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Effect of Ischemia on the Canine Large Bowel: A Comparison with the Small Intestine¹

IZUMI TAKEYOSHI, M.D., SHIMIN ZHANG, M.S., KENJIRO NAKAMURA, M.D., AKIRA IKOMA, M.D.,
YUE ZHU, M.D., THOMAS E. STARZL, M.D., PH.D., AND SATORU TODO, M.D.²

Pittsburgh Transplantation Institute and the Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

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Mucosal injury caused by ischemia and reperfusion has been well documented with the small intestine, but little is known about the colon. In the present study, the effect of warm and cold ischemia on the canine colon was studied and compared to that on the small intestine. After *in situ* flushing, the small intestine and the colon from six beagle dogs were removed and stored for 0.5, 1.5, and 3 hr at 37°C (warm ischemia) or for 1, 6, 12, 24, 36, and 48 hr at 4°C (cold ischemia). Electrophysiology, permeability, biochemistry, and histopathology of the specimens at each ischemic period and after reperfusion in the Ussing chamber were determined. Warm and cold ischemia induced duration-dependent suppression of electrophysiology in both organs, but the colonic mucosa retained higher activity of absorptive enterocytes and cryptic cells than the small intestine. Only the colon showed increased permeability of FITC-conjugated Dextran from the mucosal surface to the submucosal layer after prolonged ischemia. Changes in adenine nucleotides and purine catabolites were not markedly different between the organs. Histopathologic abnormalities during ischemia and after reperfusion were more serious with the small intestine than with the colon. Compared to warm ischemia, hypothermia lessened or delayed these morphofunctional derangements in both organs, which became universally worsened after reperfusion. Colonic mucosa receives morphofunctional derangements from ischemia and reperfusion, but the severity of the damage was much less severe in the colon than in the small intestine. © 1996 Academic Press, Inc.

INTRODUCTION

Improvements in immunosuppression, operative procedure, and posttransplant management have made clinical

intestinal transplantation feasible [1-3]. Since most patients that received intestinal transplantation lost most of their intestine and proximal colon (including the ileocecal valve), transplantation of the intestinal graft without the colon often resulted in the development of postoperative high stomal output, dehydration, and malnutrition. In several recent cases in our series, the colon has been included as a composite of intestinal grafts at transplantation to lessen these complications [4, 5]. Experimentally, it has been shown in rats that the inclusion of the ascending colon and the ileocecal valve significantly improves graft function [6, 7].

From the time of organ procurement to revascularization, the intestinal graft is subjected to various degrees of tissue damage from cold and warm ischemia. While the sensitivity of the small intestine to warm and cold ischemia has been studied extensively in terms of morphology and biochemistry [8-12], only a few reports have examined the large bowel after warm ischemia [13-17]. The aim of this experiment is to evaluate and compare the susceptibility of the small and large intestine to warm and cold ischemia using the *in vitro* Ussing chamber technique, biochemistry, and histopathology.

MATERIALS AND METHODS

Animals. Six beagle dogs of both sexes, weighing 8-13 kg, were used with approval of the University of Pittsburgh Institutional Animal Care and Use Committee. The dogs were fasted from the evening prior to surgery. Under general anesthesia with endotracheal intubation and mechanical ventilation, the abdomen was opened through a midline incision. The lower abdominal aorta was dissected and cannulated with a 12-French trocar catheter (Sherwood Medical, St. Louis, MO). One liter of lactated Ringer's solution containing 2000 units of heparin was infused via the catheter to flush the vascular beds of the small bowel and the colon. After flushing, 20 cm of the terminal ileum (to 5 cm proximal to the ileocecal valve) and 20 cm of the colon (from 5 cm distal to the ileocecal valve) were removed. The lumens of both segments were irrigated with 200 ml of the same solution containing 300 mg/liter of neomycin sulfate. Each segment was divided into two 10-cm pieces and preserved at either 37°C (warm ischemia) or 4°C (cold ischemia), immersed in the same solution. At 0.5, 1.5, and 3 hr of warm ischemia, and at 1, 6, 12, 24, 36, and 48 hr of cold ischemia, tissue samples were collected for later analysis. At each time interval, a full-thickness section of intestinal wall, 1 cm in width, was used for electrophysiologic and permeability

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² To whom correspondence should be addressed at Pittsburgh Transplantation Institute, 4C Falk Clinic, 3601 Fifth Avenue, Pittsburgh, PA 15213. Fax: (412) 624-0192.

studies and for histopathology. Mucosal tissue, 500 mg, was scraped by a glass microscope slide and then immediately frozen and stored in liquid nitrogen until biochemical analyses. Intestinal tissues taken prior to *in situ* vascular flushing were used as the control.

