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Supplementation With a New Therapeutic Oxygen Carrier Reduces Chronic Fibrosis and Organ Dysfunction in Kidney Static Preservation

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Static preservation is currently the most widely used organ preservation strategy; however, decreased donor organ quality is impacting outcome negatively. M101 is an O₂ carrier with high-oxygen affinity and the capacity to function at low temperatures. We tested the benefits of M101 both *in vitro*, on cold preserved LLC-PK1, as well as *in vivo*, in a large white pig kidney autotransplantation model. *In vitro*, M101 supplementation reduced cold storage-induced cell death. *In vivo*, early follow-up demonstrated superiority of M101-supplemented solutions, lowering the peak of serum creatinine and increasing the speed of function recovery. On the longer term, supplementation with M101 reduced kidney inflammation levels and maintained structural integrity, particularly with University of Wisconsin (UW). At the end of the 3-month follow-up, M101 supplementation proved beneficial in terms of survival and function, as well as slowing the advance of interstitial fibrosis. We show that addition of M101 to classic organ preservation protocols with UW and

Histidine–Tryptophane–Ketoglutarate, the two most widely used solutions worldwide in kidney preservation, provides significant benefits to grafts, both on early function recovery and outcome. Simple supplementation of the solution with M101 is easily translatable to the clinic and shows promises in terms of outcome.

Key words: Graft preservation, ischemia reperfusion injury, kidney transplantation, oxygen, oxygen transporters

Abbreviations: ATP, adenosine 5'-triphosphate; CS, cold storage; HBL-Hb, hexagonal-bilayer hemoglobin; HBOC, hemoglobin-based oxygen carrier; HTK, histidine tryptophane ketoglutarate; IFTA, interstitial fibrosis and tubular atrophy; IRI, ischemia reperfusion injury; LDH, lactate dehydrogenase; M101, hemarina-M101; NBT, nitroblue tetrazolium; PFCs, perfluorocarbons; SOD, superoxide dismutase; UW, University of Wisconsin.

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Introduction

Kidney transplantation remains the therapy of choice for end stage renal diseases. Ischemia reperfusion injury (IRI), inherent to transplantation, strongly correlates with delayed graft function (1,2), chronic graft failure and late graft loss (3–5). IRI is a complex process involving oxidative stress, mitochondrial uncoupling (6,7) and the coagulation cascade (8,9). It also activates innate immune response independently of allogenicity (10), with intense immune cells invasion (11,12). Hence, a better management of organ preservation could limit IRI, improving graft quality and outcome substantially.

Currently, hypothermic cold storage (CS) is the main strategy to minimize ischemic injuries (1,13). CS preservation is based on metabolism reduction with temperature; however, even slow metabolism requires O₂. Hence, the concept of introducing O₂ during CS is growing. Different approaches are developed: persufflation by retrograde venous application of O₂, the two-layer method with

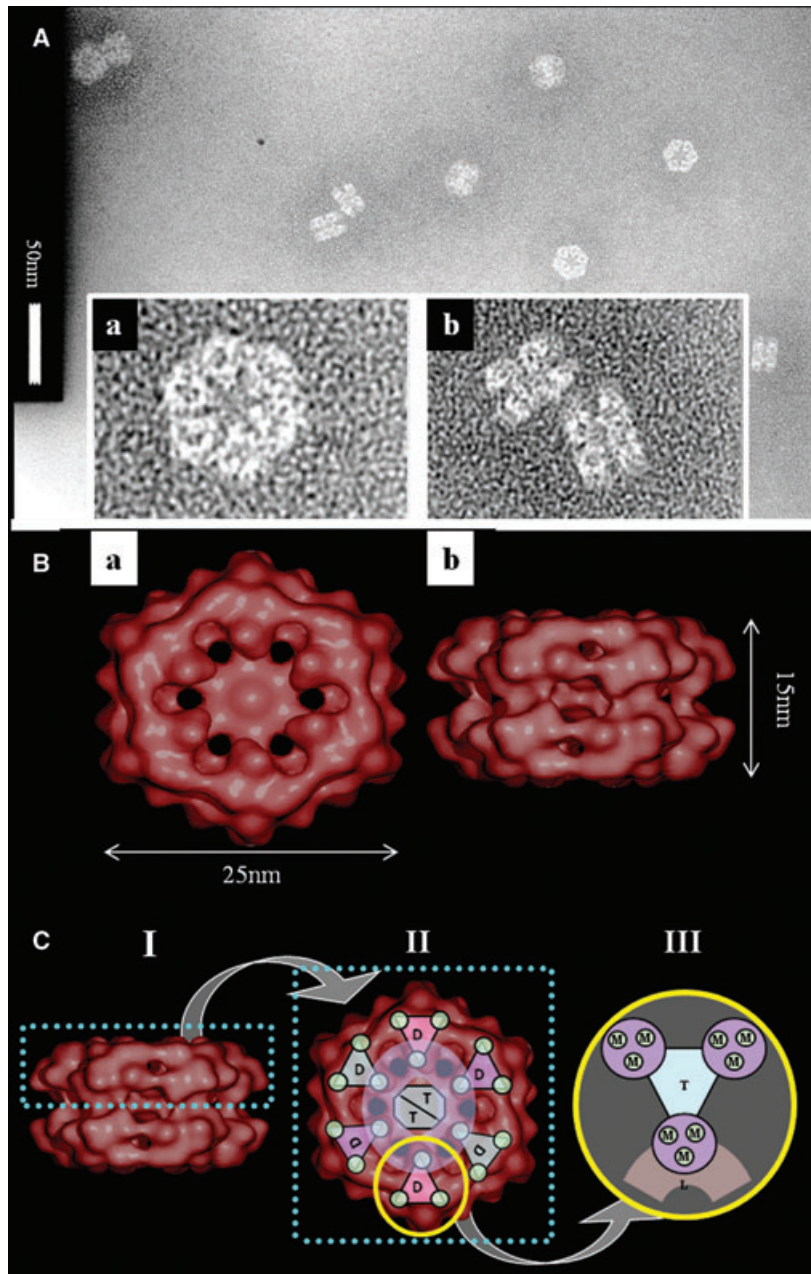


Figure 1: Structure and conformation of *Arenicola marina* hemoglobin. (A) Micrographs obtained by transmission electron microscopy (19,20). (B) Three-dimensional reconstruction obtained by frozen electron microscopy (17). Detailed are a face view (a) and a profile view (b) of the molecule. (C) Model of 3D conformation of *Arenicola marina* hemoglobin (20). I: Hexagonal bilayer of the molecule. II: Face view of hexagonal layer with dodecameric subunits (D) and trimer subunits (T). III: Dodecamer with monomers (M), trimer (T) and linkers (L). Conformation is a regular hexagonal bilayer, each consisting of a central part consisting of 2 trimers of globin (T) and a peripheral part including 6 dodecamers (D), appearing as equilateral triangles with 3 monomers of globin (M) noncovalently bonded at each edge and a trimer of globin (T) at the center of the triangle. Dodecamers are bonded with 3.5 nonglobin chains (L) ensuring conformation and stability.

perfluorocarbons (PFC) or gaseous oxygenation during CS (14–16). However, their effectiveness remains unclear and translation to the clinic could be difficult considering the important changes in protocol and equipment.

Herein, we investigate a new therapeutic molecule, Hemarina-M101 (M101) (Figure 1A), a respiratory pigment from a marine invertebrate, *Arenicola marina*, developed as an additive to preservation solutions (18–20). M101 is a giant O₂ carrier corresponding to an extracellular hexagonal-bilayer hemoglobin (HBL-Hb). HBL-Hbs are naturally extracellular heminic respiratory pigments of high molecular weight (~3600 kDa), made of complexes globin

and nonglobin linker chains (Figure 1B). Such large complexes represent a summit of complexity for oxygen-binding heme proteins with a remarkable hierarchical organization, each globin chain surrounding and protecting its own heme group, a protoporphyrin ring with an iron atom in center reversibly binding one O₂ molecule. This confers a high O₂ binding capacity: M101 can carry up to 156 molecules of O₂ (Figure 1C). M101 possesses other advantages: O₂ is released against a gradient in the absence of allosteric effectors, providing the environment with just the right amount of O₂; it functions in a large range of temperature (from 4°C to 37°C) (21,22) and it possesses intrinsic superoxide dismutase (SOD)-like activity linked to

Cu/Zn metals (18), all invaluable in IRI (23,24). M101 is also fully identified from gene to protein quaternary structure (25,26). Furthermore, M101 is nonimmunogenic (18). These suggest an important potential towards improving organ preservation by CS.

The concept of supplementing classical organ preservation protocols is rapidly gaining interest (9,27–32). Simple addition of a molecule to an established protocol presents high potential for translation to clinic, compared to more cumbersome approaches of changing solutions and/or machines. We tested the benefits of M101 supplementation *in vitro* in cold stored cultured cells using a range of solutions used in the clinic, then *in vivo* with the two most widely used static cold preservation solutions (90% of the world's market) (33): University of Wisconsin (UW) and Histidine Tryptophane Ketoglutarate (HTK). We used a pig model of kidney auto-transplantation, as this species presents an elaborate system of interlobular and segmental arteries to supply the numerous kidney lobes, a char-

acteristic shared with humans and higher mammal but absent in rodents or dogs (6), making porcine models very relevant to mimic human conditions (34,35).

