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Reduced Ischemia-Reoxygenation Injury in Rat Intestine After Luminal Preservation With a Tailored Solution

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Background. The intestine is extremely sensitive to ischemic preservation and reoxygenation injury. Current vascular perfusion and cold storage with University of Wisconsin (UW) solution neglect the intestinal lumen and the ongoing mucosal metabolism during hypothermia. This study was designed to test the effects of luminal preservation with an alternative preservation solution in addition to the common vascular flush with UW solution on graft viability after preservation and *ex vivo* reoxygenation.

Methods. Rat intestine was preserved on ice for 6 hr in UW solution or Williams Medium E with additional buffering, impermeants, and a colloid (WMEplus) after being stapled or after flushing and filling the lumen with the respective preservation solution. Tissue slices were prepared from fresh and preserved intestines and were incubated with oxygen for 6 hr at 37°C to assess the viability after reoxygenation.

Results. Directly after preservation, histologic damage was mild and unaffected by preservation strategy. Contrary to luminal preservation, closed preservation resulted in significantly decreased ATP levels compared with control. Reoxygenation aggravated damage and revealed differences between the strategies. Luminal preservation better maintained the ATP levels and histologic integrity (vs. closed preservation) for both solutions. Histomorphologic integrity was superior after preservation with WMEplus (vs. UW solution). Expression of stress responsive genes was least upregulated in the slices from tissue preserved luminally with WMEplus.

Conclusions. In conclusion, preservation and reoxygenation injury can be attenuated by luminal preservation with WMEplus.

Keywords: Intestinal transplantation, Ischemia-reoxygenation injury, Preservation solutions, Preservation techniques.

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Intestinal transplantation (ITx) was introduced in the 1990s as a promising permanent therapeutic option for patients with irreversible intestinal failure. During the past 20 years, the modifications in patient selection, surgical technique, postoperative management, and evolution in immunosuppressive protocols have significantly improved the results of ITx (*1, 2*). Nevertheless, ITx continues to be a challenging transplant procedure because long-term outcome remains inferior compared with other organ transplants. Brain death in the donor, preservation injury, surgical manipulation during retrieval and transplantation, and ischemia-reperfusion injury (IRI) compromise the mucosal barrier (*3–8*). This consequently leads to the induction of immunoinflammatory processes and bacterial translocation, which predispose the recipient to sepsis and rejection as the main causes of morbidity and mortality after ITx (*1–2, 8*). Maintenance of intestinal integrity is, therefore, critical to enhance the viability of the intestinal graft and reduce posttransplant complications.

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The intestinal mucosa is well perfused under physiologic conditions and extremely vulnerable to ischemia (9). Unfortunately, an ischemic period after retrieval during preservation is inevitable to bridge the gap between donor and recipient. The current standard for intestinal preservation is a vascular washout with University of Wisconsin (UW) solution followed by cold, static storage (CS) in UW solution. Although the gold standard for many years, it has become clear that UW solution is not optimal for the preservation of the intestinal graft (10–13). Also, vascular preservation alone without exposure of the mucosa to the preservation solution may be insufficient to maintain intestinal integrity during clinically relevant storage periods (14, 15). Recent studies suggest that the intestine benefits from luminal contact with substrates in the preservation solution that meet physiologic demands (11, 16–22). However, no consensus has been reached about the optimal solution composition and strategy for intestinal preservation.

Preservation damage is known to be aggravated by normothermic reperfusion and reoxygenation. Unfortunately, all elements of injury to the intestine will accumulate upon reperfusion during transplantation. For this reason, a transplant model complicates the distinction of responsible factors of damage. To better unravel the possibly deleterious effect of preservation and reoxygenation as such, we have used precision-cut intestinal tissue slices that enabled us to specifically study the early events of reoxygenation injury. In the past, we have successfully used reoxygenated precision-cut liver slices as an *ex vivo* model for reoxygenation of preserved liver (23). Intestinal slices contain all cell types in their natural configuration and remain functionally active in culture for at least 8 to 24 hr (24).

The aim of this study was to test the effect of luminal preservation with a modified enriched intestinal preservation solution after a common vascular washout with UW solution on the maintenance of intestinal graft viability. Intestinal integrity was evaluated directly after preservation and after reoxygenation of intestinal slices.

RESULTS

Viability of Preserved Intestinal Tissue

Nonpreserved intestine (T0) showed hardly any structural damage, reflected by a mean Park Score of 0.13 (range 0–1). Preservation clearly affected the morphology: the mean Park Score after preservation was significantly higher ($P<0.001$) than for nonpreserved control tissue, regardless of preservation solution and strategy (Table 1). No differences in Park Score were observed between the different preservation groups.

