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Glutamine treatment attenuates the development of organ injury induced by zymosan administration in mice

Stefania Mondello [a,1], Maria Galuppo [b,1], Emanuela Mazzon [c], Domenico Italiano [c], Patrizia Mondello [a], Carmela Aloisi [a], Salvatore Cuzzocrea [a,b,*]

[a] Department of Internal Medicine, University of Messina, Italy
[b] Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, Messina, Italy
[c] IRCCS Centro Neurolesi "Bonino-Pulejo", Messina, Italy

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ABSTRACT

Glutamine is the most abundant amino acid in the bloodstream. It is important in nucleotide synthesis, is anti-catabolic, has anti-oxidant properties via metabolism to glutathione, may enhance immune responsiveness and possesses immunoregulatory functions. Moreover, it reduces atrophy of intestinal mucosa in animals on total parenteral nutrition and prevents intestinal mucosal injury accompanying small bowel transplantation, chemotherapy and radiation. In the present study, we investigated the effects of glutamine on development of non-septic shock caused by zymosan. Mice received either zymosan (500 mg/kg, administered i.p., as a suspension in saline) or vehicle (saline). Glutamine (1.5 mg/kg i.p.) was administered 1 and 6 h after zymosan administration. Organ failure and systemic inflammation in mice were assessed 18 h after administration of zymosan and/or glutamine. Glutamine-treatment reduced the peritoneal exudation and the migration of polymorphonuclear cells caused by zymosan-injection and also attenuated the pancreatic and gut injury. Inflammatory and apoptotic parameters were evaluated to better investigate the effects of the glutamine-administration. So, by immunohistochemical analysis and *in vitro* assays, we have clearly showed that glutamine reduces: 1) the histological damage in pancreas and gut; 2) the inducible nitric oxide synthase expression; 3) nitrotyrosine and poly (ADP-ribose) formation; 4) TNF-α and IL-1β tissue and plasma levels; 5) FasL localization; and 6) alteration of the balance between Bax and Bcl-2. In addition, at the end of the observation period (7 days), zymosan causes severe illness in the mice characterized by a systemic toxicity, significant loss of body weight and mortality. Glutamine-treatment significantly reduced all these parameters.

1. Introduction

Glutathione is important for the integrity of the major enzymatic defense mechanisms against reactive oxygen species. To preserve glutathione content in case of oxidative stress, the supplementation of glutamine may be a safe and well tolerated method. Glutamine does not possess any antioxidant properties on its own, but as it appears to be a "conditionally essential" amino acid in states of serious illness or injury (Lacey and Wilmore, 1990), its administration is recognized to be helpful in various states of systemic inflammation (Lochs et al., 2006). It has been demonstrated that glutamine administration can protect against ischemia/reperfusion injury of gut, heart, and skeletal muscle, and one of the possible ways of action is related to the preservation of GSH content (Harward et al., 1994; Prem et al., 1999; Wischmeyer et al., 2003).

Multiple-organ dysfunction syndrome is a cumulative sequence of progressive deterioration of function occurring in several organ systems, frequently observed after septic shock, multiple trauma, severe burns, or pancreatitis (Baue et al., 1998; Bone et al., 2009). Zymosan is a non-bacterial, non-endotoxic agent derived from the cell wall of the yeast *Saccharomyces cerevisiae*. When injected into animals, it induces inflammation by inducing a wide range of inflammatory mediators (Damas et al., 1993; Deitch et al., 1990; Demling et al., 1994; Goris et al., 1991; Petit et al., 1995; van Bebber et al., 1989; von Asmuth et al., 1990).

It produces acute peritonitis and multiple organ failure characterized by functional and structural changes in liver, gut, lung, and kidneys (Deitch, 2001; Petit et al., 1995; van Bebber et al., 1989; Volman et al., 2002).

The onset of the inflammatory response caused by zymosan in the peritoneal cavity was associated with systemic hypotension, high peritoneal and plasma levels of nitric oxide, maximal cellular infiltration, exudate formation, cyclooxygenase activity and pro-inflammatory cytokine production (Cuzzocrea et al., 1999, 2001).

* Corresponding author at: Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, University of Messina, Torre Biologica, Policlinico Universitario Via C. Valeria, Gazzi, 98100 Messina, Italy. Tel.: +39 090 2213644; fax: +39 090 2213300.
E-mail address: salvator@unime.it (S. Cuzzocrea).
[1] These authors contributed equally to this work.

