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Protective effect of immunosuppressive treatment before orthotopic kidney autotransplantation

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ABSTRACT

Background: Ischemia reperfusion injury (IRI) is one of the risk factors for delayed graft function, acute rejection and long term allograft survival after kidney transplantation. IRI is an independent antigen inflammatory process that produces tissue damage. Our objective was to study the impact of immunosuppressive treatment (IS) on IRI applying only one dose of IS before orthotopic kidney autotransplantation.
Methods: Twenty-four rats allocated in four groups were studied. One group served as control (G1: autotransplanted rats without IS) and the rest received IS 12 h before kidney autotransplantation (G2: Rapamycin, G3: Mycophenolate mofetil and G4: Tacrolimus).
Results: Improved renal function and systemic inflammatory response were found among IS groups compared to the control group (Delta Urea $p<0.0001$; Delta Creatinine $p<0.0001$; Delta C3 $p<0.001$). The number of apoptotic nuclei in renal medulla in G1 was higher than in IS groups ($p<0.0001$). Tubular damage was less severe in IS groups respecting G1 ($p<0.001$). C3, TNF-α and IL-6 expression in kidney samples was reduced when IS was used compared to the control group. No differences were observed among the different immunosuppressive drugs tested. However, Heme oxygenase-1(HO-1) was increased only in Rapamycin treatment.
Conclusions: These data suggest that the use of IS administered before transplant attenuates the IRI process after kidney transplantation in an animal model.

Abbreviations: ATN, acute tubular necrosis; C3, C3 complement fraction; DGF, delayed graft function; FK506, tacrolimus; HO-1, heme oxygenase-1; HSP, heat shock protein; IL-6, interleukin 6; IRI, ischemia reperfusion injury; IS, immunosuppressive treatment; MMF, mycophenolate mofetil; SRL, sirolimus; TNF-α, tumor necrosis factor alpha.

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1. Introduction

Ischemia reperfusion injury (IRI) is one of the main risk factors for delayed graft function (DGF) after deceased donor kidney transplantation. DGF is associated with both, an increase incidence of acute rejection [1] and a decreased long term allograft survival [2].

The total ischemia time of a vascularized graft can be divided in two successive periods of time: the warm ischemic interval, before or during organ retrieval and the cold ischemia time associated with preservation and storage. Reperfusion, critical to the viability of the organ, amplifies further the ischemic damage [3]. It is expected that any intervention able to attenuate the IRI process will have an outstanding value in the renal transplant outcome [4].

The ischemia reperfusion injury is an antigen-independent inflammatory process that produces tissue damage [5,6]. We hypothesized that the employment of immunosuppressive drugs (IS) in organ donors would be associated with attenuation of this process. Treatment with Sirolimus (SRL) has shown to decrease small bowel and liver IRI in the early period of reperfusion [7,8]. On the other hand, when SRL was employed in kidney transplantation after the IRI process has already occurred, its use was associated with delayed renal function recovery [9,10]. Mycophenolate mofetil (MMF) is a powerful immunosuppressant drug currently used in organ transplantation. MMF depletes guanosine triphosphate pools in lymphocytes and monocytes and suppresses de novo synthesis of purines, exerting a selective and reversible antiproliferative activity on these cells. In addition, MMF inhibits production of cell surface adhesion molecules, which is critical for recruitment of leukocytes to inflammatory foci sites [11,12]. Lastly, pretreatment with low doses of Tacrolimus has shown to provide liver and renal protection against IRI in rats [13,14].

2. Objective

Our objective was to study the impact of immunosuppressive treatment (IS) applied before orthotopic kidney autotransplantation on IRI evolution.