Electrophysiologic study. Electrophysiologic measurements were performed using essentially the same method as described by Madara and Kirkman [18]. The ileum and colon were rinsed with lactated Ringer's solution to remove any remaining luminal contents. The mucosa and submucosa were separated from the seromuscular layer by blunt dissection. The separated submucosa and mucosa, containing no Peyer's patches, were mounted on a 13.5-mm-diameter (1.43 cm² surface area) Ussing chamber (World Precision Instruments, Inc., Sarasota, FL). When mounted in these chambers, the mucosal and submucosal membrane surfaces were perfused with a recirculating, oxygenated (95% O₂, 5% CO₂) buffer solution. The perfusion solution, with a pH of 7.4, consisted of 120 NaCl, 4.7 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 25 NaHCO₃, and 1.2 KH₂PO₄ (in mM). The recirculating reservoirs were maintained at 37°C throughout the experiment. Using two different Ussing chambers, two separate sets of electrophysiologic measurements were performed with individual samples.

For baseline potential differential (PD), resistance (R), and short-circuit current (I_{sc}) determinations, 10 mM D-glucose was added to the perfusion buffer solution recirculating each cell of the chamber. After a 30-min equilibration period, PD and I_{sc} were directly measured every 5 min for 40 min. Resistance was calculated according to Ohm's law using voltage deflection measurements of the transmucosal current (50 μA). Potential difference, I_{sc}, and R at 30 min of measurement were used as representative values of each sample. To determine electrophysiologic response to glucose stimulation, 10 mM mannitol was substituted for the glucose in the mucosal-side solution, and then PD, R, and I_{sc} were measured for the next 20 min. After completing the glucose stimulation experiment, 5 mM theophylline was added in the submucosal-side reservoir, and measurements were continued for another 20 min. Differences between the highest values after stimulation and those before stimulation were determined.

Permeability study. The permeability study was performed with the same chamber used for baseline electrophysiologic measurements. Immediately after 30 min of equilibration, 5 mg of FITC-conjugated Dextran (molecular weight, 4400; Sigma Chemical Co., St. Louis, MO) was added in the mucosal-side reservoir of the Ussing chamber. Before addition of the dye and after 40 min of measurements, 0.25 ml of solution was collected from the submucosal-side reservoir. Solution sampling prior to dye administration was done to eliminate the influence of dye contamination to the recirculating system due to frequent uses and limited cleaning periods. The con-

centration of fluorescent Dextran in each sample was measured at an excitation wavelength of 483 nm and at an emission wavelength of 517 nm using a fluorescent spectrophotometer (RF5000U, Shimadzu, Japan). The permeability of the tissue sample was calculated from the difference in dye concentrations and expressed as ng/cm² of mucosal surface area/min.

Biochemical study. Changes in tissue concentrations of adenine nucleotide (AN) and purine catabolites (PC) during warm ischemia and cold ischemia were studied using high-performance liquid chromatography (HPLC) as described before [19]. Tissue samples were homogenized with a Polytron homogenizer (Brinkmann, Inc., Westbury, NY) in 6% perchloric acid containing 0.8 nmol/liter ethylenediaminetetraacetic acid. The homogenate was centrifuged at 10,000g and 4°C for 10 min. After centrifugation, the supernatant was extracted and injected to a Waters HPLC system (Waters Chromatography Division/Millipore Corporation, Milford, MA/Model 510 pumps, Model 484 absorbance module and Model 717 WISP system). The system was equipped with a Maxima 820 Chromatography work station (Waters), a reverse phase column [E. Merck, Darmstadt, Germany; LiChrospher 100 RP-18 (5 μm), 4 × 250 mm], and a precolumn (Waters; RCSS Guard-PAK). Concentrations of the AN and PC were monitored at 254 nm (Waters 484, Tunable Absorbance Detector). Energy charge was calculated according to the formula by Atkinson [20]: (ATP + 0.5 ADP)/(AMP + ADP + ATP).

Histopathologic study. Tissues, taken at the end of each ischemic period and 70 min after oxygenation in the Ussing chamber, were fixed with 10% buffered formalin, embedded in paraffin, cut into 5-μm sections, and then stained with hematoxylin and eosin. Histopathologic analyses were performed blindly by a single pathologist without knowing the groups (warm or cold) or the ischemic periods. Modified Park's classification [8, 9] was used to evaluate the degree of tissue damage: (0) normal; (1) subepithelial detachment at villus tip; (2) extended subepithelial space; (3) epithelial lifting along villus side; (4) denudation of villi; (5) loss of villus tissue; (6) infarction of crypt layer; and (7) transmucosal infarction.

