&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>0.65 ± 0.41&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>1.03 ± 0.40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as standard error (SH) with arithmetic mean. According to the multiple comparison test (Tukey HSD), the same character indicate that the difference between the groups is not significant and different character are significant (p < 0.05).

Table 4: Statistical comparison of BUN and creatinine values

<table>
<thead>
<tr>
<th>Groups</th>
<th>BUN (mg/dL)</th>
<th>Kreatinin (mg/dL)</th>
<th>Sistatin-C (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr 1</td>
<td>n=7</td>
<td>53.36±14.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.18±0.12</td>
</tr>
<tr>
<td>Gr 2</td>
<td>n=6</td>
<td>24.17±4.67&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>1.10±0.06</td>
</tr>
<tr>
<td>Gr 3</td>
<td>n=4</td>
<td>28.0±7.31&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>1.30±0.14</td>
</tr>
<tr>
<td>Gr 4</td>
<td>n=11</td>
<td>24.85±2.89&lt;sup&gt;def&lt;/sup&gt;</td>
<td>1.10±0.03</td>
</tr>
<tr>
<td>Gr 5</td>
<td>n=7</td>
<td>36.28±9.39&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.99±0.05</td>
</tr>
<tr>
<td>Gr 6</td>
<td>n=6</td>
<td>15.90±0.61&lt;sup&gt;cdef&lt;/sup&gt;</td>
<td>1.04±0.03</td>
</tr>
<tr>
<td>Gr 7</td>
<td>n=10</td>
<td>16.57±0.44&lt;sup&gt;cdef&lt;/sup&gt;</td>
<td>1.02±0.03</td>
</tr>
</tbody>
</table>

P 0.006 0.58 0.012

Data are expressed as standard error (SH) with arithmetic mean. According to the multiple comparison test (Tukey HSD), the same character indicate that the difference between Groups is not significant and different character are significant (p < 0.05).
**Histological Findings:** In light microscopic examination, glomeruli and tubules were observed in the cortex in the kidney sections of Group 6 (Figure 1-A) and Group 7 (Figure 1-B). Dilated tubules, areas of mononuclear cell infiltration, and congestion in the vessels were found quite commonly in the cortex layer in the sections of Group 1 (Figure 1-C). In some areas belonging to Group 1, epithelial cells were poured into the tubule and hyaline material accumulation was detected (Figure 1-D). As in Group 1, dilated tubules, areas of mononuclear cell infiltration, congestion, and hyaline substance accumulation were detected in the kidney tissues of Group 5 (Figure 1-E).

In Group 2, there was a decrease in the dilated tubules and mononuclear cell infiltration areas in the cortex layer of the kidney tissue belonging to four subjects, while histological findings did not change in three subjects (Figure 1-F).

While partial improvement was observed in four subjects belonging to Group 3, dilated tubules and mononuclear cell infiltration areas were preserved in three subjects (Figure 1-G). In Group 4, nine subjects had improvement, while three subjects had dilated tubules and mononuclear cell infiltration areas (Figure 1-H).

**Findings of Apoptosis:** Tunel assay was performed to determine apoptotic cells. An apoptotic index was made by counting Tunel positive cells. In group 1, it was determined that apoptotic cells increased statistically and significantly compared to group 6. It was determined that the number of apoptotic cells in groups 2, 3, and 4 were decreased significantly compared to group 1 after the ARF model was created (Figure 2) and apoptotic index shown in table 5.

![Image](http://dx.doi.org/10.36472/msd.v10i10.1066)

**Table 5:** Apoptotic Index in Kidney Tissues

<table>
<thead>
<tr>
<th></th>
<th>Gr 1</th>
<th>Gr 2</th>
<th>Gr 3</th>
<th>Gr 4</th>
<th>Gr 5</th>
<th>Gr 6</th>
<th>Gr 7</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tunel</td>
<td>3.10 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.20 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.55 ± 0.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.80 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.11 ± 0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Data are expressed as standard error (SH) with arithmetic mean. According to the multiple comparison test (Tukey HSD), the same character indicate that the difference between the groups is not significant, and the different character are meaningful.
DISCUSSION

In this study, Etanercept was shown to have a positive effect on histopathological and biochemical changes in an ARF model created by folic acid. The presence of ARF in mice was determined by histological techniques and an increase in BUN level in accordance with studies in literature using the same ARF model [8] [9]. In a study by Yuan et al., an ARF model created with folic acid was shown to be associated with apoptosis and tubular necrosis developing following folic acid crystals that rapidly appeared in the kidney tubules. In the same study, epithelial regeneration and cortical scar tissue was shown with tubular necrosis [10].

In this present study, some animals in the groups with ARF died due to various complications. The mortality rates seen in these groups were similar to the mortality rates of a study by Wan et al., in which the same ARF model was used as this present study [11]. There were no deaths in other groups without ARF. Therefore, it can be assumed that deaths were due to complications developing in the background of ARF. Similarly, in groups without ARF, weight loss was significantly lower. Weight loss was also significantly lower in rats with increasing doses of Etanercept. These findings suggest that the severity of ARF decreases with increasing doses of Etanercept.

BUN measurement is a biochemical test used especially in the evaluation of kidney functions. Elevated levels of BUN are detected in cases of acute and chronic renal failure. In this study, Etanercept caused a decrease in BUN values at all 3 doses, significantly at 0.7mg / kg and 7mg / kg. The absence of a significant difference with the 3.5 mg / kg dose may be attributed to the decrease in the number of samples due to deaths of mice. The fact that the average BUN value determined in the 3.5 mg / kg dose group is close to half of the value in the ARF group supports this suggestion. Therefore, the detected BUN values suggest that Etanercept may have a protective effect on the development of ARF.

