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Research Article

Correction of morphofunctional disorders with asialoerythropoietin and selective inhibitor of arginase II KUD975 in cases of ischemic kidney damage in the experiment

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Abstract

Introduction: Acute kidney injury (AKI), which is based on ischemic-reperfusion damage, is a widespread life-threatening condition and remains a serious public health problem with a high mortality rate among patients. Despite significant advances in various areas of medicine, the prevention and correction of ischemic-reperfusion kidney damage are still far from being at the desired level. Pharmacological preconditioning and the use of endothelioprotectors are promising areas in this field, therefore the purpose of this study was to analyze the nephroprotective properties of asialoerythropoietin and selective inhibitor of arginase II KUD975 in ischemic kidney damage in the experiment.

Materials and methods: The study was performed on 260 white adult male Wistar rats, each weighing 180-220 g. Ischemic-reperfusion damage was simulated by applying a clamp on the renal leg for 40 minutes. To determine a degree of correction caused by morphofunctional disorders traditional functional, biochemical and morphological criteria were used.

Results and discussion: When administering asialoerythropoietin and selective inhibitor of arginase II KUD975, there is observed an improvement in the glomerular filtration and microcirculation in the kidneys, decrease in the concentration of creatinine and urea, a decrease in fractional excretion of sodium and improvement in the histological pattern at different periods. The most pronounced nephroprotective effects are observed in the combined use of the test pharmacological agents, which are superior to such used in a monotherapy. The use of glibenclamide and L-NAME against the background of the correction of the pathology caused by asialoerythropoietin completely eliminates its positive effects. When glibenclamide and L-NAME are used against the background of correction of the pathology caused by the selective inhibitor of arginase II KUD975, its positive effects are completely eliminated by L-NAME. Glibenclamide does not eliminate positive effects.

Conclusions: The results of the experiment prove the presence of pronounced nephroprotective properties of asialoerythropoietin and selective inhibitor of arginase II KUD975 in ischemic kidney damage in the experiment. The most pronounced effects are observed in the combined use of these pharmacological agents. The leading role in causing the positive effects from asialoerythropoietin is played by the activation of K+ATP channels and the activation of eNOS. The leading role in causing the positive effects from the selective inhibitor of arginase II KUD975 is played by the activation of eNOS.

Keywords

acute kidney injury, ischemic-reperfusion kidney injury, endothelial dysfunction, pharmacological preconditioning, asialoerythropoietin, selective inhibitor of arginase II KUD975

Introduction

Acute kidney damage is a common life-threatening condition that affects one in five hospital patients (Zeng et al. 2014) and remains a serious public health problem with a high patient mortality rate (Basile and Yoder 2014, Kanagasundaram 2015). Delayed risks of acute renal damage include chronic kidney disease (CKD), end-stage CKD requiring replacement therapy and transplantation (Coca et al. 2012), cardiovascular events (Odutayo et al. 2017), and deterioration in quality of life (Villeneuve et al. 2016).

The leading pathogenetic link of AKI is ischemic and reperfusion injury of the kidneys (Khvan 2013, Yu et al. 2016). Ischemia-reperfusion is a pathological condition characterized by an initial decrease in the blood supply to the organ, followed by perfusion and repeated oxygenation later. With the development of ischemia, there is a decrease in the production of adenosine triphosphate (ATP) and intracellular pH, with further development of cell overloading with calcium, the generation of reactive oxygen species and apoptosis, which is more pronounced in the epithelial cells of the proximal tubules. Their damage, in turn, leads to tubular-glomerular feedback, which activates the renin-angiotensin-aldosterone system and further aggravates vasoconstriction and a decrease in the glomerular filtration rate (Munshi et al. 2011, Chatauret et al. 2014).

An important role in the pathophysiology of AKI is played by developing disorders of microcirculation and endothelial function. Damage to the endothelium of the microvascular bed leads to the expression of new markers on their surface, which contribute to the recruitment and adhesion of leukocytes and platelets, which leads to a further decrease in perfusion and oxygen delivery and additional damage to the endothelial cells and persistence of inflammation (Molitoris 2014, Ferenbach and Bonventre 2015). Endothelial dysfunction is characterized, in particular, by impaired ability for vasodilation, which is often explained by a reduction in the production of nitric oxide. One of the enzymes that affect the functional state of the endothelium is arginase, which is present in two isoforms: arginase I and arginase II (ArgII). Arginase catalyzes the hydrolysis of L-arginine to L-ornithine and urea and, thus, competes with NOS for the common substrate, L-arginine. In one study, it was demonstrated that the expression and activity of arginase II increased significantly after ischemia-reperfusion of the kidneys, along with the progression of renal tissue damage. Pharmacological blockade or genetic deficiency of ArgII provided protection for the kidneys in this model: animals had lower levels of creatinine and plasma urea. Blocking arginases using S-(2-boroethyl)-L-cysteine (BEC) reduced the severity of histopathological changes, oxidative stress and apoptosis processes, the synthesis of pro-inflammatory cytokines; increased the formation of nitric oxide and eNOS phosphorylation and contributed to the preservation of the mitochondrial ultrastructure. Thus, inhibition of arginaseы in acute kidney injury of ischemic-reperfusion genesis is one of the promising directions for the correction of this kind of damage.

Another important link of AKI pathogenesis is mitochondrial dysfunction, expressed to a greater extent in the epithelial cells of the proximal tubules. An increase in the production of reactive oxygen species, depletion of antioxidants, a change in pyridine nucleotide ratios, fluctuations in the concentration of calcium ions and an increase in inorganic phosphate in the matrix of mitochondria leads to the opening of the mitochondrial pore. The end result of its opening is the release of factors activating apoptosis (Wayel and Heaton 2004).

One of the universal mechanisms for the prevention of ischemic and reperfusion injuries is pharmacological preconditioning (PreC). The glycoprotein hormone erythropoietin is considered as one of the most studied pharmacological agents with preconditioning properties. The main targets of erythropoietin when causing a renoprotective effect are mitochondrial K^+_{ATP} channels (ATP-dependent K^+ channels) and NO biosynthesis. Activation of mitochondrial K^+_{ATP} channels not only protects mitochondria from damage, but also affects the activity of transcription factors: increases the expression of HIF (Bahlmann and Fliser 2009), reduces the activity of NF-κB (Liao et al. 2016), and has anti-apoptotic effects (Moore and Bellomo 2011). Renoprotective effects of erythropoietin were confirmed in a clinical study: the use of erythropoietin at a dose of 300 IU/kg in patients during coronary artery bypass surgery reduced the incidence of acute kidney injury (Song et al. 2009).

