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Research Article

Correction of renal ischemia/reperfusion injury with the Combination of Infliximab and the erythropoietin-derived peptide mimetic pHBSP

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Abstract

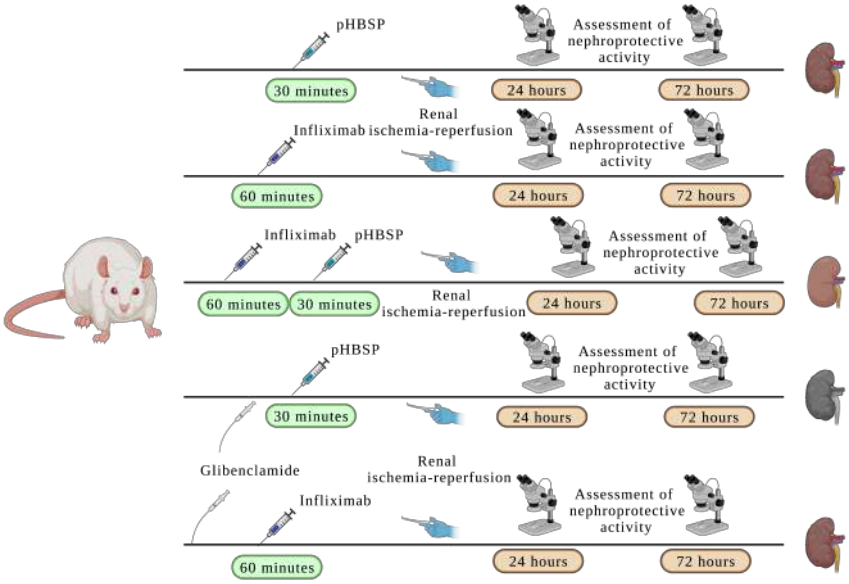
Introduction: Due to the high social and economic value of acute kidney injury, the scientific community is focused on methods of diagnosis and treatment of this pathology. A number of studies have already revealed cytoprotective effects of the helix B–derived erythropoietin peptide and infliximab in simulated ischemia/reperfusion injury of liver, myocardium, and nervous tissue. The aim of this research was to study the renoprotective effects of the combination of pHBSP and infliximab on the renal ischemia/reperfusion injury.

Materials and Methods: The experiment was performed in 230 white male Wistar rats. The animals were treated with pHBSP and infliximab. Under anesthesia, a unilateral right nephrectomy was performed and the contralateral renal pedicle was clamped. Functional tests were performed and tissue samples were taken for laboratory studies 5 minutes, 24 hours and 72 hours after reperfusion.

Results and Discussion: The results obtained confirm the dose-dependent renoprotective activity of the helix B–derived erythropoietin peptide and infliximab. The nephroprotective activity of the combination of pHBSP at a dose of 25 mcg/kg and infliximab at a dose of 10 mg/kg significantly exceeded the effect of a single-drug therapy. This is evidenced by the normalization of renal tubule function, a significant increase in the microcirculation level, the absence of rough lesion during pathomorphological examination, as well as a decrease in the expression of TNF-α by 54% and IL-1β by 65% in comparison with the ischemia/reperfusion group according to immunohistochemistry examination. The important role of ATP-sensitive potassium channel in the renoprotective activity of pHBSP has been confirmed.

Conclusion: The renoprotective activity of the helix B–derived erythropoietin peptide and infliximab has been confirmed, and the advantage of their combined administration for the correction of morphofunctional disorders in simulated renal ischemia/reperfusion injury due to the multimodal effect on pathogenetic processes has been established.

Graphical abstract:



Keywords

pHBSP, infliximab, ischemia/reperfusion, TNF-α, preconditioning, inflammation

Introduction

Oncourological morbidity in Russia remains extremely high. According to current recommendations, the optimal method of treatment at the early stages is a preserving surgery (kidney resection) under WIT conditions (Forbes et al. 2016; Ragulina et al. 2017; Jiang et al. 2019). The most threatening complication of the devascularized kidney is the acute kidney injury (Basile et al. 2014), the main pathogenetic mechanism of which is ischemia/reperfusion kidney injury (Hwang 2013).

The clinical outcome of acute kidney injury in many cases also remains unsatisfactory. In a mixed population of patients being treated in hospital, mortality can reach 72.6% (Gobe et al. 2015), which exceeds the total mortality from breast cancer and prostate cancer (Sabbisetti et al. 2014).

One of the promising methods for the prevention of ischemia/reperfusion injury is pharmacological preconditioning (Skachilova et al. 2015). In a number of major studies, a preconditioning activity of the glycoprotein hormone erythropoietin has been proved (Brookset al. 2015).

The biological effects of erythropoietin are realized by binding to specific receptors (Yakovlev et al. 2016). There are two types of receptors: homodimeric (EPOR) and heterodimeric (EPOR/βcR). In an adult, erythropoietin binding to the homodimer receptor results in apoptosis inhibition and erythropoiesis activation (Xiaoet al. 2012). Cytoprotective effects of erythropoietin are caused by activation of the heterodimer receptor (Netrebenko et al. 2021). The realization of these effects is mediated by JAK-2, STAT5, and PI3K (Grebien et al. 2008).

Being expressed in several non-hematopoietic tissues, erythropoietin plays an important role in protection against apoptosis and inflammation, and also has proliferative activity. Erythropoietin significantly reduces damage in stroke (Thériault et al. 2016; Jia et al. 2016), myocardial infarction (Arthuret al. 2014) and ischemia/reperfusion kidney injury (Golmohammadi et al. 2020). However, erythropoietin has dangerous side effects: arterial hypertension, thrombosis and stimulation of the growth and progression of malignant neoplasms (Lund et al. 2014; Yakovlev et al. 2016). Therefore, a small erythropoietin-derived peptide mimetic(pHBSP), capable of selectively binding to EPOR/βcR, was developed and synthesized (Zhang et al. 2017). In a series of experiments, pHBSP has already demonstrated a number of positive effects in simulated ischemia/reperfusion injury to the liver (Tan et al. 2018); it positively influences on the course of connective tissue diseases (Huang et al. 2018) and relieves acute lung injury (Bi et al. 2020).

Pro-inflammatory cytokines play an equally important role in the development of acute kidney injury (Netrebenko et al. 2021). One of the most significant cytokineis tumor necrosis factor alpha (Bethesda et al. 2017), which is released by macrophages and monocytes in damaged tissues and triggers pathological inflammatory reactions, stimulating the production of IL-1ß, IL-6, IL-8, enhancing this process (Netrebenko et al. 2021). Infliximab is one of the known drugs that reduce the activity of TNF-α. It has a high affinity for TNF-α and is able to effectively block it (Netrebenko et al. 2022). Infliximab is used as essential therapy in patients with Crohn's disease (Ponsioen et al. 2017) and various forms of sarcoidosis (Bakker et al. 2021). Modern

science makes steps to study the nephroprotective properties of infliximab in the renal ischemia/reperfusion injury, but the results are contradictory (Tasdemir et al. 2012).

Based on the features of pathogenetic processes in renal ischemia/reperfusion, a set of receptor mechanisms and cascades of pathological inflammatory reactions, it can be suggested that one of the promising ways of nephroprotection may be the combined administration of infliximab and pHBSP, taking into account the multimodal mechanisms of their effects and the theoretical possibility of their mutual potentiating.

The aim of the study: to make an experimental confirmation of the prospectivity of renal ischemia/reperfusion injury correction with the combination of infliximab and the erythropoietin-derived peptide mimetic.

