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Urinary Klotho measured by ELISA as an early biomarker of acute kidney injury in patients after cardiac surgery or coronary angiography

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ABSTRACT

Background. Acute kidney injury (AKI) is a common complication after cardiac surgery and percutaneous coronary interventions which markedly worsens prognosis. In recent years, new early biomarkers of AKI have been identified, but many important aspects still remain to be solved. Klotho is a pleiotropic protein that acts as a paracrine and endocrine factor in multiple organs. Reduced renal Klotho levels have been show in several animal models of AKI. No study has been published in which Klotho was tested in humans as an early marker of AKI. The aim of this work is to assess the usefulness of measuring urinary Klotho for the early diagnosis of AKI in patients with acute coronary syndrome or heart failure undergoing cardiac surgery or coronary angiography. *Methods.* Urinary Klotho was measured 12 hours after intervention in 60 patients admitted to the Intensive Care Unit with acute coronary syndrome or heart failure secondary to coronary or valvular conditions, who underwent coronary angiography (30 patients) or cardiac bypass surgery or heart valve replacement (30 patients). The primary endpoint used was the onset of AKI according to the RIFLE classification system. Human Klotho levels were measured using an ELISA assay.

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Results. We found no differences in urinary Klotho levels between AKI patients and those who did not develop AKI. Moreover, there was not significant correlation between urinary Klotho levels and the presence of AKI. *Conclusion*. Urinary Klotho measured by ELISA does not seem to be a good candidate to be used as an early biomarker of AKI.

Klotho urinario determinado por ELISA como biomarcador precoz de fracaso renal agudo en pacientes sometidos a cirugía cardiaca o angiografía coronaria

Palabras clave:
Fracaso Renal Agudo
Biomarcadores
Cirugía cardiaca
Angiografía coronaria
Klotho
ELISA

RESUMEN

Introducción y objetivos: El fracaso renal agudo (FRA) es una complicación frecuente tras la cirugía cardiaca y las intervenciones percutáneas coronarias cuya aparición empeora el pronóstico de manera marcada. En los últimos años se han identificado nuevos biomarcadores precoces de FRA, pero aún quedan muchos aspectos importantes por resolver. Klotho es una proteína pleiotrópica que actúa como un factor paracrino y endocrino en múltiples órganos. En diversos modelos animales de FRA se ha demostrado niveles disminuidos de Klotho renal. No se ha publicado ningún estudio en el que se haya probado Klotho como marcador precoz de FRA en humanos. El objetivo de este trabajo es investigar la utilidad de la determinación de Klotho en orina para el diagnóstico precoz del FRA en pacientes con síndrome coronario agudo o fallo cardiaco sometidos a cirugía cardiaca o angiografía coronaria.

Métodos: Se midió Klotho urinario 12 horas tras la intervención en 60 pacientes ingresados en la unidad de cuidados intensivos por síndrome coronario agudo o fallo cardiaco secundarios a enfermedad coronaria o valvular y a los que se realizó angiografía coronaria (30 pacientes) o cirugía cardiaca de recambio valvular o bypass (30 pacientes). El criterio de valoración primario fue la aparición de FRA según la clasificación RIFLE. Los niveles de Klotho humano se midieron utilizando un ensayo ELISA.

Resultados: No encontramos diferencias en los niveles de Klotho en orina entre los pacientes que desarrollaron FRA y aquellos que no. Además, no había correlación significativa entre niveles de klotho en orina y presencia de FRA.

Conclusión: Klotho urinario medido por ELISA no parece ser un buen candidato para ser usado como biomarcador precoz de FRA.

