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ORIGINAL ARTICLE

Effects of remote ischaemic preconditioning on intraportal islet transplantation in a rat model

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SUMMARY

Remote ischaemic preconditioning (RIPC), which is the intermittent interruption of blood flow to a site distant from the target organ, is known to improve solid organ resistance to ischaemia-reperfusion injury. This procedure could be of interest in islet transplantation to mitigate hypoxia-related loss of islet mass after isolation and transplantation. Islets isolated from control or RIPC donors were analyzed for yield, metabolic activity, gene expression and high mobility group box-1 (HMGB1) content. Syngeneic marginal mass transplantation was performed in four streptozotocin-induced diabetic groups: control, RIPC in donor only, RIPC in recipient only, and RIPC in donor and recipient. Islets isolated from RIPC donors had an increased yield of 20% after 24 h of culture compared to control donors ($P = 0.007$), linked to less cell death ($P = 0.08$), decreased expression of hypoxia-related genes (Hif1a $P = 0.04$; IRP94 $P = 0.008$), and increased intra-cellular ($P = 0.04$) and nuclear HMGB1. The use of RIPC in recipients only did not allow for reversal of diabetes, with increased serum HMGB1 at day 1; the three other groups demonstrated significantly better outcomes. Performing RIPC in the donors increases islet yield and resistance to hypoxia. Validation is needed, but this strategy could help to decrease the number of donors per islet recipient.

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Key words
experimental model, HMGB1, intraportal islet transplantation, marginal mass, remote ischaemic preconditioning

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Introduction

Intraportal islet transplantation is a therapeutic option for the management of selected patients with type 1 diabetes. However, despite recent advances, transplanted islets still suffer from multifactorial acute and chronic injury, and most patients need more than one organ donor to hope for insulin independence [1,2]. Both islet hypoxia induced by the isolation/transplantation process [3], and liver ischaemia induced by the embolization of islets [4], as testified by post-transplantation transaminitis in humans [5], have been suggested as playing an important part in early graft loss. Two independent studies have shown that *local* ischaemic preconditioning (IPC), intermittently interrupting blood flow to the target organ, of the donor pancreas increases islet yields

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after isolation [6], and that local IPC of the recipient liver improves graft function by decreasing the severity of the liver necrosis [4]. An important advantage of islet transplantation is the minimally invasive nature of the procedure in recipients, allowing fragile patients to benefit from this therapy. As such, local liver IPC of the recipient is not an option owing to the need for a laparotomy. In donors, performing local pancreatic IPC lengthens the organ retrieval process. Remote ischaemic preconditioning (RIPC), which is the intermittent interruption of blood flow to a site distant from the target organ (i.e. a limb), would be a more appropriate procedure in clinical practice. In donors, RIPC can be performed on a limb, in parallel to organ retrieval, without the presence of cumbersome clamps in the surgical site. In recipients, RIPC would still allow for the usual minimally invasive transplantation procedure. The impact of RIPC has been extensively studied in the ischaemia-reperfusion injury process of various organs, including the liver [7], however, never in the donor and/or recipient in the islet transplantation setting.

The aims of this study were to assess the impact of donor RIPC on the number, quality and function of isolated islets, and of recipient RIPC on post-transplant islet function and survival.

Materials and methods

Animals

Pancreatic islet isolation and transplantation were performed in Lewis rats (Janvier Labs, France) weighing 160–200 g and 160–180 g respectively. Animals were cared for according to international guidelines on animal care. Ethical approval was obtained from the Geneva veterinary authorities (Licenses GE/96/16 and GE/57/17).

Pancreatectomy and islet isolation

Pancreatectomy was performed as previously described [8]. Briefly, animals were anesthetized with Isofluran (Baxter, Volketswil, Switzerland) followed by an intraperitoneal (ip) injection of Ketarom [90 mg/kg Ketaminum (Graeub, Bern, Switzerland; lot n° 6680115) + 9 mg/kg Xylazinum (Bayer, Lyssach, Switzerland; lot n° KP0AC1K)]. A midline abdominal incision was performed, the common bile duct ligated distally and cannulated proximally. The animal was exsanguinated by section of the inferior vena cava, and 10 ml of Collagenase V, derived from *Clostridium histolyticum* (Sigma-Aldrich, St Louis, MO, USA), dissolved in a perfusion solution [500 ml Hank's Buffered Salt Solution (HBSS; Gibco, ThermoFisher Scientific, Waltham, MA, USA) + 2.1 ml NaHCO3 + 1.155 ml of 1M CaCl2 + 12.5 ml HEPES 1M (Gibco)], was infused in the common bile duct to obtain pancreatic distension. Two to three pancreases were pooled together in 7.5 ml, or 10 ml, respectively, of perfusion solution and enzymatic digestion was performed in a 37 °C water bath. Islet purification was achieved using a continuous Optiprep (Axis-Shield) gradient [9].

In remote ischaemic preconditioned donors, pancreatectomy was preceded by remote ischaemic preconditioning (RIPC). Briefly, under inhaled Isofluran (Baxter) anesthesia, a small incision was performed on the left hindlimb. The femoral vessels were isolated, and six cycles of 4 min of ischaemia (achieved by clamping both the femoral artery and vein), followed by 4 min of reperfusion (removal of the clamp) were carried out. Pancreatectomy was performed at the end of the last reperfusion cycle, after an ip Ketarom injection, and islet isolation was carried out as described above.

Islets were stained with dithizone (Sigma-Aldrich) and counted in absolute number and number of islet equivalents (IEQ). Islets were cultured overnight, in an 11.1 mM glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco; further referred to as islet DMEM), in a 37 °C, 5% CO_2, environment. Islets were once more counted and transplanted 24 h after isolation. Excess islets were used for *ex vivo* analyses. All *ex vivo* analyses were conducted with a minimum of six samples per group.