Material and Methods

Compliance with ethical and regulatory requirements

The study was conducted at the Research Institute of Pharmacology of Living Systems of Belgorod National Research University in accordance with regulatory legal acts and guidelines governing the conduct of experimental research in the Russian Federation. The ethical principles of the treatment of laboratory animals meet requirements of the *European Convention for the Protection of Vertical Animals Used for Experimental and Other Scientific Purposes. CETSN170.*

Experimental animals

The experiment was performed in 230 white male Wistar rats weighing 280-320g, which met all the necessary criteria and were kept in accordance with the current regulations. The experimental protocols were approved by the local independent Ethical committee of Belgorod State National Research University (Minutes No. 3.10 of 28.10.2019).

Study design

After randomization of animals by weight, the following experimental groups were formed:

1 group – Intact animals
2 group – Sham-operated animals (24 hours)
3 group – Sham-operated animals (72 hours)
4 group – Ischemia/reperfusion (24 hours)
5 group – Ischemia/reperfusion (72 hours)
6 group – Ischemia/reperfusion + pHBSP 5 mcg/kg (24 hours)
7 group – Ischemia/reperfusion + pHBSP 5 mcg/kg (72 hours)
8 group– Ischemia/reperfusion + pHBSP 25 mcg/kg (24 hours)
9 group– Ischemia/reperfusion + pHBSP 25 mcg/kg (72 hours)
10 group – Ischemia/reperfusion + infliximab 2 mg/kg (24 hours)
11 group – Ischemia/reperfusion + infliximab 2 mg/kg (72 hours)
12 group – Ischemia/reperfusion + infliximab 10 mg/kg (24 hours)
13 group – Ischemia/reperfusion + infliximab 10 mg/kg (72 hours)
14 group – Ischemia/reperfusion + EPO (24 hours)
15 group – Ischemia/reperfusion + EPO (72 hours)
16 group – Ischemia/reperfusion + pHBSP + infliximab (24 hours)
17 group – Ischemia/reperfusion + pHBSP + infliximab (72 hours)
18 group– Ischemia/reperfusion + glibenclamide (24 hours)
19 group – Sham-operated animals + glibenclamide (72 hours)
20 group – Ischemia/reperfusion + pHBSP + glibenclamide (24 hours)
21 group – Ischemia/reperfusion + pHBSP + glibenclamide (72 hours)
22 group – Ischemia/reperfusion + infliximab + glibenclamide (24 hours)
23 group – Ischemia/reperfusion + infliximab + glibenclamide (72 hours)

The activity of the erythropoietin mimetic peptide (pHBSP) (provided by a pharmaceutical company PHARMAPARK LLC) was studied at the doses of 5 mcg/kg and 25 mcg/kg; the activity of infliximab (Remicade, MSD) was studied at the doses of 2 mg/kg and 10 mg/kg; the activity of recombinant erythropoietin (Epocrine, Research Institute of Highly Pure Biopreparations, Russia) was studied at the dose of 50 IU/kg; the activity of glibenclamide (Maninil, Berlin-Chemie AG, Germany) was studied at the dose of 5 mg/kg. These doses were selected based on previously identified protective effects on ischemia/reperfusion models or were calculated taking into account the recommended human doses using conversion factors (Nagata et al. 2016; Kostina et al. 2021; Firsova et al. 2022). The dose schedule is based on the pharmacokinetic profile of the drug.

Simulation of the renal ischemia/reperfusion injury

The animals were anesthetized by intraperitoneal injection of chloral hydrate at the dose of 300 mg/kg (Bratchikov et al. 2018). After surgical field preparation, median laparotomy was performed, and a renal body with elements of a renal pedicle was pushed out on both sides (Bratchikov et al. 2018). Microcirculation in the renal parenchyma was measured according to a generally accepted method (Elagin and Bratchikov 2018). Left renal pedicle clamping for 40 minutes followed by a right nephrectomy was performed. Urine was collected during reperfusion. Twenty-four or 72 hours after reperfusion, the rats were anesthetized again; relaparotomy was performed; microcirculation parameters in the renal parenchyma were measured; blood was taken from the right ventricle for biochemical studies, and tissue samples were taken.

Measurement of biochemical and functional parameters

Serum creatinine and urea levels were measured using a biochemical analyzer URIT800 Vet (URIT Medical Electronic Co. Ltd., China). The concentrations of potassium and sodium ions in the blood serum were detected according to the standard procedure using the kits for the automatic analyzer K/N ”Ionomer ETS-59”. Endogenous creatinine clearance (glomerular filtration rate) and fractional sodium excretion were calculated using standard formulas.

Morphological examination

The kidney samples were fixed in 10% formalin. The slides were stained with hematoxylin and eosin. All

studies were performed using a Leica DM4000B microscope.

Immunohistochemistry

The immunohistochemical study was performed in serial paraffin sections with a thickness of 2-3 microns placed on adhesive glasses coated with poly–L-lysine (Super Frost Plus, "Mainzel Glazer, Germany). Antibodies to IL-1 beta (ThermoFisher, 1:100), IL-4 (ThermoFisher, 1:100), IL-6 (ThermoFisher, 1:100), IL-10 (ThermoFisher, 1:100), CD68 (514H12; LeicaRTU) were used as primary antibodies. All immunohistochemical reactions were performed manually, and reaction on CD68 was performed in automatic mode (Bond-Max immunohistosteiner "Leica", Germany). The primary antibodies were anti–rat. Secondary antibodies were a universal two-component detection system HiDef Detection™ HRP Polymer system, ("Cell Marque", USA), mouse/rabbit anti-IGG, horseradish peroxidase (HRP) and DAB substrate. The cell nuclei were stained with Mayer's hematoxylin. The evaluation of immunohistochemical reactions was based on the intensity of staining and separation of immunopositive cells according to the recommendations of D.J. Dabbs "Diagnostic immunohistochemistry" (4rd Edition, 2014).

Statistical data processing

Descriptive statistics were applied to all the data: the data were tested for the normality of distribution. The type of distribution was determined using the Shapiro-Wilk's test. The mean value (M) and the standard error of the mean (m) were calculated in a normal distribution. Taking into account the normal distribution of the results, a parametric method (Student's t-test) was used to analyze the intergroup differences. All calculations were made using the Microsoft Excel 10.0 statistical software package.

Results and Discussion

Assessment of morphofunctional disorders in the renal ischemia/reperfusion injury

Pathology simulation by the opening of the retroperitoneal space during laparotomy and applying of the atraugrip on the left vascular renal pedicle for 40 minutes leads to a complex of changes corresponding to the modern criteria for acute renal injury KDIGO 201224 and 72 hours after reperfusion. After 24 hours in the ischemia/reperfusion group, the serum creatinine level was 102.6±3.6 mmol/L, glomerular filtration rate was 0.09±0.01 ml/min, and fractional sodium excretion was 2.77±0.1%; in the group of the sham-operated animals creatinine was 45.9±0.8 mmol/L, glomerular filtration rate was 0.75±0.02 ml/min, and fractional sodium excretion was 0.37± 0.01%. Seventy-two hours after reperfusion in the ischemia/reperfusion group, serum creatinine level was 125.6±6.4 mmol/L, glomerular filtration rate was 0.06±0.01 ml/min, and fractional sodium excretion was 6.83±0.29%, in the group of the sham-operated animals creatinine was 47.0±2.3 mmol/L glomerular filtration rate was 0.77±0.04 ml/min, and fractional sodium excretion was 0.37±0.04% (Fig. 1).

