ORIGINAL ARTICLE

Efficacy of the natural oxygen transporter HEMO$_2$life® in cold preservation in a preclinical porcine model of donation after cardiac death

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SUMMARY

The growing use of marginal organs for transplantation pushes current preservation methods toward their limits, and the need for improvement is pressing. We previously demonstrated the benefits of M101, a natural extracellular oxygen carrier compatible with hypothermia, for the preservation of healthy renal grafts in a porcine model of autotransplantation. Herein, we use a variant of this preclinical model to evaluate M101 potential benefits both in static cold storage (CS) and in machine perfusion (MP) preservation in the transplantation outcomes for marginal kidneys. In the CS arm, despite the absence of obvious benefits within the first 2 weeks of follow-up, M101 dose-dependently improved long-term function, normalizing creatininemia after 1 and 3 months. In the MP arm, M101 improved short- and long-term functional outcomes as well as tissue integrity. Importantly, we provide evidence for the additivity of MP and M101 functional effects, showing that the addition of the compound further improves organ preservation, by reducing short-term function loss, with no loss of function or tissue integrity recorded throughout the follow-up. Extending previous observations with healthy kidneys, the present results point at the M101 oxygen carrier as a viable strategy to improve current organ preservation methods in marginal organ transplantation.

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Key words
cold preservation, donation after circulatory death, graft preservation, hypothermic machine perfusion, ischemia reperfusion injury, kidney transplantation, oxygen, oxygen carrier, static cold storage

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Introduction

Transplantation is the optimal therapy for end-stage organ failure. However, the growing use of this efficient therapy led to organ shortage and therefore the extension of donor criteria and the creation of extended donors (ECD) designation [1]. These include donors aged over 60, and donors aged 50–59 with at least two out of three additional risk factors prior to transplantation, including cerebrovascular accident as a cause of death and history of hypertension with serum creatinine above 1.5 mg/dl (130 μmol/l). However, this proved insufficient to fufill the ever-growing need for organs, and additional marginal donor categories have been defined, including the deceased after circulatory death (DCD) donor [2–4]. But these organs are particularly fragile and prone to

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ischemia-reperfusion injuries (IRI) [5], which exert an established negative impact on short- [6] and long-term [7] transplantation outcomes. Indeed, warm ischemia (WI), especially when combined with cold preservation, is correlated to increased rates of primary nonfunction (PNF) and delayed graft function (DGF) [8,9].

Increased rates of complications highlight the fact that current organ preservation techniques are not adapted to the new donor demographics. Thus, novel methods or therapeutics are needed to better preserve these organs and, therefore, optimize graft quality and clinical outcome.

One improvement avenue is machine perfusion (MP), a technology which has resurfaced and dominates current research on organ preservation [10]. In clinics, this technique has demonstrated benefits in terms of reducing risk of delayed graft function and graft failure as well as improving graft survival in the first year after transplantation [11,12]. However, post-transplantation complications remain important, and further improvements are needed.

In this context, bringing adequate oxygen to the organ would constitute a major improvement [13]. The combination of MP with active oxygenation *via* an oxygen carrier appears both physiologic and appealing [14–19], in addition to being a straightforward approach potentially and rapidly applicable in clinics, without major changes in protocol and devices.

We previously demonstrated the benefits of M101, a naturally occurring respiratory pigment during cold static preservation [20,21]. This molecule, extracted from a marine invertebrate, *Arenicola marina* [22–24], is a high molecular weight (≈3600 kDa) extracellular hemoglobin. This pigment is composed of 156 globins and 44 nonglobin linker chains exhibiting both a high oxygen affinity (P_{50} = ~2 mmHg, in the 4–15 °C hypothermic range, n_{50} = 2.5) and a large nominal O_2 binding capacity, with 156 O_2 binding sites [23]. Thus, its specific carrying capacity (43 μmol/g) is comparable to human hemoglobin (HbA; 62 μmol/g). Moreover, whereas M101 releases O_2 according to a simple gradient, as does HbA, it does not require any allosteric effector and operates in a broader range of temperature since *A. marina* is an intertidal poikilotherm species [22]. The Hb M101 has been developed as a class III medical device ($HEMO_2life^{®}$) for supplementing preservation solutions during organ storage before transplantation [21,24,25]. Concerning kidney preservation, beneficial effects were already demonstrated in large animal kidney transplantation models with healthy organs—subjected to 24 h preservation as well as during the clinical trial OXYOP (ClinicalTrials.gov Identifier: NCT02652520). The present study aimed at confirming the benefits of using M101 in organ transplantation, in organs that are particularly sensitive to IRI. Thus, we subjected kidneys to 60-min warm ischemia prior to preservation, a protocol previously demonstrated to increase post-transplant complications to levels encountered in ECD and DCD transplantation [7,26,27]. As clinical practice increasingly includes MP in the management of such organs, we separated our study into two preservation arms: static cold storage (CS) and hypothermic MP. The putative benefits of increasing doses of M101 were evaluated on organ function recovery and transplantation outcomes, both early and late after transplantation.

Materials and methods

Animals and ethical regulations

Large White Male pigs (3 months old), weighing 35–40 kg [28], were bred onsite at the IBiSA Platform Experimental Surgery and Transplantation (MOPICT), located at the Institut National de la Recherche Agronomique (INRA) campus (Surgères, France), where surgery, organ procurement, and euthanasia were carried out. Surgical and experimental protocols were performed in accordance with the guidelines of the French Ministry of Agriculture (FMA) for the use and care of laboratories animals, after approval by the FMA, the INRA, and the Poitou-Charentes ethical committee for animal experimentation (Accreditation number C2EA-84). A total of 60 animals were used in the study: (i) 42 in experimental arms, CS (n = 21) and MP (n = 21), (ii) 11 in the SHAM and NEP groups (5 and 6, respectively), in addition to (iii), seven animals that were lost during the CS and MP protocols, (3 and 4, respectively). The later were euthanized, under anesthesia, before the end of the protocol, for peri- and postoperation causes, unrelated to preservation method or to M101 treatment (postbiopsy bleeding, volvulus, and/or intestinal occlusion, pulmonary syndrome, infection).