3. Methods and materials

3.1. Animals

Twenty four inbred, male, Wistar rats, weighing 280–350 g were included to perform left kidney autotransplantation. Animals were allowed free access to water and standard laboratory chow ad libitum. All animal experiments were performed according to guidelines set by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

3.2. Surgical procedure

Animals were anaesthetised with ketamine (40 mg/kg) and subsequently left nephrectomy was performed tying adrenal vessels and leaving a vascular clamp in the origin of renal vessels. After opening the renal vein, leaving approximately 3 mm of renal vein from the vascular clamp, kidney was flushed through renal artery with a Ringer Lactate Solution (4 °C) until it turned homogeneous pale. Kidney was then removed with its vascular and ureteral pedicle and stored for 180 ± 15 min in the cold Ringer Lactate Solution. After nephrectomy, rats were allowed to recover in a warm blanket and O2 was supplied by a face mask. Three hours later the rat was anaesthetised again with the same dose of ketamine (40 mg/kg), re-explored and left kidney was autotransplanted in an orthotropic position performing end-to-end vascular and ureteral anastomoses. Next, the right kidney was removed, the abdominal wall was closed and the animal started the recovery period with the same care mentioned above. When the whole procedure was finished, rats were allowed free access to water and standard laboratory chow ad libitum. Animals were sacrificed 24 h after the auto transplant procedure and kidney was recovered for histological analysis.

3.3. Experimental groups

One dose of immunosuppressive drugs was administered 12 h before the surgical procedure. Doses and administration route were chosen according to previous reports [15–17]. Animals were divided in four groups:

Group 1 (Control, n = 6): no immunosuppression was administered.
Group 2 (Rapamycin, n = 6): Rapamycin (2 mg/kg) PO by gavage.
Group 3 (MMF n = 6): MMF (20 mg/kg) PO by gavage.
Group 4 (Tacrolimus, n = 6): Tacrolimus (0, 3 mg/kg) PO by gavage.

None of the groups received immunosuppressive drugs after autotransplantation. In addition, 6 rats underwent a sham procedure.

3.4. Blood measurements

Twenty four hours before and after autotransplant the following blood determinations were performed: urea, creatinine and C3 complement fraction (C3). C3 was measured by Radial Immunodiffusion and urea and creatinine by UV Kinetic and Colorimetric-Kinetic respectively (Mindray 300). Values are expressed as the difference between pre-transplant and post-trasplant for each group.

3.5. Renal histopathology

The anatomopathological samples were analyzed by a pathologist blind to group assignments. The kidneys were fixed in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. Four micrometer thick sections were cut, desparaffinized hydrated and stained with hematoxylin and eosin. The renal sections were examined on a blind fashion for the grade of cortical tubular epithelial necrosis. Counts were performed in at least 10 different fields of square micrometers and assigned for the severity of necrosis, using scores on a scale of 1 (<5%), 2 (6–25%), 3 (26–50%), 4 (51–75%) and 5 (>75%).

3.6. TUNEL assay

TUNEL assay was performed essentially according to the instructions of the manufactures (Apoptag: ONCOR, Gaithersburg, MD). Briefly, desparaffinized 4 μm-thick sections of paraffin the embedded tissue were pretreated with 20 μl/ml proteinase K (Dako, Glostrup, Denmark) for 30 min at 37 °C. After washing, sections were incubated with digoxigenin-labeled deoxyuridine triphosphate (dUTP) in the presence of terminal-deoxynucleotidyl-transferase. After the enzymatic reaction was blocked, sections were incubated with a specific peroxidase-labeled antidigoxin antibody. Peroxidase was then reduced by 0.05 diaminobenzidine (Sigma, St.Luis, MO) in 0.1 ml/L phosphate-buffered saline, pH 7.6 containing 1% H_2O_2. After washing, the sections were lightly stained with hematoxylin. Negative control reactions were performed for each reaction step. They were obtained by omission of the terminal-deoxynucleotidyl transferase, antidigoxin antibody and peroxidase substrate. Positive controls included sections of paraffin embedded lymphoma of human origin. The external medullar region was examined and the total number of labeled nuclei was counted. Ten fields of a one square mm were examined by means of a reticulated lens.

3.7. Immunohistochemistry

Sections of 4 μm thick were applied to poly-2-lysine coated slides. Sections were dewaxed in xylene, dehydrated through graded alcohols and water and immersed in 0.3% vol/vol H_2O_2 in methanol for 30 min to block endogenous peroxidase.