The dynamics of the microcirculation index in the renal parenchyma was as follows: the microcirculation level was 900±42 PU5 minutes, 881±38 PU24 hours and 890±36 PU72 hours after reperfusion in the group of sham-operated animals. Simulation of the acute kidney injury was accompanied by a statistically significant decrease in the microcirculation level 5 minutes after reperfusion to 219±12 PU with a moderate improvement in this index 24 and 72 hours after reperfusion to 430±20 PU and 410±20 PU, respectively.

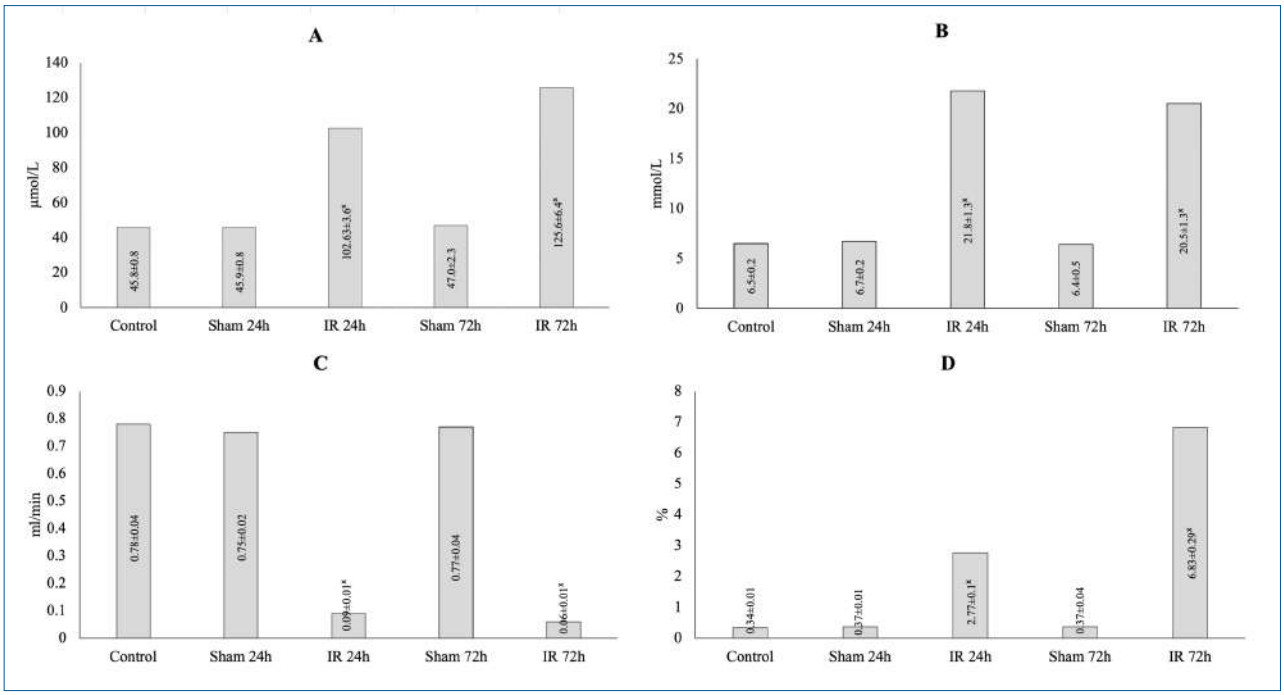


Figure 1. The values of serum creatinine (**A**), urea (**B**), glomerular filtration rate (**C**) and fractional sodium excretion (**D**) 24 and 72 hours after reperfusion in the simulated renal ischemia/reperfusion injury. **Note:** control – intact animals; sham – sham-operated animals; IR – renal ischemia/reperfusion; x – $p<0.05$ in comparison with the group of sham-operated animals.

Microscopic examination of kidney sections 24 and 72 hours after reperfusion revealed the presence of destructive changes consisting in a large number of oxyphilic masses, the predominance of shrunken glomeruli in most fields, which was confirmed by a decrease in the cross-sectional area of the glomerular vascular pole by 1.2 times compared with the group of sham-operated animals, a decrease in the height of epithelial cells of the proximal tubules by 1.4 times, most likely due to necrosis (Table 1).

Table 1. Morphometric characteristics of the structural elements of the nephron in the simulated ischemia/reperfusion (M±m)

Group	Cross-sectional area of the renal corpuscle, μm²	Height of epithelial cells of the proximal tubules, μm	Cross-sectional area of the renal corpuscle, μm²	Height of epithelial cells of the proximal tubules, μm
	24 hours		72 hours	
Sham	10496±123	11.9±0.7	10345±118	11.8±0.6
IR	8973±241^{x}	8.3±0.3^{x}	8293±227^{x}	6.4±0.5^{x}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; ^{x} – $p<0.05$ in comparison with the group of sham-operated animals.

Immunohistochemical examination of the kidneys for pro-inflammatory and anti-inflammatory cytokines revealed that in the glomeruli and tubules of nephrons of the sham-operated animals the number of cells expressing both pro-inflammatory and anti-inflammatory cytokines varies on the average from 3.0% to 6.0% (Table 2). Investigation of the intensity of the macrophages and monocytes infiltration of the kidney structural elements in sham-operated animals showed that the relative level of CD68-positive cells in interstitial tissue was 20%.

Twenty-four hours after ischemia/reperfusion surgery of the kidney, a statistically significant increase in the number of cells expressing both pro-inflammatory and anti-inflammatory cytokines was observed in all its structural elements, and the level of CD68-positive cells in interstitial tissue reached 61.8±0.42%. Seventy-two hours after ischemic reperfusion injury of the kidney, a decrease in the number of cells expressing both pro-inflammatory cytokines and anti-inflammatory cytokines was observed in all its structural elements compared to those after 24 hours (Table 2).

Thus, the proposed method of renal ischemia simulation with a subsequent reperfusion period of 24 or 72 hours is an adequate experimental model for acute renal injury and can be used to evaluate the effectiveness of new drugs.

Renoprotective effects of the erythropoietin-derived peptide mimetic in ischemia/reperfusion injury of the kidney

Administration of pHBSP at the doses of 5 mcg/kg and 25 mcg/kg restored the glomerular filtration rate to 0.27±0.01 ml/min and 0.29±0.01 ml/min respectively 24 hours after and 0.27±0.02 ml/min and 0.38±0.02 ml/min, respectively, 72 hours afterwards, which was accompanied by a decrease in serum creatinine and urea concentrations. Twenty-four hours later, a significant decrease in fractional sodium excretion by more than 2 times was revealed compared with the ischemia/reperfusion group (Fig. 2).

pHBSP administration at the doses of 5 mcg/kg and 25 mcg/kg led to a dose-dependent improvement in kidney function 72 hours after the clamps were removed from the renal pedicle manifested in a decrease in serum creatinine concentration to 88.3±3.9 mmol/L and 62.2±3.3 mmol/L and urea to 16.2±1.1 mmol/L and 9.7±0.9 mmol/L, respectively, and the fractional sodium excretion index was 2.7±0.17% and 2.1±0.16%, respectively. The renoprotective effects of pHBSP significantly exceeded the effects of a single therapy with recombinant human erythropoietin at a dose of 50 IU/kg.

