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Original Paper

Insulin Modulates Liver Function in a Type I Diabetes Rat Model

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Key Words
Insulin • Diabetes mellitus • CLP • Sepsis • Inflammation • Cytokines • Liver • Hepatic

Abstract
Background/Aims: Several studies have been performed to unravel the association between diabetes and increased susceptibility to infection. This study aimed to investigate the effect of insulin on the local environment after cecal ligation and puncture (CLP) in rats. ***Methods:*** Diabetic (alloxan, 42 mg/kg i.v., 10 days) and non-diabetic (control) male Wistar rats were subjected to a two-puncture CLP procedure and 6 h later, the following analyses were performed: (a) total and differential cell counts in peritoneal lavage (PeL) and bronchoalveolar lavage (BAL) fluids; (b) quantification of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10 and cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-2 in the PeL and BAL fluids by enzyme-linked immunosorbent assay (ELISA); (c) total leukocyte count using a veterinary hematology analyzer and differential leukocyte counts on stained slides; (d) biochemical parameters (urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) by colorimetric analyses); and (e) lung, kidney, and liver morphological analyses (hematoxylin and eosin staining). ***Results:*** Relative to controls, non-diabetic and diabetic CLP rats exhibited an increased in the concentration of IL-1β, IL-6, IL-10, CINC-1, and CINC-2 and total and neutrophil in the PeL fluid. Treatment of these animals with neutral protamine Hagedorn insulin (NPH, 1IU and 4IU, respectively, s.c.), 2 hours before CLP procedure, induced an increase on these cells in the PeL fluid but it did not change cytokine levels. The levels of ALT, AST, ALP, and urea were higher in diabetic CLP rats than in non-diabetic CLP rats. ALP levels were higher in diabetic sham rats than in non-diabetic sham rats. Treatment of diabetic rats with insulin completely restored ALT, AST, and ALP levels. ***Conclusion:*** These results together suggest that insulin attenuates liver dysfunction during early two-puncture CLP-induced peritoneal inflammation in diabetic rats.

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Introduction

Sepsis is a clinical condition caused by a systemic inflammatory response syndrome (SIRS) of the host to infection, which can manifest in varying degrees of severity. Septic shock and multiple organ dysfunction syndrome (MODS) are the most severe outcomes in patients who develop sepsis [1, 2]. Additionally, sepsis and septic shock are common causes of death in intensive care units (ICU), particularly affecting elderly, diabetic, immunocompromised, and seriously ill patients [3, 4]. Studies in humans have shown that diabetes mellitus (DM) causes immune dysfunction and reduces secretion of pro-inflammatory cytokines and chemokines such as interleukin (IL)-8, which may explain the susceptibility to infections observed in patients with DM [5, 6].

Several experimental studies in animal models have been performed to unravel the association between diabetes and increased susceptibility to infection. For instance, it has been demonstrated that the infiltration of neutrophils in lipopolysaccharide (LPS)-induced lung inflammation is reduced in diabetic rats compared to healthy animals, which is accompanied by reduced release/production and gene transcription of IL-1, tumor necrosis factor (TNF)-α, and IL-10 [7, 8]. In addition, we have previously shown that diabetic rats are more susceptible to severe sepsis (cecal ligation and puncture 12 times), but that the Acute lung injury (ALI) secondary to sepsis is less intense than in non-diabetics [9] and that insulin modulates the early phase of inflammation and myofibroblast differentiation in diabetic rats [10]. Thus, insulin may directly or indirectly regulate the behavior of leukocytes in inflammation and restore leukocyte function in diabetic animals [7–11]. Moreover, based on the immunomodulatory activity of insulin observed in experimental models and clinical trials, insulin therapy has been used for the treatment of critically ill patients [12]. Until 2001, hyperglycemia was poorly evaluated in non-diabetic patients in intensive care units (ICU) [13, 14]. Later, some authors showed that targeting normoglycemia (80–110 mg/dL) with insulin therapy improved outcome of ICU patients compared to tolerating hyperglycemia (glucose > 200 mg/dL) [13, 14]. However, this recommendation remained controversial, because normoglycemia *per se* does not necessarily benefit ICU patients and may have deleterious effects. In fact, maintaining serum glucose levels below 180 mg/dL resulted in lower mortality rates than between 81 and 108 mg/dL [15]. Also, other studies have shown that the benefits of insulin therapy are inconsistent and the increased risk of hypoglycemic episodes has also been extensively discussed [16, 17]. This study aimed to investigate the capacity of insulin to modulate organ functions on mild sepsis in a diabetic animal model. We hypothesize that insulin modulates MODS and plays a key role in the recovery of SIRS.

Materials and Methods

Animals

Forty-nine specific pathogen-free male Wistar rats weighing 200 ± 20 g at the beginning of the experiments were used. The animals were housed at 22°C under a 12-h light-dark cycle and were given *ad libitum* access to food and water. This study was carried out in strict accordance with the principles and guidelines of the National Council for the Control of Animal Experimentation (CONCEA) and approved by the Ethics Committee on Animal Use (CEUA) at the Faculty of Pharmaceutical Sciences (FCF), University of São Paulo, Brazil (protocol number: CEUA/FCF/337). Surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize animal suffering.

