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Dose-dependent Efficacy of the N-acetylglucosamine and Quercetin Combination in Rats with Renal Failure

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ABSTRACT

The study was devoted to the evaluation of dose-dependent efficacy of N-acetylglucosamine and quercetin combination in the treatment of renal failure. The combination was studied in injectable dosage form at the doses of 10, 20, 40 and 60 mg/kg in rats with chromium-induced nephropathy. The efficacy of combination was assessed by animal survival, renal excretory function, nitrogen metabolism and nephroprotective activity. Also, the ED_{50} index was calculated by Probit Analysis method. The efficacy of combination at doses of 40 and 60 mg/kg was most expressed with insignificant differences ($p>0.05$) between them. There was a significance increase ($p<0.05$) in animal survival, renal excretory function and normalization of nitrogen metabolism. This led to the nephroprotective activity of 67.8 and 70.4%, respectively. The ED_{50} index of combination was 30.2 ± 6.3 mg/kg. Thus, this test combination at a dose of 30.2 mg/kg is the promising drug for experimental treatment of kidney diseases.

Keywords: N-acetylglucosamine, Quercetin, Nephroprotective effect, Median effective dose, Renal failure

INTRODUCTION

Improving the efficacy of the renal diseases treatment is an important problem in the medical and pharmaceutical practice. The first place in this group of diseases occupied by chronic kidney disease (CKD), since it is not only the most common pathology of the urinary system, but also has a great medical

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and social significance[1]. Prevalence of CKD is 8-16% of the total population and reaches 47% among people over 70 years old[2-3]. At the same time, this pathology affects more than 500 million adults in the whole world[3].

The course of CKD leads to severe complications such as renal failure (RF), which is accompanied by a decrease of renal excretory function, development of azotemia, oxidative stress, electrolyte imbalance, and other manifestations[4]. Patients with CKD are quickly disablement and lose their social activity[1-3]. Therefore, there is an ever-increasing number of patients requiring renal replacement therapy, and this population increases by about 7% each year[4], and it is over 2.5 million people in the world[1]. In this regard, the search for drugs to improve the efficacy of CKD treatment and reduce the rate of its progression, as well as to expand the list of effective nephroprotective agents is an important task of the pharmaceutical science.

A promising approach in the solution of this problem may be the implementation of combined drugs based on membrane protects and antioxidants of natural origin, among the properties of which nephroprotective effects on different mechanisms of action are present. Based on this, the research of pharmaceutical combination on the basis of quercetin and glucosamine derivative – N-acetylglucosamine (NAG) in injectable dosage form is a great scientific interest.

Quercetin is a well-known flavonoid of plant origin with a wide range of pharmacological effects. The most significant among them are antioxidant, antihypoxic, membrane stabilizing and anti-inflammatory[5-6]. As a result, quercetin has angioprotective properties and reduces the permeability of glomerular capillaries. This complex of effects is useful in the treatment of renal diseases.

Glucosamine is a natural human metabolite almost safe for the body[7-8]. It is a part of glycosaminoglycans and glycoproteins of the biological membranes, including the glomerular basement membrane[9], which causes its nephroprotective properties. Glucosamine realizes its physiological effects through the biologically active form – NAG and it is added to the damaged membranes in this form[10]. Therefore, potentially NAG has a more expressed nephroprotective effect due to direct mechanism of action.

Based on the peculiarities of the pharmacological properties of quercetin and NAG, the combined drug on their basis is promising for the renal diseases treatment, since both components mutually complement each other's pharmacodynamics with the effects necessary for the therapy of kidney diseases. In previous experimental studies, we have proved the high efficacy of oral combinations of quercetin with some glucosamine derivatives on different models of kidney injury in rats[11].

In this regard, the scientific interest was to study the combined injection drug, which may be more effective not only in the latent course of kidney diseases, but also in acute injuries and exacerbations of chronic nephropathies. The implementation of this combination requires the investigation of dose-dependent efficacy in RF, in order to determine the optimal dose for further in-depth preclinical and clinical studies. The aim of this study was to research the efficacy of the injection combination of NAG and quercetin in different doses in rats with RF.

METHODOLOGY

Animals

Experimental study was performed using 58 randomly selected male albino rats weighing 170-190 g, which were obtained from the *vivarium* of the Central Research Laboratory of the 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 with a relative humidity of 55 ± 5% with a regular 12 h light / 12 h dark cycle[12-13]. All studies were conducted in accordance with the EU Council Directive 2010/63/EU dated 22 September 2010 on the protection of animals used for scientific purposes[14]. The experimental protocols were approved by the Bioethics Commission of the National University of Pharmacy.