Table 2. The levels of expression of pro-inflammatory and anti-inflammatory cytokines in the kidney structures (M±m)

Tissue	Group	IL-1β, %	TNF-α, %	IL-4, %	IL-10, %
		24 hours			
Glomerulus	Sham	5.8±0.3	4.9±0.3	4.2±0.2	7.8±0.4
	IR	49.9±1.1^{x}	69.7±1.3^{x}	15.5±0.8^{x}	12.4±0.4^{x}
Nephrontubules	Sham	5.9±0.3	5.7±0.3	4.0±0.2	5.8±0.2
	IR	56.5±1.5^{x}	71.1±1.2^{x}	16.2±0.9^{x}	13.7±0.3^{x}
		72 hours			
Glomerulus	Sham	5.6±0.3	5.5±0.3	4.9±0.2	8.3±0.4
	IR	45.0±0.9^{x}	63.6±1.2^{x}	11.5±0.6^{x}	20.2±0.6^{x}
Nephrontubules	Sham	4.7±0.3	5.7±0.3	4.2±0.2	6.0±0.2
	IR	49.4±1.3^{x}	62.2±1.3^{x}	15.0±0.9^{x}	10.5±0.3^{x}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; ^{x} – $p<0.05$ in comparison with the group of sham-operated animals.

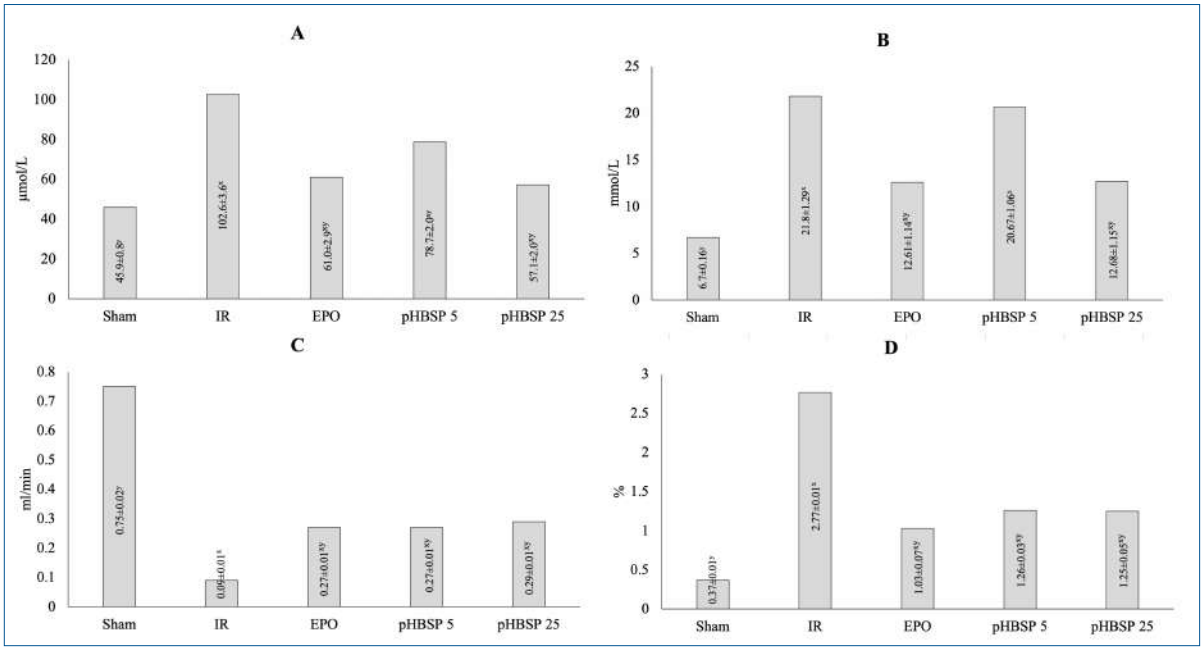


Figure 2. The effect of the erythropoietin-derived peptide mimetic (pHBSP) on the concentration of serum creatinine (A), urea (B), glomerular filtration rate (C) and fractional sodium excretion (D) 24 hours after reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia/reperfusion; EPO – erythropoietin (at the dose of 50 IU/kg); pHBSP 5 – erythropoietin-derived peptide mimetic (at the dose of 5 mcg/kg); pHBSP 25 – erythropoietin-derived peptide mimetic (at the dose of 25 mcg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group.

A single administration of pHBSP at the doses of 5 mcg/kg and 25 mcg/kg led to restoration of the microcirculation level in all control time periods, significantly exceeding the indicators of the EPO group (Table 3).

Table 3. The effect of the erythropoietin-derived peptide mimetic on the renal microcirculation (M±m)

Experimental group	Microcirculation index 5 minutes, PU	Microcirculation index 24 hours, PU	Microcirculation index 72 hours, PU
Sham	900±42^{y}	881±38^{y}	890±36^{y}
IR	219±12^{x}	430±20^{x}	410±20^{x}
IR + EPO	637±27^{xy}	733±31^{xy}	539±39^{xy}
IR + pHBSP 5	492±21^{xy}	607±28^{xy}	584±32^{xy}
IR + pHBSP 25	693±28^{xy}	771±27^{xy}	625±36^{xy}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; EPO – erythropoietin (at the dose of 50 IU/kg); pHBSP 5 – erythropoietin-derived peptide mimetic (at the dose of 5 mcg/kg); pHBSP 25 – erythropoietin-derived peptide mimetic (at the dose of 25 mcg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group.

Microscopic evaluation of the kidneys slices of the animal groups administered with pHBSP for nephroprotection revealed a significant dose-dependent improvement in the histological pattern. The number of edematous or shrunken renal corpuscles was insignificant, and the cross-sectional area of the renal corpuscles and the height of the epithelial cells increased, which indicates a reduction of the ischemia/reperfusion injury (Table 4).

Table 4. Morphometric characteristics of the structural elements of the nephron against the background of nephroprotection with pHBSP (M±m)

Group	Cross-sectional area of the renal corpuscle, µm²	Height of epithelial cells of the proximal tubules, µm	Cross-sectional area of the renal corpuscle, µm²	Height of epithelial cells of the proximal tubules, µm
	24 hours		72 hours	
Sham	10496±123^{y}	11.9±0.7^{y}	10345±118^{y}	11.8±0.6^{y}
IR	8973±241^{x}	8.3±0.3^{xy}	8293±227^{x}	6.4±0.5^{x}
IR + EPO	8938±102^{x}	8.9±0.1^{xy}	8974±98^{xy}	6.9±0.2^{x}
IR + pHBSP 5	8894±85^{x}	9.1±0.1^{xy}	9126±85^{xy}	7.1±0.1^{xy}
IR + pHBSP 25	9029±98^{x}	9.6±0.1^{xy}	9344±88^{xy}	8.1±0.2^{xy}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; EPO – erythropoietin (at the dose of 50 IU/kg); pHBSP 5 – erythropoietin-derived peptide mimetic (at the dose of 5 mcg/kg); pHBSP 25 – erythropoietin-derived peptide mimetic (at the dose of 25 mcg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group.

Twenty-four hours after reperfusion, pHBSP had a dose-dependent effect, consisting in a smaller increase in the cells expressing pro-inflammatory cytokines (Fig. 3), and an increase in cells expressing IL-10 in all parts of the nephron compared to the group of control untreated animals (Table 5). The dose-dependent effect of pHBSP on the intensity of macrophages and monocytes infiltration of the kidney tissues was also noted. pHBSP administration led to a less pronounced increase in CD68-positive cells in the interstitial tissue of the renal parenchyma. This indicator was 37.88±0.5% and 31.98±0.45%, respectively, in the groups of animals treated with pHBSP at the doses of 5 mcg/kg and 25 mcg/kg. Seventy-two hours after the clamps removal from the renal pedicle and reperfusion, the maintaining of dose-dependent effect like the one 24 hours after reperfusion was observed in the animals that had been injected with pHBSP (Table 5). The number of CD68-positive cells in the interstitial tissue was 34.94±0.47% and 32.39±0.43%.