Introduction

Acute kidney injury (AKI) is a common complication following cardiac surgery and percutaneous coronary interventions, with an estimated incidence around 30% in the former[1-3] and between 5 and 20% in the latter[4-6]. The onset of AKI significantly worsens prognosis in these patients[2,3,7]. AKI increases mortality in different clinical contexts and, additionally, patients often develop chronic kidney disease (CKD) after AKI[8-9]. The mechanisms involved in AKI include both endogenous and exogenous toxins, metabolic factors, ischaemia and reperfusion, neurohumoral activation, inflammation and oxidative stress[10]. The diagnosis of AKI is based on detecting increased serum creatinine, which occurs late and does not adequately reflect the glomerular filtration in acute patients[11]. However, experimental studies show that AKI can only be prevented or treated by means of early interventions[12]. New early biomarkers of AKI, such as NGAL (Neutrophil Gelatinase-Associated Lipocaline), KIM-1 (Kidney Injury Molecule 1), L-FABP (Liver Fatty Acid-Binding Protein), Cystatin C or IL-18 (Interleukin 18), have been identified in recent years, but many important aspects remain to be solved in the search for the ideal biomarker[13]. The study of these molecules has also provided a greater understanding of AKI pathogenesis.

The Klotho gene was identified in 1997 as an anti-aging gene[14]. This gene is expressed in multiple tissues, the kidney being the organ where it is expressed more markedly,[15] especially in the distal tubule, but also in the proximal tubule[16] and the collecting duct[17,18]. Klotho is a transmembrane protein that acts as a co-receptor for fibroblast growth factor-23 (FGF-23)[19,20]. The ectodomain may be cut and released into the extracellular space by the proteases anchored to ADAM10 and ADAM17[21] membrane. This results in soluble Klotho,

which is present in blood, cerebrospinal fluid[22] and urine[23]. There is another soluble Klotho protein which is shorter, but its function is not well known[24]. Soluble Klotho is a pleiotropic protein that acts as a paracrine and endocrine hormonal factor, both in the kidney and in other organs[25]. In the renal tubule, Klotho modulates sodium-phosphate cotransporters[23], calcium channels[26] and potassium channels[27]. Finally, Klotho is also present at nuclear and cytoplasmic levels, where it functions as an anti-aging protein[28,29].

Klotho's role in aging and in phosphocalcic metabolism continues to become more understood[16,30,31]. Its implications in the progression of CKD and its non-renal complications have also been the focus of intense study in recent years[25,32-34], as well as the relationship existing among its circulating levels, glomerular filtration and CKD prognosis[35] or its potential use as an anti-fibrotic agent[36]. With respect to AKI, the presence of decreased Klotho levels has been shown in various animal models of AKI induced by ischaemia-reperfusion, ureteral obstruction or nephrotoxic agents[23,37-43]. In 2010, Hu et al.[23] measured urinary Klotho in 17 AKI patients and found decreased levels compared to the values obtained in 14 healthy volunteers. No other studies have been published about Klotho as an AKI biomarker in humans. The objective of this paper was to assess the usefulness of measuring urinary Klotho for the early detection of AKI in patients with acute coronary syndrome or heart failure undergoing cardiac surgery or coronary angiography.

Methods

Patients

Sixty patients were enrolled from a cohort of 193 patients admitted to the intensive care unit (ICU) of the Hospital Clínico Universitario de Valencia with acute coronary syndrome (ACS) or heart failure secondary to coronary or valvular disease. All patients had undergone coronary angiography with or without angioplasty or cardiac surgery. The coronary angiography group was made up of 30 patients: 18 had AKI after the intervention and 12 did not. Another 30 patients were also selected in the cardiac surgery group: 15 with AKI and 15 without AKI (Table 1).

The exclusion criteria were: age younger than 18 years; CKD on replacement therapy and AKI secondary to cardiogenic shock during hospitalisation. All patients were prospectively monitored since their enrolment in the study. Serum creatinine was measured from before the procedure up to six days after it, and the clinical progress of each patient was monitored until discharge. The first value of serum creatinine obtained on admission was used as the baseline creatinine value. Urine samples for Klotho measurement were collected 12 hours after the intervention and processed immediately thereafter. We also obtained urine samples from 10 healthy volunteers to determine the normal values. Additionally, the following information was collected from each patient: demographic variables and comorbidities, parameters of the surgical procedure and complications during or after interventions (Table 1). The primary endpoint was the onset of AKI, defined as an increase of creatinine of 50% or more based on the RIFLE (Risk, Injury, Failure, Loss, End-stage renal disease) classification[44]. This study was approved by the Hospital Clínico Universitario de Valencia Ethics Committee and it was conducted in compliance with the principles of the Declaration of Helsinki[45].

