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Luminal Preservation Protects the Small Intestine in a Brain-dead Rat Model

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Background. Intestinal transplantation depends on donation after brain death (DBD). Luminal preservation (LP) has been beneficial against preservation injury in previous studies in animal models, but none include DBD. This study aims to investigate whether these benefits occur also with DBD. **Methods.** Wistar rats (male, N = 9) underwent brain death for 2 h. Thereafter, vascular perfusion was done with University of Wisconsin solution (UW). The small intestine was then explanted and randomized into 3 groups: control (empty segment), LP+PEG (with polyethylene glycol 3350 solution), or LP+UW (with UW), treated and tied shut. Ice-cold UW was used for cold storage. Samples were taken at procurement and after 4 (t = 4) and 8 h (t = 8) of preservation. Histopathological scorings were performed for intestinal preservation injury, subepithelial space, absence of epithelial lining, and hemeoxygenase-1 expression. **Results.** There was low-level mucosal injury (median intestinal preservation injury score 2) at procurement. At t = 4, bowels treated without LP had more damage than LP-treated samples (control score 4, LP+PEG 2 and LP+UW 2, $P < 0.001$ control versus LP+UW). At t = 8, no benefit of LP was observed (control 2, LP+PEG 3, LP+UW 2). Subepithelial space increased with time and the presence of LP; epithelial lining was better conserved in LP-treated samples. Hemeoxygenase-1 staining showed increased intensity with increased damage, irrespective of treatment. **Conclusions.** Luminal perfusion of the small intestine with UW or PEG protects the mucosa in brain-dead rats for up to 4 h. Fewer benefits of LP were found than previously described in non-DBD models. To mimic the clinical situation, DBD should be included in future animal studies on intestinal preservation.

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Intestinal transplantation is performed at a low yearly rate, and graft survival rates are still disappointing.[1,2] Early preservation and ischemia-reperfusion injury are thought to be factors involved in these poor graft survival rates.[3] Human intestines that are donations after brain death (DBDs) are viable for up to 10 h of storage,[4] which is a relatively short time compared with that of other abdominal organs that are preserved within the same procedure. The bowel has also fallen behind regarding preservation techniques in the clinics, as there have been no developments in the way the organ is treated, stored, or transported.[5]

Even though DBD provides the best quality organs,[6] these donors are presented naturally with a less than pristine condition.[7] After thorough analysis by the transplant surgeon both at procurement and implantation, the bowels might seem adequate, but the silent insults they suffer could add up after intestinal transplantation.[8]

Given the unique feature of the bowel of having a lumen, it is not surprising that luminal preservation (LP) of the

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intestinal graft is considered. The protective effects of flushing the lumen of the bowel with an ice-cold solution have been consistent in previous studies in small and large animals and trials on human tissue.[5] What is surprising is the fact that DBD has been not a part of these models. The aim of this study is therefore to see whether LP also has beneficial effects in a rat model of DBD.

MATERIALS AND METHODS

Brain Death Procedure

Surgical procedures were performed in the National University of La Plata, Argentina. Nine male Wistar rats (Central Laboratory Animal Facility of the School of Veterinary Sciences of the National University of La Plata) of 375.1 (±22.14) g were used for these experiments. Animals were housed in a climate-controlled room in 12-h light-dark cycle, fed with standard laboratory rat chow and water ad libitum. They were fasted overnight before the procedure. The experiments were approved by the local Committee for Animal Experiments, thus performed according to institutional and legislative regulations.

Rats were placed in a 5% saturated isoflurane chamber for anesthetic induction, and further maintained at 2% via a mask, which was later replaced by an endotracheal tube. Arterial (carotid) and venous (jugular) lines were placed by a ventral neck approach. Assisted ventilation was started at 50 BPM and 2.5 TV (Harvard Apparatus Rodent Ventilator Model 683).

Following a previously published method,[7,9,10] for the induction of brain death, the animal was positioned in sternal recumbency followed by trepanation of the skull. A Fogarty catheter was then placed on the brain. After reaching stability of the blood pressure above 80 mm Hg, brain death was induced with a flow rate of 1 mL/h for 30 min. Parameters were taken every 5 min for the duration of the brain-death procedure, which lasted 2 h. Hypotension was managed with hydroxyethyl starch up to a maximum of 1 mL and noradrenaline (0.01 mg/mL) up to a maximum infusion of 1 mg/kg/min. Animals were retired from the experiments if they surpassed the maximum infusion and/or remained >10 min below 60 mm Hg.