Test object and its preparation

Research object was the combination of NAG and quercetin in the injectable dosage form in a ratio of 1:1. NAG was used as a 6% solution for injections, which was developed and manufactured as a pilot series by PJSC SIC "Borschahivskiy CPP" (Ukraine). Quercetin was used as the Corvitin® (COR) medication (PJSC SIC "Borschahivskiy CPP, Ukraine), which is a freeze-dried powder for injections. COR was diluted with a solution of NAG to achieve a 1:1 ratio and added 0.9% sodium chloride solution for injections to a concentration of 20 mg/mL (for the sum of active substances) to prepare the combination immediately before use.

Experimental design

All animals were randomly divided into 6 experimental groups as follows.

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

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

Group 3 – rats with RF treated with NAG/COR at 10 mg/kg (n = 10).

Group 4 – rats with RF treated with NAG/COR at 20 mg/kg (n = 10).

Group 5 – rats with RF treated with NAG/COR at 40 mg/kg (n = 10).

Group 6 – rats with RF treated with NAG/COR at 60 mg/kg (n = 10).

Chromium-induced nephropathy was used as RF model[15]. It was induced by subcutaneous injection of 2.5% potassium chromate solution (Sigma-Aldrich, USA) in an original modification at a dose of 0.7 mL/kg on the first day of experiment[16]. After this, animals received the test NAG/COR combination at doses of 10, 20, 40 and 60 mg/kg (for the sum of active substances). All test samples were injected intramuscularly daily for 10 days. Animals of control groups were received simultaneously intramuscular injections of equivalent dose of 0.9% sodium chloride solution. The functional state of the kidneys was evaluated 10 days after the pathology induction.

Biological samples preparation and storage

The animals were sacrificed under anesthesia with ketamine/xylazine (75/10 mg/kg, i.p.) at the end of experiment[17]. Blood samples were collected from the inferior vein cava and centrifuged at 1500 g at +4°C for 10 minutes using refrigerated centrifuge Eppendorf 5702R (Eppendorf, Germany). Urine samples were collected using individual metabolic cages and centrifuged at 500 g for 10 min. The supernatants were separated and used for biochemical assays. All biological samples were frozen and stored at -80 °C.

Evaluation of the functional state of kidneys

Spontaneous daily diuresis was determined with individual metabolic cages at the end of experiment in all animals. The protein content and its daily excretion were determined in the collected urine[18]. Glomerular filtration rate (GFR) was evaluated as endogenous creatinine clearance, tubular reabsorption (TR) and urea clearance (UC) were also calculated, using the standard equations[2-3,18]:

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

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

$$UC = U_{ur} \times V / P_{ur}$$
(eq 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 assays

Biochemical studies were performed using commercial kits "Creatinine FS" (cat. 117119910021), "Urea FS" (cat. 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) to evaluate the parameters of excretory renal function and nitrogen metabolism. The creatinine and urea blood and urine levels were determined using a kinetic test without deproteinization according to Jaffe method and urease glutamate dehydrogenase enzymatic UV test respectively[18]. Urinary excretion of creatinine and urea was also calculated. The protein urine concentration was determined by photometric reaction with pyrogallol red[18].

Calculation of nephroprotective activity

The nephroprotective activity (NA) of the test combination was evaluated for its ability to reduce glomerular filtration dysfunction. The NA index was assessed by the degree of GFR amplification in comparison with untreated animals and calculated by the formula:

$$NA = (GFRt - GFRc) / (GFRi - GFRc) \times 100\% \qquad \text{(eq 4)}$$

Where GFRt is the value of GFR under the influence of the test sample, GFRc is the control pathology group GFR value and GFRi is the intact control group GFR value.

Calculation of the median effective dose (ED_{50})

Indicator ED_{50} of the test combination was calculated based on the dose-dependent NA with Probit Analysis method[19]. To do this, we used Probit Analysis according to the Bliss-Finney method in the modification of Prozorovskii[20] and MS Excel 2016 software (Microsoft Corp., USA).

