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Oxygenated Perfluorocarbons Protect the Intestine From the Ischemia/Reperfusion Injury in Rabbits

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Abstract

Objective: To investigate whether intraluminal administration of oxygenated perfluorocarbons (PFCs) protects the enterocyte from acute ischemia-reperfusion (I/R) injury. **Materials and Methods:** Twenty rabbits were divided in 4 groups: sham-operated controls (group A), acute I/R (group B), acute I/R plus infusion of oxygenated PFCs 30 minutes before ischemia (group C), and acute I/R plus infusion of oxygenated PFCs 30 minutes before reperfusion (group D). Serum creatine phosphokinase (CPK) and mucosal disaccharidase activity were examined. Intestinal biopsies were obtained for electron microscopy study. **Results:** Group B CPK mean values are 3495.2 $\pm$ 157.35 and 4855 $\pm$ 350.21 U/L. Group C: 2674.6 $\pm$ 265.87 and 3231 $\pm$ 232.30. Group D: 2382.2 $\pm$ 102.90 and 3217.6 $\pm$ 185.61 at 120 and 180 minutes ($P < .05$). At 180 minutes, maltase and sucrose values were 33.63, 51.88, 8.45, and 19.91, and 17.99, 22.87, 6.62, and 14.24 μmol/min per g for groups A, B, C, and D, respectively ($P < .05$). Histopathology showed the least cellular deterioration in PFC groups. **Conclusion:** Oxygenated PFCs protect the enterocyte during bowel I/R.

Keywords

intestinal I/R injury, perfluorocarbons, creatine phosphokinase, disaccharidase activity

Introduction

Acute mesenteric ischemia induces an inflammatory response to the sensitive intestinal mucosa. Reperfusion may increase this response and become deleterious to the intestine and to other organs. Multiple organ failure is a severe, sometimes fatal, condition, which may occur in a variety of surgical procedures and clinical conditions. Despite effective surgical treatment, the mortality rate for patients with acute occlusive mesenteric ischemia remains high (up to 80%) because of delays in diagnosis and definitive treatment.[1]

Mesenteric ischemia/reperfusion (I/R) promotes local synthesis and release of inflammatory mediators that provoke and exacerbate gut injury. Furthermore, it primes the circulation of polymorphonuclear neutrophils that produce superoxide anion that leads to remote organ injury (ie, pulmonary and hepatic).[2] At cellular level, mesenteric I/R activates a cascade of oxidative stress-sensitive protein kinases that converge on specific transcriptional factors to regulate expression of proinflammatory genes. Furthermore, I/R results in the recruitment of neutrophils to the gut and generates reactive oxygen species that further increases cellular damage of IR injury.[3]

Therapies directed to reduce I/R injury have effectuated in several experimental works and include radical scavengers and protease inhibitors.[4] Liquid perfluorocarbons (PFCs) are biologically inert compounds well known[4] for their high capability to carry respiratory gases to (and from) the tissues and already used in experimental studies of I/R injury.[5-7]

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This study aims to investigate whether intraluminal intestinal administration of PFCs could ameliorate the intestinal mucosa injury caused during an experimental model of acute I/R in rabbits.

Materials and Methods

Experimental Protocol

The procedures used in this study were approved by the University Ethics Committee and were conducted according to the Guidelines for Animal Experimentation. Twenty rabbits (white New Zealand, 6 months old and mean weight 3.200 $\pm$ 300 g) were divided randomly into 4 groups: control group (A), I/R group (B), I/R plus infusion of oxygenated PFC 30 minutes before ischemia group (C), and I/R plus infusion of oxygenated PFC 60 minutes after ischemia group (D). Pure linear chain PFC ([CF_3 (CF_2)6 CF3], MW: 438) was purchased as Perfluoron from Alcon, Fort Worth, Texas. For the PFC-O_2 group of animals, fluoron was bubbled with 100% oxygen.[8]

Anesthesia was induced and maintained with intramuscular (im) ketamine hydrochloride (20 mg/kg Imalgen; Merial, Lyon, France) plus xylazine hydrochloride (2 mg/kg Rompun; Bayer, Leverkusen, Germany).

