**Combination of Pentoxifylline and Ginko Biloba Nephroprotective Effect in Animal Models with Vancomycin-Induced Nephrotoxicity**

**ABSTRACT:** Antioxidants have been commonly used in medicine for thousands of years. Clinically, pentoxifylline and Ginkgo biloba have beneficial renal effects. Our study evaluated the nephroprotective effect of Ginkgo biloba in combination with Pentoxifilin in an experimental model of vancomycin-induced nephrotoxicity. Male Wistar rats were used in 3 groups: CONTROL, VANCO and VANCO + PTX and each group included 6 rats. Insufficient studies in the literature on the prevention of acute kidney injury by the combination of Ginkgo biloba and pentoxifylline led to the necessity to perform the study. Acute kidney injury was demonstrated by measuring serum values of classical markers such as urea and creatinine but also by measuring the urinary N-acetyl-β-d-glucosaminidase index, a topical marker in modern medicine. The significant decrease of the biochemical parameters in group III (VANCO + GBI + PTX) compared to group II (VANCO) and values similar to group I (CONTROL), demonstrates, the nephroprotective effect of the use in combination of the two substances.

**KEYWORDS:** Acute kidney injury, Vancomycin-induced nephrotoxicity, Pentoxifylline, Ginkgo Biloba.

**Introduction**

The mouse kidney contains between 12,000 and 16,000 nephrons in each kidney, with variations depending on the species, and the number of nephrons in humans varies more significantly [1]. The kidneys receive about 20% of their heart rate.

The structural and functional unit of the kidney, the nephron, has a high metabolic activity, making them susceptible to acute renal injury (AKI), which is characterized by decreased renal function [2].

Acute renal injury (AKI) involves a sudden and usually transient loss of glomerular filtration, leading to nitrogen retention, which is normally excreted in the urine.

Regardless of its etiology, AKI usually involves an extensive lesion of the renal parenchyma, which, in the event of failed repair, promotes AKI's progression to chronic kidney disease.

AKI mortality currently has high values of up to 50% and no specific therapies to alleviate AKI and avoid renal function replacement therapy by hemodialysis.

Thus, there is an unmet clinical need for new AKI treatments [3]. Since oxidative stress has been described as one of the mechanisms of AKI, several antioxidants have been studied preclinically in an attempt to prevent AKI [4]. The use of natural products with antioxidant activity has gained much attention as safe alternative therapies in the mitigation of nephrotoxicity [5], and Ginkgo biloba (GBI) is a very popular herbal product used to improve memory and cognitive function.

GBI contains on average 27% polyphenols isohamnetin, bilobalide, kaempferol quercetin, 6% lactone terpenes (ginkgolides A, B, C, J and...
M) [6], and these constituents have shown beneficial pharmacological effects in various experimental models, such as anti-inflammatory, antiapoptotic and antioxidant activities [5].

Pentoxifylline (PTX) is a drug, derived from methyl-xanthine with some anti-inflammatory, antioxidant or anti-immunity properties.

It is widely used to treat peripheral vascular disease.

PTX improves the ability of red blood cells to deliver oxygen, resulting in better hemodynamics [7].

Its effect in reducing intraglomerular pressure has led to various studies in which it has been used as a nephroprotective agent [8].

KDIGO guidelines suggest the use of newer biomarkers to identify AKI at its earliest stage before organ dysfunction occurs so that management can help prevent AKI, especially before associated complications become irreversible [9].

Urinary NAG is increased following tubular injury or dysfunction from various causes including drug toxicity, heavy metal exposure, diabetic nephropathy [10] ischemic kidney injury following cardiac surgery or renal transplantation but also glomerulopathies [11].

Urinary NAG index provides a good estimate of the excretion of the two markers over a 24-hour period.

An increase in the urinary NAG index may precede the increase in the usual parameters used for the diagnosis of renal pathologies, especially cases of acute tubular injury.

The urinary NAG index represents the mathematical ratio between urinary NAG/urinary creatinine [12].

The objectives of the article are induction of AKI on Wistar rat models using VANCO, and after installation, nephroprotective effects of GBI in combination with PTX.

All study results were evaluated by measuring a topical urinary iNAG marker, as well as classical markers (serum urea and creatinine) and correlating with histopathological changes in the kidney.

