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Essential Elements as Biomarkers of Acute Kidney Injury and Spontaneous Reversion

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Abstract Acute kidney injury (AKI) is an important health problem and can be caused by number of factors. The use of aminoglycosides, such as gentamicin, is one of these factors. Recently, an effort has been made to find biomarkers to guide treatment protocols. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to estimate the contents of Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn in serum and urine of the healthy, AKI, and **s**pontaneous recovery (SR) groups of animals. The animal model of AKI and SR was validated by measuring serum and urinary urea and creatinine. The quantitative determination of the elements showed a decrease in serum levels of Ca, and Fe in the AKI group ($P<0.01$ vs. healthy), with a return to normal levels in the SR group, without a significant difference between the healthy and SR groups. In the urine samples, there was a decrease in P and Na levels in the AKI group ($P<0.001$ and $P<0.01$ vs. healthy), but Ca levels were increased in this group compared with the healthy and SR groups ($P<0.01$). These findings indicate that mineral elements might be useful as biomarkers for AKI.

Keywords Essential elements · Acute kidney injury · ICP-OES · Gentamicin

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Introduction

Acute kidney injury (AKI) is a serious health problem that affects thousands of people around the world. Studies report overall in-hospital mortality at approximately 20% and up to 50% in intensive care unit (ICU) patients [1]. The etiology for AKI is multifactorial: ischemia/reperfusion, sepsis, and administration radiological contrast dyes and nephrotoxic drugs, such as aminoglycosides, are some of the causes. Gentamicin (GM) is an important aminoglycoside antibiotic mainly used in an ICU setting. GM is eliminated by the kidney through glomerular filtration. GM binds to negatively charged structures in the cell wall altering the permeability of the cell wall and finally causing necrosis of the tubular cells [2]. AKI is associated with changes in substrate metabolism and body composition and, specifically with some changes in protein, carbohydrate, and lipid metabolism. In addition, it promotes catabolism of skeletal muscle proteins, redistribution of elements between plasma and tissues, and acute losses of biological fluids, varying concentrations of essential elements [3].

An element to be considered essential to an organism should have physiologically important function. Essential elements have four major functions as stabilizers, structural elements, hormonal functions, and enzyme cofactors. Imbalance in the composition of these elements may cause disease [4]. Numerous enzymes have minerals as key components and are involved in important biological functions such as transport, elimination of free radicals, or hormonal activity [5]. These elements are subdivided according to their concentrations: approximately 1 mg L^{-1} in biological fluids or <100 mg kg^{-1} in tissues, in which case they are called as macroelements. Chemical elements found in concentrations less than 10 μg L^{-1} in biological fluids or less than 100 mg kg^{-1} in body tissues are defined as trace elements. Finally, elements found at concentrations less than 1 μg L^{-1} in biological fluids are known as "ultra-trace elements" [6].

Table 1 Instruments and analytical parameters for ICP-OES

R.F. generator frequency 40 MHz
Plasma torch standard 1 slit
Injector standard 2 mm
Detector CCD
Optic echelle
View axial
Power 1300 W
Plasma gas flow 15 L/min
Auxiliary gas flow 0.3 L/min
Nebulizer gas flow (Teflon Mira Mist) 0.6 L/min
Spray chamber cyclonic, baffled 12-mm-axial torch connection, 4 mm drain
Sample flow rate 1.5 mL/min

Serum creatinine and blood urea nitrogen (BUN) have typically been used to diagnose AKI, but they are not very sensitive or specific measures. In fact, they are affected by many non-renal factors such as age, sex, race, muscle mass, nutritional status, and infection [7].

A large number of animal models have been developed to mimic renal injury induced by using different agents. These AKI animal models contribute to the understanding of the initial pathophysiology of this disease and the development of therapeutic pharmaceuticals [8, 9].

The term biological markers (biomarkers) can be defined as cellular, biochemical, or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids [10]. The search for the early detection of AKI is very important to guide the treatment, inhibit the progress of disease, and decrease the mortality rate [11].

The aim of this study was to examine the potential of the elements to be biomarkers of AKI using an animal model.

Materials and Methods

Animals

Male Wistar rats weighing 200–250 g were obtained from the laboratory of the National Institute of Pharmacology and Molecular Biology (INFAR) at UNIFESP. The animals were kept in an environment controlled for temperature (22 °C ± 2 °C) and humidity (50 ± 15%), with artificial lighting (cycle of 12 h light/12 h darkness) and free access to water and ration. Animals were divided into three groups (n = 6/group). The healthy group received no treatment; the AKI group was treated intraperitoneally with 60 mg/kg/day GM over 7 days, and the spontaneous recovery (SR) group.

