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Efficiency of Canephron N in complex treatment of experimental heymann glomerulonephritis

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Abstract

Background: While the most common pharmacological treatments for autoimmune renal pathologies are well studied, herbal remedies are often overlooked. In this study, the effect of the herbal treatment Canephron® N (CAN) in a rat model of active Heymann's autoimmune glomerulonephritis (AIG) was investigated.

Methods: Forty BDIX male conventional rats and six female rats were divided into the following groups: healthy animals, AIG, AIG treated with CAN, AIG treated with prednisolone, AIG treated with prednisolone and CAN.

Results: Prednisolone or CAN monotherapy comprises various positive pharmacological effects.
Rats receiving prednisolone showed moderate increase in CD4+ lymphocyte count, increase in CD8+ cytotoxic lymphocyte count but incomplete normalization of CD4+/CD8+ lymphocyte ratio. It reduced the total count of lymphocytes. Concentration of immune complexes and cryoglobulin level decreased considerably. Prednisolone monotherapy ensured moderate reduction in nephrotic syndrome parameters.
CAN monotherapy did not affect immunological parameters and CD4+/CD8+ subpopulation ratio but reduced the level of immune complexes significantly compared to AIG. The main nephroprotective effects of the drug were normalization of diuresis and glomerular filtration rate. Significant reduction of leukocyturia was observed compared to AIG group.
The most positive effects were observed in the combined Prednisolone + CAN group. Along with positive immunological shifts in cell-bound and humoral links of immunity (prednisolone effects), renal function improved significantly: proteinuria decreased, blood creatinine and urea decreased, AOPP and PCO levels also decreased (combined effects of CAN). Qualitative differences suggest synergistic effects of complex therapy with glucocorticoid immunosuppressor and the herbal medical drug.

Conclusions: The AIG model used in this study corresponds to human membranous glomerulonephritis in terms of clinical morphology. Prednisolone monotherapy demonstrated adequate efficacy regarding immune and metabolic components of renal disease.
The herbal monotherapy was shown to partially normalize urodynamics.
Combining CAN with the immunosuppressant prednisolone promotes more positive pharmacodynamic effects on the immune and renal systems of rats.
The combined treatment may be useful in clinical nephrology practice including patients with kidney autoimmune diseases and should be investigated further.

Keywords: Canephron® N, Herbal medicinal product, Prednisolone, Renal pathologies, Glomerulonephritis

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Background

Preclinical and clinical nephrological studies focus on three subjects: 1) improvement of renal failure diagnostics [1, 2], 2) development of effective dialysis devices and detoxification regimens [3], 3) search for novel medical products for prevention and conservative treatment of renal pathologies of various geneses [4].

Autoimmune renal pathology, both in animal models and in humans, is associated with autoantibody-induced destruction of nephron structures, deposition of immune complexes, and migration of leukocytes to renal tissues [5, 6]. Currently, the most common pharmacological treatment to limit production of autoantibodies by T-helper-dependent autoreactive clones of B-lymphocytes are immunodepressant drugs such as glucocorticosteroids (prednisolone, methylprednisolone), cytostatic drugs (cyclophosphamide, chlorambucil), lymphocyte inosine monophosphate dehydrogenase inhibitors (mycophenolate mofetil), inhibitors of calcium-dependent T-cellular signal transduction pathways (cyclosporine A, tacrolimus), suppressors of transcription of discrete group of lymphokine gens, monoclonal antibodies, etc. Basic therapy of autoimmune renal pathology includes the immunodepressants prednisolone, methylprednisolone, azathioprine, cyclophosphamide, mycophenolate mofetil, cyclosporine A and tacrolimus [7]. The treatment also includes anticoagulants of the heparin group, angioprotectors [8], and herbal medicinal products [9].

Herbal medicinal products in the treatment of this pathology are often considered last. Significant experience with phytotherapy in renal and urinary diseases has been accumulated over the years [4, 9, 10]. However, the evidentiary basis, preclinical as well as clinical, of treating renal diseases with phytotherapy remains still insufficient.

