Circulating AST, H-FABP, and NGAL are early and accurate biomarkers of graft injury and dysfunction in a preclinical model of kidney transplantation

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Sources of support

Supported (in part) by unrestricted grants from Astellas and Roche, Belgium.

Histidine-tryptophan-ketoglutarate preservation solution was provided by Köhler Chemie, Alsbach-Hanhlein, Germany; University of Wisconsin solution was provided by Organ Recovery Systems, Zaventem, Belgium as SPS-1® Static Preservation Solution.

IJ is a doctoral fellow associated with the Research Foundation-Flanders, Belgium.

JP and DM are holders of a chair Abdominal Transplant Surgery from the Centrale Afdeling voor Fractionering, Vilvoorde, Belgium.

Running title: Early kidney graft injury biomarkers

Mini-Abstract

No validated biomarkers quantifying kidney graft injury exist. This study showed that plasma aspartate transaminase, heart-fatty acid binding protein and neutrophil gelatinase-associated lipocalin were earlier, more accurate markers of initial graft injury compared to creatinine (clearance), histology, and urinary biomarkers in a clinically relevant kidney transplant model.

Abstract

Objective: To investigate circulating biomarkers of initial graft injury in a porcine kidney autotransplant model.

Background: Injury endured by kidney grafts early post-transplant determines their outcome. However, creatinine (clearance) is a poor surrogate of tissue injury and urinary biomarkers are limited by graft-anuria or persistent native kidney diuresis. No validated circulating biomarkers quantifying initial graft injury exist.

Methods: Minimally injured porcine kidney grafts (n=6) were cold stored (18h) and autotransplanted. Moderately (n=6) and severely injured grafts (n=7) were exposed to 30' or 60' warm ischemia prior to storage and autotransplantation. Four biomarkers [aspartate transaminase (AST), heart-type fatty acid binding protein (H-FABP), neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl- β -glucosaminidase (NAG)] were measured post-transplant and compared with creatinine (clearance) and histology.

Results: Diuresis was delayed in moderately [2.5d (2-3)] and severely [4d (4-5)] vs. minimally injured grafts ($p<0.001$). Creatinine peaked later than AST, H-FABP, and NGAL [4d (3-5) vs. 3h (3-6), 6h (6-24), 2d (1-3), respectively] and only differentiated minimally from severely injured grafts. Peak AST, H-FABP distinguished all injury grades. NGAL discriminated initial graft injury 2d post-transplant. Peak AST, H-FABP, and NGAL correlated with peak creatinine [Pearson's coefficients: 0.70 ($p=0.001$), 0.85 ($p<0.0001$), 0.80 ($p<0.0001$)]. NAG was not different. Decreased clearance accounted for a small percentage of H-FABP, NGAL increase. Histology was not different among transplanted groups.

Conclusion: Plasma AST, H-FABP and NGAL reflect the severity of initial kidney graft injury and predict graft dysfunction earlier and more accurately than creatinine (clearance) and histology. They represent promising tools to improve patient care after kidney transplantation.

