

BASIC RESEARCH

IN VIVO OBSERVATION OF MESENTERIC LEUKOCYTE-ENDOTHELIAL INTERACTIONS AFTER CECAL LIGATION/PUNCTURE AND SURGICAL SEPSIS SOURCE CONTROL

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Nakagawa NK; Jukemura J, Aikawa P, Nogueira RA, Poli-de-Figueiredo LF, and Sannomiya P. In vivo observation of mesenteric leukocyte-endothelial interactions after cecal ligation/puncture and surgical sepsis source control. *Clinics*. 2007;62(3):321-6.

PURPOSE: Cecal ligation and puncture (CLP) has been used as a useful model for the induction of polymicrobial sepsis. Necrotic tissue resection and peritoneal lavage (REL) are the surgical procedures for controlling perforated appendicitis. The aim of this study was to evaluate leukocyte-endothelial interactions in the rat mesentery in vivo after CLP and REL.

METHODS: Thirty-seven male Wistar rats (250-300 g) underwent laparotomy and were randomly assigned to the following groups: 1) SHAM; 2) CLP: animals submitted to CLP, 3) CLP+REL: animals submitted to CLP and REL. Mesenteric leukocyte-endothelial interactions were studied by intravital microscopy assessed once in each animal (3-5 postcapillary venules, 15-25 μ m diameter) 24 hours after intervention. Follow-up was performed in all animals; this included analysis of glycemia, lactate, hematocrit, white blood cell count as well as a functional score that was the sum of scoring on the following parameters: alertness, mobility, piloerection, diarrhea, encrusted eyes, and dirty nose and tail.

RESULTS: None of the animals showed significant changes in body weight (265 ± 20 g) or in hematocrit levels ($46\% \pm 2\%$) during the experimental protocol. Compared to SHAM animals, CLP animals showed an increased number of rolling (2x), adherent, and migrating leukocytes (7x) in the mesenteric microcirculation, an increase in blood glucose (136 ± 8 mg/dL), lactate (3.58 ± 0.94 mmol/L), white cell count ($23,570 \pm 4,991$ cells/mm³) and functional alterations (score 11 ± 1), characterized by impaired alertness and mobility, and presence of piloerection, diarrhea, encrusted eyes, and dirty nose and tail. The REL procedure normalized the number of rolling, adherent, and migrated leukocytes in the mesentery; glycemia; lactate; and white blood cell count. The REL procedure also improved the functional score (7 ± 1).

CONCLUSION: Local and systemic inflammation was induced by CLP, while REL completely overcame the inflammatory process.

KEYWORDS: Intravital microscopy. Microcirculation. Mesentery. Peritonitis. Sepsis.

INTRODUCTION

Sepsis is a syndrome involving the systemic host response to an inflammatory or infectious stimulus. It is often associated with tissue injury that may lead to multiple organ failure.¹ Sepsis is still one of the leading causes of infectious

death.²⁻⁴ The introduction of antibiotics more than 50 years ago and improvement in hemodynamic support resulted in a decline of sepsis-induced mortality.³⁻⁵ However, the successful treatment of sepsis in critical care and emergency settings remains a relevant medical challenge.

Extensive experimental and clinical data have been amassed to determine the mechanism(s) of sepsis-induced multiple organ dysfunction and failure. Laparotomy complicated by sepsis is a common clinical presentation of sepsis. The rationale for selecting cecal ligation and puncture (CLP) as a septic model is that CLP induces effects analogous to a

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perforated appendicitis.^{3,6,7} The infectious milieu promotes microbial growth or impairs host defenses, which can be quickly modified by surgical source control.^{3,5} Therefore, surgical removal of the septic focus has been applied as a key component of success in therapy of ongoing sepsis.