Methods

Reagents

Unless specified, all reagents were purchased from Sigma-Aldrich (Lyon, France).

M101 production and utilization in preservation solutions

M101 (HEMO₂life, Hemarina SA, Morlaix, Brittany, France) was manufactured using standard procedures for the extraction of biologics conformed to the specifications of the health authorities. The purified protein is frozen at -80°C then thawed to 4°C before experiment and diluted in a preservation solution: UW (ViaSpan, Bristol-Myers-Squibb, Braine-l'Alleud, Belgium), HTK (Custodiol, Essential Pharmaceuticals, Newton, PA, USA), IGL (IGL-1, IGL Group, Lissieu, France), Celsior (Celsior, Genzyme, Saint-Germain-en-Laye, France), Ringer Lactate (RL, Aguetant, France) or Perfadex (Perfadex, Vitrolife, Sweden).

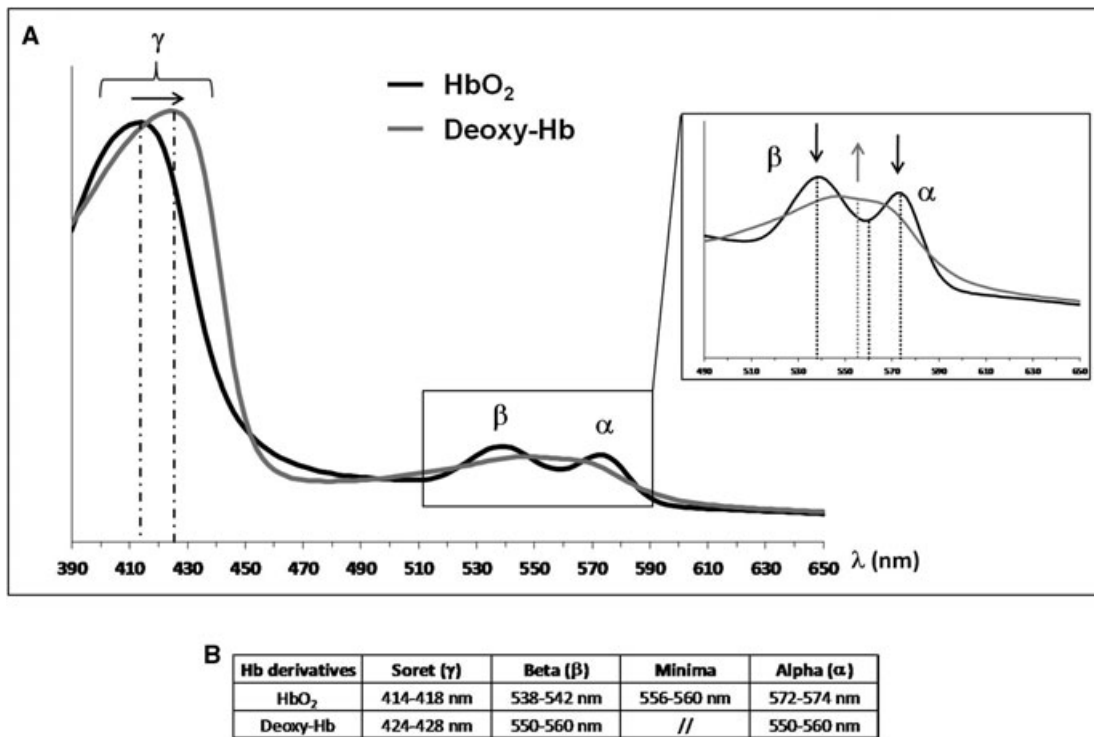


Figure 2: Spectral characteristics of oxyhemoglobin and deoxyhemoglobin derivatives of *Arenicola marina* hemoglobin. M101 is a hemic respiratory pigment. The heme pocket of hemoglobin molecules is the site of many dynamics interactions involving O₂ binding (36). If the iron atom of the hemoglobin molecule is bounded to O₂, hemoglobin is called oxy-hemoglobin or HbO₂. If the iron atom of the hemoglobin molecule is bounded to nothing, hemoglobin is called deoxy-hemoglobin or deoxy-Hb. All hemoglobin derivatives differ from each other by their light-absorption spectra, which enable to identify the hemoglobin derivatives from its spectral profile. Spectral signature of hemoglobin derivatives of *Arenicola marina* has widely been studied (37,38). The absorption is at its highest for the so-called Soret band, at 416 nm for HbO₂ and 426 nm for deoxy-Hb. It then drops off steeply until approximately 510 nm, where it picks up slightly again at two typical peaks (Beta and Alpha bands) for HbO₂ at 540 nm and 573 nm, respectively, and at a single larger band around 555 nm for deoxy-Hb. (A) Light-absorption spectra of HbO₂ (black line) and deoxy-Hb (grey line) over the 390–650 nm range. (B) Maxima and minima absorption of Soret, Beta and Alpha bands for HbO₂ and deoxy-Hb derivatives. γ = Soret band; β = Beta band; α = Alpha band.

M101 functional analyses

Oxygen binding: M101 was supplemented (1 g/L) to UW. Either N₂ gas or LLC-PK1 cells incorporated to the preparation was used to deoxygenate the solution then both preparations were hermetically sealed. The functionality of M101 was followed by spectrophotometry (36) allowing characterization of oxyhemoglobin (HbO₂) and deoxyhemoglobin (deoxy-Hb; Figure 2). Absorption spectra were recorded over the 390–650 nm range (UVmc2, SAFAS, Monaco). Dissolved O₂ (dO₂) was monitored using an O₂ sensor (Metler Toledo, France).

SOD activity: M101 SOD activity was evaluated by nitroblue tetrazolium (NBT) assay modified by Oberley and Spitz (39). Briefly, superoxide was generated by xanthine and xanthine oxidase in presence of catalase and DE-TAPAC. Reduction of NBT was detected by spectrophotometry at 560 nm. KCN was added 1 h at 4°C before starting the experiment to deactivate Cu/Zn-SOD. A Cu/Zn-SOD from bovine erythrocytes was used as a control (Calbiochem).

Decreased absorbance indicates increased scavenging activity. The percentage inhibition of superoxide anion generation was calculated using: $[(A_0 - A_1)/A_0 \times 100]$, where A₀ is the absorbance of control and A₁ is the absorbance of samples.

M101 structural analyses

M101 was supplemented (1 g/L) to solutions, its structure was followed over time by isocratic gel filtration at room temperature with an autosampler set at 4°C employing an HPLC system (Dionex, France) and a 1 cm × 30 cm Superose 6C column (fractional range 5–5000 kDa, GE Healthcare, Vélizy Cedex, France). Flow rate was 0.5 mL/min and the eluate was monitored with a photodiode array detector over the range 250–700 nm. The elution curves were acquired and processed using the Chromeleon software (Dionex, Voisins le Bretonneux, France).

The dissociation curve was obtained normalizing the area of M101 peak at time t (A_t) with the area of M101 peak at time 0 (A₀) and plotted versus time. The GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was used to fit the curve to linear ($f(A_t/A_0) = -k_d t$, $T_{1/2} = 1/(2k_d)$) or mono-exponential ($f(A_t/A_0) = a \times \exp[-k_d t]$) profile. The fit acceptability was judged with the best-correlation coefficient. The dissociation constant (k_d) and half-life (T_{1/2}) were deduced from the best-fitted curve: linear or mono-exponential.

Cell cold-storage experiments

Porcine proximal tubular cell line LLC-PK1 (CL-101, ATCC, LGC Standards, France) were cultured as previously described (40). Cold ischemia injury was simulated by storage of cell monolayer at 4°C under room atmosphere in a preservation solution (UW, HTK, IGL, Celsior, RL or Perfadex) supplemented or not with M101.

Assays were: (1) *Necrosis*: Lactate dehydrogenase (LDH) release was tested using *In Vitro* Toxicology Assay Kit; (2) *Apoptosis*: Caspase-3 activity was determined using Caspase-3 Fluorometric Assay kit (R&D Systems, Lyon, France); (3) *Viability*: Metabolic activity was determined by MTT assay and (4) *Energetic content*: Intracellular ATP was determined using Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit. Kits were used following the manufacturer's guidelines. Reactions were quantified with a multiplate reader (Victor3, Perkin-Elmer, Waltham, MA).

For each parameter, results were expressed as percentages of measured values in cold-preserved cells versus measured value in cells before injury (control).

In vivo surgical procedures and experimental groups

Large white male pigs (INRA/GEPA, Surgères, France) were prepared as previously described (4) in accordance with French guidelines of the Ethical Committee for Human and Animal Studies. The right kidney was collected, cold flushed and preserved for 24 h before transplantation, a time chosen because it is slightly longer to the cold ischemia time noticed by United Network for Organ Sharing for renal allografts (19.6 ± 8.4 h in 2000) (41). The left kidney was nephrectomized to mimic nephron mass in transplanted situation. Surgical teams were blinded to protocols. Time for vascular anastomoses was 30 ± 5 min, blood loss was minimal and no postoperative complication was observed. Four groups were studied: (1) UW: organ preservation with UW; (2) UW + M101: UW supplemented with 5 g/L M101; (3) HTK: HTK; (4) HTK + M101: HTK supplemented with 5 g/L M101. Controls were sham-operated animals.

