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Research Article

Efficacy of the N-acetylglucosamine in experimental therapy of chronic kidney disease

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Abstract

The efficacy of N-acetylglucosamine in rats with chronic kidney disease is described in this article. The results of the study show that N-acetylglucosamine significantly increased ($p<0.05$) the excretion of nitrogen compounds (creatinine by 100.4% and urea by 46.6%) and as a result de-creased the level of azotemia. The intensity of free radical oxidation was significantly decreased ($p<0.05$) (blood conjugated dienes by 32.9%, blood thiobarbituric acid reactive substances – by 14.1%, kidney conjugated dienes – by 40.1% and kidney thiobarbituric acid reactive substances – by 26.3%) and the balance of kidney antioxidant system was restored. N-acetylglucosamine was significantly superior ($p<0.05$) to comparator quercetin in renal excretory function and nitro-gen metabolism by the most of indicators and was not inferior to the influence on the free radical oxidation and kidney oxidative stress. Thus, N-acetylglucosamine is advisable to further experimental studies at i.m. administration as a chronic kidney disease treatment.

Keywords

chronic kidney disease, intramuscular administration, N-acetylglucosamine, rats

Introduction

Chronic kidney disease (CKD) is not only the most common pathology among urinary system diseases, but also has a great medical and social significance (Lerma et al. 2019). Prevalence of CKD is 8–16% of the total population and reaches 47% among people over 70 years old (Feehally et al. 2019; Gilbert et al. 2018). At the same time, this pathology affects more than 500 million adults worldwide (Gilbert et al. 2018).

Effective treatment of CKD is an unresolved problem of modern medical and pharmaceutical practice. The course of CKD leads to severe complications such as chronic renal failure (CRF), which is accompanied by a decrease in renal excretory function, the development of azotemia, oxidative stress, anemia, endothelial dysfunction, electrolyte imbalance and other manifestations (Turner et al. 2016). Patients in this group are quickly disabled and lose their social activity (Lerma et al. 2019; Feehally et al. 2019; Gilbert et al. 2018). Therefore, there is an ever-increasing number of patients requiring renal replacement therapy and this population increases by about 7% each year (Turner et al. 2016). To date, in the world there are more than 2.5 million people receiving renal replacement therapy (Lerma et al. 2019).

In this regard, the search for drugs to improve the efficacy of CKD treatment and to reduce the rate of its progression, as well as to expand the list of effective drugs with nephroprotective action is an important task of the pharmaceutical science.

A protective effect on the membranes of renal tissue and restoration of its function is a great scientific interest in the treatment of CKD. Such effect may have agents with a direct nephroprotective action, which is realized by compensating for the deficiency of macromolecules of damaged glomerular basement membranes and intercellular substance. Drugs with similar properties are absent in modern nephrology practice (Turner et al. 2016).

Therefore, our attention was attracted by the amino sugar glucosamine (GA), which is a natural metabolite of the human body (Baynes and Dominiczak 2019; Lieberman and Peet 2018). GA is a part of glycosaminoglycans and glycoproteins of biological membranes, including the glomerular basement membrane (Morita et al. 2008). The biological effects of GA are fulfilled through the active metabolite – N-acetylglucosamine (NAG) and it is added into damaged kidney membranes in this form (Chen et al. 2010). Consequently, potentially NAG may have a more expressed nephroprotective effect than GA.

The most appropriate dosage form for NAG is injectable. In this form, NAG has unconditional benefits, since the parenteral route of administration allows to neutralize the effect of the first-pass metabolism and to ensure the inflow of the entire administered unchanged dose into the blood circulation system. In contrast, after the oral administration of GA it undergoes an active liver metabolism, which causes its absolute bioavailability of only 6 – 44% (Du Souich 2014).

In previous experimental studies, we have proved nephroprotective effect of the injectable dosage form of NAG in membranous nephropathy and acute kidney injury in rats (Shebeko et al. 2019a; Shebeko et al. 2019b). All this allows us to assume the high efficacy of this agent in the CKD treatment, not only with the latent course, but also with its exacerbations.

