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Research Article

# Renoprotective effect of carbamylated darbepoetin and udenafil in ischemia-reperfusion of rat kidney due to the effect of preconditioning and inhibition of nuclear factor kappa B

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## Abstract

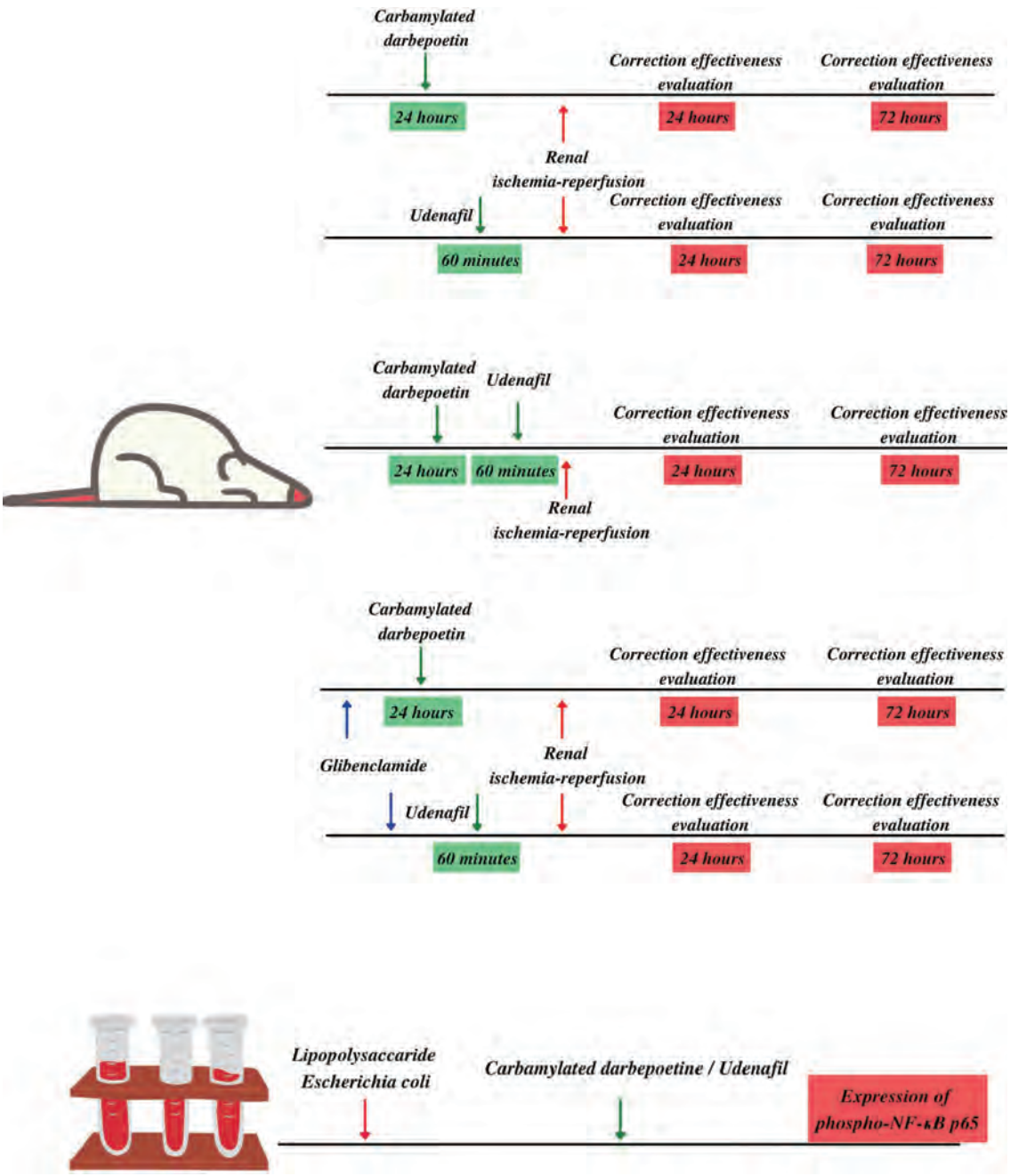
**Introduction:** Acute kidney injury is a widespread complication in hospitalized patients, with a high mortality rate and long-term complications affecting prognosis and quality of life and with high human and financial costs. In addition, to date, no clear algorithm for the prevention of this type of damage has been developed.

**Materials and methods:** The research was carried out on male Wistar rats. A 40-minute renal ischemia-reperfusion model was used to model acute kidney injury. Further, the renoprotective properties of carbamylated darbepoetin, udenafil and their combination were assessed based on the analysis of the biochemical studies' results, dynamics of the renal status and the renal microvasculature, and the pathomorphological picture. A series of experiments was also carried out to assess the contribution of adenosine triphosphate-dependent potassium channels and nuclear factor kappa B to the renoprotective properties of the said agents.

**Results and discussion:** Prophylactic administration of carbamylated darbepoetin at a dose of 50 μg/kg and udenafil at a dose of 8.7 mg/kg led to a statistically significant decrease in creatinine and blood urea nitrogen, an increase in glomerular filtration rate with a simultaneous decrease in fractional excretion of sodium, as well as an increase in the level of microcirculation in the kidneys and a decrease in the severity of damage according to the data of a pathomorphological examination at all time points of the experiment. A higher efficiency of correcting ischemic and reperfusion renal injuries was observed when using a combination of the said pharmacological agents. A series of experiments with glibenclamide demonstrated that its preliminary administration levels the renoprotective properties of carbamylated darbepoetin and udenafil. The ability of the studied pharmacological agents to reduce lipopolysaccharide-induced expression of nuclear factor kappa B in mononuclear cells was also demonstrated. The results of the research suggest that the renoprotective effects of carbamylated darbepoetin, udenafil, and their combination are realized through ATP-dependent potassium channels and nuclear factor kappa B.

**Conclusion:** Pharmacological preconditioning with carbamylated darbepoetin and udenafil reduces the severity of acute kidney injury induced by ischemia-reperfusion.

**Graphical abstract:**




## Keywords

acute kidney injury, carbamylated darbepoetin, ischemia-reperfusion, nuclear factor kappa B, preconditioning, udenafil.

## Introduction

Acute kidney injury (AKI) is one of the pressing interdisciplinary problems. According to international and national recommendations, acute kidney injury is a pathophysiological syndrome of acute kidney damage, which has a staged course from minimal changes in the renal function to its complete loss (KDIGO 2012).

Ischemic and reperfusion injuries are among the most important causes of acute kidney injury in various clinical situations (Basile et al. 2012, Makris and Spanou 2016, Dube et al. 2017). They are an independent risk factor for morbidity and mortality in vascular surgery, organ-preserving kidney surgery, transplantation and cardiac surgery (Dryazhenkov et al. 2013, Abu Jawdeh and Govil 2017, An et al. 2017, Moore et al. 2018). In addition to its

high prevalence, acute kidney injury has become one of the major public health problems with high human and financial costs, second only to the costs of treating patients with sepsis (Silver et al. 2017).

Currently, there is no generally accepted concept of kidney protection from ischemic and reperfusion injuries. Both the KDIGO international recommendations and the Russian clinical guidelines to detect and treat AKI include preventive measures aimed at maintaining an adequate level of circulating blood volume and hemodynamics, glycemic control and nutritional support (KDIGO 2012). One of the possible ways to prevent ischemic and reperfusion kidney damage is to expand the "time window" in order to reduce the severity of kidney damage. In this regard, one of the promising areas of renoprotective therapy for ischemic-reperfusion injuries is pharmacological pre- and post-conditioning. Currently, of all types of preconditioning, pharmacological preconditioning is preferred due to its higher efficiency and ease of implementation (Danilenko 2015).

One of the most studied pharmacotherapeutic agents with preconditioning properties is the glycoprotein hormone erythropoietin (EPO) (Kaplin et al. 2013, Kolesnik et al. 2015, Shabelnikova 2016). EPO exerts its hematopoietic properties through its connection with the homodimeric erythropoietin receptor (EPOR) on the precursors of the erythroid lineage in the bone marrow, accelerating the processes of erythropoiesis and increasing the number of mature erythrocytes (Watowich 2011, Kuhrt and Wojchowski 2015, Bhoopalan et al. 2020). However, there is also a heterodimeric erythropoietin complex EPOR/βCR, which consists of EPOR in combination with the ubiquitous β-common receptor (Brines et al. 2004, Rivera-Cervantes et al. 2019, Vázquez-Méndez et al. 2020). Implementation of the cytoprotective properties of EPO in various models of ischemia-reperfusion is associated primarily with the activation of signaling cascades triggered by a short contact of erythropoietin with this receptor complex (Brines and Cerami 2008, Collino et al. 2015). Renoprotective properties of erythropoietin were confirmed not only in experimental, but also in clinical studies: for example, its preliminary administration in patients before performing coronary artery bypass graft surgery led to a decrease in the severity of acute kidney injury (Moore et al. 2011, Tasanarong et al. 2013). However, there are a number of factors that limit the use of erythropoietin as a cytoprotective agent: firstly, erythropoietin has a high affinity for classical homodimeric erythropoietin receptors and a significantly lower affinity for EPOR/βCR (Brines and Cerami 2008, Kebschull et al. 2017, Zubareva et al. 2019), responsible for the cytoprotective effects. Secondly, the use of high doses of erythropoietin entails an increased risk of side effects, such as impaired hemostasis with an increased risk of thrombus formation and arterial hypertension (Agarwal 2018, Zubareva et al. 2019), which also adversely affects the erythropoietin efficacy as a preconditioning agent.

The main pathogenetic links of acute kidney injury are acute tubular necrosis (Basile et al. 2012, Makris et al. 2016), endothelial dysfunction (Bonventre and Yang 2011, Sedaghat et al. 2019), inflammation (Bonventre and Yang 2011, Han and Lee 2019), and mitochondrial dysfunction (Bhargava and Schnellmann 2017). Thus, for the successful prevention of AKI, it is necessary to search for pharmacological agents with cyto-, endothelioprotective and anti-inflammatory properties. Some of these representatives are new derivatives of erythropoietin with high affinity for the heterodimeric receptor complex EPOR/βCR and phosphodiesterase (PDE) type 5 inhibitors.

Darbepoietin is a hyperglycosylated variant of recombinant human erythropoietin with three times longer half-life period. Darbepoietin has also shown to be as effective a neuroprotective agent as erythropoietin (Reznikov et al. 2017). One of the ways to modify the erythropoietin molecule in order to potentiate the cytoprotective properties is carbamylation (Reznikov et al. 2017, Diao et al. 2019). At the same time, a number of studies confirm that carbamylated darbepoetin has neuro- (Tverskoi et al. 2018, Shirokova 2019), retino- (Peresypkina et al. 2018), and cardioprotective effects (Kolesnichenko et al. 2019), in the absence of erythropoietic properties. Thus, combination of cytoprotective and endotheliotropic properties of carbamylated erythropoietin derivatives makes the derivatives promising agents for the correction of ischemic and reperfusion renal injuries.

