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Performance-enhancing strategies for deceased donor kidneys

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Low-dose ARA290 treatment of brain dead donors does not improve renal function in an isolated perfused kidney model



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Abstract

Introduction

Erythropoietin (EPO) and carbamoylated EPO improve renal function following brain death donation. ARA290 is a drug of the latest generation of non-erythropoietic EPO derivatives. ARA290 exclusively binds to the receptor complex (EPOR₂-βCR₂) consisting of two EPO-receptors (EPOR) and two β common receptors (βCR). This receptor complex is responsible for EPO mediated cytoprotection. We investigated the protective capacities of early- and late ARA290 treatment of brain dead donors in an isolated perfused kidney model. Secondly, the effect of ARA290 addition to the perfusion solution on renal function was tested.

Material & Methods

Brain death was induced by inflating an intracranial balloon in male Lewis/HanHsd rats (n=36). ARA290 (1 nmol/kg) was administered after 30- or 120 minutes of brain death. Saline was used as vehicle treatment. Kidneys were removed after four hours of brain death. Right kidneys were used for qRT-PCR analyses, while the function of the left kidneys was measured in the isolated perfused kidney model. ARA290 was also added to the perfusion solution of one vehicle treated group. Expression of inflammatory- and acute kidney injury markers was measured by qRT-PCR analysis.

Results

Early- or late ARA290 donor treatment or addition of ARA290 to the perfusion solution did not improve glomerular filtration rate. Markers of acute kidney injury or inflammation were not affected by ARA290 treatment.

Discussion

ARA290 treatment of brain dead donors did not improve renal function or protect against brain death related injury. This also applies to the addition of ARA290 to the perfusion solution. Presumably, a dose of 1 nmol/kg ARA290 was not sufficient to induce renoprotection.

Introduction

Graft survival of brain death donor (DBD) kidneys is inferior compared to living donor kidneys. Also, the incidence of primary non function (PNF) and delayed graft function (DGF) is distinctly higher after DBD kidney transplantation compared to living donor donation¹. Since brain death donors represent over 50% of all kidney donors, strategies to improve the outcome of DBD kidney transplantation is of great importance².

The process of brain death causes systemic hemodynamic- and hormonal instability in the donor, resulting in ischemia/reperfusion (I/R) injury of donor organs³⁻⁵. Also, a systemic inflammatory response in donor organs has been observed after brain death⁶⁻⁹. These processes ultimately compromise short- and long-term outcome of renal transplantation. In this study, we applied cytoprotective treatment of the donor to increase the quality of the donor organ.

Originally, erythropoietin (EPO) is known as regulator of erythropoiesis¹⁰. Interestingly, EPO also has a endogenous protective function¹¹. In response to acute injury, EPO is locally up-regulated¹¹. This makes EPO mediated cytoprotection an interesting option for therapeutic intervention aiming at the reduction of brain death related injury. EPO mediated cytoprotection is not induced by binding of EPO to the classical EPO receptor complex (EPOR₂). To induce cytoprotection, EPO binds to a complex consisting of two EPOR and two β common receptors (EPOR₂- β CR₂)¹². The binding affinity of EPO to this protective receptor complex is approximately 80 times lower compared to binding of EPO to the EPOR₂ complex¹¹⁻¹³. Thus, considerably higher systemic doses are required to activate EPO mediated cytoprotection compared to the doses required for stimulation of erythropoiesis. This creates a major drawback for protective EPO treatment, as the risk of cardiovascular adverse effects will be increased by activation of erythropoiesis and thrombocytes¹⁴.

The protective capacities of EPO have been shown in various models of acute kidney injury^{11,15-18}. Treatment before, but also after induction of injury, improved renal function^{15,18}. Based on these pre-clinical studies, EPO treatment has been tested in four clinical trials¹⁹⁻²². Recipients of deceased donor kidneys were treated with EPO to improve short-term function and thereby reduce delayed graft function. Unfortunately, protection by EPO was not observed, while the risk of cardiovascular side effects already increased²⁰.

To prevent cardiovascular adverse effects while retaining the protective effects of EPO, non-erythropoietic EPO derivatives have been developed. Carbamoylated EPO (CEPO) was the first generation non-erythropoietic EPO derivatives and its protective capacities have been shown in several models of acute kidney injury^{12,23-26}. Besides, CEPO also improved renal function following brain death donation²⁷. The latest generation of non-erythropoietic EPO derivatives is ARA290.

