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<td>著者 (Author(s))</td>
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<tr>
<td>掲載誌・巻号・ページ (Citation)</td>
<td>The Kobe journal of the medical sciences, 49(1/2):17-24</td>
</tr>
<tr>
<td>刊行日 (Issue date)</td>
<td>2003-03</td>
</tr>
<tr>
<td>資源タイプ (Resource Type)</td>
<td>Departmental Bulletin Paper / 紀要論文</td>
</tr>
<tr>
<td>版区分 (Resource Version)</td>
<td>publisher</td>
</tr>
<tr>
<td>権利 (Rights)</td>
<td></td>
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<tr>
<td>DOI</td>
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</tr>
<tr>
<td>JaLCDOI</td>
<td>10.24546/00332872</td>
</tr>
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<td>URL</td>
<td><a href="http://www.lib.kobe-u.ac.jp/handle_kernel/00332872">http://www.lib.kobe-u.ac.jp/handle_kernel/00332872</a></td>
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PDF issue: 2019-01-04
Intraperitoneal Administration of Oxygenated Perfluorochemical Inhibits Bacterial Translocation Associated with Severe Acute Pancreatitis

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Received 22 January 2003/ Accepted 6 March 2003

Key words: perfluorochemical; severe acute pancreatitis; bacterial translocation; apoptosis; intestinal mucosa

Bacterial translocation from gut has been assumed to be an infectious source in severe acute pancreatitis. The purpose of this study was to test the effect of intraperitoneal administration of oxygenated perfluorochemical on bacterial translocation associated with rat experimental acute necrotizing pancreatitis. Severe necrotizing pancreatitis was induced by retrograde injection of 3% sodium deoxycholate into the biliopancreatic ducts of male Wistar rats. Although mortality rate was not improved by the treatment, intraperitoneal administration of oxygenated perfluorochemical, perfulorodecalin reduced incidence of bacterial translocation to the mesenteric lymph nodes from 60% to 37% 12 hours after development of pancreatitis, and significantly reduced number of bacterial colonies detected after 24 hours. The treatment did not alter the villous height and crypt depth of the ileum. In this model for pancreatitis, however, accelerated apoptosis of the intestinal epithelium was detected histochemically by TUNEL staining and biochemically by DNA fragmentation ELISA, and the apoptotic changes were significantly suppressed by the treatment. These results indicate that intraperitoneal administration of oxygenated perfluorochemical inhibits apoptosis of intestinal epithelium and bacterial translocation induced in severe acute pancreatitis.

Although acute pancreatitis is characterized by wide range of severity, mortality rate in severe acute pancreatitis is still high. Particularly, sepsis due to infection of pancreatic or peripancreatic devitalized tissues has been resistant against surgical interventions, such as necrosectomy with open or closed drainage, and has been major cause of death in this disease.

Concerning mechanism of infection in pancreatitis, bacterial translocation (BT) from gut is thought to be a major source of infection (11) (4) (7) (13). Although actual route of migration of microorganisms from the intestinal lumen to the pancreatic and/or peripancreatic sequestrum is not clearly understood, it has been reported that the increased intestinal permeability is involved in BT associated with severe acute pancreatitis (3) (5). In addition, ischemic change of the gut also has been reported to play an important role for the development of BT in this disease (3).

On the other hand, acute intestinal ischemia itself is well known to be critical, and experimental investigation of ischemic injury of the intestinal mucosa has been performed to study pathophysiology of necrotizing enterocolitis or non-occlusive mesenteric ischemia.
Perfluorochemicals (PFCs) are biologically inert liquids in which oxygen is remarkably soluble, and can dissolve 40 percent or more oxygen by volume under hyperoxygenation. They have the ability to bind oxygen reversibly, and their usefulness as oxygen carrier to tissues or organs has been demonstrated without apparent biological toxicity. Thereafter, several researchers attempted to treat or protect intestinal mucosa from ischemic damages by direct oxygenation of intestine with oxygenated PFCs, and reported that intraluminal or intraperitoneal administration of oxygenated PFCs preserved the integrity of gastrointestinal mucosa, and ameliorated local and systemic impairments and inhibited BT (1) (10) (9) (8).

The purpose of this experimental investigation is to test the effect of intraperitoneal administration of an oxygenated perfluorochemical, perfluorodecalin, on BT associated with severe acute pancreatitis. Moreover, we explore the impact of the oxygenated PFC on intestinal epithelium in acute pancreatitis.