The calculations were carried out in the next way. The percentages of activity in each group were converted to probits (y) and their weighing factors (B) according to special tables. The dose points (x) and intermediate variables were determined with necessary calculations. The relationship between dose points (x) and probits (y) was reflected by the equation:

$y=A_0 + A_1x$ (eq 5)

Coefficients A_0 and A_1 were calculated by following equations:

$A_0 = [(\Sigma B) - (\Sigma xB)A_1] / \Sigma B$ (eq 6)

$\Sigma xyB = \Sigma xB / \Sigma B \times [\Sigma yB - (\Sigma xB)A_1] + (\Sigma x^2B)A_1$ (eq 7)

The solution of these equations allowed constructing a Probit Analysis chart of the "activity-dose" dependence. Then the dose points and dose variables were found by equation 5 for ED_{16}, ED_{50} and ED_{84}, taking into account values of probits, which are 4 for ED_{16}, 5 – ED_{50} and 6 – ED_{84}, respectively.

The standard error (SE) of ED_{50} was determined by the equation:

$SE = (ED_{84} - ED_{16}) / 2\sqrt{n}$ (eq 8)

Where ED_{84} is the dose corresponding to the drug activity of 84%, ED_{16} is the dose corresponding to the drug activity of 16% and n is the number of observations.

Statistical analysis

All the results were processed by descriptive statistics and presented as the mean ± standard error of the mean (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[21-22]. 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

A high mortality was observed in the control pathology group 10 days after chromium-induced nephropathy with animal survival rates of only 50% (Figure 1). Rats were in poor physical condition, with reduced motor activity, edema and ascites.

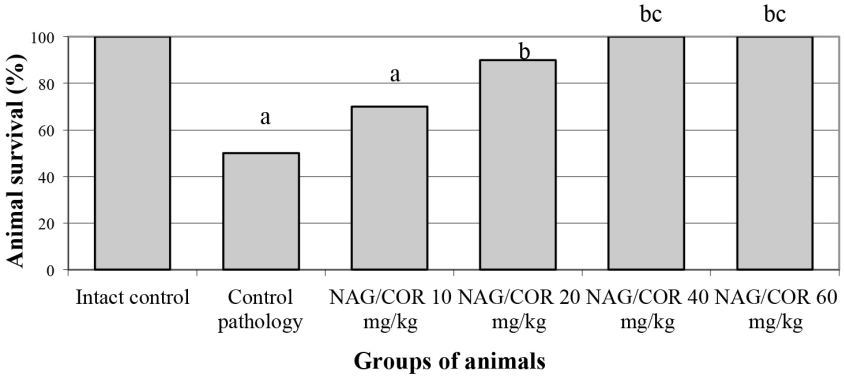

Figure 1. Influence of NAG/COR combination at different doses on the survival of rats with RF.

Data are presented as percentage of animals survived in each group. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg (Fisher's exact test).

The renal excretory function was expressly deteriorated. Daily diuresis was 1.6 times and GFR – 2.7 times lower (p<0.05) compared to intact animals and TR was increased by 1.1% (Table 1). In addition, proteinuria was observed, which reached 41.3 mg/day (Figure 2).

Table 1. Effect of NAG/COR combination at different doses on the renal excretory function in rats with RF.

Groups of animals	Daily diuresis (mL/day)	GFR (mL/day)	TR (%)
Intact control	6.7 ± 0.2	402.0 ± 16.8	98.32 ± 0.06
Control pathology	4.1 ± 0.2 [a]	150.5 ± 7.9 [a]	97.24 ± 0.16 [a]
NAG/COR 10 mg/kg	4.9 ± 0.2 [abde]	219.3 ± 5.6 [abde]	97.76 ± 0.09 [abde]
NAG/COR 20 mg/kg	5.5 ± 0.1 [abcde]	255.3 ± 9.0 [abcde]	97.80 ± 0.12 [ab]
NAG/COR 40 mg/kg	6.2 ± 0.2 [ac]	320.9 ± 11.8 [abc]	98.06 ± 0.04 [abc]
NAG/COR 60 mg/kg	6.4 ± 0.3 [ac]	327.5 ± 14.5 [abc]	98.03 ± 0.04 [abc]

*Data are expressed as mean ± SEM. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [e]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

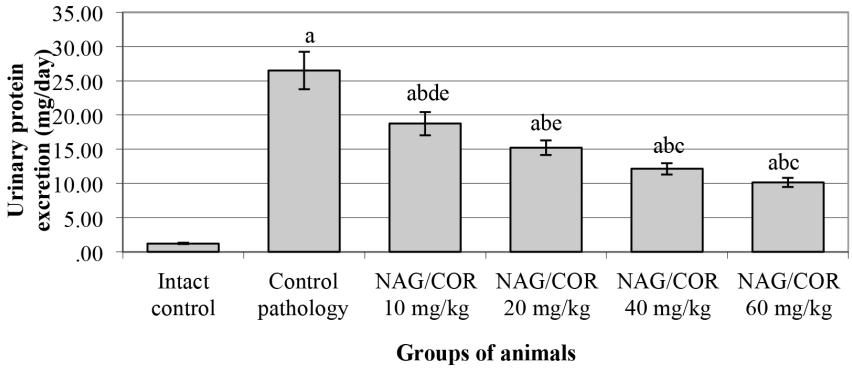

Figure 2. Influence of NAG/COR combination at different doses on the urinary protein excretion in rats with RF.