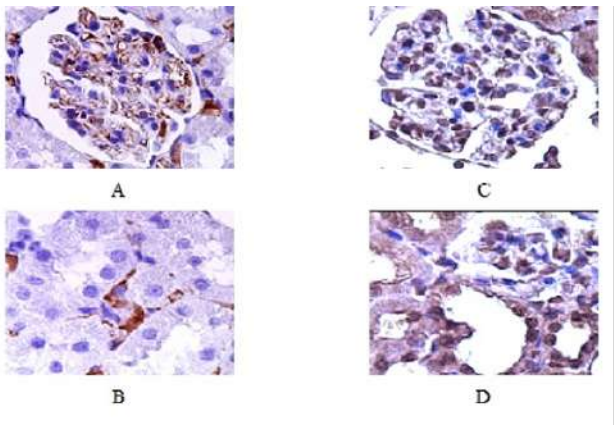


Figure 3. The effect of pHBSP 25 mcg/kg on the expression of IL-1ß (**A, B**) and TNFa (**C, D**) in the renal cells 24 hours after reperfusion. **Note:** immunohistochemical reaction with antibodies to IL-1ß and TNFa; light microscopy, magnification ×400.

Table 5. The effect of pHBSP on the expression of pro-inflammatory and anti-inflammatory cytokines in the kidney (M±m)

Tissue	Group	IL-1β, %	TNF-α, %	IL-4, %	IL-10, %
		24 hours			
	Sham	5.8±0.3	4.9±0.3	4.2±0.2	7.8±0.4
	IR	49.9±1.1^{x}	69.7±1.3^{x}	15.5±0.8^{x}	12.4±0.4^{x}
Glomerulus	IR + EPO	40.6±0.9^{xy}	62.2±1.3^{xy}	15.9±0.9^{x}	31.2±1.0^{xy}
	IR + pHBSP 5	40.6±0.9^{xy}	61.8±1.3^{xy}	14.3±0.9^{x}	33.7±1.0^{xy}
	IR + pHBSP 25	32.7±0.8^{xy}	49.4±1.1^{xy}	17.1±0.9^{x}	46.9±1.3^{xy}
Nephrontubules	Sham	5.9±0.3	5.7±0.3	4.0±0.2	5.8±0.2
	IR	56.5±1.5^{x}	71.1±1.2^{x}	16.2±0.9^{x}	13.7±0.3^{x}
	IR + EPO	47.8±0.9^{xy}	62.9±1.1^{xy}	18.7±1.1^{x}	23.3±0.8^{xy}
	IR + pHBSP 5	50.9±0.9^{xy}	61.4±1.0^{xy}	20.7±1.2^{x}	22.3±0.7^{xy}
	IR + pHBSP 25	36.9±0.8^{xy}	51.7±0.7^{xy}	18.6±1.2^{x}	46.8±0.7^{xy}
		72 hours			
	Sham	5.6±0.3	5.5±0.3	4.9±0.2	8.3±0.4
	IR	45.0±0.9^{x}	63.6±1.2^{x}	11.5±0.6^{x}	20.2±0.6^{x}
Glomerulus	IR + EPO	38.3±0.7^{xy}	56.8±1.3^{xy}	11.6±0.5^{x}	28.4±0.8^{xy}
	IR + pHBSP 5	37.6±0.8^{xy}	57.0±1.4^{xy}	13.1±0.7^{x}	25.5±0.7^{xy}
	IR + pHBSP 25	29.3±0.6^{xy}	42.4±1.0^{xy}	16.4±0.9^{xy}	40.3±1.2^{xy}
	Sham	4.7±0.3	5.7±0.3	4.2±0.2	6.0±0.2
	IR	49.4±1.3^{x}	62.2±1.3^{x}	15.0±0.9^{x}	10.5±0.3^{x}
Nephrontubules	IR + EPO	46.3±0.9^{xy}	55.7±0.9^{xy}	14.4±0.9^{x}	15.7±0.6^{xy}
	IR + pHBSP 5	43.6±0.9^{xy}	60.4±0.6^{y}	16.1±0.9^{x}	16.8±0.5^{xy}
	IR + pHBSP 25	34.1±0.7^{xy}	39.3±0.8^{xy}	16.8±1.0^{x}	37.5±0.7^{xy}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; EPO – erythropoietin (at the dose of 50 IU/kg); pHBSP 5 – erythropoietin-derived peptide mimetic (at the dose of 5 mcg/kg); pHBSP 25 – erythropoietin-derived peptide mimetic (at the dose of 25 mcg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group.

Thus, the obtained results indicate the dose-dependent renoprotective properties of the erythropoietin-derived peptide mimetic: pHBSP administration led to a decrease in the concentration of nitrogen metabolism products in blood plasma, normalization of the glomerular filtration rate and fractional sodium excretion. The level of renal parenchymal perfusion significantly increased. Also, morphological and immunohistochemical studies revealed greater protective capabilities compared to recombinant human erythropoietin.

Renoprotective effects of infliximab in ischemia/reperfusion kidney injury

The injection of infliximab at the dose of 10 mcg/kg intraperitoneally one hour before ischemia contributed to a significant decrease in serum creatinine to 63.2±2.5 mmol/L 24 hours later, and also led to an increase in glomerular filtration rate to 0.22±0.01 ml/min, which significantly differed from the ischemia/reperfusion group. Seventy-two hours after the clamps removal from the renal pedicle, a decrease in serum creatinine concentration to 108.4±5 mmol/L and 69.3±2.9 mmol/L, and an increase in glomerular filtration rate to 0.19±0.02 ml/min and 0.33±0.02 ml/min were revealed in the groups treated with infliximab at the doses of 2 mg/kg and 10 mg/kg, respectively. The concentration of urea in the blood also decreased under the influence of infliximab, reaching the levels of 20.4±1.4 mmol/L and 13.6±1.3 mmol/L24 hours later, and 15.6±1.1 mmol/L and 9.0±1.0 mmol/L 72 hours later, respectively. The obtained values in the group of infliximab 10 mg/kg significantly differed from the ischemia/reperfusion group and came close to the group of sham operated animals. Twenty-four hours after the restoration of renal blood supply, infliximab administration in both doses led to a pronounced decrease in fractional sodium excretion by more than 2 times compared with the ischemia/reperfusion group. On the 3rdday of the experiment, protection with infliximab had a positive effect on the fractional sodium excretion index, which was 2.34±0.19% and 1.5±0.14% for the doses of 2 mg/kg and 10 mg/kg, respectively, which significantly differs from the values in the ischemia/reperfusion group.

The administration of infliximab at the dose of 10 mg/kg contributed to the improvement of microcirculation in all control time periods, significantly exceeding the indicators of the ischemia/reperfusion group (Table 6).

Table 6. The influence of infliximab on the renal microcirculation (M±m)

Experimental group	Microcirculation index 5 minutes, PU	Microcirculation index 24 hours, PU	Microcirculation index 72 hours, PU
Sham	900±42^{y}	881±38^{y}	890±36^{y}
IR	219±12^{x}	430±20^{x}	410±20^{x}
IR + INF 2	418±17^{xy}	448±20^{x}	522±43^{xy}
IR+ INF 10	679±31^{xy}	743±34^{xy}	631±30^{xy}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; INF 2 – infliximab (at the dose of 2 mg/kg); INF 10 – infliximab (at the dose of 10 mg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group.