ARA290 is derived from the binding site of EPO to the EPOR₂-βCR₂ complex. It has only cytoprotective capacities and it does not affect erythropoiesis. ARA290 differs from CEPO in regard to its half-life, which is only several minutes compared to the approximately 6 hours half-life of CEPO²⁸.

In this study, we aimed to determine the renoprotective capacities of ARA290 treatment of brain death donors. ARA290 was administered to the brain donors either 30- or 120 minutes following induction of brain death. Renal function was studied during 90 minutes perfusion in an isolated perfused kidney (IPK) model. Expression of inflammatory- and acute kidney injury markers was measured after donation. Secondly, protection by addition of ARA290 to the renal perfusion solution was tested, as it might improve preservation of donor organs.

Materials & Methods

Animals

Thirty-six male Lewis/HanHsd rats (Harlan, Horst, the Netherlands, 250–300 gr.) were used for this study. They were housed individually with free access to water and rat chow. The animal experiments were approved by the animal ethics committee of the university of Groningen (DEC-RuG, 4179B, Groningen, the Netherlands). The experiments were performed according to international and Dutch guidelines of animal research. Eight animals were excluded due to complications during brain death.

ARA290

ARA290 (ARAIM Pharmaceuticals, Ossining, USA) is a small synthetic peptide consisting of eleven amino acids. It has been derived from the binding site of EPO to the protective EPOR₂-βcR₂ complex and it does not bind to the classical EPOR₂ complex. The plasma half-life is approximately two minutes.

Study design

The animals were randomized into four groups, receiving an iv injection with either saline or ARA290 at 30- or 120 minutes after induction of brain death (Table 1). After four hours of brain death both kidneys were retrieved. The right kidney was used for qRT-PCR analyses of inflammation and acute kidney injury. The renal function of left kidneys was tested in an isolated perfused kidney (IPK) model for 90 minutes²⁹. As vehicle treatment was expected not to affect renal injury or –function, ARA290 was added to the perfusion solution of the ‘early – vehicle’ group. This means that for IPK analyses the ‘late – vehicle’ group served as control for all groups. The used concentration ARA290 was 1 nmol/kg (1 nmol/kg = 1.258 ug/kg) and saline (0.9%) served as vehicle treatment. Dosage of ARA290 was chosen based on earlier renal I/R experiments.

Table 1 - Study design

Group	Treatment	Perfusion solution	Number of animals
Early – Vehicle	Saline	WME + ARA290	8
Early – ARA290	1 nmol/kg ARA290	WME	7
Late – Vehicle	Saline	WME	7
Late – ARA290	1 nmol/kg ARA290	WME	6

Brain death procedure

Brain death was induced according to earlier published protocols. Animals were anesthetized with 4% isoflurane. Arterial pressure was monitored via a femoral catheter. A tracheostomy was performed to intubate the rats. In this way animals were ventilated during brain death. After drilling a frontolateral hole, a 4-Fogarty catheter (Edwards Lifesciences, Irvine, CA) was inserted subdurally. Induction of brain death was started by slowly inflating the balloon (16 μ l/min) with saline using a pump (Terufision, Termo, Tokyo, Japan).

A marked increase in arterial pressure indicated the start of brain death and inflating of the balloon was halted. An apnea test and absence of central reflexes confirmed brain death and anesthesia was stopped. If mean arterial pressure dropped below 80 mmHg, animals were treated with hydroxyethyl starch 10% (HAES, Fresenius Kabi AG, Bad Homburg, Germany). After four hours of brain death, donors were heparinized with 500 IU heparin. Blood and urine were collected and kidneys were removed after flushing with saline. The left kidney was cannulated to determine renal function in the IPK model.

Isolated perfused kidney model

The cannulated left kidney was continuously perfused using warmed, oxygenated Williams' medium E (WME) enriched with creatinine (0.8 g/L) for 90 minutes. After starting perfusion, pressure was gradually increased to 100 mmHg, regulated by a roller pump (Ismatec mv-ca/04; Ismatec, Glattbrugg, Switzerland). Each 15 minutes, urine and perfusate samples were collected. Glomerular Filtration Rate (GFR) was calculated as (urinary creatinine (mmol) * urine volume (ml)) / (time (min) * plasma creatinine (mmol)).

Samples

Blood and urine samples were stored at -80°C. Cortical and medullary samples were snap frozen separately in liquid N₂ and stored at -80°C for qRT-PCR. Clinical parameters were measured by the laboratory center of the University Medical Center Groningen.