Alloxan-induced diabetes and insulin treatment

Rats were assigned to either a Diabetic group (n = 25) or a Non-diabetic (control) group (n = 24). Diabetes mellitus was induced by intravenous injection of 42 mg/kg of alloxan monohydrate (Sigma Chemical Co, St. Louis, MO, USA) dissolved in physiological saline (0.9% NaCl) [7, 8]. Control rats were injected with physiological saline only. Ten days later, induction of diabetes was verified by measuring blood glucose concentrations in samples from the cut tail tip using a blood glucose monitor (Accu-Check Advantage II,

Roche Diagnostica, São Paulo, SP, Brazil), and rats with levels above 200 mg/dL were included in the study. Diabetic and control rats received 4 IU and 1 IU, respectively, of neutral protamine Hagedorn (NPH) insulin (Eli Lilly, São Paulo, SP, Brazil) subcutaneously, 2 h before the cecal ligation and puncture (CLP) procedure. Because NPH insulin reaches the maximum serum concentration (C_{max}) between 6–8 h after subcutaneous administration, animals were given insulin 2 h before the CLP procedure, following the protocol of previous studies [7, 8]. The insulin dose was calculated based on previous studies from our group that determined the dose that restored inflammatory parameters that had been suppressed in diabetic rats [7, 8].

CLP-induced peritoneal inflammation

Animals in the Diabetic and Non-diabetic groups were assigned to the following subgroups: CLP (cecal ligation and puncture), CLP + Ins (cecal ligation and puncture + insulin), and Sham (false operation). Animals were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) associated with xylazine (10 mg/kg). A midline laparotomy was performed; the caecum was exposed, ligated, and punctured twice with a 22-gauge needle. The caecum was replaced in the abdomen and the incision was closed. Sham animals were subjected to midline laparotomy and manipulation of the caecum without ligation and puncture (sham operation). After surgery, animals were returned to their cages and were given free access to food and water. Six hours after the surgical procedure, animals were anesthetized, as previously described, and exsanguinated from the abdominal aorta [9]. For bronchoalveolar lavage (BAL), 10 mL of phosphate-buffered saline (PBS) was instilled intratracheally and the recovered sample was centrifuged (500 g for 15 min). For peritoneal lavage (PeL), 5 mL of phosphate-buffered saline (PBS) was instilled intraperitoneally and samples were collected and stored after massage of the abdomen. Next, PeL samples were centrifuged (500 g, 10 min, 4°C), the supernatant was stored, and the cells were re-suspended in PBS. The cell suspension was diluted 1:20 (v:v) with Turk's solution and the total cell count was determined using a Neubauer Chamber. Differential cell counts were examined on stained slides under oil immersion microscopy. A total of 100 cells were counted and classified as neutrophils or mononuclear cells based on morphological criteria. In another set of experiments, the lungs, kidney, and liver were removed 6 h after the sham or CLP procedure, rinsed, and immediately immersed in 10% buffered formalin for histopathological and morphometric analyses.

Enzyme immunoassay for cytokines and chemokines

The concentration of cytokines (IL-1β, IL-6, TNF-α, and IL-10) and cytokine-induced neutrophil chemoattractant (CINC-1 and CINC-2) in PeL and BAL supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits, according to the manufacturer's instructions (Duo-set, R & D System, Inc., Minneapolis, MN, USA).

Analysis of liver and renal parameters

The serum concentration of liver enzymes (alanine aminotransferase - ALT, aspartate aminotransferase - AST, and alkaline phosphatase - ALP) and renal parameters (urea and creatinine) were determined by colorimetric assay using commercially available kits according to the manufacturer's instructions (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil).

Histopathological analysis

Lung, kidney, and liver samples were fixed for approximately 12 h in 3.7% formalin. After fixation, the samples were dehydrated in a graded series of increasing ethanol concentrations (70% to 100%), diaphanized in xylol, and embedded in paraffin. Next, sections approximately 5 μm thick were cut and stained with hematoxylin-eosin. After staining, the material was dehydrated, diaphanized, and mounted with Entellan® between slide and cover slip to make permanent slides. Slides containing tissues were observed and photographed under a DM LS light microscope (Leica Microsystems, Wetzlar, Germany) with DF420 (Leica) image pick-up and Leica Application Suite version 3.1.0 imaging software.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM) and were analyzed using Student's t test or analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test when appropriate. The significance level was set at $p < 0.05$.

Results

Body weight gain, blood glucose levels, and blood cell count

Blood glucose levels and body weight gain were measured 10 days after diabetes induction. Body weight gain was significantly lower in diabetic rats than in non-diabetic rats (Table 1). Treatment of diabetic animals with a single dose of NPH insulin significantly reduced blood glucose levels, not sufficient to reduce glycemia to control values (Table 2).

Blood glucose levels (Table 2) and total leukocyte counts (Fig. 1) were both affected by the CLP procedure. CLP caused a reduction in blood leukocytes, both in the Diabetic and Non-diabetic groups, whereas sham surgery did not affect the number of blood leukocytes in either diabetic or non-diabetic rats (Fig. 1). Treatment of animals with a single dose of NPH insulin 2 h before the CLP procedure failed to restore blood leukocyte counts to sham levels (Fig. 1). In addition, red blood cell, hemoglobin, and platelet counts did not differ between CLP and sham animals 6 h after CLP (Table 3).

