

## Research Article

# A novel histidine–tryptophan–ketoglutarate formulation ameliorates intestinal injury in a cold storage and *ex vivo* warm oxygenated reperfusion model in rats

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**Aim:** The present study aims to evaluate protective effects of a novel histidine–tryptophan–ketoglutarate solution (HTK-N) and to investigate positive impacts of an additional luminal preservation route in cold storage-induced injury on rat small bowels.

**Methods:** Male Lewis rats were utilized as donors of small bowel grafts. Vascular or vascular plus luminal preservation were conducted with HTK or HTK-N and grafts were stored at 4°C for 8 h followed by *ex vivo* warm oxygenated reperfusion with Krebs–Henseleit buffer for 30 min. Afterwards, intestinal tissue and portal vein effluent samples were collected for evaluation of morphological alterations, mucosal permeability and graft vitality.

**Results:** The novel HTK-N decreased ultrastructural alterations but otherwise presented limited effect on protecting small bowel from ischemia–reperfusion injury in vascular route. However, the additional luminal preservation led to positive impacts on the integrity of intestinal mucosa and vitality of goblet cells. In addition, vascular plus luminal preservation route with HTK significantly protected the intestinal tissue from edema.

**Conclusion:** HTK-N protected the intestinal mucosal structure and graft vitality as a luminal preservation solution. Additional luminal preservation route in cold storage was shown to be promising.

## Introduction

Small bowel transplantation (SBT) is an established therapy for intestinal failure patients [1]. During the course of SBT, ischemia–reperfusion injury (IRI) is inevitable throughout allograft procurement, preservation and subsequent transplantation with the intestinal mucosa being highly vulnerable to IRI [2]. The consecutive severe tissue damage [3] leads to an inflammatory response including complement activation, endothelial activation, neutrophil sequestration [4] and consequently results in postoperative infection and rejection [5].

The current preservation strategy for intestinal grafts consisting of vascular perfusion followed by static cold storage (CS) with University of Wisconsin solution (UW) has not changed for decades and is still the gold standard [6,7]. Histidine–tryptophan–ketoglutarate (HTK) solution, an extracellular type and

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**Table 1 Constituents of HTK and modified histidine-tryptophan-ketoglutarate (HTK-N) solution**

Constituents (mmol/l)	HTK	HTK-N
Cl <sup>-</sup>	50	30
Na <sup>+</sup>	15	16
K <sup>+</sup>	10	10
Mg <sup>2+</sup>	4	8
Ca <sup>2+</sup>	0.01	0.02
Histidine	198	124
N- $\alpha$ -acetyl-L-histidine	–	57
Ketoglutarate	1	2
Tryptophan	2	2
Aspartate	–	5
Glycine	–	10
Alanine	–	5
Arginine	–	3
Mannitol	30	–
Sucrose	–	33
Deferoxamine	–	0.0153
LK 614	–	0.0062
Osmolality (mosm/l)	310	305
pH	7.2	7.0

All concentrations are given in mmol/l, calculated osmolality is given in mosmol/l, pH at 20°C.

low-viscosity solution with a relatively low potassium concentration was postulated superior in maintaining intestinal viability [2] and has been increasingly administered in clinical application [6,8,9]. Yet, the difference of preservation outcome between UW and HTK is still controversial and no evidence has been found which hints significant advantage of HTK in terms of histological and functional assessment [6,7,10].

Cell culture studies examining hepatocytes and endothelial cells in different preservation solutions showed the occurrence of iron-dependent hypothermic injury mediated by reactive oxygen species (ROS) in all solutions [11–13]. Furthermore, a toxic property of histidine also leading to the formation of ROS was observed [14,15]. Histidine is an important ingredient of HTK due to the substantial buffer capacity for maintaining pH during preservation [16,17]. To counteract hypothermic injury and the cytotoxicity of histidine in HTK, a novel preservation solution, HTK-N, modified on the basis of HTK, was developed with reduced histidine content. Instead, *N*- $\alpha$ -Acetyl-L-histidine, membrane-permeable iron chelators (deferoxamine and LK 614), glycine and other amino acids (AAs) were added (Table 1). *N*- $\alpha$ -Acetyl-L-histidine shares similar structure with histidine but shows almost no toxicity, partially because of its decreased cellular uptake [15]. Functioning together with the iron chelators, *N*- $\alpha$ -Acetyl-L-histidine markedly alleviates the iron-dependent injuries [11,15,18]. Since sodium influx plays a critical role in hypoxic injury [19,20], glycine and alanine were added to ameliorate hypoxic injury by inhibiting ligand-gated chloride channels and non-specific leaks of small ions including sodium [21–24]. It was reported that aspartate could improve mitochondrial function during ischemia and reperfusion and has a considerable impact on overall cellular recovery [25]. Moreover, reduced chloride content is required for mitigating hypothermic injury during preservation in some cell types [11]. Arginine is the substrate of nitric oxide synthase (NOS) [26] and is reduced during ischemia and reperfusion by activity of arginase [27]. Addition of arginine was proven to have a positive influence on IRI [28].

Compared with solid organs like liver and kidney, the unique features of the complex intestinal architecture determine that the sensitive enterocytes at the luminal surface cannot be reached by vascular preservation routes [7]. This is important since the mucosal layer with the most abundant enterocytes is the initial site of intestinal impairment during preservation [29]. Loss of intestinal barrier integrity induces bacterial translocation with the risk of local and systemic infection [30]. A series of studies documented that additional luminal preservation could elicit protective effects and thus counteract CS-induced injury [31–33]. Moreover, the addition of nutrients and AAs to normal solution and alteration of preservation parameters (e.g. addition of hydrogen and macromolecular compounds in the preservation solution) have also been proven to be beneficial [30,34–38], but the optimal components for intestinal luminal preservation remain unknown [2,7,10,39,40]. Based on these previous studies, HTK-N was now examined in intestinal luminal preservation route.

In the present study, we compared the protective impact of HTK and HTK-N on rat small bowel preservation, as well as strategies of vascular route (V) and vascular as well as luminal preservation routes (VL). For this purpose, a small bowel warm oxygenated reperfusion system was used to mimic early post-transplant conditions. This reperfusion system has already been certified technically accessible, stable and was widely used [41–45]. Both structure and function relevant parameters proved to be easily accessible.

## Materials and methods

### Animals and study design

All handling procedures and operations were performed in accordance with the German Animal Welfare Law and with approval by the administrative authority of North Rhine-Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, reference number 84/02.04.2013.A050). All animal work took place in the central animal facility of the medical faculty at the University of Münster, Münster, Germany. All animals received humane care and had an acclimatization period for more than 1 week. Animals were allowed to have free access to normal chew food (Altromin, Lage, Germany) and water. The room was in 12-h light/dark cycles and the temperature was kept at  $24 \pm 2^\circ\text{C}$ . Animals were fasted 12 h before surgery but had access to water *ad libitum*.

Male Lewis rats (Charles River, Sulzfeld, Germany) weighing 200–270 g were randomly assigned into four groups ( $n=6$  in each group) stratified by the procedure of graft procurement: (1) vascular flush with HTK (V + HTK), (2) vascular flush with HTK-N (V + HTK-N), (3) vascular plus luminal flush with HTK (VL + HTK) and vascular plus luminal flush with HTK-N (VL + HTK-N). All solutions were provided by Dr F. Köhler Chemie (Bensheim, Germany).

The study was performed in a randomized and double blinded way with unblinding by external laboratory personal after completion of all analyses.

### Small bowel procurement and preservation

All operations were conducted under sterile conditions. Under inhalation anesthesia with isoflurane (AbbVie, Ludwigshafen, Germany), a 5-cm abdominal midline incision was performed and jejunum and ileum were isolated with a vascular pedicle as described in detail by Minor et al. [45]. Vascular preservation solution (100 ml) was administered via an aortic catheter (20G, B. Braun, Melsungen, Germany) under a defined pressure of 70 mmHg and blood was evacuated via an incision on the portal vein. In vascular plus luminal preservation groups, the small bowel was additionally luminally perfused via a duodenal catheter prepared from an infusion tube (B. Braun) with a pressure of 5 mmHg. At the end of the luminal perfusion with 50 ml preservation solution, the distal and proximal ends of the small intestine were closed in sequence with bulldog clamps, reserving the preservation solution in lumen. Simultaneously, the abdomen was cooled topically with crushed frozen saline. After perfusion, the bowel was moved out of the abdominal cavity and preserved in 100 ml of the respective ice-cold preservation solution, kept in a metal container that was bathed in water-ice mixture for constant preservation temperature of  $4^\circ\text{C}$ . The CS period of 8 h was determined to be optimal according to our pilot study of histological examinations on intestinal tissue with different CS periods (data not shown).