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Some authors showed that following tissue injury or severe infections, in critically ill patients with metabolic alterations leading to muscle proteolysis activation, enhanced liver gluconeogenesis and tissue insulin resistance, glutamine depletion is proportional to severity of illness. Clinical trials in human subjects have also demonstrated that glutamine treatment decreases infectious complications (Ko et al., 2009).

The present study was performed in order to determine the pharmacological effects of glutamine (1.5 mg/kg, 1 h and 6 h after zymosan injection, i.p.) on zymosan-induced non septic-shock in mice. In particular to gain a better insight into the mechanism(s) of action of glutamine, we evaluated the following endpoints of the inflammatory response: (1) histological damage (2) nitrotyrosine and poly-ADP-ribose (PAR) formation (3) pro-inflammatory cytokine production (4) apoptosis as TUNEL staining and (5) Bax/Bcl-2 expression.

2. Materials and Methods

2.1. Animals

Male CD mice (20–22 g; Charles River; Milan; Italy) were housed in a controlled environment and provided with standard rodent chow and water. The study was approved by the University of Messina Review Board for the care of animals. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M.116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. Experimental Groups

Mice were randomly allocated into the following groups:

(1) *Zymosan + vehicle group*. Mice were treated intraperitoneally (i.p.) with zymosan (500 mg/kg, suspended in saline solution, i.p.) and with the vehicle for glutamine (saline, i.p.), 1 and 6 h after zymosan administration ($n = 10$);
(2) *Zymosan + glutamine group*. Identical to the *zymosan + vehicle group* but were administered glutamine (1.5 mg/kg, i.p.) at 1 and 6 h after zymosan ($n = 10$) instead of vehicle;
(3) *Sham + vehicle group*. Identical to the *zymosan + vehicle group* except that the saline was administered instead of zymosan ($n = 10$);
(4) *Sham + glutamine group*. Identical to the *sham + vehicle group*, except for the administration of glutamine (1.5 mg/kg, i.p.) 1 and 6 h after saline administration ($n = 10$).

Eighteen hours after administration of zymosan, animals were assessed for shock as described below. In another set of experiments, animals ($n = 10$ for each group) were randomly divided as described above monitored for loss of body weight and mortality for 7 days after zymosan or saline administration.

The dose of glutamine (1.5 mg/kg, i.p.) was chosen in agreement with previous study (Mondello et al. 2010) as well as the time of administration was chosen as previously described (Dugo et al., 2002).

2.3. Clinical Scoring of Systemic Toxicity

Clinical severity of systemic toxicity was scored for all the experimental period (7 days) in the mice after zymosan or saline injection on a subjective scale ranging from 0 to 3; 0 = absence, 1 = mild, 2 = moderate, and 3 = serious. The ranging scale was used for each of the toxic signs (conjunctivitis, ruffled fur, diarrhea and lethargy) observed in the animals. The final score will be the adding of the single evaluation (maximum value 12). All clinical score measurements were performed by an independent investigator, who had no knowledge of the treatment regimen received by each respective animal.

2.4. Measurement of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity, which was used as an indicator of PMN infiltration into the pancreas and gut, was measured as previously described (Mullane et al., 1988). Briefly, at the end of the experiments, pancreas and gut samples were weighed and homogenized in a solution containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7.4) and centrifuged for 30 min at 20,000 × *g* at 4 °C. An aliquot of supernatant was then removed and added to a reaction mixture containing 1.6 mmol/L tetramethylbenzidine and 0.1 mmol/L hydrogen peroxide (H_2O_2). The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme required degrading 1 mmol of H_2O_2 at 37 °C and was expressed in mU/100 mg wet tissue.

2.5. Assessment of Acute Peritonitis, Peritoneal Exudate Cell (PEC) Counts and Differential Staining

Eighteen hours after treatment, the mice were anesthetized with intramuscular injection of ketamine/xylazine in order to evaluate the development of acute inflammation in the peritoneum. Through an incision in the *linea alba*, 5 mL of ice-cold RPMI-1640 medium (Gibco Inc., Grand Island, NY) with 10% heparin was injected into the abdominal cavity. The peritoneal cavities were massaged for 1 min and the lavage fluid was collected with a plastic pipette and transferred into a 10 mL centrifuge tube. The amount of exudate was calculated by subtracting the volume injected (5 mL) from the total volume recovered. A part of the volume of exudates was used to perform the peritoneal exudate cell (PEC) counts and the differential staining.