Intestinal Procurement, Preservation, and Experimental Design

After 2 h of brain death, the rats were placed in dorsal recumbency, and the abdominal cavity was opened. The organs were washed with University of Wisconsin solution (UW—Belzer UW, Bridge to Life [Europe] Ltd., London, United Kingdom) at 4 °C and afterward the jejunum-ileum was explanted and cut into 4-cm-long segments. Each piece was then tied close with suture.

The control group sections, without any LP solution, were submerged in 15–20 mL of UW and stored at 4 °C until sampling. The segments included in the LP groups were treated as follows. Selection for each group was performed randomly, and after tying 1 end, they were filled with 0.25 mL (0.06 mL/cm) of either polyethylene glycol (PEG) 3350 Dalton (group LP+PEG; Colofort, Ipsen Farmaceutica B.V., Hoofddorp, The Netherlands) or UW (group LP+UW), both at ice-cold temperature, and then tied close on the other end. Afterward, they followed the same sampling protocol as the control group. Formulations of both preservation solutions are present in **Supplementary Materials and Methods, Tables S1 and S2, SDC**, http://links.lww.com/TXD/A453.

Sampling was performed at procurement (t = 0 before luminal perfusion) and after 4 (t = 4) and 8 (t = 8) h of static cold storage (CS). Samples were preserved in formaldehyde 4% for histopathology (chemical and immunohistochemical procedures).

Histopathological Analyses

Sample Processing

Samples were fixed in formaldehyde 4% for 4 d and then processed and embedded in paraffin. Later, 4-µm-thick slides were cut in series and placed on coated glasses (Starfrost, Waldemar Knittel Glasbearbeitungs GmbH, Germany).

Histochemistry

One slide per block was stained with hematoxylin-eosin (H&E) using the Ventana system (Roche Diagnostics Nederland B.V., Almere, The Netherlands) for diagnostics in the Pathology laboratory in the University Medical Center Groningen, The Netherlands.

Stained slides were scanned with the NanoZoomer 2.0HT slide scanner (Hamamatsu Photonics Europe GmbH, Herrsching am Ammersee, Germany) and analyzed with NanoZoomer Digital Pathology viewing software NDP.view2 (U12388-01, Hamamatsu Photonics Europe).

Immunohistochemistry

Slides were deparaffinised in xylene and rehydrated in graded alcohols (100% to 50%). Antibody retrieval was performed by boiling under citrate (pH 6.0). Slides were incubated at room temperature in 3% H_2O_2 to block endogenous peroxidase and afterward in blocking solution (1% BSA in phosphate-buffered solution, PBS) for 30 min per step. **Table S3, SDC**, http://links.lww.com/TXD/A453 in the Supplementary Materials and Methods section lists antibodies (antihemeoxygenase-1 and the respective secondary and tertiary antibodies) and their respective dilutions. All antibodies were diluted in blocking solution. Primary steps were performed overnight at 4 °C, secondary and tertiary steps were performed for 30 min at room temperature with washing steps in between with PBS. Finally, for immunohistochemistry, the antibodies were developed with 3,3′-diaminobenzidine and counterstained with hematoxylin. These slides were also scanned and analyzed in the same fashion as the H&E samples.

Sample Analyses and Considerations in Statistics

A junior researcher and an experienced pathologist analyzed all H&E samples with the Chiu-Park score for preservation injury,[11] details of which can be read on Table 1, and

TABLE 1.

Intestinal preservation injury score according to Chiu and Park[11]

Grade	Description
0	Normal mucosa
1	Subepithelial space at villus tip, often with capillary congestion
2	Extension of the space with moderate epithelial lifting
3	Massive epithelial lifting with a few denuded villi
4	Denuded villi with exposed lamina propria and dilated capillaries
5	Loss of villus tissue (disintegration of the lamina propria)
6	Crypt layer infarction
7	Transmucosal hemorrhage and infarction
8	Transmural infarction and ulceration

TABLE 2.