Thus, one of the most promising areas in the search for potential mechanisms and means to protect the kidneys from ischemic and reperfusion injuries is to protect the endothelium by restoring the balance of nitric oxide and launching preconditioning mechanisms. This make it possible to consider the drugs that inhibit the activity of arginase II and erythropoietin derivatives with improved pharmacokinetic properties and enhanced antihypoxic activity. as the most interesting pharmacological agents for studying.

Thereby the **objective** of this study was to analyze the nephroprotective action of asialoerythropoietin and selec-

tive inhibitor of arginase II KUD975 in the condition of experimental ischemia-reperfusion. In addition, this study looked at the role of K^+_{ATP} channels and eNOS in the nephroprotective effects of the test pharmacological agents.

Materials and research methods

Compliance with ethical and regulatory requirements in the performance of the research

The experimental part of the research was performed on the basis of the vivarium of Kursk State Medical University.

The work was organized and carried out in accordance with the following regulatory acts and guidelines governing the conduct of experimental research in the Russian Federation:

1. Order of the Ministry of Healthcare of the Russian Federation of April 1, 2016 No. 199n "On Approval of the Rules of Good Laboratory Practice"
2. GOST 33044-2014 "Principles of Good Laboratory Practice" (National Standard of the Russian Federation)
3. GOST 33217-2014 "Guidelines for the Maintenance and Care of Laboratory Animals. Rules for Maintenance and Care of Laboratory Predatory Mammals"
4. "Guidelines for Conducting Pre-clinical Trials of New Drugs" (2012) Ed. Mironova AN. Moscow, Grif and Co.
5. The ethical principles of handling laboratory animals were in accordance with "The European Convention for the Protection of Bertebral Animals Used for Experimental and Other Scientific Purposes. CETS No. 123 ".

Experimental animals

The study was performed on 260 white adult male rats of Wistar breed, each weighing 180–220 g. The experiments included the rats that had gone thrugh the quarantine regime (14 days) of the vivarium of Kursk State Medical University, without showing any signs of acute and chronic diseases.

Simulation of bilateral renal ischemia with subsequent reperfusion

After 12 hours of food deprivation, the laboratory animal was anesthetized by intraperitoneal injection of chloral hydrate (Sigma-Aldrich) at a dose of 300 mg/kg of the animal body weight. Then the animal was moved to a room with an ambient temperature of at least 250° C and was fixed on a heated veterinary table. Ischemia was reproduced by applying atraumatic vascular clamps on the renal pedicles. The correctness of the clamp application was controlled by changing the color of the kidneys. A wipe moistened with warm 0.9% sodium chloride solution was placed on the wound. Forty minutes later, the clamps were successively removed, and the microcirculation was recorded within five minutes. Next, 4-5 ml of warm 0.9% sodium chloride solution was injected into the abdominal cavity, and the wound was sutured layer-by-layer. After 24 or 72 hours of reperfusion, the laboratory animal was anesthetized by intraperitoneal injection of chloral hydrate at a dose of 300 mg/kg of the animal body weight, relaparotomy was performed, microcirculation readings were recorded, and blood was sampled from the right ventricle for biochemical studies.

Assessment of the level of microcirculation when simulating the pathology

Microcirculation in the cortical layer of the kidneys was measured using the MP100 hardware-software complex (Biopac System, Inc., USA) with a LDF100C laser Doppler flowmetry (LDF) module and a TSD143 surface sensor, which was applied on the kidney middle part and did not affect the area of the kidneyhilum. The microcirculation level was measured immediately after removing the vascular clamps from the kidney for 5 minutes, after 24 or 72 hours of reperfusion, depending on the experimental group. Registration and processing of results were performed using AcqKnowledge software 3.8.1. The values of the parameters were expressed in perfusion units (PU).

Biochemical markers of acute kidney injury

The level of serum creatinine and urea was determined by a photocolorimetric method using standard reagent kits from Diakon JSC (Russia) on a URIT800 Vet biochemical analyzer (URIT Medical Electronic Co., Ltd., China).

The concentration of sodium ions in the serum was determined by the standard method described in manuals attached to the sets for an automatic analyzer K/N "Ion meter ETs-59" (Russia).

Biochemical analysis of urine. Calculation of endogenous creatinine clearance (glomerular filtration rate) and fractional excretion of sodium.

To obtain urine samples, the animals were placed in metabolic cells with free access to water for 12 or 24 hours. Next, diuresis was measured, and samples were taken for further study.

Endogenous creatinine clearance (glomerular filtration rate (GFR) was calculated as follows (Formula 1):

$$\mathrm{GFR} = \frac{\text{urine creatinine (μmol/l)} \times \text{urine volume (ml)}}{\text{serum creatinine (μmol/l)} \times \text{time (min)}}$$

Formula 1. Formula for calculating the glomerular filtration rate

Fractional excretion of sodium (FENa) was calculated using the following formula (Formula 2):

$$\mathrm{FEna} = \frac{\text{sodium urine} \times \text{serum creatinine}}{\text{sodium serum} \times \text{creatinine urine}} \times 100\%$$

Formula 2. Formula for calculating the fractional excretion of sodium

Morphological methods for assessing changes in the kidneys

For histological examination, the obtained cadaver material was fixed in 10% neutral buffered formalin solution. When fixation is done, a tissue sample (1x1 cm) was dissected out of the biomaterial, embedded in paraffin using a standard procedure, and the sections of 5–7 μm thick were made.

The obtained histological sections were stained with hematoxylin and eosin, according to the method of van Gieson, according to Mallory.

Microscopic examination and photographing were carried out using an optical system consisting of a Leica CME microscope and a DCM-510 eyepiece camera magnifying x100, x200 and x400 times, with the images documented in the FUTURE WINJOE software supplied with the eyepiece camera.

The morphometric study included the determination of the following indicators: on micrographs, using the ImagoJ software, the height of epithelial cells in the proximal and distal parts of the nephron was measured, as well as the cross-sectional area of the renal corpuscle, vascular glomerulus and subcapsular space.

Study design. The choice of administration modes for pharmacological agents

The study of the activity of asialoerythropoietin (Protein Contour Company Ltd.) was carried out at doses of 0.4 μg/kg and 2.4 μg/kg once, 30 minutes before simulating ischemia. The dose of 0.4 μg/kg is selected based on the minimum effective dose of erythropoietin, equivalent to 50 IU of erythropoietin, and the dose of 2.4 μg/kg corresponds to the maximum recommended dose of erythropoietin in humans (300 IU/kg).