Introduction

Each step of the kidney transplant process contributes to parenchymal graft injury, culminating early after revascularization due to ischemia-reperfusion injury.¹ Clinically, initial graft injury manifests as delayed graft function (DGF), ranging from mild and short-lasting to severe and more prolonged, or occasionally to irreversible primary non-function (PNF). Parenchymal injury creates an inflammatory environment in which the graft becomes more prone to acute and chronic rejection.¹⁻² Additionally, tissue injury contributes to the later development of tubular atrophy/interstitial fibrosis and graft loss.^{1,3} Markers of initial graft injury are commonly used in solid organ transplantation (transaminase in liver,⁴⁻⁶ troponin in heart,⁷⁻⁸ amylase in pancreas transplantation⁹⁻¹⁰). They provide early, reliable information on the severity of graft injury and on its functional outcome, assisting clinicians in recipient management. Surprisingly, no markers quantifying the severity of kidney graft injury are available. Currently, initial kidney graft injury is estimated indirectly and at a late stage by assessing graft (dys)function. However, the accepted definition of DGF – need for dialysis within the first week post-transplant – is rudimentary and subjective.¹¹ DGF is diagnosed several days after the initiating injury and does not identify duration or reversibility of the dysfunction. Even standard functional markers - creatinine (clearance) - are unreliable indicators of kidney function during an episode of acute injury. Creatinine changes are not specific for parenchymal damage and occur only late after the event; creatinine clearance can remain stable in case of quite severe injury.¹²⁻¹³ Histopathology, the standard to detect tissue injury, is invasive, prone to sample error, and associated with inter-observer variability. Moreover, there is always a delay between the actual injury and its morphological expression.¹⁴ Urinary markers of kidney injury have been described.¹⁴⁻¹⁵ Although they are undoubtedly valuable tools, their use in transplant recipients is limited because of possible transient graft anuria and/or the persistent urine production by the native kidneys. However, tubular proteins released in the renal filtrate could become detectable in plasma, possibly due to protein back-leak into the circulation in case of tubular injury.¹⁶⁻¹⁷ Aspartate transaminase (AST), heart-type fatty acid binding protein (H-FABP), neutrophil gelatinase-associated lipocalin (NGAL), and N-acetyl- β -glucosaminidase (NAG) are proteins released upon cellular damage. AST and NAG are present in renal tubular cells, H-FABP in distal tubular cells, and NGAL accumulates in proximal tubules during ischemic injury.¹⁸⁻²¹

The aim of this study was to 1) explore the value of these plasma biomarkers to detect initial kidney graft injury, 2) determine whether they reflect injury severity, 3) assess whether they detect injury

earlier than standard functional parameters, and 4) establish whether they correlate with functional outcome. For this purpose a porcine model of kidney transplantation was developed in which various degrees of initial graft injury were evoked in a controllable and reproducible way.

Materials and Methods

Porcine kidney autotransplantation

Female pigs (TOPIGS-20, Lelystad, the Netherlands), 35-40kg, randomly underwent kidney autotransplantation or uninephrectomy (n=6). Minimally injured grafts (n=6) were procured, flushed with histidine-tryptophan-ketoglutarate and cold stored in University of Wisconsin solution for 18h and transplanted in the same animal after contralateral nephrectomy. The renal artery and vein were clamped in moderately (30') and severely (60') injured grafts prior to procurement. Anticoagulants were not used. As previously described, mean arterial (>85mmHg) and central venous (>10mmHg) pressures were maintained by administering colloids when necessary and all animals received a standard regimen of crystalloids and analgesics.²² A bladder catheter was surgically inserted to monitor diuresis. Animals were sacrificed 10d post-transplant. Experiments were performed according to international guidelines after approval by the Animal Care Committee (KULeuven, Belgium).²³

Sample collection

Venous blood was collected in EDTA tubes at baseline, early after intervention (15', 1h, 3h, 6h), and daily thereafter. Grafts were rinsed immediately prior to transplantation (100mL NaCl 0.9%). Six hour urine collections were performed on day 1d, 3d, 6d, 8d, and 10d after the intervention. 1 mL aliquots of plasma (centrifuged 3000 rpm, 10'), rinse and urine were stored at -25°C until analysis.

Functional parameters

Kidney function was assessed by the start of diuresis (urine output $\geq$ 40mL/day; monitored daily), plasma creatinine and creatinine clearance (urine volume x urinary creatinine)/(plasma creatinine x time urine collection)²⁴. Creatinine was determined at the hospital's central lab (kinetic Jaffé method, Hitachi/Roche Modular P). Plasma creatinine was corrected for animal weight. Baseline creatinine clearance of healthy animals was determined previously as described above.