Table 1. Body weight gain and blood glucose levels in non-diabetic and diabetic rats 10 days after diabetes induction. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.) and body weight was measured 10 days later. Blood glucose levels in the two groups were determined 10 days after diabetes induction. Values are means ± SEM. The total number of animals was 49. $^{*}P < 0.05$ Diabetic versus Non-diabetic (control) rats

	Non-diabetic (n = 24)	Diabetic (n = 25)
Body weight gain (g)	59.2 ± 0.5	-11.1 ± 4.4*
Plasma glucose (mg/dL)	110.8 ± 1.0	553.5 ± 8.6*

Table 2. Effect of CLP-induced sepsis on blood glucose levels (mg/dL) in diabetic and non-diabetic rats. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false-operated). Insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/non-diabetic rats, s.c.) was administered 2 h before CLP. Surgery (SHAM or CLP procedure). Blood glucose levels were determined before insulin treatment and 6 h after CLP. Values are means ± SEM for 8–10 animals per group. $^{*}P < 0.05$ before insulin treatment versus 6 h after CLP

	Non-diabetic			Diabetic		
	SHAM	CLP	CLP + Ins	SHAM	CLP	CLP + Ins
Before surgery	110.6 ± 1.8	112.9 ± 1.6	108.9 ± 1.9	546.6 ± 17.2	556.4 ± 16.1	557 ± 13.6
6 h after CLP	127.1 ± 1.8†	183.2 ± 17.6*	60.7 ± 4.6*	418.0 ± 6.7*	548.0 ± 20.3	253.6 ± 9.1*

Table 3. Blood parameters of non-diabetic and diabetic rats 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false-operated). Insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Red blood cells (RBC), hemoglobin (HB), and platelet (Pla). Values are means ± SEM for 3–5 animals per group

Blood parameters	Non-diabetic			Diabetic		
	SHAM	CLP	CLP + Ins	SHAM	CLP	CLP + Ins
RBC	4.1 ± 0.1	4.4 ± 1.2	6.2 ± 0.2	6.6 ± 0.1	6.4 ± 0.2	6.6 ± 0.2
Hb	12.9 ± 0.4	9.9 ± 1.9	13.5 ± 0.7	13.1 ± 0.3	12.7 ± 0.6	12.8 ± 0.4
Pa	258 ± 64	344 ± 87	447 ± 83	387 ± 11	352 ± 48	373 ± 12

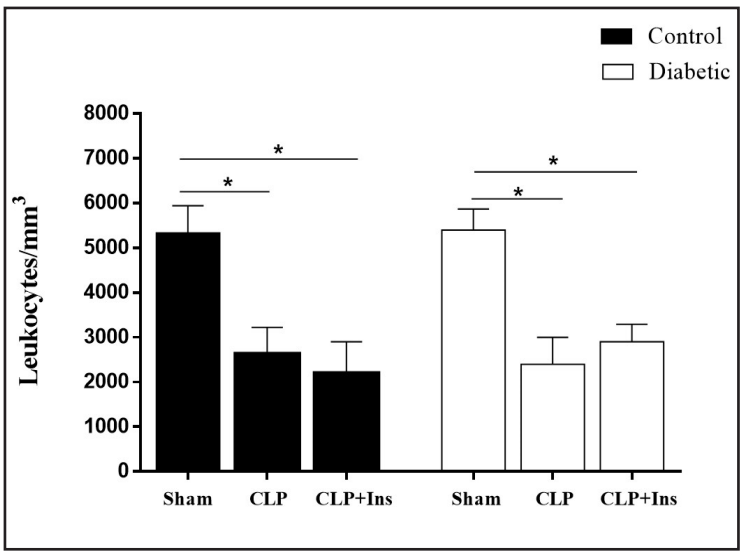

Fig. 1. Total leukocyte count in peripheral blood 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false operated) and after 6 h, total leukocyte count in the peripheral blood was determined. Data are presented as means ± SEM for 3–5 animals per group. **P* < 0.05 sham versus other groups.

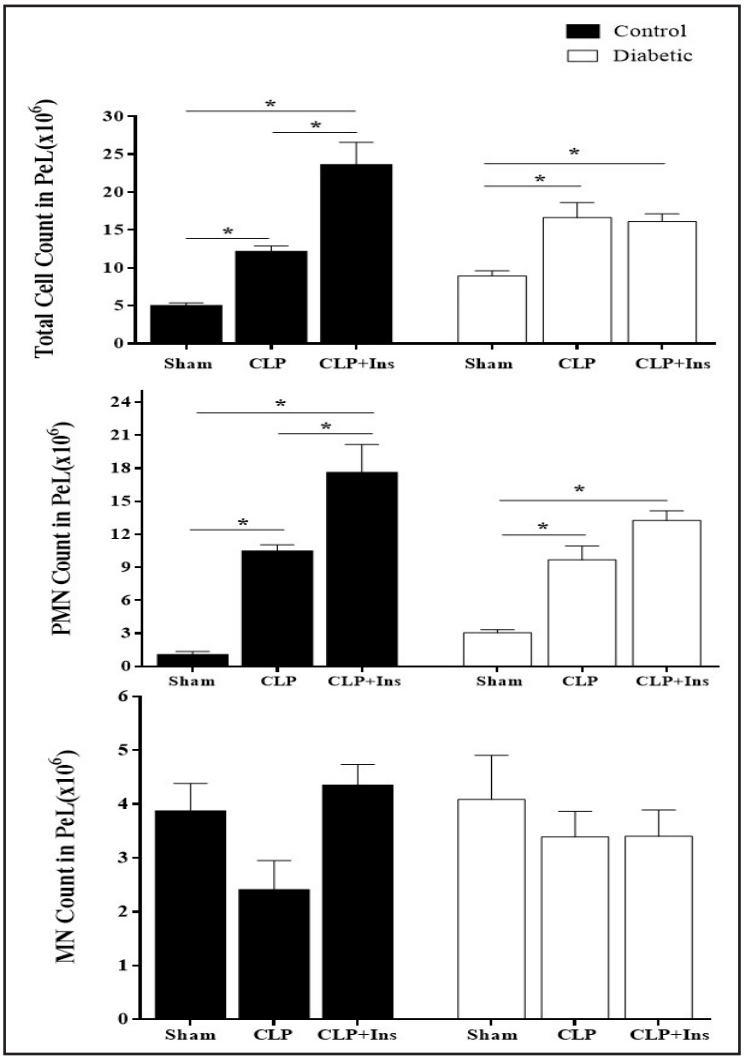

Fig. 2. Total and specific cell composition in peritoneal lavage (PeL) 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false operated) and after 6 h, peritoneal lavage (PeL) fluid was screened 6 h after CLP. Data are presented as means ± SEM for 3–5 animals per group. **P* < 0.05.