Warm ischemia, experimental design, and groups

Surgical and kidney procurement have been described in detail [29,30]. Briefly, after general anesthesia, orotracheal intubation was performed for mechanical ventilation in volume-controlled mode.

In situ warm ischemia: After a midline abdominal incision, the right renal pedicle was clamped for 60 min (WI; see Fig. 1). Then, the kidneys were excised by dissection of the vascular pedicle and ureter. After WI, kidneys were flushed gravimetrically (1 m, ~75 mmHg) with 500 ml ice-cold UW® or KPS®, for CS and MP

preservation respectively. CS kidneys were preserved in UW® solution in ice, while MP kidneys were mounted on the LifePort® machine (LifePort® Kidney Transporter 1.0; ORS, Brussels, Belgium), set at 35 mmHg of perfusion pressure and continually perfused with KPS® at 4 °C. Average preservation time was 23 h ± 30 min. For the CS arms, three groups were studied, using increasing doses (g/l) of M101 in the UW solution during storage: CS-0 (Control), CS-1 (1 g/l M101), CS-2 (2 g/l M101); $n = 6$ animals were used in each group. For the MP arms, three groups were studied, using increasing the same doses of M101 in the KPS solution during storage: MP-0 (Control), MP-1 (1 g/l M101), MP-2 (2 g/l M101; $n = 6$ *per* group, except $n = 5$ for MP-1). The experimental protocol is described in Fig. 1. Sham-operated (SHAM) and nephrectomized (NEP) animals were used as surgery and nephron mass controls, respectively ($n = 5$ and 6, respectively). Subsequent autotransplantation was carried out, with contralateral nephrectomy. Post-transplantation follow-up lasted 3 months.

HEMO$_2$life® (M101) oxygen carrier: storage and use

Purified M101 *A. marina* hemoglobin (HEMO$_2$life®; Hemarina SA, Morlaix, Brittany, France) was stored at −80 °C. Before use, M101 was thawed 60 min at room temperature and diluted at the target concentrations in UW medium (University of Wisconsin; Bridge to Life, London, UK) for CS, or in KPS (Kidney Preservation Solution; Organ Recovery Systems) for MP.

Early renal graft function assessment

Animals were placed in metabolic cages after autotransplantation to collect urine and to perform blood sampling. Biochemical parameters (e.g., serum and urinary creatinine, urinary proteins) were measured during the 3-month post-transplantation follow-up at the Poitiers Teaching Hospital technical platform (Service de Biochimie, Pôle Biospharm, Poitiers, France) on an automatic analyzer (Cobas 8000; Roche Diagnostic, Meylan, France).

Renal tissue ATP measurement after preservation

The ATP content from renal cortical tissue was determined in biopsies performed before and after warm ischemia, and at the end of the preservation phase. Samples were immediately frozen in liquid nitrogen, and ATP content of 40–70 mg tissue samples was measured with the Bioluminescent Assay kit (Sigma®), after TCA extraction. The final ATP concentration was expressed as nmol/mg of tissue.

Western blot analysis of tissue after preservation

Western blot analysis was performed to evaluate cortical expression of VEGF (C-1; sc-7269; Santa Cruz, Clini-Sciences, Nanterre, France). Briefly, flash frozen cortical tissues were dissected; lysed, and total protein content from cytosolic supernatant was measured by the BCA assay (BCA1-1KT, Bicinchoninic Acid Kit for protein determination; SIGMA-Aldrich, St-Quentin Fallavier, France). About 50 µg total protein was used into SDS-PAGE gels. VEGF expression was normalized to GAPDH [Anti-GAPDH (6C5), Calbiochem] level.

Morphological analysis

At sacrifice (M3), pieces of renal tissue were cut and immersed into 4% buffered formalin for fixation, before inclusion and slicing. After Red Sirius staining, whole sections were photographed with a macroscope (×0.63), and the percentage of interstitial fibrosis was quantified using VISOLOG software (v6.9).

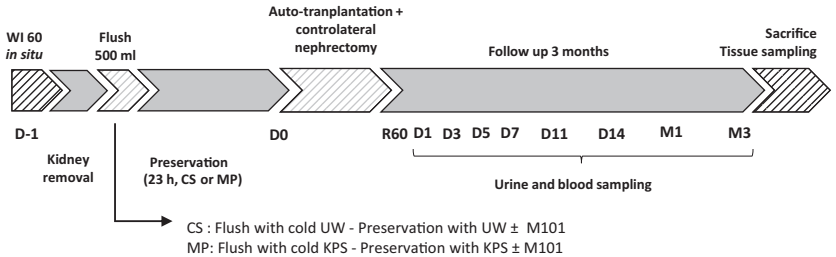

Figure 1 Experimental diagram. At day −1, WI60 was carried out *in situ*, then kidneys were removed for cold flush and submitted to CS or MP during 23 h, with or without M101 supplementation at 1 and 2 g/l. Then, at D0 contralateral nephrectomy and autotransplantation were carried out. The animals were followed up during 3-month post-transplantation. At the end of follow-up (M3), the animals were euthanized and tissue sampling was realized for western blot tissue analysis and histological analyses. CS, static cold storage; KPS, kidney perfusion solution; MP, hypothermic machine perfusion (ORS, Lifeport 1,0); R60, 60 min after reperfusion; UW, University of Wisconsin solution.