In this study, no significant difference was observed between the creatinine values. The reason of non-elevation of creatinine may be the time of taking the blood sample, which was on the 7th day of ARF. In a study with the same ARF model, the creatinine values increased on the 2nd day and reached normal limits on the 6th day [9].

As a result of the cystatin-c measurements made in this study, no significant difference was found between the groups except Group 5 and Group 3. The reason for this may be that serum values tend to improve after the 3rd day.

Immune system signaling pathways that cause cell damage are activated as a result of exposure of kidney tubules to endogenous or exogenous nephrotoxic substances. Apoptosis occurs because of processes involving the Caspase (cysteine aspartate specific protease) enzyme system. The subsequent strong inflammatory response leads to worsening of kidney tissue damage. Apoptosis and necrosis cause the development of ARF [12]. TNF-α induces apoptotic cell death and inflammation [13] [14]. TNF-β is a cytotoxic protein with TNF-α-like biological activity and is secreted from T-lymphocytes. TNF-α and TNF-β have 30% amino acid homology [15]. TNF-α specifically activates the apoptosis and Nuclear Factor Kappa-B signal pathway [16]. It produces a large number of interleukins and chemokines, especially the inflammatory cytokines, IL1, IL6, TNF-α (which has a self-enhancing effect), and granulocyte macrophage colony stimulating factor (GM-CSF) [17] [18]. It acts on TNF-α, Tumor Necrosis Factor Receptor (TNFR-1), and TNFR-2. After connecting ligands in TNFR-1, the activation of the Caspase-8 signal pathway occurs. Activated Caspase-8 further activates the pro-caspase-3 molecules and the apoptotic process is initiated. Signals emitted by TNFR-1 cause cell death from Caspase 8. As a result of the connection of TNF-α to TNFR2, a different pathway is opened from the signal generated over TNFR-1. The proteins that bind first to ligand-bound TNFR-2 are the TRAF1/2 (TNF-Receptor Associating Factor) complex. As a result of this signal pathway, Nuclear Factor Kappa-B becomes active. TNFR-2 also increases apoptosis formation and severity [19] [20].

The most common causes of ARF are acute tubular necrosis and there is apoptosis and the death of tubule cells. Etanercept inhibits the interaction of TNF-α and TNF-β with cell surface receptors. TNF-α reduces the expression of iNOS and caspase-3, preventing apoptosis and cell death. In this study, the Tunel assay, which is more sensitive in detecting apoptosis, was used. DNA fragmentation is demonstrated by Tunel assay. The free 3'-OH ends of the DNA particles are labeled with diamino benzidine. The amount of apoptosis is determined by counting the marked areas. The quality of Tunel assay images detected in this study was compatible with studies using the same technique [7] [8]. In this study, a significant decrease in apoptosis in the treatment groups indicates the protective effect of Etanercept. It is also noteworthy that there were significant dose-dependent improvements in the treatment groups. In addition to the decrease in the number of apoptosis, there were improvements in the histological view of the glomeruli and tubules with increasing doses of Etanercept. Wang et al. inhibited TNF-α using a neutralizing antibody in mice with ARF. The authors suggested that this inhibition was effective in preventing ARF, TNF-α level increased in the blood within 48 hours following folic acid administration, TNF-α levels decreased, and apoptosis was prevented as a result of antibody administration [7].

Choi et al. reported that Etanercept had a protective effect in rats with kidney damage in an ischemia reperfusion model. In this study, kidney failure of a transplantation model with ischemia reperfusion injury was established and the BUN creatinine ratio was lower in the groups that received Etanercept after the first 24 hours. In addition, TNF-α and apoptotic cell numbers decreased [21].

Suppression of the developing inflammatory response may have both a protective and a progression effect. Studies have shown that TNF-α levels start to rise rapidly in the first hours after tissue damage [22] [23]. Therefore, etanercept was applied within the first hour following the creation of the ARF model in this present study. Etanercept is administered subcutaneously in the treatment of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, and psoriasis at doses of 2x25 mg twice a week or 1x50 mg once a week [6].

In routine use, the dose of Etanercept is 0.7 mg / kg. In this study, 0.7, 3.5, and 7 mg / kg etanercept were used and the findings showed significant improvements beginning with the dose of 0.7 mg / kg. On the other hand there was no sign of

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toxicity with etanercept, there was no significant difference between the group given Etanercept without ARF and the control group in terms of the number of apoptosis, BUN, creatinine levels, and weight changes. Supporting this opinion, in a study of patients with focal cerebral ischemia, higher doses of Etanercept were used and no significant toxic effect was observed [24].

CONCLUSION

The findings of this study indicates that Etanercept is effective in preventing the development of ARF. Additionally, no nephrotoxic effects was observed. Based on these findings, a clinical study can be recommended to investigate the use of Etanercept in patients at risk of developing ARF.

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Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions: MK, HBU, MFS; Study, Design, Data Collection and/or Processing, Analysis and/or Interpretation MK; Writing, and Revisions

Ethical approval: The present study was conducted in strict accordance with the principles outlined in the Declaration of Helsinki. Ethical approval for the study was obtained from the appropriate ethics committee, and all participants provided informed consent before participating in the study. The local ethics committee approved this research (11.06.2014, 14/105).

REFERENCES