In animal models of sepsis, microcirculatory dysfunction is seen prior to the onset of shock.⁸⁻⁹ The host defense responses to sepsis may promote a generalized increase in leukocyte recruitment and accumulation in the tissues, which may lead to subsequent endothelial damage, leaky capillaries, and organ dysfunction and failure. Recruitment and accumulation of leukocytes in tissues involves a series of leukocyte-endothelial interactions in postcapillary venules mediated by the expression of adhesion molecules on the vascular endothelium and leukocytes. Rolling is the first step of leukocyte recruitment, mediated by E-, P-, and L-selectins. This early type of leukocyte-endothelial interaction is a prerequisite for adhesion of leukocytes to the endothelium mediated by the interaction of β_2 integrins (CD11/CD18) on leukocytes and intercellular adhesion molecule (ICAM)-1 on the endothelial cell. Leukocytes eventually transmigrate into the interstitial compartment and towards the site of injury.¹⁰⁻¹²

The aim of the current study was to evaluate in vivo leukocyte-endothelial interactions in rat mesenteric microcirculation by intravital microscopy in a septic state and after surgical source control.

MATERIALS AND METHODS

Experimental Protocol

This study was approved by our institutional Ethics Committee and performed according to National Institutes of Health Guidelines on the experimental use of animals. Thirty-seven male Wistar rats (weighing 250-300 g) were fasted overnight, allowed water ad libitum, and housed in standard-care facilities before the experiments. A 12-hour light/dark cycle with ambient temperature control was employed. The animals were randomly assigned to 3 groups: 1) SHAM: animals were submitted solely to laparotomy; 2) CLP: animals underwent cecal ligation/puncture, 3) CLP+REL: animals were submitted to cecal ligation/puncture and to necrotic cecal resection/peritoneal lavage, 24 hours apart. Mesenteric microcirculation was evaluated in 19 animals (SHAM, n = 6; CLP, n = 7; and CLP+REL, n = 6). Mortality rate was evaluated in 18 animals (SHAM, n = 6; CLP, n = 6; and CLP+REL, n = 6).

Laparotomy

All animals underwent anesthesia by means of an intraperitoneal sodium pentobarbital injection (50 mg/kg)

followed by a 2-cm abdominal midline incision. The cecum was exposed and returned to the abdomen, which was closed in 2 layers with 5.0 suture (Ethicon, NJ, USA). Animals were kept warm for 1 hour at 37°C and returned to their cages with free access to food and water.

Cecal Ligation and Puncture (CLP)

Animals underwent anesthesia by means of an intraperitoneal injection of sodium pentobarbital (50 mg/kg) followed by laparotomy. The cecum was exposed, isolated just distal to the ileocecal valve to avoid intestinal obstruction, and punctured twice with a 22-gauge needle. The punctured cecum was squeezed to expel a small amount of fecal material through the holes. The bowel was returned to the abdomen, which was closed in 2 layers with 5.0 suture (Ethicon, NJ, USA). Animals were kept warm for 1 hour at 37°C and returned to their cages with free access to food and water.

Necrotic Tissue Resection and Peritoneal Lavage (REL)

Animals were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), and the midline abdomen incision was opened. The necrotic cecum was resected, and the bowel was returned into the abdomen. Subsequently, peritoneal lavage with 40 mL of warmed isotonic saline solution (0.9% NaCl) was performed. The midline incision was closed in 2 layers with 5.0 suture (Ethicon, NJ, USA). The animals were kept warm- for 1 hour at 37°C and returned to their cages with free access to food and water.

Intravital Microscopy of the Mesentery

Intravital microscopy of the mesentery was performed once in each animal, 24 hours after intervention. Briefly, the animals were anesthetized with an intraperitoneal sodium pentobarbital injection (50 mg/kg). After an abdominal midline incision, the distal ileum and its accompanying mesentery were exposed for in vivo microscopic examination of the microcirculation. The animals were maintained on a specially designed stage warmed by circulating water kept at 37°C. The stage has a transparent platform on which the tissue to be transilluminated was placed. The mesentery was continuously perfused during the study period with a warmed (37°C) Ringer-Locke solution (154 mM NaCl, 5.6 mM KCl, 2 mM CaCl₂·2 H₂O, 6 mM NaHCO₃, 5 mM glucose, pH 7.20-7.40), saturated with a mixture of gases (95% N₂ and 5% CO₂). This procedure kept the microcirculatory characteristics unchanged throughout the intravital microscopic analysis.¹¹ After 10 minutes of stabilization, the mesenteric microcirculation was assessed in postcapillary venules (3-5 per animal, diameter 15-25 μ m). A CCD color camera