MATERIALS AND METHODS

Materials and chemicals.
Perfluorodecalin was purchased from F2 Chemicals Ltd. (London, U.K.). Sodium deoxycholate was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ApopTag™ Plus and Cell Death Detection ELISA PLUS were from Oncor Inc, (Gaithersburg, MD, USA) and Roche Diagnostics GmbH (Mannheim, Germany), respectively.

Animals.
Adult male Wistar rats (300-350 g) were purchased from Charles River Japan Inc. (Yokohama, Japan) and were housed under constant temperature. The rats were fed commercial rodent food and give water ad libitum more than 3 days at the shortest after purchase. The protocol for this animal study was approved by the Institutional Animal Committee of Kobe University Graduate School of Medicine.

Development of acute necrotizing pancreatitis in rats.
Under anesthesia with diethyl ether, a midline laparotomy was performed, and the biliopancreatic duct was ligated at the orifice to the duodenum in a sterile condition. Then, the biliopancreatic duct was cannulated with polyethylene tubing (PE-10, Becton Dickinson Co., Sparks, MD, USA), and 100µl of 3% (wt/vol) sodium deoxycholate dissolved in saline was injected retrogradely under low pressure with the temporary clamp of common bile duct at the hilus of the liver. Ten ml of sterile saline was injected subcutaneously immediately after the operation in all animals.

Experimental design.
Rats were randomly divided into four groups. Immediately after induction of acute pancreatitis, 10 ml of perfluorodecalin, which was oxygenated by the O2 bubbling method over 20 minutes, was injected into abdominal cavity (PFC-O2 (+) group).

We made two models for controls. After induction of acute pancreatitis, 10 ml of non-oxygenated perfluorodecalin was administered into abdominal cavity (PFC-O2(–) group). In addition, sham operation rats underwent the same procedure except for the induction of acute pancreatitis and administration of perfluorodecalin (sham group). In severe acute pancreatitis model, the rats received no treatment (SAP group).

Animals were killed after 8 hours, and full-thickness specimens of the terminal ileum were harvested for analyses of morphological alterations and apoptotic changes. Twelve and 24 hours after induction of pancreatitis, mesenteric lymph nodes (MLNs) were harvested from survived animals to determine incidence of infection.
Bacterial cultivation of the mesenteric lymph nodes.

MLNs were harvested under sterile conditions. The MLNs were processed for culture of aerobic and anaerobic organisms using a standardized method. Immediately after harvest, the MLNs were put into anaerobic chambers. Homogenized specimens were inoculated onto agar plates including BTB agar, sheep blood agar, chocolate agar (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan), brucella HK agar (Kyokuto Pharmaceutical Co., Tokyo, Japan) and GAM (Gifu Anaerobic Medium) agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). BTB agar was incubated in the aerobic chamber at 37 °C, sheep blood agar and chocolate agar were incubated in the O2/CO2 incubator and brucella HK agar was incubated anaerobic chamber for 48 hours, respectively. GAM agar was incubated in the ambient chamber at 37 °C for 72 hours. Blind visual inspection was performed. We defined bacterial colony forming as “0, ±, 1+, 2+, 3+” as follows. Colony forming over one third of the agar plate was defined “1+”, over half of the plate was defined “2+”, and over total area was defined “3+”.

Pathologic measurement of villous height and crypt depth.

Histological examination of the terminal ileum was conducted by reviewing specimens submitted for hematoxylin-eosin staining. Villous height and crypt depth were measured at three different sites of each rat. The measurement was performed in three animals in each group.

Histochemical detection of apoptotic cells by the TUNEL method.

In situ nick-end labeling technique in order to detect apoptotic cell was performed for detection of apoptosis using ApopTag™ Plus as described on manufacture’s protocol. Specimens from the ileum was fixed with 10% neutral-buffered formalin over 12 hours at room temperature, embedded in paraffin, and 4-µm-thick section were made. The paraffin sections were deparaffinized and then proteins were digested by incubation with 20 g/ml of proteinase K for 15 minutes at room temperature. The slides were washed in distilled water for 2 minutes 4 times, and covered with 2% H2O2 in PBS for 5 minutes at room temperature to inactivate endogenous peroxidase. The slides were rinsed twice with PBS, and immersed in terminal deoxynucleotidyl transferase (TdT) containing buffer (30 mM Triazma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 15 minutes to prepare digoxigenin-binding sites. An anti-digoxigenin antibody fragment carried a conjugated reporter enzyme (peroxidase) to the reaction sites. Then, localized peroxidase generated an intense signal from the chromogenic substrate, diaminobenzidine. The counterstaining was made by eosin. To compare the number of epithelial apoptotic cells, blind analysis was carried out. To avoid potential error in statistical sampling, fields were randomly selected.