### Warm oxygenated reperfusion *ex vivo*

Grafts were *ex vivo* reperfused after 8 h CS. Afterwards, Krebs–Henseleit buffer, warmed and oxygenated by a hollow fiber oxygenator (MAQUET, Rastatt, Germany), was pumped via the aortic catheter by a Harvard Peristaltic Pump (Harvard Apparatus, Holliston, U.S.A.) in a non-recirculating fashion at a constant flow rate of 5 ml/min for 30 min, according to established protocols [44]. During reperfusion, the small bowel was kept floating but totally immersed in Krebs–Henseleit buffer, and the temperature was maintained at  $37^\circ\text{C}$  by a water bath. During the whole process of reperfusion, oxygen partial pressure of the perfusate was kept over 500 mmHg. The venous effluent was collected via the portal vein catheter (Becton & Dickinson, Parsippany, U.S.A.) for further assays.

### Histology

Samples were collected immediately after reperfusion from the terminal ileum, prepared in Swiss Roll technique [46,47] fixed in 4% formaldehyde solution (Otto Fischar, Saarbrücken, Germany) for 24 h and embedded in paraffin. Four micrometer sections were stained with hematoxylin-eosin (HE) and assessed by a pathologist in a blinded fashion. Tissue injury was graded according to the Park/Chiu score [29,48,49].

## Goblet cells abundance

To evaluate the intestinal mucus-gel layer and vitality of mucosal goblet cells, 4- $\mu$ m sections of formalin fixed, paraffin embedded (FFPE) samples were stained by 3% Alcian Blue solution (Merck, Darmstadt, Germany) followed by nuclear fast red (Waldeck, Muenster, Germany) counterstaining. Photographs of five random fields from each slide were taken with a light microscope (Nikon, Tokyo, Japan) and goblet cells were identified based on mucopolysaccharide binding of the Alcian Blue stain. The results are presented as mean value of blue-stained goblet cells per high power field (HPF).

## Apoptosis

Four-micrometer sections of FFPE samples were stained with a commercially available In Situ Cell Death Detection Kit (Sigma-Aldrich, Munich, Germany) according to the manufacturer's instruction. Photographs of five random fields from each slide were taken with a light microscopy (Nikon) and results are shown as mean value of apoptotic cells per HPF.

## Tissue wet-to-dry ratio

Immediately after the 30-min reperfusion 10 cm segments from jejunum and ileum, respectively, were weighed in a standardized fashion for the value of wet weight. Afterwards, grafts were dried in an oven (Memmert, Büchenbach, Germany) at 80°C for 3 days and weighed again to determine the dry-weight value. The wet-to-dry ratio was calculated by dividing wet-weight by dry-weight.

## Electron microscopy

Two-centimeter segments were fixed in 2% (v/v) formaldehyde and 2.5% (v/v) glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, at 4°C. After being washed with phosphate buffered saline (PBS), specimens were post-fixed in 0.5% (v/v) osmium tetroxide and 1% (w/v) potassium hexacyanoferrate (III) in 0.1 M cacodylate buffer for 2 h at 4°C, followed by washing with distilled water. After dehydration in an ascending ethanol series from 30 to 100% ethanol, specimens were incubated twice in propyleneoxide for 15 min. Next, small pieces of intestinal tissue were embedded in Epon using flat embedding molds. Ultrathin sections were cut with an ultramicrotome, collected on copper grids, and negatively stained with 2% uranyl acetate for 15 min. Electron micrographs were taken with a Phillips EM-410 electron microscope using imaging plates (Ditabis, Pforzheim, Germany).

The ultrastructure of intestinal epithelia was examined in a blinded fashion. Evaluation was performed focusing on the microvilli and organelle integrity including the endoplasmic reticulum (ER) and mitochondria swelling, electron density, nucleus integrity and cell necrosis or apoptosis. The evaluation results were presented in scores: (1) mild damage, (2) moderate damage and (3) severe damage.

## Immunofluorescence (IF) staining of tight junction proteins

Four-micrometer sections of FFPE samples were deparaffinized with xylene and rehydrated in graded alcohols. Afterwards, heat-induced epitope retrieval was conducted at 95°C and slides were placed in 10 mM Tris-EDTA (pH 9.0) with 0.05% Tween-20 (Sigma-Aldrich) for 30 min and cooled down on ice for 15 min. After rinsing with PBS, slides were blocked in 5% bovine serum albumin for 1 h.

Presence of tight junction proteins was assessed by incubating slides with 1:400 dilution of Claudin-1 primary antibody (503-3414) or 1:500 dilution of Claudin-3 primary antibody (503-3834) and Claudin-5 primary antibody (503-3754; Zytomed Systems, Berlin, Germany) in a humidity chamber at room temperature for 30 min. All antibodies were diluted with antibody diluent (Dako North America, Carpinteria, U.S.A.). Two sections as negative controls were incubated with pure antibody diluent without primary antibodies. After washing with PBS, Alexa Fluor 488 goat-anti-rabbit secondary antibody (659082, Invitrogen, Camarillo, U.S.A.) was applied and slides were incubated at room temperature for 1 h in a humidity chamber. Afterwards, slides were washed again with PBS and incubated in 1  $\mu$ g/ml 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) at room temperature for 4 min. Slides were then rinsed again with PBS, coverslipped and assessed with a fluorescence microscope (Nikon). Images captured under the same conditions were analyzed by ImageJ and maximal signal intensity in each sample was measured.

## Lactate dehydrogenase (LDH) release

During reperfusion, effluent was collected from the portal vein and a commercially available Quantichrom LDH kit (BioAssay Systems, Hayward, U.S.A.) was applied to determine LDH activity in effluent as an index of tissue damage according to the manufacturer's instruction.

## Energy metabolism

Segments of intestinal grafts were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for later assays. The ATP content retained in intestinal tissue after 8 h cold storage and 30 min warm oxygenated reperfusion was determined as an indicator of ATP regeneration and graft vitality. A commercially available ATP Colorimetric/Fluorometric assay kit (BioVision, Milpitas, U.S.A.) was used according to the manufacturer's instruction. The results were corrected with the respective wet-to-dry ratio and shown in nanomoles per gram of dry-weight (nmol/g – dry tissue).

## Carbohydrate absorption

The intestinal lumen was filled with 3 ml of 5% galactose solution (Sigma-Aldrich) dissolved in 0.9% saline (B. Braun) before reperfusion, during which galactose would be absorbed and concentrated in Krebs–Henseleit buffer. Galactose concentration in effluent was determined by Galactose Colorimetric/Fluorometric Assay Kit (BioVision) according to the manufacturer's instruction and served as an index of intestinal absorption function. The optical density value of each sample was measured with a spectrophotometer (Labexim Products, Lengau, Austria).

## Oxygen consumption

During reperfusion, effluent samples were collected from the portal vein at the time point of 10, 20 and 30 min after the beginning of reperfusion. Oxygen partial pressures ( $p\text{O}_2$ ) of effluent and influent perfusate simultaneously collected from SMA were measured immediately with a blood gas analyzer (Radiometer, Copenhagen, Denmark). The total weight of the intestine was calculated with the whole length of small bowel and the wet-to-dry ratio. The oxygen consumption of the graft was calculated with the difference of influent and effluent oxygen partial pressures, perfusion flow rate, tissue dry weight and solubility coefficient ( $24\text{ }\mu\text{l}$  of  $\text{O}_2$  per milliliter buffer) [50], shown as ml  $\text{O}_2$  per minute per gram dry weight (ml/min/g dry tissue).

## Statistical analysis

A scientific graphing and statistics software, GraphPad Prism (Version 5.01, GraphPad Software, San Diego, U.S.A.), was applied for statistical analysis and charting. All data are presented as mean value with standard error of mean (mean  $\pm$  SEM) in each group unless otherwise stated. Comparisons were performed in Kruskal–Wallis one-way analysis of variance (ANOVA) between groups followed by Dunn's test for post-hoc comparisons. Differences were considered statistically significant when  $P$  value was below 0.05.