Laparotomy via midline abdominal incision was carried out. The anterior mesenteric artery was identified and clamped for 120 minutes, using 1 surgical microvascular clamp (Scalcan clamps; St. Paul, Minnesota), followed by releasing the clamp for a further 60-minute period. Mesenteric ischemia was verified by Doppler examination, inspecting the pale discoloration of the intestine and loss of pulses of the mesenteric circulation. On the other hand, reperfusion was verified again by Doppler examination, with the reset of intestine's color and pulses in the mesenteric artery. Sham operation involved the same technique and exposure without clamping the superior mesenteric artery. During the experiments, the animals received intravenous (iv) 0.9% sodium chloride solution and the mean infusion rate was approximately 60 mL/h.

A small prepyloric gastrotomy was performed. A thin catheter (20-cm length) was placed into the animal's duodenal end and was used for the administration of PFC-O_2 at a constant rate of 20 mL/h. Laparotomies were closed with continuous (3-0 polypropylene) suture (Prolene; Ethicon, Somerville, New Jersey).

Antimesenteric wedge intestinal biopsies were taken at 0, 60, and 120 minutes during the period of ischemia and at 150 and 180 minutes during the period of reperfusion. Special care was taken to preserve the intestinal lumen continuity. For each time point, small intestine biopsies were taken 20, 40, 60, 80, and 100 cm from the duodenal end. Venous blood samples were taken at the same time points for creatine phosphokinase (CPK) determination. Disaccharidase activity was measured from intestinal biopsies taken at the end (180 minutes) of the experiment. The animals were sacrificed by exsanguination.

Statistical Analysis

Continuous data were described as mean $\pm$ standard deviation (SD). Kruskal-Wallis test was used to test for differences between the groups. Repeated measures analysis of variance ([ANOVA] General Linear Model) was used to test for differences within the groups, using the Greenhouse-Geisser correction, because of sample size. Least significance difference post hoc analysis was used when significant differences were revealed between or within the groups in both the above tests. Spearman test was used to detect for significant bivariate correlations. A P value of less than .05 was considered statistically significant in all the above tests. Statistical analysis was performed using SPSS for Windows, version 13 (SPSS Inc., Illinois).

Disaccharidase Activity Assay

The intestinal mucosa was assayed for sucrase and maltase activity, according to Dahlqvist's method.[9] Mucosa was separated from the other intestinal layers by scraping, using a blade. It was then homogenized with 4 parts (v/w) of saline water in a Polytron homogenizer for 60 minutes at 5°C. The tube was chilled with crushed ice during homogenization. Nuclei and larger cell debris were removed by centrifugation at 3000 rpm for 10 minutes.

Disaccharidase activity was determined by the concentration of glucose generated, after the incubation of part of the homogenate tissue with either sucrose or maltose solution as substrate. The homogenate (100 μL) was added to 100 μL of maleate buffer (0.1 mol/L maleic acid/NaOH with 0.056 mol/L of sucrose or maltose of pH 6.0-6.5) at 37°C. The reaction was allowed to progress for 60 minutes and was stopped by boiling for 2 minutes and chilling with water.

The concentration of glucose generated was calculated with a spectrophotometric assay using the Sigma Glucose HK (hexose kinase) reagent. The chemical reactions involved are:

$$\text{glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6 phosphate} + \text{ADP},$$

$$\text{glucose-6 phosphate} + \text{NAD} \xrightarrow{\text{G-6 PDH}} \text{6-phosphonate} + \text{NADH}$$

Exactly 1.5 mL of reagent was added to 10 μL of the incubation mixture. The concentration of glucose generated was calculated based on the molar absorbance of NADH at 340 nm to standardize the enzyme activities. The results were expressed in μmol/min per g wet tissue.

Electron Microscope Study

Small intestine tissue samples were retrieved at 0, 60, and 120 minutes after the onset of ischemia and 30 and 60 minutes after the onset of reperfusion 20, 40, 60, 80, and 100 cm from the duodenal end.

