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Performance-enhancing strategies for deceased donor kidneys

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Erythropoietin mediated protection in kidney transplantation: Non-erythropoietic EPO derivatives improve function without increasing the risk of cardiovascular events

Transplant International

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Abstract

The protective, non-erythropoietic effects of erythropoietin (EPO) have become evident in pre-clinical models in renal ischaemia/reperfusion injury and kidney transplantation. However, four recently published clinical trials using high dose EPO treatment following renal transplantation did not reveal any protective effect for short-term renal function and even reported an increased risk of thrombosis.

This review focusses on the current status of protective pathways mediated by EPO, the safety concerns using high EPO dosage and discusses the discrepancies between pre-clinical- and clinical studies. The protective effects are mediated by binding of EPO to a heteromeric receptor complex consisting of two β -common receptors and two EPO receptors. An important role for activation of endothelial nitric oxide synthase is proposed.

EPO mediated cytoprotection still has enormous potential. However, only non-erythropoietic EPO derivatives may induce protection without increasing the risk of cardiovascular events. In pre-clinical models non-erythropoietic EPO derivatives, such as carbamoylated EPO and ARA290, have been tested. These EPO derivatives improve renal function and do not affect erythropoiesis.

Therefore, non-erythropoietic EPO derivatives may be able to render EPO mediated cytoprotection useful and beneficial for clinical transplantation.

Transplantation of deceased donor kidneys

Delayed graft function (DGF) and primary non function (PNF) are serious complications of renal transplantation. Overall, DGF is associated with a 41% increased risk of graft loss and a 38% increased risk of rejection¹. In Europe, deceased donor kidneys represent 73% of all transplanted kidneys in 2011². Thus, improvement of short- and long-term function of transplanted deceased donor kidneys is an important focus in transplantation research.

Renal ischaemia/reperfusion (I/R) injury is a significant cause of reduced short-term function after transplantation. Deceased organ donation can be divided into two types of donation: organs donated after brain death (DBD) and after circulatory death (DCD). Short-term function of kidneys is significantly more compromised in DCD than in DBD derived kidneys. The incidence of both DGF and PNF is 72% and 23% after DCD compared to 18% and 4% after DBD, respectively³. The increased incidence of PNF results in reduced long-term graft survival of DCD kidneys³.

Despite many important achievements in transplantation such as improved surgical techniques, better treatment of complications and a profound reduction in kidney rejection, overall graft survival has only marginally increased^{4,5}. This phenomenon is probably in part due to the current Achilles' heel in transplantation: the use of large numbers of older and high risk donor organs that have suffered from substantial I/R injury. As we suspect that future donor resources will not return to the ideal and young organ donor of the past but merely focus on marginal DCD donors, better insight in pathways of injury and repair are mandatory. Prevention and protection in high risk donor organs against ischaemic injury will be necessary to maintain and hopefully enhance the results in kidney transplantation.

A promising strategy to protect against renal I/R injury is EPO mediated cytoprotection. However, recent clinical trials did not reveal protective capacities of high dose EPO treatment following renal transplantation. In this review we will outline the renoprotective mechanism of EPO and discuss the disadvantages of high dose EPO treatment in renal transplantation. Non-erythropoietic EPO derivatives could translate EPO mediated cytoprotection to the transplantation clinic without increased risk of cardiovascular adverse events.

Cytoprotective pathway of EPO

Erythropoietin (EPO) has pleiotropic actions. Besides stimulation of erythropoiesis, EPO also has a local tissue protective function⁶⁻⁸. In numerous models of renal I/R injury use of EPO has been shown to have protective effects. EPO improved renal function and reduced inflammation, apoptosis and structural damage⁹⁻¹⁵. EPO treatment pre-ischemia, as well as treatment post-reperfusion can be cytoprotective. Protective systemic EPO doses range from 300 IU/kg to 5000 IU/kg⁹⁻¹⁵. However, a dose of 5000 IU/kg EPO appears superior in improvement of renal function after renal I/R compared to a dose of 300 IU/kg^{12,14}. No studies have been performed to compare different EPO doses following renal I/R.

Maio et al. confirmed the protective capacities against renal I/R injury in a DCD transplantation model¹⁶. Due to its 'non-erythropoietic' and cytoprotective capacities EPO became an interesting agent reducing I/R injury and improving short- and long-term function after transplantation.