ATP levels were decreased after preservation ($P<0.04$) in tissue of both closed preservation groups compared with control tissue, whereas no significant decrease was observed in the luminally preserved group (Table 1). However, there were no significant differences in ATP levels between the different preservation strategy groups when directly compared with each other.

Viability of Reoxygenated Intestinal Slices

Morphological integrity of reoxygenated slices from preserved intestinal tissue was significantly decreased (reflected by the higher total morphology score) in comparison

TABLE 1. Park scores and ATP results of control and preserved tissue

Preservation	Park score	ATP level (nmol/mg protein)
Control, not preserved (T0)	0.13 (0–1.0)	1.96 (0.7–2.7)
UW closed (T6)	2.6[a] (0–5.0)	1.4[b] (0.4–3.2)
UW luminal (T6)	2.4[a] (0.5–4.0)	2.1 (1.3–5.3)
WMEplus closed (T6)	2.6[a] (1.0–3.8)	1.29[b] (0.6–2.5)
WME plus luminal (T6)	2.2[a] (2.0–3.0)	1.31 (0.7–2.6)

Values represent the mean of intestinal tissue of eight rats with range in parenthesis.

There were no significant differences in Park Score or ATP levels between the preservation strategy groups when directly compared with each other.

[a] Values are significantly higher than control ($P<0.001$).

[b] Values are significantly lower than control ($P<0.05$).

T0, control, no preservation, direct assessment; T6, 6 hr preservation, followed by assessment; UW, University of Wisconsin; WMEplus, Williams Medium E with additional buffering, impermeants, and a colloid.

with reoxygenated slices from control tissue (Fig. 1A) for all four preservation groups. However, morphology was significantly less affected in slices from tissue preserved with WMEplus than in slices from tissue preserved with UW solution, independent of the preservation strategy ($P<0.001$, Fig. 1A, Table 2). Also, independent of the preservation solution, morphology was superior after luminal preservation than after closed preservation ($P<0.001$, Fig. 1A, Table 2). The beneficial effect of luminal preservation and WMEplus was reflected by all separate morphology parameters (Table 2) except for villus flattening, which was not prevented by either combination of strategy and preservation solution. Furthermore, viability of the muscle layer and the shape of epithelial cells were not significantly improved by luminal preservation.

The ATP content was significantly lower in reoxygenated slices from all preservation groups than in reoxygenated slices of tissue that was not preserved (Fig. 1B). After direct comparison of the preservation groups, it was noted that independent of the used preservation solution, ATP content was higher in slices after luminal preservation than after closed preservation (Table 2).

Gene Expression in Reoxygenated Slices

Remarkably, less RNA could be extracted from reoxygenated slices that were preserved with UW solution without luminal exposure than from slices from preserved tissue from the other groups (Table 2). The highest RNA recovery was obtained from slices of tissue that was luminally preserved with WMEplus. RNA yield reflects the capacity of cells to produce RNA. RNA yield closely corresponded to the outcome of other viability parameters (histology and ATP content), which indicates that more RNA could be extracted from qualitatively better tissue.