CLP-induced leukocyte migration to the PeL fluid

Total cell count in the PeL fluid was higher in non-diabetic CLP animals than in non-diabetic sham rats (Fig. 2). Neutrophil counts were elevated and accounted for 84% of all

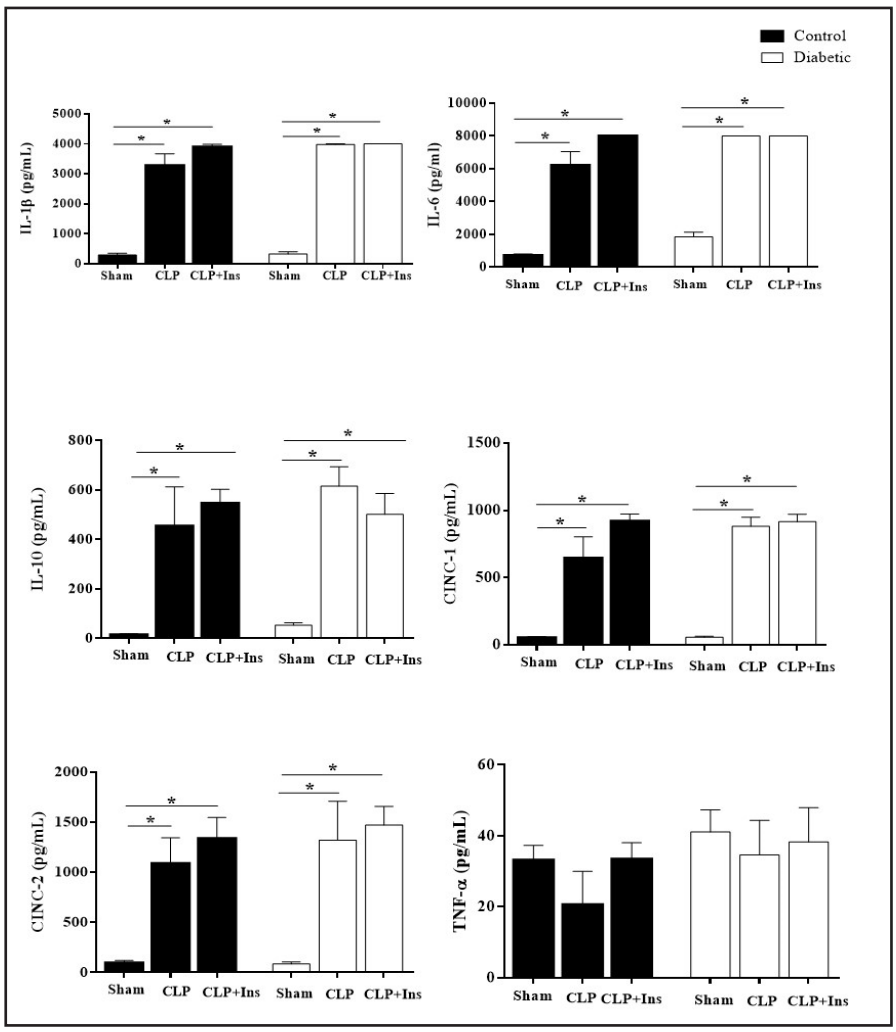

Fig. 3. Cytokine profile of peritoneal lavage (PeL) fluid 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false operated) and peritoneal lavage (PeL) fluid was screened 6 h after CLP. Values are means ± SEM for 7–10 animals per group. $^{*}P < 0.05$, $^{\#}P < 0.01$, $^{\dagger}P < 0.001$.

cells. We observed that CLP induced a greater influx of neutrophils to the PeL fluid in non-diabetic and diabetic rats and the insulin treatment increased the mobilization of neutrophils in the non-diabetic and diabetic CLP group. The number of mononuclear in the PeL fluid did not differ between groups which suggest that the CLP did not cause mobilization of these cells (Fig. 2). This result suggests that when insulin was added to the system, it caused an influx of neutrophils to the peritoneal cavity in non-diabetic and diabetic animals. Results are summarized in Fig. 2.

PeL fluid cytokine profile

PeL concentrations of the pro-inflammatory cytokines IL-1β (8.5-fold), IL-6 (6.7-fold), CINC-1 (9-fold), CINC-2 (14.5-fold) and the anti-inflammatory cytokine IL-10 (10-fold) were higher in non-diabetic and diabetic rats than in sham animals after the CLP procedure. The concentrations of all this cytokines in the PeL fluid did not differ after insulin treatment or between the non-diabetic and diabetic groups (Fig. 3). Conversely, TNF-α concentration did not differ across groups (Fig. 3).