KUD975 activity (10 mg film-coated tablets) was tested at doses of 1 mg/kg and 3 mg/kg once intragastrically, 120 minutes before ischemia simulation. The dose of 1 mg/kg was chosen based on the recalculation of the potential minimum dose recommended for use in humans, 3 mg/kg is the dose that demonstrated pronounced protective properties in other experimental models. The mode of administration is based on the pharmacokinetic profile of the drug.

Recombinant erythropoietin (Epokrin, The State Research Institute of Highly Pure Biopreparations) was administered subcutaneously at a dose of 50 IU/kg once, 30 minutes before ischemia simulation. The dose and mode of administration were selected based on the previously identified protective effects on ischemia-reperfusion models (Dolzhikova 2013).

L-norvaline was administered intraperitoneally at a dose of 100 mg/kg once, 30 minutes before simulating ischemia. The dose and mode of administration are justified by the protective effects confirmed in the previous experimental studies (Pokrovsky et al. 2013, 2014).

The blocker of ATP-dependent K^+ channels – glibenclamide (Maninil, Berlin-Chemie AG) was administered intragastrically at a dose of 50 mg/kg 30 minutes before the administration of the pharmacological agents.

The eNOS inhibitor N-nitro-L-arginine methyl ester (Sigma Aldrich, USA) was administered at a dose of 25 mg/kg 30 minutes before the administration of the pharmacological agents.

The animals were randomized by weight and formed into the following experimental groups of 10 each:

1. Sham (sham-operated) (1 day)
2. Sham (sham-operated) (3 days)
3. Control (ischemia-reperfusion) (1 day)
4. Control (ischemia-reperfusion) (3 days)
5. AsEPo (asialoerythropoietin) 0.4 μg/kg (1 day)
6. AsEPo (asialoerythropoietin) 0.4 μg/kg (3 days)
7. AsEPo (asialoerythropoietin) 2.4 μg/kg (1 day)
8. AsEPo (asialoerythropoietin) 2.4 μg/kg (3 days)
9. KUD975 1 mg/kg (1 day)
10. KUD975 1 mg/kg (3 days)
11. KUD975 3 mg/kg (1 day)
12. KUD975 3 mg/kg (3 days)
13. EPo 50 IU (recombinant erythropoietin 50 IU) (1 day)
14. EPo 50 IU (recombinant erythropoietin 50 IU) (3 days)
15. L-Norvaline 100 mg/kg (1 day)
16. L-Norvaline 100 mg/kg (3 days)
17. AsEPo 2.4 μg/kg + KUD975 3 mg/kg (1 day)
18. AsEPo 2.4 μg/kg + KUD975 3 mg/kg (3 days)
19. AsEPo 2.4 μg/kg + glibenclamide 50 mg/kg (1 day)
20. AsEPo 2.4 μg/kg + glibenclamide 50 mg/kg (3 days)
21. AsEPo 2.4 μg/kg + L-NAME 25 mg/kg (1 day)
22. AsEPo 2.4 μg/kg + L-NAME 25 mg/kg (3 days)
23. KUD975 3 mg/kg (1 day) + L-NAME 25 mg/kg
24. KUD975 3 mg/kg (3 days) + L-NAME 25 mg/kg
25. KUD975 3 mg/kg (1 day) + glibenclamide 50 mg/kg (1 day)
26. KUD975 3 mg/kg (3 days) + glibenclamide 50 mg/kg (3 days)

The protocol consisted of the following sections:

1. Simulation of bilateral ischemia of the kidneys with subsequent reperfusion within 24 or 72 hours and their correction using asialoerythropoietin and selective inhibitor of arginase II KUD975.
2. Measurement of microcirculation within the first five minutes of reperfusion.
3. Urine collection for 12 or 24 hours, followed by measuring diuresis and biochemical parameters (after 12 or 48 hours of reperfusion).
4. After 24 or 72 hours in anesthesia with chloral hydrate, the assessment of renal microcirculation, sampling venous blood for biochemical studies and calculating GFR, and also recovering kidney tissues for pathological studies.

Statistical processing of research results

Descriptive statistics methods were applied to all the data: the data was checked for normal distribution. The type of distribution was determined by the Shapiro-Wilk criterion. In the case of a normal distribution, the mean value (M) and standard error of the mean (m) were calculated. Intergroup differences were analyzed by parametric (Student's t-test) or non-parametric (Mann-Whitney test) methods, depending on the type of distribution. The statistical significance of the differences between morphological changes after their ranking was evaluated using the Mann-Whitney method of analyzing non-parametric data (Glanz 1999, Sydorenko 2003). All the calculations were performed using the statistical package of Microsoft Excel 7.0.

Results and discussion

Nephroprotective effects of asialoerythropoietin and selective inhibitor of arginase II KUD975 in experimental renal ischemia

In the control animals group (ischemia-reperfusion), 24 hours after ischemia there was no statistically significant increase in serum creatinine level; however, there was a drop in the glomerular filtration rate from 0.51±0.03 ml/min in the group of sham-operated animals to 0.17±0.02 ml/min. After 72 hours, there was a progressive decrease in the filtration capacity of the kidneys, which showed in an increase in the level of serum creatinine to 120±3.45 μmol/l and a 8.2-time decrease in the glomerular filtration rate from 0.49±0.03 ml/min in the group of sham-operated animals to 0.06±0.01 ml/min ($p<0.05$).

The administration of asialoerythropoietin at doses of 0.4 μg/kg and 2.4 μg/kg 30 minutes before the induction of ischemia led to an increase in the glomerular filtration rate to 0.32±0.04 ml/min and 0.37±0.03 ml/min after 24 hours of reperfusion, respectively, which was statistically significantly different from that in the group of control animals ($p<0.05$) (Fig. 1a). The introduction of a selective inhibitor of arginase II against the background of simulating ischemia-reperfusion of the kidneys also led to a significant increase in the glomerular filtration rate to 0.24±0.02 ml/min and 0.31±0.03 ml/min after 24 hours of reperfusion at doses of 1 mg/kg and 3 mg/kg, respectively. The test drugs had no effect on the serum creatinine level on the first day.