Real-time reverse transcription polymerase chain reaction (qRT-PCR)

RNA was extracted from snap frozen tissue using Trizol reagent according to the manufacturer's instructions (Invitrogen, Breda, the Netherlands). Total RNA was treated with DNase I to remove genomic DNA contamination (Invitrogen, Breda, the Netherlands). The integrity of total RNA was analysed by gel electrophoresis. cDNA was synthesised from 1- μ g total RNA using M-MLV (Moloney murine leukaemia virus) Reverse Transcriptase and oligo-dT primers (Invitrogen, Breda, The Netherlands).

Primer sets were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Amplification and detection were performed with the ABI Prism 7900-HT Sequence Detection System (Applied Biosystems) using emission from SYBR green master mix (Applied Biosystems). The PCR reactions were performed in triplicate. After an initial activation step at 50 °C for 2 min and a hot start at 95 °C for 10 min, PCR cycles consisted of 40 cycles at 95 °C for 15 sec and 60 °C for 60 sec. Dissociation curve analysis were performed for each reaction to ensure amplification of specific products.

Genes and primers are shown in table 2. Gene expression was normalized with the mean of β -actin mRNA content and calculated relative to controls or contralateral kidneys. Results were finally expressed as $2^{-\Delta CT}$ (CT threshold cycle), which is an index of the relative amount of mRNA expression in each tissue.

Table 2 - qRT-PCR primers

Gene	Forward	Reverse	Amplicon length (bp)
IL-6	CCAACCTCCAATGCTCTCCTAATG	TTCAAGTGCTTTCAAGAGTTGGAT	89
Kim-1	AGAGAGAGCAGGACACAGGCTTT	ACCCGTGGTAGTCCCAAACA	75
E-selectin	GTCTGCGATGCTGCCTACTTG	CTGCCACAGAAAGTGCCACTAC	73
ICAM-1	CCAGACCCTGGAGATGGAGAA	AAGCGTCGTTGTGATCCTCC	251
β-actin	GGAAATCGTGCGTGACATAAA	GCGGCAGTGGCCATCTC	74

Urinary markers of acute kidney injury

The activity of alanine aminopeptidase (AAP) was detected with the modified enzymatic assay using alanine-p-nitroanilide as substrate (Sigma-Aldrich, Zwijndrecht, the Netherlands). N-acetyl-b-D-glucosaminidase (NAG) was measured by a modified enzyme assay at pH 4.25 using p-nitrophenyl-N-acetyl-b-D-glucosaminide as substrate (Sigma-Aldrich, Zwijndrecht, the Netherlands). AAP and NAG excretion were corrected for urinary creatinine output.

Statistical analyses

All data are presented as median \pm interquartile range. Man Whitney U tests or Kruskal-Wallis H tests with Dunn's multiple comparison post-hoc analyses were used to compare treatment groups to controls. A $p < 0.05$ was considered significant.

Results

Induction of brain death by inflation of the intracranial balloon lasted approximately 30 minutes. Start of brain death was characterized by a consistent increase of mean arterial pressure. Brain death donors required 5 [3-8] ml HAES to maintain mean arterial pressure above 80 mmHg. No differences in fluid administration were observed between treatment groups and controls.

Effect on clinical parameters

At the end of four hours brain death, early- or late donor treatment with ARA290 did not affect plasma creatinine levels (Figure 1A). Also, LDH and ASAT levels did not differ between early- or late treatment and controls (Figure 1B and 1C).

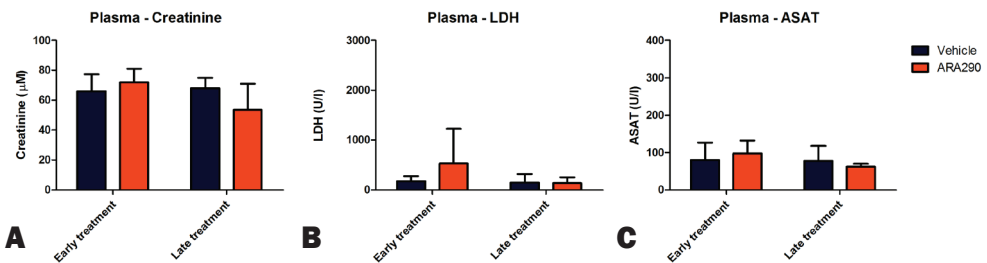


Figure 1 – Effect of ARA290 on renal function. Early or late ARA290 treatment of brain death donors did not affect plasma levels of creatinine (A), LDH (B) or ASAT (C).