(TK-C1380U, JVC Co, Tokyo, Japan) was incorporated with a triocular microscope (Axioplan 2, Carl Zeiss Co, München-Hallbergmoos, Germany) to facilitate the observation of the enlarged image (425x) on a microcomputer monitor (SyncMaster 753DFX, Samsung, Manaus, Brazil). Analyses of leukocyte-endothelium interactions were performed on-line, by using image analysis software (Axiovision 4.1, Carl Zeiss Co, München-Hallbergmoos, Germany) with an incorporated module for interactive measurements and time-lapse analysis. Images were stored, enabling off-line playback analysis. After intravital microscopy, animals were sacrificed with an overdose of sodium pentobarbital.

Rolling Leukocytes

Rolling leukocytes were defined as white blood cells that move at a velocity significantly slower than that of erythrocytes in a given microvessel.^{11,12} The number of rolling leukocytes was presented as the mean number of cells passing a designated line perpendicular to the venular axis per 10 minutes. A given section of the vascular bed was tested only once, and 3 to 5 microvessels were selected from each animal.

Adherent Leukocytes

A leukocyte was considered to be adherent to the venular endothelium if remained stationary for >30 seconds.¹³⁻¹⁵ Adherent cells were counted during a 10 minute-period in a 100 µm segment of the vessel, with 3 to 5 microvessels being selected from each animal.

Migrated Leukocytes

The number of leukocytes accumulating in the connective tissue adjacent to the chosen postcapillary venule was determined¹⁵ in a standard area of 5,000 µm²; 3 to 5 different fields were evaluated for each microvessel, and 3 to 5 microvessels were selected from each animal.

White Blood Cell Count, Hematocrit, Glycemia, and Blood Lactate

Blood samples were collected from the rats' tails. Hematocrit was measured by using microcapillary tube centrifugation. White blood cells were counted in Neubauer chambers. Glycemia was determined with a blood glucose monitor (Advantage®, Roche Diagnostics Co., IN, USA). Blood samples were collected from the abdominal aorta for lactate measurement, using a blood gas analyzer (Radiometer ABL 555, Radiometer Medical, Copenhagen, Denmark).

Functional Score

Animals were followed up at 24-hours after interventions. Functional features were scored as follows: alertness (grade: 1, normal; 2, low attention; 3, very low attention), mobility (grade: 1, normal; 2, low motion; 3, motionless), piloerection (grade: 0, none; 1, mild piloerection; 2, moderate piloerection; 3, severe piloerection), diarrhea (grade: 0, none; 1, moderate; 2, severe diarrhea), encrusted eyes, dirty nose and tail (grade: 0, none; 1, one place; 2, two places; 3, three places; 4, four places). The sum of these scores determined the overall functional score, which ranged from 2 (normal condition) to 15 (the most severe condition).

Statistical Analysis

The data are expressed as mean ± standard deviation of the mean (SD). Comparisons among groups were performed with Kruskal-Wallis analysis, and differences were tested by Dunn's test. All statistics were calculated using a standard computer software package (SigmaStat v4.1/Jandel Scientific, Corte Madera, CA, USA). Values of *P* less than .05 were considered statistically significant.

RESULTS

Rats submitted to CLP exhibited decreased alertness and mobility, presence of piloerection, diarrhea, encrusted eyes, and dirty nose and tail, which resulted in a functional score of 11, compared to the normal condition of SHAM rats (functional score, 2; Table 1). Debris around the injured tissue and ascitis were also observed in the CLP group. In addition, CLP induced increases in glycemia (136 ± 8 mg/dL, *P* < .05), blood lactate (3.58 ± 0.94 mmol/L, *P* < .05) and total white blood cell counts ($23,571 \pm 4,991$ cells/mm³, *P* < .05) compared to SHAM (Table 2). There were no significant changes in body weight, or hematocrit. When submitted to surgical source control, CLP+REL animals showed an improvement in functional condition (score 7 ± 1). Glycemia (97 ± 9 mg/dL), and lactate (1.70 ± 0.27 mmol/L). White blood cell count ($14,850 \pm 1,459$ cells/mm³) were reduced to values attained in SHAM rats. Additional experiments performed to evaluate mortality rate showed that all CLP animals (*n* = 6) died after day 5, whereas all SHAM (*n* = 6) and CLP+REL animals (*n* = 6) survived up to day 30.

