The Protective Effect of Oxygenated Perfluorocarbons (PFCs) on Intestinal Ischemia-Reperfusion Injury (I/R) in Rabbits

Achilleas Ntinas¹, Stavros Iliadis², Athanasia Alvanou Achparaki³, Dionisios Vrochides⁴, Georgios Pitoulias⁵, Georgios Papageorgiou², Charalambos Spyridis¹, Dimitrios Papadimtriou⁵, Dimitrios Karamanos,¹ and Thomas Gerasimidis¹

Abstract
Objective: To evaluate the effect of intraluminal administration of oxygenated perfluorocarbons (PFCs) on small intestine’s viability in an experimental model of acute ischemia-reperfusion (I/R).

Methods: Twenty rabbits were divided in four groups: sham-operated controls (group A), acute I/R (group B), acute I/R plus infusion of PFCs 30 min before ischemia (group C), and acute I/R plus infusion of PFCs 30 min before reperfusion (group D). Malondialdehyde (MDA) tissue levels and d-lactate blood samples were taken. All tissue sections were examined under light microscope.

Results: Mean MDA levels in group A: at 0 min 1.79 ± 0.97, at 120 min 2.25 ± 1.76 and at 180 min 3.70 ± 1.76 nmols/g. Group B: at 0 min 2.60 ± 0.58, at 120 min 4.20 ± 0.58 and at 180 min 5.48 ± 2.01. Group C: at 0 min 1.54 ± 0.85, at 120 min 1.14 ± 0.37 and at 180 min 0.59 ± 0.35. Group D: at 0 min 2.12 ± 0.62, at 120 min 3.97 ± 0.70 and at 180 min 2.32 ± 0.37. (p < 0.05). Mean d-lactate levels in group A: at 0 min 36.45 ± 1.99, at 120 min 39.10 ± 2.37 and at 180 min 40.05 ± 2.13 mg/dl. Group B: at 0 min 61.23 ± 11.03, at 120 min 74.84 ± 10.70 and at 180 min 89.90 ± 9.29. Group C: at 0 min 51.05 ± 10.36, at 120 min 56.07 ± 11.27 and at 180 min 57.20 ± 11.19. Group D: at 0 min 64.36 ± 5.26, at 120 min 72.55 ± 7.19 and at 180 min 77.02 ± 9.41. (p < 0.05). Histopathological analysis indicated a significant improvement in the groups of oxygenated PFCs compared with I/R group.

Conclusion: Intraluminal administration of oxygenated PFCs seems that protect the intestine from the I/R injury.

Keywords: Intraluminal administration of oxygenated PFCs, intestinal I/R injury, MDA, lactate

Introduction
Intestinal ischemia-reperfusion (I/R) injury is a phenomenon often confronted in various surgical procedures and many clinical conditions such as small bowel transplantation, abdominal aneurysm repair, necrotizing enterocolitis in neonates, and shock. It is associated with high morbidity and mortality rate which is above 50%.¹ In recent years, it has been made clear that the intestinal tract is one of the earliest organs involved in I/R injury. Multiple organ failure is a frequent complication following intestinal I/R injury and is a common cause of death.²

Although the mechanisms involved during I/R injury are complex, it is known that reactive oxygen metabolites play a very important role in the pathophysiology of this event. During ischemia, tissue adenosine 5'-triphosphate (ATP) degrades to hypoxanthine, following an increase in xanthine oxidase activity. Reactive oxygen radicals are generated and get involved in complex chemical interactions that lead to the immediate cellular damage of I/R injury.³,⁴ Superoxide, hydrogen peroxide, and hydroxyl radical are formed and may cause mucosal injury by direct action and by secondary activation of polymorphonuclear neutrophils (PMN).⁵,⁶ Another consequence of the ischemic bowel injury is the disruption of the gut mucosal barrier predisposing the entrance of harmful microorganisms and/or their toxins in a phenomenon called bacterial translocation. In fact, intestinal permeability seems to be increased during experimental I/R injury.⁷

¹ 5th Surgery, “Hippokration” Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece.
² Biological Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece.
³ Pathology and Histology, Aristotle University of Thessaloniki, Thessaloniki, Greece.
⁴ Department of Organ Transplantation, “Hippokration” Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece.
⁵ 2nd Surgical Department, Division of Vascular Surgery, Aristotle University of Thessaloniki, Thessaloniki, Greece.
A wide number of therapeutic approaches to reduce I/R injury including radical scavengers and protease inhibitors have been studied. Liquid perfluorocarbons (PFCs) are biologically inert compounds well known for their high capability to carry respiratory gases to (and from) the tissues and have already been used in experimental studies of I/R injury.