On the other hand, microcirculation disorders and endothelial dysfunction play an important role in the development of ischemic and reperfusion injuries (Bonventre and Yang 2011, Basile et al. 2012, Sedaghat et al. 2019). Previously, it was demonstrated that phosphodiesterase inhibitors have preconditioning properties in simulating ischemia-reperfusion in various organs and tissues, including kidney ischemia-reperfusion (Erol et al. 2015, Zahran et al. 2019, Nam et al. 2020). Udenafil is also a type 5 phosphodiesterase inhibitor used in the treatment of erectile dysfunction, but the distinctive feature of udenafil is its selectivity. The overwhelming concentration of udenafil is several times lower in relation to PDE-5 in comparison with PDE-1, PDE-2, PDE-3, and PDE-4, and 10 times lower in comparison with PDE-6 (Doh et al. 2002, Cho and Paick 2014). Udenafil hardly inhibits PDE-11, which is localized in striated muscles, testes and lungs, which does not cause any cases of myalgia, lower back pains and manifestations of testicular toxicity when taken (the drug does not inhibit spermatogenesis) (Kouvelas et al. 2009, Zhao et al. 2011). According to several multicenter, randomized, double-blind, placebo-controlled trials, udenafil has a favorable safety profile (Cho and Paick 2014).

Thus, one of the promising directions for the prevention and reduction of the severity of acute kidney injury is the study of the renoprotective effects of pharmacological agents and their combination under conditions of renal ischemia-reperfusion due to the activation of the heterodimeric EPOR/βCR receptor complex by carbamylated darbepoetin and selective blockade of type 5 phosphodiesterase with udenafil.

### Research aim

To evaluate efficiency of correcting ischemic and reperfusion renal injuries, using pharmacological preconditioning with carbamylated darbepoetin and udenafil.

## Materials and methods

Experimental work was conducted on 242 white adult male Wistar rats (weight 180–220 g). The experimental part of the study was carried out in accordance with GOST 33044-2014 "Principles of Good Laboratory Practice" the ethical principles of handling laboratory animals were observed in accordance with the "European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes. CETS No. 123".

### Simulation of bilateral renal ischemia-reperfusion

Experimental simulation was performed as follows: under anesthesia (300 mg/kg of chloral hydrate, intraperitoneally), a midline laparotomy was performed, renal pedicles were sequentially isolated, and atraumatic vascular clamps were applied, with 5 minutes apart. To choose the optimal time for the ischemic period, the animals were divided into 3 groups: group 1 – with a 30-minute ischemic period; group 2 – with a 40-minute ischemic period, group 3 – with a 60-minute ischemic period, the reperfusion period – 72 hours. Further, the operating wound was sutured in layers. After choosing the optimal model of bilateral renal ischemia-reperfusion, the severity of pathological changes and the renoprotective properties of the drugs were assessed after 24 hours or 72 hours of reperfusion. In the group of sham-operated animals, a midline laparotomy was performed, and the wound was sutured 40 minutes afterwards.

### Renal microcirculation level

The microcirculation level was assessed using a MP100 Biopac System (Biopac System Inc., USA), with the use of an LDF100C laser Doppler flowmetry (LDF) module and a TSD143 surface sensor. The level of microcirculation was assessed within the first 5 minutes of reperfusion, and then 24 or 72 hours later. Data registration and processing was carried out using AcqKnowledge version 3.8.1. The values of the indicators were expressed in perfusion units (PU).

### Laboratory methods for assessing the renal function

Concentrations of creatinine and blood urea nitrogen (BUN) in blood serum and urine were determined on a URIT800 Vet biochemical analyzer (URIT Medical Electronic Co., Ltd., China). The concentration of sodium ions in blood serum and urine was determined according to the standard technique on an automated biochemical analyzer AU480 (Beckman Coulter, Japan). To obtain urine samples, the animals were placed in metabolic cages with free access to water for 12 or 24 hours. Next, diuresis was measured, and samples were taken for further research.

The glomerular filtration rate (GFR) was calculated as follows (formula 1):

$$GFR = \frac{Urine\,creatinine\ (\mu mol/l) \times Urine\,volume\ (ml)}{Serum\,creatinine\ (\mu mol/l) \times Collection\,time\ (min)} \quad (1)$$

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

$$FeNa = \frac{Urine\,sodium \times Serum\,creatinine}{Serum\,sodium \times Urine\,creatinine} \quad (2)$$

### Morphological methods for assessing changes in the kidneys

To conduct a pathomorphological study, the obtained material was fixed in a formalin solution, after which a portion of the renal tissue was removed, embedded in liquid paraffin, and sections were made. The obtained sections were stained with hematoxylin and eosin, by van Gieson's and Mallory's methods. Microscopy and photography were carried out using a Leica CME microscope (Leica Microsystems, Germany) and a DCM-510 eyepiece camera, followed by image processing with Future WinJoe software (Future Optics, USA). The morphometric study included measuring the following parameters: the epithelial height of the nephron proximal and distal sections, the cross-sectional area of the renal corpuscle and the glomerulus.

### Stimulation and determination of the expression of phosphorylated NF-κB in peripheral blood mononuclear cells

After sampling blood into vacuum tubes with lithium heparin, mononuclear cells (MNCs) were isolated on a Ficoll density gradient according to the standard method. The isolated MNCs were added to the plate wells in RPMI-1640 medium with 10% bovine embryo serum. In order to stimulate the expression of the investigated factor, lipopolysaccharide (LPS) from *Escherichia coli* was added to the cells. The studied drugs were added at the following final concentrations: carbamylated darbepoetin – 100 ng/ml and udenafil – 400 ng/ml. Dexamethasone at a final concentration of $1 \times 10^{-4}$ mol/L was used as a reference drug. Then the plate was placed for incubation at 37 °C in a humid atmosphere of 5% CO2 for 48 hours. Then MNCs removed from the bottom of the well were transferred into test tubes, adding the supravital dye 7-aminoactinomycin, and then the tubes were placed for incubation. Then MNCs were fixed using Fix & Perm Medium A for 15 min. After that, the cells were washed, and after removing the buffer, Fix & Perm Medium B medium and phosphate-buffered saline with rabbit antibodies to Phospho-NFkB p65 (Ser536) were added to the cell pellet, thoroughly resuspended

and incubated for 30 minutes. Then the cells were washed, and Fix & Perm Medium B was added to the cell pellet with the addition of phosphate-buffered saline containing secondary Goat anti-Rabbit IgG (H+L) antibodies labeled with allophycocyanin (APC). The cells with antibodies were thoroughly resuspended on a vortex and incubated for 30 minutes. Thereafter, the cells were washed, resuspended in FACS Flow and analyzed on a FACSCanto II flow cytometer (BD, USA) with a 488 nm blue laser. The data were collected using the BD FACSDiva v.8.0.2 program.

**Research design. Choice of administration modes for pharmacological agents**

The following experimental groups were included in the research design:

1. Intact animals;
2. Sham-operated animals (reperfusion period – 24 hours);
3. Sham-operated animals (reperfusion period – 72 hours);
4. Bilateral renal ischemia-reperfusion injury (ischemia – 30 minutes; reperfusion period – 72 hours);
5. Bilateral renal ischemia-reperfusion injury (ischemia – 40 minutes; reperfusion period – 72 hours);
6. Bilateral renal ischemia-reperfusion injury (ischemia – 60 minutes; reperfusion period – 72 hours);
7. Bilateral renal ischemia-reperfusion injury (ischemia – 40 minutes; reperfusion period – 24 hours);
8. Bilateral renal ischemia-reperfusion injury + carbamylated darbepoetin 50 μg/kg (ischemia – 40 minutes; reperfusion period – 24 hours);
9. Bilateral renal ischemia-reperfusion injury + carbamylated darbepoetin 50 μg/kg (ischemia – 40 minutes; reperfusion period – 72 hours);
10. Bilateral renal ischemia-reperfusion injury + carbamylated darbepoetin 50 μg/kg (ischemia – 40 minutes; reperfusion period – 24 hours);
11. Bilateral renal ischemia-reperfusion injury + carbamylated darbepoetin 50 μg/kg (ischemia – 40 minutes; reperfusion period – 72 hours);
12. Bilateral renal ischemia-reperfusion injury + darbepoetin 0.45 μg/kg (ischemia – 40 minutes; reperfusion period – 24 hours);
13. Bilateral renal ischemia-reperfusion injury + darbepoetin 0.45 μg/kg (ischemia – 40 minutes; reperfusion period – 72 hours);
14. Bilateral renal ischemia-reperfusion injury + udenafil 8.7 mg/kg (ischemia – 40 minutes; reperfusion period – 24 hours);
15. Bilateral renal ischemia-reperfusion injury + udenafil 8.7 mg/kg (ischemia – 40 minutes; reperfusion period – 72 hours);
16. Bilateral renal ischemia-reperfusion injury + sildenafil 4.3 mg/kg (ischemia – 40 minutes; reperfusion period – 24 hours);
17. Bilateral renal ischemia-reperfusion injury + sildenafil 4.3 mg/kg (ischemia – 40 minutes; reperfusion period – 72 hours);
18. Bilateral renal ischemia-reperfusion injury + carbamylated darbepoetin 50 μg/kg + udenafil 8.7 mg/kg (ischemia – 40 minutes; reperfusion period – 24 hours);
19. Bilateral renal ischemia-reperfusion injury + carbamylated darbepoetin 50 μg/kg + udenafil 8.7 mg/kg (ischemia – 40 minutes; reperfusion period – 72 hours);
20. Bilateral renal ischemia-reperfusion injury + carbamylated darbepoetin 50 μg/kg + glibenclamide (ischemia – 40 minutes; reperfusion period – 24 hours);
21. Bilateral renal ischemia-reperfusion injury + carbamylated darbepoetin 50 μg/kg + glibenclamide (ischemia – 40 minutes; reperfusion period – 72 hours);
22. Bilateral renal ischemia-reperfusion injury + udenafil 8.7 mg/kg + glibenclamide (ischemia – 40 minutes; reperfusion period – 24 hours);
23. Bilateral renal ischemia-reperfusion injury + udenafil 8.7 mg/kg + glibenclamide (ischemia – 40 minutes; reperfusion period – 72 hours);
24. Intact group (for blood samples).

In order to study the preconditioning properties of carbamylated darbepoetin when modeling ischemia-reperfusion of the kidneys, an injection solution (Farmapark LLC, Russia) was used at doses of 50 μg/kg and 300 μg/kg once subcutaneously in the withers area 24 hours before modeling ischemia. Udenafil (Zydena, Dong-A Pharmaceutical Co. Ltd., Korea) was administered at a dose of 8.7 mg/kg once intragastrically via a tube 60 minutes before modeling ischemia. Darbepoetin (Aranesp, Amgen Europe B.V., Netherlands) was administered at a dose of 0.45 μg/kg 24 hours before the induction of ischemia, subcutaneously at the withers. Sildenafil (Viagra, Pfizer, USA) was injected at a dose of 4.3 mg/kg once intragastrically via a tube 60 minutes before ischemia simulation. Glibenclamide (Maninil, Berlin-Chemi AG, Germany) was administered intragastrically via a tube at a dose of 5 mg/kg 30 minutes before the administration of the pharmacological agents (Shabelnikova 2016). In the darbepoetin and carbamylated darbepoetin groups, additional glibenclamide was administered 24 hours after the first administration.

**Statistical processing of research results**

Descriptive statistics methods were used for all the data: the data were tested for normal distribution using the Shapiro-Wilk test. In the case of a normal distribution, the mean (M) and standard error of the mean (m) were calculated. In cases of abnormal distribution, the median (Me), 1 and 3 quartiles (Q1; Q3) were calculated. Intergroup differences were analyzed by parametric (Student's t-test)

or nonparametric (Mann-Whitney, Kruskal-Wallis tests) methods, depending on the type of distribution. All the calculations were performed using the statistical software package Microsoft Excel 2010, SPSS Statistic.