Histological examination revealed the dose-dependent nephroprotective effect of infliximab. Infliximab at the dose of 2 mg/kg scarcely led to an improvement in the microscopic pattern and morphometry indicators in comparison with the pathology simulated group, while in the course of the treatment with infliximab at the dose of 10 mg/kg, a moderate number of shrunken renal corpuscles were noted in the kidney sections, subcapsular spaces were slightly dilated 24 and 72 hours after reperfusion. This is consistent with the morphometry, according to which, an increase in the cross-sectional area of the glomerular vascular pole, as well as in the height of the epithelial cells of the proximal and distal tubules of the nephron, was revealed (Table 7).

Table 7. Morphometric characteristics of the structural elements of the nephron against the background of nephroprotection with infliximab (M±m)

Group	Cross-sectional area of the renal corpuscle, µm²	Height of epithelial cells of the proximal tubules, µm	Cross-sectional area of the renal corpuscle, µm²	Height of epithelial cells of the proximal tubules, µm
	24 hours		72 hours	
Sham	10496±123^{y}	11.9±0.7^{y}	10345±118^{y}	11.8±0.6^{y}
IR	8973±241^{x}	8.3±0.3^{xy}	8293±227^{x}	6.4±0.5^{x}
IR + INF 2	8716±113^{x}	8.9±0.1^{xy}	9092±92^{xy}	7.4±0.1^{xy}
IR+ INF 10	8994±75^{xy}	9.7±0.1^{xy}	9208±106^{xy}	8.5±0.2^{xy}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; INF 2 – infliximab (at the dose of 2 mg/kg); INF 10 – infliximab (at the dose of 10 mg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group.

Twenty-four hours after the restoration of blood supply of the renal parenchyma, a dose-dependent effect of infliximab was observed, consisting in a smaller increase in the cells expressing pro-inflammatory cytokines and an increase in the number of cells expressing IL-10 in all elements of the nephron compared to the group of control untreated animals (Table 8). There was also a less pronounced increase in the CD68-positive cells, compared with the group of control untreated animals; their level in the interstitial tissue reached 35.33±0.49% and 33.5±0.42%, respectively (Fig. 4). Immunohistochemical methods of examination of the expression of pro-inflammatory and anti-inflammatory cytokines, CD68-positive cells in kidney structures showed the maintaining of dose-dependent effect 72 hours after reperfusion like the one 24 hours after reperfusion in the animals that had been injected with infliximab (Table 8).

The obtained results confirm the renoprotective activity of infliximab: infliximab administration decreased the concentration of nitrogen metabolism indicators in blood plasma, normalized the glomerular

Table 8. The effect of infliximab on the expression of pro-inflammatory and anti-inflammatory cytokines in the kidney (M±m)

Tissue	Group	IL-1β, %	TNF-α, %	IL-4, %	IL-10, %
		24 hours			
Glomerulus	Sham	5.8±0.3	4.9±0.3	4.2±0.2	7.8±0.4
	IR	49.9±1.1^{x}	69.7±1.3^{x}	15.5±0.8^{x}	12.4±0.4^{x}
	IR + INF 2	30.4±0.6^{xy}	50.1±1.1^{xy}	22.0±1.1^{xy}	32.2±1.0^{xy}
	IR+ INF 10	25.0±0.6^{xy}	40.6±0.9^{xy}	18.4±0.9^{xy}	49.7±1.1^{xy}
Nephrontubules	Sham	5.9±0.3	5.7±0.3	4.0±0.2	5.8±0.2
	IR	56.5±1.5^{x}	71.1±1.2^{x}	16.2±0.9^{x}	13.7±0.3^{x}
	IR + INF 2	36.6±0.7^{xy}	49.8±0.9^{xy}	18.9±1.1^{x}	27.9±0.8^{xy}
	IR+ INF 10	29.9±0.6^{xy}	43.6±0.7^{xy}	18.8±1.1^{x}	50.8±0.9^{xy}
		72 hours			
Glomerulus	Sham	5.6±0.3	5.5±0.3	4.9±0.2	8.3±0.4
	IR	45.0±0.9^{x}	63.6±1.2^{x}	11.5±0.6^{x}	20.2±0.6^{x}
	IR + INF 2	25.0±0.6^{xy}	42.6±1.1^{xy}	13.1±0.9^{x}	26.2±0.7^{xy}
	IR+ INF 10	22.9±0.4^{xy}	33.9±0.9^{xy}	18.4±0.9^{xy}	41.1±1.1^{xy}
Nephrontubules	Sham	4.7±0.3	5.7±0.3	4.2±0.2	6.0±0.2
	IR	49.4±1.3^{x}	62.2±1.3^{x}	15.0±0.9^{x}	10.5±0.3^{x}
	IR + INF 2	31.6±0.7^{xy}	41.5±0.9^{xy}	17.4±0.9^{x}	22.1±0.7^{xy}
	IR+ INF 10	27.9±0.7^{xy}	36.2±0.9^{xy}	18.5±1.1^{xy}	42.1±0.7^{xy}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; INF 2 – infliximab (at the dose of 2 mg/kg); INF 10 – infliximab (at the dose of 10 mg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group.

filtration rate and fractional sodium excretion. The level of renal parenchymal perfusion significantly increased. Also, a pathomorphological study with morphometry revealed an improvement in the histological pattern of renal tissue.

The obtained immunohistochemistry results clearly demonstrate the significant role of pro-inflammatory cytokines that negatively affect the renal functions after the episode of ischemia-reperfusion. In turn, infliximab, blocking them, had a significant renoprotective effect.

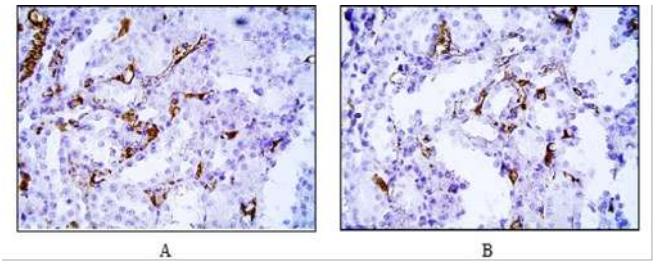


Figure 4. The effect of infliximab at the doses of 2 mg/kg (**A**) and 10 mg/kg (**B**) on the macrophage infiltration of kidney tissues 24 hours after reperfusion. **Note:** immunohistochemical reaction with antibodies to CD68; light microscopy, magnification ×400

Renoprotective properties of the combination of the helix B–derived erythropoietin peptide and infliximab

The combined administration of pHBSP at the dose of 25 mcg/kg and infliximab at the dose of 10 mg/kg in the ischemia/reperfusion kidney injury had a positive effect on the filtration function of the kidneys; the effect of the combination significantly exceeded the effect of these drugs in a single-drug therapy. So, 24 hours after reperfusion, the glomerular filtration rate reached 0.42±0.02 ml/min and was as close as possible to the group of sham operated animals. The same trend is observed for the nitrogen metabolism indicators: creatinine and serum urea, as well as fractional sodium excretion (Fig. 5). Seventy-two hours after the clamps were removed from the renal pedicle, the combined therapy with pHBSP and infliximab slightly exceeded the effectiveness of the single-drug therapy, which was reflected in a decrease in plasma creatinine and urea levels, an increase in glomerular filtration rate and a decrease in fractional sodium excretion.