The aim of this study was to evaluate the potential protective effect of intraluminal intestinal administration of oxygenated PFCs on the enteric viability in an experimental model of acute I/R.

Materials and Methods

The experimental protocol (care of the animals, model of intestinal I/R) was approved by the Institutional Scientific and Ethical Committee. A total of 20 rabbits (White New Zealand, aged 6 months and mean weight 3,200 g) were divided randomly into 4 groups: Control group (A), I/R group (B), I/R plus infusion of oxygenated PFCs 30 minutes before ischemia group (C), and I/R plus infusion of oxygenated PFCs 60 minutes after ischemia group (D). Perfluoron was used as a pure linear chain PFC [(CF3(CF2)6 CF3), MW: 438] which was purchased from Alcon, Fort Worth, Texas. For the PFC-O2 group of animals, fluoron was bubbled with 100% oxygen.

Animals were sedated with intramuscular ketamine hydrochloride (20 mg/kg Imalgan; Merial, Lyon, France) plus xylazine hydrochloride (2 mg/kg Rompun; Bayer, Leverkusen, Germany).

A midline abdominal incision was used. The small intestine was reflected to the left, and the superior mesenteric artery was exposed. The superior mesenteric artery (not the superior mesenteric vein) was occluded using 1 microvascular clamp (Scalcan, St Paul, Minnesota). After 120 minutes, the clamp was removed. The reperfusion period was set at 60 minutes. Sham operation involved the same technique and exposure without clamping the superior mesenteric artery.

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-O2 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 continuity of intestinal lumen. Biopsies of the small intestine were taken at 20, 40, 60, 80, and 100 cm from the duodenal end. Venous blood samples were taken at the same time points for d-lactate determination. At the end of the experiment, the animals were sacrificed by exsanguinations.

Statistical Analysis

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

Determination of Malondialdehyde in Liver Tissue

The determination of malondialdehyde (MDA) was carried out by a selective third-order derivative method. In brief, a 2-g sample was thoroughly homogenized (Polytron homogenizer; PCU, Bioblock, Kinematica, Lucerne, Switzerland) with 5 mL of 5% aqueous trichloroacetic acid (Merck, Darmstadt, Germany); 2 mL of 0.8% butylated hydroxytoluene (Sigma, St Louis, Missouri) in hexane (Merck; Darmstadt, Germany) was added. The solution was centrifuged. The top layer was discarded and a 2.5-mL aliquot from the bottom layer was mixed with 1.5 mL of 0.8% aqueous 2-thiobarbituric acid (Sigma) and then further incubated at 70°C for 30 minutes. Following incubation, the mixture was cooled to room temperature and submitted to conventional spectrophotometry (model UV-160A; Shimadzu, Tokyo, Japan) in the range of 400 to 650 nm, with a scanning speed of 480 nm/min. The third-order derivative spectra were obtained by electronic differentiation (derivative difference setting, 21 nm) of the conventional absorption spectra of samples from both control and PFC-O2-treated rabbits.

The MDA concentration (nmol/g wet tissue) was calculated on the basis of the third-order derivative peak height at 532 nm by referring to slope and intercept data of the computed least-square fit of a standard calibration curve prepared using 1,1,3,3-tetraethoxypropane. Butylated hydroxytoluene, 2-thiobarbituric acid, and 1,1,3,3-tetraethoxypropane were obtained from Sigma Chemical Co whereas trichloroacetic acid was obtained from Merck.