Data and statistical analysis

Results are shown as mean ± SD of $n = 6$ per group, in all but two cases (SHAM and MP 1 g/l, $n = 5$). For the statistical analysis among groups, we used the non-parametric Kruskal–Wallis rank sum test for multiple samples, followed when appropriate (P-value <0.05) by the *post hoc* Dunn's Test; Mann–Whitney test was used for two-group comparisons. Statistical tests calculations were performed with R-STUDIO (v1.1.463; see also https://rcompanion.org/) and further confirmed with the online *Astatsa* tool (http://astatsa.com/). Statistical significance was accepted for $P < 0.05$.

Results

ATP concentration and VEGF expression at the end of preservation

To evaluate the impact of oxygen provided by M101 on the organ in terms of energy metabolism and vascular bed, we determined ATP concentration and VEGF expression, both before (End-WI) and after preservation.

Cortical tissue ATP was assessed in the healthy control (no ischemia, CTL) and WI60 (End-WI) groups, as well as in CS and MP preservation groups. Figure 2 (upper panels) shows that, compared with the healthy control group (CTL), the WI60 group exhibited a 88% ATP reduction after warm ischemia (End-WI), from 2.60 to 0.33 nmol/mg tissue (Mann–Whitney $W = 25$, $P = 0.002525$).

Cold storage arm

Kruskal–Wallis rank test comparison of preservation groups and End-WI ATP yielded a significant P-value (Chi-squared statistic = 12.6208, $P = 0.00553$), and Dunn's *post hoc* test indicated an additional, significant decrease of ATP during CS preservation without M101 (CS 0 g/l), from 0.33 to 0.06 nmol/mg tissue in the untreated CS group ($P < 0.05$; Fig. 2, panel a). No difference was observed in the presence of M101.

Figure 2 presents cortical VEGF expression (panel c). Kruskal–Wallis analysis (Chi-squared statistic = 11.2956, $P = 0.01023$) showed a significant (~2-fold) increase in the untreated CS group (CS 0 g/l) versus the healthy CTL group (Dunn's $P = 0.03035$), and a significant reduction of VEGF expression in the 2 g/l M101 group to the control (CTL) level ($P = 0.002209$, Fig. 2c).

Machine perfusion arm

Kruskal–Wallis comparison of tissue ATP in the postpreservation and End-WI groups indicated significant differences between these four groups (Chi-squared = 12.15099, $P = 0.00688$); Dunn's post-test further indicated significant increases of tissue ATP in all three preservation groups versus the End-WI group from 0.32 to 5.4 ($P = 0.0130$), 5.4 ($P = 0.01569$) and 7.8 ($P = 0.004874$) nmol/mg tissue, respectively (Fig. 2, right panels, b and d). Including the healthy CTL group, a global (five groups) Kruskal–Wallis analysis (Chi-squared = 19.37589, $P = 0.000633$) showed that all three MP preservation groups tended to have 2–3 fold higher ATP levels (~5.4 to 7.8 nmol/g tissue) than the CTL group (2.6 nmol/g tissue); however, only the 2 g/l-treated group reached significantly higher levels (7.8 nmol/g tissue, Dunn's $P = 0.009641$ vs. CTL).

VEGF expression in the MP preservation arm is summarized in Fig. 2d. MP appeared to increase (~50%) the level of VEGF tissue expression, as compared with CTL (2.1 ± 0.3 vs. 1.4 ± 0.2, arbitrary units, a.u.). Likewise, the addition of M101 further increased VEGF expression level during MP perfusion (5.1 ± 1.0 and 5.9 ± 2.1 a.u., for 1 and 2 g/l, respectively). Kruskal–Wallis rank comparison was significant (Chi-squared 18.4094, $P = 0.000362$). *Post hoc* Dunn's test confirmed that while the untreated MP versus CTL groups comparison proved unsignificant (Dunn's $P = 0.211326$), both 1 and 2 g/l M101-treated groups significantly potentiated VEGF increase versus MP 0 g/l (Dunn's $P = 0.029954$ and 0.012032, respectively). Of note, the increase levels of VEGF in the MP 1 and 2 g/l groups were also significant when compared with the healthy CTL group (Dunn's $P = 0.000901$ and 0.000268, respectively).

Short-term outcomes

To evaluate putative benefits of using the oxygen carrier M101 during fragile organ preservation, kidneys were autotransplanted and renal function was evaluated in conscious transplanted pigs along a 3-month follow-up.

Cold storage arm

The kidney function recovery phase was characterized by increased serum creatinine, peaking at D3 (CS 0 and 2 g/l) or D5 (CS 1 g/l) at approximately 1200 μmol/l. Then, creatinine level gradually decreased reaching the (quasi-normal) 100–300 μmol/l range at D14 (Fig. 3a,