Ex vivo analysis of islet function

Ex vivo islet function was analyzed by glucose-stimulated insulin secretion (GSIS). On day 1 after islet isolation, 100 IEQ were plated per well and exposed for one hour to each of the following glucose concentrations in culture conditions: low glucose concentration 2.8 mM, high glucose concentration 16.7 mM, with ultimate islet lysis in HCl-ethanol solution. Insulin ELISA analysis (Mercodia) was performed on supernatants, according to the manufacturer's instructions. The stimulation index was analyzed.

RNA extraction and quantitative polymerase chain reaction (qPCR) of islets

A total of 500 IEQ lysates from control and RIPC donors were stored at −80 °C in RLT buffer (Qiagen, Hilden, Germany) + β-mercaptoethanol (BioRad, Hercules, CA, USA). RNA extraction (RNEasy Microkit; Qiagen) and

cDNA synthesis (Qiagen) were performed according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using the MESA BLUE Mastermix for SYBR Assay/No ROX (Eurogentec) and the following primers (Table 1 for sequences): RPLP1 as the housekeeping gene, insulin 1 (Ins1), insulin 2 (Ins2), amylase (Amy2a3), hypoxia-inducible factor 1-alpha (HIF1a) (NM_024359.1 sequence; Qiagen), ischaemia-responsive protein 94 (IRP94), tissue factor (F3) (NM_013057.2 sequence; Qiagen), intercellular adhesion molecule-1 (ICAM-1) (NM_012967.1 sequence; Qiagen), high-mobility group box 1 (HMGB1) (NM_012963 sequence; Qiagen).

ELISA analyses of cell culture supernatant

A total of 400 IEQ from control or RIPC donors were cultured for 24 h, after isolation, in islet DMEM. Supernatant was collected at 6 and 24 h of culture and stored at −20 °C for batch analyses. ELISA analyses were performed, according to manufacturers' instructions, for cell death (Roche, Mannheim, Germany) and high mobility group box-1 (HMGB1) content (IBL International GMBH).

HMGB1 Western blot

Isolated islets were cultured for 24 h in islet DMEM. 400 IEQ were retrieved and washed twice with cold PBS. The islet pellet was suspended in 150 µl cytoplasmic extraction buffer [10 mM HEPES (Gibco), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10% IGEPAL (Sigma-Aldrich), 0.5 mM PMSF (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich), protease inhibitor mix (Roche)], allowed to swell on ice for 10 min and centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant was collected as the cytoplasmic fraction. The pellet was suspended and washed twice with the cytoplasmic extraction buffer without IGEPAL. The pellet was then suspended in 150 µl nuclear extraction buffer [20 mM HEPES (Gibco), 0.4M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich), protease inhibitor mix (Roche)], placed on ice for 30 min and centrifuged at 13 000 *g* for 10 min at 4 °C. The supernatant was collected as the nuclear fraction. Protein content was quantified by the Bradford method. A Western blot was performed on the nuclear fraction with purified anti-histone H3 antibody (clone 1B-B2, Biolegend) and anti-HMGB1 antibody (rabbit anti-HMGB1 antibody, ChIP grade; Abcam, Cambridge, UK). The results were expressed as a ratio of HMGB1:histone.

Histology and immunofluorescence

A total of 500 IEQ from control or RIPC donors were fixed in paraformaldehyde at day 1 after isolation to perform histological analyses, and embedded in paraffin. Paraffin slides were prepared with SlowFade Gold antifade reagent with DAPI (Invitrogen, Thermofisher Scientific). Pancreatic β-cells were identified by insulin staining (Guinea pig anti-rat insulin primary antibody, and Alexa 488 goat anti-guinea pig IgG (H+L) secondary antibody; Invitrogen); apoptosis was analyzed via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (click it PLUS TUNEL, Alexa 594; ThermoFisher Scientific), according to manufacturers' instructions.

For HMGB1 localization, β-cells were stained for DAPI, insulin and HMGB1 (rabbit anti-HMGB1 antibody, ChIP grade; Abcam, and Alexa 555 donkey anti-rabbit IgG (H+L); Invitrogen).

Cured recipient pancreata were stained for insulin at day 31 to ensure absence of regenerated islets.

Slides were analyzed by confocal microscopy (Axio Imager.Z2 Basis LSM 800 microscope; Zeiss, Iena, Germany).

Induction of diabetes, intraportal islet transplantation and follow-up

Diabetes was induced in Lewis rats 4 days prior to islet transplantation with a single intra-peritoneal injection of streptozotocin (Sigma-Aldrich; lot n° WXBC2544V) at 80 mg/kg, diluted in citrate buffer solution. Diabetes

Table 1. Sequences for quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) analyses of islets.

Gene	Sequence	Forward primer	Reverse primer
RPLP1	NM_001007604	5′-TCTCTGAGCTTGCCTGCATCTACT-3′	5′-CCTACATTGCAGATGAGGCTTCCA- 3′
Insulin 1 (Ins1)	NM_019129.3	5′-AGCAAGCAGGTCATTGTTCC-3′	5′-ACGACGGGACTTGGGTGTGTA-3′
Insulin 2 (Ins2)	NM_019130.2	5′-GGAGCGTGGATTCTTCTACAC-3′	5′-TGCCAAGGTCTGAAGGTCAC-3′
Amylase (Amy2a3)	NM_031502.1	5′-GAAGCAGACCTTTCATTTTCCAAGAG-3′	5′-GCACAAAACCCCAACCTTCTCC-3′
Ischaemia-responsive protein 94 (IRP94)	AF_077354	5′-GTCTGATGGCTCCAGCTCAAAAGT-3′	5′-GGCTGTTGCTCTTCAGTATGTGGT-3′

was defined as two consecutive, nonfasting, glycaemia measurements ≥19.4 mmol/l (350 mg/dl).

