Department of Clinical Pharmacology & Clinical Pharmacy, National University of Pharmacy, Kharkiv, Ukraine

N-Acetylglucosamine is the most effective glucosamine derivative for the treatment of membranous nephropathy in rats

S. K. Shebeko, I. A. Zupanets, O. S. Popov*

Received August 14, 2019, accepted September 18, 2019

**Corresponding author: Oleksii Popov, Department of Clinical Pharmacology & Clinical Pharmacy, National University of Pharmacy, 27 Pushkinska street, Kharkiv 61057, Ukraine*
oleksii.s.popov@gmail.com

Pharmazie 74: 667-670 (2019) *doi: 10.1691/ph.2019.9733*

Nephroprotective drug development for optimizing treatment of chronic kidney disease (CKD) is an important task of pharmaceutical science. Our study evaluated nephroprotective properties of the two glucosamine derivatives N-acetylglucosamine and glucosamine hydrochloride in a 3-week-long parenteral and oral daily administration at doses of 50 mg/kg in rats with doxorubicin nephropathy. Nephroprotective activity (NA) was evaluated by determining the functional state of the kidneys, the level of azotemia and the activity of free-radical processes in the renal tissue. The results show a significant increase in renal excretory function, normalization of nitrogen metabolism and a decline of free-radical processes under the influence of both studied amino sugars in rats with doxorubicin nephropathy. I.m. route of administration yielded the highest efficacy for both amino sugars with the highest level of NA (83.3%) shown by N-acetylglucosamine. Thus N-acetylglucosamine in i.m. injections has the highest NA among the glucosamine derivatives, and is a promising agent for CKD treatment.

1. Introduction

Treatment of patients with chronic kidney disease (CKD) is an important issue in modern medical and pharmaceutical practice. According to the world statistics, the prevalence of CKD is 8–16 % of the total population (Grams and McDonald 2019) and reaches 47 % among people over 70 years old (O'Hare et al. 2018). Overall, more than 500 million of the adult population of the planet are affected by this pathology (Grams and McDonald 2019). CKD leads to the development of severe complications, such as kidney failure – thus patients of this profile quickly become disabled and lose their social activity. Therefore, there is a big – and, moreover, growing by 7% annually – demand for renal replacement therapy (Upadhyay et al. 2016). Today more than 2.5 million people in the world receive it (Grams and McDonald 2019).

In this regard, the search for effective nephroprotective drugs to optimize treatment of CKD and reduce the rate of its progression is an important task of modern medical and pharmaceutical sciences. In the pathogenetic treatment of patients of this group, we place our scientific interest on influencing membrane structures of kidney tissue and restoring its functions. Such an activity may be had by substances with direct nephroprotective action realized by compensating deficiency of macromolecules of the damaged glomerular basement membranes and intercellular matrix. Drugs with such properties are absent in modern medical practice.

Particular attention in solving this problem should be given to the creation of drugs based on membrane protectors of natural origin with nephroprotective properties. For this purpose, it is promising to study amino sugars from the group of glucosamine (GlcN) derivatives – GlcN hydrochloride and N-acetylglucosamine (GlcNAc).

GlcN is a natural human metabolite, practically safe for the organism (Elbein and Honke 2019). The nature of its nephroprotective properties lies in GlcN being a part of glycosaminoglycans and proteoglycans in the structures of biological membranes and intercellular substance, including glomerular basement membrane (Morita et al. 2008). In previous experimental studies, we have proved the efficacy of GlcN hydrochloride under conditions of oral use for glomerulonephritis in rats (Shebeko and Zupanets 2006).

The disadvantage of GlcN is a slow development of the nephroprotective effect, explained by the fact that it requires transformation into its biologically active form, GlcNAc, in order to be integrated into the macromolecular structures of biomembranes and cause pharmacological action (Chen et al. 2010; Elbein and Honke 2019). Moreover, in the case of oral administration, GlcN is being actively metabolized by the liver, which results in an absolute bioavailability from 6 to 44% (Du Souich 2014). The rest of the amino sugar is added to the energy metabolism.

