PhD THESIS ABSTRACT

The diagnosis of acute renal lesions and the evaluation of the nephroprotective effect of erythropoietin and melatonin by urinary N-Acetyl-\(\beta\)-D-Glucozaminidase activity quantification in dogs and rats

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INTRODUCTION

Acute kidney injury is a serious clinical condition commonly encountered in critical patients, being associated with increased mortality, prolonged hospitalization, and, not least, a significant increase in treatment costs. Therefore, there is a need to identify effective early diagnosis of renal failure and, last but not least, sustainable therapeutic measures with the potential to ameliorate / treat acute renal injury and its consequences.

Etiopathogenesis of acute renal failure includes numerous mechanisms - exposure to endogenous and exogenous toxins, metabolic factors, ischemia / reperfusion injury, neurohormonal activation, inflammation and oxidative stress. Among these, the ischemia / reperfusion injury is the most common cause of acute renal failure. Substances with an antioxidant and nephroprotective role such as erythropoietin and melatonin have the potential to prevent and limit the progression of such lesions or assist in the recovery and regeneration of lesioned kidney tissue following ischemic or toxic lesions. These nephroprotective mechanisms of erythropoietin and melatonin result from their ability to limit apoptosis, promote angiogenesis and neovascularization, due to anti-inflammatory effects, stimulation of tissue regeneration, and strong exogenous effects.

The identification of markers of renal lesion has become a priority of modular nephrology as a result of discoveries made in disciplines such as proteomics, genomics and metabolomics that have greatly improved the ability of researchers to understand the function and role of proteins with potential role as marker of tubular or glomerular suffering.

Research in recent years has focused on identifying molecules that are capable of indicating early and, especially, the location of renal lesions. The ideal marker of renal impairment is one that can be easily dosed in a non-invasive, low-cost way from easily available sources such as blood or urine, possessing high specificity and sensitivity, prognostic value of disease progression, and not to be affected by other
comorbidities. Investigations on the treatment of acute kidney injury and its consequences have been limited. However, in recent years, animal studies have shown that treatment with biologically active substances such as erythropoietin or melatonin seems promising for individuals at risk of acute kidney injury. These studies, as well as the need for sustained diagnostic and therapy protocols for patients with various nephropathy, stimulate us to take maximum care in designing and conducting this study that focuses on identifying the utility of the urinary N-acetyl-β-D-glucosaminidase enzyme in early diagnosis with the exact location of kidney pathologies as well as the evaluation of the nephroprotective effect of some molecules with antioxidant effect.

STRUCTURE OF THE PAPER

The Ph.D. thesis entitled "Research regarding the diagnosis of acute renal lesions and evaluation of the nephroprotective effect of erythropoietin and melatonin by urinary N-Acetyl-β-D-Glucozaminidase activity quantification in dogs and Wistar rats" contains a total of 127 pages and is structured in two parts. The first part, presenting the current state of knowledge, is divided into 5 chapters and extends to a number of 27 pages. It includes general aspects of renal anatomy and physiology in rats and domestic dogs, as well as current facts about the techniques and trends in the diagnosis of kidney diseases and, last but not least, updates on the potential of nephroprotective effect of some hormonal substances.

The second part contains 59 pages and represents the personal contribution. It is structured in 6 chapters and it contains the research conducted over the period 2014-2017. Chapter 6 illustrates the working hypothesis and research objectives while chapters 7-10 refer to 4 distinct studies, each containing objectives, materials and methods, results and discussions, and conclusions. Chapter 11 outlines the general conclusions and recommendations for the four studies. Chapter 12 presents the originality and innovative contributions to this thesis.
Chapter 7, entitled "Normal urinary NAG index values in the Wistar rat", contains the biochemical and statistical studies used to determine the normal activity of the urinary NAG index in Wistar rats, males and females under physiological conditions.