Determination of DNA fragmentation of intestinal mucosa by the ELISA.

Cell Death Detection ELISAPLUS was applied to quantification of fragmented DNA in intestinal mucosa as described on the manufacture’s protocol with some modifications. The ileum was incised longitudinally, contents were eliminated, serosal and muscular layers were gently removed and the Peyer’s patches were excluded, then each 250mg of tissue samples were homogenized with 1 ml of perfusion buffer (50 mM NaH2PO4, 120 mM NaCl, 10 mM EDTA). The homogenate was centrifuged at 13,000 g for 20 minutes and the supernatant was diluted 50 times with perfusion buffer. Then, 20 µl of dilution and 80 µl of a mixture of anti-histone-biotin and anti-DNA-peroxidase was placed onto each streptavidin-coated microtiter plate well and incubated for 2 hours. After removing the unbounded antibodies by the washing step, the amount of nucleosomes was quantified by the peroxidase retained in the immunocomplex. Retained
peroxidase activity was determined with 2,2’-Azino-di[3-ethylbenzthiazolin-sulfonate] as substrate photometrically at 405 nm with reference wave length at 490 nm.

**Statistical analysis**

All results are expressed as mean ± SEM. Statistical analyses were performed by $\chi^2$ square test, unpaired Student t test or Mann-Whitney U-test. A probability value of less than 0.05 was considered statistically significant.

Fig. 1. Bacterial culture of MLNs collected 24 hours after induction of pancreatitis. Ten ml of oxygenated PFC was administered intraperitoneally immediately after induction of pancreatitis in PFC-O$_2$ (+) group. Colony forming over one third of the agar plate was defined “1+”, over half of the plate was defined “2+”, and over total area was defined “3+”. Minimum amount of colony forming was expressed “±”. Degree of BT is different between these two groups ($p<0.05$ : Mann-Whitney U test).

Fig. 2. TUNEL stain of the terminal ileum. The terminal ileum was harvested 8 hours after induction of pancreatitis, and DNA fragmentation was examined histochemically by TUNEL method as described under “MATERIALS AND METHODS”. The cells with stained nuclei represent apoptotic cells. Sham operation group (A), SAP group (B), and PFC-O$_2$ (+) group (C) were counter stained with eosin. Magnification, ×400.
RESULTS

The mortality rate of experimental models.

All animals with sham operation survived through the periods of experiment. The mortality rate in SAP group was 2% (1/45) and 49% (19/39) at 8 and 12 hours after induction of pancreatitis, respectively. Administration of oxygenated or non-oxygenated perfluorodecalin did not decrease the mortality rate of severe acute pancreatitis. The mortality rate was 5% (2/42) and 38% (15/39) in PFC-O₂ (+) group, 22% (2/9) and 38% (15/39) in PFC-O₂ (–) group at 8 and 12 hours after operation, respectively.

Bacterial culture of the mesenteric lymph nodes.

Twelve hours after operation, no sham control animals exhibited positive bacterial culture of MLNs, whereas 9 of 15 animals (60%) in SAP group developed positive culture. Intraperitoneal administration of oxygenated perfluorodecalin decreased positive culture rate to 37% (7/12) (Table I). Furthermore, bacterial culture 24 hours after operation revealed that intraperitoneal administration of oxygenated perfluorodecalin reduced number of bacterial colonies. Difference between SAP group and PFC-O₂ (+) group was statistically significant (Figure 1). However, non-oxygenated PFC did not reduce number of bacterial colonies. In all positive cultures, only aerobic bacteria were detected.

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| MLNs were harvested aseptically 12 hours after operation. The harvested MLNs were cultured as described in “Materials and Methods”.

Pathologic measurement of villous height and crypt depth of ileum.

No morphological difference was noted on ileal mucosa by routine hematoxylin-eosin staining between sham and pancreatitis models after 12 hours. The measurement of villous height and crypt depth revealed no difference between sham (438±10 µm) and SAP (444±13 µm) groups. The results from PFC-O₂ (+) group were comparable (440±12 µm).

Histochemical detection of DNA fragmentation by the TUNEL method.

Remarkable increase of apoptotic cell ratio on ileal epithelium was noted in the pancreatitis model (30.7±3.5%) compared with the sham model (16.7±1.3%) at 12 hours after operation. The oxygenated PFC administration significantly reduced the apoptotic cell ratio (19.7±1.6%) (Figure 2). On the other hand, non-oxygenated PFC did not reduce the apoptotic ratio at all (25.7±2.3%) (Figure 3).

Detection of DNA fragmentation of intestinal mucosa by the ELISA method.