Processing of urine samples

Urine samples were centrifuged for 10 minutes at 1500 g immediately after being collected, and the supernatant was stored in 0.5-mL aliquots at –80°C for later use.

Measurement of urinary Klotho levels

Human Klotho levels were determined in urine, both in patients and in controls, using two commercial ELISA assays: one from Shanghai Sunred Biological Technology Co., Ltd,

Table 1 – Clinical and demographic characteristics of patients

	Non-AKI patients	AKI patients
Coronary angiography		
No. of patients	12	18
Age (years)	63 ± 15	72 ± 10
Sex (M/F)	8/4	14/4
Baseline eGFR (MDRD) (mL/min/1.73m^2)	78 ± 15	60 ± 18**
Baseline creatinine (mg/dL)	0.93 ± 0.23	1.16 ± 0.27*
Maximum creatinine (mg/dL)	0.94 ± 0.23	1.99 ± 0.61***
AKI (day)		4 ± 2
RIFLE (R/I/F)		12/5/1
Hospital stay (days)	13 ± 11	17 ± 13
Deaths	0	4
CARDIAC SURGERY		
No. of patients	15	15
Age (years)	68 ± 9	67 ± 15
Sex (M/F)	10/5	13/2
Baseline eGFR (MDRD) (mL/min/1.73m2)	60 ± 16	62 ± 29
Baseline Creatinine (mg/dL)	1.15 ± 0.37	1.18 ± 0.51
Maximum creatinine (mg/dL)	1.26 ± 0.35	2.14 ± 0.71***
AKI (day)		3 ± 1
RIFLE (R/I/F)		10/5/0
Hospital stay (days)	17 ± 10	27 ± 24
Deaths	0	4
Type of surgery		
Bypass	10	4
Valvular	4	10
Bypass and valvular	1	1
Time on ECC (min)	62 ± 19	98 ± 57*

AKI: Acute kidney injury; baseline eGFR (MDRD): glomerular filtration rate estimated by MDRD before the intervention; AKI (day): day of AKI diagnosis by serum creatinine; Time on ECC: time of extracorporeal circulation in minutes; Values are expressed as mean ± SD; Values from AKI patients that differ significantly from non-AKI patients are expressed with *p < 0.05; **p < 0.01; ***p < 0.001.

Table 2 – Urinary Klotho values using two commercial ELISA kits

Parameter	Control (n = 10)	Angiography		Cardiac surgery		Angiography + Cardiac surgery	
		Non-AKI (n = 12)	AKI (n = 18)	Non-AKI (n = 15)	AKI (n = 15)	Non-AKI (n = 27)	AKI (n = 33)
ELISA kit (Sun Red Biotechnologies)							
Klotho (ng/ml)	1.80 ± 0.04	1.87 ± 0.05	1.94 ± 0.05*	1.80 ± 0.08	1.97 ± 0.04*	1.85 ± 0.04	1.96 ± 0.03
Klotho (ng/mg creatinine)	1.37± 0.24	2.12 ± 0.38	2.40 ± 0.45	2.00 ± 0.23	2.51 ± 0.21**	2.04 ± 0.20	2.45 ± 0.26*
ELISA Kit (IBL International)							
Klotho (ng/ml)	0.77 ± 0.18	1.24 ± 0.34	1.51 ± 0.25	1.34 ± 0.36	1.25 ± 0.33	1.29 ± 0.25	1.38 ± 0.15
Klotho (ng/mg creatinine)	0.62 ± 0.05	1.23 ± 0.20	1.53 ± 0.30	1.25 ± 0.25	1.70 ± 0.30*	1.24 ± 0.30	1.60 ± 0.30

Values are expressed as mean ± SEM. The results are analysed by one-factor ANOVA with Bonferroni post-hoc analysis. Values from patients that are significantly different from the control group are indicated as: *$p < 0.05$; **$p < 0.01$; NON-AKI: Patients who did not develope acute kidney injury; AKI: Patients who developed acute kidney injury

with a sensitivity of 0.05 ng/mL and an assay range of 0.1–20 ng/mL, and the other assay from IBL International (Human soluble α-Klotho Assay Kit – IBL, Immuno-Biological Laboratories Co., Ltd), with a sensitivity of 0.006 ng/mL and an assay range of 0.093–6 ng/mL. The values obtained were adjusted to urine creatinine. Creatinine was measured in serum and urine using standard techniques.