Histopathology

Biopsies (14G) from the deep cortex-outer medulla were taken at baseline, after warm and cold ischemia, and after reperfusion (45', 24h, and 10d), fixed (formalin 6%), paraffin-embedded and evaluated after periodic acid-Schiff staining. Six morphological features were blindly assessed by an experienced pathologist: brush border integrity, tubular cell aspect, tubular nuclei changes, intratubular detachment, interstitial edema, and interstitial inflammatory infiltrate. These features were semiquantitatively scored: (0) no, (1) mild, (2) moderate, and (3) severe morphological changes.

Biomarker determination

AST was determined in the central lab (IFCC method, limit of detection (LOD) 4 IU/L, Hitachi/Roche Modular P). ELISA was used for H-FABP (HK403, LOD 391pg/mL, Hycult Biotech, Uden, the Netherlands) and NGAL (KIT 044, LOD 1pg/mL, Bioporto, Gentofte, Denmark). Enzymatic activity of NAG was determined by spectrophotometrically (405nm) measuring p-nitrophenol formed from adding p-nitrophenyl-N-acetyl- β -D-glucosaminide (10mM, Sigma, Bornem, Belgium) to diluted (1/10) samples and citrate buffer (100mM, pH 5) at 37°C, calculating NAG concentrations using a standard curve (LOD 0.13 IU/L). Values below LOD were replaced by LOD/2 to allow statistical analysis. Post-hoc data analysis showed that <15% of H-FABP values were below LOD. Urinary AST could not be measured in 80% of cases; NAG in urine was not detectable. The total amount of plasma biomarker detected in the first 48h following intervention was estimated by calculating areas under the curve (AUC). Plasma biomarker concentrations were corrected for kidney weight. Biomarker amount in urine was calculated from timed collections (biomarker concentration x urine volume x 24/time of collection).¹⁶

Statistical methods

Continuous variables [median (inter quartile range)] were compared with ANOVA or Kruskal-Wallis-ANOVA in case of non-normal distribution or violation of Levene's test. Tukey or Mann-Whitney U test with Bonferonni correction were used for post-hoc analysis. Differences in dependent data were evaluated with Wilcoxon matched-pairs test. Pearson's coefficients assessed correlations between biomarker and creatinine peak values. Analyses were performed using STATISTICA-9 (StatSoft Europe, Hamburg, Germany). The contribution of a sudden decrease in renal biomarker clearance to

plasma biomarker increase was estimated by correcting the AUC of the first 48h post-transplant for the AUC of the uninephrectomized animals during the same period and expressing the contribution of decreased biomarker clearance as percentage of the graft AUC. AUC's were calculated in SlideWrite Plus 7.0 (Advances Graphics Software, Rancho Santa Fe, CA, USA). P-values <0.05 were considered significant.

Results

Operative data

Variables known to influence graft function were comparable in transplanted animals (Table 1). Perfusion pressures in uninephrectomized animals were similar. Two animals were sacrificed before the end of the observation period [high gastro-intestinal bleeding (5d), bleeding due to a damaged arterial line (6d)]. Both received a severely injured graft that had regained function before sacrifice.

Graft function: diuresis, creatinine clearance, creatinine

Graft function recovered in all animals. Diuresis was immediate in minimally injured grafts, contrary to moderately and severely injured grafts in which diuresis was delayed by 2.5d (2-3) and 4d (4-5), respectively ($p<0.001$) (Fig. 1), confirming the biological difference of the transplanted groups, induced by increasing warm ischemia.

Creatinine clearance was lower in transplanted versus uninephrectomized animals (Fig. 2A). Changes in creatinine clearance were reflected by the bell-shaped creatinine course in grafts with creatinine peaking 4d (3-5) post-transplant (Fig. 2B). Although creatinine was higher in transplanted versus uninephrectomized animals at all time points, creatinine could only distinguish degrees of initial graft injury beyond the fourth day post-transplant (Fig. 2B). As expected, uninephrectomized animals experienced a sudden decrease in creatinine clearance compared to healthy animals 24h after nephrectomy ($p=0.01$), but creatinine clearance recovered after 6d. This sudden decrease in creatinine clearance was also reflected by a less pronounced creatinine rise compared with baseline values ($p<0.05$ at all time points).