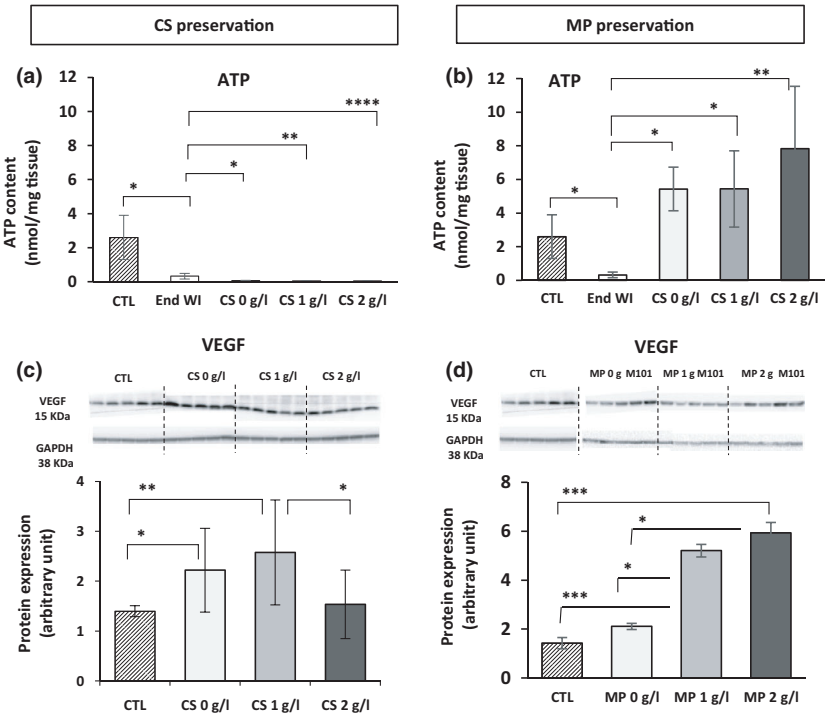

Figure 2 Cortical ATP and VEGF expression at the end of CS and MP. Cortical tissue ATP was assessed in the healthy control (no ischemia, CTL) and WI60 (End-WI) groups, as well as in CS (a) and MP (b) preservation groups. Statistical comparisons were carried out in two steps, as per protocol. Uninjured control group was compared with WI60 group, showing an 88% ATP reduction after warm ischemia, from 2.6 to 0.4 nmol/g tissue ($P = 0.0124$). CS arm: Kruskal–Wallis comparison of preservation groups and end-WI ATP yielded an F statistic of 16.1793 ($P = 0.000018$), and Tukey post-test indicated an additional, significant decrease of ATP during CS preservation (from 0.32 to 40–60 nmol/mg tissue, $P < 0.05$). MP arm: comparison of postpreservation groups and end-WI ATP yielded $F = 9.4512$ ($P = 0.0006$); Tukey post-test indicated a significant increase of tissue ATP in all three preservation groups, from 0.3 to 5.4 ($P = 0.015$), 5.4 ($P = 0.011$) and 7.8 nmol/g tissue ($P = 0.0010$), respectively. VEGF expression was measured in cortical tissue for each group in both (CS) (c) and MP (d) and in healthy control group (without ischemia, CTL). Means ± SD ($n = 5$ or 6). Kruskal–Wallis analysis (Chi-squared = 11.2956, $P = 0.01023$) indicated a significant increase in the untreated CS group (Dunn's $P = 0.03035$), and a significant reduction in the 2 g/l group ($P = 0.002209$). CS, cold storage; MP, machine perfusion. Statistics *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

upper panel). Although M101 groups appeared lower than the untreated group at D11–D14 (e.g., at D14, 282 ± 225 188 ± 48, and 104 ± 19 μmol/l, in the 0, 1, and 2 g/l M101, respectively), comparison of D0–D14 area under the curve did not reveal differences between the groups (lower panel). Table 1 reports all serum creatinine values, including the short-term window (D-1–D14), along the whole follow-up of the CS preservation groups; SHAM and NEP groups are also presented. Kidney function recovery was also evaluated on the basis of sodium excretion measurements between D1 and D7 (Fig. 3c). Fractional excretion of sodium remained in the 0.3–1.2% range throughout the protocol (0.65 ± 0.31%, $n = 8$ time-points between D-1 and M3) in the SHAM group, and in the 0.2–2.9% range in the NEP group (0.85 ± 0.59%, $n = 8$).

While, in the experimental groups, grafts exhibited a markedly reduced function at D1 (70–90% excretion), all groups recovered toward ~10% to 15% fractional excretion along the first week (Fig. 3c, upper panel). However, AUC comparison showed no difference between groups (Fig. 3c, lower panel; missing values at D1 are because of the absence of urine production at this time-point).

Machine perfusion arm

Post-transplantation serum creatinine reached similar level in the 0 and 1 g/l M101 groups (800–1000 μM; see Fig. 3b, upper panel). The control group remained in that high range between D1 and D7, evidencing some recovery only at D11 and D14. However, the 1 g/l M101 group showed a seemingly accelerated recovery (by 4–6 days). Moreover, the MP 2 g/l M101 group exhibited an marked improvement of function recovery, since at D5 serum creatinine peaked 341 ± 72 μM (vs. ~800 to 1000 μM in the other groups) followed by rapid