Systemic effects of sepsis

Figures 4 and 5 show blood liver enzymes and renal metabolites, respectively, 6 h after the CLP procedure. The concentrations of serum ALT, AST, and ALP did not change in non-diabetic rats after CLP or after treatment with a single dose of NPH insulin 2 h before

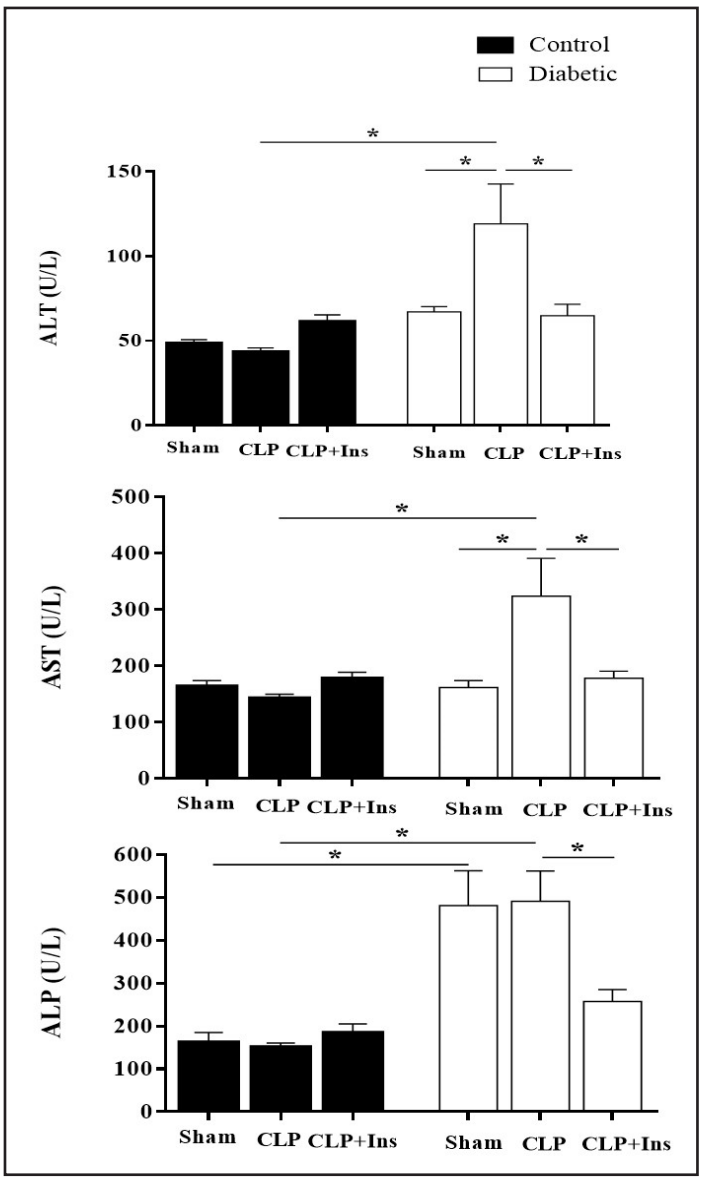

Fig. 4. Blood liver enzyme profile 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false operated) and after 6 h, the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in the peripheral blood were measured. Data are presented as means ± SEM for 8–10 animals per group. $^{*}P < 0.05$, $^{\#}P < 0.01$, $^{\dagger}P < 0.001$.

CLP surgery. However, serum levels of ALP and urea had a 2.3-fold and 1.8-fold increase, respectively, in diabetic sham animals compared to non-diabetic sham rats. Additionally, the concentrations of ALT (2.7-fold), AST (2.3-fold), ALP (2.7-fold), and urea (2.6-fold) increased significantly in diabetic CLP rats compared to CLP rats in the non-diabetic group. Treatment of diabetic CLP animals with a single dose of NPH insulin 2 h before CLP completely restored ALT, AST, and ALP (Fig. 4), but not urea levels (Fig. 5) and the creatinine levels did not differ between groups. In addition, hematoxylin and eosin staining showed minor changes in the cytoplasm of hepatocytes and the absence of neutrophil infiltration 6 h after CLP in diabetic animals (Fig. 6), which were confirmed by the negative liver and myeloperoxidase (MPO) activity (data not shown). Similarly, the evaluation of hematoxylin and eosin-stained kidney sections showed dilated glomerular capillaries, a reduction of Bowman's space, and the absence of neutrophil infiltration 6 h after CLP in diabetic animals (Fig. 7), which were confirmed by the negative kidney MPO activity (data not shown).

Tables 4 and 5 show the cell composition and the cytokine/chemokine profile in the BAL fluid. These parameters did not differ across groups which indicate that animals did not develop acute lung injury (ALI) in this model at the time of evaluation. This condition was