Histopathology

Graft histology (Table 2) showed increasing signs of tubular injury over time with a maximum 45' and 24h after reperfusion. By the end of follow-up, there were clear signs of tissue recovery. However, histology was not different between the transplanted groups.

Initial graft injury assessment by plasma biomarkers

1) Plasma AST, H-FABP, NGAL, and NAG evolution

Plasma AST of moderately and severely injured grafts increased immediately after reperfusion and returned to baseline values 48h post-transplant (Fig. 3A). A similar course was present in minimally injured grafts, only here AST initially decreased the first hour post-transplantation. Plasma H-FABP increased immediately after reperfusion and returned to normal values after 2d, 5d, and 6d post-transplant in animals receiving a minimally, moderately or severely injured graft, respectively (Fig. 3B). Plasma NGAL increased immediately post-transplant and remained higher throughout the follow-up period compared to baseline (Fig. 3C). Plasma NAG increased 3h and 1d post-reperfusion and returned to normal values 6d post-transplant in minimally and severely injured grafts, respectively (Fig. 3D).

2) Plasma AST, H-FABP, and NGAL reflect severity of initial graft injury

Plasma AST differed between the groups of injured grafts from 1h until 6h after reperfusion (Fig. 3A), plasma H-FABP between 1h and 6d (Fig. 3B), and plasma NGAL between 2d and 9d (Fig. 3C). In contrast, plasma NAG was not different between minimally and severely injured grafts (Fig. 3D), therefore NAG in moderately injured grafts was not determined.

3) Plasma AST, H-FABP, and NGAL changes precede standard functional parameter changes

Creatinine peaked much later than plasma AST, H-FABP, and NGAL (4d (3-5) versus 3h (3-6), 6h (6-24), and 2d (1-3), respectively). At its peak value, creatinine could only differentiate minimally from severely injured grafts (Fig. 4A). In contrast, peak plasma AST and H-FABP differed between minimally, moderately and severely injured grafts (Fig. 4B-C). Although plasma NGAL rose with increasing initial graft injury between 2d and 9d post-transplantation, the NGAL peak itself could not distinguish the degrees of initial graft injury (Fig. 4D).

4) Plasma AST, H-FABP, and NGAL correlate with functional parameters

Peak plasma AST, H-FABP, and NGAL strongly correlated with peak creatinine (Pearson's coefficients of 0.70 ($p=0.001$), 0.85 ($p<0.0001$), 0.80 ($p<0.0001$), respectively).

Renal biomarker clearance

Because H-FABP and NGAL are freely filtered by the glomeruli,²⁰⁻²¹ the possibility that a decreased renal clearance would lead to an accumulation of H-FABP and NGAL needed to be explored. The contribution of a sudden decrease in renal clearance, triggered by a uninephrectomy, on the increase of plasma H-FABP and NGAL in the first 48h was investigated. Decreased biomarker clearance – expressed as percentage of the graft biomarker AUC – had a minor contribution to post-transplant plasma increase in minimally, moderately and severely injured grafts for H-FABP (26%, 11%, and 7%, respectively) and NGAL (41%, 33%, and 26%, respectively) (Fig. 3B-C).

Initial graft injury assessment by urinary biomarkers

80% of urinary AST values were below the LOD. Although urinary H-FABP was higher on the first day post-transplant compared to uninephrectomized controls, this difference was not significant (21 μ g (10-42), 115 μ g (55-169), $p=0.06$). Urinary NGAL and NAG did not differ between minimally injured grafts and uninephrectomy controls ($p=0.09$, $p=1.00$, respectively). Although peak urinary H-FABP and NGAL were higher in transplanted animals compared to uninephrectomy controls ($p=0.04$, $p=0.01$, respectively), the peak values did not differ among recipients of minimally, moderately, or severely injured grafts. Peak urinary NAG was not different in transplanted animals compared to uninephrectomized animals. Furthermore, the contribution of urinary biomarkers to the early detection of initial graft injury was hampered by temporary anuria.