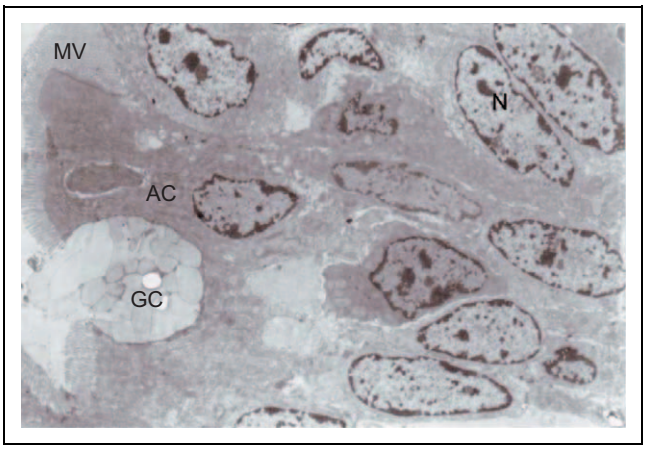

Figure 1. Ultrathin sections of small intestine. Normal morphology. MV indicates microvilli; GC, goblet cell; AC, absorptive cell; N, nucleus. Magnification ×4000.

Tissue samples intended for observation by electron microscope were cut into small tissue items (1 mm^3) and fixed with 3% glutaraldehyde at 4°C for 2 hours. They were then rinsed with Ringer's solution for 10 minutes, postfixed in 1% osmium tetroxide (OsO_4) for 90 minutes, and "in tissue" stained with 1% uranyl acetate and lead citrate for 18 hours. In addition, tissue samples were dehydrated in a degraded series of ethanol solutions, embedded in "ERON 812" resin fixative, and dehydrated again in propylene oxide for 30 minutes. Next step was polymerization at constant temperature in an oven at 70°C for 24 hours. Informative cuts of 1 μm were taken after the pyramid formation. Semi-thin tissue sections 0.5 μm thick were cut and were stained with 2% toluidine blue. Ultrathin sections 600 to 700 Å were obtained with an automatic ultramicrotome Reichert OmU2 Reichert, Vienna, Austria. These sections were stained by Reynolds lead citrate solution methods for 15 minutes. Observations were made in a passing beam electron microscope (JEOL 2000 FX II TEM, JEOL corp. Ltd, Tokyo, Japan).

Results

Electron Microscopic Findings

Group A. The architecture of the intestinal absorptive cells was normal in all experimental steps (Figure 1).

Group B. Many cells retained the absorbent structure. Cellular membranes, microvilli, the free surface, and the lateral cell connections were intact, especially at the beginning (60 minutes) of the experiment. With the progression of ischemia (120 minutes) and reperfusion (180 minutes), mitochondria showed swelling of the parent substance and the loss of their home humps. On the contrary, in different areas of the same specimens, many other absorptive cells were completely destroyed. However, their core membrane has been maintained. Many goblet intact cells, filled with secretary granules, were identified. In addition, moderate swelling, congested capillaries, and degeneration of cells were observed in the lamina propria (Figures 2 and 3).

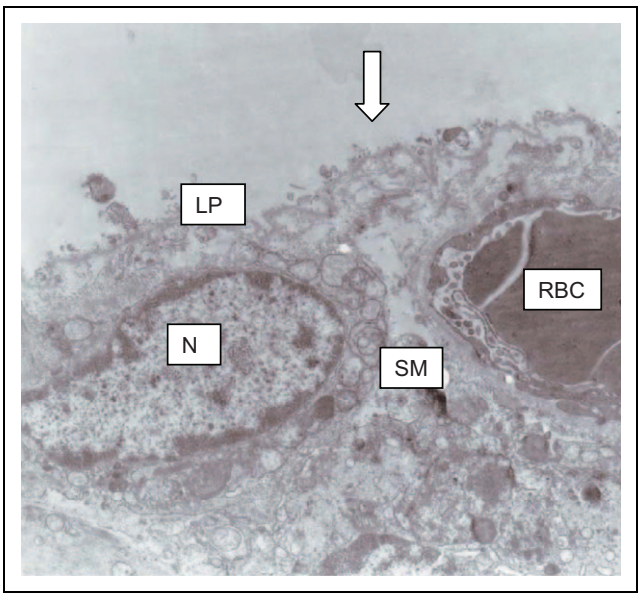

Figure 2. Group B at 120 minutes. Apoptosis (→) of the epithelial cells was presented but lamina propria (LP) was maintained. Edema and congestion of capillary vase were observed in the submucosa (SM). RBC indicates red blood cell; N, nucleus of cell.

Group C. The lesions were mild and limited. Absorptive cells retained their normal structure. Cellular membranes remained intact, as did the microvilli, the tight junctions, and the mitochondria. On the other hand, in some areas of the same specimens, only lamina propria was left, especially at the end of the experiment (180 minutes). However, this dermis showed no swelling and the surrounding cells showed no degenerative changes. The cells of the Lieberkühn glands remained normal (Figures 4 and 5).