All procedures were performed according to the recommendations of the Research Ethics Committee of UNIFESP/SP (Project No. 9287290915/CEUA). Moreover, this committee oversaw all experimental animal procedures.

Table 2 Serum creatinine and urea values of the health, AKI, and SR animals groups

	Animal groups (n = 6)		
	Health	AKI	SR
Creatinine (mg/dL)	0.60 ± 0.02	3.97 ± 0.84***	0.52 ± 0.01
Urea (mg/dL)	40.83 ± 1.32	260.4 ± 42.30***	56.33 ± 2.99

***$P < 0.001$

***$P < 0.001$

Table 3 Wavelengths adopted for elemental analysis

Element	Wavelength (nm)
Calcium	317.933
Copper	324.754
Iron	259.940
Magnesium	279.079
Manganese	257.610
Phosphorus	213.618
Potassium	766.491
Sodium	588.995
Zinc	213.856

Blood and Urine Specimen Collection

Each animal was placed in a metabolic cage for collection of urine over 24 h. All urine samples were transferred to conical polyethylene tubes (Corning, Lowell, MA, USA) and stored at −20 °C. Rats were anesthetized through intraperitoneal injection of a cocktail containing ketamine (95 mg/kg^{-1} body weight, Dopalen®, Vetbrands, Paulínia—Sao Paulo, Brazil) and xylazine (5 mg/kg^{-1} body weight, Rompun®, Bayer, Sao Paulo—SP, Brazil). Blood collection was performed by intracardiac puncture and was transferred to conical polyethylene tubes and centrifuged (2500 rpm, 10 min, room temperature). The separated serum samples were stored at −20 °C. Kidneys were collected for histopathological examination.

Table 4 Serum creatinine and urea values of the health, acute kidney injury (AKI), and spontaneous recovery (SR) animals groups

	Animal groups (n = 6)		
	Health	AKI	SR
Creatinine (mg/dL)	0.60 ± 0.02	3.97 ± 0.84***	0.52 ± 0.01
Urea (mg/dL)	40.83 ± 1.32	260.4 ± 42.30***	56.33 ± 2.99

***$P < 0.001$

Table 5 Urinary levels of creatinine and urea from health, acute kidney injury (AKI), and spontaneous recovery (SR) animals groups

	Animal groups (n = 6)		
	Health	AKI	SR
Creatinine (mg/24 h)	7.08 ± 0.12	2.93 ± 0.44**	8.52 ± 1.02
Urea (mg/24 h)	516.6 ± 18.24	170.5 ± 52.90***	611.4 ± 38.44

**P < 0.01; ***P < 0.001

Measurements of Urinary and Serum Creatinine and Urea

Measurements of creatinine and urea in both serum and urine samples were performed with a Cobas Mira Plus (Roche Diagnostic Systems, Branchburg, NJ) apparatus using a Labtest kit (Lab Test Diagnostic S/A—MG, Brazil) according to the manufacturer's instructions.

Histological Analysis

Excised kidneys were fixed in Methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid) for 3 h and routinely processed for paraffin embedding. Histopathological analysis was performed on 5-μm sections, stained with hematoxylin and eosin (n = 6 animals per group), and observed under a Leica DM1000 light microscope (Leica Microsystems, Wetzlar, Germany).

Preparation of Serum and Urinary Samples for Mineral Element Analysis

Before the preparation of the samples, all plastic bottles and glassware were cleaned by soaking in 1.50 mol/L HNO_3 during 24 h, rinsing five times with high-purity water, and dried and stored in a class 100 laminar flow hood (Hexiclean, model Clean-5).

Inside a fume hood, sample volumes between 2 and 10 mL were digested in a 250 mL beaker, on a hot plate at 200 °C (Quimis, model Q313A, Sao Paulo, Brazil), using 10 mL of 4.2 mol/L HNO_3 (ACS reagent grade, Merck, Rio de Janeiro, Brazil) plus 2.0 mL of 30% hydrogen peroxide (ACS reagent grade, Merck, Rio de Janeiro, Brazil). After the digestion, the beakers were cooled and the digests were transferred to volumetric flasks, and the volume was expanded to 5 mL with water. It was prepared two diluted solutions for each sample using HNO_3 5% solution (Suprapur® Merck, Darmstadt, Germany). In parallel, a reagent blank was prepared under the same conditions in order to correct possible error in test results that comes from the reagents themselves. High-purity deionized water obtained from Milli-Q® purification system (Millipore, Belford, MA, USA) was used in preparation of all solutions.