After 72 hours of reperfusion, a dose-dependent improvement in the filtration capacity of the kidneys was also observed, which was expressed in a statistically sig-

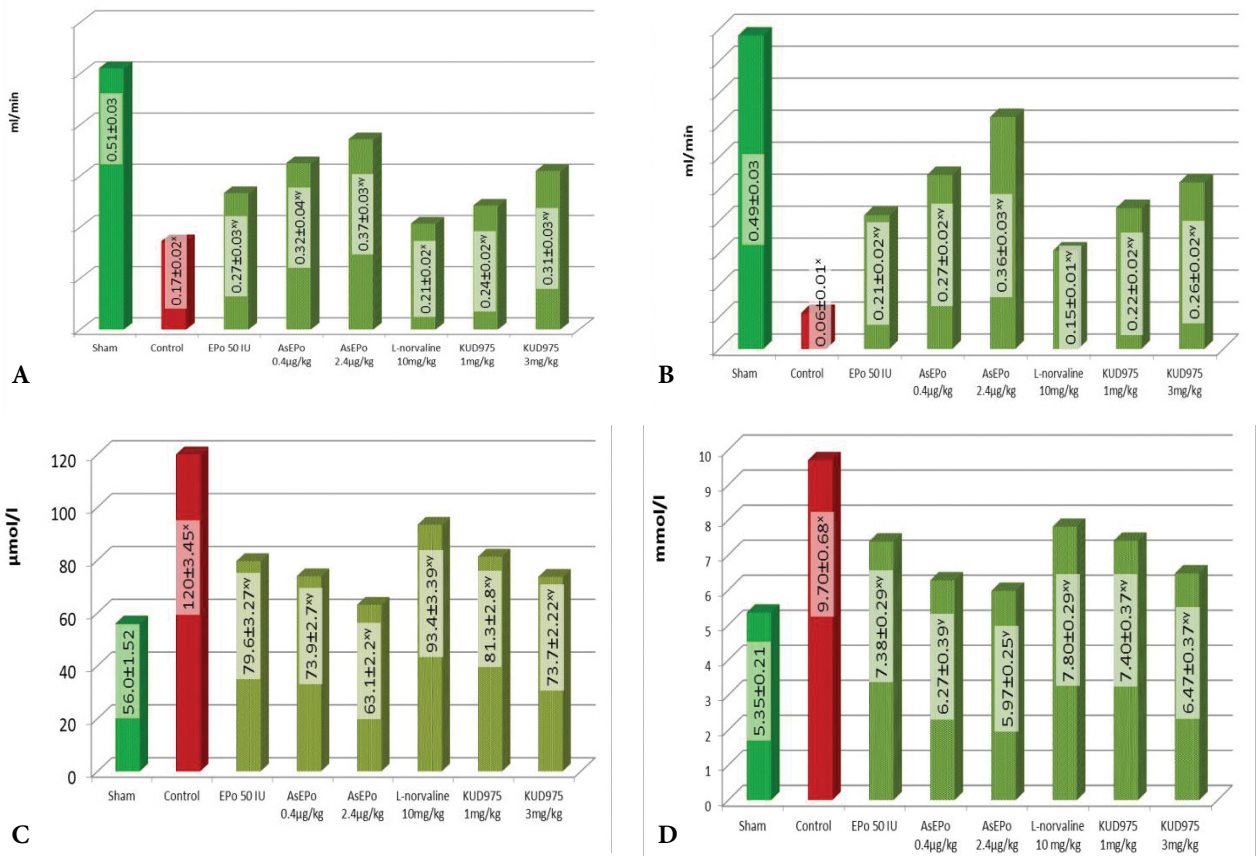

Figure 1. The effect of the test drugs on the glomerular filtration rate 24 after ischemia (A), 72 hours after ischemia (B), creatinine concentration 72 hours after ischemia (C), serum urea concentration 24 hours after ischemia (D). *Notes*: x – $p<0.05$ compared with the sham (sham-operated) group of animals; y – $p<0.05$ compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

nificant ($p<0.05$) decrease in the concentration of serum creatinine to 73.9±2.7 μmol/l and 63.1±2.2 μmol/l (Fig. 1C) and recovery of glomerular filtration rate to 0.27±0.02 ml/min and 0.36±0.03 ml/min (Fig. 1B) against the background of using asialoerythropoietin in the test doses, respectively. When using KUD975 in the test doses, there was a statistically significant decrease ($p<0.05$) in the concentration of serum creatinine to 81.3±2.8 μmol/l and 73.7±2.22 μmol/l and recovery of the glomerular filtration rate to 0.22±0.02 ml/min and 0.26±0.02 ml/min, respectively. The activity of the test drugs significantly exceeded the nephroprotective properties of the comparison drugs (Fig. 1).

A study of the serum urea concentration showed that the simulation of a 40-minute bilateral model of kidney ischemia-reperfusion led to an increase in this indicator after 24 hours of reperfusion from 5.35±0.21 mmol/l to 9.7±0.68 mmol/l (Fig. 1D), which was somewhat leveled after 72 hours, reaching a level of 8.33±0.23 mmol/l.

Under the influence of asialoerythropoietin, a statistically significant ($p<0.05$) decrease in the serum urea concentration occurred both on the first and the third day of the experiment, reaching at a single dose of 2.4 μg/kg 5.97±0.25 mmol/l and 6.76±0.33 mmol/l after 24 and 72 hours of reperfusion, respectively. Under the influence of KUD975, a statistically significant ($p<0.05$) decrease in the serum urea concentration occurred both on the first and third days of the experiment, reaching with a single prophylactic administration at a dose of 3 mg/kg 6.47±0.37 mmol/l and 6.9±0.23 mmol/l after 24 and 72 hours of reperfusion, respectively.

When assessing the functional state of the renal tubules, the rate of fractional excretion of sodium in the group of sham-operated animals increased slightly from 0.38±0.02% to 0.5±0.02%, which may be due to the mobilization of sodium in the postoperative period. Simulation of acute kidney injury of ischemic-reperfusion genesis led to an increase in FeNa to 2.24±0.12% to 7.4±0.78% after 24 and 72 hours of reperfusion, respectively (Table 1), which together with a decrease in glomerular rate filtering may indicate the development of acute tubular necrosis. Against the background of the use of asialoerythropoietin, a dose-dependent 2.2- and 2.5-time decrease in the fractional excretion of sodium was observed on the first days of the experiment, exceeding the protective effects of erythropoietin. On the 3rd day of the experiment, the correction with asialoerythropoietin made it possible to achieve the target values of fractional excretion of sodium of less than 2% to 1.57±0.09% and 1.3±0.09% at doses of 0.4 μg/kg and 2.4 μg/kg, respectively. The use of KUD975 at doses of 1 mg/kg and 3 mg/kg led to an improvement in the functional state of the renal tubules recorded by a decrease in the fractional excretion of sodium, which was more pronounced on the 3rd day of the experiment and was 2.27±0.17% and 1.8±0.09%, respectively.