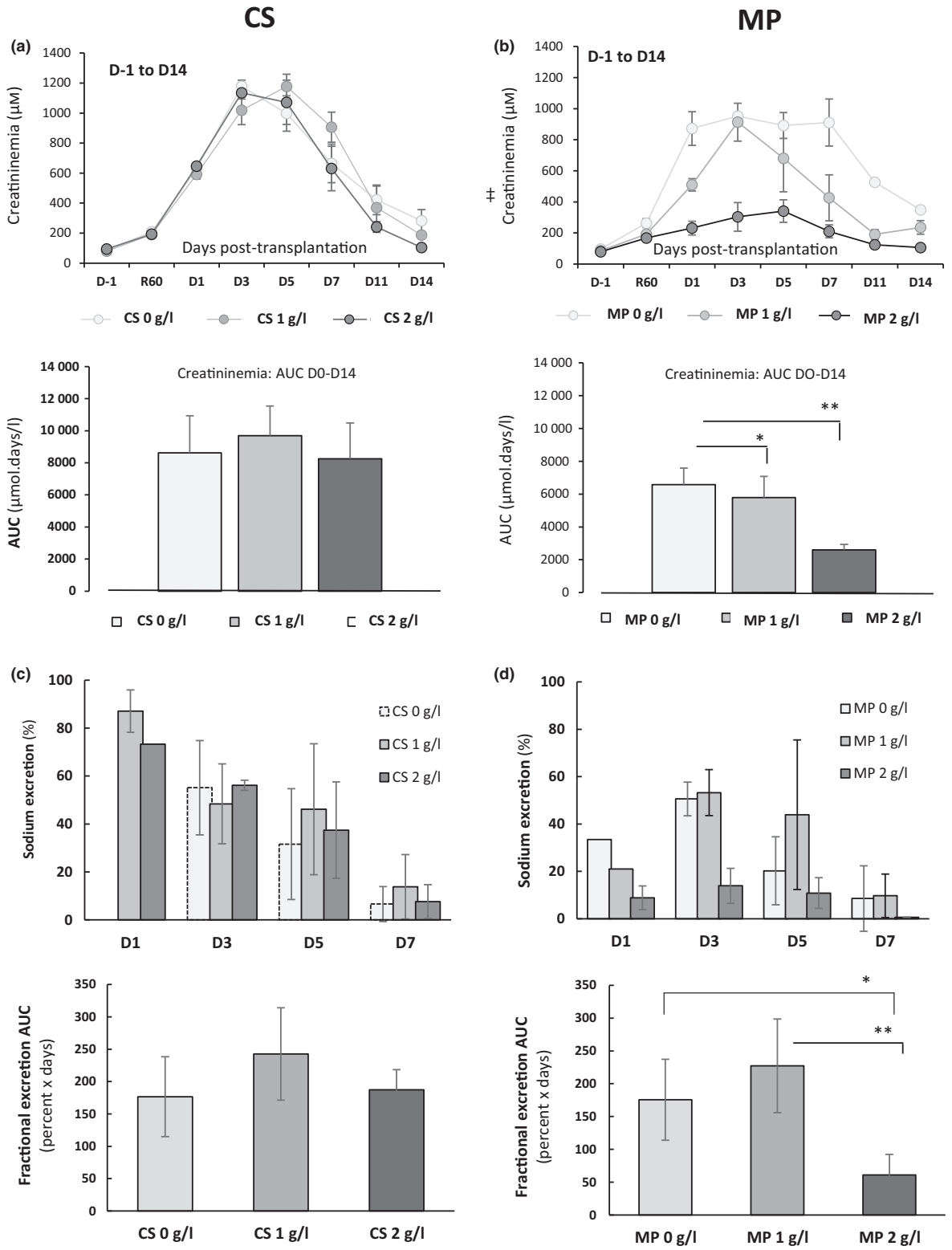

Figure 3 Transplantation outcome, short term. Transplantation outcome during the first 14 days. Graft function was evaluated through serum creatinine and sodium excretion measurements. Two control groups were added, SHAM (surgery control) and NEP (uninephrectomized). Serum creatinine level evolution (panel a for CS; panel b for MP) is displayed as the evolution over time (Top) and as the area under the curve (AUC, Bottom). Sodium excretion level evolution (c for CS; d for MP) is displayed as time evolution (Top) and AUC (Bottom). Data are shown as means ± SD; $n = 6$ (except for SHAM, $n = 5$). Statistics *P<0.05; **P<0.01. CS, cold storage; MP, machine perfusion.

recovery. AUC analysis (Chi-squared = 10.43308, $P = 0.005426$) showed that these improvements (~10% and 60%, respectively) were statistically significant for both the 1 and 2 g/l M101 groups (Dunn's $P = 0.023932$ and 0.001469, Fig. 3b, lower panel). Table 2 reports all creatininemia values during the 3-month follow-up of the MP preservation groups.

Sodium excretion (Fig. 3d, upper panel) showed a sensible loss of early function in the CTL and 1 g/l M101 groups (20–50%, at D1–D3), with no difference in recovery between the two groups, while the MP 2 g/l M101 kidneys demonstrated little loss of function at D1–D7 (peaking at only 14%, at D3). AUC comparison confirmed the significant reduction of sodium excretion with M101 2 g/l (Fig. 3d, lower panel).

Long-term outcome

Cold storage arm

Figure 5 (panel a) shows that serum creatinine at M1 and M3 was the highest in the control and CS 1 g/l M101 animals, the former consistently reaching statistical significance compared with SHAM and NEP groups (not shown on Fig. 5; see Table 1 for SHAM and NEP creatinemia values). The CS 2 g/l M101 group demonstrated reduced serum creatinine levels at these time points, significantly lower when compared with the other groups (Fig. 5a).

In terms of fibrosis, as quantified by Sirius red staining, no difference appeared between the three groups (Figs 4, upper panel, and 5c).

Machine perfusion arm

As compared with SHAM and NEP groups, the MP 0 g/l M101 control group showed significantly higher levels of serum creatinine at M1 and M3 time points (see Tables 1 and 2). Figure 5b shows that both MP 1 and 2 g/l M101 yielded markedly reduced serum creatinine versus the 0 g/l. This reduction was highly significant for the MP 2 g/l group at M1 and M3, but reached significance at M1 only with the MP 1 g/l. Of note, creatinine levels in the MP 2 g/l were not different

Table 1. Follow-up of plasma creatinine values in the static cold storage arm, and in the Sham-operated and nephrectomized control groups.

Plasma creatinine	D-1	R60	D1	D3	D5	D7	D11	D14	M1	M3
SHAM	82	na	74	82	87	85	85	82	88	97
SD	15		10	5	16	14	10	12	3	15
NEP	82	na	109	112	116	104	86	83	98	138
SD	14		6	11	8	8	7	8	10	15
Control	87	208	628	1179	998	665	422	282	199	198
SD	16	34	38	115	352	385	297	225	67	41
CS 1 g/l	80	190	591	1019	1178	907	369	188	166	137
SD	17	33	81	254	215	266	354	48	8	52
CS 2 g/l	94	193	646	1135	1072	631	241	104	80	83
SD	13	12	27	111	391	392	94	19	6	5

Creatinine values given in μmol/l, as mean ± SD ($n = 6$).