Subepithelial space score developed for this study

Grade	Description
0	No subepithelial space, although Gruenhagen's space can be present
1	SES is smaller than the epithelial monolayer's height
2	SES equals the height of between 1 and 2 enterocytes, focal
3	SES equals the height of between 1 and 2 enterocytes, broadly affected
4	SES is more than twice as the enterocyte's height, focal
5	SES is more than twice as the enterocyte's height, broadly affected
6	Focal epithelial detachment: the lamina propria is in contact with the lumen
7	Massive epithelial detachment: the lamina propria is in contact with the lumen

SES, subepithelial space.

hereon referenced to as the intestinal preservation injury (IPI) score. In short, the mucosal and submucosal lesions that are generated and progress during ischemic preservation were evaluated. These lesions were given a score from 0 (healthy) to 8 (transmural infarction), where in a score of 4 (denudation of the villi) can be considered as a point of no-return regarding recovery due to the presence of massive necrosis of the superficial half of the villi, and therefore massive surface of contact with luminal content. Nine high-power fields (HPFs, 0.9 mm^2) were taken at random and scored per their worst lesions. The absence of epithelial lining was measured at the base of the lamina propria of the entire sample, returning the percentage of absence of epithelial lining per glass slide. Finally, on the same HPF, a grading score was developed to assess the presence and amount of subepithelial space (SES score, see Table 2 and **Figure S1, SDC**, http://links.lww.com/TXD/A453 in the Supplementary Materials and Methods section). In short, the greater the space between the epithelial cells and their basal lamina, the higher the score. The number of hotspots of debris in the lamina propria was also determined, as were the number of apoptotic bodies per 10 consecutive crypts.[7,12] These last analyses are presented in the **Supplementary Results, SDC**, http://links.lww.com/TXD/A453.

The results are presented as median and interquartile range per group, except when otherwise noted. Statistical tests chosen were the Kruskal–Wallis rank sum test with the addition of Dunn's multiple comparisons tests, presenting the *P*, adjusted *P* values, respectively, and effect size (η2). Statistical significance is reached when *P* and adjusted *P* values <0.05. The software to analyze and render plots was R (version 4.0.5) with session information presented in **Supplementary Materials and Methods, SDC**, http://links.lww.com/TXD/A453.

RESULTS

Surgical Procedure

The surgical procedure proceeded without major complications. Of the 10 animals prepared for the study, 1 was excluded for presenting acute lung edema after 20 min of maintenance in brain death.

The induction of brain death took 36 ± 4.16 min, with a maximum decrease in blood pressure at 20 min (56 mm Hg). Maintenance of the individuals on brain death was of 2 h, in which the mean arterial pressure was 91.44 ± 13.43 mm Hg (mean and SD). Eight individuals required the use of noradrenaline as a vasopressor, the infused dose was 0.56 ± 0.25 μg/kg/min.

Histopathological Analyses

Histology

At procurement, the SES had already increased (IPI 2 0–2, Figures 1 and 2A and B), showing detachment and relatively minimal loss of epithelial lining (7.2% 0–66.2%, Figure 3B). The SES score (1, 0–3, Figure 3A) shows a pattern predicted by the IPI score, revealing focal points of space that is as high as up to 2 enterocytes, comparable to an IPI of 2.

At 4 h of preservation (Figure 2C), the histological structure of the control samples suffered the most damage of all groups or time points. The tips of the villi show necrosis (IPI 4, 3–4)