The effects of CLP and CLP+REL on leukocyte-endothelial interactions were investigated by means of intravital microscopic observation of the rat mesenteric microcirculation, as illustrated in Figure 1. Postcapillary venule diameters were similar among groups, ranging from 15 through 25 µm (*P* = .19).

Table 1 - Functional features 24 hours after intervention

	SHAM (n = 12)	CLP (n = 13)	CLP+REL (n = 12)
Alertness	1 ± 0	2 ± 1	1 ± 0
Mobility	1 ± 0	2 ± 1	1 ± 0
Piloerection	0 ± 0	2 ± 1	2 ± 1
Diarrhea	0 ± 0	2 ± 0	0 ± 0
Encrusted eyes /Dirty nose and tail	0 ± 0	3 ± 1	2 ± 1
Functional score	2 ± 0	11 ± 1*	7 ± 1

Kruskal-Wallis analysis and Dunn's test, **P* < .05 vs other groups. Functional score was the sum of the scores for the following parameters: alertness (1, normal; 2, low attention; 3, very low attention); mobility (1, normal; 2, low motion; 3, motionless); piloerection (0, none; 1, mild piloerection; 2, moderate piloerection; 3, severe piloerection); diarrhea (0, none; 1, moderate diarrhea; 2, severe diarrhea); encrusted eyes, dirty nose and tail (0, none; 1, one place; 2, two places; 3, three places; 4, four places). Functional scores ranged from 2 (normal condition) to 15 (the most severe condition).

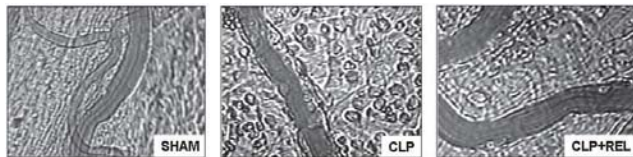


Figure 1 - Representative photomicrographs of rat mesenteric microcirculation from SHAM (SHAM-operated), CLP (cecal ligation/puncture), and CLP+REL (cecal ligation/puncture and necrotic tissue resection/peritoneal lavage) animals. Increased number of adherent and migrated leukocytes are observed in the CLP in comparison with SHAM and CLP+REL groups. Final magnification: 850x.

The number of rolling, adherent, and migrated leukocytes were markedly increased in rats submitted to CLP (*P* < .05). Rolling increased twofold, and adhesion and migration sevenfold compared with SHAM rats (Figure 2). Surgical source control reduced the number of rolling (110 ± 16 cells/10 minutes), adherent (5 ± 1 cells/100 μm venule length), and migrated leukocytes (4 ± 1 cells/5,000 μm²) to values observed in SHAM rats.

DISCUSSION

Although intra-abdominal infection has been recognized as a common disease, the mortality and morbidity for complicated cases remains high. Consequently, understanding of the pathophysiology including local, regional, and systemic host defense alterations is required to focus future efforts in the management of this disease. The current study evaluated systemic alterations after cecal ligation/puncture and surgical source control; leukocyte-endothelial interactions in mesenteric postcapillary venules - through intravital microscopy. The CLP procedure produces definite metabolic, immunologic, and physiologic alterations such as hyperglycemia; leukocytosis; increased number of rolling, adherent, and migrated leukocytes in the mesentery; decreased alertness and mobility; and presence of intense piloerection, diarrhea, en-

