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A Model of Cecal Ligation and Puncture Mimicking Perforated Diverticulitis with Purulent Peritonitis

*David Wallin[1,2], David Bock[1], Eva Angenete[1,2], Lena Öhman[3], Magnusson MM[3] and Eva Haglind[1,2]**

[1]Department of Surgery, Institute of Clinical Sciences, Sweden

[2]Department of Surgery, Sahlgrenska University Hospital, Sweden

[3]Department of Microbiology and Immunology, Institute of Biomedicine, Sahlgrenska Academy at University of Gothenburg, Sweden

Abstract

Background: Perforated diverticulitis with purulent peritonitis is an uncommon but serious clinical condition requiring emergency surgery. During the last decade laparoscopic lavage has evolved as a new treatment which seems clinically safe. However the mechanisms through which lavage works are unknown and thus improvements of the modality is difficult. An experimental model is needed for clarifying mechanisms and for optimizing the outcome of the lavage treatment.

Materials: Sprague-Dawley rats, both sexes, were used. After anesthesia Cecal Ligation and Puncture (CLP) was used and puncture size and time was varied. Samples from abdominal fluid and cecal wall were taken at the end of experiments. Determinations of proteins in abdominal fluid were performed using proximity extension immunoassay and the mouse exploratory panel. Expression of RNA in cecal wall was determined using a PCR Array for Rat Inflammatory Response & Autoimmunity.

Results: Puncture through the cecum by 1.2 needle and leaving the animal for 8 h produced macroscopic signs of peritonitis in 8/8 animals. The pattern of inflammatory proteins in abdominal fluid showed a clear separation between CLP and control animals with high values for proteins such as IL1β, IL1α, Cxcl1, TNFα and IL-17A in CLP animals but not in controls. RNA expression in cecal wall tended to cluster separately in CLP and control animals.

Conclusion: Cecal ligation and puncture mimicked the clinical condition of perforated diverticulitis with purulent peritonitis. This model can be used to clarify effect or mechanisms triggered by laparoscopic lavage.

Keywords: Rats; Cecal ligation and puncture; Peritonitis; Abdominal fluid; Protein analyses; RNA expression

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Correspondence:
Eva Haglind, Department of Surgery, Sahlgrenska University Hospital/Östra, SE- 416 85 Gothenburg, Sweden, Tel: +46705349088;
E-mail: eva.haglind@vgregion.se

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Introduction

Abdominal pain is a common cause for visits to the emergency room. Some of these patients have an inflammatory condition in the abdomen such as appendicitis or diverticulitis, both conditions with to some extent unclear pathophysiology. Most patients with appendicitis and some patients with diverticulitis require emergency surgery due to a bowel perforation. The treatment for appendicitis is to remove the appendix. Perforated diverticulitis with peritonitis was until recently routinely treated by removal of the inflamed segment of colon with formation of a stoma. In 2008 a new method for emergency treatment of patients with perforated diverticulitis and purulent peritonitis, Hinchey grade III, was reported – peritoneal lavage often called laparoscopic lavage where the abdominal cavity was rinsed with warm saline via laparoscopy until the drainage fluid was clear [1,2]. Three randomized trials and a number of meta-analyses have been carried out and in summary the method of laparoscopic lavage seems safe regarding the need for further surgery and mortality but probably resulting in more infectious complications [3-11].

Appendicitis is more common than diverticulitis, and several scoring systems including variables reflecting inflammatory responses have been presented using combinations of standard clinical signs such as fever, white blood count, neutrophilia and C-Reactive Protein (CRP) [12-14]. There are no established clinical scoring systems for diverticulitis, but several different scores to grade the inflammatory conditions in the abdomen have been suggested [15]. However, few if any studies of clinically complicated diverticulitis have focused on the details of the inflammatory

response systemically or locally in the abdomen.

The underlying path physiological mechanisms behind laparoscopic lavage, a relatively cheap, non-invasive and cost-efficient procedure remain unknown [16,17]. A deeper understanding of the mechanisms through which this new treatment works could enable extrapolation to other inflammatory conditions such as appendicitis or colon perforations from other causes. Due to the uncommon nature of diverticulitis with purulent peritonitis it is difficult to study this condition in patients. Hence an animal model would be preferable for studies of the pathophysiological mechanisms involved.