A single administration of the combination of pHBSP and infliximab restored the level of microcirculation in all control time periods, significantly exceeding the values for these drugs in a single-drug therapy (Table 9).

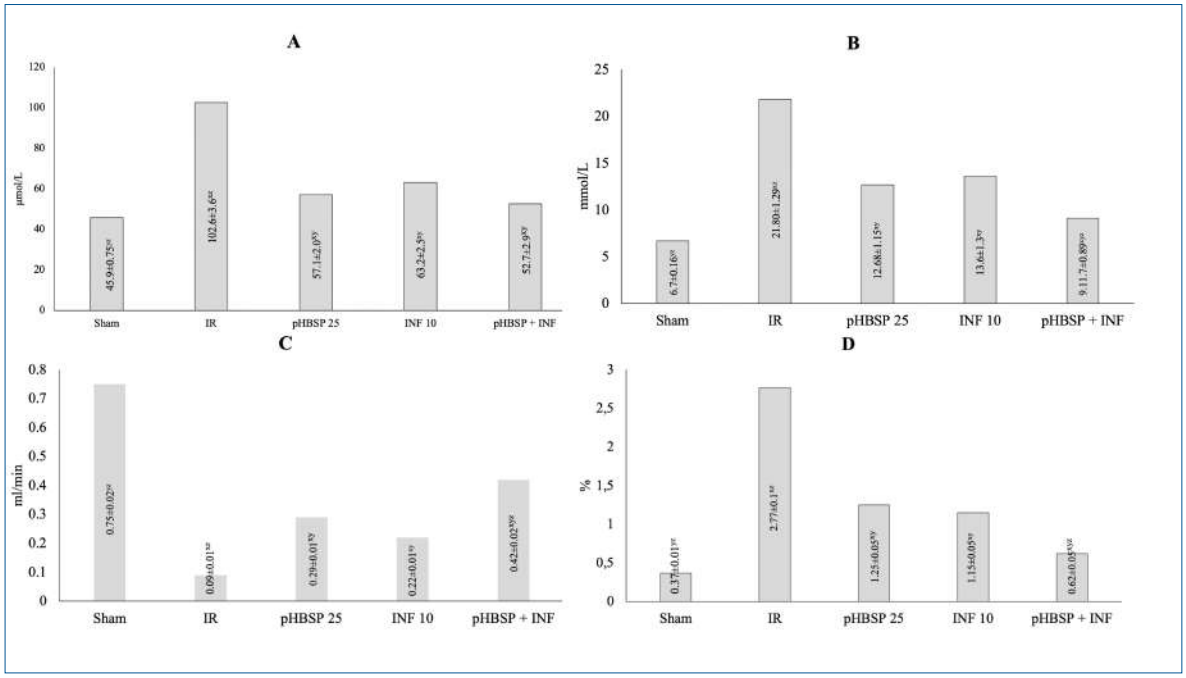


Figure 5. The effect of the erythropoietin-derived peptide mimetic (pHBSP) on the concentration of serum creatinine (A), urea (B), glomerular filtration rate (C) and fractional sodium excretion (D) 24 hours after reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia/reperfusion; EPO – erythropoietin (at the dose of 50 IU/kg); pHBSP 5 – erythropoietin-derived peptide mimetic (at the dose of 5 mcg/kg); pHBSP 25 – erythropoietin-derived peptide mimetic (at the dose of 25 mcg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group.

Histological examination of the animal kidneys treated with the combination of pHBSP 25 mcg/kg + Infliximab 10 mg/kg showed glomeruli without any signs of destruction, which is confirmed by an increase in the cross-sectional area of the renal corpuscle, renal glomerulus and subcapsular space in comparison with the single-drug therapy groups of animals (Table 10).

Table 9. The influence of the combination of pHBSP and infliximab on the renal microcirculation (M±m)

Experimental group	Microcirculation index 5 minutes, PU	Microcirculation index 24 hours, PU	Microcirculation index 72 hours, PU
Sham	900±42^{yz}	881±38^{yz}	890±36^{yz}
IR	219±12^{xz}	430±20^{xz}	410±20^{xz}
IR + pHBSP 25	693±28^{xy}	771±27^{xy}	625±36^{xy}
IR + INF 10	678±23^{xy}	743±34^{xy}	631±30^{xy}
IR + pHBSP + INF	809±41^{yz}	802±10^{y}	762±41^{xyz}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; pHBSP 25 – erythropoietin-derived peptide mimetic (at the dose of 25 mcg/kg); INF 10 – infliximab (at the dose of 10 mg/kg); x – $p<0.05$ in comparison with the group of sham-operated animals; y – $p<0.05$ in comparison with the ischemia/reperfusion group; ^{z} – $p<0.05$ in comparison with the pHBSP 25 group and $p<0.05$ in comparison with the infliximab group (10 mg/kg).

Twenty-two hours after reperfusion, the combined therapy with pHBSP and infliximab resulted in the minimal increase in the cells expressing pro-inflammatory cytokines in all structural elements of the kidney. Their level was on average 3 times lower than in the group of untreated animals.

Table 10. Morphometric characteristics of the structural elements of the nephron against the background of nephroprotection with the combination of pHBSP and infliximab (M±m)

Group	Cross-sectional area of the renal corpuscle, μm²	Height of epithelial cells of the proximal tubules, μm	Cross-sectional area of the renal corpuscle, μm²	Height of epithelial cells of the proximal tubules, μm
	24 hours		72 hours	
Sham	10496±123^{y}	11.9±0.7^{y}	10345±118^{y}	11.8±0.6^{y}
IR	8973±241^{x}	8.3±0.3^{xy}	8293±227^{x}	6.4±0.5^{x}
IR + pHBSP 25	9029±98^{x}	9.6±0.1^{xy}	9344±88^{xy}	8.5±0.2^{xy}
IR + INF 10	8994±75^{xy}	9.7±0.1^{xy}	9208±106^{xy}	8.5±0.2^{xy}
IR + pHBSP + INF	9724±122^{xyz}	10.3±0.2^{xyz}	9854±115^{xyz}	9.9±0.2^{xyz}

Note: sham – sham-operated animals; IR – renal ischemia-reperfusion; pHBSP 25 – erythropoietin-derived peptide mimetic (at the dose of 25 mcg/kg); INF 10 – infliximab (at the dose of 10 mg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group; ^{z} – $p<0.05$ in comparison with the pHBSP 25 group and $p<0.05$ in comparison with the infliximab group (10 mg/kg).

On the other hand, there was an increase in the number of IHC-positive cells expressing anti-inflammatory cytokines in the kidneys (Table 11). The minimal increase in CD68-positive cells in the interstitial kidney tissue (26.51±0.38%) was noted against the background of the combined therapy with pHBSP and infliximab 24 hours after reperfusion (Fig. 6). There was a decrease in cells expressing both pro-inflammatory and anti-inflammatory cytokines in all structural elements of the kidney 72 hours after reperfusion compared to those 24 hours later (Table 11). It was revealed that only combined therapy with pHBSP and infliximab leads to a significant increase in the expression of the anti-inflammatory cytokine IL-4, which reduces the severity of pathomorphological changes after ischemia/reperfusion kidney injury and reduces the risk of delayed fibrotic changes.