Table 1. Dynamics of fractional excretion of sodium against the background of correction with asialoerythropoietin and selective inhibitor arginase II KUD975 (M±m; n=10)

Experimental group	Day 1	Day 3
Sham	0.38±0.02%	0.5±0.02%
Control	2.24±0.12%^{x}	7.4±0.78%^{x}
EPo 50 ME	1.42±0.11%^{xy}	2.5±0.09%^{xy}
AsEPo 0.4 μg/kg	1.03±0.11%^{xy}	1.57±0.09%^{xy}
AsEPo 2.4 μg/kg	0.91±0.09%^{xy}	1.3±0.09%^{xy}
L-Norvaline 100 mg/kg	1.42±0.13%^{xy}	2.76±0.21%^{xy}
KUD975 1 mg/kg	1.16±0.12%^{xy}	2.27±0.17%^{xy}
KUD975 3 mg/kg	1.14±0.11%^{xy}	1.8±0.09%^{xy}

Notes: ^{x} – $p<0.05$ compared with the sham (sham-operated) group of animals; y – $p<0.05$ compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

The dynamics of microcirculatory disorders in the kidneys corresponded to the dynamics of biochemical and functional parameters: in the group of sham-operated animals it was 904.6±60.43 PU, 870±96.48 PU and 859±67.98 PU 5 minutes, 24 hours and 72 hours after the experiment onset, respectively. Simulation of acute kidney injury resulted in a statistically significant reduction in microcirculation to 209±24.42 PU after 5 minutes of reperfusion, followed by recovery to 418.1±46.02 PU and 315.5±13.67 PU after 24 and 72 hours of reperfusion, respectively. A single injection of asialoerythropoietin at doses of 0.4 mg/kg and 2.4 mg/kg for 30 minutes and selective inhibitor of arginase II KUD975 at doses of 1 mg/kg and 3 mg/kg for 120 minutes before ischemia resulted in the restoration of the microcirculation level at all time points of the experiment, statistically exceeding the performance of the group which received the comparison drugs ($p<0.05$) (Table 2).

A morphological study revealed a decrease in the height of the epithelium of the proximal and distal tubules in animals with ischemia-reperfusion of the kidneys both on the 1st day and on the 3rd day. When used for the correction of the test pharmacological agents, a statistically significant increase in the height of the epithelium of the proximal and distal tubules occurs, but it does not reach the target level (Table 3).

Nephroprotective effects of a combination of asialoerythropoietin and a selective inhibitor of arginase II in experimental renal ischemia

A combined therapy with asialoerythropoietin at a dose of 2.4 kg / kg and KUD975 at a dose of 3 mg/kg helped to improve the filtration capacity of the kidneys, which is superior by effecacy to the monotherapy with these drugs. So, on day 1 of the experiment, the glomerular filtration rate increased to 0.45±0.03 ml/min, being not significantly different from the indicators of sham-operated animals (Fig. 2A).

On the 3rd day of the experiment, the combination therapy with asialoerythropoietin and KUD975 exceeded the efficacy of the monotherapy with these drugs, which was expressed in the form of normalization of creatinine to 61±2.17 μmol/L and an increase in glomerular filtration rate to 0.42±0.03 ml/min.

Table 2. Dynamics of microcirculation indicators in the kidneys against the background of the correction with asialoerythropoietin and selective inhibitor of arginase II KUD975 (M±m; n=10)

Experimental group	Microcirculation rate, PU		
	5 minutes	Day 1	Day 3
Sham	904.5±60.43	870.5±96.18	859±67.98
Control	209±24.42^{x}	418.1±46.02^{x}	315.5±13.67^{x}
EPo 50 ME	459.8±24.06^{xy}	662.9±22.71^{xy}	490.5±21.81^{xy}
AsEPo 0.4 µg/kg	489.6±33.65^{xy}	636.4±20.93^{xy}	521.8±20.78^{xy}
AsEPo 2.4 µg/kg	670.4±54.19^{xy}	725.6±47.41^{y}	689.3±46.52^{y}
L-Norvaline 100 mg/kg	437.9±29.1^{xy}	657.9±18.81^{xy}	441.4±11.63^{xy}
KUD975 1 mg/kg	431.1±32.95^{xy}	691.8±2347^{y}	485±16.42^{xy}
KUD975 3 mg/kg	604.7±43.51^{xy}	718.2±44.52^{y}	653.6±61.99^{xy}

Notes: ^{x} – p<0.05 in comparison with the sham (sham operated) animals; y - p<0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

Table 3. Dynamics of the height of the epithelium of the proximal and distal tubules against the background of the correction with asialoerythropoietin and selective inhibitor of arginase II KUD975 (M±m; n=10)

Group	24 hours		72 hours	
	Epithelium height of the proximal tubules	Epithelium height of the distal tubules	Epithelium height of the proximal tubules	Epithelium height of the distal tubules
Sham	11.28±1.56	7.08±1.43	11.28±1.56	7.08±1.43
Control	8.21±0.21^{x}	6.46±0.15^{x}	6.56±0.74^{x}	4.24±0.73^{x}
EPo 50 ME	8.7±0.1^{xy}	6.58±0.09^{x}	8.25±0.1	5.65±0.09^{xy}
AsEPo 0.4 µg/kg	9.52±0.12^{xy}	6.63±0.09^{x}	9.39±0.08^{xy}	6.28±0.09^{xy}
AsEPo 2.4 µg/kg	9.72±0.11^{xy}	6.65±0.09^{x}	9.93±0.07^{xy}	6.33±0.09^{xy}
L-Norvaline 100 mg/kg	8.38±0.1^{x}	6.5±0.08^{x}	7.98±0.1^{xy}	5.55±0.1^{xy}
KUD975 1 mg/kg	9.33±0.1^{xy}	6.54±0.09^{x}	8.29±0.09^{xy}	5.96±0.09^{xy}
KUD975 3 mg/kg	9.4±0.09^{xy}	6.61±0.09^{x}	8.57±0.1^{xy}	6.19±0.1^{xy}

Notes: ^{x} – p<0.05 in comparison with the sham (sham operated) animals; y - p<0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