The standardized method Cecal Ligation and Puncture (CLP) is a common shock model in rodents used since 1980 to study sepsis and septic shock. It can vary regarding the degree of systemic reactions in the animal [18-21]. Increased amounts of inflammatory proteins have been described after CLP both systemically and locally in the abdomen [21]. To the best of our knowledge this method has never been used for the purpose of studying diverticulitis. Peritoneal fluid is continuously in exchange with plasma, containing cytokines, chemo attractants and other proteins as well as cells, including macrophages, mesothelial cells and B lymphocytes [23]. However, peritoneal response to perforations or infections in terms of changes in inflammatory proteins has not been reported from clinical situations such as diverticulitis or peritonitis. A modified CLP model could enable studies of different aspects of peritoneal lavage in a standardized setting.

The aim of this study was to develop an animal model which mimics perforated diverticulitis with purulent peritonitis.

Materials and Methods

Animals

Sprague-Dawley rats (Charles-River Laboratory, Calcao, Italy) were used, male and female, aged 8 to 10 weeks, weighing approximately 250 g.

Cecal ligation and puncture (CLP)

The animals were placed in an anesthetic box and given an induction dose of 5% Isoflurane +4 L oxygen/min. When anesthetized the animal was moved to the operating table and given a maintenance dose of 2.5% Isoflurane and 3 L oxygen/min in a mask. Through a midline incision laparotomy was performed, the cecum was identified and exteriorized. Cecum was ligated just below the ileocecal valve with a 4-0 ligature. A puncture was made through the ligated part of the cecum using a needle of varying sizes resulting in two holes or by use of a scalpel for a larger opening. Pressure was applied on the ligated and punctured cecum until a droplet of faeces was visible in the puncture. The cecum was returned to the abdominal cavity and the incision in the abdominal wall was closed in two layers. The administration of anesthetic gas was terminated and the animal was allowed to wake up. The duration of exposure to a perforated cecum was varied to establish time needed to develop purulent peritonitis. After exposure to perforation the animals were again anesthetized, the abdomen was re-opened and in the first experiments the amount and color of abdominal fluid and formation of adhesions were studied. In a later series, samples of abdominal fluid were taken after re-anesthetization, the cecum immediately distal to the ligated part, the peritoneal surface, and lungs and liver were collected. Materials were snap frozen in liquid nitrogen and later stored in -80ºC except for abdominal fluid which was stored on ice and then in -80°C.

Sham operation

The sham operation was performed as described above for CLP but after midline incision and laparotomy the abdominal wall was closed. The animals were followed for 6 h or 8 h and after that again anesthetized and the abdomen were re-opened.

Control

Animals were taken directly from the animal room, anaesthetized and then underwent laparotomy. A sample of abdominal fluid was taken using an auto pipette.

Analysis of proteins in peritoneal fluid

Samples of abdominal fluid were taken using an auto pipette, the fluid was immediately put into sample tubes immersed in ice and thereafter stored in -80ºC. The samples were analyzed regarding 92 proteins by the Proximity Extension Immunoassay (PEA, Olink Proteomics, Uppsala, Sweden) using the mouse exploratory panel. Proteins under the detection level in >60% of the samples, with no difference between the groups, were excluded from data analysis (n=8; Gcg, Clstn2, Fas, IL23r, Acvrl1, Csf2, Ghrl and IL5). However, proteins under the detection level in control animals but measurable in the CLP group were included in the data analysis (Ccl20, IL10, Itgb6, TNFrsf12a och IL6).