## Results and discussion

### Selection of the optimal duration of renal ischemia and assessment of the development of acute kidney injury in the simulation of bilateral renal ischemia-reperfusion

The results of assessing the level of serum creatinine and the survival rate of the animals of different groups after 72 hours of reperfusion are presented in Table 1.

The absence of significant differences between the group of intact and sham-operated animals testifies to the technically correct execution of the experimental model and allows using the group of sham-operated animals as a comparison group. In all the experimental groups, the level of serum creatinine was significantly different from that in the group of sham-operated animals; however, the most serious renal dysfunctions were observed in the group of 40-minute and 60-minute bilateral ischemia. But when assessing the survival rate of the animals, there was a tendency to an increase in the mortality rate with an increase in the duration of the ischemic stimulus. Thus, a 40-minute bilateral renal ischemia-reperfusion model is preferred for assessing the nephroprotective properties of drugs.

### Renoprotective effects of carbamylated darbepoetin in bilateral renal ischemia-reperfusion simulation

When the pathology was corrected with carbamylated darbepoetin at the doses of 50 μg/kg and 300 μg/kg after 24 hours of reperfusion, the creatinine level did not significantly differ from that in the group of sham-operated animals; however, due to the restoration of diuresis, GFR reached 0.29±0.02 ml/min and 0.31±0.01 ml/min, respectively, surpassing the comparison drug in efficiency. After 72 hours of reperfusion, the serum creatinine level decreased to 70.8±3.1 μmol/L and 69.9±2.57 μmol/L when 50 μg/kg and 300 μg/kg of carbamylated darbepoetin were used, respectively, with a simultaneous increase in GFR to 0.33±0.04 ml/min and 0.35±0.05 ml/min (Fig. 1).

The BUN concentration, on the contrary, was a more sensitive criterion after 24 hours of reperfusion and amounted to 6.14±0.31 mmol/L and 6.26±0.23 mmol/L, with prophylactic administration of 50 μg/kg and 300 μg/kg of carbamylated darbepoetin. Another positive effect of carbamylated darbepoetin was its ability to improve the functional state of the renal tubular apparatus, which was indirectly evidenced by a decrease in fractional sodium excretion to 1.02±0.05% and 1.31±0.11% after 24 and 72 hours of reperfusion, respectively, when using doses of 50 μg/kg (Fig. 1).

Prophylactic administration of carbamylated darbepoetin led to a decrease in the severity of microcirculatory disorders initiated by ischemia-reperfusion, manifested in recovery to 738.9±26.77 PU and 696±23.18 PU after 24 and 72 hours of reperfusion, respectively, exceeding the efficiency of the reference drug. Similar dynamics was observed when using a dose of 300 μg/kg (Table 2).

With a pathomorphological examination, there is an improvement in the picture both in the tubular and in the glomerular apparatus of the kidneys (Fig. 2).

These data are confirmed by the morphometric data: epithelial height of the proximal renal tubules increases to a level comparable to that of sham-operated animals with a simultaneous increase in the renal glomerulus area after 72 hours of reperfusion (Fig. 3).

### Renoprotective effects of udenafil in modelling bilateral renal ischemia-reperfusion

Prevention of ischemic and reperfusion renal injuries be means of udenafil led to a similar trend in the change in biochemical parameters: the level of creatinine was 59.5±1.41 μmol/L, and BUN – 6.95±0.25 mmol/L, which exceeded the effects of sildenafil after 24 hours of reperfusion (Fig. 4).

**Table 1.** Assessment of the Level of Serum Creatinine and Survival Rate of Animals with Different Duration of Renal Ischemia After 72 Hours of Reperfusion (n = 10).

| Experimental groups | Serum creatinine level, μmol/L | Survival rate, % |
|---|---|---|
| Intact | 56.9±1.63 | 100 |
| Sham-operated | 59.5±3.28 | 100 |
| Bilateral renal ischemia-reperfusion injury, ischemia 30 min | 76.9±4.67[a] | 100 |
| Bilateral renal ischemia-reperfusion injury, ischemia 40 min | 136.8±7.02[a] | 100 |
| Bilateral renal ischemia-reperfusion injury, ischemia 60 min | 203.7±6.68[a] | 60 |

**Note:** [a] – $p<0.05$ in comparison with the group of sham-operated animals.

**Table 2.** Assessment of Microcirculation by Laser Doppler Flowmetry (PE) After 5 Minutes, 24 Hours and 72 Hours of Reperfusion Against the Background of Prophylactic Use of Carbamylated Darbepoetin (M±m; n = 10).

| Experimental groups | 5 minutes | 24 hours | 72 hours |
|---|---|---|---|
| Sham-operated | 904.5±60.43 | 870.5±96.18 | 859±67.98 |
| Renal ischemia-reperfusion | 209±24.42[a] | 418.1±46.02[a] | 315.5 ±13.67[a] |
| Renal ischemia-reperfusion + darbepoetin | 483.8±19.21[a, b] | 686±27.39[a, b] | 555.2±25.46[a, b] |
| Renal ischemia-reperfusion + carbamylated darbepoetin 50 μg/kg | 667.9±40.34[a, b] | 738.9±26.77[b] | 696±23.18[a, b] |
| Renal ischemia-reperfusion + carbamylated darbepoetin 300 μg/kg | 679.1±47.45[a, b] | 749.8±32.19[b] | 700.2±21.74[a, b] |

**Note:** [a] – $p<0.05$ in comparison with the group of sham-operated animals; [b] – $p<0.05$ in comparison with the renal ischemia-reperfusion group.

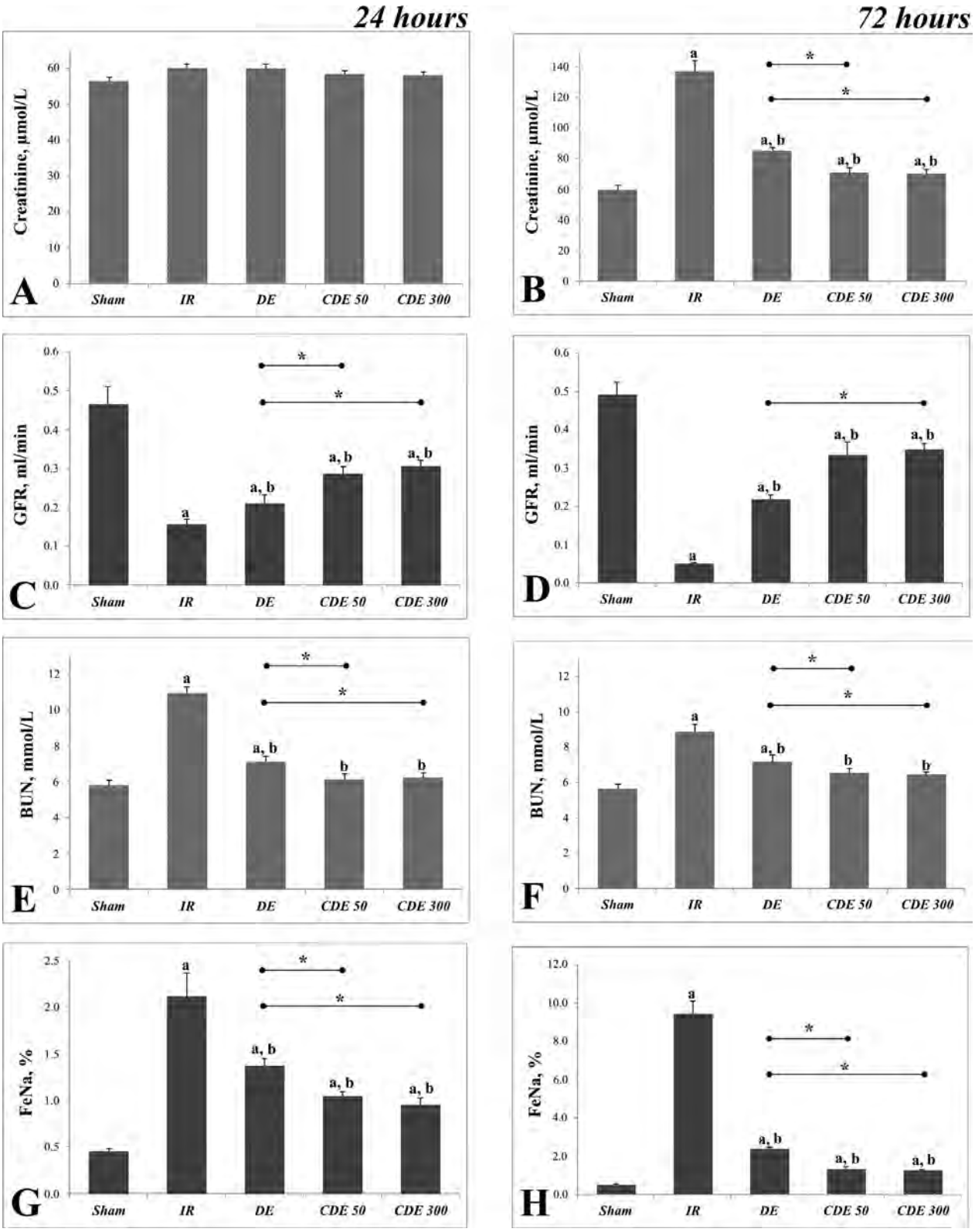



**Figure 1.** Effect of carbamylated darbepoetin at doses of 50 μg/kg and 300 μg/kg on serum creatinine concentration (**A, B**); glomerular filtration rate (**C, D**); blood urea nitrogen concentration (**E, F**) and fractional sodium excretion (**G, H**) after 24 hours and 72 hours of reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia-reperfusion; DE – darbepoetin; CDE 50 – carbamylated darbepoetin (dose of 50 μg/kg); CDE 300 – carbamylated darbepoetin (dose of 300 μg/kg); a – $p<0.05$ in comparison with the group of sham-operated animals; b – $p<0.05$ in comparison with the renal ischemia-reperfusion group; * – $p<0.05$.