Graft injury assessment prior to transplantation

AST, H-FABP and NGAL in the rinse did not differ between minimally, moderately and severely injured grafts ($p=0.38$, $p=0.93$, $p=0.47$, respectively). NAG was not detectable in the rinse.

Discussion

In a clinically relevant porcine kidney transplant model, plasma AST, H-FABP and NGAL documented the severity of initial graft injury more accurately than standard functional parameters - diuresis, creatinine (clearance) - and histology.

The identification of a 'troponin-like' biomarker of kidney injury has mainly focused on urinary markers. As recent work suggests, urinary biomarkers can predict DGF and are increased in kidneys donated after cardio-circulatory death.^{21,25-26} Although they are undoubtedly valuable tools their limitations are well illustrated in the current study where recipients of badly injured grafts remained anuric for several days. Moreover, even after the start of diuresis, no association was found between urinary markers and the severity of initial graft injury. In fact, plasma AST and H-FABP peaks provided the earliest time window to detect the severity of initial graft injury, while still strongly correlating with the creatinine peak that followed several days later, showing that they can also be used as early predictors of graft (dys)function. Because of the paramount importance of initial graft injury in early and late graft outcome,^{1,3,27} the early detection of graft injury post-transplant is essential to allow a more objective, quantifiable and reproducible diagnosis of injury and function than provided by the rudimentary surrogate DGF.

Clearly these observations from a well controlled experiment using healthy pigs as donors and recipients will have to be examined in a clinical setting where multiple variables influence production and clearance of biomarkers.

A potentially limiting factor in the use of AST, H-FABP, and NGAL as direct markers of graft injury is that they are not exclusively present in tubular cells. AST is present in most nucleated cells and is primarily used as liver injury marker. H-FABP is mainly expressed by cardiomyocytes and circulating NGAL is largely derived from activated neutrophils.²⁸⁻²⁹ However, liver and cardiomyocyte injury tests (alkaline phosphatase, bilirubin and troponin I) remained normal early post-transplant, excluding a liver or cardiac origin of AST and H-FABP (data not shown). Furthermore, release of H-FABP in the perfusate of machine-perfused kidneys demonstrates that H-FABP can be released by renal parenchymal cells.³⁰⁻³¹ Likewise, in different experiments we detected a progressive accumulation of not only H-FABP, but also AST and NGAL in the perfusate of isolated *ex vivo* machine-perfused

porcine kidneys (submitted). The early and rapid increase of NGAL after reperfusion followed by the late NGAL peak in the present study might reflect both an early NGAL release from cellular damage and a later increase reflecting tissue repair. It has indeed been suggested that an increase in NGAL levels in response to harmful stimuli acts as a defensive mechanism and might be important in the process of re-epithelialisation.^{29,32-33} Nevertheless, other sources of NGAL release such as activated neutrophils cannot be ruled out. In that light, it is interesting to note that immunohistochemistry has clearly shown the presence of NGAL in tubular cells of deceased donor kidneys the first hour after ischemic injury,³⁴ suggesting that neutrophil activation – on its own – cannot account for all the circulating NGAL detected in the experiments. The observation that NGAL can still be detected 10 days post-transplant by immunostaining, whereas no NGAL was present in baseline biopsies, strengthens this hypothesis (data not shown).