So GlcNAc is an active metabolite of GlcN, and therefore potentially has a more pronounced nephroprotective effect than GlcN in both latent and manifested kidney disease.

Taking into account the aforementioned, the aim of this work was to make a comparative study of nephroprotective properties of GlcN derivatives utilizing different routes of administration in rats with membranous nephropathy.

2. Investigations and results

2.1. Characteristics of membranous nephrorpathy in rats

We used doxorubicin-induced nephropathy as a model of membranous nephrorpathy in rats. Membranous nephropathy developed in three weeks after administration of doxorubicin in the control pathology group. Compared to the intact animals, there was a 18.0 % decrease ($P < 0.05$) in diuresis and a 58.4 % decrease in glomerular filtration rate (GFR), indicating deterioration of the renal excretory function; an increase in proteinuria to 60.2 mg/day, indicating damage of the glomerular basement membrane; and an increase in kidney weight coefficient (KWC) to 0.398 %, indicating inflammatory-destructive processes in the kidneys (Table 1). The kidney levels of endogenous GlcNAc were 2.4 times lower while thiobarbituric acid reactive substances (TBARS) were 2.2 times higher ($P < 0.05$) than in healthy rats, which can be explained by the destructive effect of free radicals on the renal tissue (Fig. 1). As a result, impaired renal function led to the development of azotemia: blood creatinine and urea were 1.7 and 2.7 times higher ($P < 0.05$) than in intact animals, respectively. Moreover, urea clearance (UC) decreased ($P < 0.05$) to 74.2 mL/day (Table 2).

Table 1: Influence of GlcNAc and GlcN at different routes of administration on the course of membranous nephropathy in rats

Group of animals	Diuresis (mL/day)	GFR (mL/day)	Proteinuria (mg/day)	KWC (%)
Intact control	6.1 ± 0.2	395.1 ± 16.4	1.1 ± 0.1	0.302 ± 0.006
Control pathology	5.0 ± 0.2 [a]	164.5 ± 9.0 [a]	60.2 ± 5.5 [a]	0.398 ± 0.010 [a]
GlcNAc i.m.	6.1 ± 0.1 [b]	392.1 ± 6.0 [b]	14.6 ± 1.0 [ab]	0.309 ± 0.005 [b]
GlcN i.m.	6.0 ± 0.1 [b]	369.6 ± 8.6 [b]	20.0 ± 0.9 [abc]	0.315 ± 0.004 [b]
GlcNAc i.p.	6.2 ± 0.2 [b]	371.6 ± 6.9 [bc]	20.2 ± 1.6 [abc]	0.319 ± 0.004 [ab]
GlcN i.p.	5.8 ± 0.1 [b]	365.1 ± 9.4 [bc]	23.9 ± 2.2 [abc]	0.326 ± 0.005 [abc]
GlcNAc p.o.	6.0 ± 0.2 [b]	370.9 ± 13.0 [b]	24.8 ± 2.3 [abc]	0.323 ± 0.003 [abc]
GlcN p.o.	5.9 ± 0.1 [b]	362.7 ± 11.2 [bc]	26.1 ± 2.4 [abcd]	0.327 ± 0.004 [abc]

GlcNAc: N-acetylglucosamine; GlcN: glucosamine; GFR: glomerular filtration rate; KWC: kidney weight coefficient. Data are expressed as mean ± SEM (n = 7 for each group). [a] $P < 0.05$ vs. intact control group; [b] $P < 0.05$ vs. control pathology group; [c] $P < 0.05$ vs. group treated with i.m. GlcNAc; [d] $P < 0.05$ vs. group treated with i.m. GlcN (ANOVA, Tukey post-hoc test).