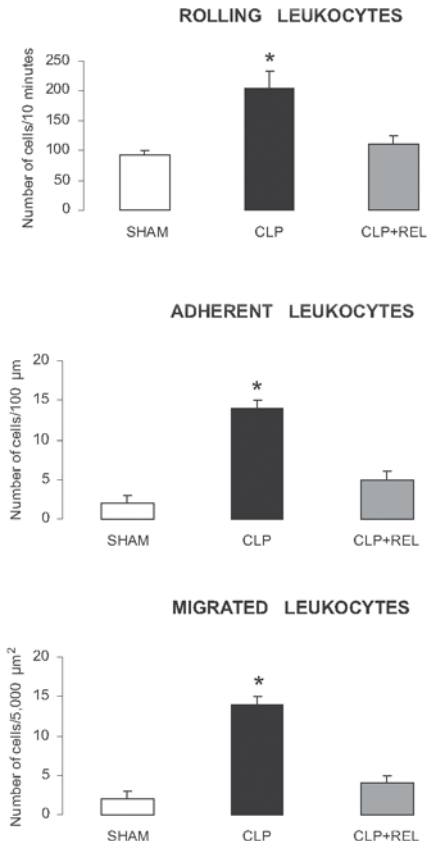


Figure 2 - Number of rolling leukocytes/10 minutes, adherent leukocytes/100 μm venule length, and migrated leukocytes/5,000 μm² in rat mesenteric microcirculation. Rats were SHAM-operated (SHAM, n = 6), submitted to cecal ligation and puncture (CLP, n = 7) and to CLP + necrotic tissue resection/peritoneal lavage (REL, n = 6). Statistical analyses were performed using Kruskal-Wallis analysis and Dunn's test and showed significant differences (**P* < .05) among CLP vs SHAM and CLP+REL groups.

crusted eyes and dirty nose and tail. Surgical source control completely overcame the inflammatory process in the mesentery by reducing leukocyte rolling, adhesion, and migration in parallel with systemic functional improvement.

Neutrophils may exert damaging effects through several mechanisms. After activation, these cells generate and release toxic oxygen metabolites, numerous proteases, and phospholipase products, all of which may result in vasomotor changes, endothelial injury, and loss of vascular integrity.¹⁶ Leukocyte-endothelial interactions are mediated by a variety of glycoproteins expressed on the surface of leukocytes and endothelial cells. The accumulation of leukocytes in inflamed tissues is preceded by leukocyte rolling and adhesion to the vascular endothelium.^{10,17} Leukocytes roll along the walls of postcapillary venules mediated by the selectin family of adhesion molecules.¹² Leukocytes become firmly adherent to the vascular wall by the interaction between ICAM-1 on endothelial cells with β₂ integrins (CD11/CD18) on leukocytes. The CLP procedure partially destroys the normal barrier of the gastrointestinal tract.¹⁸ Previous studies in rats using intravi-

Table 2 - General characteristics of study groups

	SHAM (n = 6)	CLP (n = 7)	CLP+REL (n = 6)
Body weight change/48 hours (g)	10 ± 5	-20 ± 10	10 ± 10
Glycemia (mg/dL)	101 ± 6	136 ± 8*	97 ± 9
Hematocrit (%)	46 ± 1	45 ± 1	46 ± 1
Blood lactate (mmol/L)	1.74 ± 0.48	3.58 ± 0.94*	1.70 ± 0.27
White blood cell count/mm ³	11,825 ± 1,478	23,571 ± 4,991*	14,850 ± 1,459

Kruskal-Wallis analysis and Dunn’s test, *P < .05 vs other groups. General characteristics were similar among SHAM, CLP and CLP+REL groups, except for glycemia, blood lactate, and white blood cell count, which increased in the CLP group. Data are presented as mean values ± SD.

tal microscopy have demonstrated that sepsis induces micro-circulatory derangements in the ileal mucosa¹⁹ and in other organs not directly affected by the septic process, such as the extensor digitorum longus muscle²⁰ and cremaster muscle.²¹ Normotensive sepsis induces absence of capillary flow²² and alters leukocyte-endothelium interactions.^{19,23,24} Smalley et al²⁴ found that CLP significantly increases leukocyte adherence and infiltration into the upper gastrointestinal tract 4 hours after intervention. Because any sham procedure performed in animal models of sepsis may result in a low degree of inflammation, we included a SHAM group. These animals presented results similar to those of naive animals (data not presented).