Statistics. Values were expressed as means ± standard error of the mean. Using a statistical software package (Stat-View II, Abacus Concepts, Inc.), group comparisons of electrophysiologic function and biochemistry were performed by variance analysis, and those of histopathologic changes were determined by a Wilcoxon rank sum test.

RESULTS

Warm Ischemia

Electrophysiology. Warm ischemia induced duration-dependent suppression of electrophysiologic func-

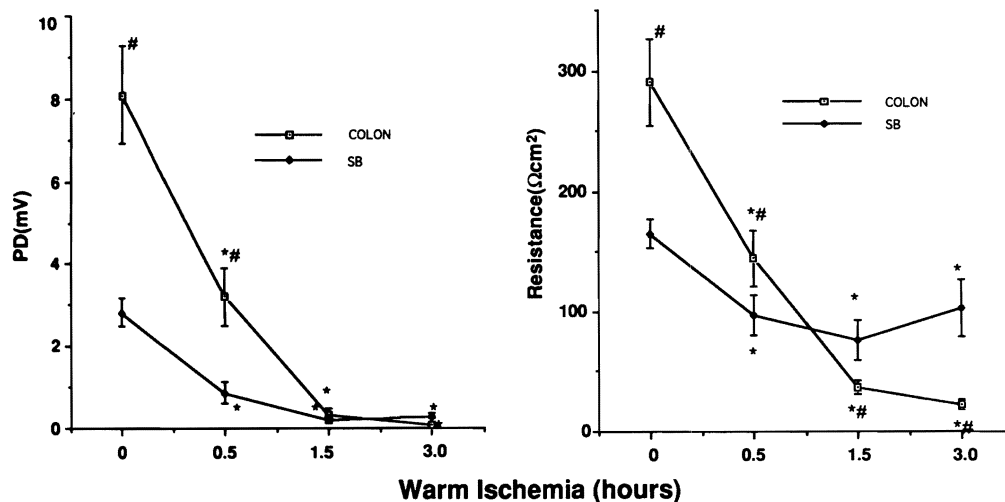


FIG. 1. The baseline potential difference (left) and resistance (right) of the colon and the small intestine after warm ischemia (**P* < 0.05 versus control; #*P* < 0.05 versus small bowel).

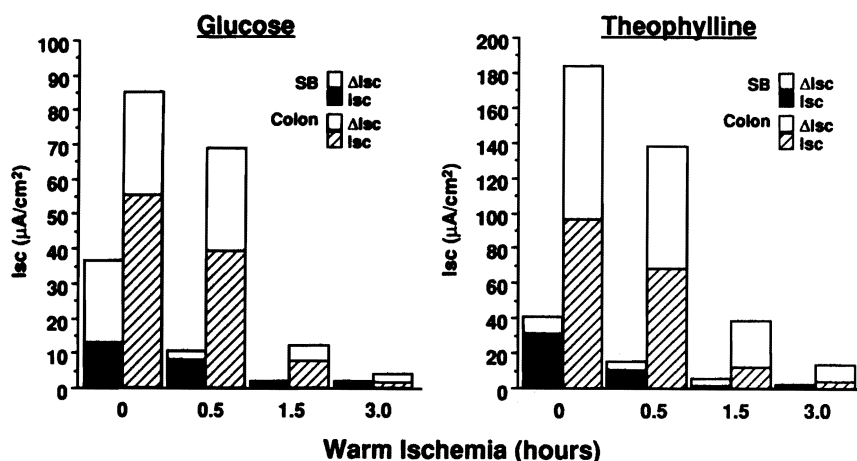


FIG. 2. Electrophysiologic response to glucose (left) or theophylline (right) stimulation of the colon and small bowel after warm ischemia.

tion in both the small intestine and the colon to differing degrees. Potential difference, which was three times higher in the colon than in the intestine before induction of ischemia, was totally abolished in both organs by 1.5 hr (Fig. 1, left). Similarly, R of the colon markedly decreased at 1.5 hr, but small intestinal R remained relatively high throughout the observation period (Fig. 1, right). Baseline Isc of both organs showed a reduction similar to that of PD. However, colonic responses to glucose administration (Fig. 2, left) and theophylline administration (Fig. 2, right) at 0.5 hr of ischemia were similar to those of the control and were still detectable after 3 hr. In contrast, responses of the small bowel were barely detectable after only 0.5 hr of warm ischemia. The colon appears to retain more electrophysiologic activity than the small intestine.

