Study of the Nephroprotective Properties of the Erythropoietin Mimetic Peptide and Infliximab in Kidney Ischemia-Reperfusion Injury in Rats

Aleksandr S. Netrebenko¹ *, V. Gureev, V¹, V. Pokrovskii, M¹, V. Gureeva, A¹, M. Tsuverkalova, Y¹, S. Rozhkov, I¹

1. Belgorod State University, 85 Pobeda St., Belgorod, 308015, Russia
Corresponding Author: aleksandr.2t@yahoo.com

Abstract
Chronic kidney disease (CKD) or acute kidney injury (AKI) causes impaired kidney function, leading to cognitive impairment, neuropathy, and cerebrovascular disease. Due to kidney damage, toxins from the blood are not able to leave the body and affect brain function through kidney-brain interaction. This study was performed to investigate the protective effects of the fungal peptide erythropoietin (pHBSP) and infliximab on ischemic renal reperfusion injury. The experiment was performed on 70 white male Wistar laboratory rats. Animals were administered with recombinant erythropoietin, erythropoietin mimetic peptide (pHBSP), and infliximab. Under anesthesia, traumatic vascular clamps were applied to the left renal pedicle for 40 minutes, and a nephrectomy was performed on the right. Functional tests and laboratory tests were performed 5 minutes and 24 hours after the reperfusion. Twenty-four hours after the surgery, the plasma creatinine and urea levels in the sham-operated animals were 45.9±0.8 mmol/L and 6.7±0.2 mmol/L, respectively. Plasma creatinine and urea levels in the control group animals were 102.63±3.6 mmol/L and 21.80±1.29 mmol/L, respectively. The administration of erythropoietin mimetic peptide (pHBSP) and infliximab to the animals with ischemia-reperfusion kidney injury has a pronounced nephroprotective effect comparable to erythropoietin. There was a significant decrease in blood levels of creatinine and urea, improvement of microcirculation in the kidney, normalization of glomerular filtration rate, and fractional sodium excretion. The results of the study show the prospects of erythropoietin mimetic peptide (pHBSP) and infliximab administration in ischemia-reperfusion kidney injury and justify the feasibility of further research in this direction.

Keywords: Erythropoietin mimetic peptide (pHBSP), Infliximab, Ischemia-reperfusion kidney injury, Rats, Microcirculation
1. Introduction

Ischemia/reperfusion injury (IRI) is caused by the limited blood supply to an organ, followed by blood flow and re-oxygenation. This complication can occur after stroke, sepsis, and organ transplantation and these phenomena lead to the formation of inflammatory cascades. IRI, a clinical syndrome with rapid renal dysfunction, contributes to a pathological condition called acute kidney injury (AKI) and increases mortality (1, 2). Activation of neutrophils, the release of reactive oxygen species, and other inflammatory mediators, including adhesion molecules and cytokines (3, 4), are affected by the pathophysiology of IRI in the kidney. Decreased levels of pro-inflammatory cytokines due to doxycycline, decreased levels of tumor necrosis factor-alpha (TNF-α) using leptin, increased levels of nitrite through antioxidants were caused by fighting IRI (5-7).

The search for new drugs with cytoprotective activity based on antioxidant properties or having anti-inflammatory properties is an urgent task of modern pharmacology (8-12). Kidney surgery is one of the areas of their practical application. To date, morbidity in oncurology remains extremely high. According to Russian Federal Service of State Statistics, from 2010 to 2018 there was an increase in the morbidity of kidney cancer from 18.7 to 24.3 thousand per year. In part, this is a consequence of early diagnosis and restoration of the prophylactic medical examination service. According to current trends, at the initial stages of the disease it is considered optimal to perform organ-preserving surgery (kidney resection), most often performed under warm ischemia. Given this fact, one of the main tasks for doctors and scientists around the world is to prevent the development of acute renal injury (ARI) caused by compression of the renal vessels (13).

The main pathogenetic link of acute renal injury is ischemia and reperfusion injury of the kidneys (14). Ischemia is a pathological state characterized by the reduced blood supply to the organ, which leads to a decrease in oxygen and nutrients in tissues and organs, as well as a decrease in the excretion of metabolic products. This is followed by reperfusion with repeated oxygenation. Pharmacological preconditioning is one of the promising mechanisms for prevention of ischemia and the reperfusion injuries (15-17).