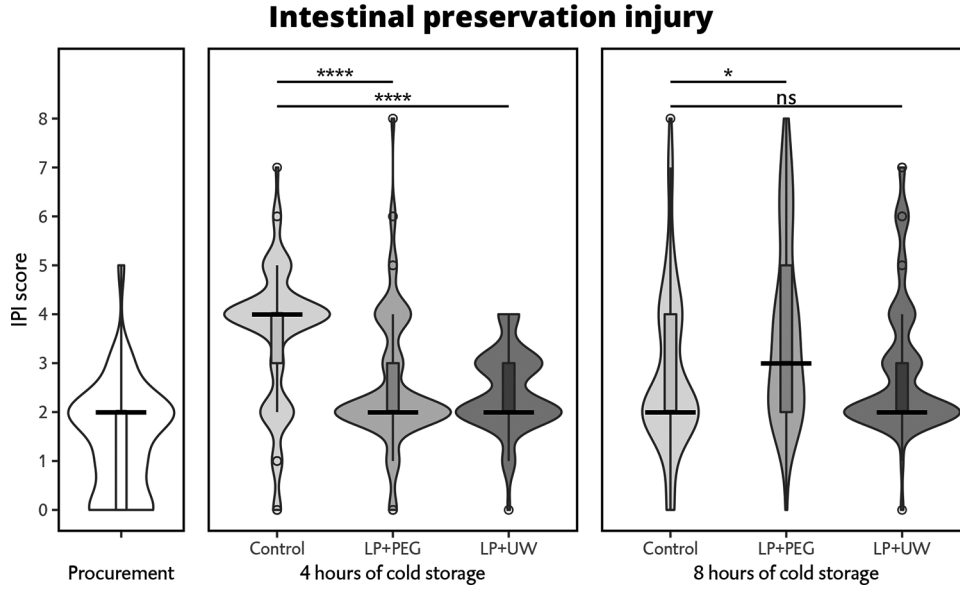

FIGURE 1. Intestinal preservation injury score shows a better histopathological appearance of luminal preservation samples after 4 h, but not after prolonged cold storage vs standard treatment. Comparisons denoted are within same time points after a Kruskal–Wallis test that returned a chi-squared = 119.05, df = 6, $P < 2.2–16$, η2 = 0.2 (large). Significance is marked with the symbols: "ns" (not significant), * ($P < 0.05$), **** ($P < 0.001$). Black horizontal bars mark the median; empty dots denote outliers.