## Results

### Histology

Grafts with HTK-N vascular preservation presented the most serious histological alteration with highest Park/Chiu score ( $6.667 \pm 0.211$ ) and the HTK vascular/luminal preservation group presented the lowest score ( $5.333 \pm 0.333$ ), although differences were small. Interestingly, statistically significant difference was found in comparison between groups of HTK-N vascular with or without luminal preservation but not between groups of HTK solution (Figure 1A–D,I).

### Goblet cells staining

To evaluate the capability to produce mucus, goblet cell abundance in tissue samples was determined (Figure 1E–H). The group of HTK-N vascular preservation presented the lowest abundance of goblet cells in tissue ( $7 \pm 2$  cells/HPF) and was significantly lower than the group of HTK-N vascular plus luminal preservation ( $52 \pm 8$  cells/HPF,  $P < 0.01$ ). No significant difference was found in other comparisons (Figure 1E–H, J).

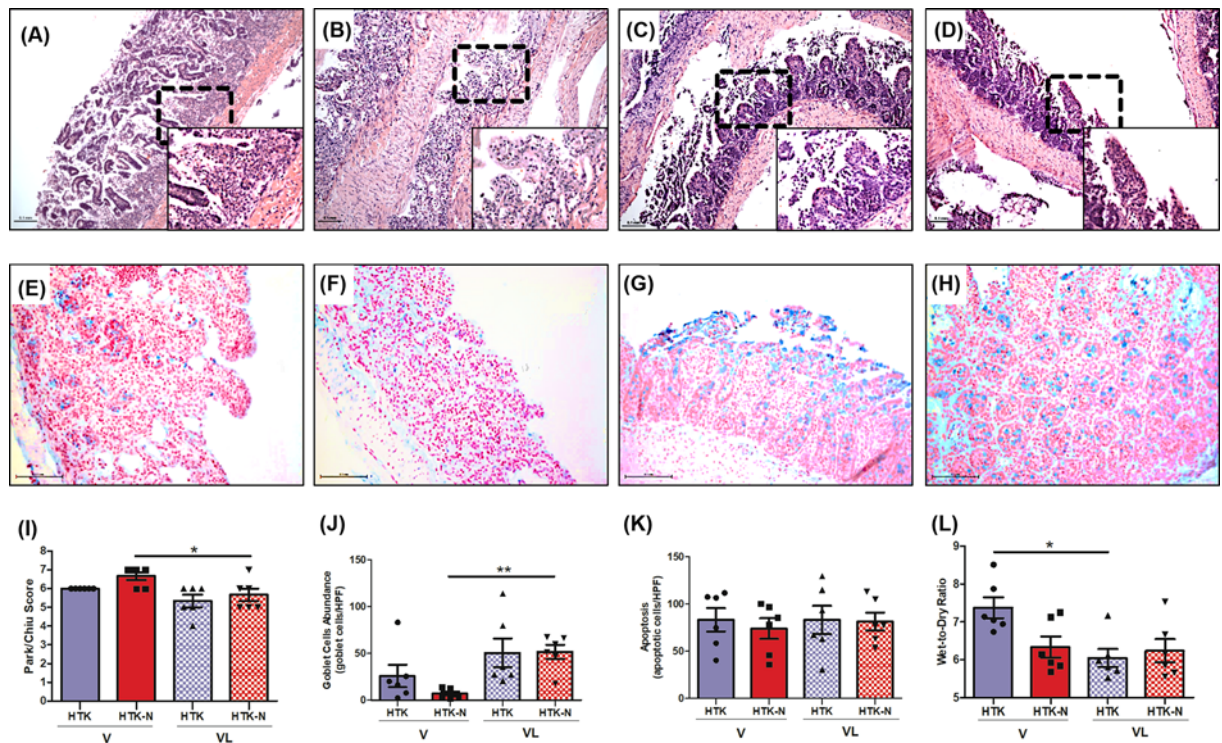
### Apoptosis

As presented in Figure 1K, small bowels with HTK-N vascular preservation showed the lowest apoptosis severity ( $74 \pm 11$  cells/HPF) while HTK vascular with or without luminal preservation groups were comparable and had the most apoptotic cells in mucosa ( $83 \pm 15$  cells/HPF,  $83 \pm 12$  cells/HPF, respectively), although there was no significant difference between groups.

### Wet-to-dry ratio

As presented in Figure 1L, samples with HTK vascular preservation presented the highest wet-to-dry ratio ( $7.375 \pm 0.277$ ) while samples with HTK vascular plus luminal preservation showed the lowest ratio ( $6.049 \pm 0.237$ ) and had





**Figure 1.** Evaluation of intestinal grafts morphological alterations and edema after 8 h cold storage and 30 min reperfusion (A–D) HE staining of tissue samples from the group of HTK vascular preservation (A), HTK-N vascular preservation (B), HTK vascular plus luminal preservation (C) and HTK-N vascular plus luminal preservation (D) (100 $\times$ , bar: 0.1 mm). (E–H) Goblet cells located in intestinal mucosa stained in light blue with Alcian Blue solution from the group of HTK vascular preservation (E), HTK-N vascular preservation (F), HTK vascular plus luminal preservation (G) and HTK-N vascular plus luminal preservation (H) (200 $\times$ , bar: 0.1 mm). (I) HE-stained tissue samples of small bowels were evaluated with the Park/Chiu score system. Grafts with HTK-N vascular preservation presented significantly higher score than grafts with HTK-N vascular plus luminal preservation ( $*P < 0.05$ ), which indicated more severe tissue damage. (J) Abundances of goblet cells in intestinal mucosa were examined and analyzed. Significant difference was found in the comparison between groups of HTK-N vascular and vascular plus luminal preservation ( $**P < 0.01$ ). (K) Abundances of apoptotic cells in intestinal mucosa were examined and analyzed. No significant difference was found in comparisons between groups. (L) Results of wet-to-dry ratio of intestinal tissue were calculated and analyzed. The group of HTK vascular plus luminal preservation presented significant lower ratio value than the counterparts with only vascular preservation ( $*P < 0.05$ ); HTK, histidine–tryptophan–ketoglutarate solution; HTK-N, modified histidine–tryptophan–ketoglutarate solution; V, vascular preservation; VL, vascular plus luminal preservation; HE, hematoxylin–eosin; HPF, high power field; mm = millimeter(s);  $n = 6$ , mean  $\pm$  standard error of mean (SEM).

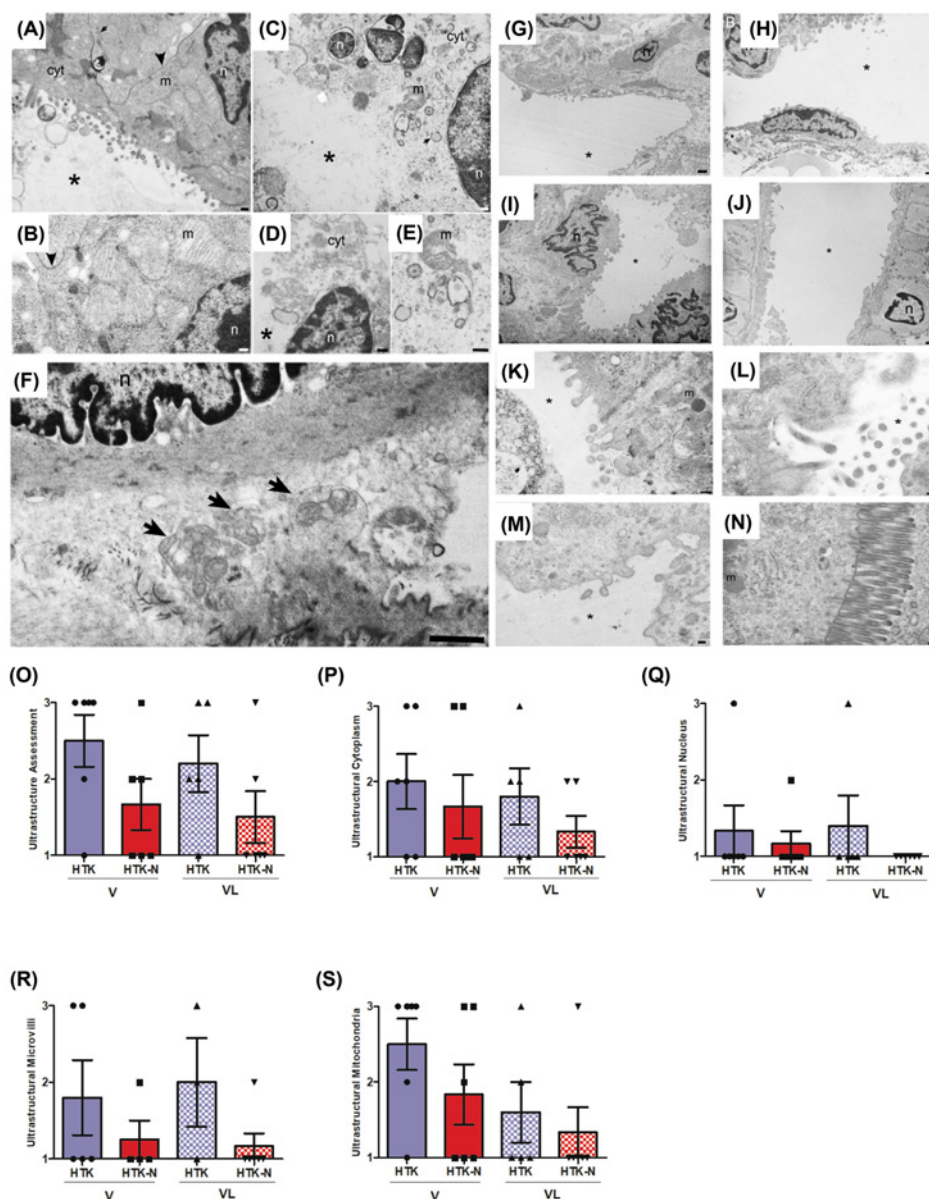
significant difference with the group of HTK vascular preservation ( $*P < 0.05$ ). No significant difference was found in comparisons between other groups.