Furthermore, transplant candidates suffer from end-stage renal failure. In contrast to AST (and NAG) whose molecular weight (>150kDa) precludes filtration by the glomeruli, H-FABP (15kDa) and NGAL (25kDa) are freely filtered and could therefore accumulate during renal failure.^{28,35} Indeed, H-FABP and NGAL baseline levels seem to be considerably higher in end-stage renal failure compared with a normal population.³⁶⁻³⁷ Additionally, NGAL in renal disease could also be released by activated neutrophils involved in the disease process, directly from diseased tubular cells, or a combination of both.^{29,38} Nevertheless, although H-FABP and NGAL increased slightly after uninephrectomy in the present study, the biomarker release from parenchymal injury was confirmed by the minor contribution of reduced renal clearance to the post-transplant biomarker increase. Therefore, in the clinical setting high biomarker levels pre-transplant could be expected to increase or stagnate in case of substantial graft injury, and decrease in case of minimal graft injury and early diuresis, as already suggested for NGAL.³⁸

In addition, hemodialysis in transplant candidates and patients experiencing DGF can potentially complicate the translation of the reported preclinical observations. Data on biomarker clearance by hemodialysis are scarce, but it seems they can be cleared by dialysis under specific conditions.^{36,39} Although the rationale of biomarker detection is diagnosis of graft injury prior to DGF and the need for dialysis, the possible effect of hemodialysis on the concentration of these markers will need to be taken into account in clinical trials.

The discrimination of DGF from PNF, the most severe and non-reversible form of DGF, would be of great value. The diagnosis of PNF is made retrospectively, when graft function does not return within 3 months post-transplant, obviously impeding the development of a PNF-model because of the prolonged follow-up and complicated challenge to dialyze pigs. The present study does not address the biomarker value in discriminating DGF and PNF, however one could speculate that the release of biomarkers would be most pronounced in PNF where the highest degree of injury is expected. However, preliminary data from a PNF-like model, currently under development at the KU Leuven, shows that although biomarkers are released in high concentrations, no-reflow phenomena and graft vascularization need to be taken into account and could lead to an underestimation of the sustained injury by an incomplete release of biomarker into the circulation. In all likelihood, this crucial question will have to be answered in a clinical trial.

In that light, the quantification and prediction of graft injury – and ideally PNF – prior to transplantation would aid the acceptance process and biomarker data could be interesting. However, the biomarker concentration in the rinse immediately prior to transplant was not associated with graft injury and function. Furthermore, perfusate H-FABP has recently been assessed as a predictor of DGF in a large clinical trial of kidneys preserved by hypothermic machine perfusion.³⁰ Although H-FABP correlated with post-transplant function, it was not able to predict graft outcome with enough certainty to allow kidney discard based upon perfusate H-FABP concentrations. Since the full effects of ischemia-reperfusion injury become obvious only after restoration of circulation, it is not unthinkable that graft viability assessment needs normothermic preservation in which injury markers would be of particular interest.

Noteworthy is mentioning that all biomarker values were corrected for kidney weight. Indeed, as these markers are - in part - released by tubular cells, their maximal concentration is determined by the tubular mass. This implies that kidneys will have to be weighed before implantation, a procedure which, albeit easy to implement, is not a standard practice.

In conclusion, plasma AST, H-FABP, and NGAL represent new diagnostic tools to evaluate initial kidney graft injury and to predict subsequent function. Given the role of initial graft injury in the early

and late post-transplant outcome, the measurement of these parameters could provide relevant information. The clinical value of these biomarkers merits assessment in everyday practice.

Acknowledgements

We would like to thank Veerle Heedfeld and Tine Wylin for their invaluable contributions to this work. We are grateful to C. Van Boven (Tramedico, St. Niklaas, Belgium) and Dr. E. Schaffner (Köhler Chemie, Alsbach-Hanhlein) for providing histidine-tryptophan-ketoglutarate solution; Organ Recovery Systems for providing SPS-1® Static Preservation Solution.

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Tables

Table 1: Operative data of animals undergoing kidney autotransplantation.