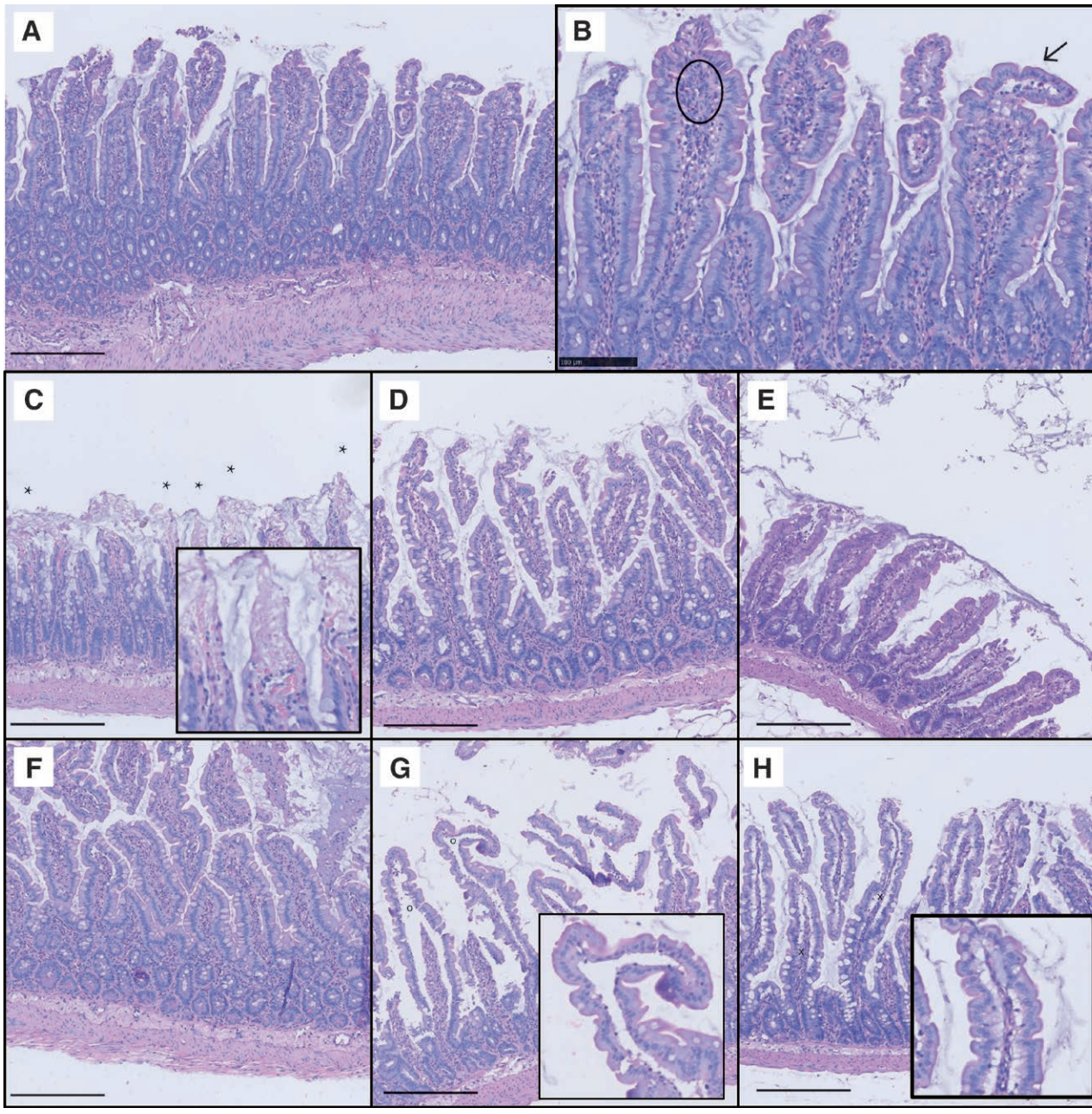

FIGURE 2. Intestinal preservation with a luminal solution prevents the appearance of necrosis at the villi tips during CS. All black bars represent 250 μm except on (B). Inner panels show an image zoomed 1.5× (A). Microphotograph of a sample taken at procurement shows increased subepithelial space in otherwise normal-looking villi, representative of the group's median IPI value of 2. B, Detail of (A), where in there is increased space at the tip of a villi (arrowhead) and evidence of debris in the lamina propria (circled). C, A representative microphotograph of a sample from the control group at 4 h of CS shows necrosis at the villi tips (stars), detailed at higher magnification in the lower right square within the panel. D, LP+PEG at 4 h of CS shows increased subepithelial space, like that of the procurement samples. E, Similar image is found in LP+UW at this time point. F, The control group shows a better-preserved mucosa at the later time point (8 h CS), similarly to what was found at procurement. G, LP+PEG samples show massive epithelial lifting with detachment from the lamina propria but no evidence of necrosis (o), detailed at higher magnification in the lower right square within the panel. H, Samples treated with LP+UW show epithelial lifting an increased subepithelial space without any detachment, with thinning of the lamina propria at the villi after extended preservation (x), detailed at higher magnification in the lower right square within the panel. CS, cold storage; IPI, intestinal preservation injury; LP, luminal preservation; PEG, polyethylene glycol; UW, University of Wisconsin solution.

and 59.6% of the epithelial lining is lost. The SES score is 1 (0–7), epithelium is conserved when there was no necrosis, the large proportion of epithelium absent is also reflected. The IPI is significantly lower in both treatment groups (2, 2–3, in both LP+PEG and LP+UW; <0.001), whereas the amount of epithelial lining absent is 21.8% and 11.4% in LP+PEG and LP+UW, respectively. Both groups also had the same SES values (2, 1–6), reflecting focal epithelial detachment instead of generalized.

At 8 h of preservation (Figure 2F), control tissue presents less damage than at 4 h regarding IPI (2, 2–4, Figure 1), with less epithelial layer lost (40%, Figure 3B). This decrease in IPI values in the control group is reflected in the statistical tests performed against the other groups, as the tissue damage evolved