Under inhaled Isofluran (Baxter) anesthesia, an abdominal midline incision was performed on recipient rats. The portal vein was exposed and islets were infused in the portal vein, using constant pressure on the syringe plunger. RIPC recipient animals underwent remote ischaemic preconditioning, as described above, before intraportal islet infusion. Transplantation was performed immediately at the end of the last reperfusion cycle.

The "marginal mass" islet transplantation model, was defined as 7000 IEQ/kg in our hands (as defined after *in vivo* testing of a different numbers of transplanted IEQ/kg). Time to reversal (reflecting the time to engraftment) was the main endpoint. Four groups were studied: control ($n = 14$), donor only RIPC (RIPC in the donor only, $n = 6$), recipient only RIPC (RIPC in the recipient only, $n = 6$), and donor/recipient RIPC (RIPC in both the donor and the recipient, $n = 12$).

The "two-to-one" full yield transplantation model was defined as the transplantation of the total islet yield from two donors to one recipient. Two groups were studied: control ($n = 11$) and donor only RIPC (RIPC in the donor only, $n = 6$).

Nonfasting glycaemia and weight of recipients were assessed thrice weekly until day 31. Diabetes reversal was defined as two consecutive nonfasting glycaemia measures ≤11.1 mmol/l (200 mg/dl).

Intraperitoneal glucose tolerance test (IPGTT)

An IPGTT was performed in all recipients at the moment of sacrifice. Briefly, a 20% glucose solution (Bichsel, Interlaken, Switzerland; lot n° A07038) was injected ip at 2 mg/kg. Glycaemia was measured at 0, 5, 10, 15, 30, 60 and 120 min.

Analysis of liver enzymes

Serum alanine aminotransferase (ALT) was analyzed using the veterinary chemistry analyzer Reflovet™ Plus (Roche). Baseline values were established on healthy and diabetic (at day 3 after diabetes induction) animals. Blood samples were taken and analyzed at days 1, 7 and 31 after transplantation.

ELISA analysis of recipient serum

To determine a possible cause of the adverse results observed in the recipient only RIPC group, serum HMGB1 levels was determined in all four recipient groups at day 1 after transplantation, and in the control and RIPC donors. The HMGB1 ELISA kit (IBL International GMBH, Hamburg, Germany) was used according to manufacturers' instructions.

Statistical analyses

Nonparametric statistical analyses were performed using Prism 7 (Graphpad, La Jolla, CA, USA). Areas under the curve were analyzed for IPGTT results; Mann–Whitney (serum liver enzyme analysis, serum HMGB1 analysis in donors, intraperitoneal glucose tolerance test in the two-to-one full yield transplantation model), Friedman (*ex vivo* analysis of islet supernatant HMGB1 content between 6 h and 24 h of culture), Wilcoxon (*ex vivo* experiments where two groups were analyzed), Mantel-Cox (reversal curve in the marginal mass model), Fisher's exact test (reversal curve in the two-to-one full yield model), two-way ANOVA (liver enzyme analysis in recipients in the marginal mass model) and Kruskal–Wallis (intraperitoneal glucose tolerance test in the marginal mass model, serum HMGB1 analysis) tests were used. All *P*-values are two-tailed; significance was set at 5%. Results in charts are expressed as median with maximum and minimum in box plots.

Results

Islet yield, metabolic function and gene expression

On the day of isolation, islet counts showed no difference in islet ($P = 0.45$) or IEQ yield ($P = 0.70$, Fig. 1a) between control and RIPC donors ($n = 10$). However, islet ($P = 0.02$) and IEQ yields ($P = 0.007$, Fig. 1b) were increased after 24 h of culture with RIPC donors ($n = 30$). Compared to control donors, performing RIPC increased IEQ yield by 20% after 24 h of culture. Overall islet size did not differ significantly between control and RIPC donors after isolation (Figure S1), as demonstrated by the IEQ/islet ratio in Fig. 1c ($P = 0.20$).

Ex vivo insulin secretion ($n = 6$) was similar between islets in both groups when stimulated ($P = 0.63$, Fig. 1d).

Culture supernatant ELISA analysis ($n = 11$) showed decreased cell death in islets isolated from RIPC donors ($P = 0.049$, Fig. 2a).

Performing RIPC on donors decreased the expression of hypoxia-related genes, Hif1a ($P = 0.04$) and IRP94 ($P = 0.008$), in islets and increased gene expression of HMGB1 ($P = 0.04$), Fig. 2b ($n = 8$). This is further confirmed by the analysis of HMGB1-protein content

Transplant International 2019; 32: 323–333

($n = 11$), where islets isolated from RIPC donors contain more HMGB1 after 24 h of culture ($P = 0.04$, Fig. 2c). Immunofluorescence analysis showed that HMGB1 is mostly found in the nucleus of islets isolated from RIPC donors, whereas it is mostly present in the cytoplasm of islets from control donors (Fig. 2d). ELISA supernatant analysis found similar HMGB1 levels 6 h after isolation between the two groups ($P = 0.43$), but a trend towards increased HMGB1 levels after 24 h of culture of RIPC donor-isolated islets ($P = 0.08$). We observed a significant increase in supernatant HMGB1 content ($n = 10$) between 6 and 24 h of culture in the RIPC donor group ($P = 0.004$, Fig. 2e).