RNA analysis of cecum

A 1 cm × 1 cm sample of the non-ligated part of cecum was taken, rinsed in water to clear it from faeces, put in a sample tube and stored at -80ºC. To extract total mRNA from the tissue Nucleospin® DNA, RNA, and protein purification kit [Macherey Nagel, Düren, Germany] was used according to the manufacturer's protocol. RNA concentration and purity was measured using a Nano Drop ND-1000 spectrophotometer [Nano Drop Technologies, Wilmington, DE, USA]. The RNA was stored at-80°C.The RT2 First Strand Kit [Qiagen, Hilden, Germany] was used to prepare cDNA and mRNA expression was analyzed the RT² Profiler™ PCR Array for "Rat Inflammatory Response & Autoimmunity"[PARN-077Z, Qiagen] in a Quantstudio 12K Flex Real-Time PCR System [Applied Bio systems, Foster City, CA, USA]. One sample in the Control group did not pass the quality check and was thus excluded. Genes where >50% of the samples had a CT level above 36 or if the RNA-level could not be detected at all (Ccl1, Ccl12, Ccl3, Ccl4, Ccl5, Ccl6, Ccr1, Ccr2, Ccr3, Ccr7, Cxcl9, Cxcr4, Il10rb, Kng1, Ltb, Nfkb1) were excluded from the multivariate analysis. B2m, HPRT1 and LDHA were used as housekeeping-genes. A normalized value was calculated as 2-(CT-value for target gene)/ Mean value of CT for HK-genes) and used in the analyses.

Protein and gene expression data analyses

Principal component analyses were performed on logarithm transformed data using the SIMCA-P+ software (Umetrics, Umeå, Sweden). A volcano plot was used for identification of differentiating proteins for which mean fold-change was calculated (CLP/control). Significance (–log10 (p value <0.05), Student's t-test) *vs.* log2 (mean fold change) were plotted; only significant variables were labeled. Visualization was performed in R 4.0.3 (24) using packages ggplot2 and ggrepel.

The experiments were approved by the Swedish Board of Agriculture (Jordbruksverket Dnr 5.8.18-17137/2018) and conducted in accordance with the ARRIVE guidelines and EU Directive 2010/63/ EU for animal experiments.

Table 1a: Initial series of experiments.

Number of animals	Time (h)	Puncture size (mm)	Abdominal fluid	Adhesions	Death
1	2	0.6	no	no	no
1	2	1.2	no	no	no
1	4	0.6	no	no	no
1	6	1.2	no	no	no
1	8	1.2	purulent	no	no
1	8	1.2	purulent	no	no
1	8	5	purulent + faeces	yes	no
1	8	5	blood + faeces	yes	yes

Table 1b: Puncture size 1.2 mm and 6 h or 8 h duration.

Number of animals	Time (h)	Puncture size (mm)	Purulent abdominal fluid, n	Adhesions, n	Death, n
9	6	1.2	4/9	0	0
8	8	1.2	8/8	0	0

Table 2: Cecal ligation and puncture (CLP) or Sham operated.

Intervention	Number of animals	Time (h)	Puncture size (mm)	Purulent abdominal fluid, n	Adhesions, n	Death, n
CLP	12	8	1.2	11/12	0	0
Sham	10	8	1.2	0/12	0	0

Table 3: Cecal ligation and puncture and control experiments for protein and RNA analyses.

Intervention	Number of animals	Time (h)	Puncture size (mm)	Purulent abdominal fluid, n	Adhesions, n	Death, n
CLP	8	8	1.2	7/8	0	0
Control	6	-	-	-	0	0

Results

Optimization of time line and punctuation approach

We first determined the optimal time line and punctuation approach. Cecal Ligation and Puncture with a 0.6 mm needle did not give rise to any visible changes in the abdominal cavity after 2 or 4 h, or after 2, 4 h or 6 h using a 1.2 mm needle. However, 8 h after a 1.2 mm puncture the amounts of abdominal cloudy fluid increased indicating peritonitis (Table 1a, 1b). Larger punctuation size, 5 mm, resulted in death of the animal within 8 h. Based on the obtained results, we performed a second series of CLP experiments using 1.2 mm puncture and 8 h duration (n=12) accompanied by sham-operated animals (n=10). All in all, 11 out of 12 CLP animals developed macroscopic signs of peritonitis, whereas no animals in the sham group developed peritonitis (Table 2). All animals in both groups survived the surgery procedure and the following 8 h.