Histopathologic Evaluation

The intestinal tissue samples were prepared in Bouin solution for 2 hours and in 10% formaldehyde solution for 24 hours consecutively. Histopathologic evaluations were performed by light microscopy with a magnification power of ×100 and ×400 after preparing the specimens with hematoxylin-eosin stain. Each specimen was evaluated by a pathologist (blind for the 4 groups) to grade the mucosal lesion using the classification of Chiu et al. In summary, a score varying from 0 to 5 was attributed to each slide to express the crescent severity of the ischemic mucosal injury. Grade 0 indicated normal mucosa and grade 1 indicated the development of a subepithelial space at
Table 1. Mean Values ± SD of Malondialdehyde

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (minute)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.79 ± 0.97</td>
<td>2.60 ± 0.58</td>
<td>1.54 ± 0.85</td>
<td>2.12 ± 0.62</td>
<td>P = NS</td>
</tr>
<tr>
<td>60</td>
<td>1.89 ± 1.05</td>
<td>2.90 ± 0.87</td>
<td>1.29 ± 0.96</td>
<td>2.55 ± 0.81</td>
<td>P = NS</td>
</tr>
<tr>
<td>120</td>
<td>2.25 ± 0.65</td>
<td>4.20 ± 0.32</td>
<td>1.14 ± 0.37</td>
<td>3.97 ± 0.58</td>
<td>P = 0.006</td>
</tr>
<tr>
<td>150</td>
<td>2.78 ± 1.48</td>
<td>5.79 ± 1.32</td>
<td>1.03 ± 0.30</td>
<td>2.68 ± 0.36</td>
<td>P = 0.003</td>
</tr>
<tr>
<td>180</td>
<td>3.78 ± 1.67</td>
<td>5.48 ± 2.01</td>
<td>0.59 ± 0.35</td>
<td>2.32 ± 0.37</td>
<td>P = 0.002</td>
</tr>
<tr>
<td></td>
<td>P = .017</td>
<td>P = NS</td>
<td>P = NS</td>
<td>P = 0.009</td>
<td></td>
</tr>
</tbody>
</table>

a The hellenic letters refer to the statistical significant difference in the same time point among the different groups while the numbers refer to the statistical significant difference in the same group.

α denotes statistical significance when compared to group A.
β denotes statistical significance when compared to group B.
γ denotes statistical significance when compared to group C.
δ denotes statistical significance when compared to group D.
ε denotes statistical significance when compared to group E.

Lactate Determination

Lactate was determined using the lactate oxidase—peroxidase—chromogen sequence. The hydrogen peroxide formed was assayed according to the Trinder type reaction.16 The intensity of the measured coloration (quinoneimine) is proportional to the quantity of lactate present in the sample. It was assayed by enzymatic spectrophotometric method at 505 nm.17

Results

Malondialdehyde in Enteric Tissue

At each time point, tissue MDA levels significantly increased in I/R group animals (B) compared to sham-operated group (A). Treatment with PFCs in group C resulted in a significant reduction in MDA values during the reperfusion period (1.54 ± at 0 minute vs 0.59 ± 0.35 at 180 minutes), whereas in group D there was a slight increase at the end of the reperfusion period (2.12 ± 0.62 at 0 minute vs 2.32 ± 0.37 at 180 minutes). Statistical significance, wherever observed, is depicted in Table 1 and Figure 1.

D-Lactate Levels

The mean d-lactate levels increased significantly in I/R group (B) at the end of the reperfusion period (89.9 ± 9.29 at 180 minutes vs 61.23 ± 11.03 at 0 minute) compared to sham group (A). d-lactate values in groups C and D, treated with PFCs, exhibited a lower increase compared to I/R group (group C: 51.05 ± 10.36 at 0 minute vs 57.20 ± 11.19 at 180 minutes, and group D: 64.36 ± 5.26 at 0 minutes vs 77.02 ± 9.41 at 180 minutes). Statistical significance, wherever observed, is depicted in Table 2 and Figure 2.