There was no significant difference in other gene expressions between the two groups (data not shown): tissue factor (F3) $P > 0.99$, ICAM-1 $P = 0.55$. There was no purity difference, with similar Ins1 and Ins2 ($P = 0.58$) and Amy2a3 ($P = 0.69$) gene expressions.

Marginal mass islet transplantation

Diabetes reversal curves were not statistically different in the control, donor only RIPC and donor/recipient RIPC groups. However, 50% of recipients were cured from diabetes in the donor only RIPC and donor/recipient RIPC groups, and 28.6% in the control group. None of the recipient only RIPC animals were cured from diabetes ($P = 0.048$ when compared with donor only RIPC, and $P = 0.052$ when compared with donor/recipient RIPC) (Fig. 3a). This is further confirmed by similar blood glucose profiles in the three groups, and persistently high blood glucose levels in the recipient only RIPC group (Fig. 3b). In addition, all groups had similar patterns of weight gain, except in the recipient only RIPC group which showed a break in weight gain starting at 3 weeks after islet transplantation (Fig. 3c).

Immunofluorescence analysis confirmed that pancreata of cured animals did not undergo β-cell regeneration (not shown).

In vivo metabolic activity

Intraperitoneal glucose tolerance tests performed at day 31 after islet transplantation showed no significant differences between groups (Fig. 3d). One recipient from the control, donor only RIPC and donor-recipient RIPC groups was excluded from analysis after accidental glucose injections in the caecum; in the recipient only RIPC group, one animal was excluded from analysis as it had to be sacrificed because of its poor tolerance to acute hyperglycaemia. When only looking at cured recipients, we observed the same results between the control, donor only RIPC and donor-recipient RIPC groups (not shown).

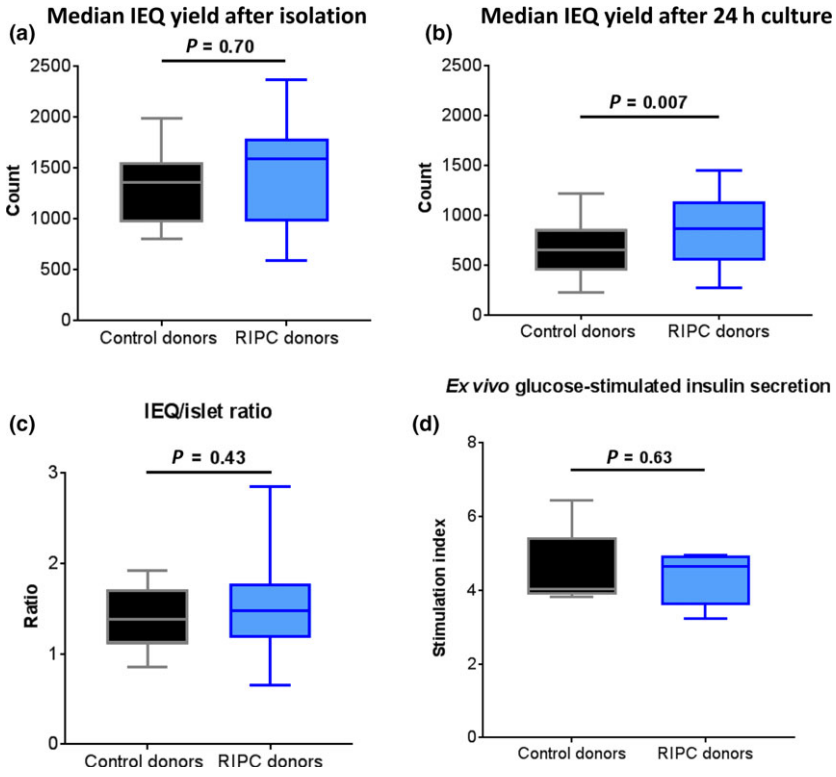

Figure 1 Comparison of islet equivalent (IEQ) yield and ex vivo metabolic activity in remote ischaemic preconditioned (RIPC) and control donors. (a) No difference of IEQ yield immediately after isolation between RIPC and control donors $P = 0.70$. (b) Increased IEQ yield after 24 h of culture in RIPC donors, $P = 0.007$. (c) IEQ to absolute islet number ratio, $P = 0.43$. (d) Glucose-stimulated insulin secretion (GSIS) analysis between remote ischaemia preconditioned (RIPC) and control donor islets $P = 0.63$.