**Material and Methods**

**Animals Studied**

Eighteen clinically healthy Wistar adult rats with a body weight of 250-350g, were used for our study.

The study of animals and the safety of experimental animals have been ensured in accordance with national legislation (Law 43/2014) and Directive 2010/63/EU.

The University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca (USAMV CN) approved the experimental procedures within the bioethics commission.

The animals were kept in the Biobase within the Faculty of Veterinary Medicine in Cluj-Napoca in adequate conditions.

The experimental animals were housed in polypropylene cages, where an optimal space/ head of animal was ensured, with environmental improvements ("enrichment of the environment"), namely the introduction of various objects in accommodation spaces, namely plastic, wooden burrows and so on.

The rats were sheltered in a real environment: humidity 40-60%, artificial cycle 12 hours/12 hours light-dark, temperature: 21-23°C, drinking water was purchased from the public network of Cluj-Napoca and food “quantity of food freely and standardized (purchased from Cantacuzino-Bucharest Institute).

**Drugs Used in the Study**

The substances used in the study are vancomycin (Vancomycin Kabi® 500mg, powder for concentrate for intravenous solution) which was obtained from FRESENIUS KABI Romania; pentoxifylline (Pentoxifylline SR Zentiva® 400mg, tablets) provided by Zentiva Romania.

One tablet was dissolved in 20ml of distilled water; tramadol (Tramadol® 50mg/ml, solution for injection) supplied by KRKA Romania, saline (0.9% NaCl, infusion solution) obtained from Braun Romania; and isoflurane (Anesteran® liquid for inhalation vapors) provided by Rompharm Romania, xylazine (Xylazin Bio® 100mg/ml, solution for injection) obtained from Bioveta.

**Biochemical Analysis**

Three experimental groups of 6 Winstar rats were used in each group.

The study was conducted over a period of 10 days and the start date was 24.03.2020.

The animals received research substances (vancomycin, pentoxifylline, gingko biloba) daily, subcutaneously and by gavage.

Rats were weighed prior to the start of the experiment to accurately calculate the doses of injectable vancomycin and the doses of pentoxifylline and Gingko biloba per individual.

The determination of urinary creatinine was performed using a commercial kit with two reagents: Reagent 1: picric acid and Reagent 2: disodium phosphate and sodium hydroxide.
This was determined by a colorimetric reaction; the method based on the Jaffe reaction and consists in quantifying the rate of formation of the color complex between creatinine and alkaline picrate at 37°C and the recorded results were expressed in g/l.

Immediately after euthanasia, urine samples were collected by cystocentesis following laparotomy.

These were collected in sterile Eppendorf tubes and centrifuged at 1000rpm for 5 minutes at 4°C.

The supernatant was used to determine urinary NAG and urinary creatine and the NAG index activity (iNAG) was subsequently calculated.

All determinations were made within a maximum of six hours of sampling.

**Preparation of Rats**

The drugs used in the experiment were Vancomycin, Pentoxifylline, Gingko Biloba (Figure 1).

In the CONTROL group the rats received 1ml of 0.9% saline (NaCl) by gavage, while the VANCO group was injected subcutaneously with Vancomycin (Vancomycin Kabi® 50mg powder concentrate solution for infusion) 200mg/kg/day and the VANCO+PTX+GBI group, was injected with Vancomycin (VANCO) 200mg/kg/day subcutaneously and was administered orally, by gavage, Pentoxifylline (Pentoxifylline SR Zentiva® 400mg) 50mg/kg/day and Gingko Biloba 100mg/kg/day.

As oral administration of the substances is a stressful operation for rodents, sedation by intramuscular injection with Xylazine (Xylazine Bio ® 100mg/ml) 5mg/kg was performed to facilitate oral administration and reduce stress.

![Figure 1. The medications used in our study.](image)

**Biological Sampling**

In this study, biological urine and blood samples.

Before euthanizing the rats, whole blood samples were taken from the infraorbital sinus, in tubes with EDTA, from which a complete hematological examination was performed.

Whole blood samples were collected in heparinized tubes, which were processed by centrifugation (temperature 4°C for 5 minutes at 3000rpm) to obtain the plasma required for dosing.

Measures have also been taken to limit the suffering of rats by analgesia, namely subcutaneous administration of Tramadol (Tramadol® 50mg/ml) 20mg/kg each day.