EPO was first discovered for its regulatory capacities of erythropoiesis. It induces proliferation and prevents apoptosis of erythroïd progenitor cells via binding to a receptor complex consisting of two EPO receptors (EPOR₂)⁸. However, in the past two decades EPO appeared to have additional distinctive cytoprotective capacities. It plays an endogenous role in limiting local inflammation and tissue damage. These cytoprotective effects are not mediated by binding of EPO to the classic EPOR₂ complex, but by binding to a tissue protective receptor complex^{17,18}. Immunoprecipitation studies showed that the EPOR is able to form a heteromeric receptor complex (EPOR₂-βCR₂) with the β common receptor (βCR)¹⁷. Binding of EPO to this receptor complex is suggested to induce the cytoprotective pathway of EPO¹⁷. In neuronal tissue, I/R injury results in up-regulation of EPOR expression starting directly after reperfusion. However, as increased EPO expression is delayed by several hours, a window of intervention is created¹⁹. Renal I/R causes up regulation of the EPOR₂-βCR₂ complex in renal tissue²⁰. The distribution of cytoprotective receptor complex in renal tissue is not known due to a lack of reliable immunohistochemical antibodies. The binding affinity of the classic EPOR₂ complex for EPO is 1-10 pmol/L, while the affinity of the EPOR₂-βCR₂ complex for EPO is 2-20 nmol/L^{21,22}. This means that significant higher doses of EPO are required to induce cytoprotection compared to stimulation of erythropoiesis.

Tissue protective signalling cascades have been described in various in vitro and in vivo models. As to the classic erythropoietic EPOR₂ complex, binding of EPO to the EPOR₂-βCR₂ complex causes phosphorylation of janus activated kinase-2 (JAK2)²³. This results in activation of two main signalling cascades: signal transducer and activator of transcription-5 (STAT5) and phosphatidylinositol 3-kinase/AKT (PI3K/AKT). These signalling pathways induce regeneration²¹, inhibit apoptosis and inhibit inflammation²¹.

PI3K/AKT is also able to increase regional blood flow by increasing endothelial nitric oxide synthase (eNOS) activity²⁴. In various renal I/R models the protective effects of EPO have been tested. EPO is able to increase phosphorylation of protective pathways as JAK2, PI3K/AKT and eNOS following renal I/R^{14,23,25}. It has been widely shown that EPO, administered pre- as well as post-reperfusion, is able to attenuate renal I/R injury⁹⁻¹⁶. Besides improvement of renal function, EPO also has anti-inflammatory and anti-apoptotic capacities. EPO reduces expression of important inflammatory markers as IL-6 and TNF- α ^{10,11}. Apoptosis and necrosis following renal I/R are reduced by EPO resulting in improved renal morphology^{10,12,26}. Structurally, EPO also decreased activity of TGF- β indicative of reduced development of fibrosis²⁷.

Endothelial nitric oxide synthase

Nitric oxide synthase activity is a physiologic regulator of renal function and determinant of glomerular haemodynamics²⁸. The direct effect of EPO on renal function can be explained by increased activity of eNOS^{9,15}. Following renal I/R injury, eNOS phosphorylation is reduced at six hours post-reperfusion and subsequently normalized after 24 hours²⁹. The direct enhancing effect of EPO on renal function is presumably the effect of increased eNOS activity. This suggests increasing eNOS phosphorylation by high-dose EPO treatment is most effective in the first six hours after reperfusion.

Growing evidence points to the important role of endothelial stimulation by protective EPO treatment. As knock-out of the EPOR is lethal due to an inefficient erythropoiesis, a transgenic EPOR knock-out has been developed in which the EPOR is only expressed in haemopoietic and vascular endothelial cells³⁰. Models of cardiac ischaemia or traumatic brain injury showed that EPO is still protective in these transgenic EPOR knock-out mice^{31,32}. However, knock-out of eNOS diminishes the protective effect of EPO^{24,31}. These studies show the dependence of eNOS enhancement and an important role of endothelial stimulation by EPO. The β CR is integrative in endothelial EPO signalling as it is essential for enhanced phosphorylation of protective signalling cascades like JAK2, AKT and eNOS in bovine aortic endothelial cells³³. In addition, to enhance PI3K/AKT, EPO may also increase eNOS phosphorylation due to an increased AMP-activated protein kinase (AMPK) activity. This regulator of energy metabolism is integrated in EPO signalling via the β CR and inhibition of AMPK reduced eNOS phosphorylation³⁴.