Group D. Absorptive cells appeared intact without cell membrane breaking. Lateral tight junctions remained intact too, just with some local interstitial edema. Microvilli were normal, although some of them became wider and shorter. Mitochondria changes were variable. Some of them were normal, while others were swollen with loss of internal crest. Some of them were completely destroyed. Finally, in very few areas of the same specimens complete destruction of the absorptive cells was observed. This was most evident by the end of reperfusion at the time points of 150 and 180 minutes (Figures 6 and 7).

Disaccharidase Activity

Brush border disaccharidase activity showed a significant increase in I/R animals (group B). At the end of the experiment (180 minutes), the mean values of maltase activity were 33.63 and 51.88 μmol/min per g, for groups A and B, respectively (expressed in μmol of glucose per minute per gram protein). Treatment with PFC resulted in a significant reduction of

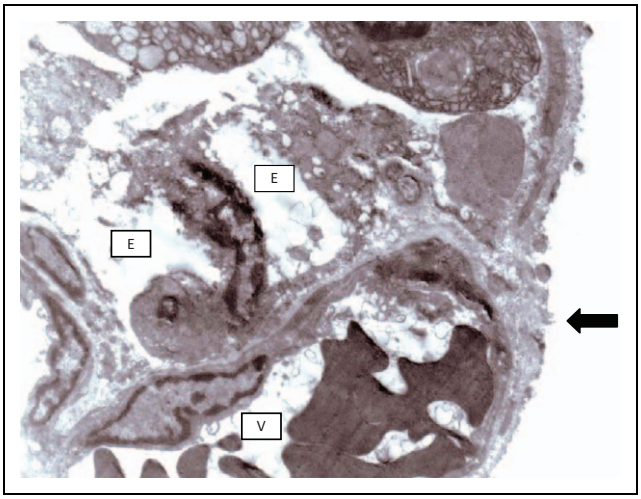

Figure 3. Group B at 180 minutes. Destruction of mucosal cells and of lamina propria (→) were observed. Edema (E) and a congested vase (V) were observed in the submucosa. Magnification ×6000.

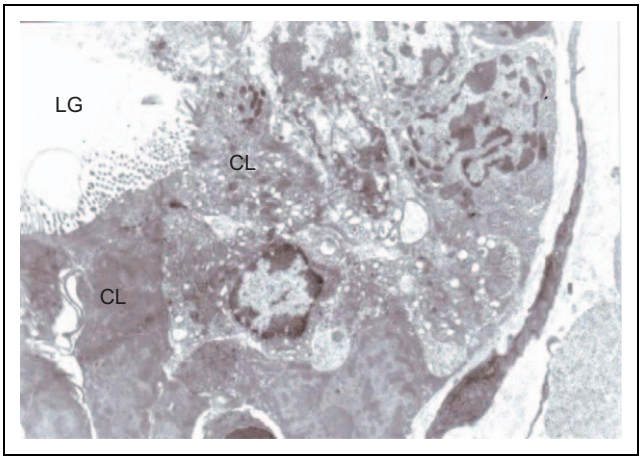

Figure 5. Group C at 150 minutes. Part of intestinal gland or crypts of Lieberkühn (CL). The crypts are normal. LG indicates lumen of the cell. Magnification ×6000.

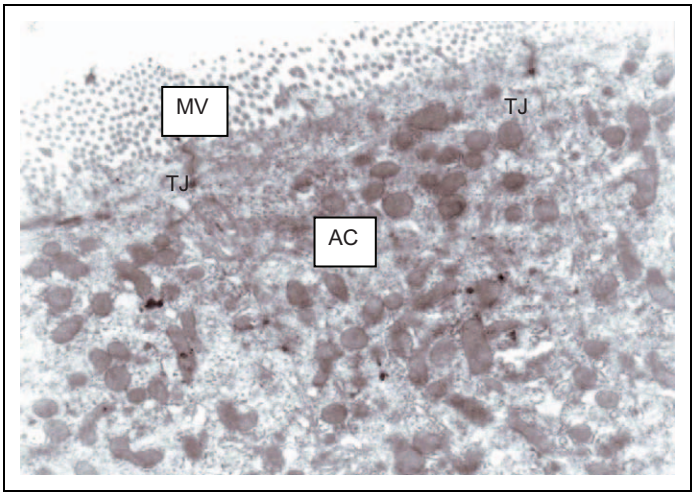

Figure 4. Group C at 180 minutes. Parts of 2 absorptive cells of the mucosa are observed. Mitochondria presented with edema and loss of their internal cristae. Tight junctions among the cells and the microvilli were preserved. Magnification ×16 000.