Functional parameters

Pigs were placed in a metabolic cage for diuresis (mL/24 h), creatinemia (μmol/L), fraction of excreted sodium (%) and proteinuria (g/24 h) measurements as previously described (3–5).

Morphological studies

Biopsies were collected 7, 14 days and 1 month after reperfusion. Brush border loss and endoluminal detachment were assessed using a semiquantitative 6-point scale: 0, no abnormality; 1, mild lesions affecting less than 25% of kidney samples; 2, lesions affecting 25–50% of kidney samples; 3, lesions affecting 51–75% of kidney samples; 4, lesions affecting more than 75% of kidney samples and 5, extensive necrosis and renal damage (42).

Quantitative determination of interstitial invasion was adapted from Banff classification (43): 0, no mononuclear inflammatory cell in tubules; 1, Foci with 1 to 4 mononuclear cells per tubular cross-section or ten tubular cells; 2, Foci with 5–10 mononuclear cells per tubular cross-section and 3, Foci with more than 10 mononuclear cells per tubular cross-section.

Tubulointerstitial fibrosis was determined using Picro Sirius staining (44).

Immunohistochemistry was used for ED1+ and CD3+ cell invasion measurement (SouthernBiotech, Birmingham, AL, USA). Quantitative evaluation was performed on 5–10 high-powered fields (200×).

Statistical methods: Means ± SEM or SD are shown. *In vitro* data were compared using Dunett's test. For *in vivo* data, the Mann-Whitney U test was used for comparisons between 2 groups (only UW vs. UW + M101 and HTK vs. HTK + M101 comparisons were conducted). Correlations were measured with Spearman's test and dependence of M101 effect on the solution used was tested using a two-way ANOVA. SPSS software (IBM, Armonk, NY, USA) and GraphPad software (GraphP) were used for statistical analyses. Significance was accepted for p < 0.050.

Results**M101 functionality**

O₂ binding: M101 in solution with UW was deoxygenated by N₂ flushing or by addition of LLC-PK1 cells. Figure 3 shows data obtained with cells since both set of experiment gave the same results. Under room atmospheric condition, M101 dissolved in UW was oxygenated (HbO₂) as shown by absorption spectrum (Figure 3A, normoxia). Then, the preparation was hermetically sealed and O₂ consumed by cells (hypoxia). After 75 min of hypoxia, M101 spectrum switched progressively towards deoxy-Hb

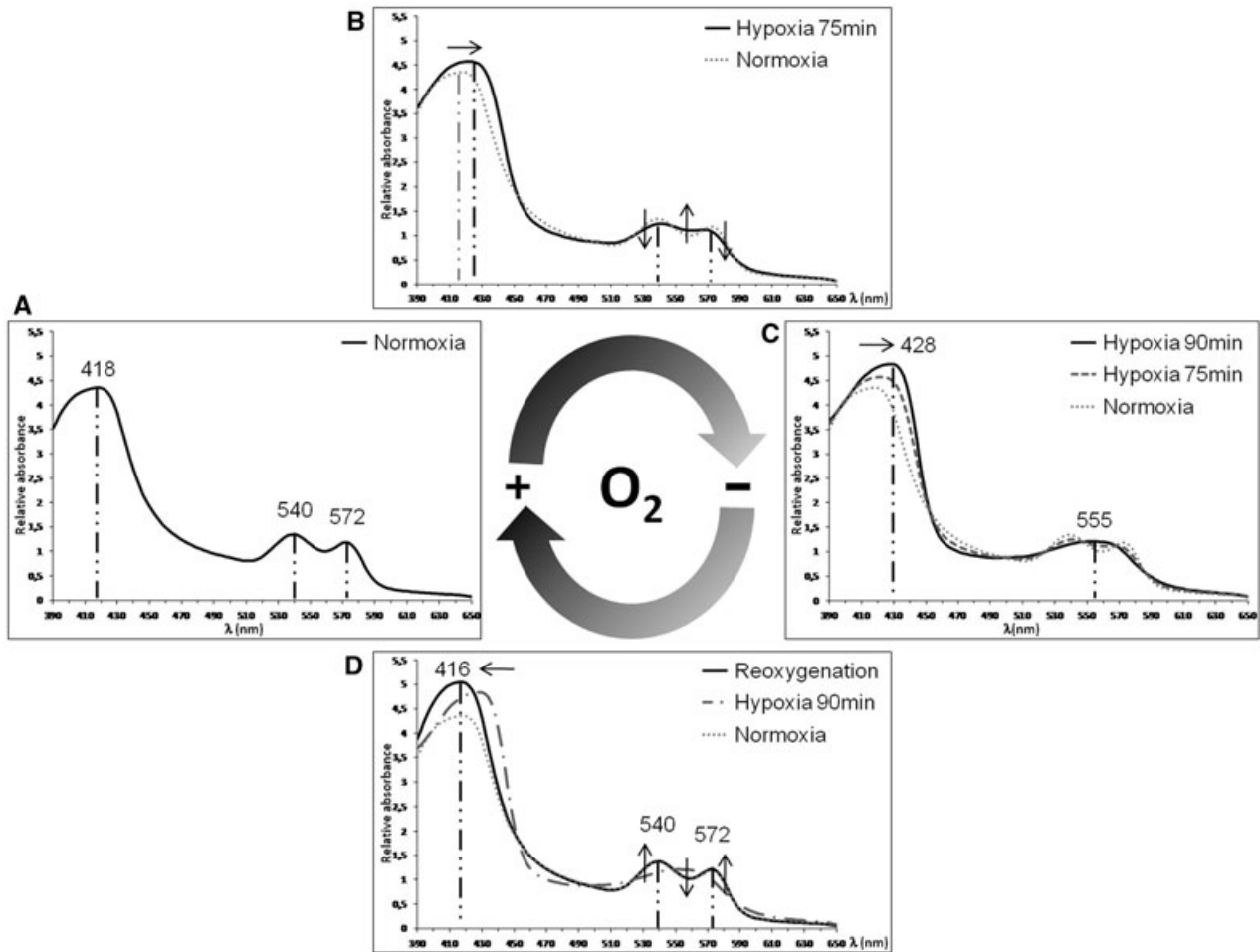


Figure 3: Spectral curves of M101 over time on the 390–650 nm range. M101 was supplemented to UW at a concentration of 1 g/L and LLC-PK1 cells were added to the preparation. Preparation was kept at 4°C and was hermetically sealed to induce hypoxia. Spectrum of M101 was followed over time. (A) Spectrum under room atmospheric condition (normoxia). (B) Spectra after 75 min of hypoxia (—) and under normoxia (···). (C) Spectra after 90 min of hypoxia (—), 75 min of hypoxia (---) and under normoxia (···). (D) Spectra after reoxygenation (—), 90 min of hypoxia (---) and under normoxia (···). 416, 418, 428 nm: maxima of Soret band (γ); 540 nm: maxima of Beta band (β); 572 nm: maxima of Alpha band (α); 555 nm: maxima of β/α band.

conformation with a shift of Soret band, a decrease of Beta and Alpha bands and an increase of absorbance around 555 nm (Figure 3B). Complete deoxygenation appeared after 90 min of hypoxia (Figure 3C): spectrum showed Soret with a maximum at 428 nm and a plateau with a maximum at 555 nm, the characteristic spectrum of deoxy-Hb derivative. This state was reversible as oxygenation of the preparation resulted in the initial spectrum of M101 (Figure 3D). The measure of dO₂ correlated with the light-absorption spectra (data not shown).

SOD activity: M101 was an effective antioxidant with total inhibition of NBT formation (Figure 4A, $93.5 \pm 1.1\%$). This activity is relative to a Cu/Zn-SOD since KCN, a specific inhibitor of Cu/Zn-SOD enzymatic activity, totally inhibited the scavenging ability of M101 (Figure 4B).

M101 is stable in commercial preservation solutions

The stability of M101 was assayed in UW, HTK, IGL, Cel-sior, RL and Perfadex. Dissociation constant and half-life show that M101 is stable for long periods of time (Table 1).

M101 protects cells in vitro against cold preservation lesions

Conservation of kidney epithelial cells in the gold standard solution, UW, was very deleterious (Figures 5–8). Assessment of cell viability by LDH release demonstrated a loss of cell viability after 12 h of CS (Figure 5A). No significant activation of caspase-3 was detected (Figure 5B). Metabolic activity (Figure 5C) and ATP content (Figure 5D) were reduced concomitantly to LDH release.