Recently, a new interaction between the β CR and the vascular endothelial growth factor receptor-2 (VEGFR2) has been described by Sautina et al. NO induction by EPO depends on β CR as well as VEGFR2³⁵. This finding underlines the importance of endothelial stimulation for immediate improvement of renal function and supports that EPO is able to preserve density of peritubular capillaries following renal I/R injury³⁶. Affinity of the interaction between β CR and VEGFR₂ for EPO has not been investigated yet.

In several *in vitro* studies, the role of the β CR in cytoprotection by EPO has been shown to be essential^{17,35,37}. In a model of spinal cord injury in β CR knock-out mice, EPO did not induce cytoprotection¹⁷. However, Kanellakis et al. showed that darbepoietin, a long-working EPO analogue, still is protective against cardiac I/R injury in β CR knock-out mice³⁸. Thus, EPO mediated cytoprotection may not be solely dependent of the EPOR₂- β CR₂ complex or β CR-VEGFR2 interaction.

Apparently, EPO is able to activate several protective signalling pathways. Further studies are necessary to determine the exact role of each pathway. It is however evident that tissue protection is mediated by other receptor complexes than stimulation of erythropoiesis. Enhanced eNOS activation appears to be crucial for improvement of renal function as EPO is not able to ameliorate renal function in eNOS knock-out mice. eNOS activity can be increased by EPO treatment via three signalling cascades. Figure 1 illustrates a scheme of proposed renoprotective pathways. The erythropoietic receptor complex has no protective function, although stimulation of this complex may be responsible for the increased risk of cardiovascular adverse events.

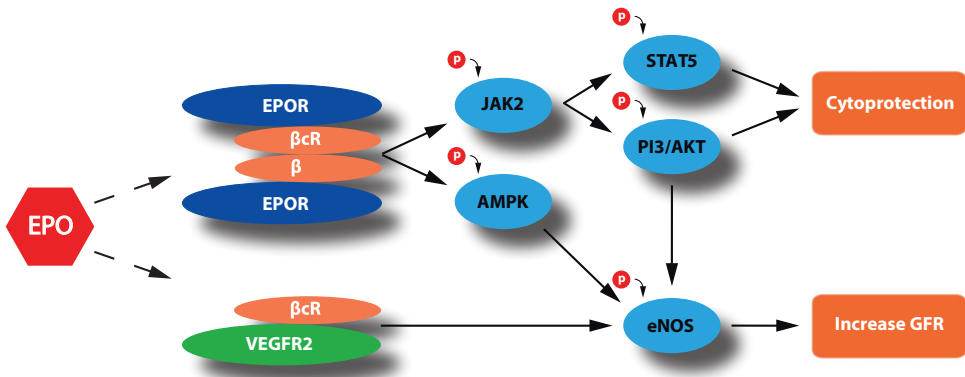


Figure 1 - Proposed renoprotective pathway of EPO. EPO is able to activate either the EPOR₂- β CR₂ complex or an interaction between β CR-VEGFR2. Binding of EPO to the EPOR₂- β CR₂ complex activates anti-inflammatory, anti-apoptotic and pro-survival pathways. PI3/AKT and AMPK, activated by the EPOR₂- β CR₂ complex, and β CR-VEGFR2 interaction, are responsible for increased eNOS phosphorylation by EPO. The direct stimulative effect on renal function is presumably the effect of enhanced eNOS activity.

Clinical EPO treatment after clinical renal transplantation

Encouraged by pre-clinical results, four clinical trials were initiated in the Netherlands³⁹, Germany⁴⁰, France⁴¹ and the USA⁴². The protective effect of EPO following transplantation of deceased donor kidneys has been investigated in one open label and three double blind, randomized controlled trials. All studies aimed to improve short-term function after transplantation. The end points were incidence of DGF or renal function at one month after transplantation. Major differences were seen in inclusion criteria for donor types. In two studies both types of deceased donors were included^{40,42}. Martinez et al. included all recipients of a deceased donor kidney with a risk of DGF $\geq 60\%$ based on the DGF risk index⁴¹. Aydin et al. only included DCD donor kidneys³⁹. Statistical power was determined 80% based on either reduced incidence of DGF^{39,40,42} or improved renal function⁴¹. Aydin et al. did not meet its powered inclusion as the Data and Safety Monitoring Board stopped the trial because of the slow inclusion rate. However, using the actual DGF rate of 81% in the placebo group, power was recalculated at 98%³⁹. Characteristics of these studies are shown in table 1.