*Data are expressed as mean ± SEM. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [e]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

The kidney dysfunction led to a decrease in the excretion of nitrogenous compounds and an increase in the blood residual nitrogen level. The blood creatinine and urea were 3.1 and 3.2 times higher (p<0.05) than in intact rats, respectively (Table 2). Their urinary excretion was increased, which can be regarded as an organism compensatory reaction to the nitrogen compounds retention and autointoxication (Table 2). But this was not enough to rebalance nitrogen metabolism.

Table 2. Influence of NAG/COR combination at different doses on the nitrogen metabolism in rats with RF.

Groups of animals	Blood level		Urine excretion	
	Creatinine (μmol/L)	Urea (mmol/L)	Creatinine (μmol/day)	Urea (mmol/day)
Intact control	60.6 ± 2.7	4.7 ± 0.3	24.1 ± 0.5	0.78 ± 0.05
Control pathology	188.8 ± 8.8 [a]	15.1 ± 0.8 [a]	28.2 ± 0.7 [a]	0.83 ± 0.06
NAG/COR 10 mg/kg	148.3 ± 7.6 [abde]	12.3 ± 0.4 [abde]	32.4 ± 1.3 [ab]	0.93 ± 0.06 [de]
NAG/COR 20 mg/kg	132.1 ± 6.1 [abde]	10.6 ± 0.5 [abcde]	33.6 ± 1.7 [ab]	1.07 ± 0.05 [ab]
NAG/COR 40 mg/kg	107.1 ± 5.5 [abc]	8.5 ± 0.4 [abc]	34.0 ± 1.3 [ab]	1.12 ± 0.05 [abc]
NAG/COR 60 mg/kg	99.6 ± 5.5 [abc]	8.2 ± 0.4 [abc]	32.1 ± 1.2 [ab]	1.10 ± 0.04 [abc]

*Data are expressed as mean ± SEM. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [e]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

Corresponding changes were also observed in UC, which reflects the rate of blood purification from urea. In untreated rats, this index dropped to 54.9 mL/day, which was 3.0 times lower (p<0.05) than in intact rats (Figure 3).

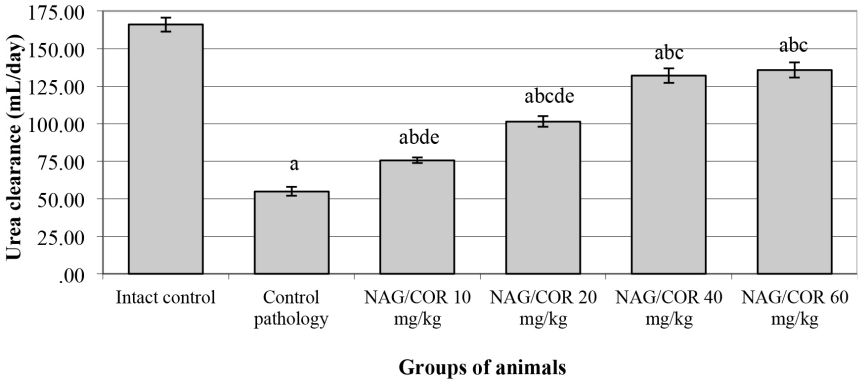

Figure 3. Effect of NAG/COR combination at different doses on the urea clearance in rats with RF.

*Data are expressed as mean ± SEM. [a]p<0.05 compared to intact control group, [b]p<0.05 compared to control pathology group, [c]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [e]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

The described pattern is typical for chromium-induced nephropathy, which develops as a result of toxic effect of chromium compounds on the proximal nephron tubule, induces tubular necrosis and subsequent RF[15].