Permeability. Permeability of FITC-conjugated Dextran across mucosal specimens is shown in Fig. 3. During a 40-min perfusion of normal tissues, 0.65 ± 0.30 and 0.23 ± 0.20 ng/cm²/min of fluorescent dye were found to permeate the small intestine and the colon, respectively, accounting for 0.0008 and 0.0003% of the

total amount of the dye injected into the mucosal-side reservoir. No changes in permeability were found in ischemic small intestines, but the colon developed a significant increase in permeability after 1.5 hr of warm ischemia (Fig. 3, left).

Biochemistry. Levels of ATP and energy charge in mucosal tissues of the normal small intestine and normal colon were similar (Table 1). After the onset of warm ischemia, ATP and energy charge dropped significantly at 0.5 hr and were markedly depressed throughout the entire ischemic period. The rate of energy charge reduction was slightly less in the small bowel compared to that of the colon. In contrast, tissue concentrations of AMP and xanthine (X) showed significant elevations in the small intestine by 3 hr, while the colon had greater concentrations of hypoxanthine (HX).

Histopathology. Compared to the control, warm ischemia caused duration-dependent mucosal injury of equal severity in both organs (Fig. 4A). After reperfusion, histological abnormalities were always augmented in both organs. Mucosal damage after 0.5 hr

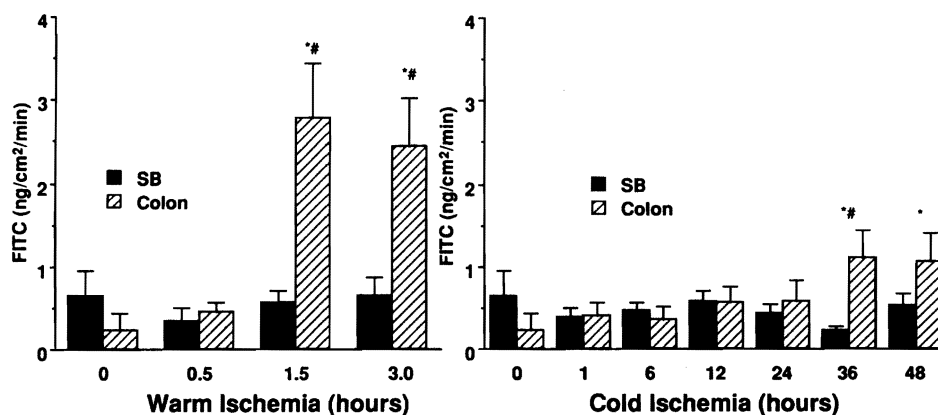


FIG. 3. Permeability of the colon and the small intestine after warm (left) or cold (right) ischemia of the colon and small bowel (* $P < 0.05$ versus control; * $P < 0.05$ versus small bowel).

TABLE 1
Adenine Nucleotides and Purine Catabolites in Intestinal and Colonic Mucosa at Various Durations of Warm and Cold Ischemia