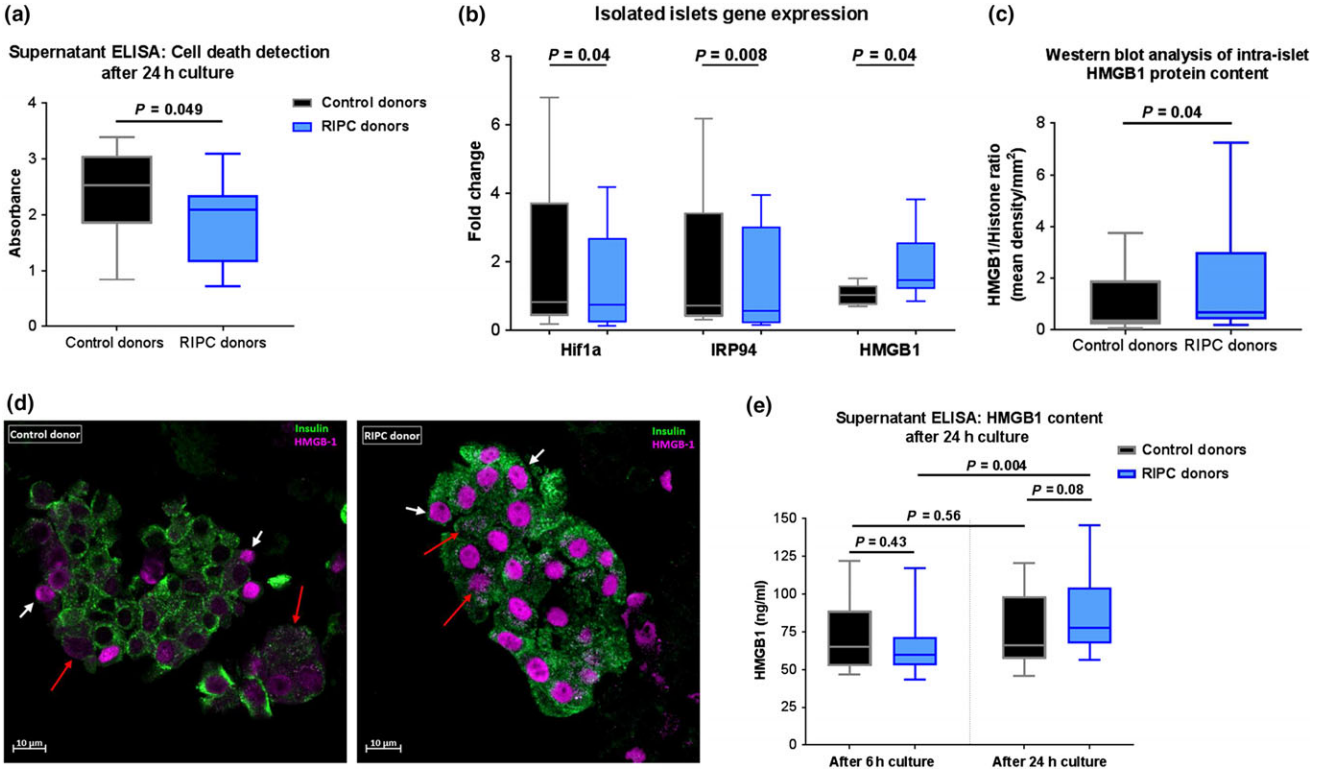

Figure 2 *Ex vivo* analyses of islet cell death, gene expression, and high mobility group box-1 (HMGB1) protein content. (a) Decreased markers of cell death in cell culture supernatant of RIPC donor-isolated islets compared to controls, $P = 0.08$. (b) Quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) analysis of the expression of hypoxia-inducible factor (Hif1a) ($P = 0.04$), ischaemia responsive protein 94 (IRP94) ($P = 0.008$), and high mobility group box-1 (HMGB1) ($P = 0.04$) between RIPC and control donor islets. (c) Western blot analysis of islet HMGB1 protein content, with increased RIPC donor-isolated islet HMGB1 content, $P = 0.04$. (d) Immunofluorescence staining (HMGB1 and insulin) of isolated islets from control or RIPC donors. Short white arrows point to nuclear HMGB1; long red arrows point to cytoplasmic HMGB1 in dying cells. (e) Supernatant content of HMGB1 shows a trend towards increased levels in the supernatant from RIPC islets at 24 h of culture, $P = 0.08$.

Liver function tests

To define the impact of RIPC on liver ischaemia-reperfusion injury, we assessed the serum levels of liver enzymes after transplantation. Compared to baseline levels, serum ALT was increased in rats 3 days after streptozotocin injection ($P < 0.0001$, Figure S2a). Overall, mid- to long-term impaired blood glucose control was associated with increased ALT levels, as can be observed between cured versus not-cured recipients after islet transplantation (Figure S2a).

This point in mind, we further analyzed all recipients and did not observe an early ALT increase after transplantation (Figure S2b). However, when compared to control recipients, performing RIPC in recipients only was subsequently associated with increased ALT levels at days 7 and 31 ($P = 0.002$, and $P = 0.02$). Conversely, compared with the recipient only RIPC group, the donor/recipient RIPC group displayed decreased ALT levels at day 31 ($P = 0.002$).

Of note, when analyzing cured recipients only, we also observed a better profile in the donor/recipient RIPC group at day 31 (control-donor/recipient RIPC $P = 0.04$; donor only RIPC donor/recipient RIPC $P = 0.005$; data not shown).

Serum HMGB1 ELISA analyses

In an effort to explore the cause behind the worse post-transplant outcomes in the recipient only RIPC group, we performed an HMGB1 ELISA analysis of recipient sera at day 1 after transplantation. There was an increase in serum HMGB1 in the recipient only RIPC group compared to all three other groups (control $P = 0.02$, donor only RIPC $P = 0.03$ group, and donor/recipient RIPC group $P = 0.07$, Fig. 4).

There was no correlation between HMGB1 and ALT levels at day 1 after transplantation (Figure S3a).

To determine whether the increase in early circulating HMGB1 was as a result of the RIPC process in the