Taking into account the aforementioned facts, the aim of this work was to study the efficacy of NAG at parenteral administration under development of experimental CRF.

Materials and methods

Chemicals and drugs

The research object was NAG in the form of 6% solution for injections, which was developed and manufactured as a pilot series by PJSC SIC "Borschahivskiy CPP" (Ukraine). This drug was diluted with 0.9% solution of sodium chloride for injections up to the concentration of 20 mg/ml immediately before use. The test NAG samples were i.m. injected at the dose of 50 mg/kg, which was studied previously in the models of membranous nephropathy and acute kidney injury in rats (Shebeko et al. 2019a; Shebeko et al. 2019b).

Quercetin was chosen as the reference drug. It was used in the form of the Corvitin® (COR) medication produced by PJSC SIC "Borschahivskiy CPP" (Ukraine), which is a freeze-dried powder for injections. The efficacy of quercetin in the injectable dosage form was confirmed in experimental studies on various models of nephropathy in rats (Shebeko et al. 2018; Yang et al. 2018; Layal et al. 2017). When choosing a comparator drug, we proceeded from the maximum similarity of the test object, namely: natural origin, presence of similar pharmacological effects (antioxidant, membrane-protective, anti-inflammatory) and dosage form for parenteral administration.

Immediately before use, COR was diluted with 0.9% sodium chloride solution for injections up to the concentration of 10 mg/ml followed by i.p. injection in the dose of 34 mg/kg, corresponding to median effective dose for nephroprotective effect (Shebeko et al. 2018).

Test animals and treatment

Experimental study was performed using 38 random-bred male albino rats weighing 170–190 g, which were obtained, from the vivarium of the Central Research Laboratory, National University of Pharmacy (Kharkiv, Ukraine). The animals received standard rat diet and water ad libitum. The rats were housed under standard laboratory conditions in a well-ventilated room at 25±1 °C and a relative humidity 55±5 % with a regular 12 h light / 12 h dark cycle (Guide for the care and use of laboratory animals 2011; Sharp and Villano 2016). All studies were conducted in accordance with EU Council Directive 2010/63/EU dated 22 September, 2010 on compliance with the laws, regulations and administrative provisions of the EU Member States on the protection of animals used for scientific purposes. The experimental protocols were approved by the Bioethics Commission of the National University of Pharmacy (Kharkiv, Ukraine).

All animals were randomly divided into 4 experimental groups as follows:

Group 1 – intact control (healthy animals receiving vehicle, n=8).

Group 2 – control pathology (untreated animals receiving vehicle, n=10).

Group 3 – animals with CRF treated with NAG i.m., 50 mg/kg (n=10).

Group 4 – animals with CRF treated with COR i.p., 34 mg/kg (n=10).

Experimental design

As a CRF model was used mercuric chloride nephropathy in rats (Balakumar et al. 2008), which was induced by s.c. injection of 0.1% mercury (II) chloride solution (Sigma-Aldrich, USA) at a dose of 4 mg/kg for three days (Shtrygol´ et al. 2009). With such stimulation scheme, nephropathy unfolds slowly and in 2–3 weeks acquires a chronic character with all inherent manifestations. After that, the animals in the corresponding groups received NAG at a dose of 50 mg/kg and COR at a dose of 34 mg/kg. All test samples were administered as injectable solutions daily for 3 weeks. Animals of control groups received i.m. equivalent dose of 0.9% sodium chloride solution. 21 days after the induction of pathology in rats, the functional

state of the kidneys was evaluated. Subsequently, animals were sacrificed under general anesthesia with ketamine/xylazine (75/10 mg/kg, i.p.) (Flecknell 2015) to obtain the blood and renal tissue for biochemical assays.