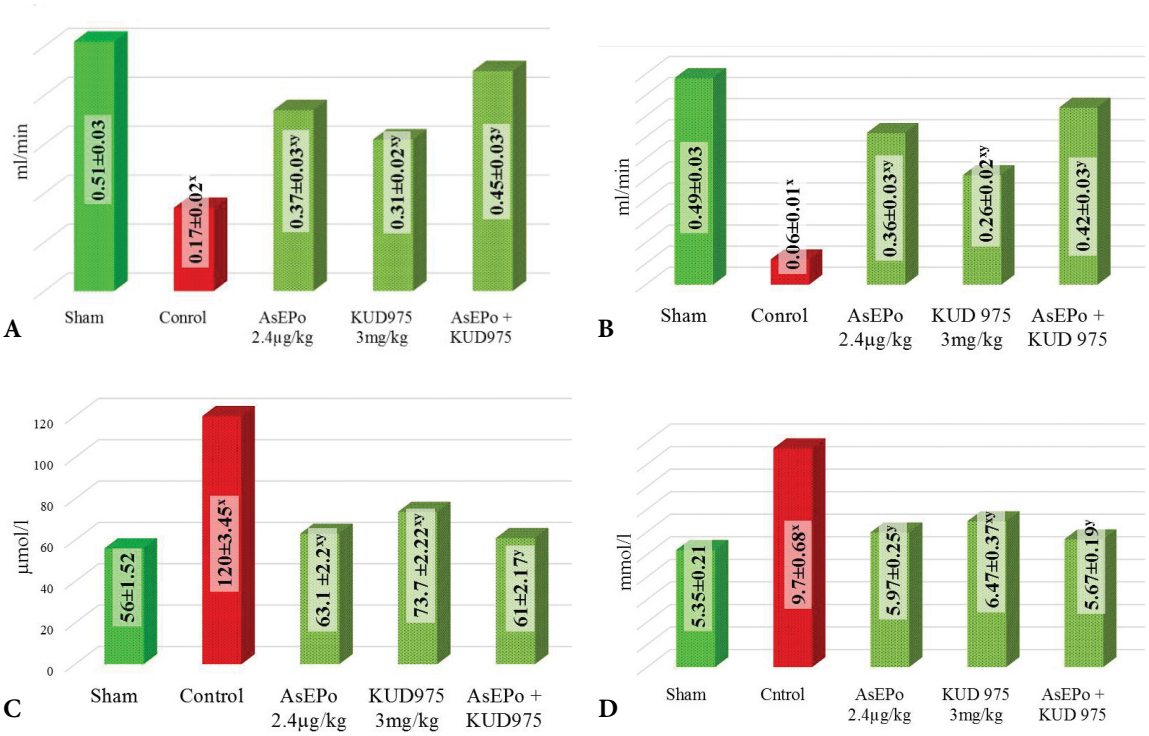

Figure 2. The effect of the test drugs on the glomerular filtration rate after 24 after ischemia (A), after 72 hours (B), creatinine concentration after 72 hours (C), serum urea concentration after 24 hours (D). *Notes:* x – p<0.05 in comparison with the sham (sham operated) animals; y – p<0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

The serum urea concentration against the background of combined pharmacotherapy using asialoerythropoietin and selective inhibitor of arginase II KUD975 decreased and reached 5.67±0.19 mmol/L and 6.13±0.16 mmol/L after 24 and 72 hours of reperfusion, respectively. The efficacy of pharmacotherapy surpassed that of monotherapy regimens with these drugs.

Against the background of correction with asialoerythropoietin and selective inhibitor of arginase II KUD975, tubular dysfunction was leveled, resulting in a decrease in the fractional excretion of sodium to 0.65±0.04% and 0.8±0.04% after 24 and 72 hours of reperfusion, respectively. These indicators were significantly different from those of animals in the groups with correction with asialoerythropoietin or KUD975 (Table 4).

A single prophylactic use of a combination of asialoerythropoietin and selective inhibitor of arginase II KUD975 resulted in the restoration of the level of microcirculation at all time points of the experiment, exceeding the indicators of the group using these drugs in a monotherapy (Table 5).

The morphological study revealed that an increase in the height of the epithelium of the proximal and distal tubules in animals with a combined use of asialoerythropoietin and selective inhibitor of arginase II KUD975 is even more pronounced than in animals treated with a monotherapy (Table 6).

The role of eNOS and K^+_{ATP} channels in inducing the nephroprotective effects of asialoerythropoietin and selective inhibitor of arginase II in experimental ischemia-reperfusion of the kidneys

The administration of the K^+_{ATP} blocker of glibenclamide channels completely eliminated the positive effects of asialoerythropoietin, which was expressed in a decrease in the glomerular filtration rate and had no nephroprotective effects of the selective inhibitor of arginase II. The use of an endothelial NO synthase inhibitor, L-NAME 30 minutes before the administration of the pharmacological agents leveled the positive effects of both asialoerythropoietin and KUD975 to the level of the indicators of the GFR group with simulated ischemia-reperfusion (Fig. 3A).

Table 4. Dynamics of the fractional excretion of sodium against the background of the correction with asialoerythropoietin and selective inhibitor of arginase II KUD975 (M±m; n=10)

Experimental group	Day 1	Day 3
Sham	0.38±0.02%	0.5±0.02%
Control	2.24±0.12%^{x}	7.4±0.78%^{x}
AsEPo 2.4 μg/kg	0.91±0.09%^{xy}	1.3±0.09%^{xy}
KUD975 3 mg/kg	1.14±0.11%^{xy}	1.8±0.09%^{xy}
AsEPo 2.4 μg/kg + KUD975 3 mg/kg	0.65±0.04%^{xy}	0.8±0.04%^{xy}

Notes: ^{x} – p<0.05 in comparison with the sham (sham operated) animals; y – p<0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

Table 5. Dynamics of indicators of microcirculation in the kidneys against the background of the correction with asialoerythropoietin and selective inhibitor of arginase II KUD975 (M±m; n=10)

Experimental group	Microcirculation rate, PU		
	5 minutes	Day 1	Day 3
Sham	904.5±60.43	870.5±96.18	859±67.98
Control	209±24.42^{x}	418.1±46.02^{x}	315.5±13.67^{x}
AsEPo 2.4 μg/kg	670.4±54.19^{xy}	725.6±47.41^{y}	689.3±46.52^{y}
KUD975 3 mg/kg	604.7±43.51^{xy}	718.2±44.52^{y}	653.6±61.99^{xy}
AsEPo 2.4 μg/kg + KUD975 3 mg/kg	803.4±25.23^{y}	775.5±29.13^{y}	801.3±20.16^{y}

Notes: ^{x} – p<0.05 in comparison with the sham (sham operated) animals; y – p<0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

Table 6. Dynamics of the height of epithelium of the proximal and distal tubules against the background of the correction by the combination of asialoerythropoietin and selective inhibitor of arginase II KUD975 (M±m; n=10)

Group	24 hours		72 hours	
	Epithelium height of the proximal tubules	Epithelium height of the distal tubules	Epithelium height of the proximal tubules	Epithelium height of the distal tubules
Sham	11.28±1.56	7.08±1.43	11.28±1.56	7.08±1.43
Control	8.21±0.21^{x}	6.46±0.15^{x}	6.56±0.74^{x}	4.24±0.73^{x}
AsEPo 2.4 μg/kg	9.72±0.11^{xy}	6.65±0.09^{x}	9.93±0.07^{xy}	6.33±0.09^{xy}
KUD975 3 mg/kg	9.4±0.09^{xy}	6.61±0.09^{x}	8.57±0.1^{xy}	6.19±0.1^{xy}
AsEPo 2.4 μg/kg + KUD975 3 mg/kg	10.32±0.14^{xy}	6.9±0.08^{y}	10.66±0.11^{y}	6.85±0.11^{y}