## Electron microscopy

The ultrastructural alterations of intestinal epithelial cells from four groups were assessed with transmission EM (Figure 2). HTK vascularly or vascularly plus luminally preserved grafts showed increased ultrastructural injury compared with their counterparts with HTK-N ( $2.5 \pm 0.342$  vs.  $1.667 \pm 0.333$ ,  $2.2 \pm 0.374$  vs.  $1.5 \pm 0.342$ , respectively), but no significant difference was determined between groups (Figure 2O).

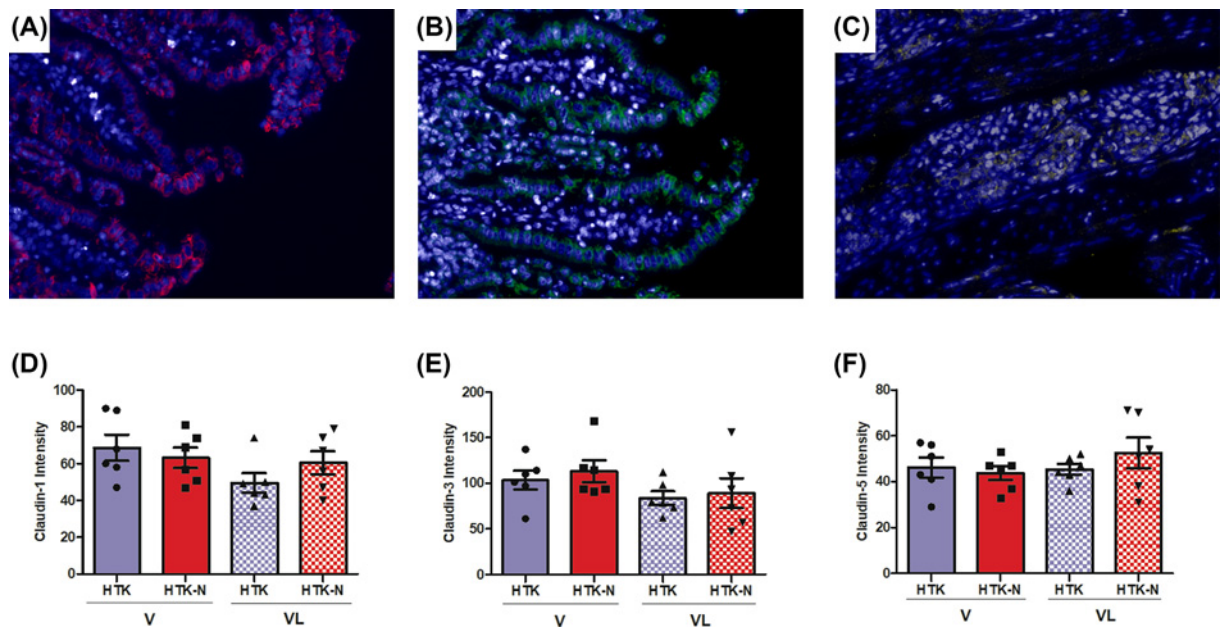
Regarding the electron density of epithelial cytoplasm, the group of HTK-N vascular plus luminal preservation showed less damage ( $1.333 \pm 0.211$ ) than the other three groups, although there was no significance (Figure 2P).

Similarly, the alterations of epithelial nuclei also manifested the positive impacts of HTK-N to HTK with both vascular and vascular plus luminal preservation ( $1.167 \pm 0.167$  vs.  $1.333 \pm 0.333$ ,  $1 \pm 0$  vs.  $1.4 \pm 0.4$ , respectively). However, the comparisons showed no significant difference (Figure 2Q).