Biological samples preparation and storage

Blood samples were collected from the inferior vena cava and were centrifuged at 1500 g at +4 °C for 10 min using refrigerated centrifuge "MPW-350R" (MPW, Poland). Urine samples were collected using individual metabolic cages and were centrifuged at 500 g for 10 min. 10% kidney homogenate was prepared in a cooled phosphate buffer (pH 7.4) using a Potter-Elvehjem type glass-teflon homogenizer in an ice-bath, and it was centrifuged at 10000 g at +4 ° C for 10 min. The supernatants were separated and used for the biochemical assays for evaluation of renal excretory function and oxidative stress. All biological samples were frozen and stored at -80 °C.

Assessment of renal functional state

At the end of the study in animals spontaneous daily diuresis and the amount of consumed fluid were determined by individual metabolic cages, and then the relative diuresis was calculated. The protein content and its daily excretion were determined in the collected urine. Glomerular filtration rate (GFR) was evaluated as endogenous creatinine clearance, tubular reabsorption (TR) and urea clearance (UC) were also calculated, using the standard formulas (Feehally et al. 2019; Gilbert et al. 2018; Kamyshnikov 2016):

$$GFR = U_{cr} \times V / P_{cr} \quad (1)$$

$$TR = (1 - P_{cr} / U_{cr}) \times 100\% \quad (2)$$

$$UC = U_{ur} \times V / P_{ur} \quad (3)$$

where U_{cr} is the urine creatinine concentration, V is the daily diuresis, P_{cr} is the plasma creatinine concentration, U_{ur} is the urine urea concentration and P_{ur} is the plasma urea concentration.

Biochemical evaluation of renal excretory function

To evaluate the parameters of renal excretory function and nitrogen metabolism, biochemical assays were performed using commercial kits "Creatinine FS" (cat. No 117119910021), "Urea FS" (cat. No 131019910021) and "Total protein UC FS" (cat. No 102109910021) manufactured by "DiaSys Diagnostic Systems GmbH" (Germany) using the automatic biochemical analyzer "Express Plus" (Bayer Diagnostics, Germany). Creatinine was determined in the animal's blood and urine using a kinetic test without deproteinization according to Jaffe method. Blood and urine urea was determined using urease – glutamate dehydrogenase enzymatic UV test. Urinary excretion of creatinine and urea was also calculated. The concentration of urine protein was determined by a photometric test with pyrogallol red reaction (Kamyshnikov 2016).

Lipid peroxidation measurement

In order to evaluate the intensity of the lipid peroxidation (LPO) processes in rats, the determination of the primary and secondary products of LPO: conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) in the blood and kidney homogenate was performed (Ahmad 2016). The content of CD in homogenate was determined spectrophotometrically at a wavelength of 233 nm in a cyclohexane medium, with the prior extraction of chloroform: methanol (2:1) (Devasagayam et al. 2003; Sen et al. 2000). TBARS were determined by the reaction of thiobarbituric acid with heating in a water boiling bath with the following spectrophotometric determination of colored products at 532 nm (Devasagayam et al. 2003; Grotto et al. 2009). All spectrophotometric measurements were performed using the UNICO SQ-2800 spectrophotometer (United Products & lnstruments Inc., USA).

Antioxidant enzymes assay

To assess the state of the kidney antioxidant system (AOS), the content of reduced glutathione (GSH) in kidney homogenate and the activity of superoxide dismutase (SOD) and catalase (CAT) enzymes were determined (Ahmad 2016). For this purpose commercial kits (Elabscience Biotechnology Inc., USA) were used according to the manufacturer's instructions.

GSH was determined using a "Reduced Glutathione (GSH) Colorimetric Assay Kit" (cat. No. E-BC-K051) by the reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) by spectrophotometry at an absorbance of 420 nm (Tipple and Rogers 2012).

SOD activity was determined using the "Superoxide Dismutase (SOD) Typed Colorimetric Assay Kit" (cat. No. E-BC-K022) according to the hydroxylamine method with spectrophotometer at 550 nm (Martin 1990). It utilizes a hydroxylamine for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit (U) of SOD is defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radicals.