Antigens were reduced by microwaving at 750 W for 15 min in 0.01 mol/L trisodium citrate buffer, pH 6.0, then well rinsed in standard PBS and non specific binding was blocked with 10% equine serum in PBS. Sections were incubated with primary antibodies of monoclonal origin against C3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with polyclonal from goat against tumor necrosis factor alpha (TNF-α); Interleukin 6 (IL-6), HSP70 (K-20) and HO-1(C-18) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After being rinsed with PBS, sections were incubated with biotinilated secondary antibodies. Then sections were rinsed with PBS and incubated with avidin–biotin horseradish peroxidase complex according to the manufacturer's instructions (Vectastain, Universal Quick Kits; Vector Laboratories Ltd., Peterborough, UK). Peroxides were visualized by incubating the sections in 3.3′ diaminobenizidine (Sigma Chemical Co., Poole, UK) and hydrogen peroxide. Negative control experiments were performed by omitting the incubation with the primary antibodies. The presence of C3, TNF-α, IL-6, HSP70 and HO-1 were assessed in ten consecutive cortex and medulla fields. The extension rather than intensity of these markers was evaluated using a semi quantitative score. According to the percentage of tubule-interstitial area affected, the scale is presented as follows: 1 (<5%), 2 (6–25%), 3 (26–50%) 4 (51–75%) and 5 (>75%).

3.8. Statistical analysis

Analysis of variance (ANOVA) was used to evaluate parametric variables. Dunnet's Test was used to compare each treatment group with the control group. ANOVA and Student–Newman–Keuls Test was used to compare IS groups. Non-parametric variables were analyzed with Kruskal–Wallis non-parametric ANOVA. Data are expressed as mean + SEM. Statistical significance was determined as $p<0.05$.

4. Results

4.1. Urea, creatinine and C3 determination

Twenty four hours after the auto transplant procedure rats were sacrificed. Animals pretreated with immunosuppression showed better renal function and inflammatory response than rats in the control group (G1). Urea, creatinine and C3 levels in treatment groups (IS) were significantly lower when compared with Group 1 levels (Table 1). Nevertheless, there was no difference among the 3 drugs employed for urea ($p=0.18$); creatinine ($p=0.36$) and C3 ($p=0.31$). No differences were observed in urea, creatinine and C3 in the Sham group between pre-transplant and post-procedure (data not shown).

Table 1
Blood measurements in control vs. treatment groups (IS).

Groups	Urea (mg/dl)	Creatinine (mg/dl)	C3 (pg/ml)
	Mean ± ES (Min–Max)		
Control	268.8 ± 14.6 (235–315)	4.08 ± 0.34 (3.11–5.10)	423.1 ± 33.3 (272.5–510.3)
Mycophenolate mofetil	210.3 ± 14.6 (155–242)	1.88 ± 0.08 (1.68–2.18)	194.8 ± 11.9 (167.3–234.0)
Rapamycin	210.2 ± 11.1 (166–236)	2.07 ± 0.06 (1.89–2.28)	193.5 ± 9.8 (174.3–240.0)
Tacrolimus	183.7 ± 5.5 (160–201)	1.77 ± 0.05 (1.55–1.88)	175.4 ± 7.0 (149.7–202.0)
ANOVA	**p = 0.0006**[a]	**p<0.0001**[a]	**p<0.0001**[a]

[a] Control group vs. Mycophenolate mofetil, Rapamycin and Tacrolimus. Values are expressed as the difference between pre-transplant and post-trasplant for each group. No differences were found among Mycophenolate mofetil, Rapamycin and Tacrolimus.

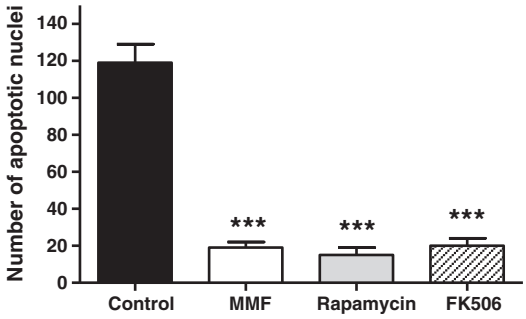

Fig. 1. Apoptosis in outer and inner medulla in the control group vs. immunosuppressive treatment groups. In the latter, rats received Mycophenolate mofetil, Rapamycin or Tacrolimus 12 h before autotransplantation. The difference between control and treatment groups was statistically significant ($p<0.0001$). No differences among immunosuppressive drugs were shown ($p>0.05$).