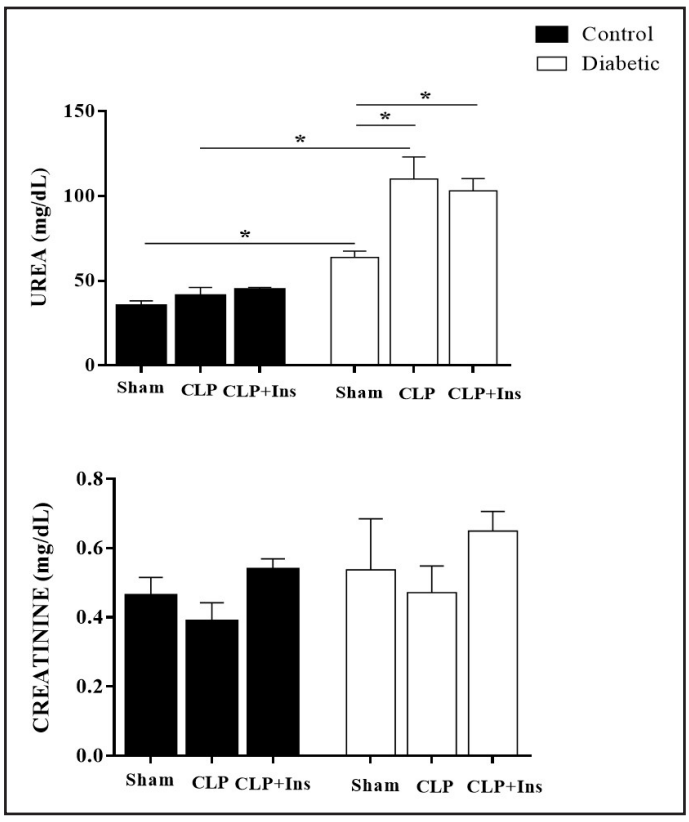

Fig. 5. Blood renal profile 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false operated) and after 6 h, urea and creatinine levels in the peripheral blood were measured. Data are presented as means ± SEM for 8–10 animals per group. $^{*}P < 0.05$, $^{\#}P < 0.01$, $^{\dagger}P < 0.001$.

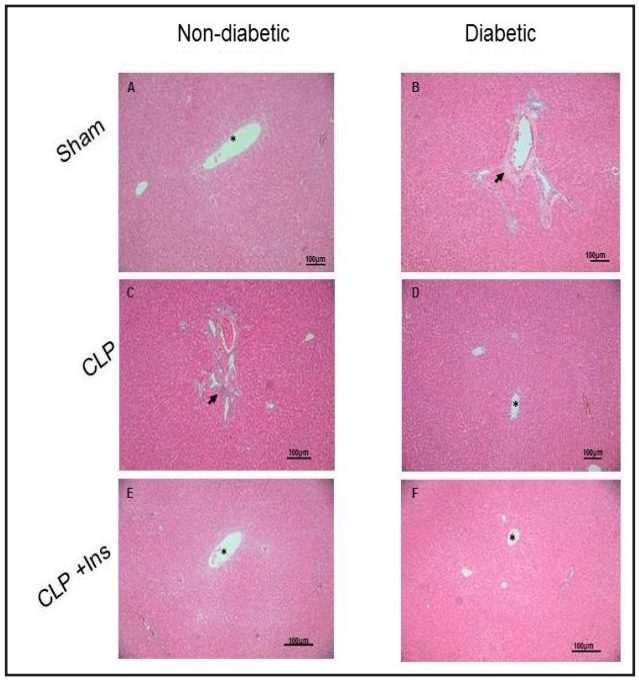

Fig. 6. Microphotographs of liver tissue 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false operated) and after 6 h, the liver was collected, processed, and stained with hematoxylin-eosin. (A) Non-diabetic SHAM, (B) Diabetic SHAM, (C) Non-diabetic CLP, (D) Diabetic CLP, (E) Insulin-treated non-diabetic CLP, and (F) Insulin treated diabetic CLP animals. Absence of neutrophil infiltrate, central vein (*) and portal triad (Arrows). (bars A–F = 100μm). Images are representative of four animals per experimental group.

confirmed in hematoxylin and eosin-stained lung tissue sections (Fig. 8) and by the negative lung MPO activity (data not shown).