On the third day of the experiment, preliminary administration of udenafil at a dose of 8.7 mg/kg led to a decrease in the level of serum creatinine to 72.4±2.28 μmol/L, and BUN – to 6.44±0.26 mmol/L. An increase in the glomerular filtration rate to 0.25±0.02 ml/min and 0.28±0.02 ml/min on the first and third days of the experiment, respectively,

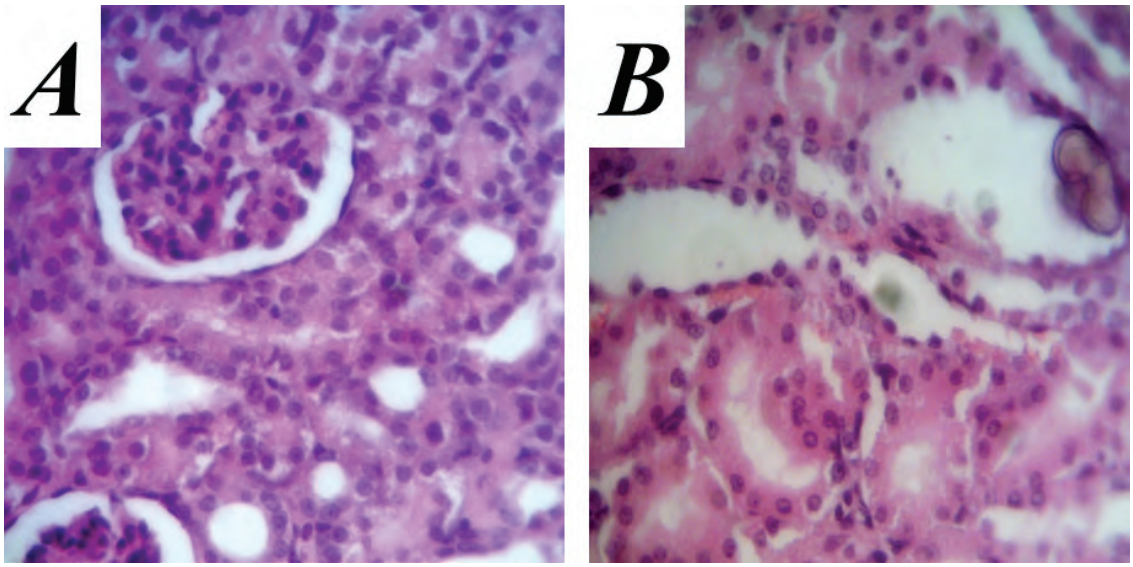



**Figure 2.** Micrograph of the cortical substance (**A**) and tubular structures of the kidney (**B**) after 72 hours of reperfusion against the background of prophylactic use of carbamylated darbepoetin at a dose 50 μg/kg (stained with hematoxylin-eosin, magnification of ×400). **Note:** A large number of unchanged renal corpuscles are detected in the renal cortex. There are no signs of interstitial edema. There are single shrunken glomeruli. The proximal tubules with an indefinite diffuse lumen are formed with epithelial cells with a well-defined brush border on the apical surface. In the distal tubules, the lumen is well defined, the intercellular contacts are preserved. Locally, areas with dilated tubules and obturation of the lumen by dead epithelial cell pellet detached from the basement membrane are visualized.

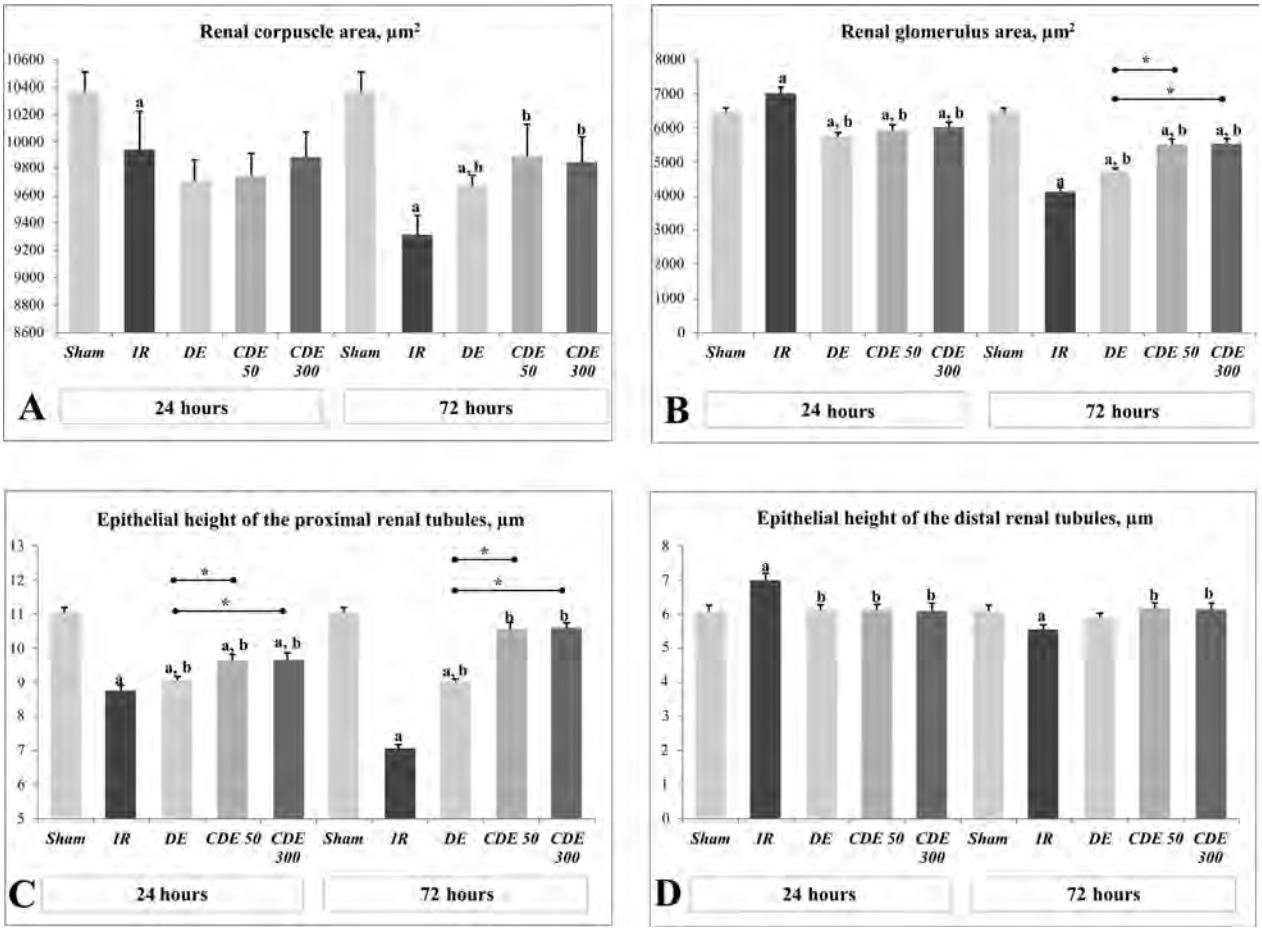



**Figure 3.** Effect of carbamylated darbepoetin (50 μg/kg and 300 μg/kg) on the renal corpuscle area (**A**), renal glomerulus area (**B**), the epithelial height of the proximal renal tubules (**C**), the epithelial height of the distal renal tubules (**D**) after 24 and 72 hours of reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia-reperfusion; DE – darbepoetin; CDE 50 – carbamylated darbepoetin (dose of 50 μg/kg); CDE 300 – carbamylated darbepoetin (dose of 300 μg/kg); a – $p<0.05$ in comparison with the group of sham-operated animals; b – $p<0.05$ in comparison with the renal ischemia-reperfusion group; * – $p<0.05$.

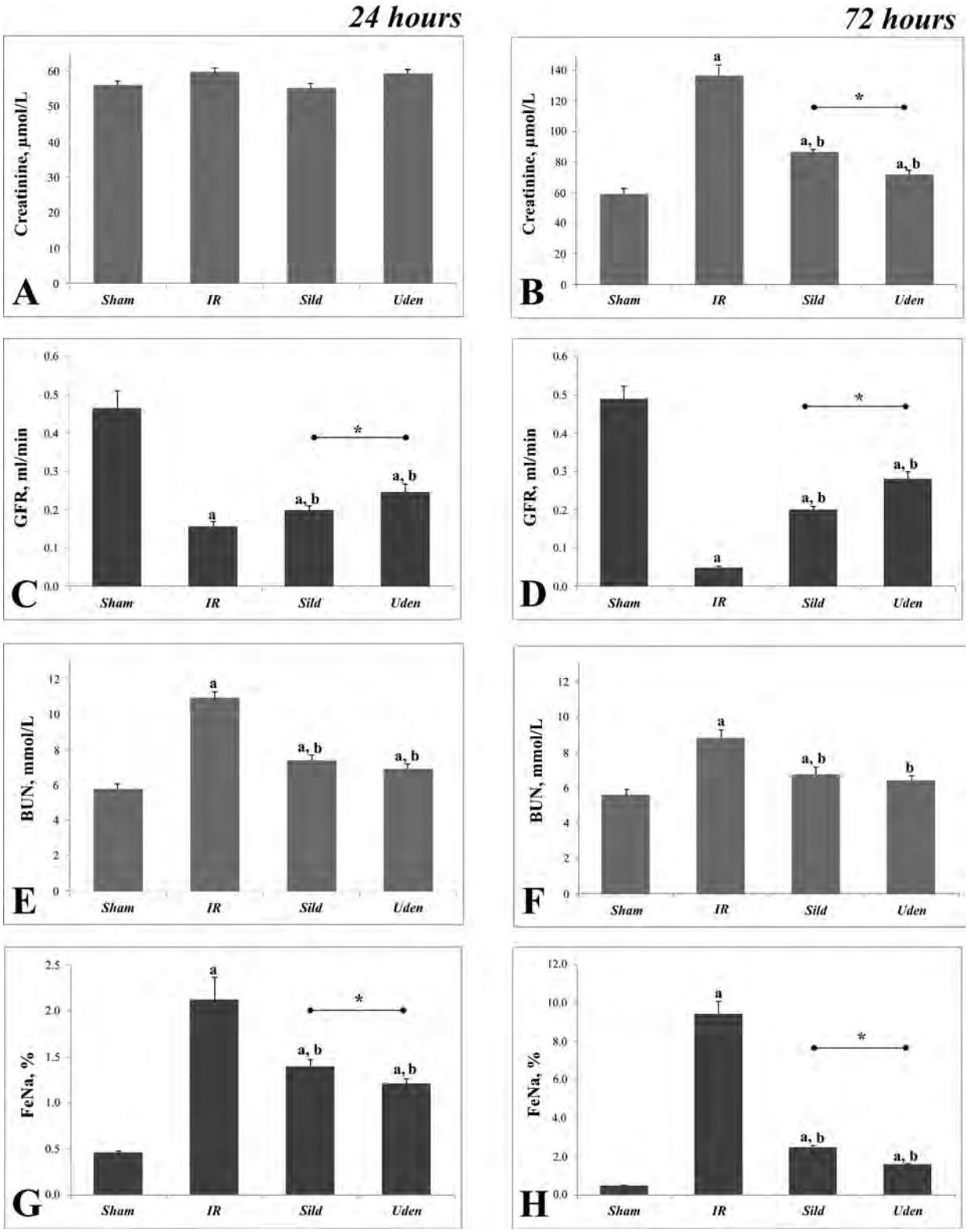



**Figure 4.** Effect of udenafil at a dose 8.7 mg/kg on serum creatinine concentration (**A, B**); glomerular filtration rate (**C, D**); blood urea nitrogen concentration (**E, F**) and fractional sodium excretion (**G, H**) after 24 hours and 72 hours of reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia-reperfusion; Sild – sildenafil (dose of 4.3 mg/kg); Uden – udenafil (dose of 8.7 mg/kg); a – $p<0.05$ in comparison with the group of sham-operated animals; b – $p<0.05$ in comparison with the renal ischemia-reperfusion group; * – $p<0.05$.

also confirmed the renoprotective effects of udenafil. The FeNa values below 2% were also recorded only in the group of laboratory animals with prophylactic use of udenafil (1.21±0.05% and 1.57±0.08% after 24 and 72 hours of reperfusion), which indicates a higher efficacy of an udenafil therapy compared with a sildenafil therapy.