In general, gene expression was least affected (most resembled expression in slices from control tissue) when luminal preservation with WMEplus was applied (Table 3). Furthermore, Table 3 shows the contribution of the preservation solution and preservation strategy to the differences in gene expression. The expression of villin, a protein that is exclusively expressed in the epithelial cells, was unaffected by luminal preservation with WMEplus but significantly down-
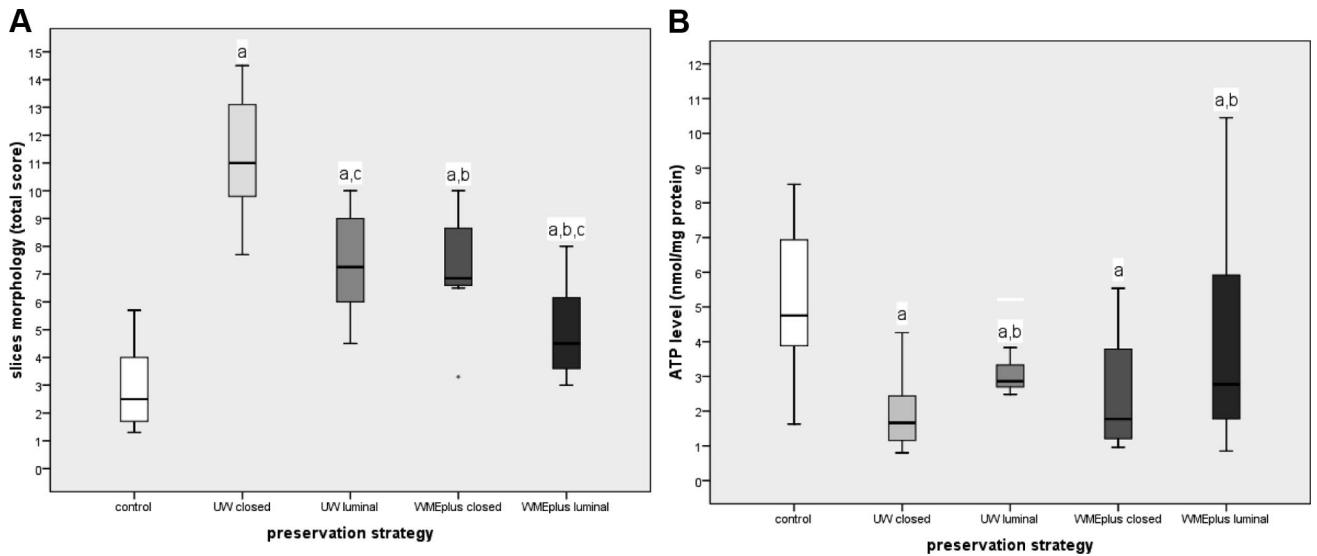
FIGURE 1. Viability of intestinal slices after reoxygenation. (A) Morphology score: scores of separate morphologic parameters were added up to a total morphology score (maximum score 18) to indicate overall viability of the slices. (a) Values are significantly higher than control value ($P<0.05$). (b) Values are significantly lower than for slices from tissue preserved with University of Wisconsin (UW) solution, independent of the preservation strategy ($P<0.001$). (c) Values are significantly lower than for slices of tissue after closed preservation, independent of the preservation solution ($P<0.001$). (B) ATP level: values represent mean ATP level (nmol/mg protein) of tissue of eight experiments (intestines of eight rats). (a) ATP levels are significantly lower than control ($P<0.05$). (b) ATP levels were significantly higher than after closed preservation, independent of the preservation solution ($P<0.001$). The band in the middle of the *boxes* reflects the median of the data set. The upper edge indicates the 75th percentile of the data set, whereas the lower edge indicates the 25th percentile. The length of the *box* represents the inter quartile range (IQR). The whiskers mark the highest and lowest values within a distance of 1.5 IQR to the *box*.

regulated in the other preservation groups. However, direct comparison of the preservation solutions and preservation strategies only showed a beneficial effect of the luminal preservation strategy.

TABLE 2. Effect of preservation strategy and solution on slice viability after reoxygenation

Viability parameter	Luminal vs. closed[a]	WMEplus vs. UW[b]	Interaction[c]
Morphology			
Total morphology score	$P=0.0001$	$P=0.0001$	No
Viability enterocytes	$P=0.0031$	$P=0.0017$	No
Shape epithelium	No	$P<0.0001$	No
Viability Stroma	$P<0.0001$	$P=0.023$	No
Villi flattening	No	No	No
Viability crypts	$P=0.0018$	$P=0.0007$	No
Viability muscle layer	No	$P=0.023$	No
RNA concentration	$P=0.0003$	$P=0.0001$	No
ATP content	$P=0.0003$	No	No

[a] $P<0.05$ indicates superiority of slices of luminally preserved tissue compared with slices of tissue that was not luminally exposed to the preservation solution.
[b] $P<0.05$ indicates superiority of slices of tissue preserved with WMEplus compared with slices of tissue that was preserved with UW.
[c] Interaction was found between preservation strategy and preservation solution.
UW, University of Wisconsin; WMEplus, Williams Medium E with additional buffering, impermeants, and a colloid.

The expression of genes known for their response to cellular stress, inducible nitric oxide synthase (iNOS), interleukin (IL)-6, heme oxygenase (HO)-1, and heat shock protein 70 (Hsp70) was most pronouncedly up-regulated in slices preserved according to the clinical standard (closed preservation with UW solution). This induction was significantly lower after preservation with WMEplus, and in the case of iNOS and HO-1, this induction was also significant after luminal preservation. Furthermore, the mRNA expression of tight junction proteins was up-regulated in slices from preserved tissue compared with control. Again, expression most closely resembled that of slices from control tissue when WMEplus in combination with luminal preservation was applied.

DISCUSSION

Ischemic preservation and reoxygenation on reperfusion negatively affect the intestinal graft and result in inflammatory and immunological complications after ITx. Standard hypothermic preservation causes substantial damage to the vulnerable intestinal graft, particularly because vascular washout and CS with UW solution seem to insufficiently protect this organ (20). Development of a specific intestinal preservation solution and protective conditions is essential to improve the long-term outcome of ITx. We assessed the potential of luminal preservation and the use of an alternative CS solution to protect intestinal graft quality.