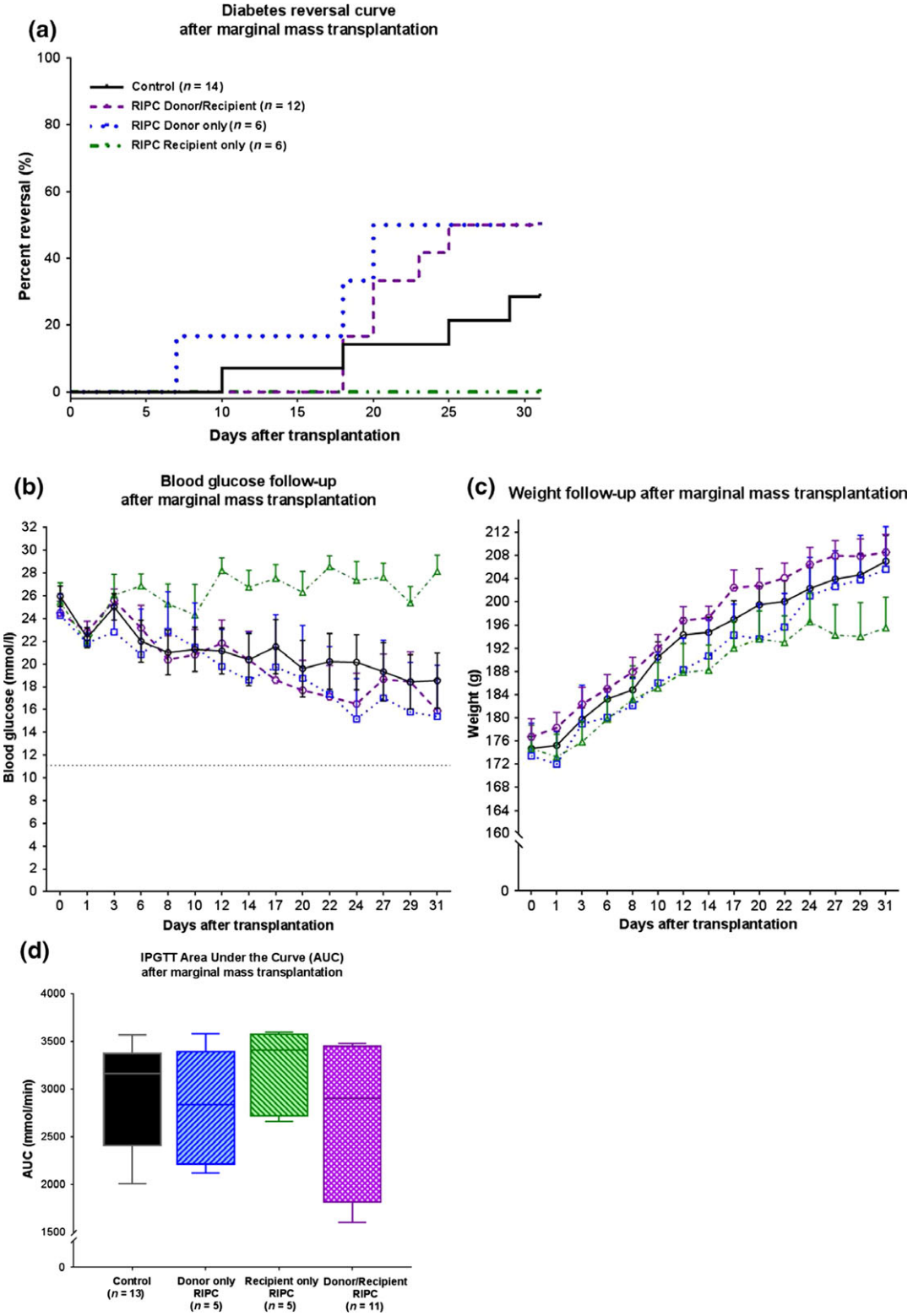

Figure 3 Follow-up and *in vivo* metabolic activity of marginal mass recipients. (a) Time to diabetes reversal (recipient only remote ischaemic preconditioning (RIPC) versus donor only RIPC $P = 0.048$; recipient only RIPC versus donor/recipient RIPC $P = 0.052$). (b) Glycaemia follow-up, high blood glucose throughout follow-up in the recipient only RIPC group. (c) Weight follow-up, break in the weight gain curve in the recipient only RIPC group starting at 3 weeks after transplantation. (d) Area under the curve (AUC) of the intra-peritoneal glucose tolerance test in all groups: control versus donor only RIPC $P = 0.78$; control versus recipient only RIPC $P = 0.31$; control versus donor/recipient RIPC $P = 0.49$; donor only RIPC versus recipient only RIPC $P = 0.28$; donor only RIPC versus donor/recipient RIPC $P = 0.79$; recipient only RIPC versus donor/recipient RIPC $P = 0.13$.

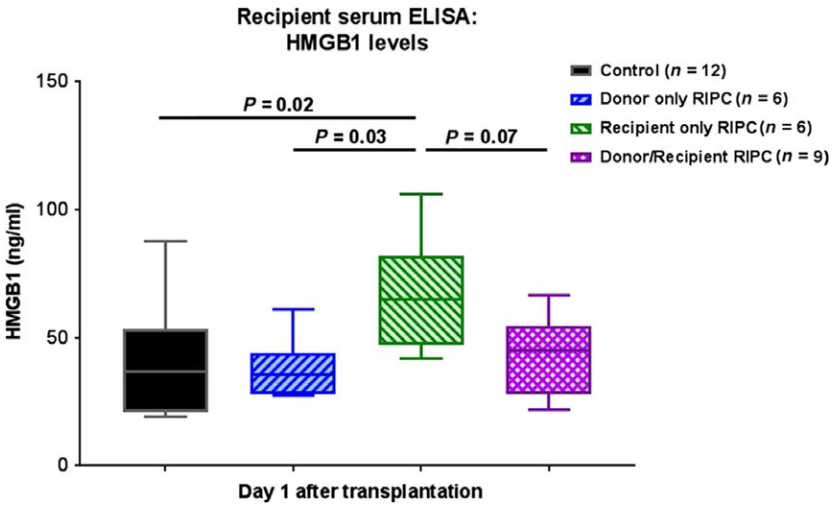

Figure 4 Serum analysis of high mobility group box-1 (HMGB1) content in recipients at day 1 after transplantation. Control versus recipient only remote ischaemic preconditioning (RIPC) $P = 0.02$, donor only RIPC versus recipient only RIPC $P = 0.03$, recipient only RIPC versus donor/recipient RIPC $P = 0.07$.

recipient, ELISA analyses of serum from control donors or RIPC donors (5 min after the final reperfusion) were performed ($n = 12$). However, there was no immediate increase in HMGB1 levels ($P = 0.93$, Figure S3b).