Table 2. Follow-up of plasma creatinine in the hypothermic machine perfusion preservation arm.

Plasma creatinine	D-1	R60	D1	D3	D5	D7	D11	D14	M1	M3
Control	95	270	787	1007	909	899	543	346	257	199
SD	20	45	27	42	149	264	30	31	40	30
MP 1 g/l	85	181	540	901	783	426	190	236	145	113
SD	10	20	72	298	518	332	70	97	36	32
MP 2 g/l	79	168	231	304	341	211	125	106	93	86
SD	11	12	45	92	72	42	13	10	6	9

Creatinine values are given in μmol/l, as mean ± SD ($n = 6$, except for the MP 1 g/l group, $n = 5$). Sham and Nep groups the same as for the CS arm (see Table 1).

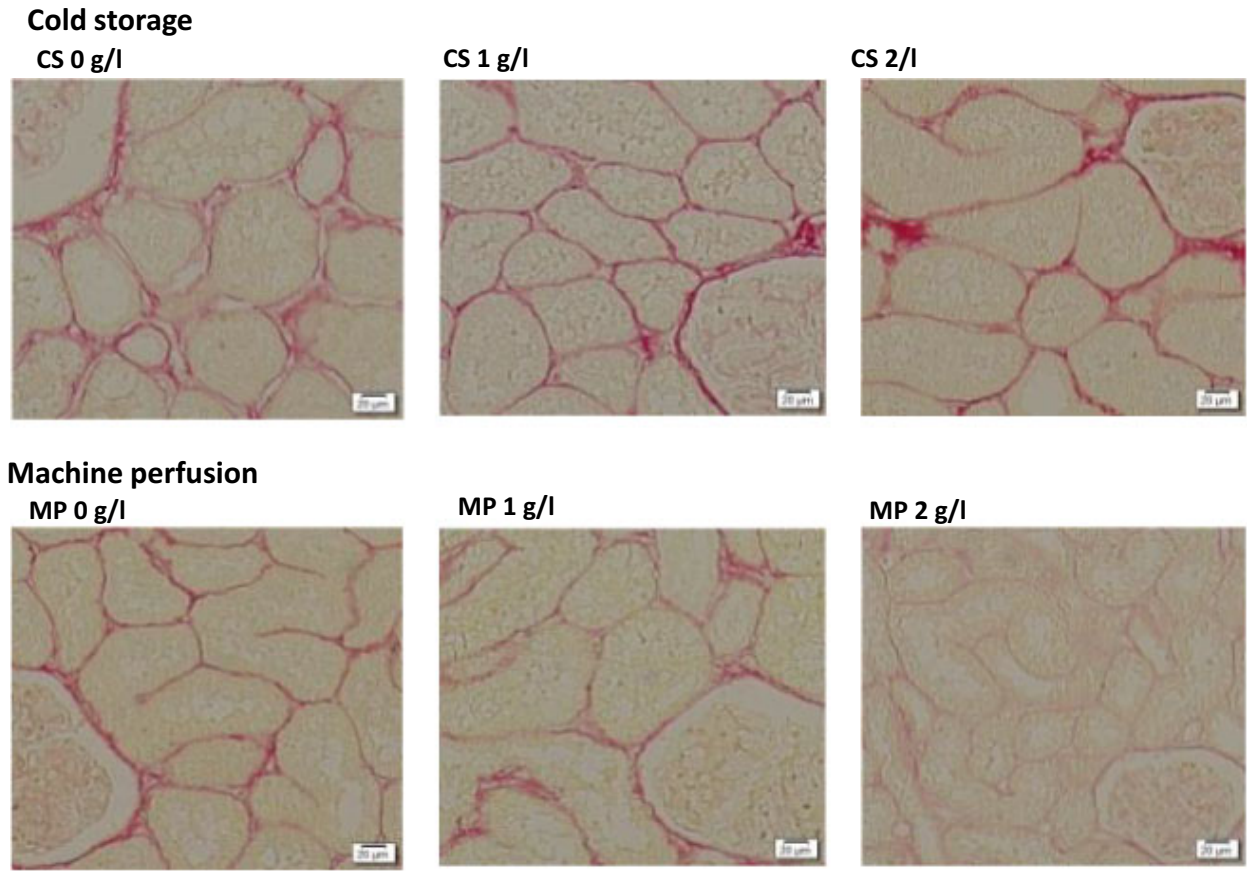

Figure 4 Transplantation outcome, long term. Fibrosis was expressed as the percentage of Sirius red staining. Representative photographs, for each CS and MP groups, of Red Sirius-stained renal sections, upon which staining quantification was performed (×0.63 objective, 2–5 mm size, main panels, with Visilog software; see Methods); average values are given in Fig. 5c & 5d. Insets represent zooms over a chosen region close to a glomerulus (bar, 60 μm). CS, cold storage; MP, machine perfusion.

from those of SHAM or NEP animals, indicating complete recovery. Finally, fibrosis was almost absent in the MP 2 g/l M101 group as evidenced by Sirius red staining (Fig. 5d, see also Fig. 4, lower panel).

Discussion

Current organ preservation rely on hypothermia to slow metabolism down and commensurately reduce oxygen need. However, even at a temperature as low as 4 °C, cells still require oxygen to insure basic metabolic needs and thus only withstand limited duration of ischemia. This is especially true for marginal organs. We previously showed that the use of the M101 oxygen carrier ($Hemo_2Life^{®}$) could improve organ preservation quality, in an autotransplanted pig model [20,21]. We expand this analysis in a more severe model of kidney transplantation, in which the organ is subjected to warm ischemia prior to collection and preservation, a procedure known to impact graft outcome [28].