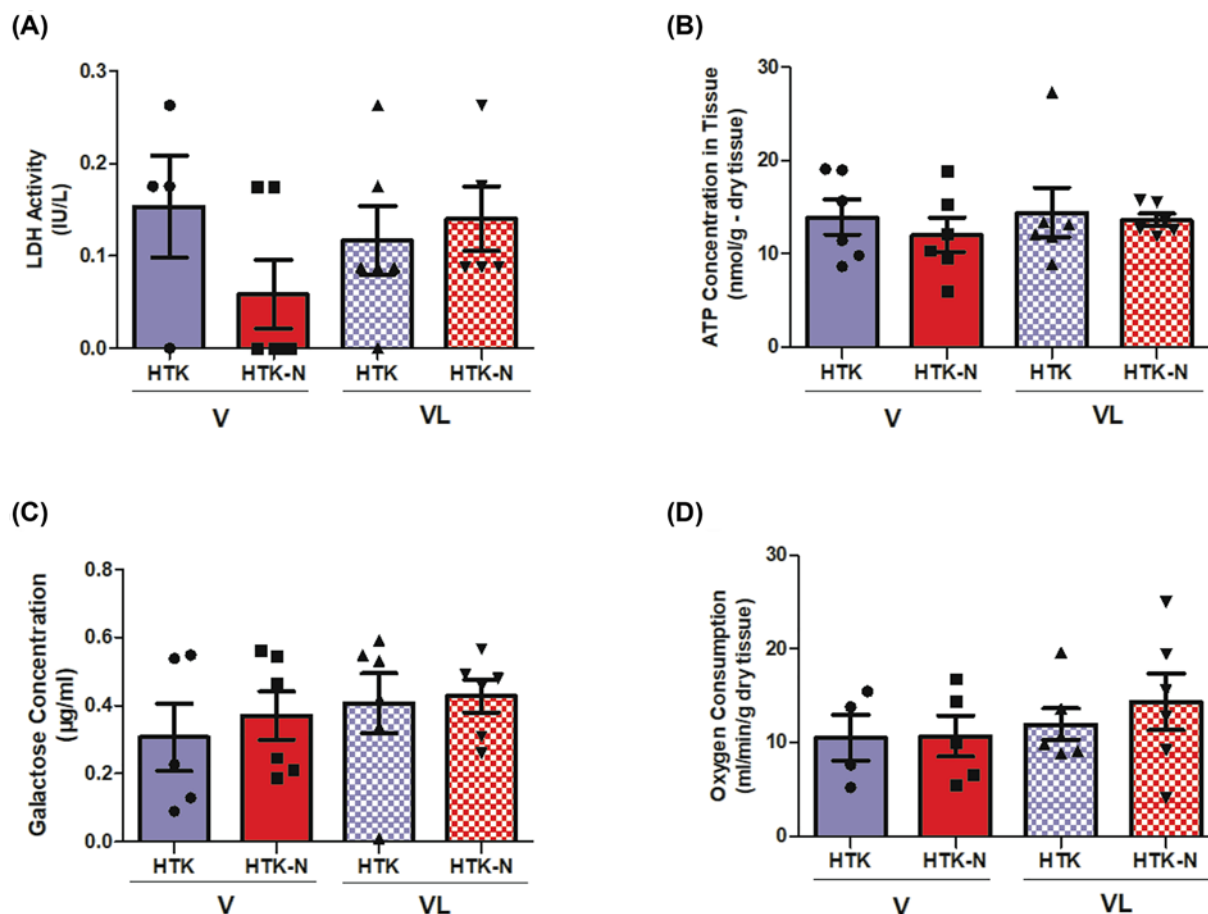


**Figure 2. Transmission electron micrographs and evaluations of the small intestinal epithelium**

Small bowel epithelial ultrastructure was examined and assessed with transmission electron microscopy after 8 h cold storage and 30 min warm oxygenated reperfusion. (A and B) Brush border and apical cytoplasm (cyt) of normal intestinal epithelial cells with highly organized mitochondria (m) with intact membrane systems (B) and clearly visible cell-cell contacts (arrowhead). The rough endoplasmic reticulum (arrow) showed the characteristic tube-like morphology and the nuclear envelope was clearly visible. (C–E) In contrast, conditions for damaging the cytoplasm were disrupted and disorganized with a complete loss of the brush border and a completely missing plasma membrane. The lumen of the endoplasmic reticulum (arrow) was enlarged and the highly structured membrane system of the mitochondria was disrupted (C and E). The nuclear membrane was partly disrupted and enlarged (D) and nucleus in some cells were clearly fragmented (C and F). In the case of damage including mitochondrial stress autophagosomes containing mitochondria (arrows) were visible. (G and K) Ultrastructural overviews of epithelia in the group of HTK vascular preservation. (H and L) Ultrastructural overviews of epithelia in the group of HTK-N vascular preservation. (I and M) Ultrastructural overviews of epithelia in the group of HTK vascular plus luminal preservation. (J and N) Ultrastructural overviews of epithelia in the group of HTK-N vascular plus luminal preservation. The morphological alterations of ultrastructures were evaluated and scored in general (O) or specifically by cytoplasm (P), nucleus (Q), microvilli (R) and mitochondria (S). \* = intestinal lumen, bars in (A–E) 300 nm; bar in (F) 1  $\mu$ m. nm = nanometer(s),  $\mu$ m = micrometer(s); HTK, histidine–tryptophan–ketoglutarate solution; HTK-N, modified histidine–tryptophan–ketoglutarate solution; V, vascular preservation; VL, vascular plus luminal preservation;  $n=5$  in group VL + HTK;  $n=6$  in the other three groups, mean  $\pm$  standard error of mean (SEM) (O–S).







**Figure 4. Evaluation of tissue damage and intestinal vitality**

(A) LDH activity in effluent drained from the portal vein during 30 min warm oxygenated reperfusion was tested as an index of tissue damage during cold storage and reperfusion ( $n=4$  in group V + HTK,  $n=5$  in group VL + HTK-N,  $n=6$  in the other two groups). (B) ATP content in small bowel tissue after 8 h cold storage and 30 min warm oxygenated reperfusion was analyzed to reflect tissue energy metabolism ( $n=6$ ). (C) Galactose concentration in effluent absorbed from lumen and drained from portal vein during 30 min warm oxygenated reperfusion was tested ( $n=5$  in group V + HTK and  $n=6$  in other three groups). (D) Volume of oxygen consumed by the intestinal grafts during 30 min warm oxygenated reperfusion following 8 h cold storage was determined, presented as milliliters oxygen per minute per gram of tissue dry weight (ml/min/g dry tissue;  $n=4$  in group V + HTK,  $n=5$  in group V + HTK-N,  $n=6$  in the other two groups). No significant difference was found in comparisons between groups in these four assays; HTK, histidine–tryptophan–ketoglutarate solution; HTK-N, modified histidine–tryptophan–ketoglutarate solution; V, vascular preservation; VL, vascular plus luminal preservation; LDH, lactate dehydrogenase; U/l, unit(s) per liter; nmol/g-dry tissue, nanomole(s) per gram dry tissue;  $\mu\text{g/ml}$ , microgram(s) per milliliter; ml/min/g dry tissue, milliliters oxygen per minute per gram of tissue dry weight; mean  $\pm$  standard error of mean (SEM).

## LDH release

In the group of HTK vascular preservation LDH activity in effluent was higher ( $0.154 \pm 0.55$  IU/l) than in the other groups while HTK-N vascularly preserved grafts showed the least LDH release ( $0.058 \pm 0.037$  IU/l). No significant difference was found between groups (Figure 4A).

## Energy metabolism

The tissue with HTK-N vascular preservation retained the lowest ( $12.03 \pm 1.85$  nmol/g-dry tissue), grafts preserved vascularly plus luminally with HTK the highest ATP content ( $14.44 \pm 2.666$  nmol/g-dry tissue), although values were relatively low under all conditions and differences were both, small and not significant (Figure 4B).

## Carbohydrate absorption

Small bowels with only HTK vascular preservation presented the lowest galactose concentration ( $0.307 \pm 0.099$   $\mu\text{g/ml}$ ) in the effluent drained from the portal vein during warm oxygenated reperfusion among these four groups while grafts with HTK-N vascular plus luminal preservation showed the highest results ( $0.428 \pm 0.048$   $\mu\text{g/ml}$ ), but there was no statistical significance (Figure 4C).

## Oxygen consumption

Oxygen consumed by small bowels with HTK or HTK-N vascular preservation presented similar results ( $10.5 \pm 2.446$  ml/min/g dry tissue,  $10.67 \pm 2.187$  ml/min/g dry tissue, respectively) and less than the other groups with additional luminal preservation, but no significant difference was found between groups (Figure 4D).

## Discussion

The present study applied the novel preservation solution HTK-N in rat small bowel CS and evaluated its capacity to protect the integrity of intestinal structure and function. In addition, the present study first compared HTK and HTK-N in two different preservation routes to assess whether vascular or vascular plus luminal preservation strategies are superior in protecting intestinal grafts from IRI.

With partial substitution of histidine with N- $\alpha$ -acetyl-L-histidine and the complement of iron chelators and AAs, HTK-N was expected to be a promising alternative to the currently used standard preservation solutions UW and HTK. Previous studies documented nontoxic property of HTK-N in a variety of organs and grafts including liver, kidney, lung, pancreas, intestine and heart [51–57]. In addition, animal models showed protective effects of HTK-N on different organs including liver [18,51,52,58], lung [53] and heart [54,59,60]. On the contrary, no noticeable superiority of HTK-N to HTK was found in studies on kidney [55], pancreas [56] and vascularized tissue isograft [61].

With the special focus on the small bowel, HTK-N was recently used in a rat small bowel transplantation model [57]. It was shown that HTK-N elicited a significant improvement in intestinal microcirculation during the early post-transplant stage following 24h CS, an effect that was attributed to the addition of iron chelators. In line with this, we conducted the present study to further discover whether HTK-N is superior to the conventional HTK regarding the protection on the structural and functional integrity of small bowels, but only limited evidence was achieved indicating the advantage of HTK-N compared to HTK in protecting intestinal grafts in both vascular and vascular plus luminal routes. One might speculate that the extreme susceptibility of small bowel to ischemia and reperfusion restricts the protective effect of this novel preservation solution, leading to unsatisfactory outcome despite the significant improvement on the microcirculation presented in the previous study. However, the study by Lautenschläger et al. used a heterotopic, syngeneic small-bowel transplantation model with 24 h CS and 180 min of reperfusion, i.e. a far longer cold storage period than the one used here. Alternatively, the combination of a relatively short reperfusion period (30 min) and the saline reperfusion solution (lacking blood cells) might not be sufficient to show the full reperfusion injury, especially, with regard to the histological alterations that are known to occur relatively late. The better preservation of cellular ultrastructure (Figure 2O–S) with HTK-N than with HTK suggests superior preservation on the cellular level that might not yet be evident in routine HE staining. A further difference to the study by Lautenschläger et al. is that their study concentrating on microcirculation predominantly assessed vascular/endothelial preservation, whereas our study predominantly assessed the integrity of the intestinal epithelium. Intestinal epithelial cells and vascular endothelial cells might differ in the predominant injurious mechanisms and thus in preservation effects. Of note, wet-to-dry weight ratio, a marker of edema formation, was decreased by almost 50% with vascular HTK-N compared with vascular HTK (Figure 1L), suggesting improved vascular integrity after preservation with HTK-N.