4.2. Histology

The number of apoptotic nuclei in renal medulla in Group 1 (control group) was higher comparing with IS groups (G1: 117.91 + 9.76; G2: 13.31 + 4.19; G3: 18.8 + 2.9 and G4: 20.1 + 3.09 $p<0.0001$). Once again, no differences were found among the immunosuppressive drugs used (G2, G3 and G4 $p=0.34$) (Fig. 1). However, Group 2 (Rapamycin) showed a trend toward a lower number of apoptotic nuclei cells with respect to groups 3 (MMF) and 4 (Tacrolimus), but it didn't reach a statistically significant difference.

Tubular damage was less severe in treatment groups with respect to the control group (G1) (Table 2). Although necrosis scores didn't differ among the three immunosuppressive drugs (G2, G3 and G4 $p=0.3$), the use of Tacrolimus (group 4) was associated with a tendency towards a lower level of acute tubular necrosis (ATN) compared with the other treatment drugs ($p=0.31$).

4.3. Local inflammatory molecules

Finally, local expression of C3, TNF-α and IL-6 was higher in the control group (Scale 5), than in IS groups (Scale 1 and 2; $p<0.05$) (Fig. 2). We didn't observe any differences among the different drugs employed ($p>0.05$).

The expression of HSP70 and HO-1 was also determined by inmmunohistochemistry. HO-1 was markedly increased 24 h after transplantation in animal treated with Rapamycin compared to control animals or compared with the others immunosupressive treatments ($P<0.001$). However, there were no differences in HO-1 expression in Mycophenolate mofetil or Tacrolimus groups compared to the control animal ($P>0.05$). In the case of HSP70 no differences were observed between any groups analyzed ($p>0.05$) (Fig. 3).

5. Discussion

In this study, the impact of immunosupressive treatment before kidney autotransplantation was investigated. A single dose of immunosuppressive drug before orthotopic kidney autotransplantation was associated with significant, clinical and histological improvement, along with reduction in systemic and *in situ* inflammatory response. In treatment groups (IS), urea and creatinine decreased significantly compared with the control group when immunosuppressive drugs were administered 12 h before orthotopic autotransplant. This model reproduces the sequence of renal transplantation but assumes the exclusion of any antigenic response in the IRI process [18–21].

Table 2
Acute tubular necrosis score in control vs. treatment groups (IS).

Histology Score	Groups			
	Control	Mycophenolate mofetil	Rapamycin	Tacrolimus
Median	5.0	4.0	3.5	3.0
Min–Max	(5–5)	(3–4)	(3–4)	(2–4)
Kruskal–Wallis	–	–	–	$p=0.0015$[a]

No differences were found among Mycophenolate mofetil, Rapamycin and Tacrolimus ($p=0.3$).

[a] Control group vs. Mycophenolate mofetil, Rapamycin and Tacrolimus.