We first evaluated ATP level in cortical tissue, as an indicator of energetic status. While CS severely depleted energy level, MP was able to significantly increase ATP, above concentrations recorded at the end of warm ischemia. While the later effect is, at least in part, because of established effects of machine preservation [10,11], the addition of 2 g/l M101 yielded higher levels than the control group, suggesting cross-potentiation of machine and M101 benefits. Of note, whereas ATP level in the MP 2 g/l M101 was significantly higher than the healthy control (~threefold), the apparent increase versus the untreated MP group (~40%), did not reach significance. This could be because of the limited statistical power: ~25% statistical power for an expected size effect of 40%, with $n = 6$ *per* group. Secondly, since the vascular network is a major target of ischemia–reperfusion injury [31,32], we determined the pro-angiogenic factor VEGF expression in the cortical tissue, as an indirect indicator of the microvascular status. We observed modest changes in the CS groups, although an increase

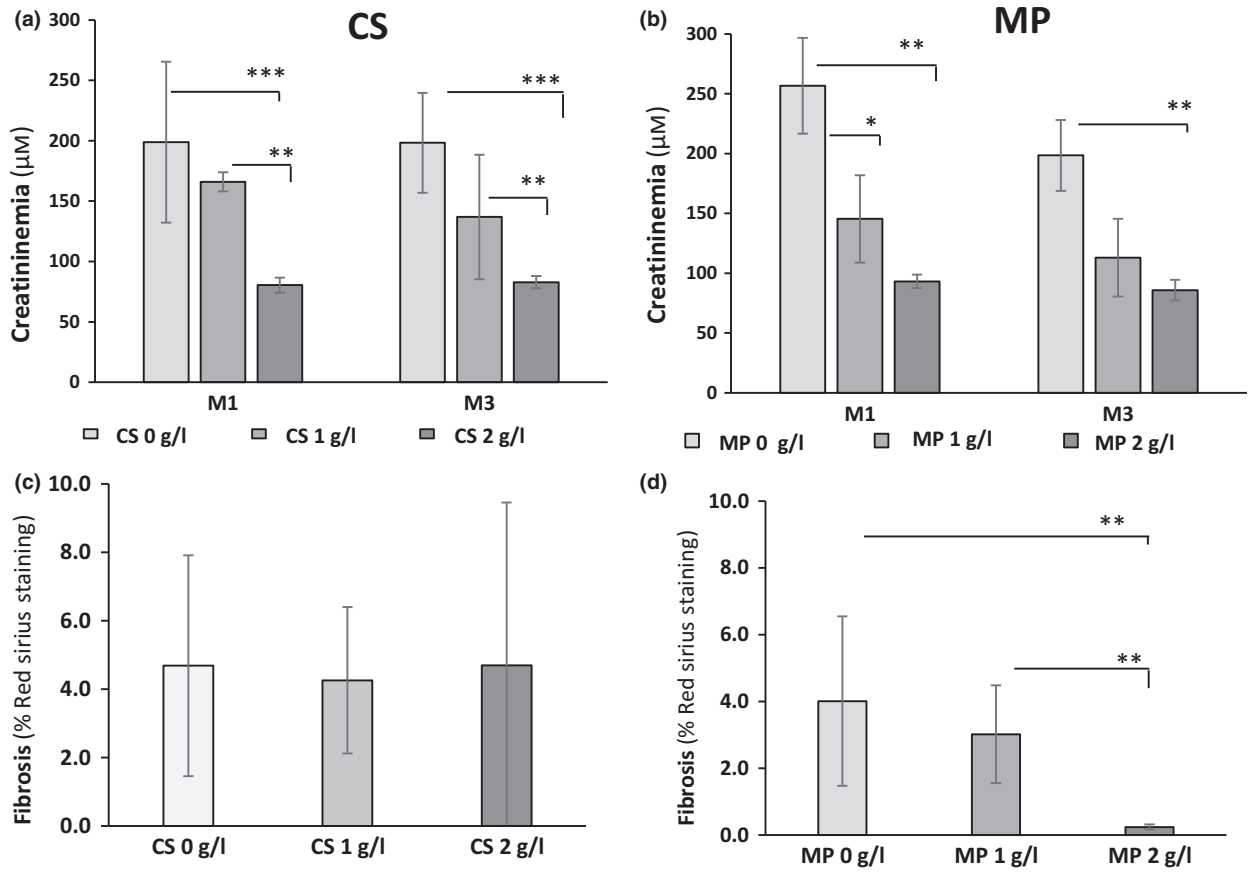

Figure 5 Transplantation outcome, long term. Transplantation outcome after 1 month (M1) and 3 months (M3). Serum creatinine was measured (panel a for CS, panel b for MP) as well as the extent of interstitial fibrosis development (panel c for CS, panel d for MP). Values are given as means ± SD (n = 5 or 6). CS Statistics: *P < 0.05; **P < 0.01; ***P < 0.001. CS, cold storage; MP, machine perfusion.

versus control was detected in untreated kidneys, and a further decrease with 2 g/l M101. While VEGF increase is *a priori* expected to be beneficial in terms of the vascular network, no correlation emerged between VEGF and function, suggesting that either this increase was not sufficient to bestow organ protection or that this mechanism was hampered during CS. This is concordant with previous results from our group showing the detrimental effect of combined WI and CS on HIF pathway regulation [30].