Renal function, defined as GFR in the isolated perfused kidney model, was not significantly affected by ARA290 donor treatment compared to controls (Figure 2A and 2B). However, late treatment at 120 minutes after start of brain death, tended to improve renal function after 30 minutes perfusion (Figure 2A). No influence on GFR by addition of ARA290 to the perfusion solution was observed compared to controls.

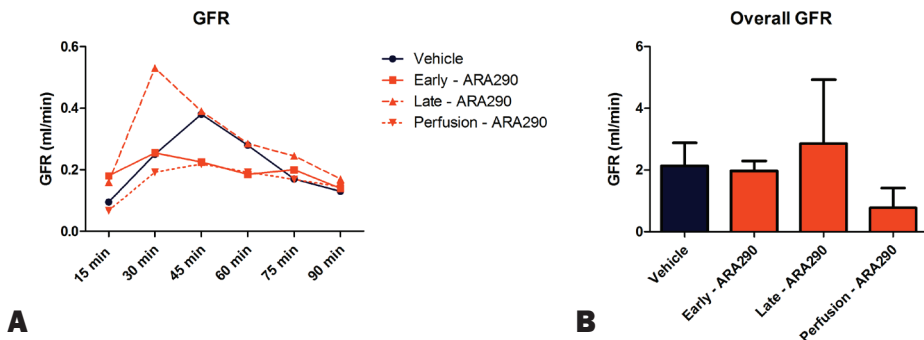


Figure 2 – Effect of ARA290 on renal function. Figure A represents precise GFR during isolated perfusion, while figure B shows overall GFR of the 90 minutes perfusion period. No significant differences were observed between treatment groups and controls.

Effect on acute kidney injury

Urinary excretion of AAP and NAG have been measured in the isolated perfused kidney model. AAP and NAG are brush border proteins and these molecule are excreted in acute kidney injury. Early- or late ARA290 donor treatment or addition of ARA290 to the perfusion solution did not reduce excretion of urinary AAP or NAG excretion (Figure 3A and 3B).

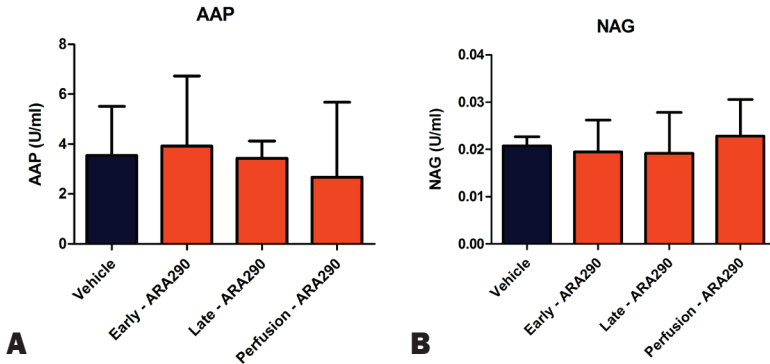


Figure 3 – Effect of ARA290 urinary AAP and NAG excretion. AAP (A) or NAG (B) were not affected by ARA290 donor treatment or addition to perfusion solution.

Kidney injury molecule-1 (Kim-1) is a cortical marker of acute kidney injury. ARA290 donor treatment or addition to perfusion solution did not result in reduced Kim-1 protein expression.

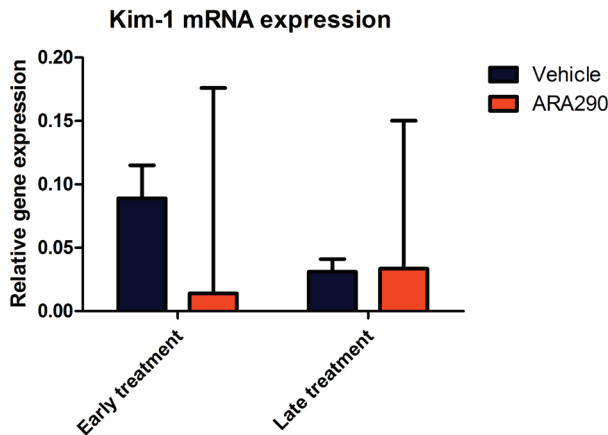


Figure 4 – Effect of ARA290 Kim-1 mRNA expression. ARA290 treatment did not reduce Kim-1 mRNA expression as marker of acute kidney injury.