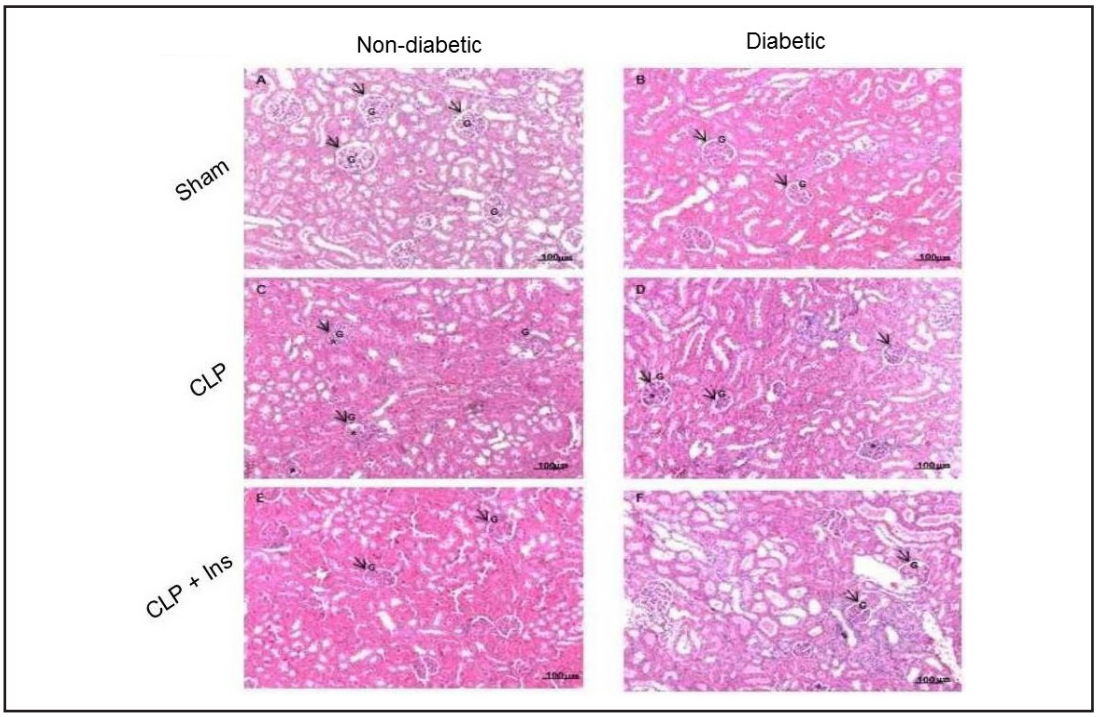

Fig. 7. Microphotographs of kidney tissue 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/Kg, i.v.). Ten days later, insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false operated) and after 6 h, the kidney was collected, processed, and stained with hematoxylin-eosin. (A) Non-diabetic SHAM, (B) Diabetic SHAM, (C) Non-diabetic CLP, (D) Diabetic CLP, (E) Insulin-treated non-diabetic CLP, and (F) Insulin-treated diabetic CLP (F) animals. (G) Glomerular enlargement (*) capillaries. Bowman's space reduction (arrows) and absence of neutrophilic infiltrate (A–F bars = 100μm). Images are representative of four animals per experimental group.

Table 4. Cell composition in the bronchoalveolar lavage (BAL) fluid of non-diabetic and diabetic rats. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false-operated). Insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Bronchoalveolar lavage (BAL) was screened 6 h after CLP. Values are means ± SEM for 7–10 animals per group

Cell composition in BAL ($x10^6$)	Non-diabetic			Diabetic		
	SHAM	CLP	CLP + Ins	SHAM	CLP	CLP + Ins
Total	1.1 ± 0.2	1.2 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.3
Neutrophils	0.1 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.7 ± 0.1	0.3 ± 0.1
Mononuclear cells	1.0 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.2 ± 0.1	0.8 ± 0.2

Discussion

The CLP model is one of the most widely used models of sepsis and septic shock and resembles human sepsis on many parameters [18]. In this model, the severity of sepsis correlates with the number of caecum punctures. We have previously shown in a CLP model of sepsis that evaluation of animals six hours after CLP indicated that sepsis was successfully induced after 12 punctures, because the lungs had clear signs of inflammatory alterations [9, 10]. However, local peritoneal inflammation could not be evaluated. Herein, we have chosen to use two punctures to ensure measurable alterations in the local peritoneal inflammation

Table 5. Cytokine and chemokine profile in the bronchoalveolar lavage (BAL) fluid of non-diabetic and diabetic rats. Rats were rendered diabetic with alloxan (42 mg/kg, i.v.). Ten days later, non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false-operated). Insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Bronchoalveolar lavage (BAL) was screened 6 h after CLP. Values are means ± SEM for 7–10 animals per group

	Non-diabetic			Diabetic		
	SHAM	CLP	CLP + Ins	SHAM	CLP	CLP + Ins
IL-1β	161 ± 23	207 ± 27	110 ± 33	156 ± 36	118 ± 34	186 ± 41
IL-6	43 ± 09	45 ± 14	42 ± 04	64 ± 16	76 ± 27	55 ± 05
TNF-α	35 ± 05	47 ± 06	30 ± 04	35 ± 04	34 ± 03	30 ± 03
CINC-1	125 ± 25	172 ± 66	130 ± 30	131 ± 44	91 ± 36	99 ± 19
CINC-2	403 ± 47	238 ± 75	309 ± 68	427 ± 51	286 ± 36	412 ± 54
IL-10	53 ± 12	82 ± 12	57 ± 08	57 ± 11	45 ± 10	56 ± 08

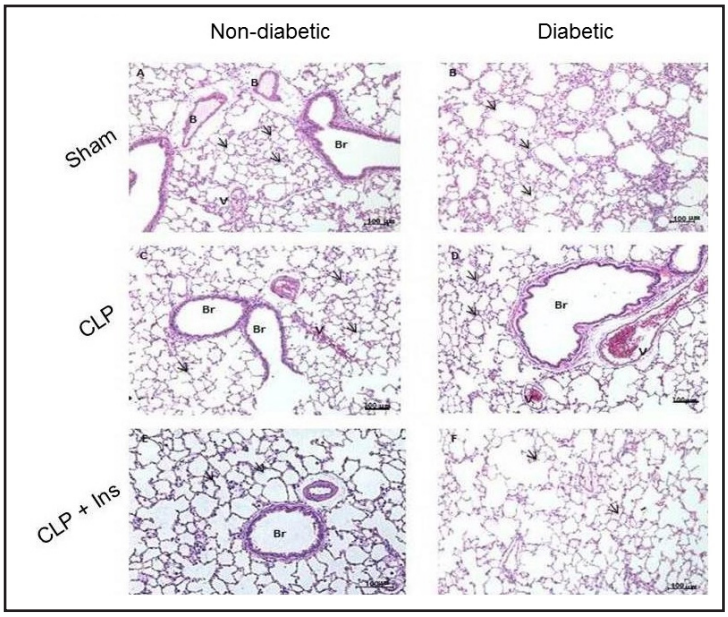

Fig. 8. Microphotographs of lung tissue 6 h after CLP. Rats were rendered diabetic with alloxan (42 mg/Kg, i.v.). Ten days later, insulin (CLP + Ins, NPH, 4 IU/diabetic rats or 1 IU/control rats, s.c.) was administered 2 h before CLP. Non-diabetic and diabetic rats were subjected to cecal ligation and puncture (CLP, with a 22G needle) or SHAM (false operated) and after 6 h, the kidney was collected, processed, and stained with hematoxylin-eosin. (A) Non-diabetic SHAM, (B) Diabetic SHAM, (C) Non-diabetic CLP, (D) Diabetic CLP, (E) Insulin-treated non-diabetic CLP, and (F) Insulin-treated diabetic CLP animals. (A–F bars = 100 μm). Absence of neutrophilic infiltrate; Br = bronchioles; B = bronchus; V = blood vessel; and arrows = alveoli. Images are representative of four animals per experimental group.

and analyze if systemic response was affected by studying blood parameters (red blood cells, hemoglobin, and platelets) and organ function (liver, kidneys, and lungs).