Table 1 - Study characteristics

	Aydin et al.³⁹	Hafer et al.⁴⁰	Martinez et al.⁴¹	Sureskumar et al.⁴²
Design	RCT Double blind Single-center	RCT Double blind Single-center	Open label Randomized Multicenter	RCT Double blind Single-center
Treatment	Epoetin- β	Epoetin- α	Epoetin- β	Epoetin- α
Dose	33000 IU	40000 IU	30000 IU	40000 IU
Control treatment	Saline	Saline	N/A	Saline
Timing	3-4 hours pre-Tx 24 hours post-Tx 48 hours post-Tx	Reperfusion 3 days post-Tx 7 days post-Tx	Pre-surgery 12 hours post-Tx 7 days post-Tx 14 days post-Tx	Reperfusion
Donor type	DCD	Deceased donors	Deceased donors, DGF risk > 7	Deceased donors
Number of patients	C: 45 vs. EPO: 47	C: 44 vs. EPO: 44	C: 52 vs. EPO: 52	C: 36 vs. EPO: 36
Follow-up	12 months	12 months	3 months	1 months
Renal function	GFR C: 57 ml/min EPO: 68 ml/min	eGFR C: 44 ml/min EPO: 41 ml/min	eGFR C: 44 ml/min EPO: 43 ml/min	eGFR C: 37 ml/min EPO: 37 ml/min

Table 2 - Incidence of DGF

	EPO group	Control group
Aydin et al.³⁸	76,2%	78,3%
Hafer et al.³⁹	23%	32%
Martinez et al.⁴⁰	32%	38.8%
Sureshkumar et al.⁴¹	41,7%	47,2%

High dose EPO treatment after renal transplantation did not significantly reduce incidence of delayed graft function (DGF).

None of the clinical studies showed a significant reduction of DGF (Table 2) or immediate improvement of renal function. The large differences in incidence of DGF between the four studies can be explained by inclusion of different donor types. As secondary end points these studies used markers of renal function. Aydin et al. showed a significant increase of endogenous creatinine clearance at 12 months post-transplantation (EPO vs. placebo: 68 ± 23 mL/min vs. 57 ± 25 mL/min) whilst the other studies did not observe any difference in renal function.

An important finding is the increased risk of thrombosis during the first year following transplantation by EPO treatment (EPO vs. placebo: 24,4% vs. 6,4%) in the report by Aydin et al. The other studies did not show any differences in adverse events although in three studies EPO was shown to increase haemoglobin levels after transplantation³⁹⁻⁴².

Translation of protective EPO treatment

In pre-clinical studies the cytoprotective capacities of EPO have been thoroughly tested in renal I/R or transplantation models as discussed earlier⁹⁻¹⁶. However, the translation appears difficult. Apart from healthy animals and no immunosuppressive treatment in experimental models, there are several factors that may explain the lack of protection and clinical improvement in the recent trials³⁹⁻⁴².

Timing

Currently, treatment to reduce ischaemia/reperfusion injury is ethically and practically best applicable in the recipient. Although protective treatment of donors is increasingly being considered if it does not harm the donor. Most renal I/R injury emerges during the reperfusion phase. Thus, cytoprotective treatment early in the reperfusional phase potentially improves function after transplantation. Based on these practical, ethical and mechanistical reasons, protective EPO treatment focusses on the recipient.

In the four clinical trials, timing of the EPO treatment considerably differs (Table 1). Pre-clinical studies showed that high-dose EPO administration is protective when administered between thirty minutes pre-ischaemia and 6 hours post-reperfusion^{12,14}.

There are no studies showing the protective capacities of EPO when administered more than six hours post-reperfusion. Although timing of treatment in rodents cannot be directly translated to the human, there is definitely no evidence for clinical high dose EPO treatment from two to fourteen days after transplantation.

Dosing

Dosing of protective EPO treatment is a difficult issue to allow translation of this treatment from animal work to the clinical situation. In most pre-clinical I/R models EPO doses of 1000 IU/kg or higher were tested⁹⁻¹⁵. However, in the clinical trials doses ranging from 30000 to 40000 IU were used independently of the weight of the recipient³⁹⁻⁴².