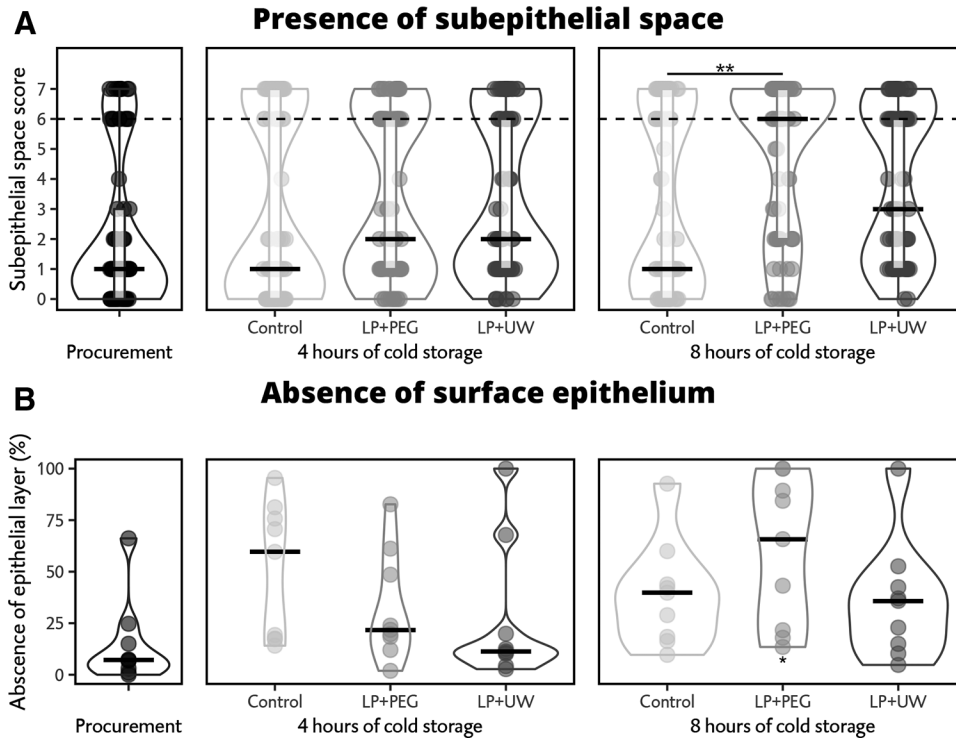

FIGURE 3. Analyses of the structural state of the surface epithelium shows no clear benefit of luminal preservation. A, Presence of subepithelial space (SES) according to the developed score on each HPF analyzed for IPI. B, Proportion of detached epithelial lining retrieved by measuring. Procurement samples generally show increased amount of SES associated to an IPI of 2 and a low proportion of epithelial lifting. After 4 h of cold storage, control samples show increased lifting and detachment of the epithelial lining, but the areas without necrosis remained with the same amount of SES. LP-treated samples show more SES but less detachment, reflected by the upper limit of SES score of 6 (focal detachment) and low proportion. After prolonged storage, control samples remained with a low SES score but with higher amount of overall detachment of the epithelium, whereas treated samples showed increased scoring and proportioned lifting. A, Interrupted line demarks the score's limit of a present and attached epithelium: above this line the detachment is focal (SES = 6) or massive (SES = 7) within the studied area. The black bar denotes the median values in all graphs. Statistical significance: * ($P < 0.05$) is marked in graph (B) against procurement samples; ** ($P < 0.005$) denotes the difference between the SES values of control vs PEG, the only relevant difference found. A, Kruskal–Wallis chi-squared = 15.38, df = 6, P = 0.018, $\eta 2 = 0.17$ (large). B, Kruskal–Wallis chi-squared = 44.489, df = 6, P = 5.91-08, $\eta 2 = 0.069$ (moderate). HPF, high-power field; IPI, intestinal preservation injury; LP, luminal preservation; PEG, polyethylene glycol.

in segments treated with PEG in relation to time, albeit within relatively healthier values (3, 2–5, $P = 0.013$). This was accompanied with an increase in the absence of epithelial lining (66%, $P = 0.017$ in comparison to procurement, but not different to what is found at the same time in other groups). The LP+UW group had the lowest interquartile range of IPI (2, 2–3) and proportion of absent epithelial lining (36%), but the statistical analyses did not yield significant differences. SES scores were the highest in the LP+PEG group (6, 1–6, $P = 0.003$ versus control) reflecting focal loss of epithelial lining, mostly at the tip of the villi, which is associated to the massive lifting denoted by the IPI score. There is a lower SES score in the control than in the LP+UW-treated samples (1, 0–7 and 3, 1–6, respectively).

Immunohistochemistry

Hemeoxygenase-1 is an enzyme that is activated during periods of oxidative stress.[13] Due to the ischemia suffered by the organ during preservation, immunohistochemistry against HO-1 can provide clues as to how the most damaged cells react to the effects of brain death and LP.