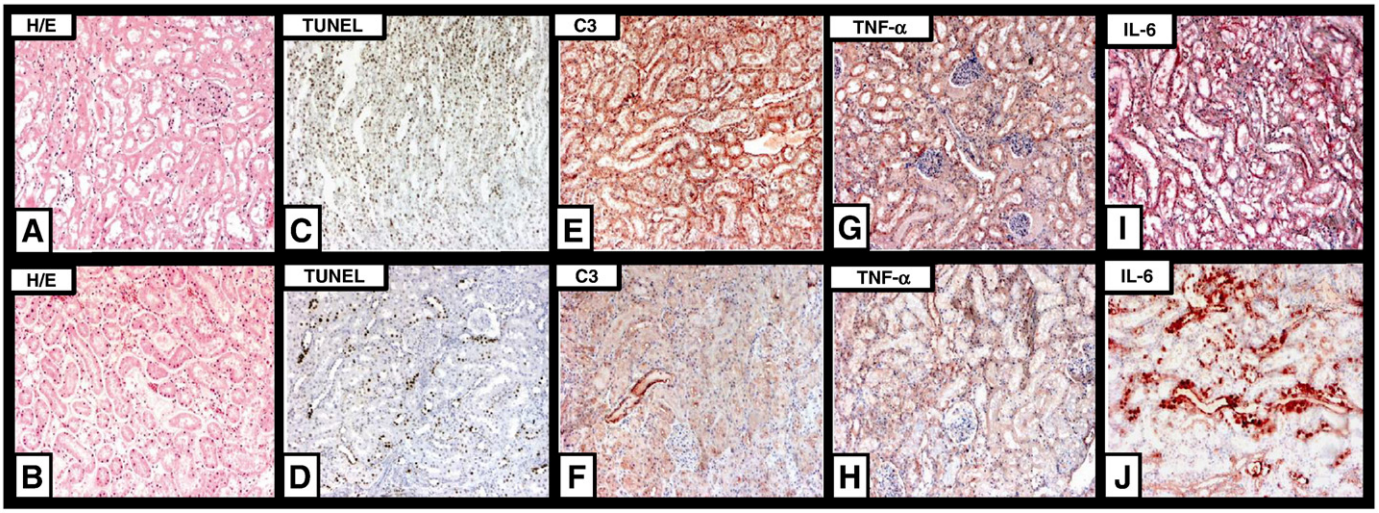

Fig. 2. Renal damage after 24 h of ischemia reperfusion injury in control vs. treatment groups. Immunosuppressive drugs (Rapamycin, Mycophenolate mofetil and Tacrolimus) were given in a single dose 12 h before autotransplantation. Hematoxylin eosin stain: Autotransplant rats (A) showing more than 75% of tubular cells damage in renal cortex vs. Tacrolimus treated rats (B) where epithelial damage is less than 50%. TUNEL staining of the kidney sample after 24 h of reperfusion: Control animals (C) and Rapamycin treated animals (D). Renal cortex and outer medulla (E to J): Immunohistochemical demonstration of C3 (E, F), TNF-α (G, H) and IL-6 (I, J) in renal tissue from ischemia reperfusion rats. Control group (group 1) E, G, I vs. treated animals: F (Tacrolimus); H (Mycophenolate) and J (Rapamycin).

Ischemic damage is responsible for a large share of the high incidence of DFG and the increased incidence of acute rejection. Ischemia reperfusion injury is considered an inflammatory process. Endothelial injury, leukocyte infiltration and tubular epithelial cell are implicated in tissue damage by inducing inflammation [22–24]. Inflammatory mediators such as IL-6, C3 and TNF-α are markers of tissue damage in IRI [25]. Especially, high circulating levels of IL-6 have been considered a marker of inflammatory response severity [26,27]. In our study, we measured local expression of IL-6, which was significantly reduced in IS groups.

C3 and TNF-α play a key role in the process of apoptosis and necrosis associated with ischemia reperfusion injury [28–30]. In our work systemic C3 was up regulated in the control group, contrasting with the significantly lower levels found in IS groups. The reduction in plasmatic levels of C3 in IS groups could be related to lower expression observed *in situ*. In a model of warm ischemia, Thurman et al. showed even higher systemic levels of C3, although the measurement was taken in a different time frame (8 h post-IRI) [31]. An up-regulated, *in situ*, expression of C3 and caspase 3 can be seen as soon as 2 h following IRI

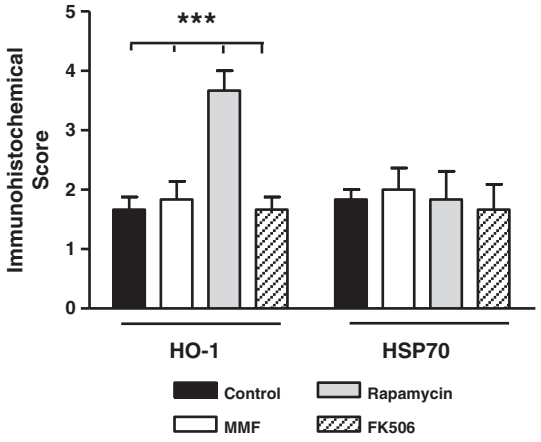

Fig. 3. Score of HO-1 and HSP70 after autotransplantation in control group vs. immunosuppressive treatment groups. The score of HO-1 and HSP70 were graded on a scale from 1 to 5 by using microscopic criteria as described in Materials and Methods. The difference between Rapamycin group and all the other groups was statistically significant ($p > 0.0001$).