Light Microscopy

No mucosal damage was detected in any of the group A animals (Figure 3). However, a high degree of epithelial cells sloughing from the villi tips (up to 100%) till the middle of the crypts was observed in group B animals. In addition, there was villi flattening and intense neutrophil infiltration of the submucosa. These injuries occurred mainly at 120 minutes, 150 minutes, and 180 minutes and corresponded to a 4 and 5 degree of injury (Figures 4 and 5). In group C animals, the mucosal damage was considerably decreased. Partial epithelial cell sloughing from the villi tips was observed (50% to 70%). Enteric glands remain unattached (Figures 6 and 7). These injuries correspond to a 2 and 3 degree of injury. Finally, in group D animals, the intestinal mucosa injuries were similar to those of group C (2 and 3 degree; Figures 8 and 9). The quantification of the mucosal injuries described above, stratified by group and by time point, is depicted in Table 3 and Figure 10.
Table 2. Mean Values ± SD of d-Lactate

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>36.45±1.99</td>
<td>61.23±2.34</td>
<td>11.03±2.55</td>
<td>64.36±3.45</td>
</tr>
<tr>
<td>60</td>
<td>37.91±2.20</td>
<td>66.77±1.34</td>
<td>10.35±2.00</td>
<td>67.02±1.77</td>
</tr>
<tr>
<td>120</td>
<td>39.1±2.37</td>
<td>74.84±2.95</td>
<td>10.70±2.45</td>
<td>72.55±1.71</td>
</tr>
<tr>
<td>150</td>
<td>39.74±2.55</td>
<td>83.04±1.35</td>
<td>10.67±2.00</td>
<td>75.75±1.55</td>
</tr>
<tr>
<td>180</td>
<td>40.05±2.13</td>
<td>89.9±2.34</td>
<td>9.29±2.20</td>
<td>77.02±1.41</td>
</tr>
</tbody>
</table>

*The hellenic letters refer to the statistical significant difference in the same time point among the different groups while the numbers refer to the statistical significant difference in the same group.

α denotes statistical significance when compared to group A.
β denotes statistical significance when compared to group B.
γ denotes statistical significance when compared to group C.
δ denotes statistical significance when compared to group D.
ε denotes statistical significance when compared to group E.

Discussion

The events that follow I/R injury are complex and not fully understood. Mechanisms differ according to the length of ischemia, the specific tissue involved, and the species studied.

Oxygen-free radicals (OFRs), mainly derived from xanthine dehydrogenase/xanthine oxidase system, are the primary etiological agents of intestinal I/R injury. Reperfusion significantly exacerbates ischemia-induced mucosal injury via the formation of OFRs, such as superoxide anion, hydroxyl radical, hydrogen peroxide, and peroxynitrite. Oxygen free radicals cause biological damage by stimulating the free chain reaction, known as lipid peroxidation, during which the superoxide attacks the fatty acid side chains of the membrane phospholipids and causes organelle and cell death. This reperfusion damage frequently exceeds the original ischemic insult. The small intestine is sensitive to ischemic insult, which can cause intestinal mucosal barrier dysfunction, resulting in bacterial translocation.

In an attempt to improve survival after acute mesenteric I/R injury, a number of experimental studies have been conducted. Several pharmacological agents that might attenuate reperfusion injury of the intestinal mucosa have been tested.

However, these ameliorative strategies, including ischemic preconditioning, antioxidants, free radical scavengers, nitric oxide supplementation, anticomplement therapy, antileukocyte therapy, glutamine supplementation, and glycine supplementation, are still inadequate.

Previous publications examine the impact of the above-mentioned agents on I/R injury, when administered to participants at different time points. In our study, with choice of 120 minutes of ischemia and 60 minutes of reperfusion, we tried to simulate the real clinical conditions. Probably, more investigations are necessary to clarify whether this period of I/R is suitable.
Fluorinated molecules such as PFCs and their derivatives represent a very interesting and stimulating class of chemicals in the physical chemistry and polymer science due to their specific and unusual properties. 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 are chemical and biochemical inert. The chemical structure and the weak intermolecular interactions are responsible for the specific properties of PFCs namely the low surface tensions (<20 mN m⁻¹), dielectric constants and refractive indices, the high densities, viscosities, and gas solubilities that are the largest known for liquids. The solubility of such gases is related to the molecular volume of the gas, which occupies the intermolecular spaces of the PFCs and depends on the partial gas pressure (i.e., Pₒ₂). The O₂ solubility in PFCs is 40% to 45%, which is 20 times greater than that of blood plasma under identical conditions. In intestinal lumen environment (very low Pₒ₂ levels), the PFCs release oxygen progressively and pass by diffusion through the membranes in the intracellular compartment. It has been shown that intraluminal administration of oxygenated PFCs preserve mucosal integrity and function. Delivery of oxygen to the ischemic intestine before irreversible changes take place is the critical point in this pathway. Therefore, no additional injury is incurred at the reperfusion, if the ischemic tissue has been exposed to oxygen.¹²

D-lactate is a marker of mesenteric ischemia. It is produced by bacteria indigenous to the gastrointestinal tract, including Escherichia coli, Lactobacillus sp, Klebsiella, and Bacteroides

Figure 4. Intestinal villi of group B at 120 minutes. Flattening of the villi and infiltration by neutrophils of the lamina propria was observed (grade 4; EO × 5).