For the mineral elemental analysis, a Perkin Elmer ICP-OES Optimal DV 7000 was used and calibrated with analytical solutions prepared by suitable dilution of stock solution (ICP phosphorous and 21 multi-element standard solution Inorganic Ventures, Christiansburg, USA) in HNO_3 5% solution (Suprapur® Merck, Darmstadt, Germany). Between the analysis of each sample, all system was rinsed with ultrapure water and HNO_3 5% solution in order to remove any contamination remained. This method for analyzing the samples was based on the method EPA/600/R-94/111, 1994. The measurement conditions for ICP-OES are shown in Tables 1, 2, and 3.

Statistical Analysis

Multiple mean comparisons were performed using a one-way ANOVA followed by Bonferroni's test using the GraphPad

Table 6 Comparison of mineral elements concentrations (mg/L) in serum samples of health, acute kidney injury (AKI), and spontaneous recovery (SR) animals groups (n = 6)

	Health		AKI		SR		Health vs. AKI	Health vs. SR	AKI vs. SR
Elements	Mean ± SE		Mean ± SE		Mean ± SE		P	P	P
Ca	223.43	26.93	132.91	9.21	210.01	25.28	<0.01	NS	<0.05
Cu	4.05	0.76	3.51	0.49	3.00	0.16	NS	NS	NS
Fe	81.55	7.55	42.16	2.73	70.94	4.04	<0.01	NS	<0.05
K	536.56	48.01	397.17	18.09	381.78	13.58	<0.05	<0.05	NS
Mg	68.68	7.28	67.05	2.84	45.96	2.05	NS	<0.05	<0.05
Mn	1.11	0.28	0.33	0.03	0.61	0.01	<0.05	NS	NS
Na	4833.25	419.89	2524.08	308.71	2928.94	81.40	<0.001	<0.01	NS
P	185.88	4.89	100.25	24.58	116.52	5.49	<0.001	<0.001	NS
Zn	4.98	1.35	2.41	0.25	4.30	0.22	<0.05	NS	NS

NS statistical difference is not significant

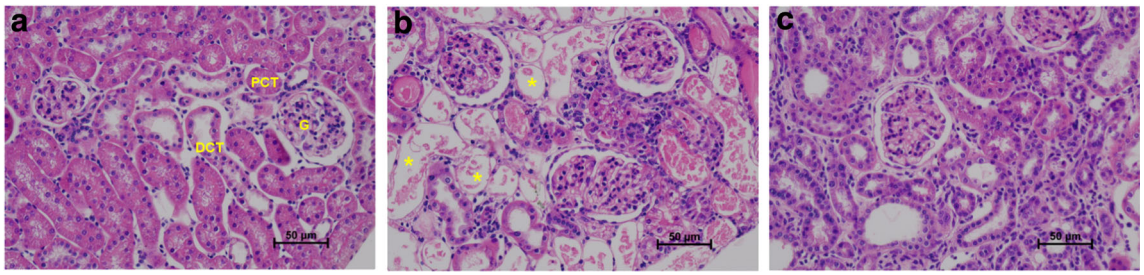

Fig. 1 Histopathological analysis of the kidneys of healthy, AKI, and SR groups stained with hematoxylin & eosin. **a** Section of healthy kidney; cortical parenchyma to consist of dense rounded structures, the glomeruli (*G*), distal contorted tubule (*DCT*), proximal tubule (*PT*). **b** Section of AKI kidney; ATN (*), glomeruli with loss of integrity. **c** Late stage of tissue regeneration. Scale bar 50 μm

Prism program Version 5.0 Windows (GraphPad® Software, San Diego, CA, USA), http://www.graphpad.com. Differences with a P value of $P < 0.05$ were considered statistically significant.

Results

Initially, the animal models of AKI and SR were validated by measuring serum and urinary urea and creatinine. The results are shown in Tables 4, 5, and 6.