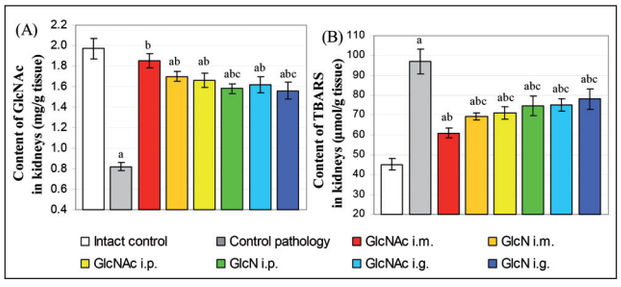

Fig. 1: Influence of GlcNAc and GlcN at different routes of administration on the kidney content of endogenous GlcNAc (A) and TBARS (B) in rats with membranous nephropathy. GlcNAc: N-acetylglucosamine; GlcN: glucosamine; TBARS: thiobarbituric acid reactive substances. Data are expressed as mean ± SEM (n = 7 for each group). [a] $P < 0.05$ vs. intact control group; [b] $P < 0.05$ vs. control pathology group; [c] $P < 0.05$ vs. group treated with i.m. GlcNAc (ANOVA, Tukey post-hoc test).

Table 2: Influence of GlcNAc and GlcN at different routes of administration on nitrogen metabolism in rats with membranous nephropathy

Group of animals	Blood creatinine (μmol/L)	Blood urea (mmol/L)	UC (mL/day)
Intact control	47.55 ± 1.55	4.10 ± 0.23	144.8 ± 6.0
Control pathology	80.39 ± 5.87 [a]	10.96 ± 0.80 [a]	74.2 ± 3.1 [a]
GlcNAc i.m.	50.37 ± 3.03 [b]	4.90 ± 0.34 [b]	130.9 ± 3.5 [b]
GlcN i.m.	53.04 ± 2.02 [b]	5.65 ± 0.13 [ab]	127.4 ± 3.5 [ab]
GlcNAc i.p.	53.18 ± 3.81 [b]	5.75 ± 0.30 [ab]	125.2 ± 4.4 [ab]
GlcN i.p.	56.18 ± 3.57 [ab]	6.05 ± 0.29 [abc]	123.0 ± 4.1 [ab]
GlcNAc p.o.	53.55 ± 4.88 [b]	5.81 ± 0.53 [ab]	122.7 ± 5.1 [ab]
GlcN p.o.	56.50 ± 5.15 [b]	6.2 ± 0.57 [ab]	118.7 ± 4.9 [ab]

GlcNAc: N-acetylglucosamine; GlcN: glucosamine; UC: urea clearance. Data are expressed as mean ± SEM (n = 7 for each group). [a] $P < 0.05$ vs. intact control group; [b] $P < 0.05$ vs. control pathology group; [c] $P < 0.05$ vs. group treated with i.m. GlcNAc (ANOVA, Tukey post-hoc test).

2.2. Nephroprotective effect of GlcNAc in i.m. injections

The greatest efficacy in the presented study was shown by GlcNAc, especially following i.m. injection. This manifested in a significant increase ($P < 0.05$ vs. control pathology group) in diuresis by 22.0 % and in GFR to 392.1 mL/day, which corresponds to the intact control group. The proteinuria level was 4.1 times lower than in untreated animals and KWC index decreased by 22.4 %, indicating normalization of kidney functional state (Table 1). Also, i.m. GlcNAc decreased destructive free-radical processes in the kidneys, as evidenced by a significant increase ($P < 0.05$) of endogenous GlcNAc to 1.85 mg/g and a decrease in the TBARS level by 37.4 % (Fig. 1). In addition, there was a significant decrease ($P < 0.05$) in the blood creatinine and urea by 37.3 % and 55.3 %, respectively, and a corresponding increase in the UC to 130.9 mL/day, indicating normalization of nitrogen metabolism (Table 2). All these data allowed us to calculate the index of nephroprotective activity (NA), which equaled 83.3 % (Fig. 2).