CAT activity assay was performed using the "Catalase (CAT) Colorimetric Assay Kit" (cat. No. E-BC-K031) with spectrophotometric determination of hydrogen peroxide (H_2O_2) which forms a stable complex with ammonium molybdate absorbing at 405–410 nm (Goth 1991; Hadwan and Abed 2016). The amount of CAT that decomposes 1 μmol of H_2O_2 per minute at 37 ° C is defined as 1 U.

The kidney homogenate was assayed for protein concentration according to the Lowry method using Folin & Ciocalteu's reagent and bovine serum albumin (Sigma-Aldrich, USA) as standard (Waterborg 2002). All the

results correlated with the concentration of protein in the homogenate and counted on 1 mg of protein.

Statistical analysis

All the results were processed by descriptive statistics and presented as the mean ± standard error of the mean (M±SEM) excluding the survival rate. Statistical differences between groups were analyzed using one-way ANOVA followed by Dunnett`s post-hoc test and using Fisher's exact test for survival analysis (Islam and Al-Shiha 2018; Quirk et al. 2015). Utilized computer software included IBM SPSS Statistics v. 22 (IBM Corp., USA) and MS Excel 2016 (Microsoft Corp., USA).The level of statistical significance was considered as $p < 0.05$.

Results and discussion

The results of the experiment indicate that severe CRF develops within 3 weeks under the nephrotoxic influence of mercuric chloride in untreated animals. Rats were in poor physiological state, with reduced motor activity, edema and ascites. There was a high mortality rate, with animal survival of only 50% (Fig. 1).

Renal excretory function in rats of this group was significantly decrease ($p<0.05$) compared to intact animals. Daily and relative diuresis was decreased by 45.9% and 20.0%, respectively. GFR index was reduced to 69.6 ml/day and TR – by 3.3%. (Table 1). The level of proteinuria increased to 31.3 mg/day (Fig. 2).

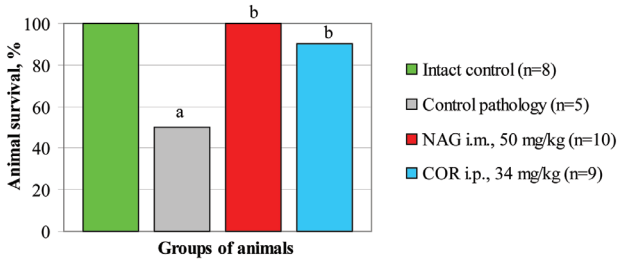

Figure 1. Influence of NAG and COR on survival of rats with CRF. Notes. [a] – $p < 0.05$ compared to the intact control group; [b] – $p < 0.05$ compared to the control pathology group (Fisher's exact test); n – amount of animals at the end of experiment.

Table 1. Indicators of the renal excretory function under the influence of NAG and COR in rats with CRF, M±SEM.

Group of animals	Daily diuresis, ml/day	Relative diuresis, %	GFR, ml/day	TR, %
Intact control (n=8)	6.1±0.2	51.1±2.3	413.4±19.8	98.50±0.06
Control pathology (n=5)	3.3±0.2 [a]	40.9±1.4 [a]	69.6±3.0 1	95.21±0.20 [a]
NAG i.m., 50 mg/kg (n=10)	5.7±0.1 [b]	50.0±0.6 [b]	328.6±8.8 [ab]	98.26±0.07 [ab]
COR i.p., 34 mg/kg (n=9)	6.9±0.2 [abc]	52.5±0.7 [bc]	286.9±10.7 [abc]	97.60±0.03 [abc]

Notes. [a] – $p < 0.05$ compared to the intact control group; [b] – $p < 0.05$ compared to the control pathology group; [c] – $p < 0.05$ compared to the NAG-treated group (ANOVA, Dunnett's post-hoc test); n – amount of animals at the end of experiment.