This study endorses that actual graft damage is not revealed directly after a clinically relevant preservation span of 6 hr, irrespective of the preservation solution and strategy, but
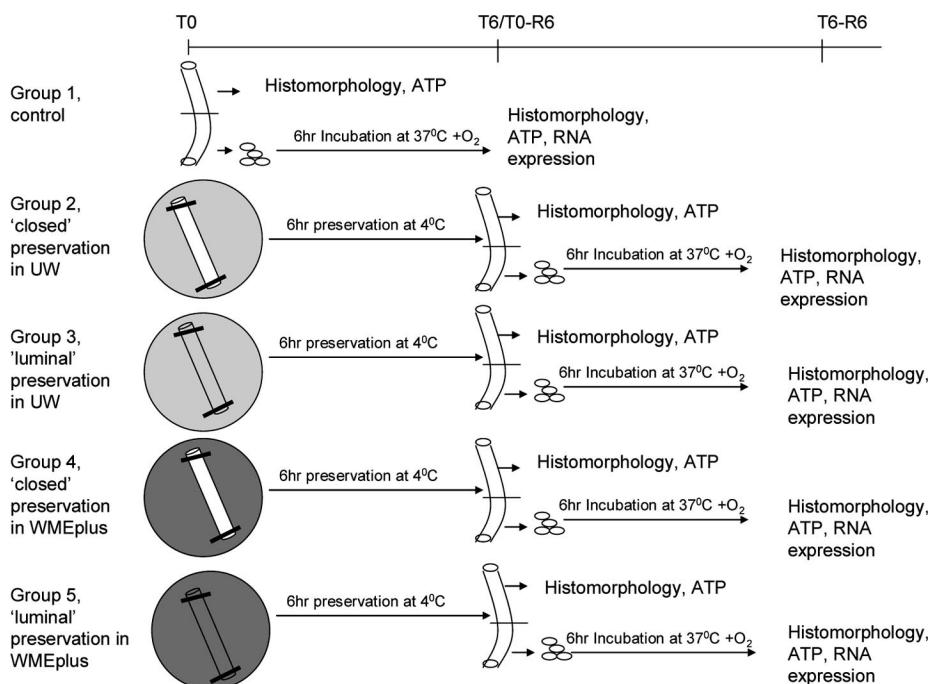
FIGURE 2. Experimental set up. For all experimental groups, a vascular flush with 20 mL of ice-cold (4°C) of Wisconsin (UW) solution was applied before preservation. The jejunum was retrieved and divided into five equal pieces, which were randomly assigned to five different groups to exclude confounding by anatomical factors. Group 1: fresh control without preservation. Group 2: UW closed preservation: The intestinal lumen was directly stapled with vascular clips (Titanium Clips, Horizon, Teleflex Medical, NC) followed by cold, static storage (CS) in UW solution for 6 hr. Group 3: UW luminal preservation: The intestinal lumen was flushed with 15 to 20 mL of UW solution. Then the distal lumen was closed, and the intestine was filled (1–2 mL, hydrostatic pressure maximum 10 cm H_2O). Finally, the proximal lumen was closed, followed by CS in UW solution for 6 hr. Group 4: Williams Medium E with additional buffering, impermeants, and a colloid (WMEplus) closed preservation: the intestinal lumen was directly stapled with vascular clips followed by CS in WMEplus for 6 hr. Group 5: WMEplus luminal preservation: the intestinal lumen was flushed with 15 to 20 mL of WMEplus. Then the distal lumen was closed, and the intestine was filled. Finally, the proximal lumen was closed, followed by CS in WMEplus for 6 hr. T0 reflects the time point at which preservation starts, whereas T6 reflects the end of the 6-hr preservation period. After reoxygenation, T0–R6 reflects the time point at which slices from nonpreserved tissue have been reoxygenated for 6 hr, whereas T6–R6 reflects 6-hr reoxygenation of slices derived from tissue that was preserved for 6 hr.

becomes manifest after reoxygenation (25). We were able to reduce the encountered preservation and reoxygenation injury by luminal preservation with an alternative solution (WMEplus) for hypothermic preservation of the intestine after the standard vascular washout with UW solution. WMEplus is based on WME (with L-glutamine), a relatively cheap, ready-to-use medium, that contains many ingredients that have previously shown to be beneficial for intestinal preservation. To increase its potential as a preservation solution, we added PEG (35 kDa), raffinose, lactobionate, and the powerful HEPES buffer. Because the composition of WMEplus differs in many aspects from UW solution (Table 4), we can only speculate about which ingredients are responsible for the apparent superiority of WMEplus in this study. Earlier studies have identified colloid agents, amino acids (AAs), and buffering capacity as the possible crucial factors responsible for the protective effect of luminal preservation with tailored solutions (16, 22, 26).