Protein analysis of abdominal fluid

A third set of experiments was performed including CLP animals and a control group comprising animals undergoing laparotomy but not CLP (Table 3). Samples from abdominal fluid from the CLP group (n=5) and the control group (n=3) were analyzed for protein content. A Principal Component Analysis (PCA) based on abdominal fluid proteins (n=79) showed a clear separation between CLP and control animals (Figure 1A). Further, a volcano plot showed that a large number of proteins such as IL1β, IL1α, Cxcl1, TNF α and IL-17A were higher in CLP animals as compared to control animals, whereas only a few variables such as Ccl2 and Tnfsf12 were found in lower abundance (Figure 1b).

RNA analysis of cecum

A Principal Component Analysis (PCA) based on RNA expression in cecal biopsies showed that CLP and control animals tended to cluster separately (Figure 1a). Four out of five analyzed CLP animals clustered together, whereas the fifth animal clustered together with the controls (Figure 2a). The gene expression of TNF, IL1A, IL1B, CXCL1 and IL-17A in CLP and control animals is displayed in Figure 2b.

Discussion

In this exploratory study we demonstrate that cecal ligation and puncture with a 1.2 mm needle and duration of trauma for 8 h give rise to a reproducible model of purulent peritonitis without mortality. In the model the abdominal fluid displayed a plethora of inflammatory proteins reflecting the protein profile in purulent peritonitis.

The use of a 1.2 mm needle to perforate the ligated cecum gave rise to a purulent peritonitis while use of a 5 mm incision gave rise to faecal peritonitis and was discarded as a possible method. Significant differences in outcome using different needle sizes to perforate the cecum has been reported earlier, although the outcome in that study was mortality and not degree of peritonitis [19].

Our results indicated that it took between 6 and 8 h to develop purulent peritonitis after cecal ligation and puncture by 1.2 mm. As peritonitis, not septic shock or death was the purpose a puncture of 1.2 mm and 8 h was suitable.

The analyses of abdominal fluid regarding protein concentrations indicated that cecal ligation and puncture with purulent peritonitis had a clear effect on the protein expression in the abdominal cavity. Proteins known to be important for an inflammatory response, such as pro-inflammatory cytokines TNFα, IL1β and IL-17A along with chemo attractants such as CXCL1 were in higher abundance in CLP animals than controls. In a murine model of peritonitis inflammatory