As a result, impaired renal function led to the development of azotemia. Blood creatinine and urea were 4.0 and 4.1 times higher ($p<0.05$) than in healthy rats, respectively. UC level significantly decreased ($p<0.05$) to 36.3 ml/day (Table 2). Corresponding changes were also observed in the parameters of nitrogen compounds elimination: creatinine excretion was significantly reduced ($p<0.05$) by 33.1%, and urea – by 13.1% (Fig. 3).

The development of nephropathy was accompanied by the activation of free radical oxidation and formation of kidney oxidative stress. Accumulation of primary and secondary LPO products was determined in the blood and kidneys of untreated animals. The blood CD and TBARS levels were 2.1 and 1.5 times higher ($p<0.05$) than in intact rats, respectively. In the kidney homogenates, CD and TBARS indices were 2.5 and 2.0 times higher, respectively (Table 3). At the same time, AOS imbalance occurred in the renal tissue. GSH content was significantly reduced ($p<0.05$) by 34.4%, SOD activity – by 29.5%, and CAT activity was increased by 42.2% compared to intact animals (Fig. 4).

There was an expressed positive effect on the course of CRF, when NAG was applied to treat animals. Under its influence, the functional state of rats normalized and mortality disappeared (Fig. 1). There was a significant increase ($p<0.05$) in the renal excretory function compared to untreated animals: daily diuresis was increased by 72.7%, relative diuresis – by 22.3%, TR – by 3.2%, and GFR index was 4.7 times higher (Table 1). In addition, NAG contributed to a significant decrease ($p<0.05$) in proteinuria by 50.0% (Fig. 2).

Positive changes occurred in the nitrogen metabolism. NAG significantly increased ($p<0.05$) the urinary excretion of creatinine by 100.4% and urea by 46.6% compared to the control pathology group (Fig. 3). As a result, blood creatinine and urea levels were 2.4 and 2.5 times lower, respectively. UC index was 3.6 times higher ($p<0.05$) than in untreated animals (Table 2).

Additionally, NAG exhibited a pronounced antioxidant effect. Under its influence, the LPO products in blood and kidney tissue were significantly lower ($p<0.05$) compared to untreated animals: blood CD was decreased by 32.9%, blood TBARS – by 14.1%, kidney CD – by 40.1% and kidney TBARS – by 26.3% (Table 3). There was a significant increase ($p<0.05$) in kidney GSH content and SOD activity by 48.5%

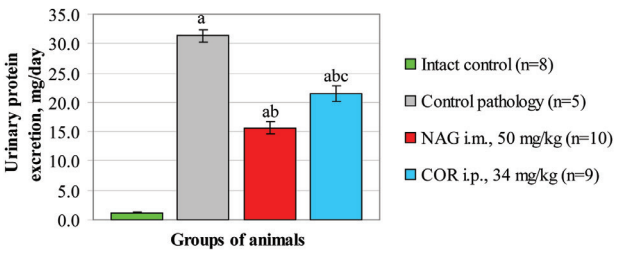

Figure 2. Influence of NAG and COR on proteinuria in rats with CRF, M±SEM. Notes. [a] – $p < 0.05$ compared to the intact control group; [b] – $p < 0.05$ compared to the control pathology group; [c] – $p < 0.05$ compared to the NAG-treated group (ANOVA, Dunnett's post-hoc test); n – amount of animals at the end of experiment.

Table 2. Indicators of nitrogen metabolism in rats with CRF under the influence of NAG and COR, M±SEM.

Group of animals	Blood creatinine, μmol/l	Blood urea, mmol/l	UC, ml/day
Intact control (n=8)	51.2±1.6	4.95±0.22	170.0±5.3
Control pathology (n=5)	203.1±14.5 [a]	20.31±0.88 [ab]	36.3±1.6 [a]
NAG i.m., 50 mg/kg (n=10)	86.0±2.7 [ab]	8.25±0.24 [ab]	131.2±4.2 [ab]
COR i.p., 34 mg/kg (n=9)	96.8±3.9 [abc]	9.06±0.26 [abc]	108.9±4.3 [abc]

Notes. [a] – $p < 0.05$ compared to the intact control group; [b] – $p < 0.05$ compared to the control pathology group; [c] – $p < 0.05$ compared to the NAG-treated group (ANOVA, Dunnett's post-hoc test); n – amount of animals at the end of experiment.