Test NAG/COR combination showed a positive dose-dependent effect on the course of RF. Under its influence at a dose of 10 mg/kg, the functional state of rats was improved, and survival increased to 70%, which, however, was unreliably (Figure 1). There was a significant increase (p<0.05) in the renal excretory function compared to untreated animals: diuresis was increased by 19.5% and GFR – by 45.7% (Table 1). Also, there was a significant decrease (p<0.05) in urinary protein excretion by 29.2% (Figure 2). Additionally, creatinine and urea blood levels were significantly decreased (p<0.05) by 21.5% and 18.5%, respectively (Table 2). The UC index was significantly increased (p<0.05) by 37.7% (Figure 3). As a result, the NA index was 27.3% (Figure 4).

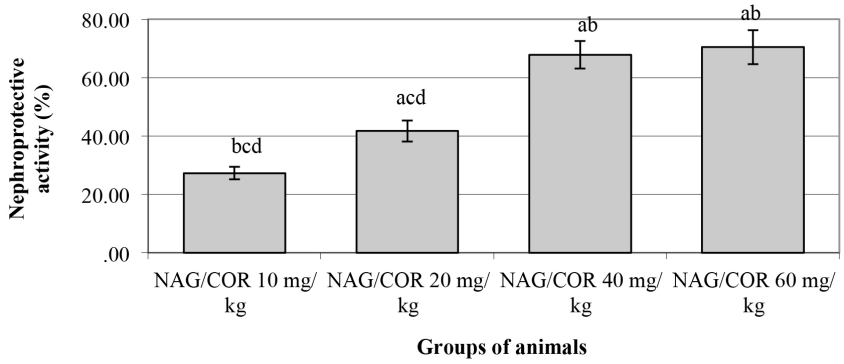

Figure 4. Nephroprotective activity of NAG/COR combination at different doses in rats with RF.

*Data are expressed as mean ± SEM. [a]p<0.05 compared to group treated with NAG/COR at 10 mg/kg, [b]p<0.05 compared to group treated with NAG/COR at 20 mg/kg, [c]p<0.05 compared to group treated with NAG/COR at 40 mg/kg, [d]p<0.05 compared to group treated with NAG/COR at 60 mg/kg (*ANOVA, *Dunnett's* post-hoc test*).*

The use of test combination at a higher dose level of 20 mg/kg led to increased efficacy, which was reliably in the most cases. The animal survival rate was 90% unlike the previous group (Figure 1). Daily diuresis and GFR were significantly increased ($p<0.05$) by 34.1% and 69.6% compared to untreated animals, respectively (Table 1). The proteinuria level was 1.7 times lower (Figure 2). This test sample significantly reduced ($p<0.05$) the creatinine and urea blood levels by 30.0%, and also increased their excretion by 19.1% and 28.9%, respectively (Table 3). In addition, UC was 1.8 times higher ($p<0.05$) than in untreated animals (Figure 3). The NA index reached to 41.7%, which was credibly higher ($p<0.05$) than at the dose of 10 mg/kg (Figure 4).

An increase in the NAG/COR dose level up to 40 mg/kg resulted in a significant enhancing of efficacy, which was reliable ($p<0.05$) compared to dose level of 20 mg/kg in the most of assessment parameters. Under the influence of NAG/COR at 40 mg/kg, the functional state of rats normalized, and mortality disappeared. The survival rate was at the level of intact group – 100% (Figure 1). Renal excretory function was significantly increased ($p<0.05$) compared to the control pathology group: diuresis was 1.5 times higher, GFR was 2.1 times higher and TR was increased by 0.84% (Table 1). Also, the level of proteinuria was 2.2 times lower (Figure 2). This dose reliably increased ($p<0.05$) urinary creatinine and urea excretion. As a result, the blood creatinine and urea were 1.8 times lower ($p<0.05$) than in untreated animals (Table 2), and there was a

significant 2.4-fold increase in UC (Figure 3). The obtained results allowed to calculate the NA index, which was 67.8% (Figure 4).

A similar level of influence on RF was observed with the test combination at a dose of 60 mg/kg. At the same time, differences in all indicators of efficacy compared to the dose of 40 mg/kg were insignificant, despite a 1.5-fold higher dose. The NA index in this case was 70.4%, which was significantly higher ($p<0.05$) than in doses of 10 and 20 mg/kg and did not differ ($p>0.05$) from the dose of 40 mg/kg (Figure 4).

These results are expected and correspond to scientific data. Both components of the combination have a positive effect on the course of experimental renal pathology. Thus, the efficacy of quercetin has been confirmed in some studies on various models of kidney injury in different dosage forms, including injections[11,23-25]. The glucosamine derivatives also showed the expressed nephroprotective effect in the experiment. It was proved that glucosamine is embedded to the damaged renal tissue and increases the content of endogenous hexosamines[26]. These results correlate with another studies, which showed the efficacy of glucosamine derivatives in the treatment of kidney fibrosis in mice[27], contrast-induced acute kidney injury[28] and renal ischemia/reperfusion injury in rats[29].