It was observed that GM treatment resulted in a significant increase in serum creatinine and urea concentrations (6.6 and 6.4 times, respectively) of the AKI animals when compared to the healthy animals ($P < 0.001$ vs. healthy group). After completing 20 days of treatment (period of spontaneous reversion), the SR animals group showed serum creatinine and urea concentrations that had returned to baseline. (Table 4).

Treatment with GM promoted a significant decrease in the urinary creatinine and urea concentrations (2.4 and 3.0 times, respectively) in the AKI animal group when compared to the healthy rats ($P < 0.01$ and $P < 0.001$ vs. healthy group, respectively). After completing 20 days of treatment, the SR group showed a resolution of AKI, as urinary creatinine concentrations returned to baseline levels (Tables 5 and 6).

Kidney Histopathological Analysis

The histopathological analysis of kidneys from the experimental groups corroborated urea and creatinine results. Figure 1a shows a normal morphology and preserved the architecture of the renal parenchyma, characterized by the presence of simple cuboidal epithelial tissue in the renal tubules. Figure 1b shows acute tubular necrosis (ATN) and altered glomeruli with loss of integrity, and Fig. 1c shows late stage tissue regeneration.

Mineral Element Analysis

Quantitative determination was performed for nine mineral elements: calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), and zinc (Zn); the values are expressed in micrograms per mL. The mean profile of serum and urinary mineral elements in healthy, AKI, and SR groups are shown in Tables 5, 6, and 7.

Almost all mineral elements were significantly decreased in the serum of the AKI group. However, after resolution of disease, only Ca and Fe returned to normal levels (Tables 5 and 6).

Table 7 Comparison of mineral elements concentration (mg/L) in urinary samples health, acute kidney injury (AKI), and spontaneous recovery (SR) animals groups ($n = 6$)

	Health		AKI		SR		Health vs. AKI	Health vs. SR	AKI vs. SR
Elements	Mean ± SE		Mean ± SE		Mean ± SE		*P*	*P*	*P*
Ca	20.03	0.72	138.16	22.67	23.24	1.60	<0.01	NS	<0.01
Cu	2.30	0.33	0.74	0.12	1.22	0.18	<0.01	NS	NS
Fe	2.11	0.33	2.40	0.55	0.66	0.15	NS	NS	<0.05
K	7281.06	865.93	2668.01	517.23	6303.53	1125.54	<0.05	NS	NS
Mg	254.75	14.67	111.69	17.89	147.77	18.65	<0.001	<0.01	NS
Mn	0.11	0.02	0.06	0.01	0.09	0.02	NS	NS	NS
Na	1935.11	276.59	612.30	248.11	1304.10	430.37	<0.01	NS	<0.01
P	1694.53	131.77	504.88	71.08	1494.76	113.87	<0.001	NS	<0.001
Zn	0.35	0.02	0.28	0.05	0.40	0.05	NS	NS	NS

NS statistical difference is not significant

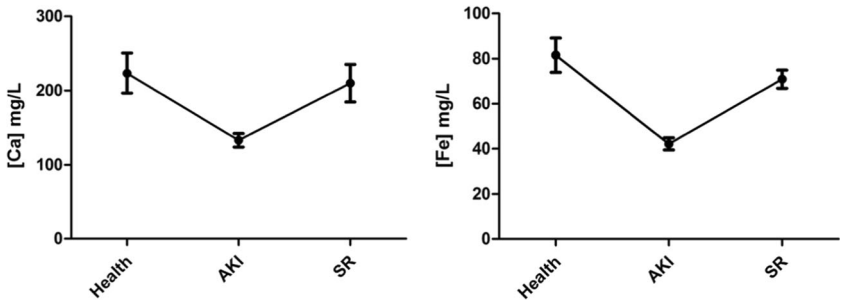

Fig. 2 Serum concentrations of Ca and Fe for healthy, acute kidney injury (*AKI*), and spontaneous recovery (*SR*) groups. Results represent the mean ± SE ($n = 6$) of three experiments

Significant difference in urinary levels of Ca, Na, and P was found between health and AKI groups and with the reversion of kidney injury, the concentration of these elements returned to physiological levels (Table 7).

Quantitative determination of mineral elements showed a decrease in serum levels of Ca and Fe in the AKI group ($P < 0.01$ vs. healthy) and returned to normal in the SR group, with no significant difference between the healthy and SR groups, as shown in Fig. 2.