The herbal medicinal product Canephron N (CAN) produced by Bionorica SE combines three herbal components: rosemary, lovage and centaury [11]. Pharmacological effects of CAN consist of anti-inflammatory, diuretic and nephroprotective effects [12]. The main advantages of this drug are the availability on the pharmaceutical market, high efficacy in various renal and urinary pathologies, safety and the possibility to use it in children and during pregnancy. Safety and efficacy of CAN has been indicated in pregnant women with late gestational toxicosis [13, 14]. The main studies, both clinical [15, 16] and preclinical [17], prove efficacy of the drug in tubular pathology, such as pyelonephritis and tubulointerstitial nephritis, as part of combination therapy with antibacterial drugs (pefloxacin, amoxiclav, roxithromycin) [18].

The aim of this study was to investigate the nephroprotective effects of CAN in complex pharmacotherapy in a rat model of active Heymann's autoimmune glomerulonephritis (AIG). The study comprised the following groups: 1) healthy animals, 2) animals with AIG, 3) animals with AIG treated with CAN (for 60 days), 4) animals with AIG treated with the standard immunodepressant prednisolone (for 60 days), and 5) animals with AIG treated with prednisolone in combination with CAN (for 60 days). Evaluation of pharmacological effect of the treatments was performed using a series of laboratory tests recommended for preclinical studies [19].

Methods

Animals

Work with the laboratory animals was in accordance with the Guide for the Care and Use of Laboratory Animals (USA, National Academy Press, Washington, D.C., 1996); the Guide for the Care and Use of Laboratory Animals (FELASA, 2010); Laboratory Animals (guidelines, Russian Academy of Medical Sciences, Moscow, 2003) [20]. Experiments were carried out with 40 BDIX male conventional rats and 6 female rats [21] weighing 200–220 g purchased from the Pavlov Institute of Physiology Russian Academy of Sciences (Saint Petersburg, Russia). The experimental protocol followed in this study was fully approved by the Bioethics Committee Animal Care of Institute of Toxicology.

Modeling of autoimmune glomerulonephritis (AIG)

To induce active Heymann's glomerulonephritis, maternal kidneys were isolated and homogenized, freed from connective tissue and mixed with complete Freund's adjuvant (1:1) [22–24]. Thus maternal renal antigen containing glycoprotein gp330 was obtained. At the age of 3 months rats of the f 1 generation were immunized by two intraperitoneal administration of maternal renal antigen (10 mg/200 g body weight) with a 14-day interval. 28 days after the second immunization severity of proteinuria was assessed (proteinuria in experimental rats was at least 0.5 g/mmol creatinine/day) and rats were divided into the following groups: healthy animals not receiving immunization (Healthy $n = 8$), pathology control (AIG, $n = 8$), prednisolone therapy (Prednisolone, $n = 8$), CAN treatment (CAN, $n = 8$), prednisolone + CAN treatment (Prednisolone + CAN, $n = 8$). Prednisolone was administered at 10 mg/kg/day, CAN oral drops at 3.0 ml/kg/day [25]. Drug administration was performed via nontraumatic gavage (p.o.) once daily for 60 days. 60 days after end of treatment, 24-hour urine was collected from rats, and subsequently animals were euthanized under anesthesia (zolazepam + tiletamine 1:1, 20 mg/kg) via instantaneous decapitation with blood sampling for biochemical and immunological tests.

Parameters and tests

Twenty-four-hour urine volume was determined at the end of experiment (day 60) by placing all rats for 24 hours into metabolic cages (Tekniplast Gazzada, Italy) with free

access to water (100 ml per each rat) without food. The main markers of renal pathology (nephrotic syndrome) were protein and erythrocyte levels in the urine. Urine was analyzed using Aution Sticks 10EA test strips on the AutionEleven AE-4020 (Arkray Factory, Inc. (Japan)). Collected urine samples were analyzed for count of erythrocytes, leukocytes, casts (supravital staining using Sternheimer-Malbin method with microscopy in × 100 and × 400 magnification) [26, 27], protein level (using pyrogallol red binding method at 600 nm) and creatinine level (using Jaffe pseudokinetic reaction at 505 nm) [28]. Proteinuria in 24-hour urine was calculated as g of protein per mmol of creatinine. Ready-to-use kits for clinical chemistry manufactured by Abris + and Olvex Diagnosticum (Russia) were used. Glomerular filtration rate (GFR ml/min) was calculated using the equation described by Methods in renal toxicology [29].