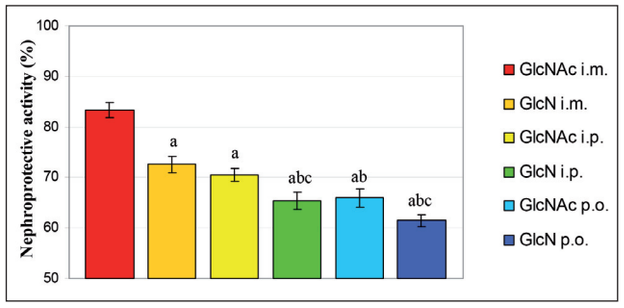

Fig. 2: Total nephroprotective activity of GlcNAc and GlcN at different routes of administration in rats with membranous nephropathy. GlcNAc: N-acetylglucosamine; GlcN: glucosamine. Data are expressed as mean ± SEM (n = 7 for each group). [a] $P < 0.05$ vs. group treated with i.m. GlcNAc; [b] $P < 0.05$ vs. group treated with i.m. GlcN; [c] $P < 0.05$ vs. group treated with i.p. GlcNAc (ANOVA, Tukey post-hoc test).

2.3. Nephroprotective effect of GlcN in i.m. injections

In the course of the study, GlcN showed a lower level of activity than GlcNAc. After i.m. administration of GlcN, the renal excretory function did restore, but to a lesser extent: diuresis increased ($P < 0.05$ vs. untreated animals) by 20.0 %, GFR increase to 369.6 mL/day, while proteinuria decreased to 20.0 mg/day, and KWC index decreased by 20.9 % (Table 1). In the kidney homogenates, endogenous GlcNAc index was 2.1 times higher ($P < 0.05$) compared to control pathology group, while TBARS content decreased by 28.8 % (Fig. 1). Additionally, creatinine and urea blood levels significantly decreased ($P < 0.05$) by 34.0 % and 48.4 %, respectively, and UC index increased by 71.7 % (Table 2). All these data allowed us to calculate the index of NA, which equaled 72.6 % (Fig. 2).

2.4. Nephroprotective effects of GlcNAc and GlcN in i.p. injections

In i.p. injections, both amino sugars showed a lower level of efficacy than in i.m. injections. GFR and UC were 2.2–2.3 and 1.7 times higher ($P < 0.05$), respectively, while proteinuria level was 2.5–3.0 times lower compared to the untreated animals (Table 1, 2). The kidney endogenous GlcNAc was 1.9–2.0 times higher ($P < 0.05$ vs. control pathology group) and TBARS content decreased by 26.9 % and 23.0 % (Fig. 1). As a result, the index of NA equaled 70.6 % for GlcNAc and 65.4 % for GlcN (Fig. 2).

2.5. Nephroprotective effects of oral GlcNAc and GlcN

The studied amino sugars showed an even lower level of efficacy when administered orally. GFR and UC were 2.2–2.3 and 1.6–1.7 times higher ($P < 0.05$) than in the untreated animals, and proteinuria reduced to 24.8 mg/day (on GlcNAc) and 26.1 mg/day (on GlcN) (Tables 1, 2). The kidney level of endogenous GlcNAc was 1.9–2.0 times higher ($P < 0.05$) than in control pathology group, and TBARS decreased by 22.7 % and 19.5 %, respectively (Fig. 1). The obtained results allowed to calculate the NA index, which was 66.0 % for GlcNAc and 61.5 % for GlcN (Fig. 2).

3. Discussion

Under the influence of both amino sugars, we observed not only nephroprotective but also hypoazotemic action of varying degrees of magnitude depending on the route of drug administration. In general, GlcNAc showed a higher level of efficacy than GlcN, and, according to some of the studied indices, it had significant advantages at the same route of administration. This can be explained by the fact that GlcNAc is an active metabolite of GlcN and has a more pronounced nephroprotective effect, realized *via* direct mechanism of action. In contrast to GlcN, GlcNAc is capable of attaching unchanged to glycosaminoglycans and proteoglycans of the damaged basement membranes and intercellular substance, while GlcN can do that only after acetylation (Elbein and Honke 2019).