[32]. In the case of TNF-α prior studies demonstrated that it is an inflammatory mediator that induces apoptosis in renal epithelial cells [33–36]. In our model there were significantly lower TNF-α found in IS groups compared with the control group. This finding might be associated with the lesser amount of apoptotic nuclei in outer and inner medulla [31]. The immunosuppressive treatment decreased the number of apoptotic nuclei in outer and inner renal medulla. There was a tendency towards a lower amount of apoptosis when Rapamycin was administered as compared with MMF and Tacrolimus. Although Rapamycin might delay kidney recovery from acute renal failure [16,37], in our model, this drug attenuated the inflammatory process and actually improved renal function. This could have been related to the administration of Rapamycin previous to the transplant. So, according to our results Rapamycin could be used before transplant to reduce the inflammation associated with IRI; but it shouldn't be employed in the presence of acute tubular necrosis after renal transplantation.

In agreement with our results, Yang B et al. [38] found a decreased level of apoptosis associated with the use of Tacrolimus, MMF and Rapamycin in a model of warm ischemia. However, these drugs were given after the surgical procedure and not before. On the contrary, Serr et al. showed increased apoptosis using Sirolimus in a model of pancreatic warm ischemia. In this case, Sirolimus was administered before and after the surgical procedure [39].

The three immunosuppressant drugs employed in this model have anti-inflammatory properties but their exact mechanism is not completely understood [40]. It has been suggested that induction of heat shock protein (HSP) 70 by Tacrolimus inhibits the expression or release of TNF-α. This is one of the protective mechanisms used by HSP to counter inflammation [41,42] but in our model none of the drugs could increase the expression of HSP70 (Fig. 3). Moreover, other HSP could be involved in renal protection, as it was postulated by Yem et al. [42]. Wagner et al. demonstrated that Heme oxygenase-1 (HO-1) another HSP, attenuates ischemia/reperfusion-induced apoptosis and improves survival in rat renal allografts [43]. In the present work we found that Rapamycin treatment increased HO-1 expression in agreement with *Gonçalves* work [44]. Also the number of TUNEL positive cells was significantly reduced in the Rapamycin group. So the induction of HO-1 by Rapamycin preconditioning could be a good approach to preserve graft function and increase survival by preventing apoptosis.

On the other hand, donor treatment with MMF was associated *in vitro* with a decreased number of leukocytes adhering to the graft,

which is consistent with decreased cell adhesion molecules *in vivo* [15,45]. Furthermore, the use of MMF as pretreatment demonstrated not only attenuation of ischemia reperfusion injury but also avoidance of adverse effects on renal function recovery.

Acute tubular injury was attenuated with the use of immunosuppressive drugs. Although none of the drugs was statistically significantly better than the others, the use of Tacrolimus was associated with a tendency towards a lower level of ATN. In the same way, Yang CW and col. [17] have shown a reduction in the IRI process using preconditioning with low doses of FK506 in a kidney rat model. In addition, Shihab et al. [46] demonstrated that pharmacologic preconditioning of rat donors with Tacrolimus significantly improved kidney function and histology 3 days after transplant. In this model the 3-h cold ischemia time employed was considered appropriate to reproduce the IRI process because tubular injury in the control group was higher than 75%. This is in agreement with the 2-h cold ischemia time used by Reutzel-Selke et al. in their model [47].

As far as we have noticed, this is the first study to compare the impact of immunosuppressive treatment given before orthotopic kidney autotransplantation on the IRI process. Tacrolimus, MMF and Rapamycin given before transplant were associated with clinical and histological improvement, as well as with reduction in the inflammatory response. In our study, the different drugs tested were equivalent as far as outcome measures were concerned. Employing immunosuppressive treatment on deceased donors before recovery of organs might decrease damage associated with IRI and consequently improved recipient graft function. In fact, systemic inflammatory response triggered by brain death could also be reduced by immunosuppressive drugs [48,49]. In this way, the two most important antigen-independent risk factors for allograft loss, brain death and IRI, could be mitigated by the administration of immunosuppressive drugs to the donor. Graft immunogenicity would be reduced with the resulting positive effects on delay graft function, acute rejection and long term allograft survival.

6. Conclusion

Collectively, we show that immunosuppressive treatment before orthotopic kidney autotransplantation is associated with clinical and histological improvement, along with reduction in systemic and *in situ* inflammatory response. This finding suggests a potential use of immunosuppressive drugs before transplantation to decline damage associated with IRI.

Conflict of Interest

Authors: declared no conflicts of interest.

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