The PEC counts were done in a hemocytometer by mixing 100 μL of peritoneal cell exudate and 100 μL of eosin. The PEC was spun in a cytocentrifuge at 50 × *g* for 5 min onto a slide for the differential count. The slides were carefully removed and allowed to air dry briefly. Peritoneal exudate cell cytospins were stained with Wright–Giemsa stain. Peritoneal exudate cell cytospins were also stained with neutrophil/mast cell-specific chloroacetate esterase staining and macrophage/monocyte-specific alpha naphthyl butyrate esterase stains for the differential count.

2.6. Measurement of Nitrite/Nitrate Concentrations

Plasma samples and the remainder part of the exudates collected 18 h after zymosan or saline administration were used to assess nitrite/nitrate production, an indicator of nitric oxide synthesis, as previously described (Cuzzocrea et al., 1999, 2004a). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

2.7. Measurement of Malondialdehyde Levels

Levels of malondialdehyde (MDA) in pancreas and gut samples were determined as an indicator of lipid peroxidation following a protocol described previously (Ohkawa et al., 1974). Briefly, pancreas and gut samples were weighed and homogenized in a 1.15% (wt/vol) KCl solution. A 100 mL aliquot of homogenate was then removed and added to a reaction mixture containing 200 mL 8.1% (wt/vol) lauryl sulfate, 1.5 mL 20% (vol/vol) acetic acid (pH 3.5), 1.5 mL 0.8% (wt/vol) thiobarbituric acid, and 700 mL distilled water. Samples were then boiled for one hour at 95 °C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 650 nm. MDA levels were expressed as mmol/L MDA/100 mg wet tissue.