In several major studies, it was proved that erythropoietin has precondition properties (18, 19). The biological effects of erythropoietin are realized by binding to specific receptors that are expressed in the bone marrow, vascular endothelium, kidneys, nervous system, placenta, gastric mucosa, and skeletal muscles (20, 21). There are two types of receptors: homodimeric and heterodimeric. Binding to the homodimeric receptor inhibits apoptosis and activates erythropoiesis (22). Cytoprotective
effects of erythropoietin are caused by activation of the heterodimeric receptor (23, 24). The realization of these effects is mediated by JAK-2, STAT5, PI3-K, and NFkB (25). However, the affinity of erythropoietin to the homodimeric receptor is significantly higher than to the heterodimeric one (26). Thus, significantly higher systemic doses of erythropoietin than conventional therapeutic doses are required to realize the cytoprotective effect (27). In practice, treatment with high doses of erythropoietin led to an increased risk of thrombotic events in the first year after transplantation (erythropoietin 24.4% compared to placebo 6.4%) (28). To prevent these adverse events, erythropoietin derivatives that activate only the cytoprotective effect and do not affect erythropoiesis have been developed. pHBSP is a synthetic peptide that selectively binds to a heterodimeric receptor. It has already been shown that pHBSP is not erythropoietic (29). In the experiment, we set out to identify and study the nephroprotective effects of erythropoietin mimetic peptide (pHBSP) in ischemia-reperfusion kidney injury.

Pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), play an equally important role in the pathophysiology of kidney ischemia-reperfusion injury (30). Free radicals cause the accumulation of white blood cells in the tissues. Activated neutrophils produce enzymes such as myeloperoxidase and release more free radicals (31). In pharmacology, drugs that reduce the activity of the tumor necrosis factor-alpha are known. Infliximab is one of them (32). It has a high affinity for the tumor necrosis factor-α, as well as decreases the concentration (binds and inhibits the synthesis) of IL-1, IL-6, IL-8, monocyte chemoattractant-1, nitric oxide, metalloproteinases (collagenase, stromelysin), and other inducers of inflammation and tissue destruction.

Thus, this study was performed to investigate neuroprotective effects of the erythropoietin mimetic peptide and infliximab in simulated ischemia-reperfusion kidney injury.

2. Materials and Methods
The experiment was performed in 70 white male Wistar laboratory rats weighing 280-320g. The animals were divided into 7 groups, each with 10 animals.
1. Sham operated animals.
2. Ischemia/reperfusion (I/R) – control.
3. Ischemia/reperfusion + recombinant erythropoietin (EPO) (Epocrine®, FSUE "State Research Institute of Especially Purified Bioproducts" FMBA of Russia at the dose of 50 IU/kg intraperitoneally once 30 minutes before the ischemia simulation.
4. Ischemia/reperfusion + erythropoietin mimetic peptide (pHBSP) at the dose of 5 µg/kg, intraperitoneally 30 minutes before the ischemia simulation.
5. Ischemia/reperfusion + peptide erythropoietin mimetic peptide (pHBSP) at the dose of 25 µg/kg, intraperitoneally 30 minutes before the ischemia simulation.
6. Ischemia/reperfusion + Infliximab (Remicade®, MSDIreland (Brinney) at the dose of 2 mg/kg intraperitoneally 1 hour before the ischemia simulation.
7. Ischemia/reperfusion + Infliximab (Remicade®, MSDIreland (Brinney) at the dose of 10 mg/kg intraperitoneally 1 hour before the ischemia simulation.

Midline laparotomy was performed under anesthesia (chloral hydrate, 300 mg/kg intraperitoneally) after preoperative showering. Next, the loops of the intestine were mobilized, the kidneys with elements of the renal pedicle were isolated. Atraumatic vascular clamps were applied to the left renal pedicle for 40 minutes. The effectiveness of the ischemia was assessed by changing the kidney color. Right nephrectomy was performed.

The level of microcirculation in the cortex of kidneys was recorded using the MP100 hardware and software complex (BiopacSystem, Inc., USA) with the laser doppler flowmetry (LDF) module LDF100C and the TSD143 surface sensor, which was applied to the middle part of the kidney without affecting the hilum of kidney. The estimation of microcirculation was performed 5 minutes after removing the clamps from the vascular pedicle. The results were recorded and processed using the AcqKnowledge software version 3.8.1. The values were expressed in perfusion units (PU). Next, 4-5 ml of warm 0.9% sodium chloride solution was instilled into the abdominal cavity and the wound was closed in layers.