Interestingly, Wei et al. [50] tested another novel preservation solution (Polysol) in rat small bowel CS. In the present study, Polysol exhibited remarkably superior protection from intestinal IRI compared with the clinical gold standard UW, as well as HTK and Celsior. In consideration of the ingredient characteristics of Polysol, the authors emphasized the rich AAs supplementation, antioxidant complements and histidine reduction. Analogously, HTK-N contains a lower dose of histidine than HTK while components of various AAs and antioxidants play the protective roles in different mechanisms. However, the authors presumed glutamic acid, which is absent in HTK-N, is momentous in not only supplying the energy sources and maintaining ATP level, but also antagonizing the mucosal atrophy. In addition, Polysol contains polyethylene glycol (PEG) as the colloid component. Valuckaite et al. [62] tested PEG in another study by comparing HTK with or without PEG in mouse intestinal preservation. PEG was proven to be valid in preventing tissue edema, sustaining the mucosal barrier and consequently ameliorating bacterial translocation and aggression during ischemia and reperfusion stages. However, no ingredient in HTK-N plays the homoplastic role of

colloid giving the advantage of low viscosity of the solution for the application via the vascular route. Whether the addition of a colloid for luminal application would be of benefit requires further studies. However, compared with HTK, edema formation after preservation with HTK-N appeared to be decreased (Figure 1L).

Another purpose of the present study was to investigate the impact of additional luminal perfusion and preservation with HTK or novel HTK-N. In the present study, the most noticeable and interesting result is the abundance of goblet cells in mucosa that still possessed the capacity of mucus secretion. Mucus plays a critical role in the establishment of intestinal mucosal barrier by separating the epithelial cells from the luminal content, especially bacteria [63,64]. In addition, mucus is also a part of the innate immune defense, contributing to concentrate the antibacterial peptides secreted by Paneth cells [65]. We found that samples with HTK-N vascular plus luminal preservation retained significantly more goblet cells than samples with only HTK-N vascular preservation; besides, similar situation happened on the HTK-preserved grafts, although no significant difference was found. On the other hand, morphological parameters were analogous. Park/Chiu score is a systemic measurement that focuses on the epithelial/subepithelial damage and mucosal barrier integrity [48,49]. It was proven that mucosal injury worsens upon the reperfusion after CS, which causes further impairment of mucosal barrier integrity [66]. Hence, the experimental design with a sequence of CS and reperfusion is more reasonable to evaluate the protective effects of organ preservation strategies than a process of only CS. In the present study, the intestinal tissue histological and ultrastructural assessment as well as wet-to-dry ratio, which reflects the tissue edema severity [44,62], elucidated the superiority of HTK-N in luminal route.

In conclusion, the present study applied a novel solution HTK-N in small bowel CS and evaluated its effects on protecting the morphological integrity, mucosal permeability and graft vitality, but no significantly different result was found between samples with HTK or HTK-N. This solution might need further additives to meet the unique requirement of intestine (nutrients supply, colloid complement, etc.). In addition, studies with longer observation periods after reperfusion are essential in order to evaluate the full reperfusion injury and the consequences of the ultrastructural, especially mitochondrial alterations observed here. As a major result of the present study additional luminal preservation with HTK or novel HTK-N presented remarkable superiority in tissue morphology and vitality than vascular preservation alone, thus corroborating the necessity of additional luminal route as a promising intestinal preservation strategy.

## Competing Interests

U.R. is a scientific consultant of Dr F. Köhler Chemie, Germany. She is one of the inventors of Custodiol-N (HTK-N). The company Dr F. Köhler Chemie holds a patent on this solution; however, the design, performance, data interpretation and manuscript writing were under the complete control of the authors and have never been influenced by the company. All other authors declare to have no conflict of interest.

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## Author Contribution

Z.C., L.K., T.V., D.A.F., A.M. and M.H. performed research/study. B.H. and U.H. collected data. L.K., F.B., U.R. and N.S. designed research/study and discussed data. L.K., Z.C., T.V. and A.M. analyzed data and Z.C., F.B. and L.K. wrote the paper; all authors corrected the final manuscript.

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## Abbreviations

CS, cold storage; HTK, histidine–tryptophan–ketoglutarate solution; HTK-N, novel histidine–tryptophan–ketoglutarate solution; IRI, ischemia–reperfusion injury; LDH, lactate dehydrogenase; NOS, nitric oxide synthase; PEG, polyethylene glycol; ROS, reactive oxygen species; SBT, small bowel transplantation.

## References

- 1 Fishbein, T.M. (2009) Intestinal transplantation. *N. Engl. J. Med.* **361**, 998–1008, <https://doi.org/10.1056/NEJMr0804605>
- 2 Roskott, A.M., Nieuwenhuijs, V.B., Dijkstra, G., Koudstaal, L.G., Leuvenink, H.G. and Ploeg, R.J. (2011) Small bowel preservation for intestinal transplantation: a review. *Transpl. Int.* **24**, 107–131, <https://doi.org/10.1111/j.1432-2277.2010.01187.x>