In the MP groups, whereas preservation did not significantly modify VEGF tissue levels, expression was strongly increased by M101 (1 and 2 g/l) suggesting that the M101 may positively influence the vascular network. This observation confirms our previously reported benefits of MP on the vascular network function, relating to the NO° pathway [31].

In terms of short-term functional outcomes, as evaluated by serum creatinine, the M101-treated groups proved superior to the untreated group. A previous study showed that in progressive, but not in stable proteinuric kidney disease, VEGF expression was attenuated despite an activation of intracellular hypoxia response and VEGF signaling pathways [33]. Moreover, we have shown that the early expression of VEGF is an indicator of a favorable long-term evolution of transplanted organs [30,34]. To determine the impact of reperfusion on this pathway and to properly establish the clinical impact of M101 on kidney outcome, a second experimental design was established, in which kidneys were transplanted and animals were followed up for 3 months. HIF1α expression was also evaluated in both arms, but no variations could be observed (data not shown). In particular, this could be due to the faster HIF1 activation kinetics and relaxation (minutes–hours), relatively to the longer protocol time scale (>22 h). Of note, VEGF-HIF1 decoupling has been observed in other experimental models [33].

Short-term impact of M101 supplementation on graft recovery: in CS conditions, we observe that M101 tended to improve the quality of short-term recovery (creatininemia, D11–D14), with no effect on tubular

function in this model. This is in qualitative agreement with our previous demonstrations of M101-induced protection of healthy kidneys with 1, 2, and 5 g/l [20,21] At this stage, the optimal concentration of M101 for ischemia-injured kidneys in CS remains to be determined, as well as the administration conditions (e.g., should M101 be added to the flushing before preservation?), and the preservation setup *per se*. Indeed, to rigorously evaluate putative effects of an oxygen carrier, in addition to a "sealed" container, one would have to consider numerous physical factors able to influence passive and carrier-dependent oxygenation, including the preservation solution volume, the solution/gaseous phases volumic ratio and the exchange surface area. Those issues have not been taken into account in this study. Nevertheless, it is worthwhile to mention that exploratory experiments (reported in Appendix S1: see figure S1 for oxygen monitoring, and figure S2 for the experimental setup), using sealed containers with kidneys in static cold preservation, with or without M101 molecule (1 g/l) showed that, without M101, oxygen pressure in the solution decreased to zero in ~24 h, whereas in the presence of M101, it remained around 50 mmHg for 30 h, before decreasing toward zero within another 20 h or so. This evidences a progressive release of oxygen by the carrier in response to graft consumption.

In MP conditions, the use of M101 at 1 g/l imparted a first level of improvement in serum creatinine evolution, which further increased at 2 g/l. This observation is in agreement with established beneficial effects of M101 [15,21], suggesting a protective effect of M101 in MP preservation [31],and advocates for a combined use of MP and M101.

In order to evaluate late outcomes and to complete analysis, animals were monitored up to 3 months, with M1 and M3 being sampled. In CS, kidney function improvements brought about by M101 were both clear-cut and significant, confirming the apparent effect observed earlier. However, in these conditions, M101 treatment did not reduce interstitial fibrosis. Considering our earlier studies with nonischemic kidneys [20,21], and provided that experimental settings are appropriate (see above comment), it is possible that higher doses of the compound are needed for WI-injured kidneys to obtain benefits on both function and histology outcomes after CS preservation. In MP preservation on the other hand, 1 g/l M101 was able to improve function, while 2 g/l M101 at did completely prevent the development of interstitial fibrosis, and normalize graft function.

It is difficult to propose a simple explanation for the beneficial effects of M101 reported here. On one hand, in CS no ATP resynthesis was detected during preservation, while definite functional improvements were shown. On the other hand, in MP preservation, markedly elevated levels of ATP were observed, together with clear-cut benefits in terms of function and tissue integrity. We hypothesize that, the dynamic regimen provided by MP allowed M101 to be properly distributed within the vascular compartment and, consequently, to deliver its oxygen more efficiently to the graft. Obviously, this cannot be the case (or at any rate much less efficiently) under static CS conditions. Since oxygen release regimen and efficiency will depend on graft consumption and on system configuration (oxygen reserve in solution and carrier-bound, sealing, exchange surfaces, volumes), oxygen measurements in dedicated experiments are needed.

Our results, obtained in a model of severe IRI [7,15,26], suggest that an oxygen carrier such as M101 could be a valuable therapeutic option allowing to improve kidney protection during the hypothermic preservation window. While static CS is not recommended for injured kidneys [10,11,35], the present results support the notion that the use of M101 could improve function recovery, particularly in situations where no other option is available. On the other hand, the MP arm revealed that this technology, currently recommended for high-risk organs [36], can be potentiated by M101. Indeed, we demonstrate that the simple addition of the molecule can drastically improve outcomes, reducing early post-transplantation dysfunctions, with no loss of tissue integrity throughout the follow-up. Provided that appropriate safety controls check, the critical improvements observed with M101 during MP plead for a translational application in the clinical setting.

Authorship

JK: performed experiments and first-pass data analysis, and revised the manuscript. RT: wrote the first draft, and performed statistical analyses. PH: performed calculations and wrote and revised the manuscript. AK and CS: performed experiments and revised the manuscript. JMG: performed histological analyses. FZ, EL, ED, and TH: initiated the study. FZ and TH: directed the study.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest. Franck Zal is the cofounder and CEO/CSO of HEMARINA and possesses shares, with Elisabeth Leize, in the company. Eric Delpy works for Hemarina but does not hold shares.

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SUPPORTING INFORMATION

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

Appendix S1. Protocol summary.

Figure S1. Oxygen partial pressure in the preservation solution (UW) as a function of time, with and without M101 (Hemo$_2$Life®).

Figure S2. The experimental setup.

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