Neutrophil Gelatinase Associated Lipocalin, a new biomarker candidate in perfusate of machine perfused kidneys: a porcine pilot experiment

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Abstract

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The enduring donor shortage has led to the increasing use of expanded criteria donors and kidneys donated after cardiac death, triggering the revival of machine perfusion preservation. Indeed, machine perfusion does not only preserve these kidneys better than static cold storage, it also has the potential to evaluate them. The presence of certain biomarkers, among them aspartate aminotransferase (AST) and heart-type fatty acid binding protein (H-FABP), has been demonstrated in the perfusate of human kidneys. This potentially makes them useful as biomarkers of graft quality. Neutrophil gelatinase associated lipocalin (NGAL) is another biomarker candidate. NGAL is believed to be released upon injury of renal tubular cells. However, because it is also released from neutrophils, it is currently unclear whether NGAL is a direct or indirect, inflammatory-mediated marker of kidney injury. To resolve this issue we set up a pilot experiment in which we studied the concentrations of AST, H-FABP, and NGAL in the perfusate of six porcine kidneys that were exposed to incremental periods of warm ischemia and subsequently machine perfused for 22 hours. An *ex vivo* porcine model was chosen because preclinical large animal work remains necessary to refine machine perfusion technology and the presence of these markers in perfusate of porcine kidneys has not been shown previously. All three biomarkers were detectable in the cold acellular perfusate and their release seemed proportional to the degree of warm injury endured, albeit this has to be confirmed in a larger sample population. In conclusion, NGAL is directly released by ischemically damaged kidneys, independent of neutrophil activation. In addition to NGAL, the determination of AST and H-FABP in perfusate of machine perfused porcine kidneys is also feasible. As such the determination of these markers can be added to the arsenal of research tools available in preclinical preservation research.

Introduction

Kidney grafts are inevitably exposed to a certain period of warm and cold ischemia, leading to ischemic acute kidney failure associated with tubular cell injury.¹ This cellular injury can translate into early graft failure, clinically observable as primary non-function or delayed graft function. Especially expanded criteria donor kidneys and kidneys donated after cardiac death are at high risk to develop this type of early graft failure.²⁻³ Nevertheless, the enduring donor shortage has led to an increased use of these kidneys, emphasizing the importance to accurately assess kidney graft quality prior to transplantation. It would be advantageous if we could assess the risk of early graft failure by measuring biomarkers of cellular injury to determine the degree of tubular cell injury in the graft. Neutrophil gelatinase-associated lipocalin (NGAL) is a new candidate biomarker of kidney injury because it is highly accumulated in the proximal tubule during acute tubular injury.⁴⁻⁶

In this light, it is particularly interesting that the change in donor profile has led to a revival of machine perfusion (MP) preservation. Indeed, MP does not only reduce delayed graft function and graft loss⁷⁻⁹, it also holds the promise to serve as a quality assessment tool because perfusion dynamics and perfusate biomarkers can be monitored during preservation.¹⁰ NGAL is currently under investigation as a biomarker of kidney injury in urine and plasma.⁴ However, because NGAL is also released by activated neutrophils, it remains unclear whether NGAL directly reflects cellular injury or rather the inflammation that concurs with injury.⁶ Additionally, its potential as a perfusate biomarker has not yet been established.

Although certain markers of cellular injury, among them aspartate aminotransferase (AST) and heart-type fatty acid binding protein (H-FABP), have already been investigated in the perfusate of human kidneys¹¹⁻¹², preclinical preservation research to further fine tune the MP technology and its use as a quality assessment tool remains necessary. Because of the anatomical and physiological resemblances between porcine and human kidneys¹³, this type of research is mostly performed in porcine models.¹⁴⁻¹⁹

To explore whether NGAL could serve as a new biomarker of cellular injury in perfusate of a machine perfused kidney and to study the feasibility of measuring biomarkers in an *ex vivo* porcine model, we set up a pilot experiment in which AST, H-FABP, and NGAL were determined in the perfusate of machine perfused porcine kidneys.

Materials and Methods

Experimental set-up

Female pigs (TOPIGS 20, Lelystad, The Netherlands), 30-50 kg, were anaesthetized as previously described.²⁰ Kidneys were procured either immediately (n=2) or after exposure to 1 hour (n=2) or 3 hours (n=2) of warm ischemia induced by clamping of the renal artery and vein. After procurement, kidneys were flushed with 500 mL of histidine-tryptophan-ketoglutarate and machine perfused on a LifePort Kidney Transporter (Organ Recovery Systems, Zaventem, Belgium) with Belzer's machine perfusion solution, available as KPS-1 (Organ Recovery Systems, Zaventem, Belgium). Experiments were performed according to international guidelines²¹ and the study protocol was approved by the Animal Care Committee of the KU Leuven, Belgium.