Here, HO-1 is present constitutively as a cytoplasmic staining in both epithelial cells and populations within the lamina propria that could be of immune nature (Figure 4). This pattern's intensity increased where more severe structural damage was present in remaining cells. Areas with debris and hotspots in the lamina propria were also positive for HO-1. Also, all samples with epithelium presented foci of intense staining at the apical side of the enterocyte membranes when cells were not detached from the basal lamina. Intense focal points were present at the basal portion of the cellular membrane of enterocytes that are detached of the basal lamina. These patterns were independent of treatment conditions.

DISCUSSION

This study shows the potential benefits of LP to improve graft quality before transplantation in a rat model of DBD. The strength of this model lies on the information given by the addition of brain death to LP of the intestine that mimics the current clinical scenario.

Compared with results of previous studies in LP, particularly in animal models,[5] the effects of brain death seem to generate irregular injuries along the whole intestine, an effect reflected in the seemingly better-preserved sections that underwent control treatment for a prolonged period (8 h of CS). The present study shows higher damage already at the beginning of the donation procedure than what other studies without DBD have presented, with the absence of epithelial lining and increased presence of debris in the lamina propria (**Supplementary Results, Figure S2, SDC,** http://links.lww.com/TXD/A453). The literature is scarce with regard to the state of the intestinal mucosa at the beginning of the