WMEplus contains the colloid PEG instead of hydroxyethyl starch in UW solution to reduce osmotic cell swelling. The efficacy of hydroxyethyl starch is controversial (27, 28). The water-soluble PEG macromolecule with abundant hydroxyl groups is assumed to effectively retain water. Furthermore, PEG binds to enterocyte-attached sphingolipids that may stabilize the epithelium and prevent shifts of luminal contents (e.g., water and electrolytes) into the tissue. In addition, PEG acts as a free radical scavenger (29–31). Recently, Oltean et al. (32) reported that intraluminal preservation with a PEG-containing solution decreased preservation injury.

Fujimoto et al. showed improved viability of intestinal grafts directly after preservation by using an AA-rich solution. AAs are postulated to play a cytoprotective role by catering for metabolic and synthetic elements of intestinal metabolism (16, 22). This benefit was most pronounced after luminal exposure to AA during preservation (16, 17, 33) and was attributed to a better maintenance of energy levels. Especially the most vulnerable epithelial cells at the villus top seem to benefit from luminally supplied nutrients (17). WME (with L-glutamine) contains 20 different AA. Particularly glutamine has been proposed to be favorable for intestinal preservation, because it is the main energy substrate of the enterocyte. The addition of glutamine to the preservation solution requires high buffering capacity to counteract unphysiologic pH shifts when glutamine metabolism is sustained in a system devoid of hepatic detoxification (16). For this reason, we have added the powerful sulfonic buffer HEPES to WMEplus. Future studies should

TABLE 3. Gene expression in slices of preserved tissue

	UW closed	UW luminal	WMEplus closed	WMEplus luminal	Luminal vs. closed[a]	WMEplus vs. UW[b]	Interaction[c]
House-keeping gene							
GAPDH	**1.4** (0.7–3.2)	**1.2** (0.7–3.2)	**1.2** (0.9–1.3)	**1.2** (0.8–1.8)	No	No	No
Epithelial cell marker							
Villin	**0.4** (0.1–1.0)	**0.5** (0.2–1.6)	**0.4** (0.1–0.8)	**0.9** (0.1–2.5)	P=0.007	No	No
Proinflammatory marker							
IL-6/GAPDH	**4.6** (0.2–11.9)	**3.1** (0.4–6.3)	**2.3** (0.2–5.9)	**1.5** (0.3–4.2)	No	P=0.0082	No
Tight junction proteins							
Occludin/villin	**1.9** (0.9–5.3)	**1.4** (0.9–2.7)	**1.5** (0.8–2.5)	**1.3** (0.8–2.5)	No	No	No
ZO1/villin	**3.8** (1.5–7.0)	**2.9** (1.7–2.0)	**2.5** (1.5–3.4)	**2** (0.9–3.7)	No	P=0.0065	No
Claudin-3/villin	**2.5** (1.2–6.1)	**2** (1.2–2.9)	**1.7** (1.1–2.4)	**1.4** (0.9–2.3)	No	P=0.0059	No
Cellular stress marker							
HSP-70/GAPDH	**10.4** (1.2–51.8)	**10.1** (1.5–50.5)	**5.9** (1.5–22.3)	**3.4** (0.7–8.6)	No	P=0.04	No
HO-1/GAPDH	**5.4** (1.8–8.0)	**3.1** (2.0–6.4)	**3.7** (1.7–8.5)	**1.9** (0.8–4.7)	P=0.0006	P=0.006	No
iNOS/villin	**23.5** (0.8–66.2)	**11** (0.3–63.4)	**14.4** (0.3–69.0)	**4.1** (0.2–14.2)	P=0.0014	P=0.014	No

Values represent average fold expression (in bold) and range of the measured values. mRNA expression of control slices of not-preserved tissue incubated for 6 hr is set to 1. ■, mRNA expression significantly up-regulated in comparison with control slices; ■, mRNA expression not significantly different from control slices; ■, mRNA expression significantly down-regulated in comparison with control slices.

[a] Gene expression was significantly less affected in slices of luminally preserved tissue than in slices of tissue that was not luminally exposed to preservation solution, *P* value is given.

[b] Gene expression was significantly less affected in slices of tissue preserved in WMEplus than in slices of tissue that was preserved with UW, *P* value is given.

[c] Interaction between preservation strategy and preservation solution.

UW, University of Wisconsin; WMEplus, Williams Medium E with additional buffering, impermeants, and a colloid; IL, interleukin; HSP-70, heat shock protein 70; HO-1, heme oxygenase 1; iNOS, inducible nitric oxide synthase.

identify the beneficial effect of each ingredient, or group of ingredients, of WMEplus by systematic comparison of a range of solutions with different compositions.

Luminal preservation is postulated as an effective strategy to reduce intestinal graft damage (*11, 14, 16, 17, 32, 33*). We demonstrated that luminal preservation improved energy levels of preserved tissue directly after preservation and after reoxygenation compared with closed preservation. The luminal uptake of additional cytoprotective agents such as AA may not be the only explanation for the increased viability because luminal preservation with UW solution also improved energy levels. Possibly, both the dilution of enteric cytotoxic intestinal contents by luminal flushing and the faster cooling during luminal preservation contributed to improved graft preservation, independent of solution composition, as suggested before (*14, 20*). Luminal preservation is clinically feasible by nasogastric administration to the donor simultaneously with cold vascular perfusion.