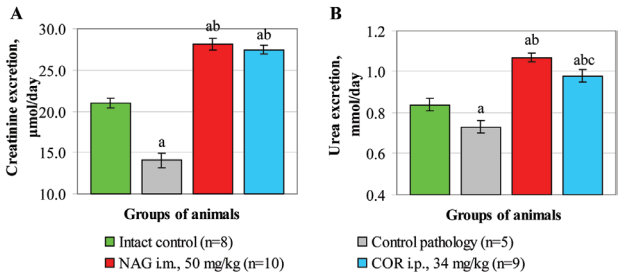

Figure 3. Urinary excretion of creatinine (**A**) and urea (**B**) under the influence of NAG and COR in rats with CRF, M±SEM. Notes. [a] – $p < 0.05$ compared to the intact control group; [b] – $p < 0.05$ compared to the control pathology group; [c] – $p < 0.05$ compared to the NAG-treated group (ANOVA, Dunnett's post-hoc test); n – amount of animals at the end of experiment.

Table 3. Influence of NAG and COR on the content of LPO blood and renal tissue products in rats with CRF, M±SEM.

Group of animals	Blood CD, μmol/l	Blood TBARS, μmol/l	Kidney CD, nmol/mg protein	Kidney TBARS, μmol/mg protein
Intact control (n=8)	51.2±1.6	2.54±0.16	40.0±2.2	0.41±0.02
Control pathology (n=5)	106.6±4.6 [a]	3.75±0.16 [a]	99.5±4.3 [a]	0.80±0.03 [a]
NAG i.m., 50 mg/kg (n=10)	71.5±2.8 [ab]	3.22±0.13 [ab]	59.6±2.3 [ab]	0.59±0.02 [ab]
COR i.p., 34 mg/kg (n=9)	65.7±2.4 [ab]	2.75±0.10 [bc]	54.8±2.0 [ab]	0.55±0.02 [ab]

Notes. [a] – $p < 0.05$ compared to the intact control group; [b] – $p < 0.05$ compared to the control pathology group; [c] – $p < 0.05$ compared to the NAG-treated group (ANOVA, Dunnett's post-hoc test); n – amount of animals at the end of experiment.

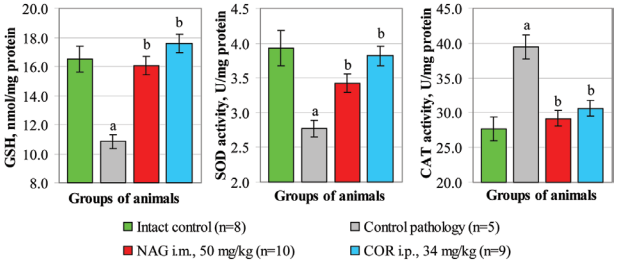

Figure 4. Influence of NAG and COR on kidney AOS indices in rats with CRF, M±SEM.Notes. [a] – $p < 0.05$ compared to the intact control group; [b] – $p < 0.05$ compared to the control pathology group (ANOVA, Dunnett's post-hoc test); n – amount of animals at the end of experiment.

and 23.5% compared to control pathology group, respectively. Except this, CAT activity decreased by 26.0%, indicating a normalization of the balance of kidney AOS (Fig. 4).

The reference drug COR has shown a lower level of efficacy. Under its influence, the animal survival was increased to 90% (Fig. 1). Daily, relative diuresis and GFR index were 2.1, 1.3 and 4.1 times higher ($p<0.05$) than in untreated rats (Table 1). Also, there was a reliable decrease ($p<0.05$) in proteinuria (Fig. 2). COR significantly increased ($p<0.05$) the creatinine and urea excretion, and reduced their blood levels by 52.3% and 55.4%, respectively. UC index also was 3.0 times higher ($p<0.05$) than in control pathology group (Table 2).