The blood was sampled into tubes with the anticoagulant lithium heparin for immunological tests and into clot activator tubes for biochemical tests (Vacuette).

Lymphocyte immunophenotyping

To evaluate count of CD4+ and CD8+ T-lymphocytes, murine monoclonal antibodies were used: PE labeled anti-rat CD45, FITC labeled anti-rat CD3, APC labeled anti-rat CD4, PerCP labeled anti-rat CD8a (BD Pharmingen). 10 μl of antibody mixture were added to 50 μl of blood and incubated for 30 min at room temperature. Then 450 μl of 1 % BD FACSC™ Lysing Solution (BD, USA) was added and incubated for 30 min. Cytometry was carried out on flow cytofluorimeter BD FACSCalibur™ (BD Biosciences, USA) using CellQuestPrO universal software [30]. At least 10 thousand cells were measured in each sample.

Level of immune complexes (CIC) was defined using turbidimetry in the presence of 3.5 % PEG-6000 in borate buffer at 450 nm [28, 31]. Cryoglobulin level was determined after a 7-day serum incubation at +4 °C in the presence of sodium azide, cryoprecipitate centrifugation, precipitate dissolved in 0.1 M sodium hydroxide and spectrophotometry at 260 and 280 nm [31].

Serum level of renal damage biomarkers, i.e. urea (blood urea nitrogen, BUN) and creatinine, was determined using a UV-kinetic method, and cholesterol at 500 nm by means of Randox Laboratories Ltd. (UK) kits [28]. The level of advanced oxidation protein products (AOPP) as a marker of chronic renal disease was assessed at 340 nm using the Witko-Sarsat method [32], carbonyl protein (PCO) binding to 2,4-dinitrophenylhydrazine at 370 nm [33]. Measurements were performed using Synergy2 (BioTek Instruments, Inc., USA) microplate reader. Correctness of measurements was ensured using a control serum (Calibration sera level 1–3) by Randox Laboratories Ltd. (UK) and urine materials (Liquichek urinalysis level 1–2) by Bio-Rad (USA).

Statistical tests were performed using Statistica 8.0 Software for Windows. The nonparametric Kruskall-Wallis test and Mann–Whitney U test were used. Data are presented as mean ± standard error of the mean (SEM). Significance of differences was assessed at $p \leq 0.05$ [34].

Results and discussion

Animals with AIG showed immunopathological shifts characteristic for the autoimmune pathology: statistically significant reduction of blood CD8+ T-cells, of CD4+ T-helper cells and increase of immune-regulatory index ($p \leq 0.05$). CD4+ T cells play a critical role in the induction of Heymann's nephritis. Concentration