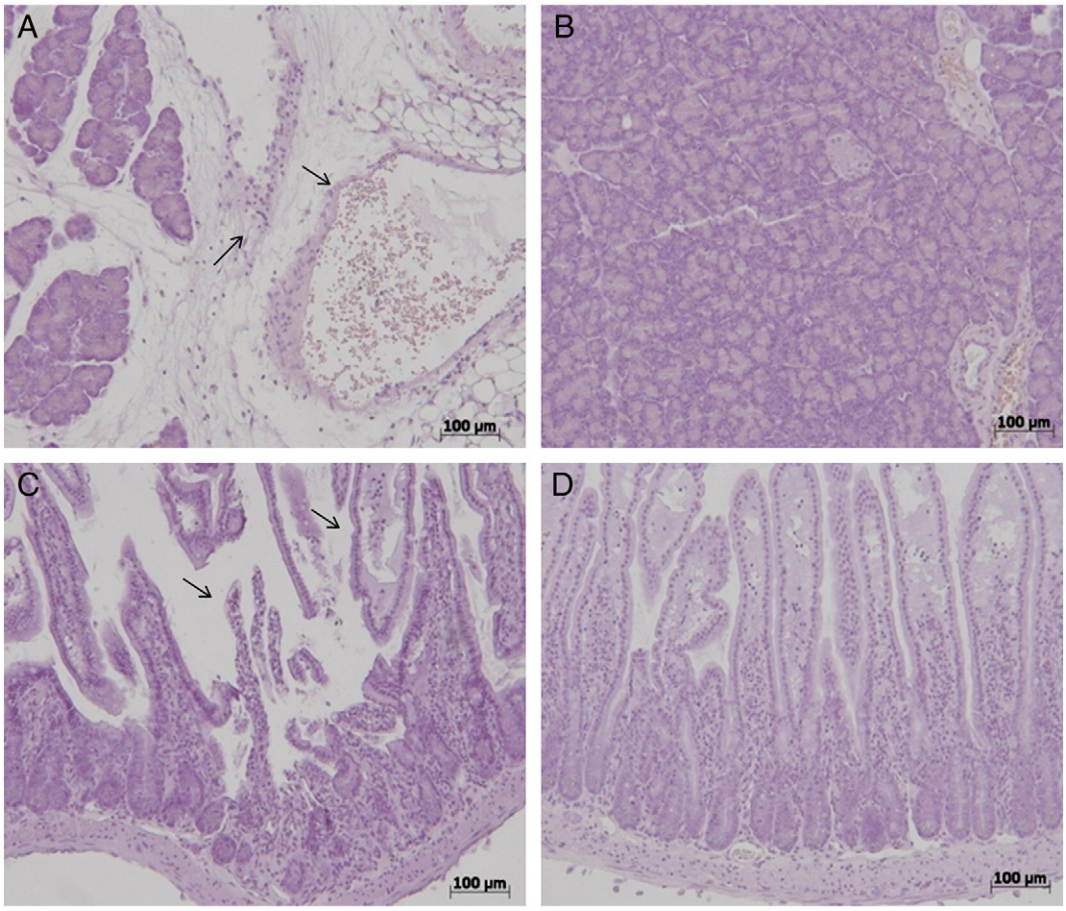


Fig. 1. Pancreas (A) and distal ileum (C) sections from zymosan-administered mice revealed morphological alterations and inflammatory cell infiltration. Pancreas (B) and distal ileum (D) sections from zymosan-administered mice treated with glutamine demonstrated reduced morphological alterations and inflammatory cell infiltration. Figures are representative of at least 3 experiments performed on different experimental days.

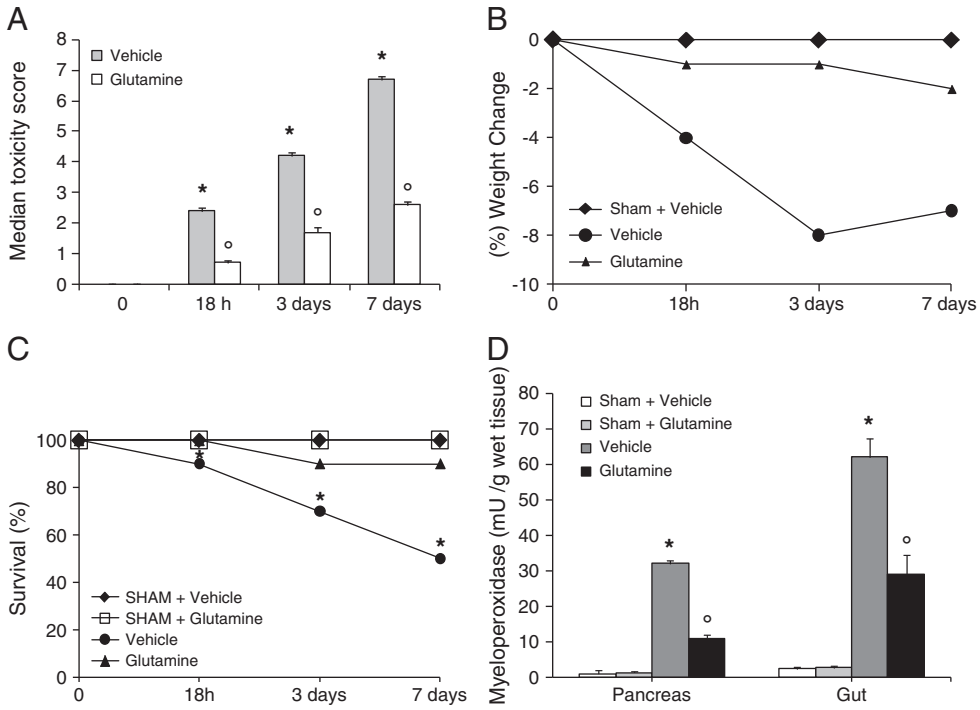


Fig. 2. Glutamine-treatment reduces toxicity score (A), body weight change (B), mortality (C) and MPO levels (D) after zymosan-injection. Data are means ± S.E.M. of 10 mice for each group. *$P<0.05$ *vs* sham, °$P<0.05$ *vs* zymosan + vehicle.