Assuming the average recipient weighs 75 kg, this means that recipients were treated with a dose of approximately 500 IU/kg. This is a relatively low dose to induce EPO mediated tissue protection as binding affinities of the erythropoietic- and the tissue protective receptor complex are different. As mentioned earlier, the binding affinity of the classic EPOR₂ complex for EPO is considerably higher than the affinity of the EPOR₂-βCR₂ complex^{21,22}. This means that distinctly higher systemic EPO levels are required to induce the tissue protective receptor complex compared to stimulation of the erythropoiesis. Clinical EPO treatment to stimulate erythropoiesis is dosed at 75-300 IU/kg. This means that the dose used in the clinical trials, 500 IU/kg, is comparatively low to induce renoprotection.

Non-erythropoietic EPO derivatives

In animal models no increased risk of high dose, protective EPO treatment is observed since follow-up is relatively short in pre-clinical models. Besides, recipients of a renal transplant often suffer any kind of co-morbidity, while in pre-clinical studies healthy animals are used. However, based on pre-clinical trials high-dose EPO treatment was thought to be safe. As mentioned above, the used dose EPO in clinical renal transplantation trials was 2-10 times lower than dosages in animal models. However, Aydin et al. already observed an increased risk of thrombosis within the first year following transplantation³⁹. In renal transplantation EPO doses used post-transplantation did not reach protective levels, although the risk of side effects already increased. An increased serum EPO concentration raises the haematocrit and markedly enhances platelet and endothelial activation^{8,43}. These mechanisms are causative for the increased risk of cardiovascular adverse events. In cancer patients it has also been shown that EPO treatment to stimulate erythropoiesis already increased thromboembolic events and mortality⁴⁴.

Thus, safety concerns about high dose EPO treatment in renal transplantation are justified and increasing the EPO dose to induce cytoprotection is irresponsible. Besides the risks of cardiovascular events, several clinical trials in anaemic cancer patients suggested a stimulating effect of EPO on tumour progression. Aapro et al. elegantly reviewed meta-analyses and there is no evidence for enhanced tumour progression by EPO⁴⁵.

To overcome the shortcomings of cytoprotective EPO treatment, non-erythropoietic EPO derivatives have been developed. Tissue protection is mediated by a specific receptor complex and this created an opportunity to develop these non-erythropoietic EPO derivatives. All non-erythropoietic EPO derivatives, which have been tested in models of acute renal injury, will be discussed: asialo-erythropoietin (asialo-EPO), carbamoylated EPO (CEPO), glutaraldehyde EPO (GEPO) and ARA290. These derivatives do not bind to the classic EPOR₂ complex. Thus, erythropoiesis or platelet activation is not stimulated. In this way cytoprotection can be induced without increasing risk of cardiovascular adverse events. The effect of non-erythropoietic EPO derivatives on tumour progression has not been investigated. However, an enhancing effect of non-erythropoietic EPO derivatives on cancer is unlikely, as the proposed mechanism of tumour progression by EPO is mediated by the classic EPOR₂ complex⁴⁵ which is not activated by non-erythropoietic EPO derivatives.

Continuous exposure of precursor red blood cells to EPO is required for stimulation of erythropoiesis, while cytoprotection can be induced by brief exposure. Based on this principle, an EPO derivative with a very short half-life could be protective and would not stimulate erythropoiesis. Enzymatic desialylation of EPO results in asialo-EPO possessing a half-life of several minutes. In renal I/R asialo-EPO attenuated renal dysfunction and improved survival²⁶. Although, asialo-EPO does not stimulate erythropoiesis, asialo-EPO still has the same affinity for the classic EPOR₂ complex as EPO^{18,46}. Therefore, redundant effects of asialo-EPO via this receptor complex cannot be excluded.

CEPO is synthesized by cyanide carbamoylation and GEPO is based on glutaraldehyde modification^{18,47,48}. These EPO derivatives distinctly differ on molecular level of EPO and asialo-EPO. Carbamoylation and glutaraldehyde modification reduce the charge of lysine residues on EPO molecules. This prevents stimulation of erythropoiesis⁴⁹. In vitro and in vivo experiments showed that CEPO and GEPO do not affect erythropoiesis^{18,48}. The half-life of CEPO and GEPO is approximately 6 hours, comparable to the half-life of EPO¹⁸. In several models of renal I/R injury and brain death, protective capacities of CEPO have been observed. Depending on AKT phosphorylation, CEPO improves renal function. Apoptosis, tubular injury and structural damage were reduced by CEPO treatment^{27,36,50-53}.