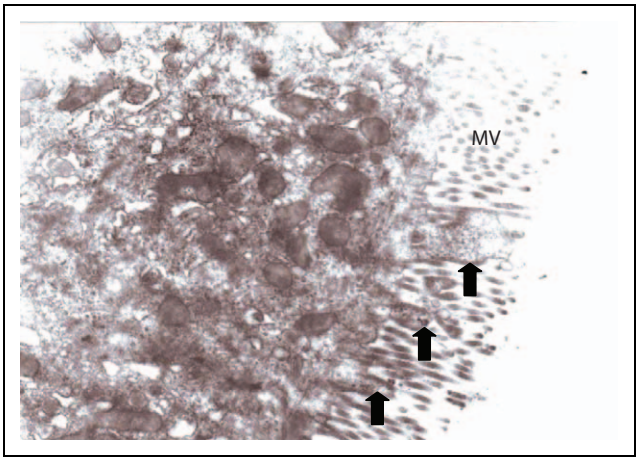

Figure 6. Group C at 150 minutes. Part of an absorptive cell of the intestinal mucosa. MV indicates microvilli. Some of the microvilli presented with increase of their width and decrease of their height (→). Magnification ×6000.

maltase values in both groups C and D that is 8.45 and 19.91 μmol/min per g, respectively. The same observations were made for the mean values of sucrase. The mean values of sucrase activity at 180 minutes were 17.99, 22.87, 6.62, and 14.24 μmol/min per g, for groups A, B, C, and D, respectively. Statistical significance, wherever observed, is reported in Table 1.

Creatine Phosphokinase Levels

Group A mean CPK values were 1926.60 ± 47.79, 2439.60 ± 240.90, and 2701.8 ± 182.25 U/L at 0, 120, and 180 minutes, respectively. Group B mean CPK values were 1961.6 ± 151.8, 3495.2 ± 157.35, and 4855 ± 350.21 at 0, 120, and 180 minutes, respectively. Group C mean CPK values were 1635.2 ± 178.94, 2674.6 ± 265.87, and 3231 ± 232.30 at 0, 120, and 180 minutes. Finally, group D mean CPK values were 1630.8 ± 130.21, 2382.2 ± 102.90, and 3217.6 ± 185.61 at 0, 120, and 180 minutes. The mean CPK levels increased significantly in group B during reperfusion compared to group A ($P < .05$). The mean CPK levels in groups C and D tended to return back to baseline ($P < .05$). All statistical observations regarding CPK values are depicted in Tables 2 and 3.

Discussion

It is well known that I/R syndrome is associated with high morbidity and mortality.[1] The only way to reduce the morbidity and mortality associated with this disease process is to diagnose and treat it early, before irreversible signs of bowel ischemia develop. Intestinal I/R injury is a frequent consequence of hypovolemic shock and bloodstream infection.[3] Inadequate

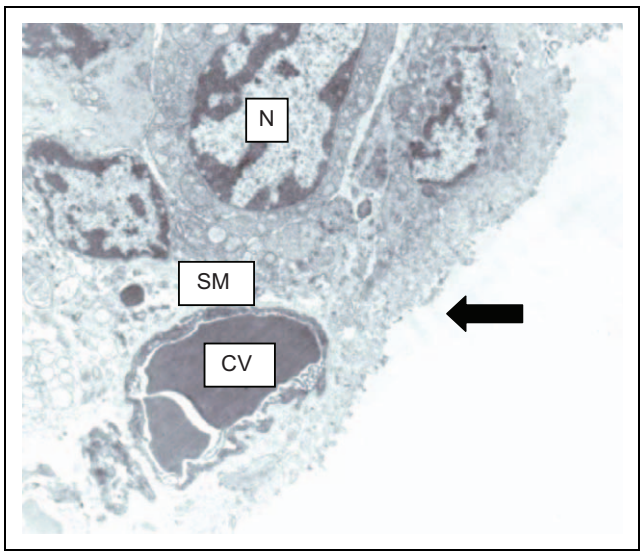

Figure 7. Group D at 180 minutes. Total apoptosis (→) of absorptive cells and of lamina propria are presented. Cells of the submucosa (SM) presented with the least intense deterioration, while a congested capillary vase (CV) is presented. N indicates nucleus of the SM cells.

perfusion of the gastrointestinal (GI) tract may also occur during states of fluid and electrolyte imbalance or when cardiac output is marginal. In order to treat patients with acute mesenteric ischemia successfully, we have to expand our knowledge of the early molecular pathways involved in the activation and proliferation of both local and systemic inflammation.