Reference values have a major importance in describing the diversity of healthy individuals variables. These are population reference ranges (RIs) that comprise 95% of the healthy population. The determination of biological markers, like urinary NAG and creatinine, is considered a simple, rapid and non-invasive method for detecting and monitoring renal tubular function under different conditions. Calculation of the urinary NAG index provides a good estimate of the excretion of the two markers over a 24 hour period. An increase of the urinary NAG index may precede increases in standard parameters used in the diagnosis of renal disease, especially in cases of acute tubular lesions. With the purpose of detecting deviations of the urinary NAG index, this study established the reference values in Wistar rats. Urine samples were collected from 100 healthy Wistar rats, 50 males and 50 females. NAG and creatinine were determined, and subsequently the NAG index was calculated for reference value establishing. The mean value of NAG index was found to be 5.81±1.68 (U / g) for healthy females and 4.10±0.90 (U / g) for healthy males.

Chapter 8, entitled "The evaluation of urinary activity of the N-acetyl-Beta -D-glucosaminidase index as a marker of renal impairment in rat experimental nephropathies" contains research on obtaining an effective and predictable experimental model of acute renal injury in vivo Wistar rat and urinary activity evaluation of the NAG index, induced by renal ischemia/reperfusion and gentamicin sulfate nephrotoxicity.

The identification of a suitable prevention method which facilitates limiting the deleterious effects of acute kidney injuries is highly required. In order to identify a proper treatment for acute kidney injuries, a suitable experimental model that replicates the structural, metabolic and inflammatory lesions that occur in the natural acute injured kidney is highly necessary. Intense urinary NAG activity can be found in a variety of renal disease such as toxic nephropathies, ischemic renal injury following cardiac surgery or renal transplantation but also in glomerular disease especially in
diabetic nephropathy. Rises in urinary NAG enzyme activity strongly suggests tubular cell damage and support NAG enzyme as a biomarker of renal tubular injury. The aim of this paper is to obtain a stable in vivo acute kidney injury experimental model, in Wistar, rats and to evaluate the urinary activity of N-acetyl-β-D-glucosaminidase (NAG) enzyme, blood levels of urea and creatinine and microstructural renal alterations induced by ischemia/reperfusion injury respectively gentamicin nephrotoxicity. For this purpose we have used a rat experimental model. Adult male Wistar rats weighing 250-300 g were randomly divided into 3 groups with 8 rats in each group. Group 1 served as a model for the renal ischemia/reperfusion injury experiment, group 2 served for toxic kidney injury experimental model and group 3 served as control group. All individuals in both groups 1 and 2 presented marked elevations in blood urea and creatinine at the moment of euthanasia (day 3 for group 1 and day 9 for group 2) compared to the control group where biochemical values remained within normal limits. Urine analysis of both group 1 and 2 showed marked urinary NAG index activity which suggests acute tubular injury, suggestion confirmed by histological evaluation of the renal parenchyma sampled from this subjects

Chapter 9, entitled "The quantification of the nephroprotective effect of erythropoietin and melatonin in gentamicine toxic-induced nephropathy" contains research on the nephroprotective effect of erythropoietin and melatonin in gentamicin sulfate-induced nephrotoxicity.

Melatonin (MLT) is an epiphysial chronobiotic hormone, also known for its antioxidant effects. Besides erythropoiesis, erythropoietin (EPO) possesses other biological functions (neuroprotection, nephroprotection). We focused on the assessment of MLT and EPO nephroprotective effects in a gentamicin toxicity model. Forty adult male Wistar rats were divided into 5 groups consisting of 8 individuals each; chemicals were administrated daily by i.p. route. Control group received normal saline. GM group received 100 mg/kg/d GM. EPO+GM group received EPO 100 UI/kg/d and GM 100 mg/kg/d. MLT+GM received MLT dissolved in methanol 20 mg/kg/d and GM 100 mg/kg/d. Group GM+EPO+MLT received MLT dissolved in methanol 20 mg/kg/d, EPO 100mg/kg/d and GM 100 mg/kg/d. Both MLT and EPO prevented kidney damage reflected by lower urinary iNAG activity, reduced kidney structural
damage and increased urinary density, and lower blood urea and creatinine concentrations. However the best protective effect was provided by MLT and EPO association.