	Warm ischemia						Cold ischemia					
	Control	0.5 hr	1.5 hr	3 hr	1 hr	6 hr	12 hr	24 hr	36 hr	48 hr		
ATP	C 22.11 ± 6.56	2.33 ± 1.00*	0.88 ± 0.14*	0.64 ± 0.06*	9.50 ± 1.20*	9.91 ± 1.87*	8.59 ± 1.54*	7.97 ± 2.24*	3.46 ± 1.89*	4.48 ± 0.65*		
SB	27.42 ± 1.91	3.57 ± 0.40*	1.91 ± 0.55*	1.37 ± 0.28*	13.27 ± 2.62*	8.96 ± 2.52*	9.51 ± 2.34*	6.90 ± 0.92*	3.60 ± 0.59*	2.66 ± 0.96*		
ADP	C 11.15 ± 1.55	5.41 ± 1.71	1.88 ± 0.26***	2.02 ± 0.39**	13.92 ± 4.36	9.17 ± 3.03	9.50 ± 2.53**	7.79 ± 2.09	7.21 ± 2.78	8.63 ± 1.24		
SB	9.28 ± 2.26	13.31 ± 0.87	10.17 ± 2.09	12.56 ± 1.79	18.41 ± 4.46*	15.68 ± 1.45	16.84 ± 3.20	12.76 ± 2.20	10.38 ± 1.18	8.91 ± 2.24		
AMP	C 5.68 ± 0.561	35.19 ± 8.41*	31.82 ± 4.50*	21.12 ± 2.59*	65.23 ± 7.42*	55.94 ± 5.89*	50.51 ± 4.00*	48.69 ± 8.17*	47.74 ± 6.03*	29.51 ± 3.20*		
SB	4.23 ± 1.25	49.26 ± 1.63*	28.81 ± 4.71*	14.86 ± 2.22	55.17 ± 7.98*	43.42 ± 4.94*	55.37 ± 2.05*	59.66 ± 3.13*	39.04 ± 3.05*	42.41 ± 4.65		
Energy charge	C 68.0 ± 5.6	11.4 ± 5.7*	6.0 ± 1.0***	7.0 ± 1.0***	18.9 ± 1.9*	19.4 ± 4.4*	19.2 ± 1.9*	18.4 ± 3.8*	13.0 ± 5.0*	20.02 ± 1.0*		
SB	80.3 ± 4.3	16.0 ± 1.0*	17.6 ± 3.9*	23.0 ± 1.0*	26.2 ± 5.5*	25.0 ± 5.2*	21.1 ± 3.4*	16.7 ± 1.3*	16.5 ± 1.2*	13.0 ± 2.7*		
HX	C 0.75 ± 0.27	14.67 ± 2.27*	22.07 ± 1.40***	29.05 ± 3.20***	8.01 ± 2.07*	9.22 ± 1.70*	9.79 ± 1.20*	9.65 ± 1.32*	8.43 ± 2.13*	12.86 ± 1.95*		
SB	0.841 ± 0.24	14.64 ± 0.81	13.22 ± 2.08*	16.72 ± 1.45*	6.77 ± 0.70*	7.06 ± 0.51*	7.06 ± 1.04*	10.14 ± 1.09*	9.01 ± 1.50*	9.11 ± 1.07*		
X	C 0.50 ± 0.25	11.93 ± 3.39*	23.53 ± 3.13*	38.66 ± 6.14***	1.54 ± 0.32	2.07 ± 0.33	2.78 ± 0.34	5.46 ± 1.41*	5.44 ± 0.99***	6.86 ± 0.31***		
SB	0.24 ± 0.21	8.61 ± 1.64*	29.60 ± 1.51*	56.91 ± 10.66*	0.53 ± 0.09	1.32 ± 0.31	1.83 ± 0.26	7.22 ± 0.72*	10.62 ± 1.36*	12.08 ± 1.42		

Note. ATP, ADP, AMP, HX, X: nmol/mg protein. Energy charge: %.

* $P < 0.05$ vs control.

** $P < 0.05$ vs SB.

ischemia and reperfusion was significantly worse in the small intestine than in the colon, but there was no important difference after 1.5 hr.

Cold Ischemia

Electrophysiology. Cold preservation slowed the decrease in electrophysiologic functions by approximately 8 times in the small intestine and by approximately 16–24 times in the colon. Potential difference, which was undetectable in both organs after 1.5 hr of warm ischemia (Fig. 1A), was undetectable after 12 hr in the small bowel and at 24 to 36 hr in the colon (Fig. 5, left). Changes in R after cold ischemia showed a trend similar to those seen after warm ischemia (Fig. 5, right). Baseline Isc had a duration-dependent decline in both organs, but response to the administration of glucose (Fig. 6, left) and theophylline (Fig. 6, right) was more prominent with colonic tissues for up to 48 hr of cold ischemia. Thus, colonic mucosal cells retain more electrophysiologic activity during cold ischemia.

Permeability. Similar to warm ischemia (Fig. 3, left), cold ischemia did not cause any change in mucosal permeability of the small intestine even after 48 hr of cold ischemia. Although significantly less than that of warm ischemia, there was an increase in colonic permeability after 36 hr of cold ischemia.

Biochemistry. ATP and energy charge degradations were delayed under cold ischemia in both organs (Table 1). Although ATP and energy charge decreased rapidly within 1 hr, the levels were much higher for up to 48 hr than levels seen during warm ischemia, being 10 to 20% of initial values. The degradation of ATP during cold ischemia compared to warm ischemia was reflected by a greater concentration of ADP and AMP, and a lesser accumulation of HX and X.

Histopathology. Compared to the control, cold ischemia induced duration-dependent histologic damage in both organs. The damage was worse in the small bowel than in the colon at 12 hr of ischemia. After reperfusion, further derangements of mucosal structure developed in both organs. The severity of mucosal damage after reperfusion was greater in the small intestine than in the colon at 6 hr and at 12 hr. At 24 and 36 hr, histologic damage after ischemia and reperfusion was similar between the two organs, but the colon was worse at 48 hr.