The local production of proinflammatory cytokines, chemokines, and reactive oxygen species (ROS) during reoxygenation of the ischemic tissue is a crucial early event in the cascade that leads to tissue IRI (*34, 35*). These signaling factors trigger a specific stress response aiming to counteract the physiologic challenges provoked by reoxygenation. Precision-cut slices, which contain all intestinal cell types (including resident macrophages) in their physiologic matrix, have enabled us to mimic this early phase of IRI, under *ex vivo* well-controlled conditions.

Several transcription factors are known to be activated by reoxygenation-associated ROS formation. One of these transcription factors is nuclear factor-E2-related factor 2 (Nrf2). Activation of Nrf2 has protective effects aiming to counteract an ischemic insult by up-regulation of the expression of the antioxidant HO-1 (*36*), stress response protein Hsp70 (*37, 38*), and tight junction proteins (*39*), which play a central role in maintaining the integrity of the intestinal physical barrier (*6, 7*), necessary to prevent (systemic) inflammation after ITx (*3, 35, 40*). We found that the gene expression of Hsp70, HO-1, and tight junction proteins such as ZO-1, claudin-3, and occludin-1 was generally up-regulated in reoxygenated slices from preserved intestinal tissue. The most marked up-regulation was consistently shown in reoxygenated slices of tissue preserved with UW solution, without luminal exposure. Because these slices also showed most morphologic damage and had the lowest ATP content, we concluded that most ischemic stress as a result of ROS formation was induced in the latter preservation group.

Another key factor in the origin of IRI is the nuclear factor (NF)-κB, which induces many proinflammatory reactions that serve as an adaptive mechanism on the one hand but also reflect tissue damage on the other hand. A number of studies have shown that suppression of NF-κB protects against IRI (*41–44*). Among the proteins that are up-regulated because of induction by NF-κB are the proinflammatory cytokine IL-6 (*45*) and iNOS, which both play a role in IRI (*3*). We demonstrated that the expression of iNOS and IL-6 is lower in tissue that is preserved with WMEplus compared with UW solution. Furthermore, iNOS is significantly less up-regulated after luminal preservation compared with closed preservation. The lowest expression of both Nrf2- and NF-κB-driven stress responsive genes was consequently demonstrated in tissue that was luminally preserved with WMEplus, which also showed the best morphology and high-

TABLE 4. Composition of the tested preservation solutions

Components (mmol/L if not defined)		WMEplus	UW
Physical characteristics	pH	7.4–7.5	7.4
	Osmolarity (mOsm/L)	333	320
	Viscosity at 5°C (cP)		5.7
Colloid/ impermeants	HES (250 kDa) (g/L)		50
	PEG (35kDa) (g/L)	16	
	Lactobionate	24	100
	Raffinose	40	30
Buffers	NaHC0$_3$	20.8	
	NaH$_2$PO$_4$	0.8	
	KH$_2$PO$_4$		25
	HEPES	8	
Anorganic salts	Calcium chloride	1.44	
	Cupric sulphate	0.00008	
	Ferric sulphate	0.00008	
	NaCl	94	
	KCl	4.2	
	Magnesium sulphate	0.64	5
	Manganese sulfate	0.8×10^{-6}	
	Zincsulfate	0.8×10^{-6}	
	NaOH	26	27
	KOH		100
	Total potassium	4.2	125
	Total sodium	142	27
	Total calcium	1.44	—
Antioxidants	Allopurinol		1
	Glutathion	0.00016	3
	α-Tocopherol	1.1×10^{-5}	
	Ascorbic acid	0.008	
Others	Glucose	8.9	
	Adenosine		5
	Sodium pyruvate	0.18	
	Methyllineolate	0.00008	
Amino acids	L -Alanine	0.8	
	L-Arginine	0.22	
	L-Asparagine-H$_2$O	0.104	
	L-Aspartate	0.18	
	L-Cysteine	0.26	
	L-Cysteine 2HCl	0.064	
	L-Glutamic acid	0.27	
	L-Histidine	0.08	
	L-Glutamine	2.4	
	L-Glycine	0.48	
	L-Isoleucine	0.30	
	Leucine	0.46	
	L-lycine-HCl	0.4	
	L-Methionine	0.08	
	L-Phenylalanine	0.12	
	L-Proline	0.21	
	L-Serine	0.76	

(*Continued*)