Figure 5. Intestinal villi of ischemia-reperfusion (I/R) group at 180 minutes. Note the ulcers and the injuries of lamina propria and crypts (grade 5; EO, × 5).

Figure 6. Intestinal villi of group C at 120 minutes. Note the partial epithelial cell sloughing from the tips of the villi (grade 2; EO, × 5).

Figure 7. Development of Gruenhagen space at the tip of a villus in group C rabbits at 180 minutes (grade 3; EO, × 5).
sp. As mesenteric ischemia causes mucosal injury and subsequently bacterial proliferation, low serum levels of D-lactate increase due to the products of bacterial metabolism that enter the circulation. Mammals do not have the enzyme system required to metabolize D-lactate. This substance is not degraded by the liver and therefore enters the peripheral circulation early. D-lactate levels could be used as a marker of intestinal injury. In an experimental model of acute I/R, Günel et al. observed that D-lactate levels elevated significantly compared with animals in sham-operated group.

The increased serum D-lactate levels in the animals subjected to I/R without prior treatment compared with the sham operation group were statistically significant (P < .001). Administration of PFCs prior to I/R caused a statistically significant decrease (P < .001) in the D-lactate values (group C).

Compared with I/R group, the administration of PFCs after the onset of ischemia also resulted in a significant reduction of D-lactate levels although the values were still higher than the base values recorded in the sham operation group. The reason D-lactate levels were not significantly elevated in groups C and D, especially after reperfusion is that PFCs have a protective role scavenging oxygen-free radicals from ischemic tissue. Malondialdehyde is a hydrophilic final and stable product of lipid peroxidation and is derived from the disruption of super acids of the polyunsaturated fatty acids (PUFAs). It is a sensitive indicator of I/R injury in the intestinal tissue. Our study shows that in groups C and D, where PFCs were administered, the mean values of MDA are considerably decreased in comparison with group B, of only I/R (P < .05). Between the groups C and D (PFCs groups), the MDA values are lower in group C, where PFCs were administered 30 minutes prior to mesenteric ischemia.
ischemia. The comparison between the groups A and D showed no statistically significant difference. We did not find any work in the bibliography that evaluates the I/R injuries of the small intestine following intraluminal administration of oxygenated PFCs by measuring MDA.

Light microscopy revealed that intestinal histological alterations remain minimal after 120 minutes of ischemia and 60 minutes of reperfusion, when intraluminal oxygenated PFCs were used. The administration of PFCs resulted in the reduction in the scale of mucosal damage, according to Chiu’s classification.\(^{15}\) The major amelioration was observed in group C, where the mucosal injury remained stable during reperfusion. In addition, minor amelioration was observed in group D in comparison with group C. This observation was even more prominent when group D was compared with group B (no PFCs). These results support the findings of O’Donnell et al\(^9\) who reported that oxygenated PFCs maintain mucosal integrity throughout I/R and have anti-inflammatory effects.

In summary, better results were observed in group C, where the delivery of oxygen by PFCs occurred prior to the ischemic insult. These findings suggest that the early provision of oxygen to the ischemic tissue, before irreversible changes take place reduces I/R injuries. Moreover, the protective effect of PFCs is more prominent in group D. The experimental model of group D is closer to clinical scenarios (i.e., patient with acute mesenteric ischemia presenting to the emergency department).

If the findings of this study are verified by other investigators, a trial for the application of PFCs in a clinical setting of intestinal ischemia needs to be designed and conducted.

**Declaration of Conflicting Interests**

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

**Funding**

The whole project was funded by Aristotle University of Thessaloniki and there was no pharmaceutical industry funding.

**References**