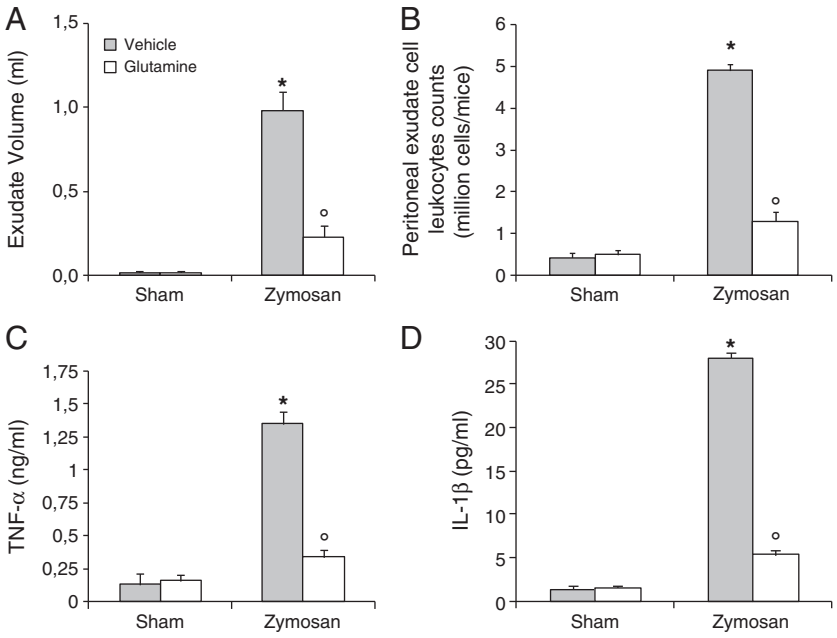


Fig. 3. Effects of glutamine-treatment on inflammatory response in the peritoneal cavity. The increase in volume exudates (A) and peritoneal exudates cell leukocyte counts (B) in peritoneal cavity at 18 h after zymosan was reduced by glutamine treatment. Moreover, we evaluated the effects of glutamine on plasma tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) production. A substantial increase in TNF-α (C) and IL-1β (D) production was found in tissues collected from zymosan-treated-mice compared to sham mice. Plasma levels of TNF-α and IL-1β were significantly attenuated by the treatment with glutamine after zymosan-injection (C and D, respectively). Data are mean ± standard deviation from $n = 10$ mice for each group. $^{*}P<0.05$ *vs* sham, $^{\circ}P<0.05$ *vs* zymosan + vehicle.