TABLE 4. Continued

Components (mmol/L if not defined)		WMEplus	UW
	Threonine	0.27	
	Tyrosine	0.15	
	Tryptophan	0.04	
Vitamins	Ascorbic acid	0.008	
	Biotin	0.0016	
	Choline-Cl	0.008	
	Ca-pantothenate	0.0016	
	Ergocalciferol	0.0024	
	Folic acid	0.0016	
	Menadione-Na(SO$_3$)$_2$	3.2×10^{-5}	
	Niacinamide	0.0064	
	Pyridoxal-HCl	0.004	
	Ribovlavin	0.00024	
	Thiamine-HCl	0.00024	
	Vitamin A-acetate	0.00024	
	Vitamin B12	0.00008	
	i-Inositol	0.008	
	Vitamin E	1.1×10^{-5}	

Components of WME are derived from Invitrogen (http://products.invitrogen.com/) and bold indicates extra additions to WME for this study. Components of UW are derived from the instruction leaflet supplied with the solution.

UW, University of Wisconsin; WMEplus, Williams Medium E with additional buffering, impermeants, and a colloid.

est ATP content, indicating that the least stress was induced in this group.

A limitation of this study is the lack of ultimate proof using a transplant model. However, because the cascade of injury after transplantation and *in vivo* reperfusion is rather complex, we have deliberately chosen to first study the effect of preservation in combination with reoxygenation in a multicellular reperfusion model without any alloreactive features. In conclusion, our data consistently demonstrate that luminal preservation and preservation with WMEplus independently reduce cellular stress and subsequent loss of viability of preserved intestinal tissue after *ex vivo* reoxygenation. Our next experiments will concern assessment of the proposed strategy in a transplant model to further refine intestinal preservation and reduce intestinal graft injury, ultimately improving the outcome after ITx.

MATERIALS AND METHODS
Animals
Adult male Wistar rats (n=8, HsdCbp:Wu, 330–400 g, Harlan, Horst, The Netherlands) were housed under standard conditions with free access to drinking water and rat chow. The experiments were conducted in accordance with institutional and legislatory regulations.

Surgical Procedure
Rats were anesthetized under 5% isoflurane/O$_2$, followed by 2.5% to maintain anesthesia. A midline laparotomy was performed to expose the aorta at the level of the celiac trunk. The supraceliac aorta was clamped, and 20 mL of ice-cold (4°C) UW solution was administered retrogradely through the infrarenal aorta through a 20-G canula. The suprahepatic vena cava was

transected to facilitate the outflow of blood and perfusate. Starting from 15 cm distally to the stomach, 30 cm of small intestine (jejunum) was excised. Afterward, rats were killed.

Preservation Solutions

UW solution (Viaspan, Belzer, Du Pont, Bristol, United Kingdom) and Williams Medium E with additional buffering, impermeants, and a colloid (WMEplus) were used as preservation solutions. WMEplus was prepared by adding 20 g/L polyethylene glycol (PEG; 35 kDa), 50 mmol/L raffinose, 30 mmol/L lactobionate, and 10 mmol/L HEPES (Sigma-Aldrich, St Louis, MO) to Williams Medium E (WME; with L-glutamine, Invitrogen, Paisley, United Kingdom). Finally, four parts of the solution were diluted with one part of distilled water to reach a final osmolarity of 330 mOsm/L. The pH was set to 7.4–7.5 by adding NaOH (26 mmol/L). Solution composition is specified in Table 4.

Experimental Groups

The excised jejunum of one rat was divided into five pieces of 5–6 cm, which were randomly assigned to serve as fresh control without preservation (group 1) or to be preserved according to one of the four different preservation protocols (groups 2–5) as illustrated in Figure 2. CS was performed on melting ice for 6 hr in 5 mL of preservation solution.

Ex Vivo Reoxygenation

Precision-cut intestinal slices were prepared from control and preserved intestinal segments as described previously (24, 46) and incubated at 37°C for 6 hr in 12-well culture plates in 1.3-mL Williams Medium E, supplemented with L-glutamine, extra D-glucose (final concentration 25 mM), gentamicin (50 μg/mL, Invitrogen, Paisley, United Kingdom), and fungizone (2.5 μg/mL, Invitrogen, Paisley, United Kingdom) (24, 46), in an atmosphere of 95% oxygen/5% CO_2 (dissolved oxygen in the medium was 95% of maximal saturation as measured by van Midwoud et al. [47]). For each outcome parameter, three slices were incubated, except for mRNA analysis by reverse-transcriptase polymerase chain reaction for which six slices were used.

Outcome Parameters

The viability of fresh control tissue and preserved intestinal segments was determined by the assessment of histomorphologic integrity and ATP level. In reoxygenated slices, histomorphologic integrity, ATP levels, and mRNA expression of several stress-responsive genes (Tables 3 and 5) were determined.