Urine collection urine was carried out using special metabolic cages. The animal was placed in a cage for 24 hours with free access to water.

Twenty four hours after reperfusion, the rats were re-anesthetized by intraperitoneal injection of chloral hydrate at the dose of 300 mg/kg of animal body weight, re-laparotomy was performed, microcirculation were estimated, and blood was sampled from the right ventricle for biochemical studies.
Endogenous creatinine clearance (glomerular filtration rate) was calculated using the following formula:

\[
\text{GFR} = \frac{\text{Urine creatinine (μmol/l) \times Urine volume (ml)}}{\text{Serum creatinine (μmol/l) \times Collection time (min)}}
\]

Fractional sodium excretion (FEna) was calculated as follows:

\[
\frac{\text{Urine sodium (mmol/l)} \times \text{Serum creatinine(μmol/l) \times 100%}}{\text{Serum sodium (mmol/l)} \times \text{Urine creatinine (μmol/l)}}
\]

3. Results and Discussion

Twenty four hours after the surgery, the plasma creatinine and urea levels in the sham operated animals were 45.9±0.8 mmol/L and 6.7±0.2 mmol/L, respectively. Plasma creatinine and urea levels in the control group animals were 102.63±3.6 mmol/L and 21.80±1.29 mmol/L, respectively. In the group of animals administered with the reference drug erythropoietin these indicators were lower: 61.01±2.88 mmol/l and 12.61±1.14 mmol/L, respectively (p<0.05).

The administration of the erythropoietin mimetic peptide (pHBSP) (25 µg/kg) leaded to a statistically significant decrease in the plasma level of creatinine (57.10±2.03 mmol/L) and urea (12.68±1.15 mmol/L) (p<0.05). The effect of the erythropoietin mimetic peptide (pHBSP) (5 µg/kg) was less pronounced: the values were 78.73±2.0 mmol/L and 20.67±1.06 mmol/L, respectively (p<0.05).

The administration of infliximab at the dose of 10 mg/kg was accompanied by a marked decrease in the parameters of nitrogen metabolism in blood plasma: creatinine 63.21±2.48 mmol/l and urea 13.62±1.25 mmol/l compared with the animals of the control group (p<0.05). There were no significant differences in the levels of creatinine and urea when infliximab was administered at the dose of 2 mg/kg compared to the control group (Figures 1, 2).
Figure 1. Values of serum creatinine concentration 24 hours after the reperfusion in simulated ischemia-reperfusion injury of kidney

*Note: x – p<0.05 in comparison with the sham-operated animals. y – p<0.05 in comparison with the ischemia/reperfusion group*

The glomerular filtration rate was maximal in the group of sham-operated animals (0.75±0.02 ml/min), and minimal in the group of ischemia/reperfusion (0.09±0.01 ml/min).
Figure 2. Values of serum urea concentration 24 hours after the reperfusion in simulated ischemia-reperfusion injury of kidney.

Note: $^x$ – $p<0.05$ in comparison with the sham-operated animals. $^y$ – $p<0.05$ in comparison with the ischemia/reperfusion group

In animals that were administered with erythropoietin before ischemia, the glomerular filtration rate was 0.27±0.01 ml/min, which is comparable to the value in the pHBSP group (25 µg/kg) - 0.29±0.01 ml/min. After administration of pHBSP at the dose of 5 µg/kg, there was also an increase in the glomerular filtration rate to 0.27±0.01 ml/min, associated with an increase in the volume of diuresis. The glomerular filtration rate in the Infliximab group (10 mg/kg) was 0.22±0.01 ml/min. Infliximab at the dose of 2 mg/kg did not have a pronounced effect on the glomerular filtration rate, which was 0.10±0.01 ml/min (Figure 3).

Assessment the functional state of the renal tubules revealed a normal fractional sodium excretion index (FEna) in the group of sham-operated animals - 0.37±0.01%. Simulation of ischemia-reperfusion kidney injury lead to an increase in FEna to 2.77±0.1%, which, together with a drop in glomerular filtration rate, is evidence of the development of acute tubular necrosis.