Administration of oxygen to the intestinal lumen is shown to protect the mucosa and reduce the mortality rate in experimental models.[10] However, the mechanism of oxygen transport in these reports is difficult to apply, mainly due to clinical adverse events and disorders resulting from fluid and electrolyte disturbances.[10] Gao et al[11] showed that the intraluminal administration of hyperoxygenated electrolyte or dextrose solutions in the intestine had protective effect on mucosal integrity without creating any further complications.

Previous experimental studies have shown that intraluminal administration of oxygenated PFCs protects the intestinal mucosa from injuries caused by ischemia alone or by the combination of ischemia with reperfusion. Oldham et al[12] and Papadimitriou et al[6] examined the injuries caused by mere ischemia. They observed that with PFC's intestinal viability was prolonged after acute ischemia even after 8 hours. On the other hand, O'Donnell et al[5] experimented on an ischemia (60 minutes) and reperfusion (120 minutes) model. In this study, administration of oxygenated PFCs during the phase of ischemia alone ameliorates the mucosal injury caused to the intestine by the I/R syndrome. Papadimitriou et al[6] and O'Donnell et al[5] established the dosage of intraluminally administered PFCs. On the other hand, extraluminal administration of oxygenated PFCs failed to prevent I/R intestinal injury. In particular, Vejchapipat et al,[13] despite placing the small intestine in a bag containing oxygenated PFCs solution, after 30 minutes of ischemia and 60 minutes of reperfusion, did not observe any improvement in the intestinal mucosa lesions.

The difference between the present and the aforementioned studies is the duration of ischemia and reperfusion. In addition, the biochemical and pathological markers used to measure the intestinal injury are not the same.

Perfluorocarbons are fluorocarbons, compounds derived from hydrocarbons by replacement of hydrogen atom by fluorine atoms. They are made from carbon and fluorine alone and include octafluoropropane, perfluorohexane, and perfluorodecalin. Perfluorocarbons are arranged in a linear, cyclic, or polycyclic shape. They represent an interesting category of chemicals due to their specific and unusual properties. They are used in tissue oxygenation fluids (blood substitutes and oxygen therapeutics), anti-tumural agents, perfusates for isolated organs, surgical tools for ophthalmology, lubrication and cushioning for articular disorders, cell culture media supplements, and drug formulations.[10]

Perfluorochemicals are nonpolar highly fluorinated compounds and as a result of the strong intramolecular bonding (C-F bonds are 485 kJ/mol, that is 84 kJ/mol more than a regular C-H bond). They have chemical and biochemical inertness. The chemical structure and the weak intermolecular interactions are responsible for the specific properties of PFCs namely the low surface tensions (<20 mN/m), dielectric

Table 1. Mean Values ± SD of CPK[a]

	Groups				
Time (min)	A	B	C	D	
0	$1926.6^{4,5} \pm 47.79$	$1961.6^{2,3,4,5} \pm 151.8$	$1635.2^{2,3,4,5} \pm 178.94$	$1630.8^{2,3,4,5} \pm 130.21$	$P = NS$
60	$2190^{5} \pm 154.93$	$2354.8^{1,3,4,5} \pm 111.16$	$2261.4^{1,5} \pm 299.05$	$1869.4^{1,3,4,5} \pm 135.26$	$P = NS$
120	$2439.6^{\beta,5} \pm 240.90$	$3495.2^{\alpha,\gamma,\delta,1,2,4,5} \pm 157.35$	$2674.6^{\beta,1,5} \pm 265.87$	$2382.2^{\beta,1,2,4,5} \pm 102.90$	$P = .029$
150	$2538.6^{\beta,1,5} \pm 205.20$	$4168,8^{\alpha,\gamma,\delta,1,2,3} \pm 153.58$	$3060.2^{\beta,1} \pm 300.71$	$2904^{\beta,1,2,4,5} \pm 217.90$	$P = .01$
180	$2701.8^{\beta,1,2,3,4} \pm 182.25$	$4855^{\alpha,\gamma,\delta,1,2,3} \pm 350.21$	$3231^{\beta,1,2,3} \pm 232.30$	$3217.6^{\beta,1,2,3,4} \pm 185.61$	$P < .001$
	$P = .02$	$P = .001$	$P = .001$	$P = .007$	