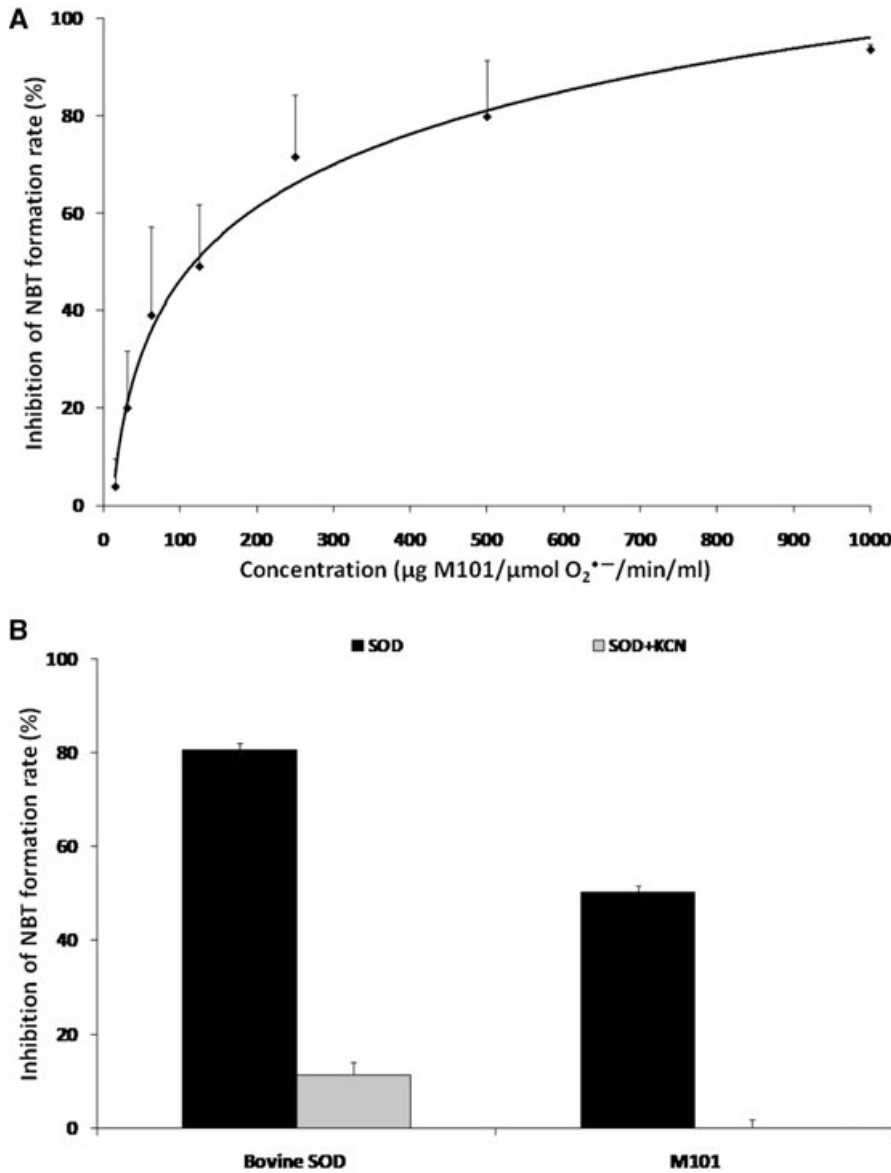


Figure 4: Inhibitory effect of M101 on the reduction of nitroblue tetrazolium by superoxide anion radical generated by the hypoxanthine-xanthine oxidase system. (A) Percentage of inhibition of NBT formation with an increase of M101 under a constant superoxide (O₂^{•-}) generation. (B) Percentage of inhibition of NBT formation with M101 or bovine SOD from erythrocyte used as control. Total SOD activity (SOD, black bars) was compared to specific Cu/Zn-SOD activity determined in presence of KCN (SOD + KCN, light gray bars). Shown are mean ± SD; n = 3.

M101 was protective against these events (Figure 6): as little as 0.312 g/L was sufficient to significantly improve structural and metabolic integrity and energetic content after 24 h. Total protection was reached at 1.25 g/L of M101 (LDH release: 6 ± 8%; MTT test: 71 ± 13%; ATP content: 78 ± 23%). Cellular energetic content was upregu-

lated with concentrations higher than 2.5 g/L (ATP content >120% compared to control). M101 also protected renal cells in a time and concentration dependent manners (Figure 7): cellular integrity was totally preserved (LDH release < 20%) for 24 h at 1.25 g/L, 36 h at 2.5 g/L, 48 h at 5 g/L and 72 h at 10 g/L.

Table 1: M101 Stability in commercial preservation solution

Solution	M101 Stability						
	Buffer ²	UW ²	HTK ¹	IGL ²	Celsior ²	RL ¹	Perfadex ¹
k _d (h ⁻¹)	0.00015	0.00017	0.00171	0.00107	0.00056	0.00140	0.00036
T _{1/2} (h)	3333	2941	292	467	893	357	1389

k_d = dissociation constant; T_{1/2} = half-life.

¹Stability of M101 was assayed for 24 h at 4°C in different commercial solutions in comparison with the conditioning buffer (Buffer).

²Stability of M101 was assayed for 48 h at 4°C in different commercial solutions in comparison with the conditioning buffer (Buffer).

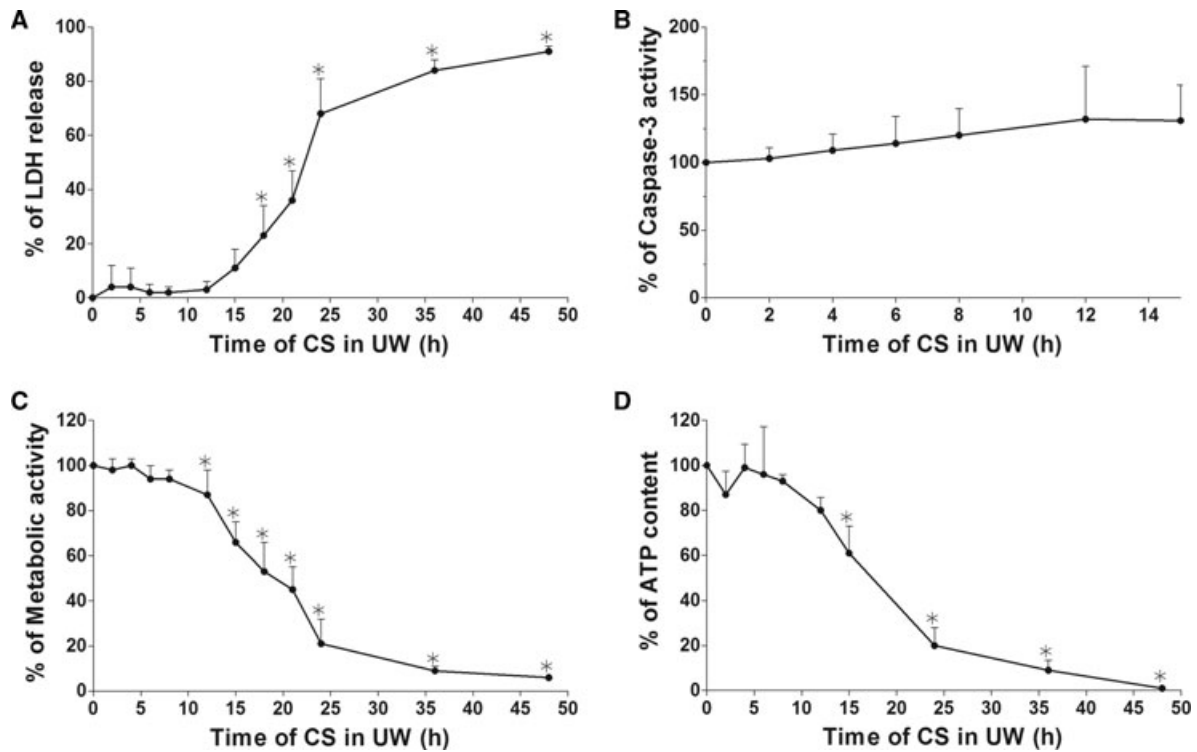


Figure 5: Cold-stored kidney epithelial cells preserved in UW for 48 h. LLC-PK1 cells were stored at 4°C for up to 48 h in UW. Cell damage was assayed by percentages of LDH release (A), caspase-3 activity (B), metabolic activity (C) and cellular ATP content (D). CS = cold storage. Shown are mean ± SD. Statistics: * = p < 0.05 versus time 0; n = 3.

Experiments were reproduced with other solutions (Figure 8): RL (Figure 8A), Perfadex (Figure 8B), HTK (Figure 8B), IGL (Figure 8D) and Celsior (Figure 8E). As for UW, conservation in these solutions induced cell structural and/or functional damages (respectively, Figures 8a and b). Two kinds of results were obtained: (1) similarly to UW, cells cold-stored in RL and, in a lesser extent, in Perfadex presented both structural and functional injuries; (2) cells cold-stored in HTK, IGL or Celsior presented only functional injuries. In every solution, M101 supplementation protected both cell integrity and cell functionality (LDH release <20% in all conditions and MTT test: 50–100%).