Perfusate collection

Perfusate samples were collected at the following time points: 30 minutes after the start of MP, hourly until 6 hours of MP and at 22 hours of MP. The perfusate was aliquoted in 1 mL microtubes after centrifugation (3000 rpm, 10 min) and stored at -25°C until analysis.

Biomarker determination

We measured perfusate concentrations of AST, H-FABP, and NGAL. AST was determined in the central lab of the University Hospitals Leuven (IFCC method, detection limit 4 U/L, Hitachi/Roche Modular P, Roche Diagnostics, Belgium). H-FABP was measured by ELISA according to manufacturer's instructions (dilution 1/2, H-FABP ELISA HK403, detection limit 391 pg/mL, Hycult Biotech, Uden, The Netherlands). NGAL was determined by ELISA according to manufacturer's instructions (dilution 1/25, NGAL ELISA KIT 044, detection limit 1 pg/mL, Bioporto, Gentofte, Denmark). Values below the

limit of detection were replaced with a value equal to the detection limit divided by 2. A post-hoc analysis of the data showed that <15% of data were below the detection limit. Perfusate biomarker concentrations were corrected for kidney weight.

Statistical methods

Continuous data are presented as mean $\pm$ standard deviation. Logarithmic curves were fitted to the data in each experimental group, R and R² are given to reflect the strength of the relationship between observed and predicted values. SPSS 16.0 for Windows was used for data-analysis.

Results

AST, H-FABP, and NGAL could be detected in the acellular perfusate of machine perfused kidneys. The biomarker concentration increased with time during MP (Fig. 1). The course of the concentration increase could be fairly well described by a logarithmic curve fitting as reflected by the R and R² values in Fig. 1. Biomarker concentrations tended to be higher in warm ischemically damaged kidneys (Fig. 1).

Discussion

In this pilot experiment, we showed that AST, H-FABP, and NGAL can be measured in cold, acellular perfusate of porcine kidneys. The presence of these markers of cellular injury in the perfusate of an isolated perfused kidney corroborates that they are in fact released by the kidney cells. Indeed, none of these three biomarkers are specific to the kidney. AST is a large cytosolic enzyme (>150 kDa) present in most cells, but is mainly used to assess the extent of liver injury. H-FABP is a 15 kDa cytoplasmic fatty acid binding protein expressed in distal tubular cells but is also abundantly present in myocardial cells.²² NGAL is a 25 kDa protein that is highly accumulated in the proximal tubule during acute tubular injury⁵⁻⁶ and its synthesis is markedly up-regulated within hours after ischemia reperfusion injury.²³ However, the main source of circulating NGAL are activated neutrophils.⁶ The

presence of NGAL in the acellular and therefore neutrophil-free perfusate supports the hypothesis that circulating NGAL in kidney transplant recipients might also – partly – reflect cellular kidney injury.²⁴

Warm ischemia induces cellular injury that is reflected by the release of markers of cellular injury. Our data seem to indicate that the biomarker concentrations of kidneys exposed to warm ischemia are higher compared to the concentrations observed in kidneys that were not exposed to warm ischemia. This observation – which needs confirmation in a larger population – is in agreement with reports showing that higher H-FABP concentrations were found in perfusate of discarded kidneys and of uncontrolled DCD kidneys (Maastricht category II) compared to controlled DCD kidneys (Maastricht category III).^{12, 25} In prospectively collected perfusate samples, H-FABP also proved to be an independent predictor of delayed graft function.¹¹

Additionally, the release of these markers in a porcine model of MP-preservation research had not been shown previously. The similarities between human and porcine kidneys¹³ make it tempting to assume that markers determined in the perfusate of human kidneys will also be present in the perfusate of porcine kidneys. However, inter-species differences do exist and therefore confirmation was a prerequisite before this assessment tool can be used in preclinical porcine preservation research.

In conclusion, the presence of NGAL in the perfusate of machine perfused kidneys suggests that NGAL is indeed released from injured kidney cells, independent of neutrophil activation. In addition, the determination of AST and H-FABP in perfusate of machine perfused porcine kidneys is also feasible, adding this to the arsenal of research tools available in preclinical preservation research.

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Figure Legend

Figure 1: Course of AST, H-FABP, and NGAL in the perfusate of individual porcine kidneys. A logarithmic curve was fitted through the biomarker concentrations of kidneys exposed to no warm ischemia (n=2), 1 hour of warm ischemia (n=2), 3 hours of warm ischemia (n=2). R and R² for each curve fitting are given.

WI: warm ischemia

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