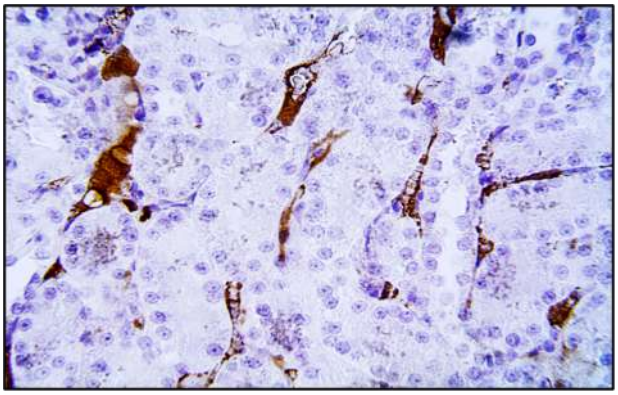


Figure 6. The effect of infliximab at the doses of 2 mg/kg (A) and 10 mg/kg (B) on the macrophage infiltration of kidney tissues 24 hours after reperfusion. **Note:** immunohistochemical reaction with antibodies to CD68; light microscopy, magnification ×400.

Table 11. The effect of the combination of pHBSP and infliximab on the expression of pro-inflammatory and anti-inflammatory cytokines in the kidney (M±m)

Tissue	Group	IL-1β, %	TNF-α, %	IL-4, %	IL-10, %
		24 hours			
Glomerulus	Sham	5.8±0.3	4.9±0.3	4.2±0.2	7.8±0.4
	IR	49.9±1.1^{x}	69.7±1.3^{x}	15.5±0.8^{x}	12.4±0.4^{x}
	IR + pHBSP 25	32.7±0.8^{xy}	49.4±1.1^{xy}	17.1±0.9^{x}	46.9±1.3^{xy}
	IR + INF 10	25.0±0.6^{xy}	40.6±0.9^{xy}	18.4±0.9^{xy}	49.7±1.1^{xy}
	IR + pHBSP + INF	17.6±0.6^{xyz}	32.9±0.8^{xyz}	20.0±1.2^{xy}	57.9±1.1^{xyz}
Nephrontubules	Sham	5.9±0.3	5.7±0.3	4.0±0.2	5.8±0.2
	IR	56.5±1.5^{x}	71.1±1.2^{x}	16.2±0.9^{x}	13.7±0.3^{x}
	IR + pHBSP 25	36.9±0.8^{xy}	51.7±0.7^{xy}	18.6±1.2^{x}	46.8±0.7^{xy}
	IR + INF 10	29.9±0.6^{xy}	43.6±0.7^{xy}	18.8±1.1^{x}	50.8±0.9^{xy}
	IR + pHBSP + INF	19.5±0.6^{xyz}	35.9±0.9^{xyz}	21.5±1.3^{xy}	63.6±0.9^{xyz}
		72 hours			
Glomerulus	Sham	5.6±0.3	5.5±0.3	4.9±0.2	8.3±0.4
	IR	45.0±0.9^{x}	63.6±1.2^{x}	11.5±0.6^{x}	20.2±0.6^{x}
	IR + pHBSP 25	29.3±0.6^{xy}	42.4±1.0^{xy}	16.4±0.9^{xy}	40.3±1.2^{xy}
	IR + INF 10	22.9±0.4^{xy}	33.9±0.9^{xy}	18.4±0.9^{xy}	41.1±1.1^{xy}
	IR + pHBSP + INF	15.4±0.6^{xyz}	21.6±0.7^{xyz}	18.1±0.9^{xy}	51.3±0.9^{xyz}
Nephron tubules	Sham	4.7±0.3	5.7±0.3	4.2±0.2	6.0±0.2
	IR	49.4±1.3^{x}	62.2±1.3^{x}	15.0±0.9^{x}	10.5±0.3^{x}
	IR + pHBSP 25	34.1±0.7^{xy}	39.3±0.8^{xy}	16.8±1.0^{x}	37.5±0.7^{xy}
	IR + INF 10	27.9±0.7^{xy}	36.2±0.9^{xy}	18.5±1.1^{xy}	42.1±0.7^{xy}
	IR + pHBSP + INF	20.1±0.6^{xyz}	24.4±0.9^{xyz}	20.9±1.2^{xy}	58.2±0.7^{xyz}

Note: sham – sham-operated animals; IR – renal ischemia/reperfusion; pHBSP 25 – erythropoietin-derived peptide mimetic (at the dose of 25 mcg/kg); INF 10 – infliximab (at the dose of 10 mg/kg); ^{x} – $p<0.05$ in comparison with the group of sham-operated animals; ^{y} – $p<0.05$ in comparison with the ischemia/reperfusion group; ^{z} – $p<0.05$ in comparison with the pHBSP 25 group and $p<0.05$ in comparison with the infliximab group (10 mg/kg).

The obtained results evidence the advantage of the combined administration of pHBSP and infliximab for the nephroprotection in simulated ischemia/reperfusion kidney injury surpassing in effectiveness the protective effects of pHBSP and infliximab in a single-drug therapy, due to the multimodal effect on pathogenetic processes involving in ischemia/reperfusion kidney injury.

The immunohistochemistry results confirmed the mechanism of renoprotective activity of infliximab and pHBSP: these substances block the macrophage and monocyte infiltration of kidney tissues, which leads to a significant decrease in the expression of pro-inflammatory cytokines in the structural elements of the nephron and contribute to the retention of the renal structure and function after simulated ischemia/reperfusion injury.

Determination of the role of ATP-sensitive potassium channels in the nephroprotective effect of the helix B–derived erythropoietin peptide and infliximab in simulated renal ischemia/reperfusion

The inhibition of ATP-sensitive potassium channels with glibenclamide led to a pronounced subsidence of the nephroprotective effects of pHBSP, which was confirmed by an increase in plasma creatinine levels to 91.9±4.1 mmol/L and 109.8±5.6 mmol/L, and urea to 19.4±1.6 mmol/L and 17.8±1.9 mmol/L 24 hours and 72 hours after reperfusion, respectively. Similar dynamics were noted for glomerular filtration rate, which was 0.14±0.01 ml/min and 0.13±0.01 ml/min, and fractional sodium excretion, which was 2.25±0.1% and 5.82±0.42% 24 hours and 72 hours after reperfusion, respectively. The level of microcirculation at all time points in the groups of animals treated with glibenclamide together with pHBSP was comparable to that of the ischemia/reperfusion group. The administration of glibenclamide together with pHBSP significantly worsened the histological pattern and the results of morphometry: microscopic examination revealed pronounced destructive changes, multiple local deposits of oxyphilic masses between the renal cortex and renal medulla, as well as in the tubule lumen and collector tubules. Shrunken glomeruli were found in most fields. The tubule lumen is dilated, and severe intracellular edema was noted. There was no significant dependence of the protective activity of infliximab on ATP-sensitive potassium channels.

It should be concluded that ATP-sensitive potassium channels play an important role in the realization of renoprotective effect of pHBSP in the simulated ischemia/reperfusion kidney injury, unlike infliximab, which showed nephroprotective activity through other ways independent of ATP-sensitive potassium channels.

Conclusion

The results of the performed study reliably confirm the renoprotective properties of pHBSP and infliximab, and also verify the advantage of their combined administration for correction of morphofunctional disorders in simulated ischemia/reperfusion kidney injury. The results of immunohistochemical study confirmed the mechanism of the renoprotective effect of infliximab and pHBSP: these substances reduce the macrophage and monocyteinfiltration of kidney tissues, which leads to a significant decrease in the expression of pro-inflammatory cytokines in the structural elements of the nephron and contributes to the retention of the renal structure and function after simulated ischemia/reperfusion injury.

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Conflict of Interests

The author declares no conflict of interests.

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