DISCUSSION

In the present study, we used the *in vitro* Ussing chamber technique to simulate reoxygenation of enteric tissues after various durations of warm and cold ischemia. Changes in electrophysiology, permeability, biochemistry, and histopathology before and after reoxygenation with asanguinous perfusion fluid were determined. Although this method cannot reproduce events similar to those which occur in the grafts after actual transplantation, particularly due to the blood-

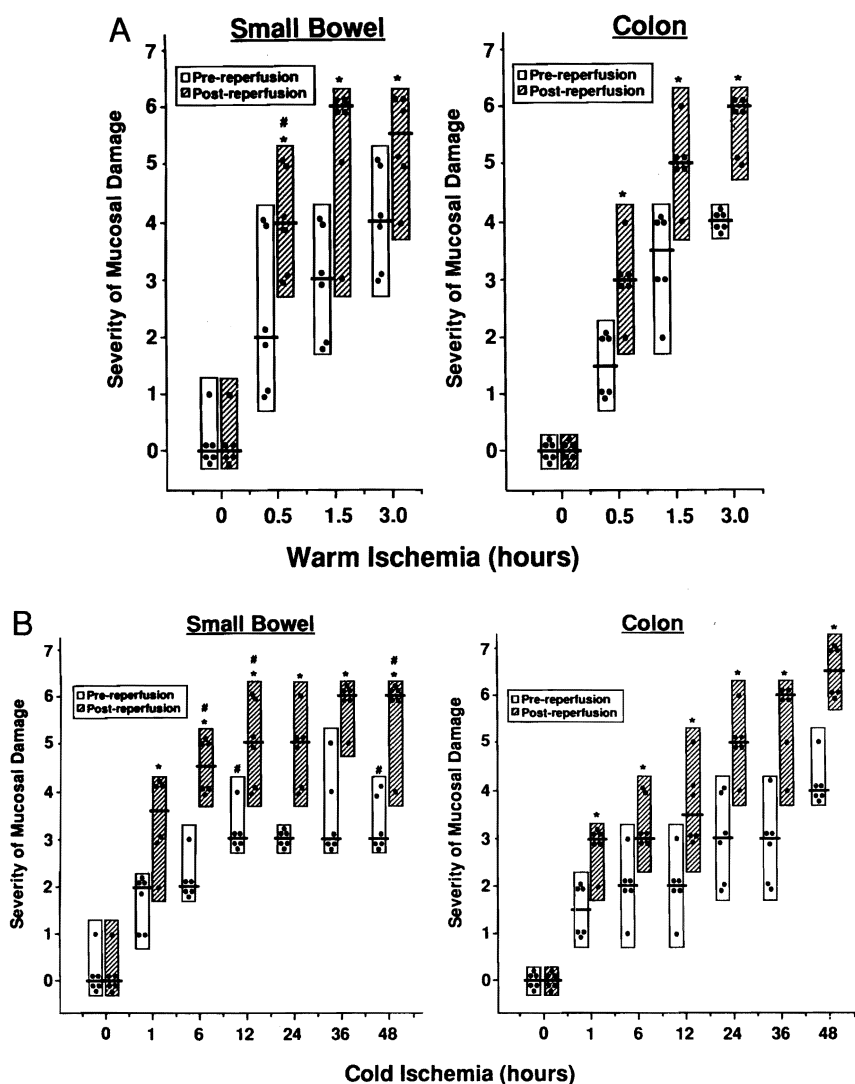


FIG. 4. Histopathologic severity of the mucosal damage in the colon (left) and small intestine (right) at the end of warm ischemia (A) and cold ischemia (B). Horizontal bar indicates median score (* $P < 0.05$ versus control; # $P < 0.05$ versus colon).

less perfusion system [21], it allows direct evaluation of tissue damage caused by ischemia and reperfusion in a carefully controlled environment.