Udenafil also contributed to the restoration of the microcirculation level to 619.2±27.91 PU, 701.6±34.83 PU,

**Table 3.** Assessment of Microcirculation by Laser Doppler Flowmetry (PE) After 5 Minutes, 24 Hours and 72 Hours of Reperfusion Against the Background of Prophylactic Use of Udenafil (M±m; n = 10).

| Experimental groups | 5 minutes | 24 hours | 72 hours |
|---|---|---|---|
| Sham-operated | 904.5±60.43 | 870.5±96.18 | 859±67.98 |
| Renal ischemia-reperfusion | 209±24.42[a] | 418.1±46.02[a] | 315.5 ±13.67[a] |
| Renal ischemia-reperfusion + sildenafil | 447.5±28.65[a, b] | 691±16.67[a, b] | 504.5±21.38[a, b] |
| Renal ischemia-reperfusion + udenafil | 619.2±27.91[a, b] | 701.6±34.83[a, b] | 664.4±22.65[a, b] |

**Note:** [a] – $p<0.05$ in comparison with the group of sham-operated animals; [b] – $p<0.05$ in comparison with the renal ischemia-reperfusion group.

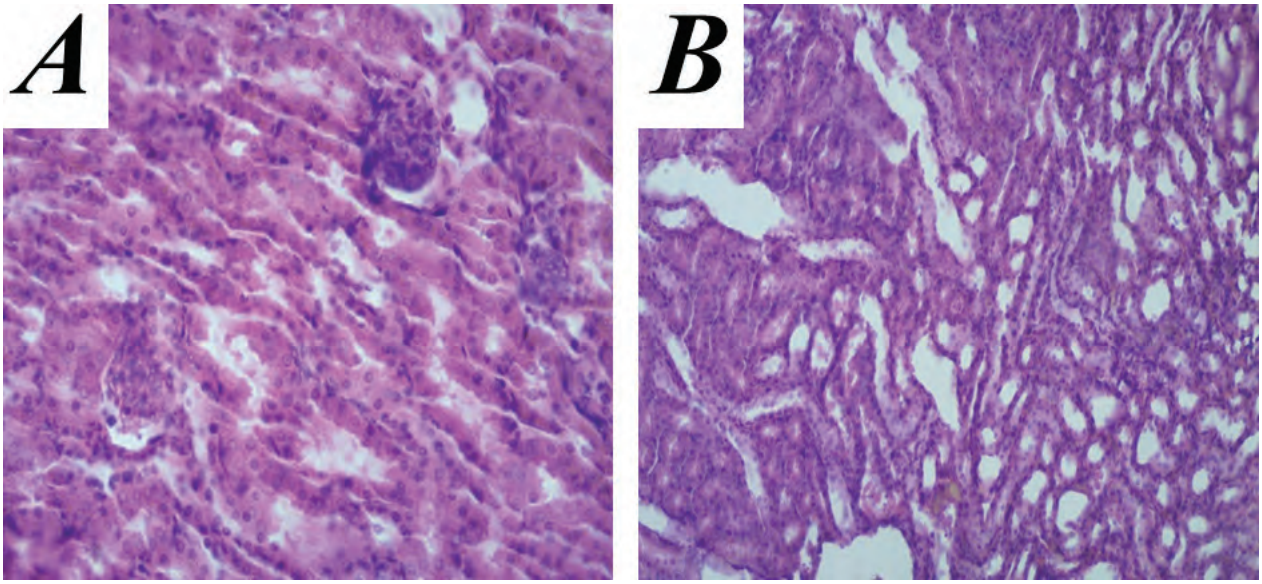



**Figure 5.** Micrograph of the cortical substance (**A**) and tubular structures of the kidney (**B**) after 72 hours of reperfusion against the background of prophylactic use of udenafil at a dose 8.7 mg/kg (stained with hematoxylin-eosin, magnification of ×200). **Note:** There is detected moderate mesangial stromal edema. There are a few shrunken vascular glomeruli of the kidney. The brush border on the apical surface of the epithelial cells of some tubules is flattened; the cytoplasm is vacuolated and swollen. Reactive changes in the damaged epithelium of the tubules are observed: the brush border on the apical surface of some epithelial cells is flattened; the cytoplasm is vacuolated and swollen; some cells detach from the basement membrane and shed into the lumen of single tubules; some tubules are significantly expanded in diameter; there are small areas with necrotic tubules.

664.4±22.65 PU after 5 minutes, 24 hours, and 72 hours of reperfusion, respectively, which were statistically significantly different from the indicators in the control group and in the sham-operated rats ($p<0.05$). On the other hand, udenafil was superior to the reference drug sildenafil in terms of the efficacy of correction of microcirculatory disorders (Table 3).

A pathomorphological examination also showed a decrease in the severity of tubular and glomerular injuries (Fig. 5).

This was confirmed by the morphometric data: epithelial height of the proximal renal tubules increased to 9.36±0.13 μm, with a simultaneous increase in the renal glomerulus area after 72 hours of reperfusion to 4813.04±110.21 $\mu m^2$ after prophylactic administration of udenafil versus 4487.13±87.09 $\mu m^2$ – after administration of sildenafil (Fig. 6).

### Renoprotective effects of the combination of carbamylated darbepoetin and udenafil in the simulation of bilateral renal ischemia-reperfusion

A combined therapy with carbamylated darbepoetin at a dose of 50 μg/kg and udenafil at a dose of 8.7 mg/kg was more effective and was characterized by a decrease in the level of BUN to 5.78±0.17 mmol/L, reaching the parameters of the group of sham-operated animals with similar values of serum creatinine (56.8±1.23 μmol/L) after 24 hours of reperfusion. After 72 hours of reperfusion, a similar dynamics was observed: the efficiency of the combined pharmacotherapy exceeded the monotherapy modes, reaching the parameters of sham-operated animals: a decrease in the concentration of serum creatinine reached 64.7±1.59 μmol/L, and BUN – to 6.12±0.23 mmol/L.

The glomerular filtration rate noticeably increased with the combined administration of carbamylated darbepoetin and udenafil: up to 0.39±0.03 ml/min and 0.43±0.02 ml/min on Days 1 and 3 of the experiment, respectively, statistically significantly exceeding the efficiency of monotherapy modes with the indicated pharmacological agents ($p<0.05$), without reaching, however, the indicators of sham-operated animals.

The dynamics of fractional sodium excretion was similar: it decreased to 0.8±0.02% and 0.78±0.05% after 24 hours and 72 hours of reperfusion, respectively. Despite the statistically significant differences from the group of sham-operated animals, the absence of oliguria in this

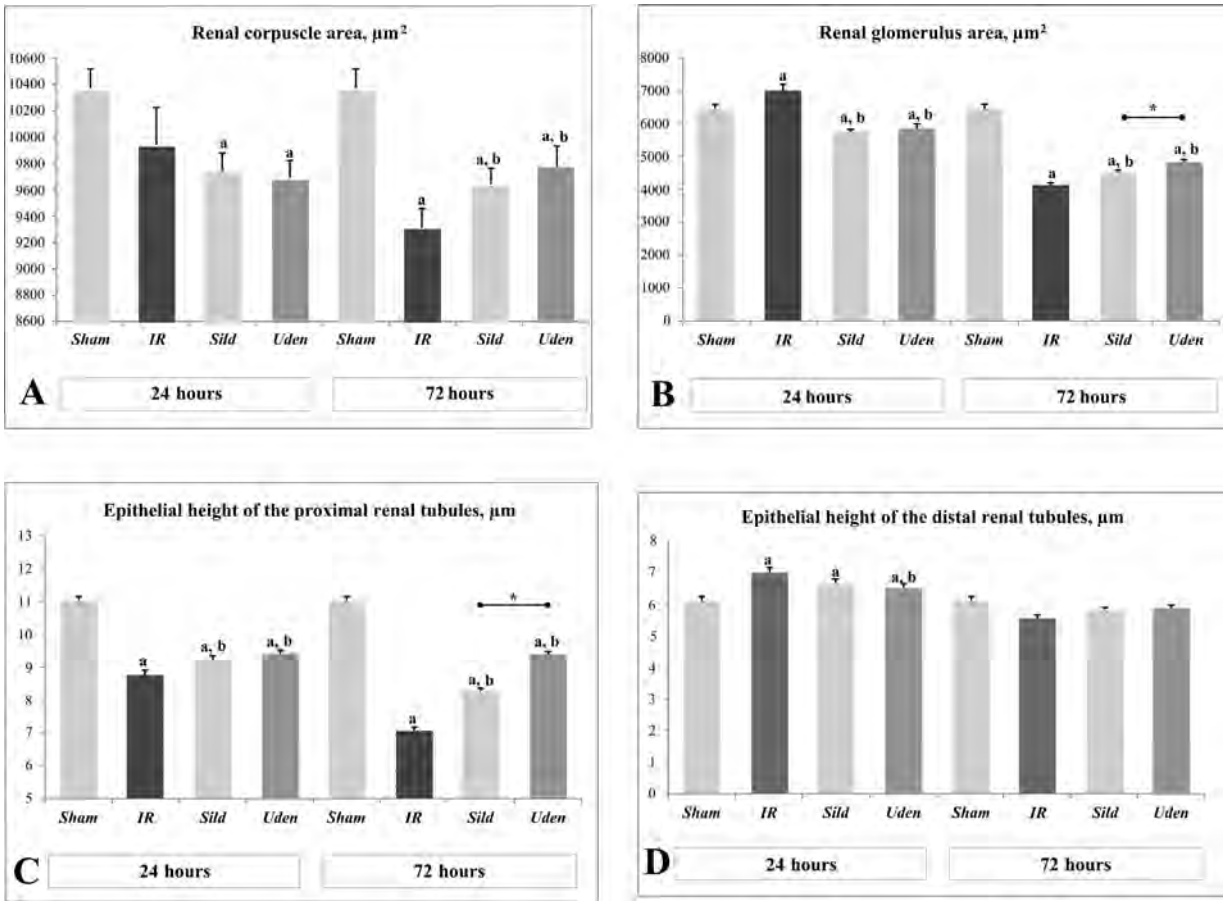



**Figure 6.** Effect of udenafil on the renal corpuscle area (**A**), renal glomerulus area (**B**), the epithelial height of the proximal renal tubules (**C**), the epithelial height of the distal renal tubules (**D**) after 24 hours and 72 hours of reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia-reperfusion; Sild – sildenafil (dose of 4.3 mg/kg); Uden – udenafil (dose of 8.7 mg/kg); a – $p<0.05$ in comparison with the group of sham-operated animals; b – $p<0.05$ in comparison with the renal ischemia-reperfusion group; * – $p<0.05$.

**Table 4.** Assessment of Microcirculation by Laser Doppler Flowmetry (PE) After 5 Minutes, 24 Hours and 72 Hours of Reperfusion Against the Background of Prophylactic Use of Combination of Carbamylated Darbepoetin and Udenafil (M±m; n = 10).

| Experimental groups | 5 minutes | 24 hours | 72 hours |
|---|---|---|---|
| Sham-operated | 904.5±60.43 | 870.5±96.18 | 859±67.98 |
| Renal ischemia-reperfusion | 209±24.42[a] | 418.1±46.02[a] | 315.5 ±13.67[a] |
| Renal ischemia-reperfusion + carbamylated darbepoetin 50 µg/kg | 667.9±40.34[a, b] | 738.9±26.77[b] | 696±23.18[a, b] |
| Renal ischemia-reperfusion + udenafil | 619.2±27.91[a, b] | 701.6±34.83[a, b] | 664.4±22.65[a, b] |
| Renal ischemia-reperfusion + carbamylated darbepoetin 50 µg/kg + udenafil | 692.9±20.08[a, b] | 764.3±48.46[b] | 804.3±29.8[b] |

**Note:** [a] – $p<0.05$ in comparison with the group of sham-operated animals; [b] – $p<0.05$ in comparison with the renal ischemia-reperfusion group.

category of animals may indicate the normal function of the renal tubular apparatus.