Notes: ^{x} – p<0,05 in comparison with the sham (sham operated) animals; y – p<0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

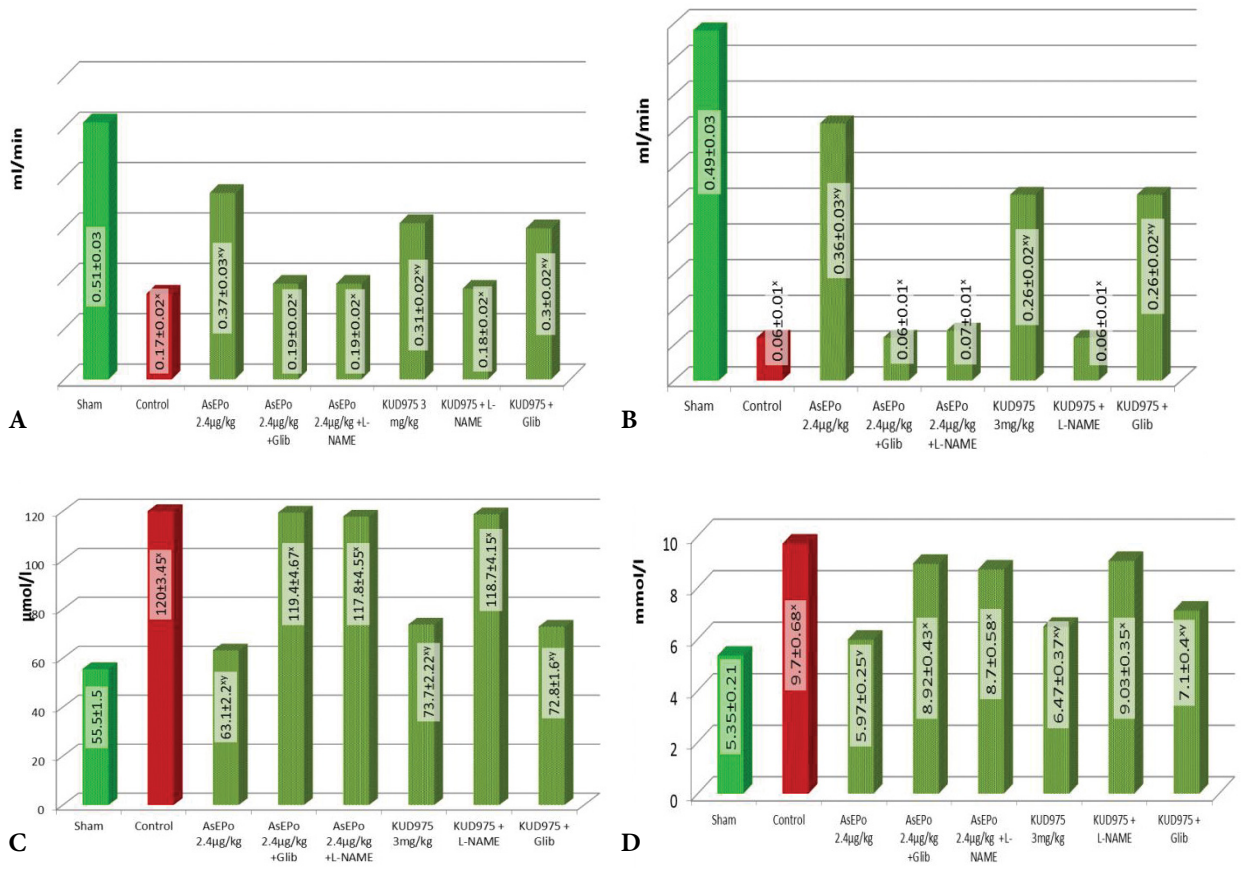

Figure 3. Effect of the test drugs against the background of glibenclamide and L-NAME on the glomerular filtration rate 24 after ischemia (A), after 72 hours (B), creatinine concentration after 72 hours (C), serum urea concentration after 24 after ischemia (D). *Notes:* x – $p<0.05$ compared with the sham (sham-operated) group of animals; y – $p<0.05$ compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – assayed erythropoietin.

After 72 hours of reperfusion, a similar trend was observed: against the background of using glibenclamide, the protective effects of asialoerythropoietin were leveled, which manifested itself in the growing creatinine level and a decrease in glomerular filtration rate to the level of the indicators of the control group. In the group with the introduction of glibenclamide and KUD975, the index of glomerular filtration rate did not significantly differ from those of the group with pathology simulation and the use of KUD975, reaching 0.3±0.02 ml/min. Preliminary administration of L-NAME led to the elimination of the nephroprotective properties of both asialoerythropoietin and selective inhibitor of arginase II (Fig. 3B).

The serum urea concentration also increased against the background of prior administration of the eNOS inhibitor, both in the groups treated for correction with asialoerythropoietin and in the groups using KUD975; however, the blockade of K+ATP channels did not lead to an increase in the level of urea in the group against the background of the KUD975 correction.

When assessing the functional state of the renal tubules, there was an increase in damage to the tubular apparatus in the group with the preliminary administration of glibenclamide during the use of asialoerythropoietin, and no dynamics against the use of the selective inhibitor of arginase II. The blockade of NO synthesis led to the leveling of the effects of both classes of drugs, which was expressed in an increased fractional excretion of sodium both after 24 and 72 hours of reperfusion (Table 7).

At all time intervals, when measuring the level of microcirculation against the background of the introduction of glibenclamide, the disappearance of the protective effects of asialoerythropoietin was observed, expressed in the growth of microcirculatory disorders to reach the indicators of the control group. In the group with a prophylactic administration of KUD975, K+ATP channels had no contribution to the realization of nephroprotective properties. Preliminary administration of L-NAME at a dose of 25 mg/kg led to the elimination of the nephroprotective properties of both asialoerythropoietin and selective inhibitor of arginase II (Table 8).

The morphological study revealed that the height of the epithelium of the proximal and distal tubules during the correction with asialoerythropoietin against the background of glibenclamide and L-NAME approaches the parameters of the animals in the control group. When correcting by means of selective inhibitor of arginase II KUD975 against the background of glibenclamide, the effect of the therapy is maintained; whereas when correcting by means of selective inhibitor of arginase II KUD975 against the background of L-NAME it is leveled (Table 9).