Our results suggest that insulin differentially modulates the various types of cells, in non-diabetic and diabetic CLP groups it rescue the local peritoneal neutrophil migration during the early course of two-puncture CLP-induced local peritoneal inflammation. Insulin also affected CLP-induced liver dysfunction in diabetic rats, because treatment of diabetic rats with insulin completely restored serum ALT, AST, and ALP levels.

Neutrophils are the first line of defense against infection and here we showed that the CLP procedure an increased total neutrophil count in the PeL fluid of both non-diabetic and diabetic animals compared to non-diabetic and diabetic sham animals. Interestingly, treatment of animals with insulin differentially affected neutrophils and mononuclear cells. Although the leukocyte migration profile was similar in the Non-diabetic and Diabetic CLP

groups, it showed that insulin treatment had a greater effect on neutrophils and caused no mobilization of mononuclear cells. In addition, CLP in both non-diabetic and diabetic rats induced an increase PeL concentration of the pro-inflammatory cytokines IL-1β, IL-6, CINC-1 and CINC-2 and also of the anti-inflammatory cytokine IL-10 after the CLP procedure compared to sham rats. These early-response pro-inflammatory cytokines amplify the inflammatory response in other tissues, such as airway epithelial cells [19] and the anti-inflammatory cytokine IL-10, which is among the most potent anti-inflammatory agents induced in response to sepsis [20]. Pro-inflammatory cytokines probably drive neutrophil influx to the inflamed tissue first [7, 8]. However, a single dose of insulin seems to affect leukocyte mobility, causing an influx of cells to and from the tissue, but does not seem to change the concentrations of cytokines in the PeL fluid [21].

Serum markers of renal (urea and creatinine) and liver (ALT, AST, and ALP) function were measured to determine organ dysfunction in this model. The results show that the concentrations of serum ALT, AST, and ALP did not change in non-diabetic rats after CLP or treatment with insulin. Conversely, insulin completely rescued liver enzymes in all CLP diabetic animals, probably by affecting hepatic glucose levels and lipid metabolism [22-24]. Another explanation for the dysfunction of organs located away from the focus of infection may be inappropriate neutrophil activation, the accumulation of neutrophils within the microcirculation next to certain organs, and neutrophil migration to regions other than the primary focus [25]; however, liver neutrophil infiltration was not observed in our study. The elevated ALP levels in sham diabetic animals may be an indicator of early liver obstruction and, when sepsis is induced, persistent elevation of this enzyme with a simultaneous elevation in aminotransferase (ALT/AST) levels is a strong indicator of this disease [26]. Because sepsis is a multifaceted condition, it is possible that we may have induced mild systemic disease rather than severe sepsis in our study.

Urea was also higher in CLP diabetic animals and was not restored to normal levels after insulin treatment. A possible explanation for this result is that sepsis intensifies the kidney damage caused by diabetes. Therefore, persistent hyperglycemia induces oxidative stress and inflammation [27, 28], which in turn induces mitochondrial dysfunction in the kidney. This mitochondrial dysfunction is only corrected by normoglycemia, but that was not our model's focus. In our study, the dose of insulin used [7–8] was not sufficient to ameliorate renal function maybe because the toxic glucose concentration persisted after insulin treatment, causing an overload in the renal cortex and mitochondrial malfunction, which culminates with organ dysfunction [29, 30].

ALI was not observed in our study, because the number of neutrophils and cytokines harvested from the BAL fluid was similar in all experimental groups and no morphological alterations were found in the lungs. In addition, lung neutrophil infiltration and MPO activity were also negative. The lack of lung inflammation may be explained by the number of punctures used in the CLP procedure [9, 10]. For instance, in a CLP model that used 12 punctures, the inflammatory process initiated only at the molecular and cellular levels, but had no effect on respiratory function [9, 10]. In addition, our results suggest that the effect of insulin on different tissues and cells is unique. Insulin treatment completely rescued CLP-induced liver injury in diabetic rats but failed to rescue local peritoneal inflammation, even though insulin modulates inflammatory cells. Based on previous studies [7–11], we hypothesize that insulin may control the influx and outflux of cells in the same tissue in diverse ways, for example by modulating the influx of neutrophils to the inflammatory site while sending mononuclear cells throughout the body as sentinels to prevent systemic inflammation. However, a complete and positive resolution of the inflammatory process may depend on the ability of the host to respond appropriately to pathogenic challenges [11]. A dysregulation of the mechanisms that trigger the innate immune response against bacterial pathogens contributes to the pathophysiological consequences of bacterial sepsis [5, 6].

It has long been recognized that certain infections occur almost exclusively in diabetic patients and that many of these patients have a poorer prognosis following the onset of infection [5, 6, 11]. Adequate insulin concentrations are essential for the maintenance of

normal function of endothelial cells and neutrophils during the inflammatory process. In conclusion, our results suggest that insulin attenuates liver dysfunction during two-puncture CLP-induced peritoneal inflammation in diabetic rats.

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Disclosure Statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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