Combined administration of carbamylated darbepoetin and udenafil led to a decrease in the severity of microcirculatory disorders initiated by ischemia-reperfusion, manifested in recovery to 764.3±48.46 PU and 804.3±29.8 PU after 24 hours and 72 hours of reperfusion, respectively, exceeding the efficiency of monotherapy modes with the indicated pharmacological agents (Table 4).

The pathological picture was close to that of sham-operated animals (Fig. 8).

This was also confirmed by the morphometric data of the tubular and glomerular apparatus of the kidneys (Fig. 9).

## The role of ATP-dependent potassium channels in the realization of the renoprotective effects of carbamylated darbepoetin and udenafil in experimental renal ischemia-reperfusion

The preconditioning properties of carbamylated darbepoetin and udenafil, realized through ATP-dependent potassium channels, are confirmed by a series of experiments with the preliminary introduction of glibenclamide. Thus, sequential administration of glibenclamide and carbamylated darbepoetin led to an increase in serum creatinine after 72 hours of reperfusion to 90.5±3.16 µmol/L, and udenafil – to 93.7±2.11 µmol/L. The BUN concentration also significantly increased in all the experimental groups with pre-administration of glibenclamide, being

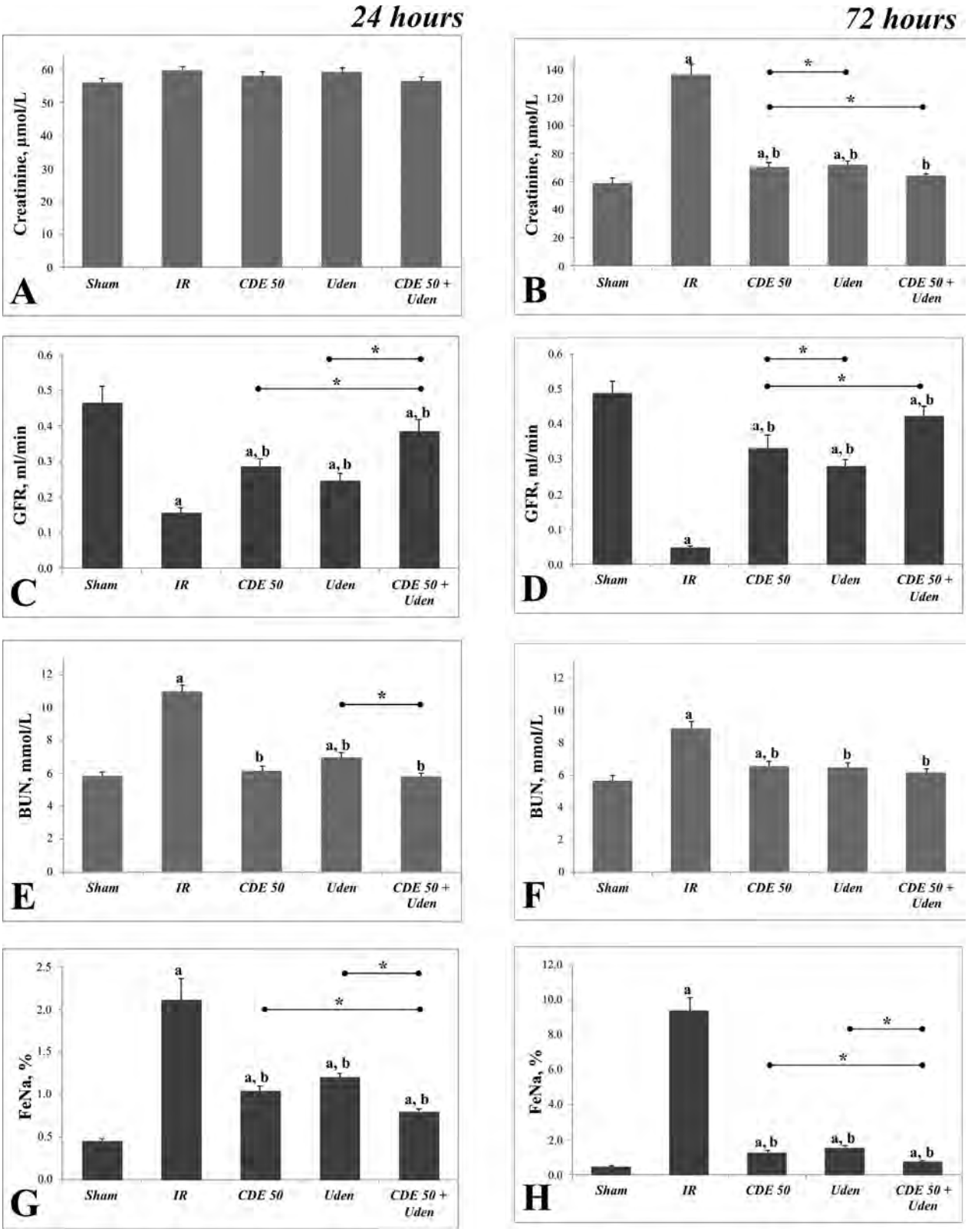



**Figure 7.** Effect of combined therapy with carbamylated darbepoetin at a dose of 50 μg/kg and udenafil at a dose of 8.7 mg/kg on serum creatinine concentration (**A, B**); glomerular filtration rate (**C, D**); blood urea nitrogen concentration (**E, F**) and fractional sodium excretion (**G, H**) after 24 hours and 72 hours of reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia-reperfusion; CDE 50 – carbamylated darbepoetin (dose of 50 μg/kg); Uden – udenafil (dose of 8.7 mg/kg); a – $p<0.05$ in comparison with the group of sham-operated animals; b – $p<0.05$ in comparison with the renal ischemia-reperfusion group; * – $p<0.05$.

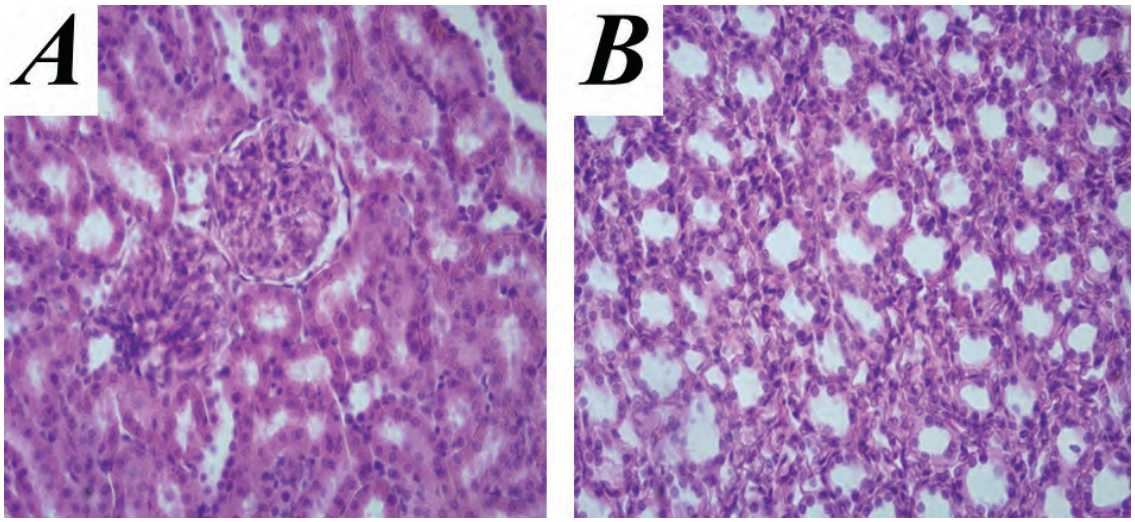



**Figure 8.** Micrograph of the cortical substance (**A**) and tubular structures (**B**) of the kidney 72 hours of reperfusion against the background of prophylactic use of combination of carbamylated darbepoetin at a dose 50 μg/kg and udenafil at a dose 8.7 mg/kg (stained with hematoxylin-eosin, magnification of ×400). **Note:** A large number of unchanged renal corpuscles are detected in the renal cortex. There are no signs of interstitial edema. The proximal tubules with an indefinite diffuse lumen are formed by prismatic epithelial cells with a well-defined brush border on the apical surface. In the distal tubules, the lumen is well defined, the intercellular contacts are preserved. There are locally visualized areas with flattened cells with hyperchromic nuclei and figures of mitosis.

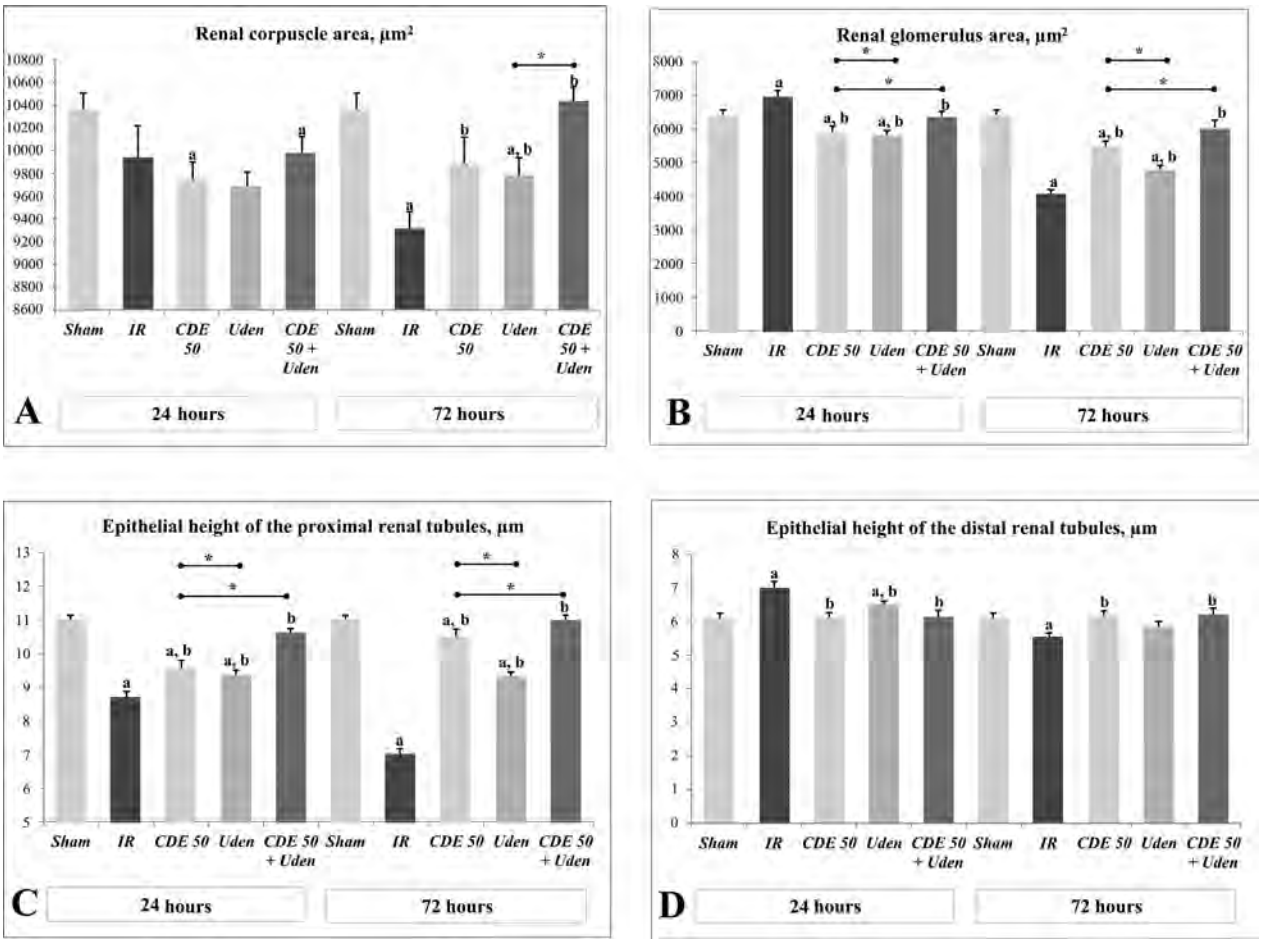



**Figure 9.** Effect of combined therapy with carbamylated darbepoetin at a dose of 50 μg/kg and udenafil at a dose of 8.7 mg/kg on the renal corpuscle area (**A**), renal glomerulus area (**B**), the epithelial height of the proximal renal tubules (**C**), the epithelial height of the distal renal tubules (**D**) after 24 hours and 72 hours of reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia-reperfusion; CDE 50 – carbamylated darbepoetin (dose of 50 μg/kg); Uden – udenafil (dose of 8.7 mg/kg); a – $p<0.05$ in comparison with the group of sham-operated animals; b – $p<0.05$ in comparison with the renal ischemia-reperfusion group; * – $p<0.05$.

statistically significantly different from the values of the groups without glibenclamide ($p<0.05$).