Two-to-one full yield transplantation

To strive for clinical relevance, where recipients most often receive islets from two or more donors to attain insulin independence, we performed an experiment where islets isolated from two donors, either control or having undergone RIPC, were transplanted into one recipient. The donor only RIPC group (100% reversal) demonstrated a trend towards improved diabetes reversal compared to the control group (55% of recipients) ($P = 0.10$). Furthermore, glycaemic control was improved in the donor only RIPC group (Fig. 5a). The IPGTT showed a trend towards improved metabolic control in the donor only RIPC group ($P = 0.22$, Fig. 5b); only five of the six recipients in the donor only RIPC group were analyzed as one intraperitoneal injection was accidentally performed in the caecum.

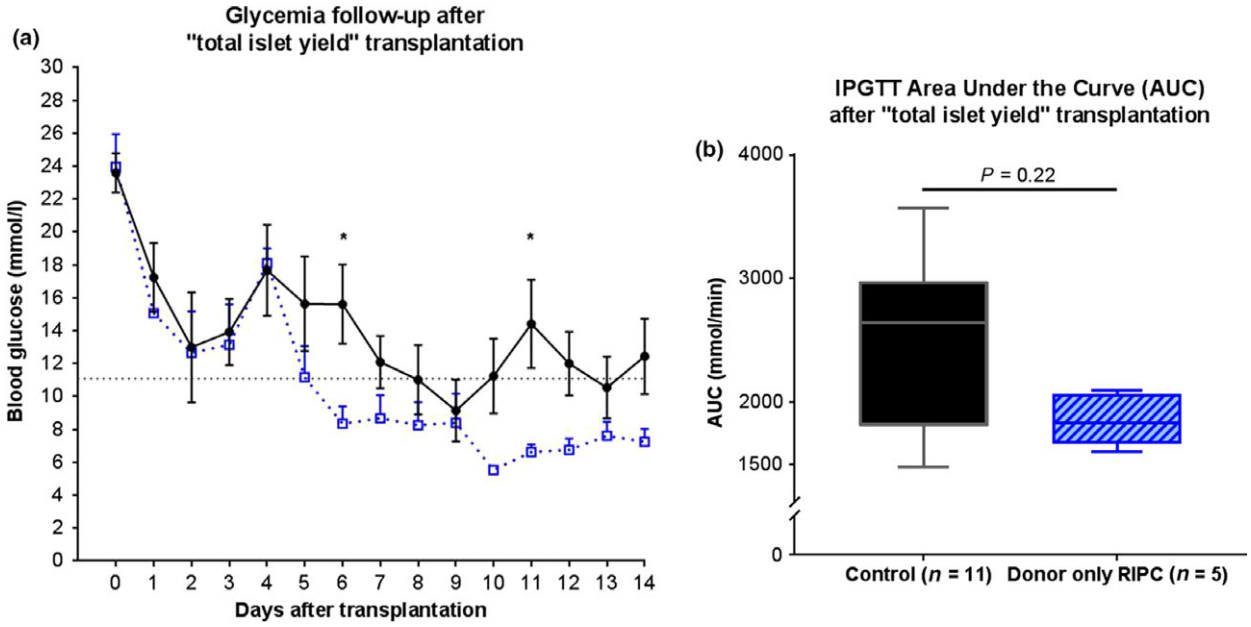

Figure 5 Follow-up and *in vivo* metabolic activity of "full yield" recipients. (a) Glycaemia follow-up. Improved glycaemia control in the donor only RIPC group (at day 6 $P = 0.02$, day 11 $P = 0.04$) (b) Area under the curve (AUC) of the intra-peritoneal glucose tolerance test (IPGTT). Control versus donor only RIPC $P = 0.22$.

Discussion

Performing remote ischaemic preconditioning (RIPC) in donors confers protection to the isolated islets, with an increased resistance to post-isolation hypoxia. The net result translates into a 20% increase in islet recovery after 24 h of culture compared to controls. This effect is supported by a decrease in the expression of Hif1a and IRP94, two markers linked to hypoxia and/or ischaemia [10–12]. Our results are comparable to those of a previous study on the impact of local ischaemic preconditioning (IPC), of the pancreas, on isolated islets [6]. As in our study, this procedure allowed the recovery of 20% more islets in the IPC group compared to the control group after overnight culture, with improved *in vivo* metabolic control in the IPC group in a full mass model. The advantage of RIPC is that it can be carried out in parallel to organ retrieval without increasing the duration of surgery. Performing RIPC in organ donors, specifically for better islet resistance to hypoxia, would not be detrimental to other retrieved organs; indeed, most studies to date demonstrate a somewhat beneficial effect of such an intervention to solid organs in the transplantation setting [13–16].

In the marginal mass model, we did not find any difference in metabolic function between the control group and the donor only RIPC group. However, when performing "two-to-one" full yield transplantation we observed improved metabolic control in the donor only RIPC group. These data suggest that although the islets are not more metabolically active, the RIPC procedure in donors allows for increased numbers of transplantable islets. This result is particularly encouraging, and a similar experiment should be carried out in an allogeneic animal model to strive towards translation to clinical practice.