Studies on electrophysiologic function of intestinal and colonic mucosa showed an inverse relationship with the duration of ischemia. As ischemia progresses, electric activity, barrier function, villus absorptive function, and cryptic cell function are negatively affected. However, from the present experiment, electrophysiologic function of the colonic mucosa appears more resistant to ischemic insults than small intestinal tissues. Colonic PD levels remained consistently higher than those of the small intestine until the activities were lost in both organs at 1.5 hr of warm ischemia and at 24 hr of cold ischemia. Similarly, Isc of the colon was higher than that of the small intestine throughout the ischemia period. More importantly, even when the electrophysiologic functions of absorptive enterocytes, determined by glucose stimulation, and cryptic cells,

determined by theophylline stimulation, in the small intestine were abolished at longer periods of ischemia, colonic epithelial cells still responded to administration of both agents. An exceptional observation with our electrophysiologic measurements was the rather sustained small intestinal R even after prolonged ischemia. Although difficult to explain, this may indicate better barrier function of the small intestine during ischemia, or the influence of a thicker submucosal layer of the small intestinal specimen.

Barrier function of small intestinal and colonic mucosa was directly estimated by using FITC-conjugated Dextran as a marker. Disruption of the mucosal barrier, or the movement of micro- and macromolecules from the lumen to the circulation across enterocytes, has been reported under various conditions [22]. Minimal but similar movement of the marker substance through the enteric mucosa of the normal dog was demonstrated in this study, as described in the normal rat

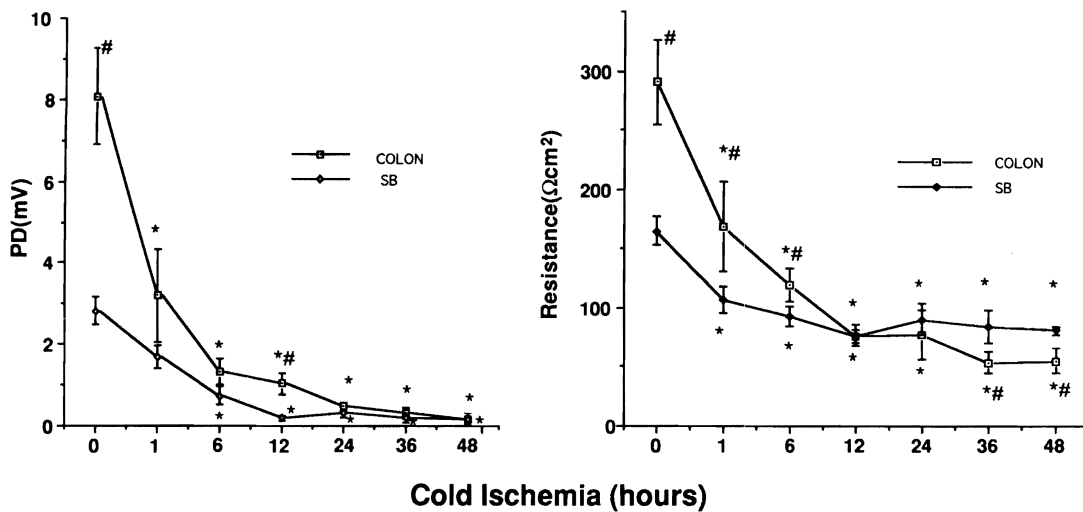


FIG. 5. The baseline potential difference (left) and resistance (right) of the colon and the small intestine after cold ischemia (* $P < 0.05$ versus control; # $P < 0.05$ versus small bowel).

intestine [23]. Pantzar *et al.*, using the same Ussing chamber technique, demonstrated that 0.03–0.04% of the FITC-conjugated Dextran injected into the mucosal-side reservoir was recovered from the serosal-side solution after 60 min perfusion. Permeability of the normal canine intestine was much less than that of the normal rat. Permeability after perfusion of ischemic specimens showed differences between the organs. Although the small intestine had significant histologic mucosal damage, and the circulation-to-mucosa permeability has been demonstrated to increase with ischemia [10], no evident changes in the permeability were noted with our ischemic small intestines. In contrast, the colon developed increased permeability after 1.5 hr of warm ischemia and 36 hr of cold ischemia. This was an unexpected observation, because the colon has generally been thought less permeable to molecules than

the small intestine [24], as seen with normal tissues in this study. Although the results with the permeability study and electrophysiologic measurement were similar, reasons for the difference between the two organs are unclear. At least, an artifact in preparing thinner colonic specimens can be excluded since all of the colonic mucosa with increased permeability had a standard error similar to that of the other specimens.