In the present study, increased number of rolling, adherent, and migrated leukocytes were observed in vivo in the mesentery of rats submitted to CLP. The increased recruitment of leukocytes to the site of injury represents a mechanism triggered by the host to produce an efficient defense against invading pathogens. However, if sepsis is not controlled, it may lead to multiple organ failure. Mortality in the CLP group was observed after the 5th day, but not in SHAM and CLP+REL animals, which survived up to the 30th day.

An adequate surgical source control of the septic focus should be used to control the immune suppressive response to the ongoing sepsis. Therapeutic strategies such as drainage of infected fluids, debridement of infected soft tissues,

and removal of necrotic tissues may play a crucial role in avoiding the development and progression of multiple organ failure.^{3,5} In the present report, REL normalized leukocyte-endothelium interactions in the mesentery as indicated by the decreasing numbers of rolling, adherent, and migrated leukocytes in comparison to CLP. Surgical source control was effective in reducing inflammation after CLP, and in improving functional parameters, which were considered to indicate the resolution of the induced sepsis.

In conclusion, the current experimental rat model allowed in vivo observations of leukocyte-endothelium interactions in the mesentery by intravital microscopy in parallel with documenting functional alterations after cecal ligation/puncture and surgical source control.

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RESUMO

Nakagawa NK; Jukemura J, Aikawa P, Nogueira RA, Polide-Figueiredo LF and Sannomiya P. Avaliação in vivo da interação leucócito-endotélio mesentérico após ligadura e punção cecal e remoção cirúrgica do foco sééptico. Clinics. 2007;62(3):321-6.

OBJETIVO: O procedimento de ligadura cecal e perfuração (CLP) tem sido usado como um modelo útil de indução de sepse polimicrobiana. A ressecção do tecido necrosado e lavagem peritoneal (REL) são procedimentos cirúrgicos freqüentemente utilizados para controlar uma

apendicite perfurada. O objetivo desse estudo foi avaliar *in vivo* as interações leucócito-endotélio no mesentério de ratos após a CLP e REL.

MÉTODOS: Trinta e sete ratos Wistar machos (250-300 g) foram submetidos à laparotomia e aleatoriamente divididos em grupos: 1) SHAM, 2) CLP: ratos submetidos à CLP, 3) CLP+REL: animais submetidos à CLP e REL. As interações leucócito-endotélio no mesentério foram estudadas através de microscopia intravital somente uma vez em cada animal (3-5 vênulas pós-capilares, 15-25 µm diâmetro), 24-horas após as intervenções. A evolução clínica foi realizada em

todos os animais, incluindo glicemia, lactato, hematócrito, número total de células brancas e um escore funcional, o qual foi considerado como a somatória dos seguintes parâmetros: estado de alerta, mobilidade, piloereção, diarreia, olhos encrustados, e nariz e cauda sujos.

RESULTADOS: Os animais não apresentaram alterações significantes no peso (265 ± 20 g) e hematócrito ($46 \pm 2\%$) ao longo do estudo. Comparados ao SHAM, os animais CLP apresentaram aumento no número de leucócitos em rolamento (2x), aderidos (7x) e migrados (7x) na microcirculação mesentérica, aumentos da glicemia (136 ± 8 mg/dL), lactato ($3,58 \pm 0,94$ mmol/L), leucocitose (23.570

± 4.991 células/mm³) e alterações clínicas (escore 11 ± 1), caracterizadas por comprometimento do estado de alerta e mobilidade, e presença de piloereção, diarreia, olhos encrustados, nariz e cauda sujos. REL normalizou o número de leucócitos em rolamento, aderidos e migrados no mesentério, a glicemia, o lactato e o número de leucócitos circulantes. REL também melhorou o escore clínico (7 ± 1). **CONCLUSÃO:** A CLP induziu inflamação local e sistêmica. A REL resolveu, por completo, o processo inflamatório.

UNITERMOS: Microcirculação. Mesentério. Microscopia intravital. Peritonite. Sepsis.

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