- 3 Grootjans, J., Lenaerts, K., Buurman, W.A., Dejong, C.H. and Derikx, J.P. (2016) Life and death at the mucosal-luminal interface: New perspectives on human intestinal ischemia-reperfusion. *World J. Gastroenterol.* **22**, 2760–2770, <https://doi.org/10.3748/wjg.v22.i9.2760>
- 4 Grootjans, J., Lenaerts, K., Derikx, J.P., Matthijsen, R.A., de Bruine, A.P., van Bijnen, A.A. et al. (2010) Human intestinal ischemia-reperfusion-induced inflammation characterized: experiences from a new translational model. *Am. J. Pathol.* **176**, 2283–2291, <https://doi.org/10.2353/ajpath.2010.091069>
- 5 Mori, D.N., Kreisel, D., Fullerton, J.N., Gilroy, D.W. and Goldstein, D.R. (2014) Inflammatory triggers of acute rejection of organ allografts. *Immunol. Rev.* **258**, 132–144, <https://doi.org/10.1111/imr.12146>
- 6 Klein, A.S., Messersmith, E.E., Ratner, L.E., Kochik, R., Baliga, P.K. and Ojo, A.O. (2010) Organ donation and utilization in the United States, 1999–2008. *Am. J. Transplant.* **10**, 973–986, <https://doi.org/10.1111/j.1600-6143.2009.03008.x>
- 7 Oltean, M. (2015) Intestinal preservation for transplantation: current status and alternatives for the future. *Curr. Opin. Organ Transplant.* **20**, 308–313, <https://doi.org/10.1097/MOT.0000000000000187>
- 8 Abu-Elmagd, K.M., Costa, G., Bond, G.J., Soltys, K., Sindhi, R., Wu, T. et al. (2009) Five hundred intestinal and multivisceral transplantations at a single center: major advances with new challenges. *Ann. Surg.* **250**, 567–581
- 9 Fischer-Frohlich, C.L., Konigsrainer, A., Schaffer, R., Schaub, F., Pratschke, J., Pascher, A. et al. (2012) Organ donation: when should we consider intestinal donation. *Transpl. Int.* **25**, 1229–1240, <https://doi.org/10.1111/j.1432-2277.2012.01556.x>
- 10 Parsons, R.F. and Guarrera, J.V. (2014) Preservation solutions for static cold storage of abdominal allografts: which is best? *Curr. Opin. Organ Transplant.* **19**, 100–107, <https://doi.org/10.1097/MOT.0000000000000063>
- 11 Rauen, U., Kerkweg, U. and de Groot, H. (2007) Iron-dependent vs. iron-independent cold-induced injury to cultured rat hepatocytes: a comparative study in physiological media and organ preservation solutions. *Cryobiology* **54**, 77–86, <https://doi.org/10.1016/j.cryobiol.2006.11.008>
- 12 Rauen, U., Polzar, B., Stephan, H., Mannherz, H.G. and de Groot, H. (1999) Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species. *FASEB J.* **13**, 155–168, <https://doi.org/10.1096/fasebj.13.1.155>
- 13 Rauen, U. and de Groot, H. (2004) New insights into the cellular and molecular mechanisms of cold storage injury. *J. Invest. Med.* **52**, 299–309, <https://doi.org/10.1136/jim-52-05-29>
- 14 Rauen, U. and de Groot, H. (2008) Inherent toxicity of organ preservation solutions to cultured hepatocytes. *Cryobiology* **56**, 88–92, <https://doi.org/10.1016/j.cryobiol.2007.09.003>
- 15 Rauen, U., Klempt, S. and de Groot, H. (2007) Histidine-induced injury to cultured liver cells, effects of histidine derivatives and of iron chelators. *Cell. Mol. Life Sci.* **64**, 192–205, <https://doi.org/10.1007/s00018-006-6456-1>
- 16 Teloh, J.K., Dohle, D.S., Petersen, M., Verhaegh, R., Waack, I.N., Roehrborn, F. et al. (2016) Histidine and other amino acids in blood and urine after administration of Bretschneider solution (HTK) for cardioplegic arrest in patients: effects on N-metabolism. *Amino Acids* **48**, 1423–1432, <https://doi.org/10.1007/s00726-016-2195-2>
- 17 Fridell, J.A., Mangus, R.S. and Tector, A.J. (2009) Clinical experience with histidine-tryptophan-ketoglutarate solution in abdominal organ preservation: a review of recent literature. *Clin. Transplant.* **23**, 305–312, <https://doi.org/10.1111/j.1399-0012.2008.00952.x>
- 18 Wu, S., Wohlschlaeger, J., de Groot, H. and Rauen, U. (2009) Evaluation of a modified HTK solution containing the new iron chelator LK 614 in an isolated rat liver perfusion model. *J. Invest. Surg.* **22**, 340–347, <https://doi.org/10.1080/08941930903214735>
- 19 Carini, R., Bellomo, G., Grazia De Cesaris, M. and Albano, E. (1997) Glycine protects against hepatocyte killing by KCN or hypoxia by preventing intracellular Na<sup>+</sup> overload in the rat. *Hepatology* **26**, 107–112, <https://doi.org/10.1002/hep.510260114>
- 20 Fuckert, O., Rauen, U. and De Groot, H. (2000) A role for sodium in hypoxic but not in hypothermic injury to hepatocytes and LLC-PK1 cells. *Transplantation* **70**, 723–730, <https://doi.org/10.1097/00007890-200009150-00003>
- 21 Frank, A., Rauen, U. and de Groot, H. (2000) Protection by glycine against hypoxic injury of rat hepatocytes: inhibition of ion fluxes through nonspecific leaks. *J. Hepatol.* **32**, 58–66, [https://doi.org/10.1016/S0168-8278\(00\)80190-7](https://doi.org/10.1016/S0168-8278(00)80190-7)
- 22 Nagatomi, A., Sakaida, I., Matsumura, Y. and Okita, K. (1997) Cytoprotection by glycine against hypoxia-induced injury in cultured hepatocytes. *Liver* **17**, 57–62, <https://doi.org/10.1111/j.1600-0676.1997.tb00781.x>
- 23 Carini, R., De Cesaris, M.G., Bellomo, G. and Albano, E. (1999) Intracellular Na<sup>+</sup> accumulation and hepatocyte injury during cold storage. *Transplantation* **68**, 294–297, <https://doi.org/10.1097/00007890-199907270-00023>
- 24 Dong, Z., Patel, Y., Saikumar, P., Weinberg, J.M. and Venkatachalam, M.A. (1998) Development of porous defects in plasma membranes of adenosine triphosphate-depleted Madin-Darby canine kidney cells and its inhibition by glycine. *Lab. Invest.* **78**, 657–668
- 25 Weinberg, J.M., Venkatachalam, M.A., Roeser, N.F. and Nissim, I. (2000) Mitochondrial dysfunction during hypoxia/reoxygenation and its correction by anaerobic metabolism of citric acid cycle intermediates. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 2826–2831, <https://doi.org/10.1073/pnas.97.6.2826>
- 26 Durante, W., Liao, L., Iftikhar, I., O'Brien, W.E. and Schafer, A.I. (1996) Differential regulation of L-arginine transport and nitric oxide production by vascular smooth muscle and endothelium. *Circ. Res.* **78**, 1075–1082, <https://doi.org/10.1161/01.RES.78.6.1075>
- 27 Jung, C., Gonon, A.T., Sjoquist, P.O., Lundberg, J.O. and Pernow, J. (2010) Arginase inhibition mediates cardioprotection during ischaemia-reperfusion. *Cardiovasc. Res.* **85**, 147–154, <https://doi.org/10.1093/cvr/cvp303>
- 28 Sukhotnik, I., Helou, H., Mogilner, J., Lurie, M., Bernsteyn, A., Coran, A.G. et al. (2005) Oral arginine improves intestinal recovery following ischemia-reperfusion injury in rat. *Pediatr. Surg. Int.* **21**, 191–196, <https://doi.org/10.1007/s00383-004-1318-0>
- 29 Chiu, C.J., McArdle, A.H., Brown, R., Scott, H.J. and Gurd, F.N. (1970) Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch. Surg.* **101**, 478–483, <https://doi.org/10.1001/archsurg.1970.01340280030009>
- 30 Salehi, P., Zhu, J.Z., Castillo, E.G., Avila, J., Lakey, J. and Churchill, T.A. (2003) Preserving the mucosal barrier during small bowel storage. *Transplantation* **76**, 911–917, <https://doi.org/10.1097/01.TP.0000085291.36510.53>
- 31 Oltean, M., Hellstrom, M., Ciuce, C., Zhu, C. and Casselbrant, A. (2015) Luminal solutions protect mucosal barrier during extended preservation. *J. Surg. Res.* **194**, 289–296, <https://doi.org/10.1016/j.jss.2014.10.001>