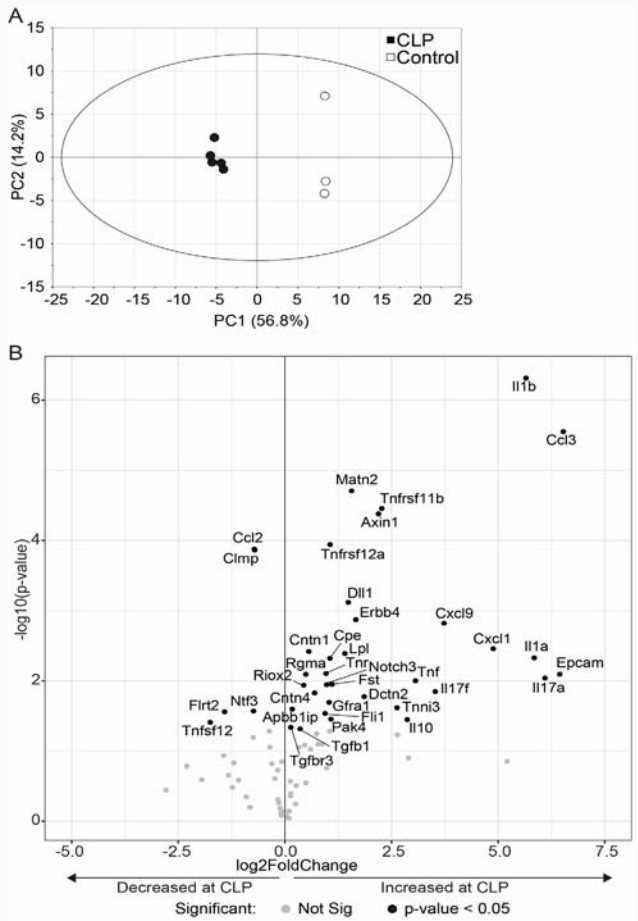

Figure 1: Protein expression in the abdominal fluid of CLP and control animals. Abdominal fluid was taken from animals 8hours after CLP using a 1.2 mm puncture (n=5) and from control animals undergoing laparotomy but not CLP (n=3). Protein content was analyzed using Proximity Extension Immunoassay. (A) Principal component analysis and (B) a volcano plot based on the expression of 92 proteins. The Volcano plot displays log2 fold change of the mean protein levels (CLP/control) versus significance (Student's t-test). Significantly altered proteins in (B) are annotated and indicated in black (p<0.05).

response through IL6 and TNFα in peritoneal fluid was found together with systemic inflammatory response [25].

The results regarding RNA expression in cecal biopsies displayed a somewhat indistinctive pattern, likely due to the limited number of samples passing the quality check. Nevertheless, our results suggest that also the inflammatory RNA profile, similar to the protein profile, differs between CLP and control animals. To our knowledge there are no earlier reports of RNA expression in animal models such as CLP or bacterial peritonitis or in patients.

Strengths of the CLP model is that it is simple and easily reproducible and that the ligated and perforated cecum mimics a perforated diverticulum including peritonitis. Hence, this model may be used to study laparoscopic lavage as a treatment of perforated diverticulitis. A limitation is that a ligated and mechanically perforated piece of bowel (cecum) is not entirely comparable to a perforation caused by an inflammation (diverticulitis). Further, results generated in a rat model may not be translated to the human setting [26]. Possibly the most important difference in the model compared with the clinical situation is that ischemic tissue is left in the rat abdomen and thus only short term effects of lavage will be possible to study.

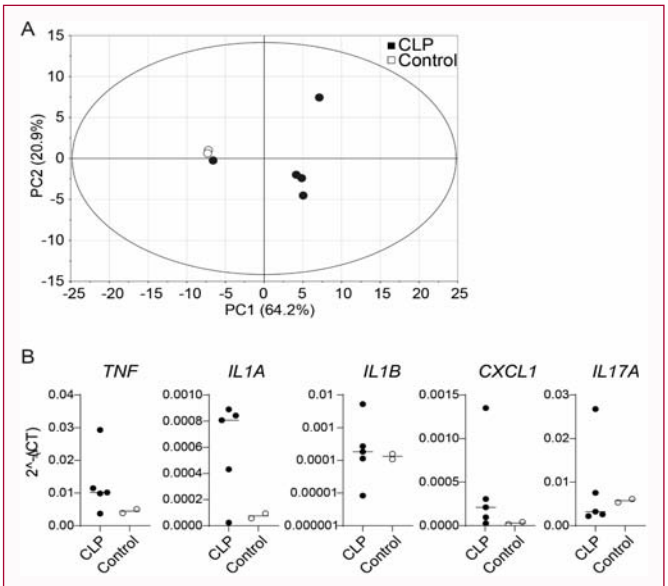

Figure 2: Cecal gene expression in CLP and control animals. Biopsies from cecum were taken from animals 8 h after CLP (n=5) using a 1.2 mm puncture and from control animals (n=2) undergoing laparotomy but not CLP. The PCR array "Rat Inflammatory Response & Autoimmunity" was used to quantify gene expression. (A) Principal component analysis based on the expression of 68 genes. (B) Gene expression of TNF, IL1A, IL1B, CXCL1 and IL17A in CLP and control animals.

Cecal ligation and puncture is a well-established model [27] that could also mimic perforated diverticulitis in terms of peritonitis. In addition to being used for the studying sepsis it has been used for the study of peritonitis and peritoneal lavage in rats [28-32].

Conclusion

In conclusion we have established a model of cecal ligation and puncture in order to mimic the clinical condition of a perforated diverticulum with purulent peritonitis. This model will be used to clarify effect or mechanisms triggered by laparoscopic lavage.

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