Effect on renal inflammation

The effect of ARA290 on three mRNA markers of inflammation has been measured. After four hours of brain, death interleukin-6 (IL-6) mRNA levels, a general marker of inflammation, were not affected by early- or late ARA290 donor treatment (Figure 5A). Renal, endothelial inflammation was represented by mRNA levels of endothelial leukocyte adhesion molecule-1 (E-selectin) and intercellular adhesion molecule-1 (ICAM-1). After 4 hours brain death early- or late ARA290 donor treatment did not reduce mRNA levels of E-selectin or ICAM-1 compared to controls (Figure 5B and 5C).

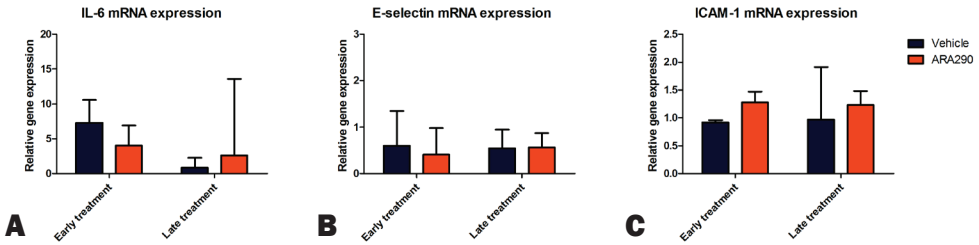


Figure 5 – Effect of renal inflammation. Early- or late ARA290 donor treatment did not affect renal inflammation.

Discussion

This study shows that early- or late ARA290 donor treatment did not improve renal function in an IPK model. Furthermore, ARA290 treatment of brain dead donors did not reduce inflammation or acute kidney injury. Addition of ARA290 to the perfusion solution did not reveal performance-enhancing capacities.

Our present results are in contrast to recent observations of Nijboer et al. who showed that brain death donor treatment with EPO and CEPO improves renal function as measured in the isolated perfused kidney model²⁷. Furthermore, in that study, renal expression of inflammatory genes and infiltration of polymorphonuclear cells were more effectively reduced by CEPO compared to EPO treatment²⁷. Surprisingly, in our study, ARA290, the newest generation non-erythropoietic EPO derivatives, did not show such renoprotective effects.

The ineffective results of this study may be explained by the used dose of ARA290 (1nmol/kg = 1.3 µg/kg), which was chosen based on renal I/R experiments in mice. In that study, doses of 1 µg/kg and 10 µg/kg protected against renal I/R injury²⁸. However, possibly the dose of 1 nmol/kg was too low to induce renoprotection in our brain death donor model in rats. EPO mediated cytoprotection is induced by binding to the EPOR2-βCR2 complex¹². Due to a lower binding affinity, this receptor complex requires higher systemic doses compared to stimulation of erythropoiesis. ARA290 is derived from the binding site of the EPOR₂-βCR₂ complex and does not bind the classical, erythropoietic EPOR2 complex²⁸. Therefore, ARA290 could be titrated to renoprotective doses without increasing risk of cardiovascular events.

Next to donor treatment, also perfusion solutions have to be optimized, especially since normothermic perfusion is of increasing interest³⁰⁻³³. Contemporary perfusion solutions, like UW, have been developed for cold storage. However, normothermic perfusion requires additional substances since the renal metabolic rate will be higher. Addition of EPO derivatives to perfusion solutions could be particularly interesting for normothermic machine perfusion, as the protective effects of EPO and EPO derivatives have been shown in several I/R models. During perfusion, EPO treatment might activate anti-inflammatory pathways and enhance renal function due to increased phosphorylation of endothelial nitric oxide synthase^{11,34}. However, in literature, no studies have been performed testing the protective capacities of EPO addition to perfusion solutions. We tested the renoprotective capacities of ARA290 addition to WME and in this isolated perfused kidney model, kidneys were normothermically perfused. However, no protective effect by addition of ARA290 to WME was observed.

In conclusion, we showed that early- or late low-dose ARA290 treatment of brain dead donors did not result in renoprotection in an isolated perfused kidney model. We propose to perform this experiment again with higher doses of ARA290, as EPO mediated renoprotection in brain death donation by EPO and CEPO donor treatment has been demonstrated in a former study²⁷.

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