In an effort to understand the mechanism behind the improved resistance to hypoxia, we found that islets isolated from RIPC donors have an increase in HMGB1 gene expression and protein content. HMGB1 is a protein with complex roles in a multitude of vastly different and paradoxical pathways, which can explain some of the presently observed features. When HMGB1 is present in the nucleus, it acts as a DNA chaperone, and a nuclear overexpression of HMGB1 has been shown to trigger anti-apoptotic pathways [17–20]. Through immunofluorescence analysis, we observed that islets isolated from RIPC donors have more nuclear HMGB1 than those from control donors, suggesting conserved islet integrity, further hinted at by decreased cell death in the culture medium of islets isolated from RIPC donors.

Performing RIPC in recipients only has surprisingly proven to be extremely harmful to the islet graft. This was quite unforeseen as a previous study had shown a beneficial impact of local liver IPC, by intermittent clamping of the portal vein, on intraportally transplanted islets [4]. Our data show significantly increased HMGB1 levels at day 1 in recipients having undergone RIPC and transplanted with islets from control donors. In this context, the dual role of HMGB1 may help to understand the observed negative effect. When cells die, HMGB1 is trafficked from the nucleus to the cytoplasm, and is later released in the extracellular compartment (either passively by necrotic cells; or actively by activated macrophages and NK cells, and mature dendritic cells, refs. 17,20,21). The RIPC procedure itself can lead

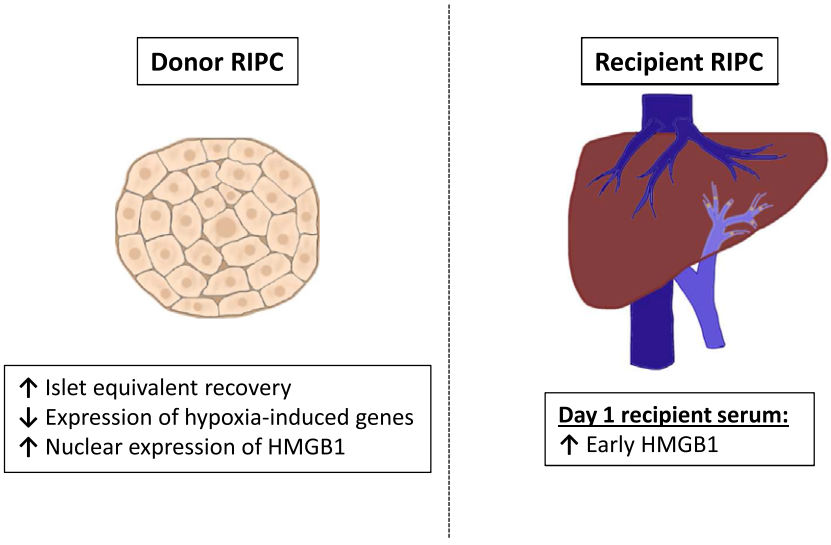

Figure 6 Summary of the effects of remote ischaemic preconditioning (RIPC) on donors and islets, and on recipients.

to an increase in serum levels of HMGB1 [22]. In addition, the higher serum HMGB1 levels in this group could be the consequence of an acute release by damaged islets. However, the observed increase in serum HMGB1 was not correlated with ALT levels suggesting that it was likely not of liver origin.

Whatever its origin, acute extracellular HMGB1 acts as a damage associated molecular pattern (DAMP), promoting inflammation by binding to the receptor for advanced glycation products (RAGE) and toll-like receptors (TLR), notably TLR2 and TLR4 [23], and is known to be involved in the early loss of transplanted islets [24–26]. We can therefore hypothesize that, in the setting of intraportal islet transplantation, performing RIPC in recipients creates a harmful cycle where the acute increase in circulating HMGB1, induced by the remote ischaemic preconditioning procedure, harms the intraportally transplanted islets, which in turn passively release their high content of HMGB1 [24] and so on, thus maintaining a local inflammatory state.

Overall, performing RIPC in the donors confers increased protection against short-term post-isolation hypoxia, with a 20% increase in islet yield after 24 hours of culture, and improves glycaemic control when transplanted in a full mass syngeneic setting. However, when performed immediately prior to transplantation, recipient RIPC seems to be extremely harmful to the intraportally transplanted islets, and should not be tried in the clinical setting (Fig. 6). Islets isolated from RIPC donors seem to have further unexplained protective effects, notably in mitigating the harmful effects of recipient RIPC, as was observed in the donor-recipient RIPC group.

Most islet recipients need two to three transplantations to hope for insulin-independence [2]. Therefore, although islets isolated from two pancreata would still be needed in order to attain the required islet equivalent mass for clinical transplantation, RIPC could increase the number of islet recipients by decreasing the number transplantations needed to achieve insulin independence. Validation of donor only RIPC in allogeneic animal models is needed before moving on to clinical studies.

Authorship

VD: designed and performed the experiments, analyzed the data, wrote the paper. SL, CT: designed the experiments, crucial scientific input. QG, FS, AK-Q: collected the data, performed sample analysis. VL: crucial scientific input. AP, LAO, TB: crucial revision and editing of the paper.

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Conflicts of interest

The authors have declared no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Control versus remote ischaemic preconditioned (RIPC) donor islet sizes expressed as a percentage of the total number of islets.

Figure S2. Liver enzyme follow-up after intraportal islet transplantation in all four groups.

Figure S3. (a) Correlation analysis between serum high-mobility group box-1 (HMGB1) and alanine aminotransferase (ALT) levels at day 1 after transplantation. No correlation was found ($P = 0.36$). (b) Donor serum high mobility group box-1 (HMGB1) ELISA analyses: no difference in immediate HMGB1 levels between the two groups, $P = 0.93$.

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