Irreversible organ damage from prolonged ischemia has long been considered to be associated with the decrease of ATP and energy charge in the tissue. In addition, purine catabolites, particularly HX, which accumulate as degradation products of adenine nucleotides, are thought to play an important role in reperfusion injury by producing superoxide radicals [10, 19]. Although the reversibility of mucosal damage was not determined, similar changes in adenine nucleotides

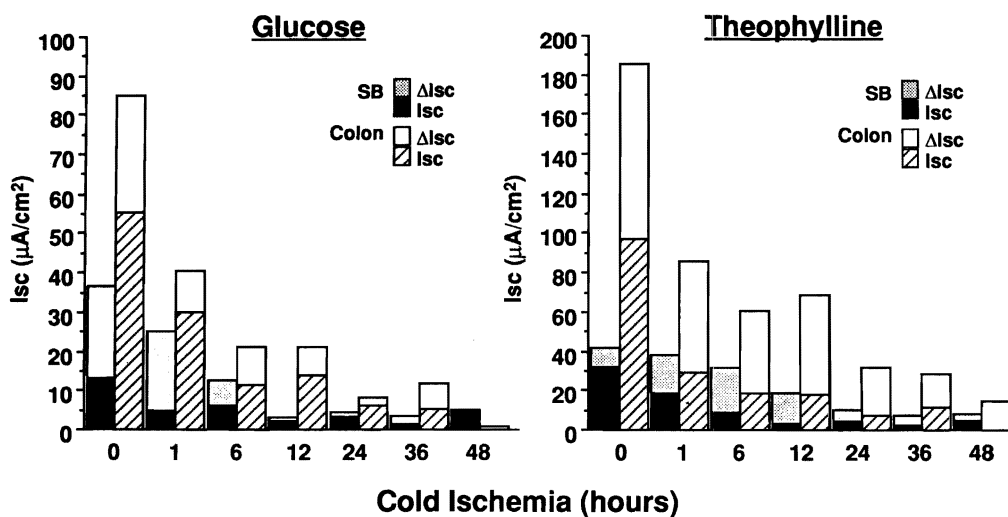


FIG. 6. Electrophysiologic response to glucose (left) or theophylline (right) stimulation of the colon and small bowel after cold ischemia.

and purine catabolites after warm and cold ischemia were obtained with the small intestine and the colon. Under warm ischemia, intestinal mucosa retained consistently higher ATP and energy charge than colonic mucosa, and the latter had greater hypoxanthine levels. These biochemical findings did not correlate with the more severe histologic mucosal damage seen in the small intestine during ischemia and after reperfusion. Indeed, Canada *et al.* [25] indicated that xanthine was the only biochemical marker that correlated with the duration of ischemia, but energy charge was of no value in indicating the extent of injury in the small intestine. Hypothermic storage significantly retarded adenine nucleotide and purine catabolite changes.

Because of the architectural difference between the organs, it may not be appropriate to compare the degree of histopathologic damage to the mucosa, but the small intestine developed extensive structural abnormalities at earlier periods of warm and cold ischemia than the colon. This finding is consistent with the study reported by Robinson *et al.* [16], who compared functional and morphological changes in canine ileum and colon following *in situ* 2-hr strangulation ischemia. According to Robinson, immediately postischemia, both organs showed serious histologic damage, no amino acid and sugar transport, diminished Na⁺-K⁺-ATPase levels, and liberation of acid phosphatase into the circulation, but the ileum was more sensitive to acute ischemia.

The surgical procedure, transportation, dissection, and mounting, followed by 70 min perfusion with an asanguinous buffer solution, caused no notorious insults to the mucosal structure, since normal specimens had no appreciable alterations except for one intestinal specimen. After reperfusion of the ischemic samples, the severity of mucosal damage was augmented in both organs. Reperfusion injury developed during reoxygenation. Although reperfusion injury of organs is a well-established concept [10, 11], several studies failed to demonstrate enhanced intestinal injury after *in vivo* warm ischemia and reperfusion [26, 27]. Recently, Park *et al.* [8, 9] confirmed the phenomenon histologically after warm and cold ischemia of the rat intestine. Our study is the first demonstration of intestinal reperfusion injury *in vitro*, particularly with the colonic mucosa. Neutrophils, and xanthine oxidase and hypoxanthine, have been considered factors involved in the reperfusion injury [10, 11], but they had little effect in our investigations with cold ischemia because we used blood-free perfusion, and xanthine oxidase did not increase during cold ischemia [28, 29]. Further study is needed to clarify the cause of intestinal reperfusion injury under bloodless perfusion.

In summary, our results suggest that the colon is more resistant to ischemia than the small intestine. This implies that, from a technical perspective, the colon can be safely included in the intestinal graft without fear of increased risk of ischemia-related morbidity. At the same time, however, if the colon truly has a

greater chance of increased permeability after ischemia, inclusion of the colon for intestinal transplantation requires reevaluation to avoid harmful bacterial translocation, which is now under investigation.

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