Statistical analysis

We analysed our results with GraphPad PRISM (version 4.0) software. A Kolmogorov-Smirnov test was conducted to verify that the variables followed a normal distribution. For the comparison of means with more than two variables, a one-factor ANOVA was performed with a post-hoc Bonferroni analysis, and for the comparison of two means, a Student's or Mann-Whitney test was used in case of non-normal distribution. The correlations between the presence of AKI and urinary Klotho levels were conducted by Spearman's correlation. The correlations between the Klotho levels measured with the two ELISA assays, and between the Klotho level (ng/mg creatinine) and the serum delta creatinine (maximum serum creatinine – baseline creatinine), were conducted by Pearson's bivariate correlation, with the SPSS vs. 19 analysis program. The significance level was set at $p < 0.05$.

Results

Patients characteristics

The clinical and demographic characteristics of patients are shown in Table 1. The diagnosis of AKI using creatinine required 3 ± 1 days in cardiac surgery patients and 4 ± 2 days in angiography patients. With respect to mortality, eight patients died: four from the cardiac surgery group and the other four from the angiography group, all of whom had developed AKI. AKI patients had a mean hospital stay longer than patients who did not develop AKI in both groups of patients, although the differences were non-significant. In the angiography group, pre-intervention serum creatinine was significantly higher ($p < 0.05$) in AKI patients than in non-AKI patients, whereas the baseline estimated glomerular filtration rate (eGFR) was significantly lower in AKI patients ($p < 0.01$). No significant differences were found in these values in the group of patients with cardiac surgery (Table 1). In this group, the patients who developed AKI had been on extracorporeal circulation (ECC) for a longer period ($p < 0.05$) than patients who did not develop AKI (Table 1).

Urinary Klotho values

Urinary Klotho protein levels were significantly elevated in AKI patients both in the angiography group and the cardiac surgery group, when compared to the control group ($p < 0.05$) (Table 2). When correcting Klotho concentrations based on urine creatinine levels, only the AKI patients from the surgery group differed from the controls ($p < 0.01$), but there were no significant differences with respect to non-AKI patients (Table 2). If the patients from both groups are considered together, urinary Klotho levels (corrected for creatinine) were significantly higher in AKI patients than in healthy controls ($p < 0.05$), but there were no significant differences compared to non-AKI patients (Table 2). A correlation was not found between Klotho levels and the presence of AKI ($r = 0.182$, $p = 0.67$) when the Spearman's test was conducted. Additionally, we examined whether there was a correlation between the Klotho level (ng/mg of urine creatinine) and delta serum creatinine (maximum serum creatinine – baseline creatinine) for both ELISA assays. No significant correlations were found when the patients from both groups were considered together or separately, regardless of the assay used.

There was no significant correlation between urinary Klotho levels (ng/mL) measured by the two ELISA assays ($r = -0.079$; $p = 0.614$) or after correction based on urine creatinine ($r = -0.043$; $p = 0.792$).

Discussion

In this study, we assessed the usefulness of measuring Klotho protein in urine samples by ELISA for the early (12 hours post-intervention) detection of AKI in a group of patients with acute coronary syndrome or heart failure who had undergone cardiac surgery or coronary angiography, either with or without angioplasty or stenting. We found no significant differences in Klotho levels between the patients who developed AKI and those who did not. We also found a small but significant increase in urinary Klotho levels in AKI patients following cardiac surgery compared to healthy control subjects. The Klotho protein was measured in urine with two different ELISA kits for human Klotho.

In the literature, there are no other studies testing urinary Klotho as an early biomarker of AKI in humans. There is only one study[23] in which urinary Klotho was measured in 17 AKI patients by immunoblotting using anti-Klotho antibodies, and the authors reported decreased Klotho in AKI patients compared to 14 healthy controls, although the urine samples were collected at a late stage of renal failure progression. It has been shown in animal models that Klotho is underexpressed in the renal tissue in AKI secondary to ischaemia-reperfusion, ureteral obstruction, sepsis or nephrotoxic agents[23,37-43], and that the overexpression of the transmembrane protein or the administration of soluble protein has a protective effect against the renal damage induced by ischaemia and reperfusion[23,28] or nephrotoxins[42]. In 2010, Hu et al.[23] measured Klotho expression in renal tissue as well as the levels of Klotho protein in plasma and urine in an animal model of ischaemia/reperfusion-induced AKI and found that Klotho was decreased at all levels.