The administration of glibenclamide neutralized the renoprotective properties of carbamylated darbepoetin and udenafil, which manifested itself in a statistically significant increase in GFR on the first and third days of the experiment. So, after 24 hours of reperfusion, the blockade of K+ATP channels reduced the GFR level in the group of carbamylated darbepoetin to 0.21±0.02, udenafil –to 0.18±0.02, which was comparable with the indicators of the control group of animals. Preconditioning properties of carbamylated darbepoetin and udenafil were also confirmed by the dynamics of changes in the fractional excretion of sodium upon preliminary administration of glibenclamide (Fig. 10).

A blocker of ATP-dependent potassium channels also aggravated microcirculatory disorders against the background of correction with carbamylated darbepoetin at a dose of 50 μg/kg and udenafil at a dose of 8.7 mg/kg, which indicates a significant contribution of these channels to the implementation of the preconditioning properties of the studied drugs (Table 5).

There was also an increase in tubular and glomerular disorders according to the data of the pathomorphological examination (Fig. 11).

### Role of nuclear factor kappa B in the realization of the renoprotective effects of carbamylated darbepoetin and udenafil in experimental renal ischemia-reperfusion

When studying the effect of the pharmacological agents on the expression of the phosphorylated p65 subunit of nuclear factor kappa B, it was found that carbamylated darbepoetin and udenafil inhibit the lipopolysaccharide-stimulated activity of this factor, which is manifested in a decrease in the expression of the active form of phospho-NF-κB p65 in MNCs to 74.14 (54.76; 103.51)% and 63.72 (46.61; 67.01)%, respectively (Fig. 12).

A universal mechanism, which to some extent is a component of acute kidney injury in various conditions, is ischemia and the subsequent reperfusion.

Erythropoietin was one of the first pharmacological agents used to prevent and treat AKI. However, the data obtained in clinical studies did not correspond to the dynamics reflected in animal studies (Elliott et al. 2017). The possible reasons for the low efficiency of erythropoietin in humans are its low affinity for the heterodimeric erythropoietin receptor, which implements cytoprotective effects, as well as the occurrence of side effects (primarily, thrombosis) due to the use of high doses (Brines and Cerami 2008, Kebschull et al. 2017, Zubareva et al. 2019, Agarwal 2018). In this regard, an opportunity to study a new erythropoietin derivative with a high affinity for heterodimeric erythropoietin receptors and without erythropoietic properties looked promising.

Preventive administration of carbamylated darbepoetin at the doses of 50 and 300 μg/kg 24 hours before ischemia led to a significant increase in GFR both after 24 hours and 72 hours of reperfusion, reaching 0.33±0.04 ml/min and 0.35±0.02 ml/min by the third day of the experiment, respectively, with a decrease in serum creatinine of more than 1.9 times. A decrease both in the BUN concentration and in fractional sodium excretion was recorded at all time points of the experiment. There was also a statistically significant increase in the hemodynamic parameters; however, the best indicators were achieved by the first day of the experiment, which was expressed in the restoration of the level of microcirculation to 738.9±26.77 PU and 749.8±32.19 PU, when using 50 μg/kg and 300 μg/kg of carbamylated darbepoetin, respectively, which corresponded to the level of microcirculation in the sham-operated animals. Also, both doses of the investigated pharmacological agent improved the pathomorphological picture. The morphometric data supplemented the data of the pathomorphological picture.

A significant contribution of the nitric oxide system to the pathogenesis of acute kidney injury (Wang et al. 2004, Maringer and Sims-Lucas 2016, Sedaghat et al. 2019) and to the implementation of the preconditioning properties of pharmacological agents in ischemia-reperfusion (Park et al. 2003, Yamasowa et al. 2005) makes it possible to investigate drugs with pleiotropic properties, already available on the pharmaceutical market. Phosphodiesterase-5 inhibitors are among these pharmacological agents.

Preventive administration of the PDE-5 inhibitor, udenafil, at a dose of 8.7 mg/kg led to the implementation of its renoprotective effects under the conditions of experimental simulation of ischemia-reperfusion, expressed in a decrease in plasma concentrations of creatinine and BUN, an increase in the calculated GFR, a decrease in fractional sodium excretion, and restoration of the renal microcirculation level, as well as an improvement in the pathomorphological picture and morphometric data after 24 and 72 hours of reperfusion, surpassing sildenafil in efficiency, however, not reaching the indicators of the sham-operated animals.

**Table 5.** Assessment of Microcirculation by Laser Doppler Flowmetry (PE) After 24 Hours and 72 Hours of Reperfusion in the Experimental Groups (M±m; n = 10).

| Experimental groups | 5 minutes | 24 hours | 72 hours |
|---|---|---|---|
| Sham-operated | 904.5±60.43 | 870.5±96.18 | 859±67.98 |
| Renal ischemia-reperfusion | 209±24.42[a] | 418.1±46.02[a] | 315.5 ±13.67[a] |
| Renal ischemia-reperfusion + carbamylated darbepoetin. 50 μg/kg | 667.9±40.34[a, b] | 738.9±26.77[b] | 696±23.18[a, b] |
| Renal ischemia-reperfusion + carbamylated darbepoetin. 50 μg/kg + glibenclamide | 466.5±16.51[a, b] | 624±27.28[a, b] | 537.2±15.2[a, b] |
| Renal ischemia-reperfusion + udenafil | 619.2±27.91[a, b] | 701.6±34.83[a, b] | 664.4±22.65[a, b] |
| Renal ischemia-reperfusion + udenafil + glibenclamide | 469.7±15.03[a, b] | 585.9±18.25[a, b] | 490±21.49[a, b] |

**Note:** [a] – $p<0.05$ in comparison with the group of sham-operated animals; [b] – $p<0.05$ in comparison with the renal ischemia-reperfusion group.

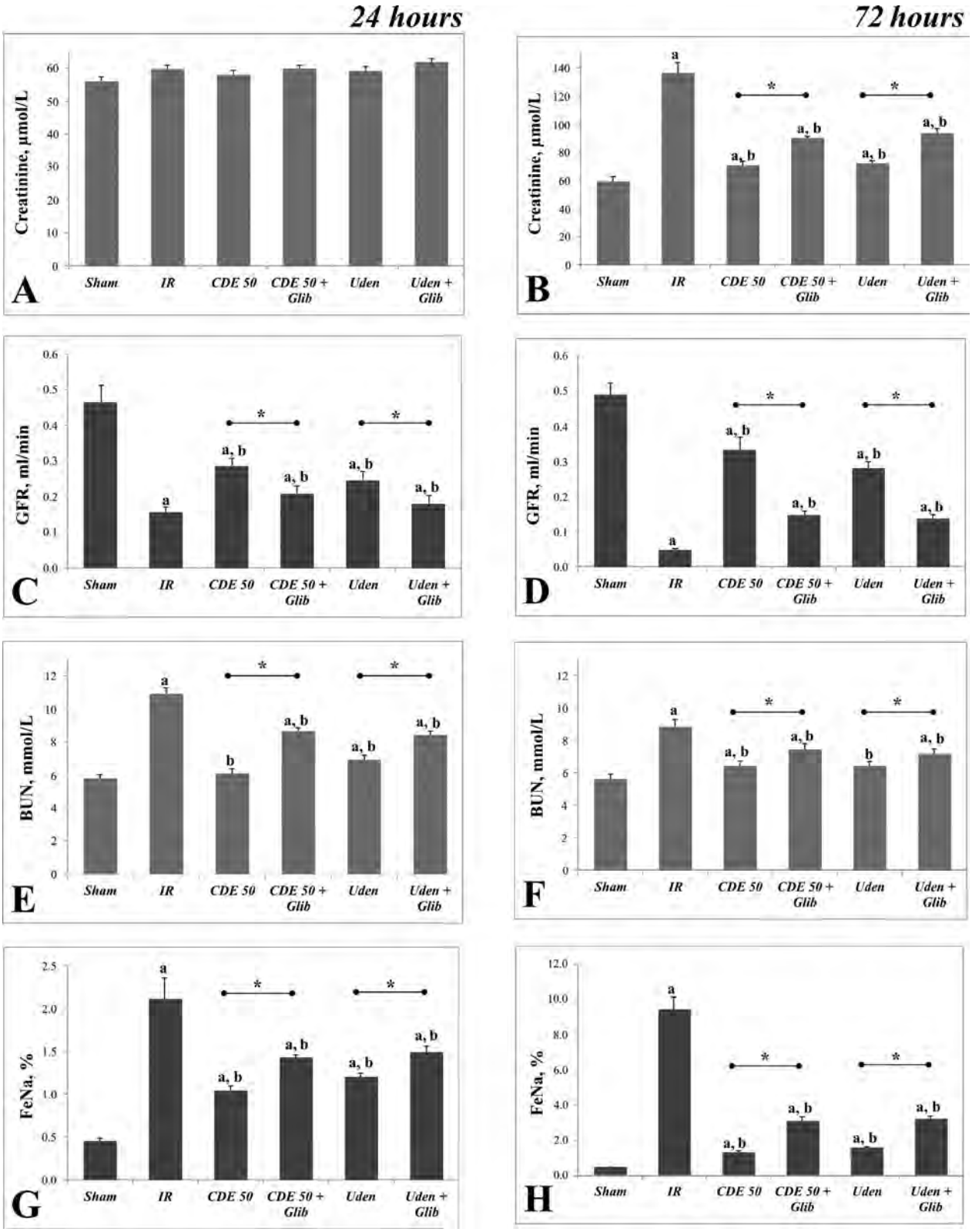



**Figure 10.** Effect of preliminary administration of glibenclamide on serum creatinine concentration (**A, B**); glomerular filtration rate (**C, D**); blood urea nitrogen concentration (**E, F**) and fractional sodium excretion (**G, H**) after 24 hours and 72 hours of reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia-reperfusion; CDE 50 – carbamylated darbepoetin (50 µg/kg); Uden – udenafil (dose of 8.7 mg/kg); Glib – glibenclamide (dose of 5 mg/kg); a – $p<0.05$ in comparison with the group of sham-operated animals; b – $p<0.05$ in comparison with the renal ischemia-reperfusion group; * – $p<0.05$.