During the experiment, it was revealed that the administration of pHBSP at the dose of 5 µg/kg and 25 µg/kg had a positive effect on the renal tubules: the fractional sodium excretion was 1.26±0.03% and 1.25±0.05%, respectively.
Against the background of the infliximab administration, a significant decrease in the fractional sodium excretion was also noted in comparison with the ischemia/reperfusion group, and the doses 2 mg/kg and 10 mg/kg had a comparable effect: the fractional sodium excretion was 1.17±0.05% and 1.15±0.05%, respectively (Figure 4).

Figure 3. Glomerular filtration rate values 24 hours after the reperfusion in simulated ischemia-reperfusion injury of kidney

Note: \( ^x \) – \( p<0.05 \) in comparison with the sham-operated animals. \( ^y \) – \( p<0.05 \) in comparison with the ischemia/reperfusion group
Figure 4. Values of fractional sodium excretion 24 hours after the reperfusion in simulated ischemia-reperfusion injury of kidney

Note: $x - p<0.05$ in comparison with the sham-operated animals. $y - p<0.05$ in comparison with the ischemia/reperfusion group

Assess the microcirculation showed the greatest effectiveness of pHBSP at the dose of 25 µg/kg: the value in both control points was as close as possible to the value of microcirculation in the kidneys of sham-operated animals. The administration of infliximab at the dose of 10 mg/kg also helped to keep the high level of microcirculation, but the effect was slightly less than pHBSP at the dose of 25 µg/kg. pHBSP administration at the dose of 5 µg/kg increased the level of microcirculation by 2 times compare to only ischemia/reperfusion group, but was significantly behind pHBSP 25 µg/kg and infliximab 10 mg/kg. There was not revealed significant differences in the level of microcirculation between infliximab at the dose of 2 mg/kg and the pathology group (Table 1).

Table 1. The effect of pHBSP and infliximab on renal microcirculation in simulated ischemia/reperfusion injury, PU
<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Before ischemia</th>
<th>5 minutes after</th>
<th>24 hours after</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated animals</td>
<td>898±44&lt;sup&gt;y&lt;/sup&gt;</td>
<td>900±42&lt;sup&gt;y&lt;/sup&gt;</td>
<td>881±38&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>I/R</td>
<td>904±45&lt;sup&gt;y&lt;/sup&gt;</td>
<td>219±12&lt;sup&gt;x&lt;/sup&gt;</td>
<td>430±20&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>EPO</td>
<td>899±37&lt;sup&gt;y&lt;/sup&gt;</td>
<td>637±27&lt;sup&gt;xy&lt;/sup&gt;</td>
<td>733±31&lt;sup&gt;xy&lt;/sup&gt;</td>
</tr>
<tr>
<td>pHBS (5 μg/kg)</td>
<td>905±44&lt;sup&gt;y&lt;/sup&gt;</td>
<td>492±21&lt;sup&gt;xy&lt;/sup&gt;</td>
<td>607±28&lt;sup&gt;xy&lt;/sup&gt;</td>
</tr>
<tr>
<td>pHBS (25 μg/kg)</td>
<td>895±15&lt;sup&gt;y&lt;/sup&gt;</td>
<td>693±28&lt;sup&gt;xy&lt;/sup&gt;</td>
<td>771±27&lt;sup&gt;xy&lt;/sup&gt;</td>
</tr>
<tr>
<td>Infliximab (2 mg/kg)</td>
<td>900±57&lt;sup&gt;y&lt;/sup&gt;</td>
<td>249±13&lt;sup&gt;x&lt;/sup&gt;</td>
<td>448±20&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>Infliximab (10 mg/kg)</td>
<td>903±72&lt;sup&gt;y&lt;/sup&gt;</td>
<td>674±28&lt;sup&gt;xy&lt;/sup&gt;</td>
<td>743±34&lt;sup&gt;xy&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: <sup>x</sup> – <i>p</i> < 0.05 in comparison with the sham-operated animals. <sup>y</sup> – <i>p</i> < 0.05 in comparison with the ischemia/reperfusion group

Currently, such kidney surgery as transplantation and resection, have firmly and permanently entered the practice of modern medicines. In this regard, the problem of preventing the development of the most dangerous complication of renal ischemia, acute renal injury, becomes more relevant than ever. In our study, we proved that pHBS and infliximab significantly contribute to the preservation of normal functional activity of the kidneys after simulated ischemia-reperfusion injury.