We had already studied the usefulness of different biomarkers as early predictors of AKI in this cohort of patients and had shown that NGAL[46,47] was a strong predictor. Based on the evidence obtained in animal models of AKI, we expected to find decreased levels of Klotho protein in the urine of patients with AKI. There are several explanations for these dissimilar results. Firstly, all the experiments have been conducted in rats and mice. It is obvious that animal experiment conditions are not comparable to those in a clinical context and that the results obtained cannot be directly extrapolated. Moreover, it is possible that Klotho protein does not behave in the same manner in humans as in animal models. Another possible explanation lies in sample collection time. Hu et al.[23] found decreased Klotho levels in urine one day after ischaemia-reperfusion, but in our study the samples were collected 12 hours after the intervention. They also measured urinary Klotho in 17 patients with AKI and found that the levels were much lower than those obtained from healthy volunteers. However, the population in their study was very heterogeneous (including prerenal AKI and sepsis-induced AKI, lupus, obstruction, liver failure, transplantation, uremic haemolytic syndrome and pregnancy) and data about the sample collection time were not provided. Notwithstanding, when the samples were collected, serum creatinine was 3.76 ± 0.58 mg/dL, so they were collected very late. It is also possible that urinary Klotho levels do not reflect Klotho expression well at renal tissue level. We know that the circulating levels do not relate to the expression in renal tissue in CKD. Data from animal experimentation clearly indicate the existence of Klotho deficit at renal level in CKD[48]. However, the measurement of Klotho protein in plasma provides dissimilar results. Hu et al.[48] found very low Klotho levels in renal tissue, plasma and urine in mice with CKD, whereas Sugiura et al.[49] found elevated levels in plasma from patients with CKD. Devaraj et al.[50] reported decreased levels in diabetic patients and high levels in CKD patients and, furthermore, those values correlated with plasma creatinine. They hypothesised that the Klotho protein could be synthesised at an extrarenal level to provide renal protection by anti-oxidant and anti-inflammatory mechanisms. Nevertheless, Seiler et al.[35] measured plasma Klotho levels in a cohort of 312 patients with Stage 2–4 CKD and did not find a correlation with glomerular filtration. Plasma Klotho levels did not correlate with renal function and did not predict progression in patients with CKD. It is possible that, in patients with AKI, the Klotho protein is also being synthesised at the extrarenal level to provide renal protection and that it is appearing in the urine due to the glomerular filtration. Hu et al.[23] and Moreno et al.[40] found decreased Klotho levels in plasma from animal models of AKI. So far, no study has measured Klotho in plasma from humans with AKI. Even if Klotho levels were not increased in plasma, it is possible that it could be synthesised at the extrarenal level and pass into urine. Therefore, plasma or urine levels may not reflect what is going on at tissue level. Further studies would be required to clarify this issue.

Obviously, another possible explanation for the results is the existence of methodological differences. To obtain more robust results, two different human ELISA kits from different manufacturers were used to measure urinary Klotho levels, but no differences were found in patients with or without acute kidney injury using either kit. The little consistency between both assays is also notable. It is possible that different assays recognise different parts of the molecule and that some assays recognise the entire molecule while others recognise fragments. Heijboer et al.[51] assessed three different ELISA assays, including the IBL one, and found major quality differences among them, IBL being clearly superior to the other two evaluated assays.

Conclusion

Despite the evidence in animal experimentation that Klotho is underexpressed in the kidney after either toxic or ischaemic AKI, in this study we did not find any correlation between the presence of AKI and urinary Klotho levels 12 hours after the intervention in a group of patients admitted to the ICU for acute coronary syndrome or heart failure who had undergone cardiac surgery or coronary angiography, either with or without angioplasty or stenting. Although these results need to be confirmed, urinary Klotho measured by ELISA does not seem to be a good candidate to be used as an early biomarker of AKI.

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