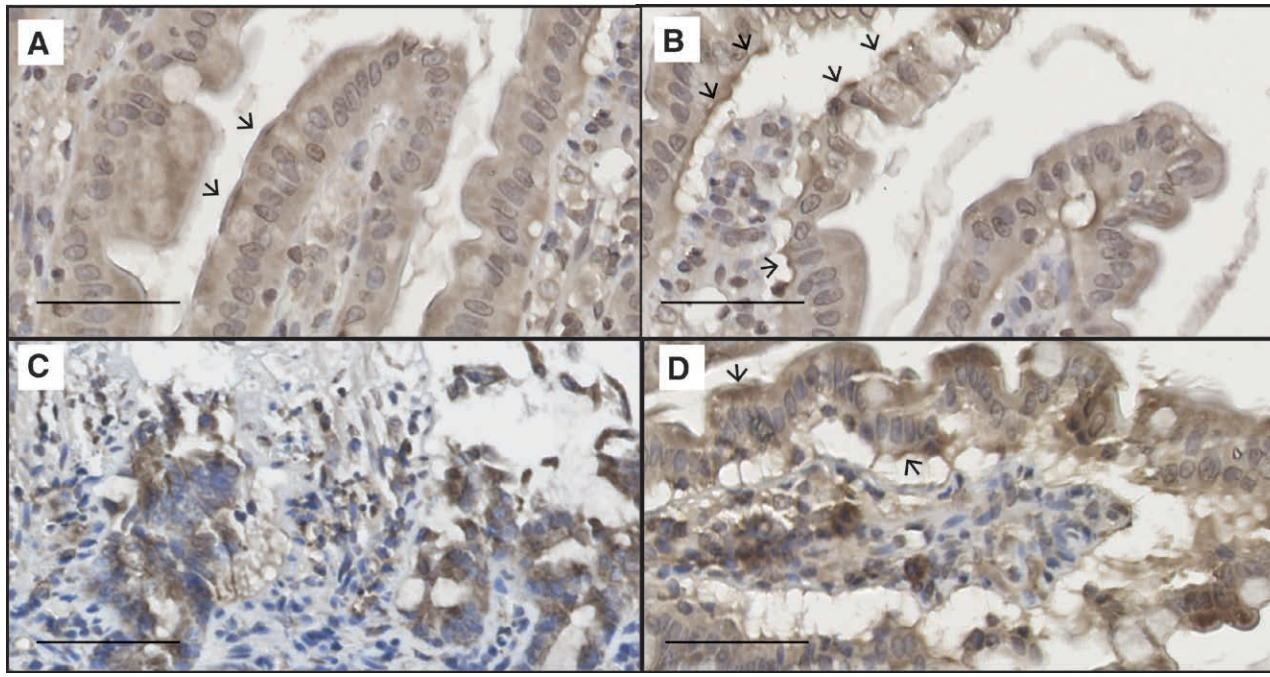

FIGURE 4. HO-1 staining is increasingly present with different patterns associated to injury severity. Bars represent 50 μm. A, Sample (procurement, IPI score 0) presenting constitutive cytoplasmic staining in enterocytes, also presenting increased intensity staining foci in the apical membrane of some cells (arrows). B, Sample (IPI 2, procurement) with additional foci in the basal portion of the cell membrane where the cells are detached from the lamina propria (arrows). C, A sample from the control group at 4 h of preservation shows increased cytoplasmic staining intensity in the remaining enterocytes and a staining pattern that follows the debris hotspots seen in H&E slides within the lamina propria (circle, see also Figure 2B). D, A sample after 8 h of LP (IPI 3, group LP+PEG) shows increased staining intensity in cells of the lamina propria and both apical and basal foci of increased staining intensity in the enterocyte membranes (arrows). All figures at 400× magnification. H&E, hematoxylin-eosin; HO-1, hemeoxygenase-1; IPI, intestinal preservation injury; LP, luminal preservation; PEG, polyethylene glycol.

preservation period, although the effect of DBD on the intestine has been studied.[10,14] The damage perceived in samples at procurement can be related to the catecholamine discharge after brain death, which occurs also in human subjects.[15] The effect of maintenance therapy with noradrenaline during the brain-death period of 2 h is also translated from the clinical settings,[15] giving further evidence of the necessity of the inclusion of DBD in studies to understand and treat intestinal injury before donation. With current protocols, the damage at procurement is unavoidable, thus must be considered as baseline injury that must be at least maintained during CS. IPI increases time-dependently,[11] but the results from these experiments do not show such an effect in the control group. Control samples have lower scores and more epithelial lining present (ie, better histological structure) after the extended preservation period of 8 h than after 4 h CS. LP is beneficial to the structure of the intestine, but because the experiments showed severe damage (necrotic tissue) after 4 h in the control samples, these effects might not be as strong as reported elsewhere when animals donate immediately after scarification (ie, in the absence of brain death).

The intensity and location of the staining for stress marker HO-1 from the beginning of the preservation period shows that it is of great relevance to include brain death in the models used to study (intestinal) preservation. Conclusions taken from studies in the field show that LP is beneficial because it could dilute the toxins present in the lumen while protecting the epithelial barrier.[16,17] Also, the addition of a cold solution can quicken the fall in temperature of the graft and the donor itself, depending on when the solution is delivered.[18] This can in turn help to reduce the warm ischemia periods to a minimum, thus further improving graft viability.

The choice for either luminal UW or PEG is based on previous studies that have shown beneficial effects on the graft's mucosa, but also on the fact that they are readily available in most transplant centers. The former is the gold standard vascular perfusion solution,[19] whereas the latter is used in all hospitals as lavage fluid before endoscopies. The results depicted in this study show a slightly more beneficial effect of UW after an extended period of CS, although PEG maintains the mucosa with little evidence of debris. Extrapolation to a clinical situation might mean that either solution could be chosen regarding the expected CS times and costs, seemingly without losing the desired protective effect.

This study has some clear disadvantages when trying to analyze the effect of LP on the intestinal graft. The reperfusion injury that is a natural part of the transplantation is missing, as is a model of transplantation where in one can attest for the effects on graft function and immune sufficiency. These methods are actually missing in most of these models that focus on preservation, which assume that reperfusion injury will mostly mean an increase in up to 2 points in the IPI score.[11] The development of an experimental model based on machine perfusion can give a more complete approach into LP. Insight into the effects of the modulation of storage temperatures (hypothermic, hypo normothermic, normothermic perfusion), dynamics (static or dynamic perfusion), and the effects of reperfusion on the graft within 1 experiment could confirm or discard this theory within the context of LP.

CONCLUSIONS

The results presented in this study point toward a beneficial effect of LP during CS. Both UW and PEG maintain the

histological structure by protecting it from suffering further necrotic damage and improving the proportion of attached epithelial lining in comparison to controls. Additionally, intestinal grafts preserved via the lumen and vessels with UW continue to show less damage for an extended period of 8 h, whereas both solutions seem to decrease the amount of apoptosis occurring in the lamina propria. The effects of brain death are visible throughout the whole period of preservation, undermining previously published effects of this therapy, and therefore should not be ignored for future studies on organ preservation, luminal, or otherwise.

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