In vivo kidney graft function recovery is faster with M101

Pigs transplanted with UW + M101-preserved kidney resumed urine production at day 1 (versus day 2 for UW), and had a faster recovery to stabilized urine production levels by day 4 (Figure 9A, p = 0.016). HTK groups had equivalent diuresis recoveries (Figure 9B). Serum creatinine levels in UW group showed high levels peaking at day 3 and slowly decreasing afterwards. UW + M101 animals showed significantly lower levels peaking at day 1 and recovered pre-transplant levels by day 7 (p = 0.009 at all time points, Figure 9C). HTK animals showed a high serum creatinine peak at day 1, followed by a slow recovery remaining above

pretransplant levels while HTK + M101 animals had a significantly lower peak at day 1 (p = 0.009) and a faster recovery to pretransplant level by day 11 (Figure 9D). Sodium reabsorption measurements showed significantly superior performance in M101 preserved kidneys compared to solution alone, confirming better recovery (Figures 9E and F).

Tissue integrity is better preserved in M101 grafts

Evaluation of brush border loss and cell detachment, typical IRI tubular lesions, revealed important damage in UW grafts at days 7 and 14, stabilizing at month 1 (Figure 10; Table 2, top panel). UW + M101 kidneys showed less extended lesions. HTK groups showed a similar trend towards amelioration of histological lesions by M101.

Inflammation is less severe in M101 kidneys

There was an important immune response development in UW grafts throughout the duration of the follow-up (Table 2, middle–bottom panel). Kidneys preserved in UW + M101 showed little immune infiltration early on, and reduced signs of inflammation later. HTK groups both showed low-level infiltration. At 3 months, invasion of both innate (ED1+) and adaptive (CD3+) immune cells revealed decreased levels of invasion in M101 preserved kidneys compared to the solution alone.

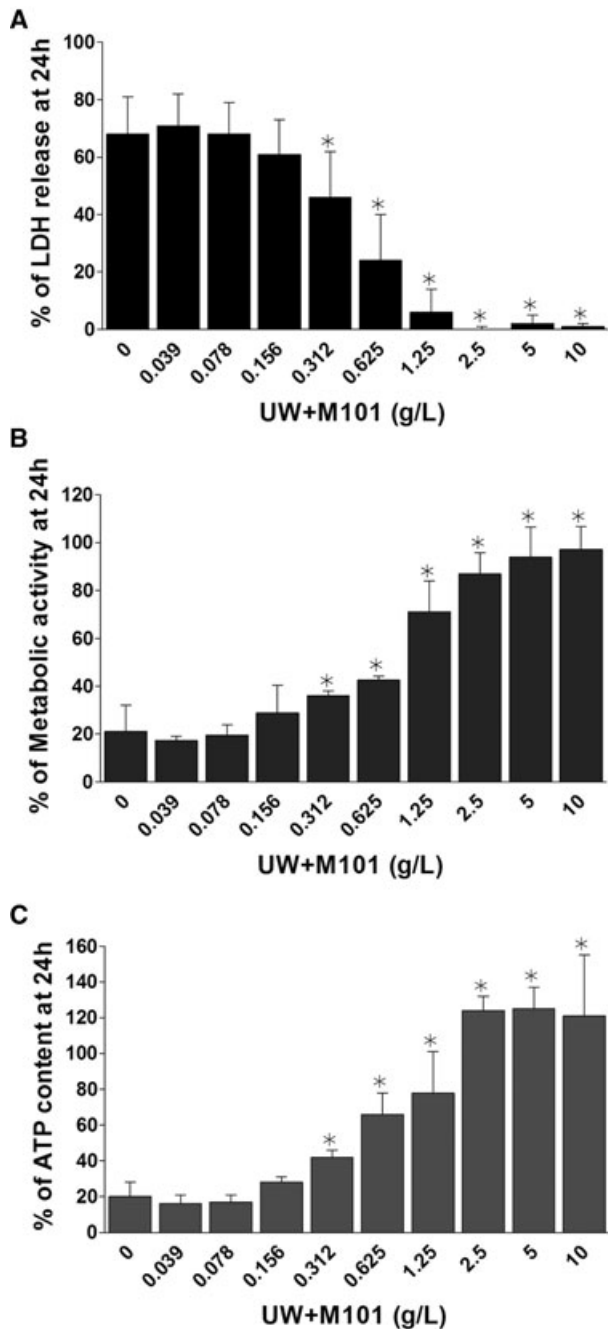


Figure 6: Cold-stored kidney epithelial cells preserved in UW supplemented with M101 for 24 h. LLC-PK1 cells were stored at 4°C for 24 h in UW supplemented with M101 in concentrations ranging from 0 to 10 g/L. Cell damage was assayed by percentages of LDH release (A), metabolic activity (B) and cellular ATP content (D). Shown are mean ± SD. Statistics: * = p < 0.05 versus UW alone; n = 3.

Outcome is improved by M101 supplementation

Pigs were euthanized at 3 months, time at which we previously demonstrated development of chronic fibrosis in this model (Figure 11) (45,46). Development of interstitial fibrosis and tubular atrophy (IFTA) in UW grafts was extensive (23%), while M101 supplementation significantly reduced it (11%, p = 0.049; Figure 11E). HTK kidneys also demonstrated important IFTA development (25%). Here also, addition of M101 significantly reduced damage development (10%, p = 0.038).

Histological damage was associated with chronic loss of function, as both HTK and UW groups showed high serum creatinine (Figure 11F) and proteinuria (Figure 11G) levels. For both solutions, M101 supplementation significantly reduced these levels.

M101 supplementation correlates with better early and 3-month outcomes

Further statistical analysis showed that M101 supplementation was negatively correlated with creatinine levels at day 3 (R² = 0.75, p = 0.0001) and sodium excretion levels at day 3 (R² = 0.74, p = 0.0001). Moreover, we determined that M101 supplementation was also negatively correlated with chronic outcome (3 months): creatinine (R² = 0.75, p = 0.0001), proteinuria (R² = 0.55, p = 0.013) and fibrosis (R² = 0.78, p = 0.0001). Two-way ANOVA revealed that there was an interaction between M101 and the solution used for acute outcome: creatinemia (p = 0.001) and sodium reabsorption (p = 0.04) at day 3; while chronic M101 effects at 3 months were independent of the solution used (data not shown).

Discussion

Herein, we describe the benefits of M101 supplementation on cold stored cells *in vitro* and on kidney grafts stored with either UW or HTK. M101 supplementation increased cell resistance to IRI and correlated with improved early function recovery and better long-term outcome with less chronic fibrosis and amelioration of function.

M101 is a new therapeutic molecule, an extracellular respiratory pigment from marine organism, developed as an additive to preservation solutions. This O₂ carrier has peculiar assets for organ preservation such as high oxygen affinity, ability to function at low temperatures and anti-oxidative activity. We demonstrated the oxygenation ability of M101 in CS conditions. O₂ binding and release occurs passively in a simple O₂ gradient and in absence of allosteric effector. M101 is characterized by a P₅₀, corresponding to 50% of the O₂ saturation. When the PO₂ is below the P₅₀, oxygen is released passively from M101 and consumed by cells or tissues. This is a dynamic equilibrium process dependant on cellular needs, when a O₂ site becomes free, it will be reused by a new O₂ molecule. We also confirmed that this

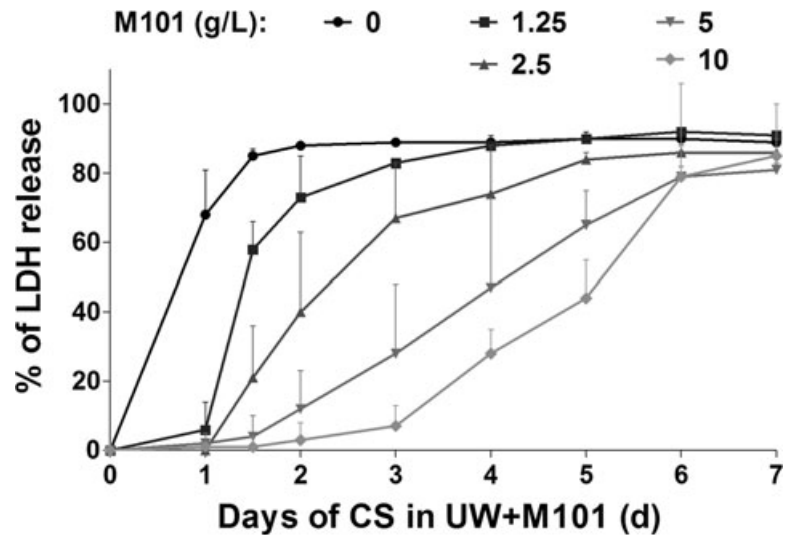


Figure 7: Cold-stored kidney epithelial cells preserved in UW supplemented with M101 up to 7 days. LLC-PK1 were stored at 4°C up to 7 days in UW supplemented with M101 in concentrations ranging from 0 to 10 g/L. Cell damage was assayed by percentage of LDH release. CS = cold storage. Shown are mean ± SD; n = 3.

molecule possesses a Cu/Zn-SOD activity, which is of high value in the context of IRI (18,23,24).

In vitro, progressive CS causes a significant and progressive (1) increase in cell LDH release, (2) reduction in metabolic activity and (3) reduction in energetic content. Twenty-four hours of CS resulted in almost complete loss of structural viability, functional viability and energetic content. The majority of cell death was likely due to necrosis, as shown by LDH test, rather than apoptosis since caspase-3 activity did not change. M101 protected renal cells in a time- and dose-dependent manner, independently of the solution used. LDH release in HTK, IGL and Cel-sior was at low levels without M101 so the addition of M101 had no additional effect on this parameter. The benefit of M101 is more evident by MTT assay, which is in accordance to a study showing superiority of MTT assay sensitivity over LDH release assay on the evaluation of metabolic activity (47).