On the last day of the study (day 11), all rodents were euthanized by prolonged narcosis with Isoflurane (Anesteran ®) using the anesthesia chamber, followed by biological sampling and incineration of the corpses in the USAMV crematorium.

**Histopathology Kidney**

Histopathology: The kidneys were cut longitudinally (including the renal cortex, medulla, and renal pelvis), and after complete fixation by immersion in 10% NBF, the samples were dehydrated in ethanol in increasing concentrations (70-100%) and usually incorporated in paraffin.

Finally, the paraffin blocks were sectioned and stained with hematoxylin eosin (H&E).

**Statistical-Mathematical Analysis**

The statistical results for the biological parameters were processed with the Excel program Microsoft Office 2019, where the arithmetic mean was used.

The control groups were compared with the experimental groups for each biological parameter.

**Results**

All rats subjected to acute renal injury remained alive until the end of the experiment and the weight of the treated rats did not vary significantly from the CONTROL group.

The results obtained at the end of the experiment are represented by hematological and biochemical parameters.

In all groups, the blood count reflected normal values, similar to those found in the control group.

The biochemical parameters were reported and interpreted in Table 1.
Table 1. Biochemical, blood and urinary parameters, from the 11th day of the study, from lots I CONTROL and II VANCO-values obtained, mean, standard deviation, coefficient of variation and t test.

<table>
<thead>
<tr>
<th>LOTS</th>
<th>Urea mg/dL 10-33</th>
<th>Creatinine mg/dL 0,5-2,2</th>
<th>Urinary iNAG U/g</th>
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</thead>
<tbody>
<tr>
<td>I CONTROL</td>
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<td>31,3</td>
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<td>31,5</td>
<td>0,44</td>
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<td>31,2</td>
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<td>5,5</td>
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<tr>
<td>27,8</td>
<td>0,37</td>
<td>6,13</td>
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<tr>
<td>29,1</td>
<td>0,43</td>
<td>5,57</td>
<td></td>
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<tr>
<td>MEDIA±D.S.</td>
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<td>0,42±0,06</td>
<td>5,95±0,37</td>
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<tr>
<td>CV %</td>
<td>5,41</td>
<td>16,35</td>
<td>6,31</td>
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<tr>
<td>II VANCO</td>
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<tr>
<td>45,5</td>
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<td>48,2</td>
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<td>MEDIA±D.S.</td>
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<td>0,83±0,05</td>
<td>283,13±70,68</td>
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<tr>
<td>CV %</td>
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<tr>
<td>T TEST</td>
<td>0,00000000445</td>
<td>0,000000859</td>
<td>0,000207</td>
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<tr>
<td>III VANCO+PTX+GBI</td>
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<tr>
<td>40,1</td>
<td>0,46</td>
<td>66,7</td>
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<tr>
<td>MEDIA±D.S.</td>
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<td>0,37±0,06</td>
<td>68,58±10,41</td>
</tr>
<tr>
<td>CV %</td>
<td>16,48</td>
<td>8,46</td>
<td>15,18</td>
</tr>
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</table>

Figure 2. Blood and urinary biochemical parameters from the 11th day of the study (lot average values), from the CONTROL, VANCO and VANCO+PTX+GBI lots.

We presented the serum creatinine values from group I (CONTROL) and group II (VANCO) in Figure 2.

The concentration of serum creatinine mediated within the VANCO group showed an increase of 2.57 times compared to the CONTROL group being statistically significant (p<0.05) and in the VANCO+PTX+GBI group we observed slightly lower values compared to the group CONTROL.

We presented in Figure 3 the values of serum urea in the VANCO group, which show an increase of 1.6 times compared to the CONTROL group being significantly higher (p <0.05), and in the VANCO+PTX+GBI group there is an increased coefficient of variation of 16, 48% and mean values (35.36mg/dl) discreetly similar to the CONTROL group (30.48mg/dl).

By comparing these three groups, the aim was to obtain experimental models of AKI, induced by the administration of VANCO, but also the evaluation of renal function after the administration of nephroproectors.

AKI in the VANCO group was also confirmed by the presence of histopathological lesions in which the urinary tract showed cell necrosis, cytoplasmic vacuolation and occupation of the tubular lumen with necrotic detritus (Figure 6) compared to the CONTROL group where the renal tubules show normal morphology (Figure 5).
Figure 3. Serum urea values from the 11th study day (batch average values), within the CONTROL, VANCO, VANCO+PTX+GBI batches.