Furthermore, CEPO also improves angiogenesis, improves renal blood flow and prevents reduced density of peritubular capillaries^{36,51,52}. GEPO has only been tested in one I/R model, showing preserved renal function and reduced histological damage^{18,48}.

The third and newest generation of non-erythropoietic EPO derivatives is ARA290, also known as pyroglutamate helix B surface peptide (pHBSP). ARA290 is derived from the binding site of EPO to the EPOR₂-βCR₂ complex. It mimics the 3-dimensional structure of the ligand binding to EPOR₂-βCR₂ complex and possesses a half-life of approximately two minutes³⁷. This means that ARA290 is not able to bind the erythropoietic EPOR₂ complex. The protective capacities of ARA290 have been shown in models of haemorrhagic shock and neuronal injury⁵⁴⁻⁵⁷. In renal I/R cytoprotection by ARA290 has been shown in rodent and porcine models^{20,25,37,58}. Post-reperfusion administration of ARA290 to six hours post-reperfusion improved short-term renal function, reduced inflammation, reduced apoptosis and reduced structural damage^{20,25,58}.

Mechanistically, ARA290 is able to increase AKT and eNOS phosphorylation²⁵. Inhibition of PI3/AKT diminishes the protective effect of ARA290, indicating the importance of this pathway²⁰. As mentioned before, Yang et al. showed that renal I/R upregulates EPOR₂-βCR₂ expression in renal tissue at 48 hours post-reperfusion. Interestingly, ARA290 prevents this increase of receptor expression. ARA290 in combination with Wortmannin, a PI3/AKT pathway inhibitor, doubled EPOR₂-βCR₂ expression compared to I/R injury²⁰. This suggests the EPOR₂-βCR₂ complex is part of a physiologic cytoprotective effect and therefore, inhibition of one of its down-stream pathways results in a further increase of the expression of the cytoprotective receptor complex. We showed in a porcine I/R model that ARA290 is able to improve the glomerular filtration rate in the first 7 days post-reperfusion. Furthermore, ARA290 prevented structural damage. In the first 24 hours post-reperfusion ARA290 increased urinary nitrite + nitrate concentrations, suggesting increased nitric oxide synthase activity⁵⁸.

The half-life of the four different EPO derivatives is important for determining the timing of treatment. CEPO and GEPO possess a half-life of several hours¹⁸, while the half-life of asialo-EPO and ARA290 is only minutes^{37,46}. In ischaemia/reperfusion injury most damage occurs early in the reperfusion phase and eNOS phosphorylation is reduced in the first six hours post-reperfusion. Therefore, the most optimal window of treatment is in the first six hours post-reperfusion. Depending on the different pharmacokinetics of the non-erythropoietic EPO derivatives, the timing of treatment should be chosen carefully as differences in half-life will affect the moment of treatment.

Asialo-EPO, CEPO, GEPO and ARA290 show protective effects in renal I/R injury comparable to cytoprotective EPO treatment. The major benefit of non-erythropoietic EPO derivatives is that they do not influence the erythropoiesis or platelet activation^{37,50}. Therefore, titration to high, cytoprotective levels is possible without an increased risk of cardiovascular events. CEPO and ARA290 are most interesting derivatives as these molecules have no affinity for the classic EPOR₂ complex and the renoprotective capacities have already been shown in several renal I/R experiments.

Conclusions

EPO mediated cytoprotection is promising. However, increased risk of cardiovascular events is a serious concern of high-dose EPO treatment. Especially as cytoprotective levels have not been reached in clinical trials, although the risk of thrombosis already increased. Non-erythropoietic EPO derivatives may be the solution. In pre-clinical models, derivatives like CEPO or ARA290 retained their protective capacities without influencing erythropoiesis. These EPO derivatives could be titrated safely to protective levels in the transplantation clinic. Cytoprotective treatment should be timed early in the reperfusion phase.

Only non-erythropoietic EPO derivatives, like CEPO or ARA290, may induce protection without increasing the risk of cardiovascular events. Non-erythropoietic EPO derivatives, administered early post-reperfusion, may be able to improve short-term renal function. Hereby, incidence of DGF and PNF following renal transplantation could be reduced. Pre-clinical results warrant further investigation of the renoprotective effects of non-erythropoietic EPO derivatives in renal transplantation.

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