M101 supplementation increased cellular ATP content, reflecting superior maintenance of energetic metabolism, likely decreasing the need to switch from mitochondrial respiration to anaerobic glycolysis and protecting the mitochondria. Maintenance of high ATP levels during preservation by M101 may also benefit the restoration of energy homeostasis upon reperfusion due to less metabolic stress on oxidative pathways.

In vitro results lead us to adopt 5 g/L *in vivo*, allowing for possible discrepancies between *in vitro* 2D culture and *in vivo* 3D tissue. In the later setting, we characterized the effects of M101 supplementation in a porcine autologous transplantation model particularly well adapted for studying IRI, the target of M101. An allograft setting may have biased the findings, for instance with side effects from immunosuppression.

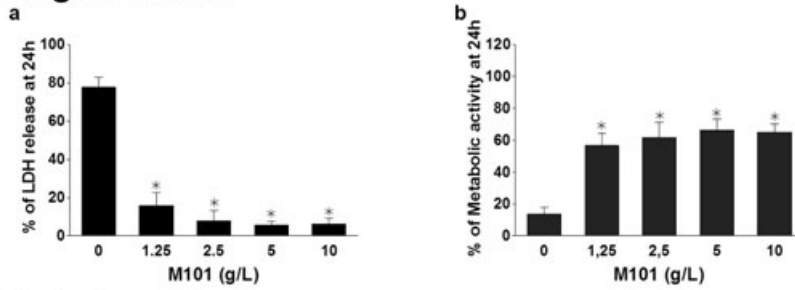
We preserved kidneys with either HTK or UW, the most widely used solutions in the world (Eurotransplant and UNOS data [14]), for 24 h. As current preservation time average 18 h, with a trend towards increasing, our choice of 24 h appears justified. Furthermore, 24 h provides the amount of IR damage adapted for a proof of concept. While we did not evaluate *in vivo* benefits on shorter preservation times, demonstration of M101 benefits on shorter preservation time are likely, considering the impact of the molecule *in vivo* after 24 h preservation and the protection demonstrated on *in vitro* studies for shorter preservation times.

The differences between UW and HTK have been the object of several publications and since this study was not designed to compare these solutions, we will focus our discussion on the impact of M101 on their performance separately. Use of M101 was correlated with improved early recovery of function, particularly in grafts preserved with UW. Since the intensity of IRI is directly correlated to the occurrence of delayed graft function in patients (1,2), it is expected that M101 will be efficient in reducing IRI *in vivo* in preserved kidney graft.

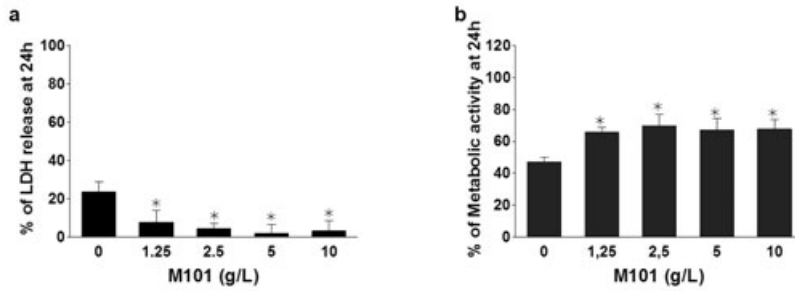
Histologically, IRI is characterized by extensive tubular damage, such as loss of brush border, and intense immune cell infiltration (11,12). Indeed, IRI was shown to be capable to induce inflammatory processes in an antigen-independent fashion (10). M101 supplementation was effective in protecting against these damages early on, likely through direct cell protection as observed *in vitro*. As stressed cells produce danger signals inducing inflammation and cell invasion (48), reduction of these mechanisms by M101 supplementation was likely due to better cell protection.

IFTA is the pivotal marker of chronic loss of graft function, and ultimately loss of the grafts itself (49). We

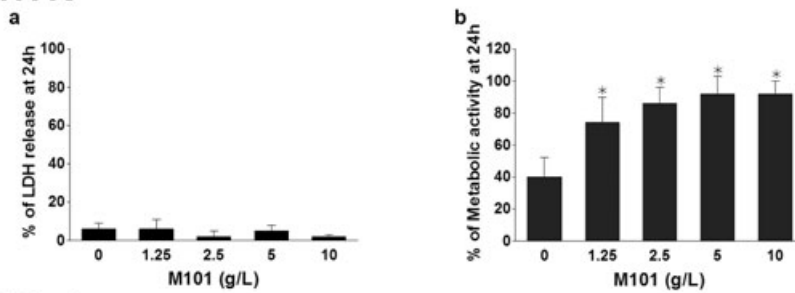
A - Ringer Lactate



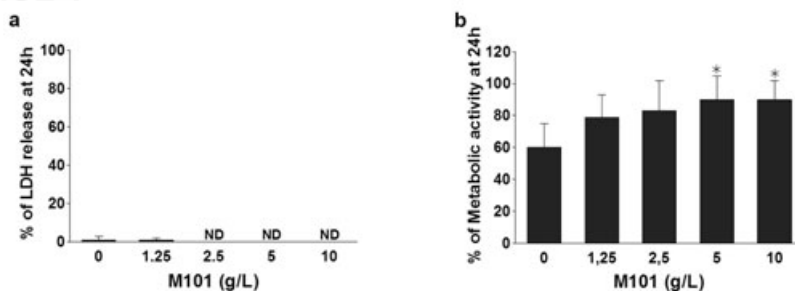
B - Perfadex



C - HTK



D - IGL-1



E - Celsior

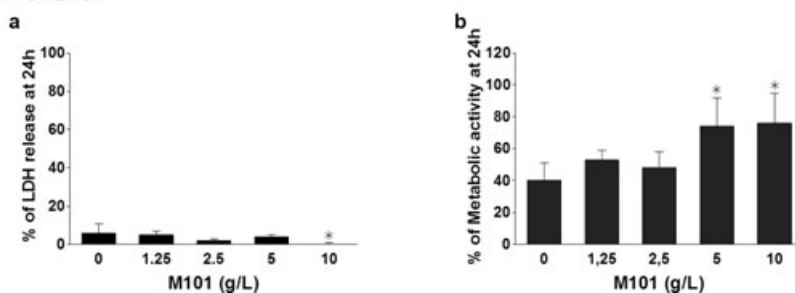


Figure 8: Cold-stored kidney epithelial cells preserved for 24 h in different commercial preservation solutions supplemented with M101. LLC-PK1 cells were stored at 4°C for 24 h in Ringer Lactate (A), Perfadex (B), HTK (C), IGL-1 (D) and Celsior (E), supplemented with M101 in concentrations ranging from 0 to 10 g/L. Cell damage was assayed by percentages of LDH release (a) and metabolic activity (b). Shown are mean ± SD. ND = not detected. Statistics: * = p < 0.05 versus cells cold-stored in the preservation solution alone; n = 3.