Figure 4. Urinary iNAG values on the 11th study day in the CONTROL, VANCO, VANCO+GBI+PTX lots.

The urinary iNAG values in the VANCO group in Figure 4 showed an increase of 47.58 times compared to the CONTROL group, and in the VANCO+GBI+PTX group there is a significant decrease of the average of approximately 4.12 times compared to the VANCO group, but it still shows higher values than in the CONTROL group.

The coefficient of variation in the VANCO+GBI+PTX batch is 15.18%.

Figure 5. Histopathological sections through the kidney from an individual in the CONTROL group. Normal urinary tubules. Control group, x20ColH&E.

Figure 6. Histopathological sections through the kidney from an individual in the VANCO group. Urinary tract with cell necrosis and cytoplasmic vacuolations and occupation of the tubular lumen with necrotic detritus x20H&E.

Figure 7. Histopathological sections through the kidney from an individual in the VANCO+PTX+GBI. Proximal twisted tubes with ischemia x20H&E.
Discussion

In our study, the results obtained and their interpretations were found in several aspects of nephroprotection and acute renal injury.

The results of our study show that administration of VANCO in Wistar rats produced a typical pattern of acute kidney injury, which was characterized by a marked increase in biomarkers, namely: creatinine increased 2-fold, serum urea increased approximately 1.6-fold compared to CONTROL group, and the VANCO group showed approximately 47-fold higher urinary iNAG values compared to the CONTROL group and these results correlated strongly with histopathological changes.

The nephrotoxicity of VANCO has been intensively studied in recent years both in animal models, where in an experimental study by Miao H dose of 300-500mg/kg administered for 4 days were sufficient for induction of AKI being demonstrated using classical markers (urea, creatine), where elevated values were recorded [12] but also studies on human subjects from a centre, including 3719 patients from 30 out of 34 administrative regions at provincial level in China, investigated the burden and characteristics of AKI associated with VANCO in China.

The study covers patients from different areas in China and the results showed that the incidence of AKI associated with VANCO was 14.3%, however, this could be an underestimate as 998 patients were excluded due to insufficient SCR measurements, which is consistent [13].

AKI is confirmed in the VANCO group by serum creatinine values where it doubled compared to the CONTROL group, and urinary iNAG recorded 47 times higher values compared to the CONTROL group so it is consistent with other studies where NAG indicates earlier onset of AKI and is a more sensitive biomarker than serum creatinine [14].

AKI in the VANCO group was also confirmed by the presence of histopathological lesions where the urinary tubules showed cell necrosis, cytoplasmic vacuolization and occupation of the tubular lumen with necrotic detritus being in agreement with the study performed by Ngoentra T and collaborators where renal histopathological samples showed necrotic cells probably tubular, cell degeneration.

Tubular cells often showed acute lesions including flattening, loss of differentiation, cytoplasmic vacuolization or disintegration of the cell membrane [15].

The VANCO+GBI+PTX group showed a significant decrease in biochemical parameter values following PTX and GBI administration.

The study by Hashemi M et al evaluated the role of administering PTX in rat groups where AKI was induced by renal ischemia, showing a significant reduction of cell apoptosis [16].

Other studies have evaluated the effect of Ginko biloba in lipopolysaccharide-induced AKI where the inflammatory response was reduced as well as tubular apoptosis.

GBI also suppressed the lipopolysaccharide-induced inflammatory response exerting a renoprotective role in AKI [17].

Conclusions

Serum creatinine, serum urea and iNAG were significantly higher in the VANCO group compared to the CONTROL group for which we can conclude that AKI was successfully installed.

The originality of the work consists in the combination of Pentoxifylline and Ginko biloba for AKI remission, where the nephroprotective effect was demonstrated by decreasing the biochemical parameters in the VANCO+GBI+PTX group compared to the VANCO group.

Further studies are needed as the combination of Ginko biloba and pentoxifylline for the improvement of AKI has not been documented in the literature.

Authors’ Contribution

Romeo Popa and Daniel Cosmin Caragea equally contributed to the manuscript.

Conflict of Interests

The authors declare that they have no conflict of interests.

References


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