- 32 Roskott, A.M., Nieuwenhuijs, V.B., Leuvenink, H.G., Dijkstra, G., Ottens, P., de Jager, M.H. et al. (2010) Reduced ischemia-reoxygenation injury in rat intestine after luminal preservation with a tailored solution. *Transplantation* **90**, 622–629, <https://doi.org/10.1097/TP.0b013e3181ebf796>
- 33 Olson, D.W., Jijon, H., Madsen, K.L., Al-Saghi, M., Zeng, J., Jewell, L.D. et al. (2003) Human small bowel storage: the role for luminal preservation solutions. *Transplantation* **76**, 709–714, <https://doi.org/10.1097/01.TP.0000079455.62493.E2>
- 34 Shigeta, T., Sakamoto, S., Li, X.K., Cai, S., Liu, C., Kurokawa, R. et al. (2015) Luminal injection of hydrogen-rich solution attenuates intestinal ischemia-reperfusion injury in rats. *Transplantation* **99**, 500–507, <https://doi.org/10.1097/TP.0000000000000510>
- 35 Oltean, M., Joshi, M., Herlenius, G. and Olausson, M. (2010) Improved intestinal preservation using an intraluminal macromolecular solution: evidence from a rat model. *Transplantation* **89**, 285–290, <https://doi.org/10.1097/TP.0b013e3181c9905a>
- 36 Tsujimura, T., Salehi, P., Walker, J., Avila, J., Madsen, K., Lakey, J. et al. (2004) Ameliorating small bowel injury using a cavitary two-layer preservation method with perfluorocarbon and a nutrient-rich solution. *Am. J. Transplant.* **4**, 1421–1428, <https://doi.org/10.1111/j.1600-6143.2004.00541.x>
- 37 Olson, D., Stewart, B., Carle, M., Chen, M., Madsen, K., Zhu, J. et al. (2001) The importance of impermeant support in small bowel preservation: a morphologic, metabolic and functional study. *Am. J. Transplant.* **1**, 236–242, <https://doi.org/10.1034/j.1600-6143.2001.001003236.x>
- 38 Fujimoto, Y., Olson, D.W., Madsen, K.L., Zeng, J., Jewell, L.D., Kneteman, N.M. et al. (2002) Defining the role of a tailored luminal solution for small bowel preservation. *Am. J. Transplant.* **2**, 229–236, <https://doi.org/10.1034/j.1600-6143.2002.20307.x>
- 39 Latchana, N., Peck, J.R., Whitson, B.A., Henry, M.L., Elkhannas, E.A. and Black, S.M. (2015) Preservation solutions used during abdominal transplantation: Current status and outcomes. *World J. Transplant.* **5**, 154–164, <https://doi.org/10.5500/wjt.v5.i4.154>
- 40 Leuvenink, H.G., van Dijk, A., Freund, R.L., Ploeg, R.J. and van Goor, H. (2005) Luminal preservation of rat small intestine with University of Wisconsin or Celsior solution. *Transplant. Proc.* **37**, 445–447, <https://doi.org/10.1016/j.transproceed.2004.11.059>
- 41 Boyle, E.C., Dombrowsky, H., Sarau, J., Braun, J., Aepfelbacher, M., Lautenschlager, I. et al. (2016) Ex vivo perfusion of the isolated rat small intestine as a novel model of Salmonella enteritis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **310**, G55–G63, <https://doi.org/10.1152/ajpgi.00444.2014>
- 42 Gonzalez, L.M., Moeser, A.J. and Blikslager, A.T. (2015) Animal models of ischemia-reperfusion-induced intestinal injury: progress and promise for translational research. *Am. J. Physiol. Gastrointest. Liver Physiol.* **308**, G63–G75, <https://doi.org/10.1152/ajpgi.00112.2013>
- 43 Lautenschlager, I., Dombrowsky, H., Frerichs, I., Kuchenbecker, S.C., Bade, S., Schultz, H. et al. (2010) A model of the isolated perfused rat small intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* **298**, G304–G313, <https://doi.org/10.1152/ajpgi.00313.2009>
- 44 Minor, T., Vollmar, B., Menger, M.D. and Isselhard, W. (1998) Cold preservation of the small intestine with the new Celsior-solution. First experimental results. *Transpl. Int.* **11**, 32–37
- 45 Minor, T., Klauke, H. and Isselhard, W. (1997) Assessment of intestinal integrity after ischemic preservation by luminal and vascular perfusion in vitro. *Eur. Surg. Res.* **29**, 246–253, <https://doi.org/10.1159/000129530>
- 46 Moolenbeek, C. and Ruitenbergh, E.J. (1981) The “Swiss roll”: a simple technique for histological studies of the rodent intestine. *Lab. Anim.* **15**, 57–59, <https://doi.org/10.1258/002367781780958577>
- 47 Nowacki, T.M., Remaley, A.T., Bettenworth, D., Eisenblätter, M., Vowinkel, T., Becker, F. et al. (2016) The 5A apolipoprotein A-I (apoA-I) mimetic peptide ameliorates experimental colitis by regulating monocyte infiltration. *Br J Pharmacol* **173**, 2780–2792, <https://doi.org/10.1111/bph.13556>
- 48 Quaedackers, J.S., Beuk, R.J., Bennet, L., Charlton, A., oude Egbrink, M.G., Gunn, A.J. et al. (2000) An evaluation of methods for grading histologic injury following ischemia/reperfusion of the small bowel. *Transplant. Proc.* **32**, 1307–1310, [https://doi.org/10.1016/S0041-1345\(00\)01238-0](https://doi.org/10.1016/S0041-1345(00)01238-0)
- 49 Park, P.O., Haglund, U., Bulkley, G.B. and Falt, K. (1990) The sequence of development of intestinal tissue injury after strangulation ischemia and reperfusion. *Surgery* **107**, 574–580
- 50 Wei, L., Hata, K., Doorschodt, B.M., Buttner, R., Minor, T. and Tolba, R.H. (2007) Experimental small bowel preservation using Polysol: a new alternative to University of Wisconsin solution, Celsior and histidine-tryptophan-ketoglutarate solution? *World J. Gastroenterol.* **13**, 3684–3691, <https://doi.org/10.3748/wjg.v13.i27.3684>
- 51 Bahde, R., Palmes, D., Gerns, O., Minin, E., Stratmann, U., de Groot, H. et al. (2008) Attenuated cold storage injury of rat livers using a modified HTK solution. *J. Surg. Res.* **146**, 49–56, <https://doi.org/10.1016/j.jss.2007.08.011>
- 52 Liu, Q., Bruns, H., Schultze, D., Xue, Y., Zorn, M., Flechtenmacher, C. et al. (2012) HTK-N, a modified HTK solution, decreases preservation injury in a model of microsteatotic rat liver transplantation. *Langenbecks Arch. Surg.* **397**, 1323–1331, <https://doi.org/10.1007/s00423-012-1022-9>
- 53 Pizanis, N., Petrov, A., Heckmann, J., Wiswedel, I., Wohlschlager, J., de Groot, H. et al. (2012) A new preservation solution for lung transplantation: evaluation in a porcine transplantation model. *J. Heart Lung Transplant.* **31**, 310–317, <https://doi.org/10.1016/j.healun.2011.11.009>
- 54 Turk, T.R., Su, S., Rauen, U., Feldkamp, T., de Groot, H., Kribben, A. et al. (2012) Reduction of chronic graft injury with a new HTK-based preservation solution in a murine heart transplantation model. *Cryobiology* **64**, 273–278, <https://doi.org/10.1016/j.cryobiol.2012.02.011>
- 55 Golriz, M., Fonouni, H., Kuttymuratov, G., Esmailzadeh, M., Rad, M.T., Jarahian, P. et al. (2017) Influence of a modified preservation solution in kidney transplantation: A comparative experimental study in a porcine model. *Asian J. Surg.* **40**, 106–115, <https://doi.org/10.1016/j.asjsur.2015.07.001>
- 56 Esmailzadeh, M., Fonouni, H., Golriz, M., Majlesara, A., Kuttymuratov, G., Bergmann, F. et al. (2016) Evaluation of the modified HTK solution in pancreas transplantation—An experimental model. *Asian J. Surg.* **39**, 66–73, <https://doi.org/10.1016/j.asjsur.2015.03.017>
- 57 Lautenschlager, I., Pless-Petig, G., Middel, P., de Groot, H., Rauen, U. and Stojanovic, T. (2018) Cold storage injury to rat small-bowel transplants—beneficial effect of a modified HTK solution. *Transplantation* **102**, 1666–1673, <https://doi.org/10.1097/TP.0000000000002318>
- 58 Stegemann, J., Hirner, A., Rauen, U. and Minor, T. (2010) Use of a new modified HTK solution for machine preservation of marginal liver grafts. *J. Surg. Res.* **160**, 155–162, <https://doi.org/10.1016/j.jss.2008.10.021>
- 59 Wu, K., Turk, T.R., Rauen, U., Su, S., Feldkamp, T., de Groot, H. et al. (2011) Prolonged cold storage using a new histidine-tryptophan-ketoglutarate-based preservation solution in isogeneic cardiac mouse grafts. *Eur. Heart J.* **32**, 509–516, <https://doi.org/10.1093/eurheartj/ehq135>
- 60 Loganathan, S., Radovits, K., Hirschberg, K., Korkmaz, S., Koch, A., Karck, M. et al. (2010) Effects of Custodiol-N, a novel organ preservation solution, on ischemia/reperfusion injury. *J. Thorac. Cardiovasc. Surg.* **139**, 1048–1056, <https://doi.org/10.1016/j.jtcvs.2009.09.034>

- 61 Messner, F., Hautz, T., Blumer, M.J.F., Bitsche, M., Pechriggl, E.J., Hermann, M. et al. (2017) Critical Ischemia Times and the Effect of Novel Preservation Solutions HTK-N and TiProtec on Tissues of a Vascularized Tissue Isograft. *Transplantation* **101**, e301–e310, <https://doi.org/10.1097/TP.0000000000001845>
- 62 Valuckaite, V., Seal, J., Zaborina, O., Tretiakova, M., Testa, G. and Alverdy, J.C. (2013) High molecular weight polyethylene glycol (PEG 15-20) maintains mucosal microbial barrier function during intestinal graft preservation. *J. Surg. Res.* **183**, 869–875, <https://doi.org/10.1016/j.jss.2013.02.035>
- 63 Birchenough, G.M., Johansson, M.E., Gustafsson, J.K., Bergstrom, J.H. and Hansson, G.C. (2015) New developments in goblet cell mucus secretion and function. *Mucosal. Immunol.* **8**, 712–719, <https://doi.org/10.1038/mi.2015.32>
- 64 Turner, J.R. (2009) Intestinal mucosal barrier function in health and disease. *Nat. Rev. Immunol.* **9**, 799–809, <https://doi.org/10.1038/nri2653>
- 65 Johansson, M.E. and Hansson, G.C. (2011) Microbiology. Keeping bacteria at a distance. *Science* **334**, 182–183, <https://doi.org/10.1126/science.1213909>
- 66 Oltean, M. and Churchill, T.A. (2014) Organ-specific solutions and strategies for the intestinal preservation. *Int. Rev. Immunol.* **33**, 234–244, <https://doi.org/10.3109/08830185.2013.853764>