In the urine samples, there was a decrease in P and Na levels in the AKI group ($P < 0.001$ and $P < 0.01$ vs. healthy), but there was an increase in Ca levels in this group compared with the health and SR groups ($P < 0.01$) (Fig. 3).

Discussion

Gentamicin is a very useful antibiotic for gram-negative bacterial disease; however, it is very nephrotoxic and frequently causes AKI in patients [12]. In this study, an animal model of nephrotoxicity and spontaneous reversion of AKI was used in order to reveal potential mineral biomarkers.

The animal model was validated by measuring classical biochemical tests such as blood urea and creatinine levels and using histopathological findings. Among nine elements in the serum samples, Ca and Fe levels were decreased in the AKI group and returned to normal levels in the SR group. Hypocalcemia can develop during the course of renal failure. One of the reasons is that injured or dying renal tubular cells accumulate cytosolic calcium, thus reducing blood levels [13]. Low serum levels of Fe in the AKI group can be explained by inflammatory mediators that provoke an imbalance in erythropoietin (EPO) production. EPO is a glycoprotein hormone produced by interstitial fibroblasts in the renal cortex. Lack of EPO production results in anemia in these animals, with a subsequent decrease in Fe levels [14].

In the urinary samples, P, Na, and Ca levels varied among the experimental groups, which make them useful as potential biomarkers. The kidneys help control the amount of P and Na in the body by cotransporters in the proximal tubular cells. As this portion of the kidney is affected in AKI, it is reasonable to conclude that this reduction in urinary levels of P and Na is a consequence of injury to the renal tubular cells [15, 16]. Hypercalciuria, or excessive urinary Ca excretion, is a consequence of renal tubular acidosis in AKI [16].

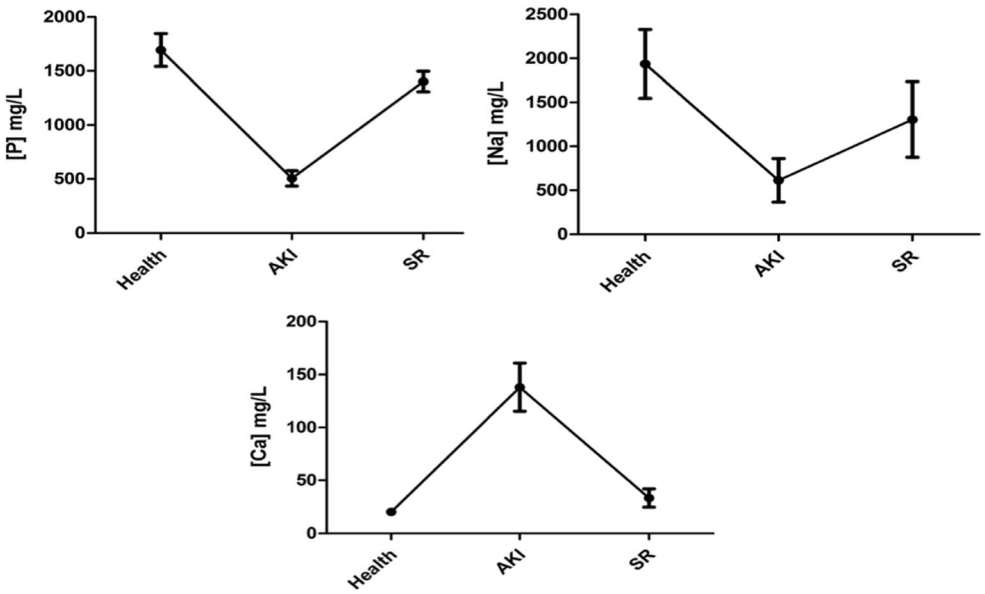

Fig. 3 Urinary concentrations of P, Na, and Ca for healthy, acute kidney injury (*AKI*), and spontaneous recovery (*SR*) groups. Results represent the mean ± SE ($n = 6$) of three experiments

The present study reveals differences in the concentrations of selected elements in serum and urine in AKI and SR stages. These findings indicate that some elements might be useful as biomarkers.

Conclusion

The research in this field could lead to a better understanding of the pathophysiology of AKI and result in valuable practical information that can be applied to clinical medicine.

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Compliance with Ethical Standards All procedures were performed according to the recommendations of the Research Ethics Committee of UNIFESP/SP (Project No. 9287290915/CEUA). Moreover, this committee oversaw all experimental animal procedures.

Conflict of Interest The authors declare that they have no conflicts of interest.

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