The fragmented DNA ELISA also indicated that the oxygenated PFC administration blocked apoptosis accelerated on ileal mucosa in pancreatitis models at 8 hours after operation (Figure 4). Amounts of fragmented DNA were significantly increased in mucosal epithelium of the terminal ileum in the rats with pancreatitis compared to those in the rats with sham operation. Optical density representing amount of DNA fragmentation in sham operated group was 0.237±0.029 while 0.509±0.057 in SAP group (p<0.01). Administration of the non-oxygenated PFC did not decrease fragmented DNA collected from ileal mucosa (0.524±0.070). In contrast, the oxygenated PFC significantly decrease fragmented DNA on ileal mucosa (0.348±0.041) compared with SAP models (p<0.05) (Figure 4).
Fig. 3. Apoptotic index of ileal epithelial cells analyzed by TUNEL method. The terminal ileum was harvested 8 hours after induction of pancreatitis, and DNA fragmentation was examined histochemically by TUNEL method as described under “MATERIALS AND METHODS”. Apoptotic index was calculated as a ratio of number of TUNEL-positive cells to number of total cells. *, significantly different from sham operation group (p<0.05). **, significantly different from SAP group (p<0.05) (unpaired Student t test).

Fig. 4. ELISA of DNA fragmentation in the ileal mucosa. The terminal ileum was harvested 8 hours after induction of pancreatitis, and fragmented DNA associated with mucosal layer was quantified using ELISA as described in “MATERIALS AND METHODS”. *, significantly different from sham operation group (p<0.05). **, significantly different from SAP group (p<0.05) (unpaired Student t test).
OXYGENATED PERFLUOROCHEMICAL AND PANCREATITIS

DISCUSSION

We have demonstrated that protective effect of intraperitoneal administration of oxygenated PFC on BT with rat experimental acute pancreatitis. Moreover, the PFC administration inhibited acceleration of apoptosis on the ileal intestinal epithelial cells in the same condition. There are issues to be discussed from the results obtained as follows.

The first issue is the mechanism of inhibitory effect of oxygenated PFC on BT associated with pancreatitis. As demonstrated in “Results”, non-oxygenated PFC itself did not reduce frequency of BT to MLNs 12 hours after induction of pancreatitis, and did not alter apoptosis induced after 8 hours. These findings indicate that oxygenation itself has beneficial effect(s). Previous papers reporting beneficial effects of PFC on intestinal ischemia demonstrated evident morphological preservation of intestinal mucosa from ischemic injury (1) (10) (9) (8). In the present study, however, we could not detect apparent morphological alterations including villous height 12 hours after induction of pancreatitis. Wong et al. have recently reported that apoptosis is accelerated on ileal intestinal epithelial cells in rat with necrotizing pancreatitis 12 hours after induction of pancreatitis (14). The authors assumed that the accelerated apoptosis of intestinal epithelium contributes to the damage in intestinal barrier integrity. We also have found accelerated apoptosis in the epithelium of the terminal ileum 8 hours after induction of pancreatitis, and it was inhibited by oxygenated PFC administration. Accordingly, we can assume that the intraperitoneal administration of oxygenated PFC preserves intestinal barrier function by suppressing apoptosis accelerated in severe acute pancreatitis.

Secondly, we should consider the optimal route of PFC administration. Among the literatures reporting beneficial effects of oxygenated PFCs on intestinal injuries after ischemia or ischemia/reperfusion, most investigators adopted the intraluminal pathway except Ohara et al. who adopted peritoneal lavage with oxygenated PFC (1) (10) (9) (8). If the target of the therapy is mucosal epithelium of the intestine, intraluminal administration might be ideal approach. On the other hand, it seems important to deliver oxygenated PFC to distal intestines in order to inhibit BT because terminal ileum and colon are reported to be major sources of translocated bacteria in severe acute pancreatitis (12). However, the delivery of oxygenated PFC to distal intestines seems to be difficult by intraluminal approach in acute pancreatitis, because intestinal motility is known to be impaired in acute pancreatitis (6). Thus, we selected the intraperitoneal approach in this study. Considering that restoration of intestinal motility reduced incidence of BT in acute pancreatitis (2), the intraluminal administration of both an agent restoring intestinal motility and oxygenated PFC might be theoretically ideal.

In conclusion, intraperitoneal administration of oxygenated PFC inhibits acceleration of apoptotic change of intestinal mucosal epithelium, and BT associated with severe acute pancreatitis. Although further investigation is required to explore an optimal mode for the administration of PFC, preservation of intestinal integrity using oxygenated PFC might be promising approach to prevention from serious infection associated with severe acute pancreatitis.

REFERENCES