Variable	Minimally	Moderately	Severely	
Number	6	injured grafts 6	7	p-value
CIT (h)	18.2 (17.9-18.2)	18 (17.2-18.2)	18.1 (17.8-18.2)	0.59
Donor procedure time (min)	132 (125-145)	142 (138-165)	164 (141-172)	0.09
Recipient procedure time (min)	173 (159-192)	187 (173-195)	190 (179-210)	0.17
Anastomotic time (min)	41 (37-45)	42 (35-51)	39 (39-55)	0.61
CVP at reperfusion (mmHg)	13 (12-14)	14 (12-15)	13 (11-15)	0.85
MAP at reperfusion (mmHg)	112 (89-114)	104 (94-115)	91 (88-132)	0.86

Values are presented as median (inter quartile range)

CIT: cold ischemic time

CVP: central venous pressure

MAP: mean arterial pressure

Minimally injured grafts were only exposed to cold storage; moderately and severely injured grafts were exposed to warm ischemia prior to cold storage by clamping the renal artery and vein for 30' or 60', respectively.

Table 2: Histological assessment of the kidney in uninephrectomized and transplanted animals.

Variable	Uninephrectomy	Minimally injured grafts	Moderately injured grafts	Severely injured grafts	p-value
Number	6	6	6	7	
Baseline	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	1
After WI	-	-	0 (0-0)	1 (0-1)	1
After CI	-	0 (0-1)	2.5 (1-3)	3 (0-5)	0.16
45' after uninephrectomy/transplantation	0 (0-1)	9 (7-9)	9.5 (9-11)	9 (8-10)	0.48
24h after uninephrectomy/transplantation	0.5 (0-1)	9 (7-9)	9.5 (9-11)	9 (8-10)	0.48
End of follow-up	1 (0-2)	2 (0-5)	4 (0-6)	4 (1-6)	0.75

Values are presented as median (inter quartile range).

Six morphological features (brush border integrity, aspect of the tubular cells, changes to the tubular nuclei, intratubular detachment, interstitial edema, and interstitial inflammatory infiltrate) were assessed for morphological changes on periodic acid-Schiff stained sections according to a semiquantitative score: (0) no abnormality, (1) mild, (2) moderate, and (3) severe morphological changes.

p-value denotes the comparison between the transplanted groups

WI: warm ischemia induced by clamping of the renal artery and vein

CI: cold ischemia

Figures

Figure 1: Dot plot representing the start of urine production post-transplantation according to severity of the induced initial graft injury.

Kruskal-Wallis $p=0.0003$; * $p=0.002$; # $p=0.001$; § $p=0.002$

Figure 2: Evolution of kidney function over time assessed by creatinine clearance (panel A) and by plasma creatinine (panel B). Data are presented as mean with standard deviation. Transplantation/uninephrectomy was performed at Time 0. Plasma creatinine concentrations are corrected for animal weight.

* $p<0.05$ for comparison between the transplanted groups; § $p<0.05$ for comparison between all 4 groups.

Figure 3: Evolution of plasma biomarker concentrations over time.

Data are presented as mean with standard deviation. Transplantation / uninephrectomy was performed at Time 0. Concentrations H-FABP, and NGAL in uninephrectomized animals were measured until 48h after the procedure to determine the contribution of a sudden decrease of plasma biomarker clearance to the total amount of biomarker detected in the plasma. Biomarker concentrations are corrected for kidney weight. Creatinine lagging behind the biomarker concentrations is illustrated by the creatinine course of severely injured grafts.

* $p<0.05$ for comparison between the transplanted groups

Figure 4: Box plot of plasma biomarker concentrations at their peak.

Biomarker concentration peaks were detected at following time points:

Creatinine peak at 4d (3-5) (panel A), AST peak 3h (3-6) (panel B), H-FABP peak at 6h (6-24) (panel C), NGAL peak at 2d (1-3) (panel D).

Biomarker concentrations are corrected for kidney weight, creatinine values are corrected for animal weight.

* $p<0.01$ for minimally vs. severely injured grafts; # $p<0.05$ for minimally vs. moderately injured grafts;

° $p<0.05$ for moderately vs. severely injured grafts

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