The effect of pHBS can be explained by its ability to selectively bind to the heterodimeric erythropoietin receptor, which leads to the activation of pleiotropic effects: anti-ischemic, anti-apoptotic, and anti-inflammatory (19). This contributes to cytoprotective effects in the renal parenchyma, reducing the formation of humoral factors leading to glomerular and tubular dysfunction and activation of eNOS (24).

It is also known that cell damage is aggravated by reperfusion: reactive oxygen species and pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), trigger a pathological cascade of damage to the renal parenchyma (33). The nephroprotective effects of infliximab can be explained by its high affinity for the tumor necrosis factor - α, as well as its ability to reduce the concentration (binds and inhibits the synthesis) of IL−1, IL−6, IL−8, monocyte chemoattractant-1, nitric oxide, metalloproteinases (collagenase, stromelysin), and other inducers of inflammation and tissue destruction (33). This, in turn, leads to the normalization of the functional activity of the kidneys in ischemia-reperfusion injury.

Thus, our data suggest that pHBS and infliximab may be useful for preventing acute renal injury in surgery with warm renal ischemia. Further researches are needed to confirm our results.
In conclusion, administration of pHBSP at the doses of 5 \( \mu \text{g/kg} \) and 25 \( \mu \text{g/kg} \) 30 minutes before ischemia, a dose-dependent improvement in the filtration function of the kidneys was observed in comparison to the group of control animals (I/R), which manifested in a decrease in the concentration of serum creatinine to 78.73±2.0 mmol/l and 57.10±2.03 mmol/l, while the glomerular filtration rate was 0.27±0.01 ml/min and 0.29±0.01 ml/min, respectively (p<0.05). There was also a 2.2-fold decrease in fractional sodium excretion compared to the ischemia/reperfusion group.

A comparable nephroprotective effect was provided by infliximab at the dose of 10 mg/kg when administered 1 hour before ischemia: creatinine was 63.21±2.48 mmol/L, urea - 13.62±1.25 mmol/L, glomerular filtration rate-0.22±0.01 ml/min, and fractional sodium excretion-1.15±0.05%. The dose of infliximab 2 mg/kg influenced only on the level of fractional sodium excretion (1.17±0.05%), apparently preventing pathological changes of the part of the nephron tubules.

A statistically significant (p<0.05) dose-dependent improvement in microcirculation was observed after pHBSP administration at the dose of 5 \( \mu \text{g/kg} \) and 25 \( \mu \text{g/kg} \), infliximab at the dose of 10 mg/kg.

Aleksandr S. Netrebenko – postgraduate student of the Department of Pharmacology and Clinical Pharmacology, Belgorod National Research University. ORCID: 0000-0003-2212-0508, e-mail: alexnetrebenko@mail.ru.

Vladimir V. Gureev – PhD, professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod National Research University. ORCID ID:0000-0003-1433-1225. e-mail: produmen@yandex.ru

Mikhail V. Pokrovskii – PhD, professor of the Department of Pharmacology and Clinical Pharmacology, chief of the Research institute of the pharmacology of living systems, Belgorod National Research University. ORCID: 0000-0002-2761-6249. E-mail: mpokrovsky@yandex.ru

Anastasiya V. Gureeva – 4th year student of the medical faculty of Kursk State Medical University. ORCID:0000-00031719-7316. e-mail: nastasyi.207@gmail.com

Yuliya M. Tsvverkalova - postgraduate student of the Department of Pharmacology and Clinical Pharmacology, Belgorod National Research University. ORCID ID:0000-0001-8489-247X, e-mail: cvd404@mail.ru.

Ilya S. Rozhkov - postgraduate student of the Department of Pharmacology and Clinical Pharmacology, Belgorod National Research University. e-mail:medik768@yandex.ru. ORCID: 0000-0002-9092-229X
References

17. Skachilova SY, Danilenko L, Kesarov O, Kochkarova I. Pharmacological protection of the ischemic myocardium by derivatives of 3-(2, 2, 2-trimethylhydrazinium) propionate and evaluation of their antioxidant activity. Res Results Pharmacol. 2015;1(1 (1)).


27. Korokin MV, Soldatov VO, Tietze AA, Golubev IV, Belykh AE, Kubekina MV, et al. 11-amino acid peptide imitating the structure of erythropoietin α-helix b improves endothelial function, but stimulates thrombosis in rats. Фармация и фармакология. 2019;7(6 (eng)).