**Chapter 10**, entitled "Evaluation of the effects of ultrasound guided renal biopsy on kidney function in dogs", contains research into the adverse effects of renal biopsy in dogs, evidenced both by classical methods of kidney function diagnosis and by measuring the urinary NAG index activity.

Ultrasound guided renal biopsy is an essential diagnostics method which, by facilitating histopathological examination can increase the accuracy of the differential diagnosis between acute and chronic nephropathies and will help the clinician perform an etiologic diagnosis, issue a prognosis and orient the therapy of the majority of parenchymal nephropathies.

Due to the relative invasiveness and potential adverse effects, the use of renal biopsy is limited among practitioners. In this study we evaluate the intensity of renal damage induced by renal cortex sampling and the clinical consequences of such a procedure.

We examined 28 dogs, mixed breed and variable ages, 11 (39, 29 %) males and 17 (60, 71 %) females that were referred to our clinic and underwent ultrasound guided renal biopsy in order to establish a definite diagnosis. Patients were presented with a variety of diffuse nephropaties: kidney lymphoma: 1 (3.57%), glomerulonephritis: 13 (46.43%), tubulointerstitial nephritis: 11 (39.29 %) and nephrocalcinosis: 3 (10.71 %) of which 18 (64.29 %) were in acute kidney failure and 10 (35.71 %) were chronic renal patients.

The type and the severity of renal lesions were correlated with changes in urinary NAG index (iNAG), and specific serum renal damage markers such as urea, creatinine, phosphorus and ionized calcium.

To quantify the side effects of percutaneous renal biopsy the magnitude of post biopsy haematuria and changes in urinary iNAG activity were evaluated. The results indicate a significant post biopsy increase in urinary iNAG activity in all patients that underwent this procedure (100.08±34.45 (U/g) pre-biopsy vs.
147.65±33.26 (U/g) post-biopsy iNAG, p<0.001) suggesting an intensification in renal tubular damage consecutive to kidney puncture and sampling.

**GENERAL CONCLUSIONS**

1. The normal values of urinary NAG index in Wistar rats differ significantly depending on the gender of the individuals.

2. Female Wistar rats exhibit higher NAG urinary when compared to males (5.81 ± 1.68 vs. 4.10 ± 0.90 U / g), with an average difference of 0.82 (U / g).

3. Both renal ischemia/reperfusion injury and gentamicin-induced nephrotoxicity are feasible methods for obtaining in vivo models of acute renal injury capable of inducing a significant increase in urinary NAG activity.

4. Significant increases in urinary NAG index activity following temporary occlusion of urinary pediculum and following administration of nephrotoxic doses of gentamicin suggest massive renal tubular damage.

5. The urinary activity of NAG index may substantially complement conventional methods of diagnosis of kidney lesions, in addition to offering the possibility to include or exclude kidney tubular lesions.

6. Combined erythropoietin and melatonin therapy offers favorable effects in the prevention and counteraction of renal lesions induced by toxic doses of gentamicin.

7. In the dog, ultrasound guided renal biopsy induces significant increases in urinary NAG index suggesting acute tubular disease.

**RECOMMENDATIONS**

1. We recommend dosing the urinary activity of NAG index as a measure of early diagnosis in the treatment with aminoglycoside antibiotics as well as when administrating other potentially nephrotoxic drugs.

2. We recommend the use of combined erythropoietin and melatonin therapy, which may complement the treatment protocols in patients with renal impairment or may represent a measure of prevention of renal impairment in the categories at risk (nephrotoxic, shock conditions, renal surgery, other systemic diseases with renal impairment).