Failure to reach the target values against the background of a monotherapy with carbamylated darbepoetin and udenafil, primarily creatinine, necessitated to study the efficiency of a combined correction of ischemic- and reperfusion-induced kidney injuries. After 24 hours of reperfusion, preliminary administration of carbamylated

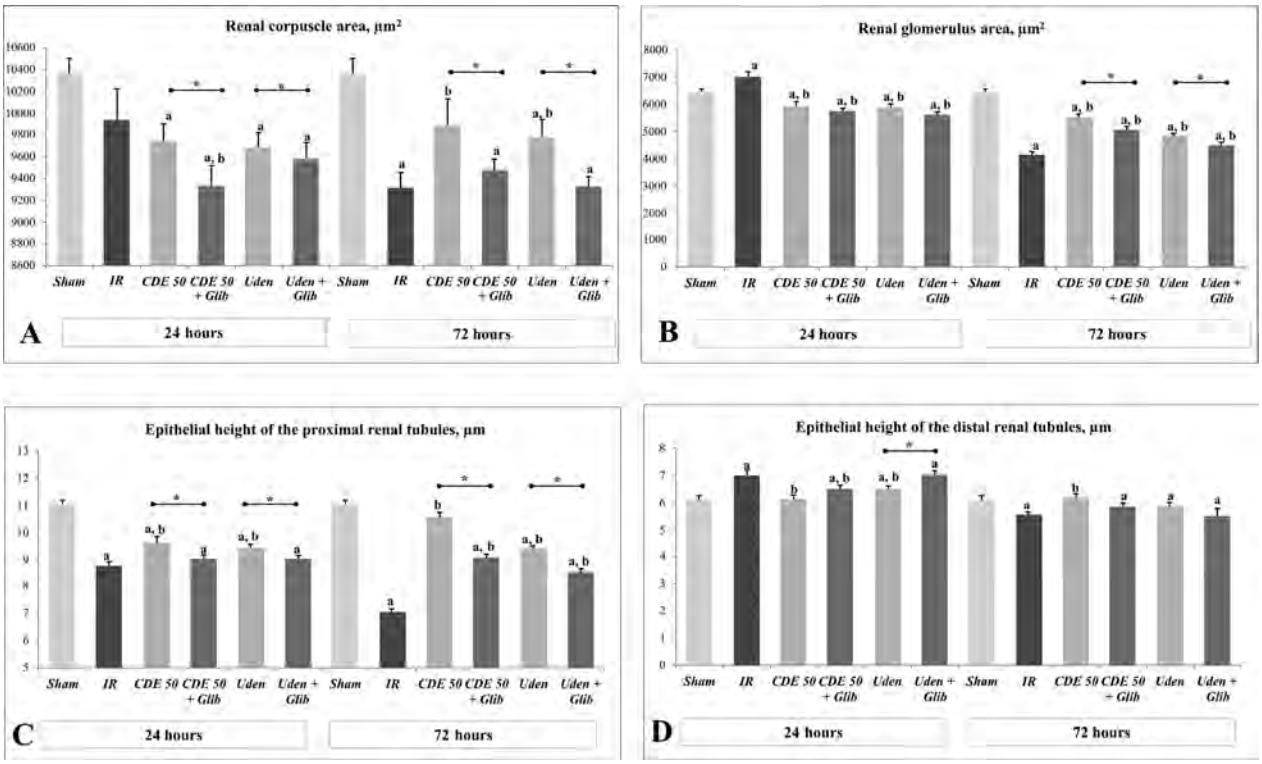



**Figure 11.** Effect of preliminary administration of glibenclamide on the renal corpuscle area (**A**), renal glomerulus area (**B**), the epithelial height of the proximal renal tubules (**C**), the epithelial height of the proximal renal tubules (**D**) after 24 and 72 hours of reperfusion. **Note:** sham – sham-operated animals; IR – renal ischemia-reperfusion; CDE 50 – carbamylated darbepoetin (50 μg/kg); Uden – udenafil (dose of 8.7 mg/kg); Glib – glibenclamide (dose of 5 mg/kg); a – $p<0.05$ in comparison with the group of sham-operated animals; b – $p<0.05$ in comparison with the renal ischemia-reperfusion group; * – $p<0.05$.

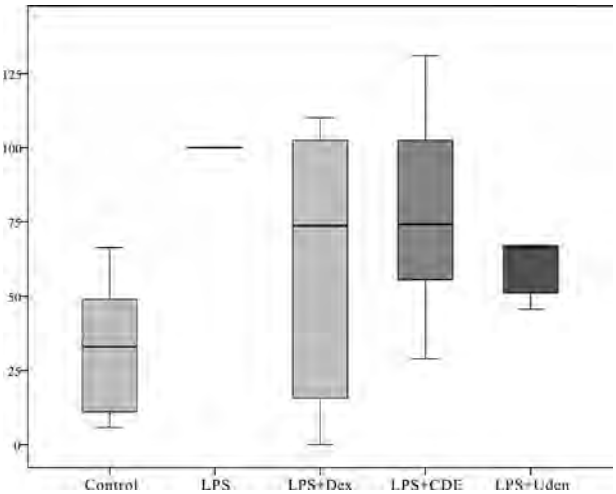



**Figure 12.** Effect of carbamylated darbepoetin and udenafil on the expression of the phosphorylated p65 subunit of nuclear factor kappa B in MNCs. **Note:** LPS – lipopolysaccharide-stimulated MNCs; Dex – dexamethasone; CDE – carbamylated darbepoetin; Uden – udenafil; a – $p<0.05$ in comparison with the control group; b – $p<0.05$ in comparison with the lipopolysaccharide-stimulated MNCs.

darbepoetin at a dose of 50 μg/kg and udenafil at a dose of 8.7 mg/kg led to a significant increase in the GFR index to 0.39±0.03 ml/min, which was comparable to that of the sham-operated animals and exceeded the efficiency of monotherapy modes with these drugs. By Day 3, the concentration of creatinine decreases more than twofold, thus surpassing the modes of monotherapy with the indicated pharmacological agents with a simultaneous increase in GFR to 0.43±0.02 ml/min. BUN concentrations decreased in all time periods of the experiment against the background of preventive administration of carbamylated darbepoetin and udenafil. FeNa also decreased by 2.6 and 12 times on the first and third days, respectively. Renal hemodynamic disorders were also successfully neutralized by the combination under study: after 24 and 72 hours of reperfusion, the level of microcirculation was 764.3±48.46 PU and 804.3±29.8 PU, respectively, which corresponded to the parameters of the sham-operated animals. The pathological picture was close to that of the sham-operated animals. The next series of experiments revealed the preconditioning properties of carbamylated darbepoetin and udenafil by means of preliminary blockade of ATP-dependent potassium channels through preliminary administration of glibenclamide at a dose of 5 mg/kg: this led to the elimination of the positive effects of carbamylated darbepoetin and udenafil, presumably due to blockade of ATP-dependent potassium channels.

Inflammation is an integral part of ischemic and subsequent reperfusion injury. One of the regulators of such inflammation is the nuclear factor kappa B system. Most of the genes that are under the transcriptional control of NF-κB encode the synthesis of biologically active substances involved in the immune response and inflammatory reactions (Johnson et al. 2017, Kezić et al. 2017).

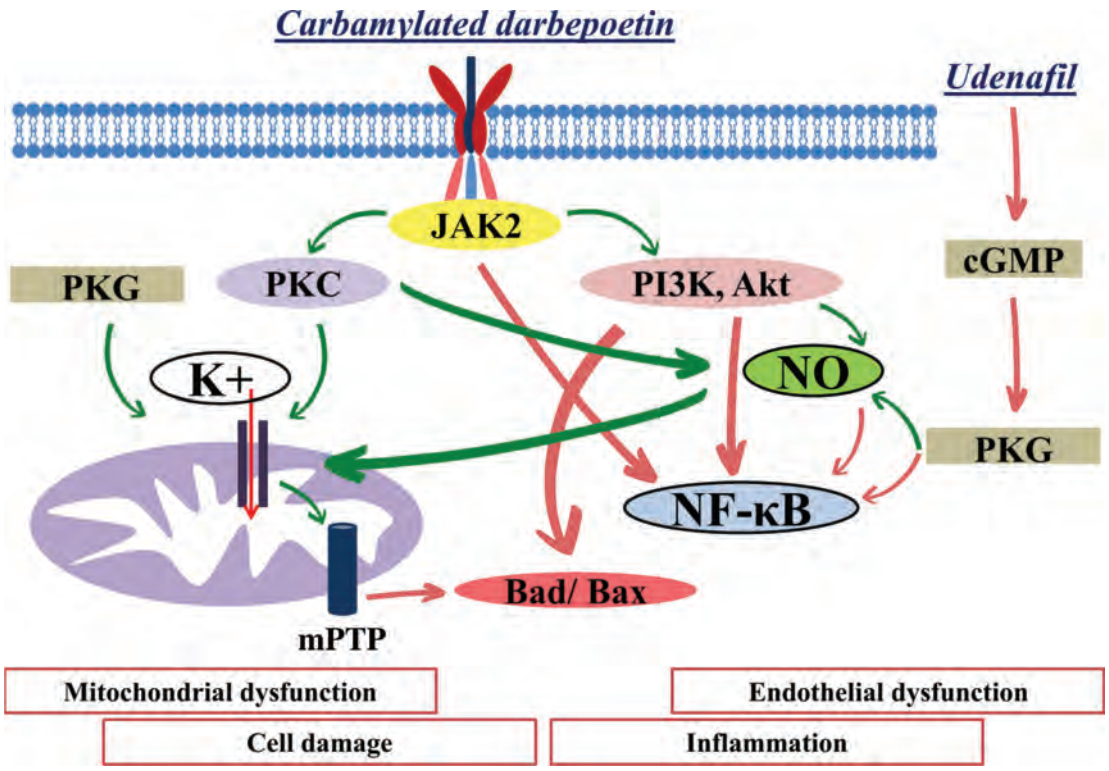



**Figure 13.** Potential mechanism of action of carbamylated darbepoetin and udenafil in bilateral renal ischemia-reperfusion. **Note:** JAK2 – Janus kinase 2; PI3K – Phosphoinositide 3-kinases; Akt – Protein kinase B; PKC – protein kinase C; PKG – protein kinase G; cGMP – cyclic guanosine monophosphate; NO – nitric oxide; mPTP – mitochondrial permeability transition pore; Bad – B-cell lymphoma 2 associated agonist of cell death**;** Bax – B-cell lymphoma 2- associated X protein; NF-κB – nuclear factor kappa B; $K^+$ – ATP-sensitive potassium channel

A series of experiments to study the inhibitory activity of udenafil and carbamylated darbepoetin against nuclear factor kappa B on rat mononuclear cells demonstrated a decrease in the expression of the active form of phospho-NF-κB p65 to 74.14 (54.76; 103.51)% and 63.72 (46.61; 67.01)% in the groups of carbamylated darbepoetin and udenafil, respectively, which is evidence of the realization of the renoprotective properties of these pharmacological agents by means of anti-inflammatory action through the nuclear factor kappa B system.

## Conclusion

The presented data demonstrate renoprotective properties of pharmacological preconditioning with carbamylated darbepoetin and udenafil under the conditions of simulating bilateral 40-minute ischemia-reperfusion of rat kidneys.

## Conflict of interests

The authors declare no conflict of interests.

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