In previous experiments, we studied nephroprotective properties of oral GlcN and proved its efficacy in treatment of glomerulonephritis and membranous nephropathy (Shebeko and Zupanets 2006; Zupanets and Shebeko 2006). We showed that GlcN integrates into the damaged structures of kidney tissue and increases the content of endogenous hexosamines therein (Zupanets and Shebeko 2006). These results correlate with other studies, which showed the efficacy of glucosamine 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). Since, in the present experiment, the efficacy of GlcNAc exceeds GlcN, we conclude that the presented data is very promising.

When comparing different routes of administration of amino sugars, it turns out that the highest level of NA was observed in animals treated with i.m. injections: i.m. GlcNAc significantly exceeded itself at other routes of administration. Moreover, GlcNAc significantly exceeded GlcN by the level of effect on proteinuria and kidney TBARS under the conditions of i.m. injection, and also was better than both oral and i.p. GlcN in all investigated parameters.

Consequently, the i.m. route of administration for GlcN derivatives has unconditional advantages, since it allows to neutralize the effect of first-pass metabolism and to ensure that the total dose of the administered hexosamine stays unchanged. The most suitable GlcN derivative for i.m. administration is GlcNAc, since it is an active metabolite while GlcN itself requires biotransformation for inclusion into macromolecules of biomembranes and intercellular matrix.

On the other hand, the i.p. route of administration for GlcN derivatives, despite being an acceptable alternative to i.v. administration in experimental studies, should be considered inappropriate. This is because in the present study, it did not have any advantages over oral administration (explained by the equally intense liver metabolism) and inconvenience of administration. But this does not apply to i.v. administration of amino sugars, since it, as well as i.m. administration, is not characterized by the effect of first-pass metabolism and needs further study.

Thus, the obtained experimental data show that GlcNAc has a pronounced nephroprotective and hypoazotemic activity in rats with membranous nephropathy and is the most promising derivative of GlcN for treatment of kidney diseases, including CKD. It is advisable to use this hexosamine in i.m. injections, but it is also possible to use it orally in accordance with the goal of treatment.

4. Experimental

4.1. Animals and treatment

The experimental study was performed on 56 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 (National Research Council (US) 2011; Sharp and Villano 2013). All studies were conducted in accordance with EU Council Directive 2010/63/EU 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 8 experimental groups of 7 rats as follows.

Group 1 – intact control (healthy animals receiving i.m. vehicle).
Group 2 – control pathology (untreated animals receiving i.m. vehicle).
Group 3 – animals with nephropathy treated with GlcNAc (i.m.) at 50 mg/kg.
Group 4 – animals with nephropathy treated with GlcN (i.m.) at 50 mg/kg.
Group 5 – animals with nephropathy treated with GlcNAc (i.p.) at 50 mg/kg.
Group 6 – animals with nephropathy treated with GlcN (i.p.) at 50 mg/kg.
Group 7 – animals with nephropathy treated with GlcNAc (p.o.) at 50 mg/kg.
Group 8 – animals with nephropathy treated with GlcN (p.o.) at 50 mg/kg.

4.2. Test objects and their preparation

The objects of the study were GlcNAc and GlcN in the form of hydrochloric salt (Sigma-Aldrich, USA), from which solutions were aseptically made using 0.9 % sodium chloride solution for injections. Solutions were made immediately before use with a concentration of 20 mg/mL for i.m. and 10 mg/mL for i.p. and p.o. administration.