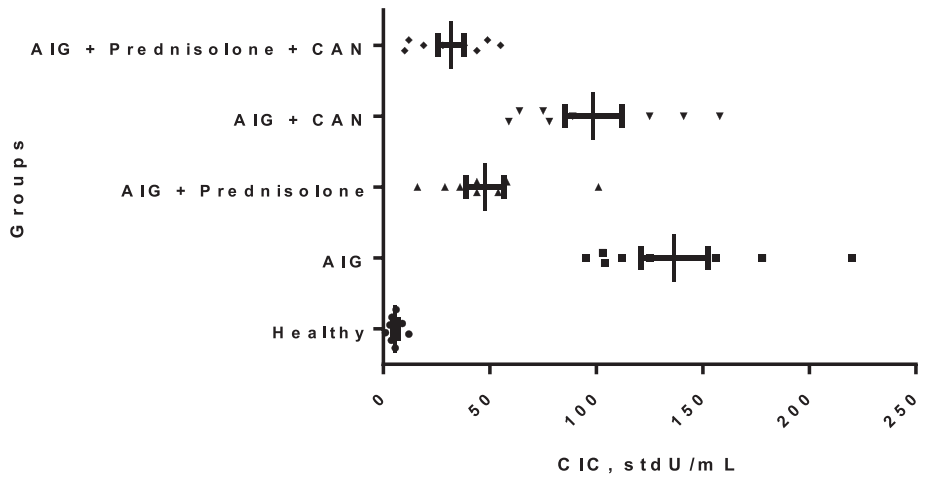

Fig. 1 Evaluation of monotherapy and combination of prednisolone with CAN on the circulating immune complexes level (Mean±SEM) in a rat model of active Heymann autoimmune glomerulonephritis: 1 – Healthy; 2 – AIG; 3 – AIG + Prednisolone; 4 – AIG + CAN; 5 – AIG + Prednisolone + CAN; n=8 in each group. Scatter dot plot. *P* value < 0,05

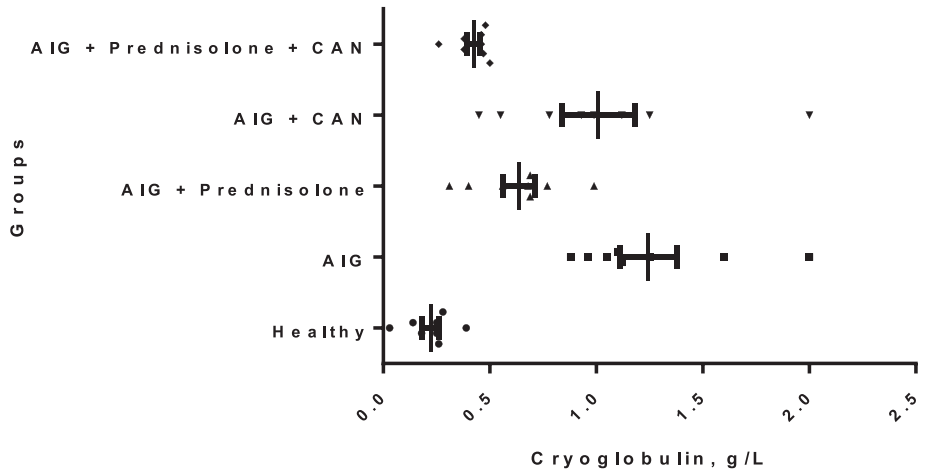

Fig. 2 Evaluation of monotherapy and combination of prednisolone with CAN on the serum cryoglobulin level (Mean±SEM) in a rat model of active Heymann autoimmune glomerulonephritis: 1 – Healthy; 2 – AIG; 3 – AIG + Prednisolone; 4 – AIG + CAN; 5 – AIG + Prednisolone + CAN; n=8 in each group. Scatter dot plot. *P* value < 0,05

of serum circulating immune complexes (CIC) increased significantly (approximately 25-fold), and the cryoglobulin level was increased 5.4-fold when compared to healthy rats (Figs. 1 and 2).

Renal inflammation was indicated by enhanced proteinuria, leukocyte- and hematuria, and the presence of erythrocytic and waxy sediments (casts) in the urine of the rats.

Total blood cholesterol, creatinine and urea increased significantly (nephrotic syndrome). The AIG model was also characterized by the accumulation of advanced oxidation protein products (AOPP) and carbonyl protein groups in blood, thus confirming the development of chronic renal pathology in rats.

The effects of the experimental therapy on immunological parameters are presented in Table 1, biochemical blood parameters in Table 2, and parameters of renal function in Table 3.

Prednisolone or CAN monotherapy comprises various positive pharmacological effects. Rats receiving prednisolone showed moderate increase in CD4+ lymphocyte count up to the normal values, increase in CD8+ cytotoxic lymphocyte count but incomplete normalization of CD4+/CD8+ lymphocyte ratio. However, prednisolone reduced the total count of lymphocytes. Concentration of immune complexes and cryoglobulin level decreased considerably. Prednisolone monotherapy ensured moderate reduction in nephrotic syndrome parameters – urea and creatinine, total cholesterol and AOPP as well as PCO, primarily albumin. Partial normalization of glomerular filtration rate took place along with reduced proteinuria, erythrocyte- and leukocyturia; count of waxy and red blood cell casts in urine also reduced significantly.