Under the influence of COR, blood CD and TBARS indices were significantly decreased ($p<0.05$) compared to untreated animals and their kidney levels were reduced by 44.9% and 31.3%, respectively (Table 3). The kidney GSH and SOD activity indices were significantly increased ($p<0.05$) by 62.6% and 37.9%, respectively, and the CAT activity was decreased by 22.3%, indicating the restoration of kidneys AOS balance (Fig. 4).

The high efficacy of NAG nephroprotective action is due to the fact that it is an active metabolite of GA (Baynes and Dominiczak 2019) and has a direct protective effect on damaged membranes and intercellular substance of the renal tissue by attaching to their macromolecules in unchanged form (Chen et al. 2010). The hypoazotemic action of NAG is due to the improvement of intraglomerular hemodynamics, which leads to increased urine excretion of nitrogen metabolism products under the influence of filtration processes.

The basis of antioxidant action of NAG is the protective effect on the kidney membranes, which prevents their lipoperoxidation, decline of the kidney AOS and contributes to the preservation of its enzymes activity. This kind of NAG pharmacodynamics has a great importance in the CKD treatment, since free radical oxidation and oxidative stress are a significant link in the pathogenesis of this pathology (Krata et al. 2018; Daenen et al. 2019).

NAG from the GA derivatives group was little studied as an agent for kidney pathology treatment. In previous experiments, we studied the nephroprotective properties of NAG in various routes of administration and proved that this is the most effective GA derivative in rats with membranous nephropathy. We showed that NAG integrates into the damaged structures of kidney tissue and increases the content of endogenous hexosamines therein (Shebeko et al. 2019a). In next study, we proved its high efficacy in rats with acute kidney injury (Shebeko et al. 2019b). These results correlate with other studies, which showed the efficacy of GA in the treatment of kidney fibrosis in mice (Park et al. 2013), contrast-induced acute kidney injury in rats (Hu et al. 2017) and the efficacy of its conjugates in rats with renal ischemia/reperfusion injury (Wang et al. 2014; Fu et al. 2016).

The comparator COR showed a positive effect on the CRF as expected, because it contains quercetin. This is

consistent with other studies, in which the nephroprotective properties of quercetin have been studied and showed promising prospects for its use in kidney diseases treatment (Shebeko et al. 2018; Yang et al. 2018; Layal et al. 2017; Vargas et al. 2018). But COR showed mainly an antioxidant effect. This is due to the fact that, first of all, quercetin is a known natural antioxidant (Ahmad 2016; Sen et al. 2000; Anand David et al. 2016).

According to the results obtained, NAG was significantly superior ($p<0.05$) to COR in normalizing renal excretory function and nitrogen metabolism by the most of indicators. Because unlike NAG, quercetin does not directly affect the renal tissue, and its action is realized through antioxidant, antihypoxic, anti-inflammatory and other effects. But the most important thing is that NAG was not inferior to the influence of COR on the free radical oxidation and oxidative stress in the kidney.

Therefore, NAG has a more balanced pharmacodynamic complex for the CKD treatment, in which nephroprotective, hypoazotemic and antioxidant effects should be distinguished. In this regard, the results have a great significance for nephrology, since they discover wide perspectives for the use of a new nephroprotective drug – the injectable dosage form of NAG.

Conclusion

Under the conditions of CRF in rats, amino sugar NAG at i.m. administration has a significant nephroprotective, hypoazotemic and antioxidant effect. A great advantage of the drug is i.m. route of administration, since it causes the receipt of the entire dose of hexosamine in active form to the blood circulation system and kidney tissue. NAG credible exceeds the nephroprotective effect of comparator COR in rats with CRF and is not inferior to its antioxidant activity. NAG in the injectable dosage form is a promising agent for the CKD treatment, which should be confirmed in further studies.

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