4.3. Experimental design

We used doxorubicin-induced nephropathy to trigger membranous kidney injury in rats (Balakumar et al. 2008; Hart et al. 2008). This model ensues as a result of destructive action of free radicals on the kidney membranes; it manifests, primarily, in the deterioration of the renal excretory function (Lee and Harris 2011). Pathology was induced in rats on the first day of the experiment by i.p. injection of doxorubicin hydrochloride (Sigma-Aldrich, USA) at a dose of 10 mg/kg (Shtrygol´ et al. 2009). Starting from the second day of the experiment, the animals received appropriate test samples at a dose of 50 mg/kg, which corresponds to the effective dose of GlcN on the model of autoimmune glomerulonephritis (Shebeko and Zupanets 2006). All test samples were administered as solutions daily for three weeks. Animals of control groups received (i.m.) equivalent dose of 0.9 % solution of sodium chloride. Three weeks after the reproduction of the pathology in rats, the functional state of the kidneys was evaluated. Subsequently, rats were sacrificed under general anesthesia with ketamine/xylazine (75/10 mg/kg, i.p.) (Flecknell 2015) to obtain the blood and the renal tissue for biochemical assays.

4.4. Biological samples preparation and storage

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

4.5. Evaluation of the functional state of kidneys

Spontaneous daily diuresis was determined with individual metabolic cages at the end of the experiment in all animals (Hart et al. 2008). Kidneys were subjected to macroscopic analysis and weighed; the right kidney weight was used for KWC standard method calculation relative to body weight. The protein content and its daily excretion were determined in the collected urine. GFR was evaluated as endogenous creatinine clearance; UC was calculated by standard method (Topf and Lesley 2019; Koeppen and Stanton 2019):

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

$$UC = U_{ur} \times V / P_{ur} \quad (2)$$

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.

4.6. Biochemical assays

Biochemical studies to evaluate parameters of renal excretory function and nitrogen metabolism 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) in the automatic biochemical analyzer Express Plus (Bayer Diagnostics, Germany). Creatinine and urea levels in blood and urine were determined using a kinetic test without deproteinization according to Jaffe method and urease glutamate dehydrogenase enzymatic UV test, respectively. Urinary excretion of creatinine and urea was also calculated. The protein urine concentration was determined by Pyrogallol Red – molybdate method (Hart et al. 2008). In addition, in order to evaluate the degree of destruction of the renal tissue, we determined the content of endogenous GlcNAc in the kidney homogenate by Elson-Morgan method in our modification (Zupanets and Shebeko 2005). To evaluate kidney free-radical processes, we determined the content of TBARS in the kidney homogenate by the reaction of interaction with thiobarbituric acid using the standard spectrophotometric method (Devasagayam et al. 2003; Grotto et al. 2009). All spectrophotometric measurements were performed using the UNICO SQ-2800 spectrophotometer (United Products & Instruments Inc., USA).

4.7. Calculation of nephroprotective activity index

The NA of test amino sugars was evaluated by an integral index, which was calculated as the arithmetic mean of the activities on five different parameters, which taken together fully reflect the course of nephropathy: proteinuria (1), GFR (2), UC (3), kidney content of endogenous GlcNAc (4) and TBARS (5). Each of these activities was calculated by the following formula:

$$\text{Activity} = (X_c - X_i) / (X_c - X_t) \times 100\% \quad (3)$$

where X_c is the index in the control pathology group, X_i is the index in the intact control group and X_t is the index in the group treated with the test drug.

4.8. Statistical analysis

All the results were processed by descriptive statistics and presented as the mean ± standard error of the mean (SEM). Statistical differences between groups were analyzed using one-way ANOVA followed by Tukey post-hoc test (Quirk et al. 2015; Islam and Al-Shiha 2018). Utilized computer software included IBM SPSS Statistics v. 22 (IBM Corp.) and MS Excel 2016 (Microsoft Corp.). The level of statistical significance was considered as $P < 0.05$.

Conflicts of interest: None declared. No funding source had involvement in the design, analysis, or writing of this manuscript.

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