CAN monotherapy did not affect immunological parameters and CD4+/CD8+ subpopulation ratio but reduced the level of immune complexes significantly compared to AIG. Biochemically, the drug produced moderate positive effect on creatinine level only. The main targets of nephroprotective effect of the drug were the kidneys, i.e. normalization of diuresis and glomerular filtration rate, probably due to creatinine elimination via tubular secretion. Significant reduction of leukocyturia

Table 1 Effect of therapy with prednisolone, CAN and their combination on the main immunological markers of autoimmune renal pathology (mean ± SEM, $n = 8$ in each group)

Groups of animals	Study parameters			
	CD3+, $\times 10^9$/L	CD4 + CD3+, %	CD8 + CD3+, %	Immune-regulatory index (CD4+/CD8+)
Healthy	5,7 ± 0,1	55,2 ± 0,9	44,1 ± 1,1	1,26 ± 0,04
AIG	5,5 ± 0,1	51,0 ± 1,6*	25,6 ± 1,3*	2,01 ± 0,07*
AIG + Prednisolone	4,9 ± 0,1 */†	52,1 ± 1,1	30,2 ± 1,3*/†	1,74 ± 0,07*/†
AIG + CAN	5,6 ± 0,1#	51,9 ± 1,2	26,2 ± 1,4*	2,00 ± 0,09*
AIG + Prednisolone + CAN	5,1 ± 0,1*/†	54,2 ± 0,7	34,5 ± 0,6*/†/#	1,58 ± 0,04*/†

* -differences are significant vs. healthy rats, $p \leq 0,05$
† –differences are significant vs. AIG group, $p \leq 0,05$
# -differences are significant vs. Prednisolone group, $p \leq 0,05$

Table 2 Effect of therapy with prednisolone, CAN and their combination on biochemical blood parameters in autoimmune glomerulonephritis (mean ± SEM, $n = 8$ in each group)

Groups of animals	Study parameters				
	BUN, mmol/L	Creatinine, μmol/L	Cholesterol, mmol/L	AOPP, μmol/L	PCO, nmol/mg
Healthy	4,3 ± 0,2	50,4 ± 3,5	1,22 ± 0,08	58,0 ± 10,1	0,79 ± 0,12
AIG	9,3 ± 0,6*	168,4 ± 11,9*	2,59 ± 0,17*	238,1 ± 13,1*	3,84 ± 0,31*
AIG + Prednisolone	6,4 ± 0,5*/†	101,3 ± 10,8*/†	1,97 ± 0,15*/†	154,6 ± 11,0*/†	2,59 ± 0,18*/†
AIG + CAN	8,6 ± 0,8*#	118,1 ± 9,3*/†	2,39 ± 0,23*	204,0 ± 20,3*/#	3,38 ± 0,17*/#
AIG + Prednisolone + CAN	5,3 ± 0,4*/†	86,2 ± 6,1*/†	1,72 ± 0,11*/†	105,7 ± 11,98*/†/#	1,99 ± 0,11*/†/#

* -differences are significant vs, healthy rats, $p \leq 0,05$
† –differences are significant vs, AIG group, $p \leq 0,05$
# -differences are significant vs, Prednisolone group, $p \leq 0,05$

was observed compared to AIG group which, however, did not exceed prednisolone monotherapy. This could be explained by anti-inflammatory effects of the drug. Antioxidant effects were supported by the tendency for blood AOPP and PCO reduction in CAN treatment.