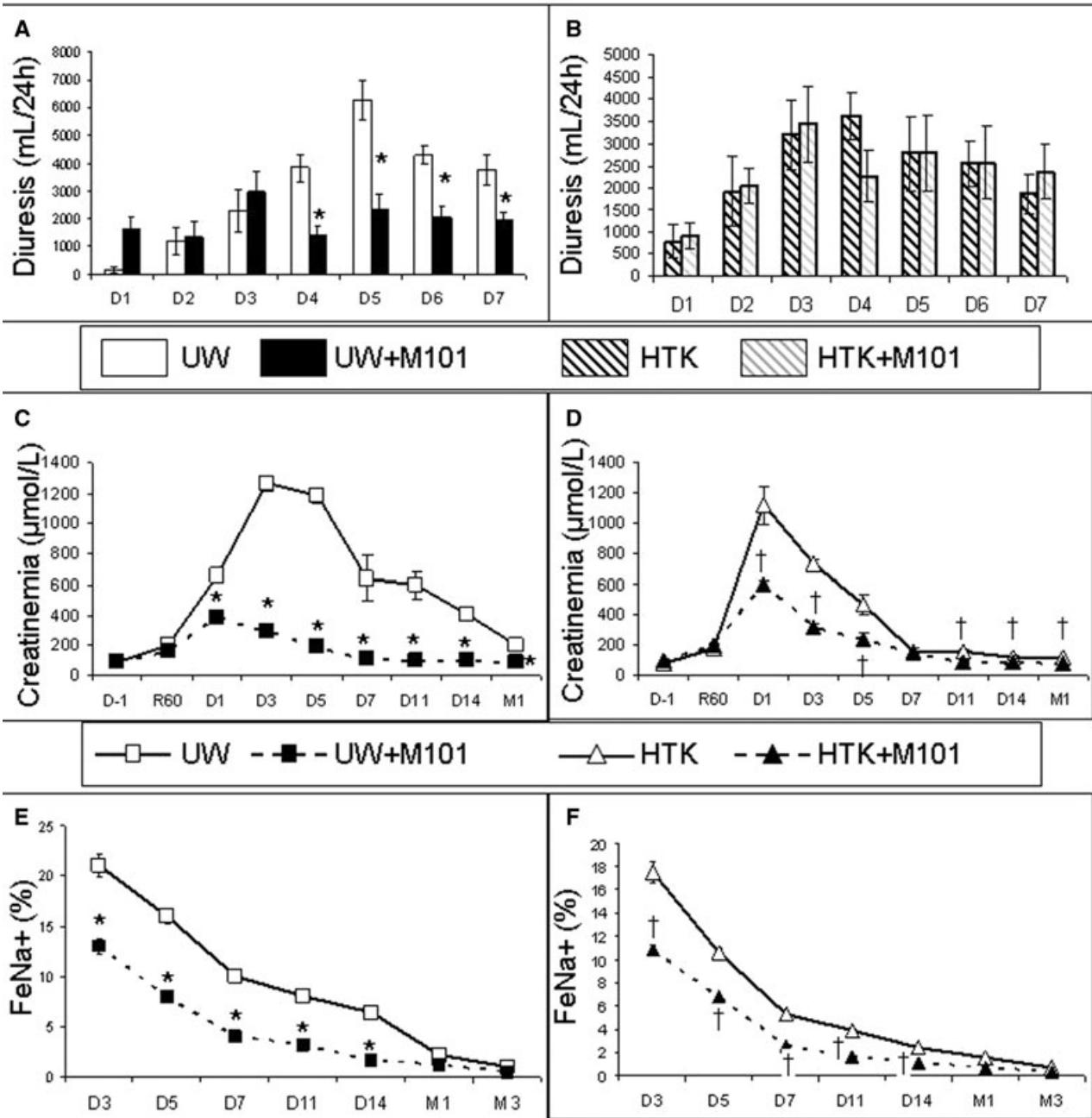


Figure 9: Kidney function following reperfusion. Porcine kidneys were cold-flushed and preserved for 24 h with UW (A-C-E) or HTK (B-D-F) supplemented with M101 at a concentration of 0 g/L (UW and HTK groups) or 5 g/L (UW+M101 and HTK+M101 groups). After transplantation, kidney function was assayed by diuresis (A-B), serum creatinine (C-D) and fraction of excreted sodium (E-F). Follow-up was performed the day before transplantation (D-1) and after transplantation (at 1h = R60; from day 1 to days 14 = D1 to D14; at months 1 and 3 = M1 and M3). Shown are mean ± SEM. Statistics: * = p < 0.05 versus UW; † = p < 0.05 versus HTK; n = 5.

therefore measured the development of IFTA in parallel with function loss at 3 months and determined that classical preservation solutions could not avoid extensive fibrosis development as well as loss of function. However, supplementation with M101 at the time of preservation correlated with diminished development of fibrosis, pre-

serving function and improving outcome. In our *in vivo* model, free of any effects due to immunosuppression (50), we observed the summated effects of damage sustained by organ preservation and reperfusion (51) and our data outlines the importance of an optimized preservation protocol.

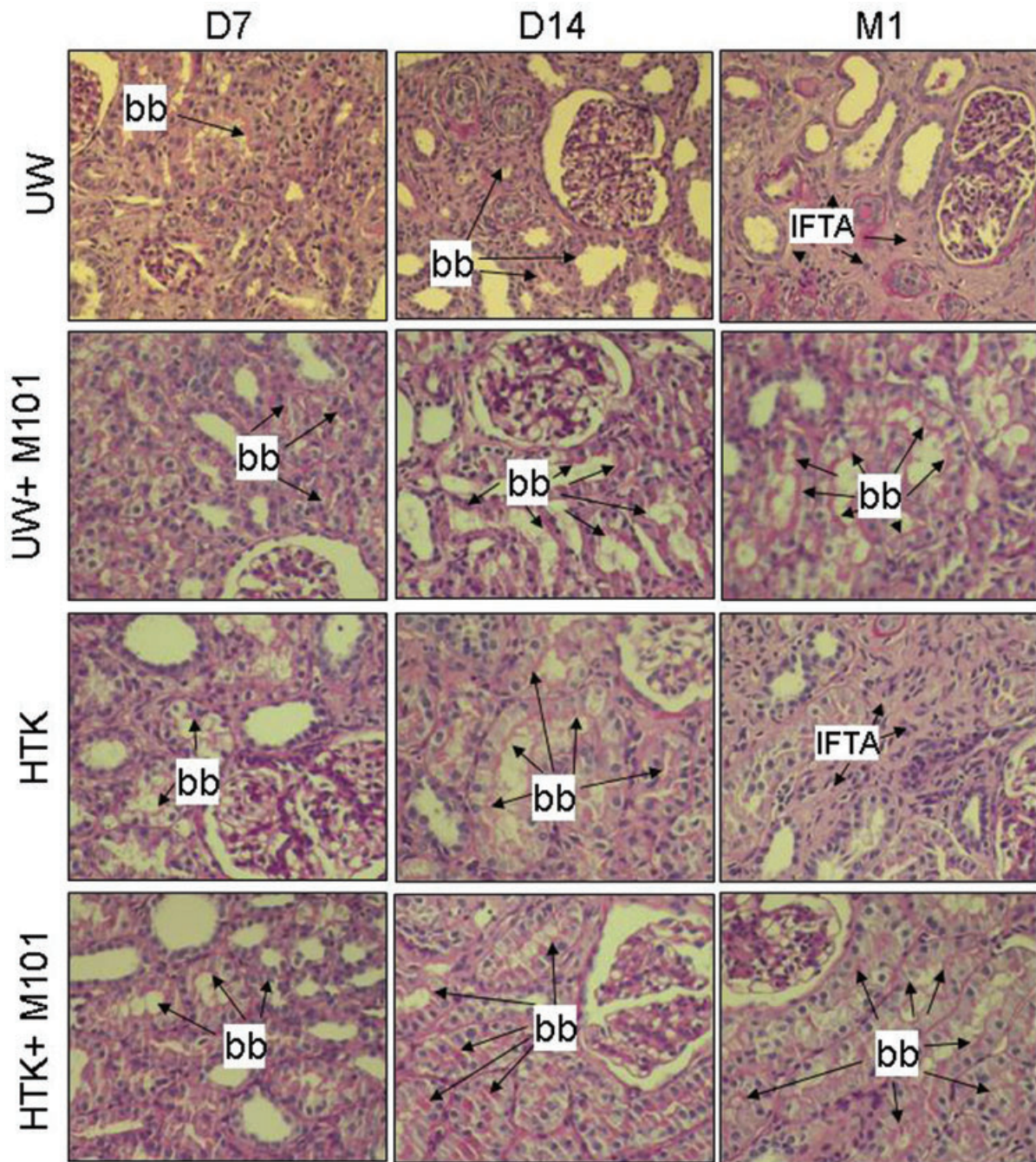


Figure 10: Evolution of kidney histology. Representative PAS staining of kidney biopsies at days 7, 14 and month 1 posttransplantation for UW, UW+M101, HTK, HTK+ M101 groups. Arrows showed brush border loss (bb) and interstitial fibrosis/tubular atrophy (IFTA).

Providing O₂ to ischemic tissue during CS could however be a “double-edged sword,” as O₂ can also fuel oxidative stress if the cell is not capable of restoring oxidative respiration (52). Herein, we showed that the use of an O₂ carrier during static preservation was protective, allowing cold-preserved cells to maintain their stock of ATP, hence presenting a balanced energy

metabolism at reperfusion and being able to handle the sudden influx of O₂ without excessive oxidative stress activation.

Alternative carriers are possible, such as PFCs (14,16), which through the use of the two-layers method (53) showed interesting potential in pancreas (54) and small

Table 2: Histological evaluation of kidney grafts

Groups	Histological Score						
	Brush Border Loss			Endoluminal detachment			
	D7	D14	M1	D7	D14	M1	
UW	3.5 ± 1.1	2.6 ± 0.9	2.1 ± 0.3	3.1 ± 0.3	2.5 ± 0.6	2.0 ± 0.1	
UW + M101	2.0 ± 1.4	2.0 ± 1.5	1.8 ± 0.4	0	0	0	
HTK	3.5 ± 0.6	2.3 ± 1.2	1.8 ± 0.4	1.0 ± 0.1	0	0	
HTK + M101	3.0 ± 1.6	1.5 ± 0.6	1.4 ± 0.5	1.0 ± 0.5	0	0	
Groups	Inflammation						
	Immune Infiltration			Tubulitis			
	D7	D14	M1	D7	D14	M1	
UW	3.1 ± 0.4	3.2 ± 0.3	2.6 ± 0.4	3.2 ± 0.4	3.1 ± 0.2	3.0 ± 0.3	
UW+M101	0.0 ± 0.0 ¹	1.0 ± 0.6	1.0 ± 0.0	0	0	1.0 ± 0.1	
HTK	1.5 ± 0.6	1.3 ± 1.2	1.2 ± 0.4	0.8 ± 0.9	0.7 ± 0.6	1.2 ± 0.4	
HTK+M101	1.5 ± 0.9	1.2 ± 0.5	1.2 ± 0.4	1.0 ± 0.5	0.5 ± 0.6	1.0 ± 0.1	
		CD3+ at 3 month			ED1+ at 3 month		
UW		17.0 ± 2.0			12.5 ± 1.5		
UW+M101		6.9 ± 0.4 ¹			9.5 ± 0.4		
HTK		23.0 ± 1.4			17.3 ± 3.2		
HTK+M101		14.2 ± 3.9 ²			6.6 ± 1.2 ²		

¹p < 0.05 to UW.