Abbreviations: CPK, serum creatine phosphokinase; SD, standard deviation; NS, not significant.
[a] Hellenic letters denote statistical significance at the same time point among different groups. Numbers denote statistical significance within the same group.
[α] Compared to group A.
[β] Compared to group B.
[γ] Compared to group C.

Table 2. Mean Value of Maltase

	Groups				
Time	A	B	C	D	
180 min	33.63	51.88	8.45	19.91	$P < .001$

Table 3. Mean Value of Sucrase

	Groups				
Time	A	B	C	D	
180 min	17.99	22.87	6.62	14.24	$P < .001$

constants and refractive indices, the high densities, viscosities, and gas solubilities that are largely known for liquids. The solubility of such gases is related to the molecular volume of the gas, according to Henry's Law, which occupies the intermolecular spaces of PFCs and depends on the partial gas pressure (ie, P_{O_2}).[10] The O_2 solubility in PFCs is 40% to 45%, which is 20 times greater than in blood plasma under identical conditions. In intestinal lumen environment (very low P_{O_2} levels), PFC releases oxygen progressively and passes by diffusion through the membranes in the intracellular compartment.

In all groups, serum CPK showed an almost exponential increase. That was more prominent after 120 minutes. Group B CPK value was significantly higher when compared not only with group A but also with groups C and D ($P < .05$). Caglayan et al[14] observed in their animal I/R model that CPK levels increased significantly during the ischemia and were maintained at high levels during reperfusion. In the present study, the administration of oxygenated PFCs prevents the excessive increase of CPK that is observed in the I/R group. Since the CPK values between groups C and D did not show any statistical significance, there might not be clinical significance in the precise timing of the administration of PFCs. Finally, in another study reported by Fujino et al,[15] where the small intestine was placed in a bag containing oxygenated PFCs, the CPK levels were lower when compared to the control group.

The small intestine epithelium undergoes constant renewal. The I/R injury is known to provoke sequential events beginning with desquamation of villus cells, followed by a transient increase of crypt cell production and ending with the migration and differentiation of the replenished villus cells for functional recovery, provided that the intestinal injury is not irreversible. Dissacharidase activity is an important marker of small intestine mucosal functionality.[16] The current study implicates that the mucosal injury was worse in group B, since the mean values of sucrose and maltase were higher in comparison with the other groups. Administration of PFCs prior to reperfusion led to a statistically significant decrease in the mean values of dissacharidases in groups C and D. In the latter groups, continuous intraluminal flow of oxygenated PFCs maintained intestinal enzyme function despite I/R insult, as shown by their significantly lower mean values, when compared to those of group B. The beneficial action of PFCs is accentuated when groups B and D are compared. This is especially true after reperfusion, in the period between 150 and 180 minutes. Our findings are consistent with the observations of O'Donnell et al who reported that delivery of oxygen before irreversible changes take place protects intestinal mucosa from I/R injury.

Electron microscopy revealed destruction of mucosal cells in group B in both 120 minutes of ischemia and 180 minutes of reperfusion. The majority of cells showed swelling of mitochondria and endoplasmic reticulum, apoptosis of cristae, scarce and lodging microvilli, and opening of tight junctions. Similar mucosal cell changes were observed in group C but only at the end of the experiment. At earlier time points, the changes were less intense, when compared to group B. Group D showed the least prominent tissue destruction among the groups that received PFCs. Mucosal appearance was comparable to normal. The intestinal mucosal cells preserved structural and intracellular integrity with tight junctions and cellular organelles remaining intact.

It seems that administration of PFCs attenuates the unfavourable course of the I/R syndrome, since it constrains the bowel mucosa injuries. Intraluminal use of PFCs could be useful as an adjunctive tool in the treatment of acute mesenteric ischemia, increasing the time frame for surgical intervention.

Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

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