The most positive effects were observed in the combined Prednisolone + CAN group. Along with positive immunological shifts in cell-bound and humoral links of immunity (prednisolone effects), renal function improved significantly: proteinuria decreased, blood creatinine and urea decreased due to GFR increase, AOPP and PCO levels also decreased (combined effects of CAN). Qualitative differences suggesting synergistic effects of complex therapy with glucocorticoid immunosuppressor and the herbal medical drug included significant differences in terms of CD8+ lymphocytes ($p = 0.024$), cryoglobulins ($p = 0.021$), advanced oxidation protein products ($p = 0.016$), carbonyl groups ($p = 0.015$), erythrocyturia ($p = 0.011$) and leukocyturia ($p = 0.001$) vs. group of animals receiving prednisolone monotherapy.

Conclusions

The AIG model used in this study corresponds to human membranous glomerulonephritis in terms of clinical morphology [24, 25]. This experimental work showed a negative selection of T-killer cells (cytotoxic lymphocytes) associated with active development of autoimmune renal pathology. Prednisolone (reference immunodepressant) monotherapy demonstrated adequate efficacy regarding immune and metabolic components of renal disease.

Nephroprotective effects of CAN have been identified in the experimental Heymann autoimmune glomerulonephritis. The herbal monotherapy was shown to partially normalize urodynamics, increase rate of glomerular filtration, mildly decrease blood creatinine, and significantly reduce the severity of leukocyturia due to anti-inflammatory effects of the herbal medicinal product. Combining CAN with the immunosuppressant prednisolone promotes more positive pharmacodynamic effects on the immune and renal systems of rats. Synergistic effects of "Prednisolone + CAN" combination were demonstrated in the enhanced reduction of hematuria and leukocyturia, reduced level of uremic toxins (products of deep protein oxidation and carbonyl proteins) and blood cryoglobulins, and also increased CD8 + –lymphocyte count.

Analysis of the resulting data indicates an increase in effectiveness when treating glomerulonephritis with a combination of immunosuppressive drugs with herbal medicines. The combined treatment may be useful in clinical nephrology practice including patients with kidney autoimmune diseases and should be investigated further.

Competing interests
The authors declare that they have no competing interests.

Table 3 Effect of therapy with prednisolone, CAN and their combination on renal function in autoimmune glomerulonephritis (mean ± SEM, $n = 8$ in each group)

Groups of animals	Study parameters					
	Diuresis, mL/day	GFR, mL/min	Protein, g/mmol Cr	Erythrocytes, $\times 10^3$/mL	Leukocytes, $\times 10^3$/mL	Casts, units/mL
Healthy	13,3 ± 0,6	4,50 ± 0,32	0,13 ± 0,01	1,87 ± 0,69	7,6 ± 1,3	10,0 ± 4,2
AIG	5,6 ± 0,5*	0,63 ± 0,13*	1,05 ± 0,14*	27,25 ± 5,47*	324,4 ± 50,6*	102,5 ± 25,9 *
AIG + Prednisolone	10,5 ± 0,9*/†	2,98 ± 0,45*/†	0,43 ± 0,03*/†	9,25 ± 1,25*/†	197,0 ± 29,.2*/†	23,6 ± 3,2†
AIG + CAN	9,7 ± 0,9*/†	1,37 ± 0,14*/†/#	0,70 ± 0,05*/#	15,12 ± 0,79*/#	149,5 ± 46,6*/†	48,2 ± 5,3*/#
AIG + Prednisolone + CAN	12,5 ± 1,1†	3,24 ± 0,23*/†	0,29 ± 0,05*/†	5,00 ± 0,75*/†/#	82,6 ± 7,7*/†/#	19,0 ± 3,8†

* -differences are significant vs. healthy rats, $p \leq 0,05$
† –differences are significant vs. AIG group, $p \leq 0,05$
# -differences are significant vs. Prednisolone group, $p \leq 0,05$

Authors' contributions
KVS carried out the modeling disease, urinalysis and clinical chemistry studies, analyzed and interpreted the data, and wrote the first draft of the manuscript. KIS carried out the immunological studies. EL oversaw the study design and advised on the doses and regimens. TS was responsible for study monitoring and compliance with Good Laboratory Practice guidelines analyzed and interpreted the data. All authors read and approved the final manuscript.

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