²p < 0.05 to HTK; n = 5.

Top panel: Typical IRI histological lesions, brush border loss and endoluminal detachment, were assayed for UW, UW+M101, HTK and HTK + M101 groups after 7 days, 14 days and 1 month posttransplantation. Scale: 0, no abnormality; 1, mild lesions affecting less than 25% of kidney samples; 2, lesions affecting 25–50% of kidney samples; 3, lesions affecting 51–75% of kidney samples; 4, lesions affecting more.

Middle panel: Inflammation was evaluated by measurement of tubulitis and immune infiltration and invasion for UW, UW + M101, HTK and HTK + M101 groups after 7 days, 14 days and 1 month posttransplantation. Quantitative determination was adapted from Banff classification: 0, no mononuclear inflammatory cell in tubules; 1, Foci with 1 to 4 mononuclear cells per tubular cross-section or 10 tubular cells; 2, Foci with 5 to 10 mononuclear cells per tubular cross-section; and 3, Foci with more than 10 mononuclear cells per tubular cross-section. Shown are mean ± SD.

Bottom panel: Inflammation was evaluated by measurement of CD3+ and ED1+ after 3 months posttransplantation: 3-month samples were specifically stained by immunofluorescence and the average number of positive cells by high-powered field (200×) was determined. Shown are mean ± SEM.

bowels (55) preservation. However, PFCs have the inconvenient of being hydrophobic, lipophilic and difficult to sterilize (56). Moreover, O₂ binding by PFCs needs high O₂ partial pressures, which implies a continuous O₂ supply during the preservation period and which raises the problem of hyperoxia and oxidative stress (57), possibly complicating their use in the clinic. Furthermore, recent data on pigs showed that the two layer method was not advantageous for kidney preservation (58). Another method consisting of active gaseous oxygenation by retrograde persufflation is also possible and has been shown in a small study to improve initial organ function (59); however, it might be difficult to set up in the clinic. These techniques provides O₂ in excess which could induce oxidative stress while a carrier such as M101 has the unique property of providing O₂ against a gradient, according to the physiological need of the cell and in a reversible process as we showed with functionality studies. Hemoglobin-based oxygen carriers are polymerized hemoglobin solutions from human or bovine origin first developed as blood substitute (60,61). However, they have not been used in organ preservation

and their use appears difficult, as the function of warm-blooded hemoglobin is uncertain at reduced temperatures. Nonprotein O₂ carriers are also included in Lifor (Lifeblood Medical, Freehold, NJ, USA), a new preservation solution, and showed promises in improving resistance to IRI in a rat model of warm ischemia (62). However, protection during hypothermia remains to be demonstrated and thus does not justify a complete change of preservation solution policy in the clinic, while the simple addition of an additive such as M101 has a higher translatability potential.

Moreover, M101 is naturally suited for hypothermic delivery of O₂. Indeed, it is derived from a marine organism, produced in a safe and secure environment, subjected to important changes in temperatures and environmental conditions. Evolution has therefore honed it into a most efficient way to transport O₂ in an extracellular manner, at low temperatures and with the ability to withstand a harsh environment such as hypoxia. In effect, M101 has been designed by evolution to maintain life in hypoxic and hypothermic conditions for long periods of time, hence is

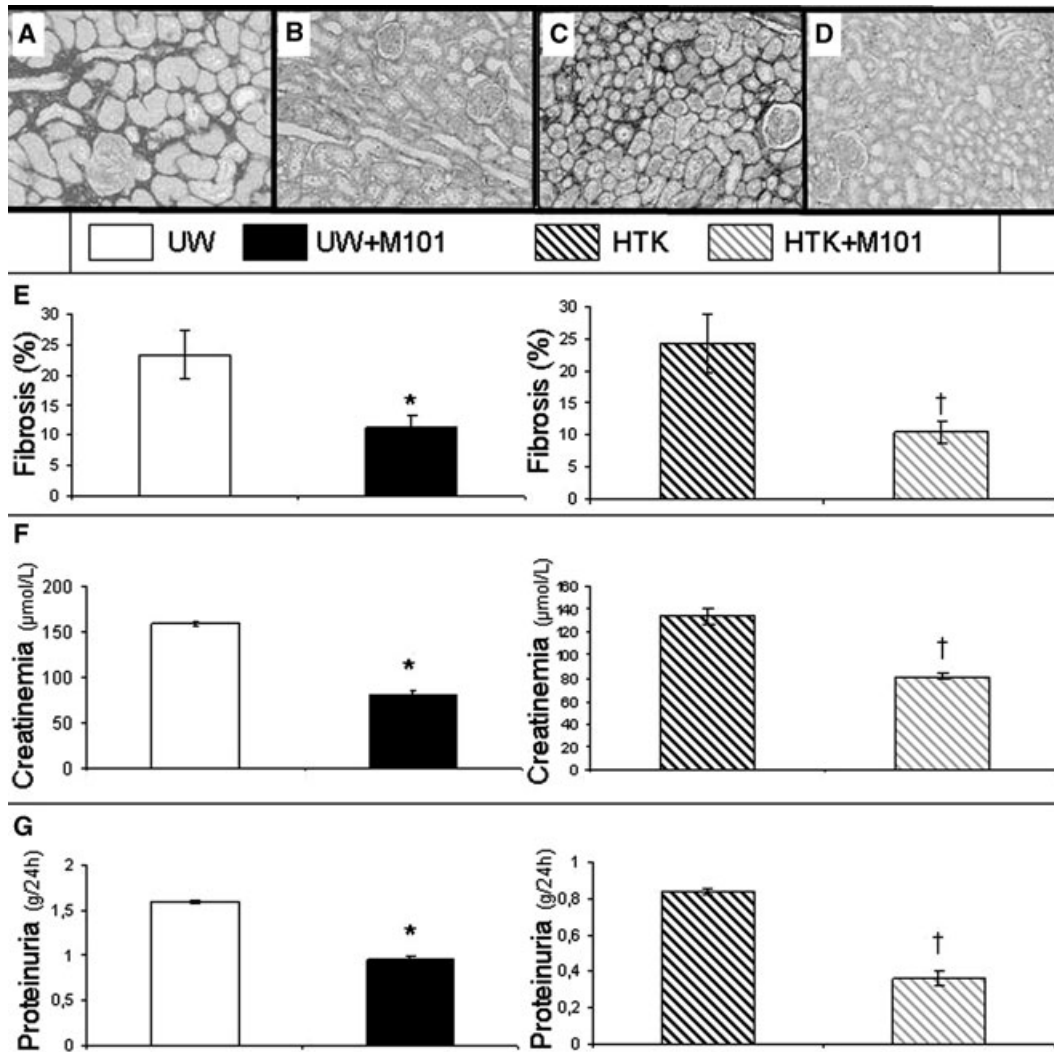


Figure 11: Kidney outcome at 3 months. A–D: Representative red Sirius staining for UW (A), UW+ M101 (B), HTK (C) and HTK+ M101 (D) groups at 3 months posttransplantation. E: Quantification of fibrosis development in each group. F: Serum creatinine in each group. G: Proteinuria in each group. Shown are mean \pm SEM. Statistics: * = $p < 0.05$ versus UW; † = $p < 0.05$ versus HTK; $n = 5$.

perfectly suited for organ preservation. M101 is thus stable in a wide range of organ preservation solutions of various ionic compositions and osmolarities, and provides both O_2 carrying and SOD abilities (18). This technology harnesses the product of millions of years of evolution to target the heart of IRI: lack of O_2 .

There is currently a rise of interest in regards to machine preservation of grafts (63,64) as it provides important improvement of graft quality and outcome (65). Although its mechanism remains unclear, the washout of metabolites and cellular waste produced during ischemia is a likely hypothesis. The presence of these products at reperfusion is indeed likely associated with the intense activation of the innate immune pathway, in accordance with the danger signal pathway (66,67). In this setting, the addition of

M101, to be cycled through the organ to deliver oxygen to its most remote territories, could be a valuable addition and provide additive or synergistic protection for the organ. This important issue is to be investigated in a future study.

In conclusion, we demonstrated herein the beneficial use of a novel O_2 carrier in two of the most used preservation solutions. We have demonstrated that the use of M101 in CS protocol is correlated with better short-term function recovery and reduced development of IFTA, main cause of graft loss. It is hoped that, in future, this technique will improve organ preservation not only for conventional organs but also for extended criteria and deceased after cardiac death organs, particularly as organ shortage leads centers towards increasingly marginal donors.

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Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. 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. Co-authors from Inserm, CNRS and UPMC do not have shares in the capital of the company. Co-authors from Hemarina were employed by the company at the time of these studies; furthermore, Franck Zal and Morgane Rousselot are the founders and the main shareholders of HEMARINA. Other authors from HEMARINA are only employed by the company.

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