The high efficacy of the combination is due to the fact that quercetin and NAG have nephroprotective effect with different mechanisms of action. The injection route of administration brings advantages for both components, since it allows to avoid the first pass metabolism and to ensure that the total dose of active substances reached the blood circulation and renal tissue in non-metabolized form.

At the next stage of the study, we calculated the ED_{50} index for NAG/COR combination under condition of RF based on the dependence of test drug activity on the administered dose by Probit Analysis.

Using the special table data, the percentages of activity were converted to probits (y) and their weighing coefficients (B). The dose points (x), intermediate variables were determined with necessary calculations. All the results obtained are presented in Table 3.

Table 3. Doses, activity levels and intermediate variables for calculating ED_{50} of NAG/COR combination using Probit Analysis.

Dose (mg/kg)	NA (%)	Dose point (x)	Probit (y)	Weighing factor (B)	xB	x^2B	yB	xyB
10	27.3	1	4.39	4.3	4.3	4.3	18.88	18.88
20	41.7	2	4.82	4.8	9.6	19.2	23.14	46.27
40	67.8	4	5.47	4.5	18.0	72.0	24.62	98.46
60	70.4	6	5.52	4.5	27.0	162.0	24.84	149.04
Sum				18.1	58.9	257.5	91.47	312.65

The results of calculations allowed to construct a chart of Probit Analysis of the "activity-dose" dependence (Figure 5). Final results of calculations are presented in Table 4.

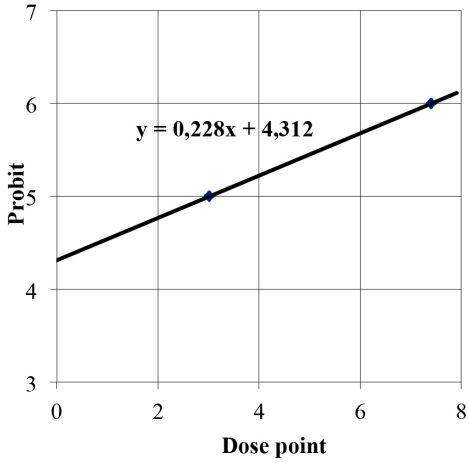

Figure 5. Chart of Probit Analysis of "activity-dose" dependence of NAG/COR combination.

Table 4. Results of calculations for determining ED50 of NAG/COR combination using Probit Analysis.

A_1	A_0	Equation of „probit-dose" dependence	Dose point ED_{50}	Dose point ED_{16}	Dose point ED_{84}	ED_{50} (mg/kg)	SE (mg/kg)
0.228	4.312	y = 0.228x + 4.312	3.02	-1.37	7.41	30.2	6.3

As a result of calculations, the ED_{50} index of test combination was 30.2 ± 6.3 mg/kg (Table 4).

The daily dose for clinical application of the combination was determined based on observed ED_{50} with conversion to humans following the FDA recommendations[30]:

Daily Dose = (30.2 mg/kg / 6.2) x 60 kg = 292.3 mg

Thus, the recommended daily dose of NAG/COR combination is 292.3 mg (for a median patient body weight of 60 kg) or 4.9 mg/kg.

ED_{50} index of the combination of quercetin and some glucosamine derivatives in oral dosage form is 80 mg/kg, determined in a previous experimental study on chromium-induced nephropathy in rats[11]. The result obtained in this study was 2.7 times lower, so the injection solution of NAG/COR combination is much more effective. These data have a great value for clinical nephrology, since they discover wide perspectives for the use of a new nephroprotective drug for CKD treatment – the NAG/COR combination in injectable dosage form.

Combination of NAG and quercetin in the injectable dosage form in a ratio of 1:1 leads to a significant increase in efficacy under conditions of RF in rats, which causes the expressed positive effect on the course of nephropathy. The NA of this combination has an expressed dose-dependence in the dose range of 10-40 mg/kg. The ED_{50} of the test combination is 30.2 ± 6.3 mg/kg, and it is the most optimal dose for further preclinical in-depth studies in order to justify its use in CKD therapy. The starting dose of the combination for clinical application extrapolated from the experimental data is 292.3 mg/day or 4.9 mg/kg/day, and it is the most appropriate for clinical trials as a kidney diseases treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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