The results obtained indicate the dose-dependent nephroprotective properties of asialoerythropoietin. The protective effects of asialoerythropoietin are explained by its ability to bind to heterodimeric receptors for erythropoietin (Carelli et al. 2011, Yakovlev et al. 2016) and exert an anti-apoptotic, antioxidant effect (Kaneko et al. 2013, Gaddam et al. 2013), a preconditioning effect (Kapitsinou and Haase 2015, Oba et al. 2015, Heyman et al. 2016, Khaksari et al. 2017) and an ability to restore the system of NO synthesis (Elshiekh et al. 2017).

Selective inhibitor of arginase II KUD975 also showed dose-dependent nephroprotective effects. They are associated with blockade of arginase II. When this occurs, eNOS is activated, which leads to the correction of endothelial dysfunction (Suwanpradid et al. 2014, Rath et al. 2014, Pandey et al. 2014, Nara et al. 2015, Krause et al. 2015, Steppan et al. 2016).

The high efficacy of the combined therapy can be explained by the effect of drugs on various pathogenetic links of ischemic and reperfusion kidney injuries with activating the mechanisms of pharmacological preconditioning and implementation of endothelium protective effects.

The leveling of the positive effects of asialoerythropoietin against the background of using glibenclamide and L-NAME, which could have been anticipated in advance, is explained by the fact that its mechanism of action has endothelium-protective properties and an ability to activate K+ATP channels. When using selective inhibitor of arginase II KUD975 against the background of glibenclamide, the leveling of its nephroprotective effects does not happen, because they are induced through the activation of eNOS, which is confirmed by the leveling of positive effects against the background of using L-NAME.

Table 7. Dynamics of fractional excretion of sodium in the experimental groups (M±m; n=10).

Experimental group	Day 1	Day 3
Sham	0.38±0.02%	0.5±0.02%
Control	2.24±0.12%^{x}	7.4±0.78%^{x}
AsEPo 2.4 μg/kg	0.91±0.09%^{xy}	1.3±0.09%^{xy}
AsEPo 2.4 μg/kg + glibenclamide	2.03±0.13%^{x}	6.59±0.45%^{x}
AsEPo 2.4 μg/kg + L-NAME	1.94±0.14%^{x}	5.94±0.34%^{x}
KUD975 3 mg/kg	1.14±0.11%^{xy}	1.8±0.09%^{xy}
KUD975 3 mg/kg + L-NAME	2.13±0.18%^{x}	6.77±0.54%^{x}
KUD975 3 mg/kg + glibenclamide	1.01±0.07%^{xy}	1.84±0.11%^{xy}

Notes: ^{x} – p<0.05 in comparison with the sham (sham operated) animals; y – p <0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

Table 8. Dynamics of indicators of microcirculation in the kidneys against the background of correction with asialoerythropoietin (M±m; n=10)

Experimental group	Microcirculation rate, PU		
	5 minutes	Day 1	Day 3
Sham	904.5±60.43	870.5±96.18	859±67.98
Control	209±24.42^{x}	418.1±46.02^{x}	315.5±13.67^{x}
AsEPo 2.4 μg/kg	670.4±54.19^{xy}	725.6±47.41^{y}	689.3±46.52^{y}
AsEPo 2.4 μg/kg + glibenclamide	215.9±18.9^{x}	421.4±24.62^{x}	329.2±28.05^{x}
AsEPo 2.4 μg/kg + L-NAME	227.2±18.8^{x}	451.8±30.70^{x}	334.7±20.25^{x}
KUD975 3 mg/kg	604.7±43.51^{xy}	718.2±44.52^{y}	653.6±61.99^{xy}
KUD975 3 mg/kg + L-NAME	220.9±22.47^{x}	437.9±31.39^{x}	323±25.43^{x}
KUD975 3 mg/kg + glibenclamide	615.2±28.74^{xy}	717.6±25.73^{y}	652.1±46.33^{xy}

Notes: ^{x} – p<0.05 in comparison with the sham (sham operated) animals; y – p <0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

Table 9. Dynamics of the epithelium height of the proximal and distal tubules when correcting with asialoerythropoietin and selective inhibitor of arginase II KUD975 against the background of glibenclamide and L-NAME (M±m; n=10)

Group	24 hours		72 hours	
	Epithelium height of the proximal tubules	Epithelium height of the distal tubules	Epithelium height of the proximal tubules	Epithelium height of the distal tubules
Sham	11.28±1.56	7.08±1.43	11.28±1.56	7.08±1.43
Control	8.21±0.21^{x}	6.46±0.15^{x}	6.56±0.74^{x}	4.24±0.73^{x}
AsEPo 2.4 μg/kg	9.72±0.11^{xy}	6.65±0.09^{x}	9.93±0.07^{xy}	6.33±0.09^{xy}
AsEPo 2.4 μg/kg + glibenclamide 50 mg/kg	8.66±0.16^{x}	6.57±0.09^{x}	6.79±0.11^{x}	4.37±0.11^{x}
AsEPo 2.4 μg/kg + L-NAME 25 mg/kg	8.79±0.19^{x}	6.62±0.09^{x}	6.85±0.11^{x}	4.57±0.13^{x}
KUD975 3 mg/kg	9.4±0.09^{xy}	6.61±0.09^{x}	8.57±0.1^{xy}	6.19±0.1^{xy}
KUD975 3 mg/kg + L-NAME 25 mg/kg	8.68±0.2^{x}	6.45±0.09^{x}	6.84±0.11^{x}	4.48±0.11^{x}
KUD975 3 mg/kg + glibenclamide 50 mg/kg	9.13±0.15^{xy}	6.6±0.09^{x}	8.27±0.13^{xy}	6.03±0.09^{xy}

Notes: ^{x} – p<0.05 in comparison with the sham (sham operated) animals; y – p<0.05 compared with the control group (ischemia-reperfusion); EPo – erythropoietin; AsEPo – asialoerythropoietin.

Conclusions

The obtained results testify to the dose-dependent nephroprotective properties of asialoerythropoietin and selective inhibitor of arginase II KUD975, the efficacy of which exceeds the effect of the comparison drugs.

Prevention of ischemic and reperfusion kidney injuries with a combination of asialoerythropoietin at a dose of 2.4 μg/kg and a selective inhibitor of arginase II KUD975 at a dose of 3 mg/kg is superior by efficacy to the monotherapy regimens with these drugs.

The results obtained indicate that the nephroprotective properties of asialoerythropoietin are induced through activation of the K^+_{ATP} channels and the NO system, which was demonstrated in the experiments with glibenclamide and the eNOS blocker, respectively. The mechanism of the nephroprotective effects of the selective inhibitor of arginase II is also closely related to the nitric oxide system and does not depend on K^+_{ATP} channels.

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