Histologic Examination of Tissue

Full-thickness samples of control and preserved intestinal tissue were fixed in 4% buffered formalin, dehydrated, embedded in paraffin, cut (3–5

μm), and stained with hematoxylin-eosin. Histologic damage was assessed using the Park Score (9).

Histologic Examination of Slices

Histomorphologic appearance of the slices after reoxygenation was assessed using a scoring system that was developed to evaluate the integrity of cultured intestinal slices. The structure of the slices was evaluated by ascribing a score between 0 (no changes) and 3 to 6 aspects: viability and shape (columnar or flat) of the epithelial cells, viability of the stroma, crypts, and muscle layer, and flattening of the villi. Scores of the six separate morphologic parameters were added up to a total morphology score between 0 and 18, reflecting the overall integrity of the slices. All histologic samples (tissue and slices) were evaluated by a pathologist blinded to the assignment of experimental groups.

ATP Measurement in Tissue and Slices

ATP samples were immersed in 1-mL ice-cold 70% ethanol, containing 2 mM ethylenediaminetetraacetic acid (pH 10.9), directly snap-frozen in liquid nitrogen, and stored at −80°C. The ATP content was determined after centrifugation in the supernatant as described previously (24). The protein content of tissue samples was determined in the pellet to normalize the ATP concentration. ATP values of the slice were corrected with the average protein content of three slices from nonpreserved tissue. For this purpose, the pellet was dissolved with 5 M NaOH and then diluted 50 times with MilliQ water after which the protein content was determined colorimetrically using BioRad protein assay dye reagent (Bio-Rad, Munich, Germany).

Gene Expression Levels

After reoxygenation, six slices were collected together in one sample vial, snap-frozen in liquid nitrogen, and stored at −80°C. After thawing, RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was prepared as described previously (5). Polymerase chain reaction was performed according to the Table 5. All assays were performed at least in duplicate. Dissociation curve analyses were performed for each reaction to check the formation of one specific product. For each gene, the expression was normalized with the mean computed tomographic threshold value of villin (for genes expressed in epithelial cells) or GAPDH (other genes). Results were expressed as $2^{-[\Delta\Delta C_T]}$, which is an index of the amount of mRNA expressed relative to the chosen house-keeping gene (GAPDH or villin) and the expression in slices derived from nonpreserved tissue.

Statistics

Data were analyzed by fitting mixed-effect models (48). For the ATP concentration and the RNA expression calculations, values were first trans-

TABLE 5. Sequences of primers/genes of interest

	Forward primer	Reverse primer
GAPDH[a,b]	CGCTGGTGCTGAGTATGTCG	CTGTGGTCATGAGCCCTTCC
Villin[a,b]	GCTCTTTGAGTGCTCCAACC	GGGGTGGGTCTTGAGGTATT
IL-6[a]	ATGTTGTTGACAGCCACTGC	ACAGTGCATCATCGCTGTTC
iNOS[a]	CGTTCGATGTTCAAAGCAAA	CCCTGGACTTCTCACTCTGC
Occludin[b]	ATTGAGCCCGAGTGGAAAGG	AGAGTGCAGAGTGGAGAGCTGATTAA
ZO1[b]	AACGCTATGAACCCATCCAG	CGGTTTGGTGGTCTGAAAGT
Claudin-3[b]	CTCCGGTTGCCACCTGATTAC	TCCATTCGACTTGGACAGTTCC
HSP-70[b]	GGTTGCATGTTCTTTGCGTTTA	GGTGGCAGTGCTGAGGTGTT
HO-1[b]	CTCGCATGAACACTCTGGAGAT	GCAGGAAGGCGGTCTTAGC

The following amplification conditions were used for real time polymerase chain reaction.
[a] 10 min at 95°C and then 40 cycles of amplification at 95°C for 15 sec, 56°C for 15 sec, and 72°C for 40 sec followed by a dissociation stage at 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec.
[b] 2 min at 50°C and 10 min at 95°C. This was followed by 40 cycles amplification consisting of denaturation for 15 sec at 95°C followed by annealing and extension for 1 min at 60°C followed by a dissociation stage at 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec.
IL, interleukin; HSP-70, heat shock protein 70; HO-1, heme oxygenase 1; iNOS, inducible nitric oxide synthase.

formed to the natural logarithm. The fitted models consisted of random intercepts that were related to the individual rats (intestines) and the replicates (of slices or pieces) measured for each outcome parameter within the rat (intestine). The calculated fixed effects express the influence of the preservation solution or strategy on the outcome parameters. To be able to distinguish between the "solution effect" (UW solution vs. WMEplus) and the "strategy effect" (closed vs. luminal preservation), a possible interaction between these parameters was determined. When no interactions were found, it could be concluded that the solution effect was independent of the strategy that was used and vice versa. Statistical package R (library NLME) was used for computations. P values less than 0.05 were considered to be significant.

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