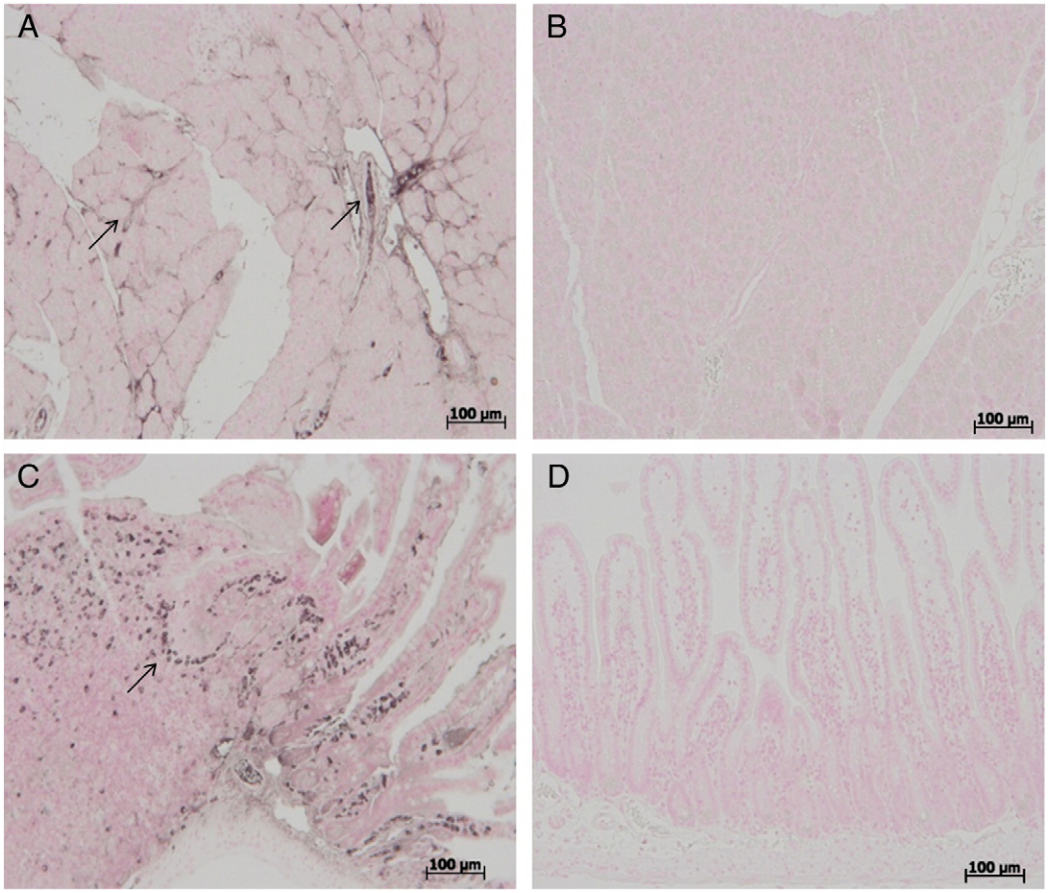


Fig. 4. Immunohistochemical localization in pancreas and gut. 18 h following zymosan injection, a positive TNF-α staining was found in pancreas (A) and gut (C). There was no detectable immunostaining for TNF-α in pancreas (B) and gut (D) of zymosan-treated mice when mice were treated with glutamine. Figures are representative of at least 3 experiments performed on different experimental days.

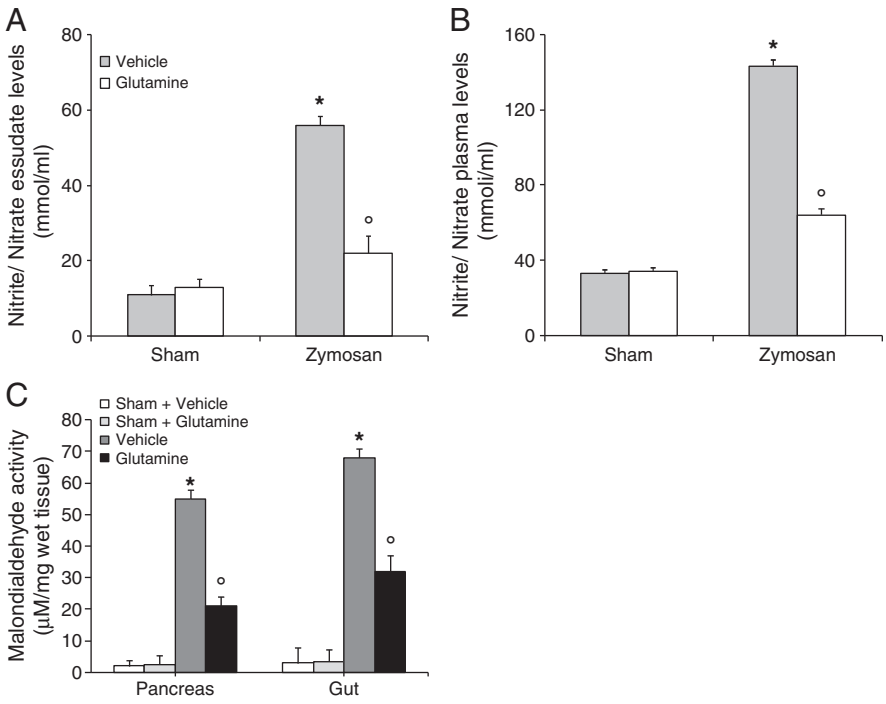


Fig. 5. Effect of glutamine on peritoneal exudates (A), plasma nitrate/nitrite (B) and MDA levels. Nitrate/nitrite levels were significantly increased both in peritoneal exudate and in plasma of zymosan-treated mice in comparison to vehicle group (sham group). Glutamine reduced the zymosan-induced increase of nitrate/nitrite levels in peritoneal exudate (A) and in plasma (B). Moreover, in pancreas and gut, zymosan-injection causes an increase in MDA levels. On the contrary, treatment with glutamine produced a significant attenuation of MDA levels in pancreas and gut (C). Data are mean ± standard deviation from $n = 10$ mice for each group. $^{*}P<0.05$ *vs* sham, $^{\circ}P<0.05$ *vs* zymosan + vehicle.

2.8. Immunohistochemical Localization of TNF-α, iNOS, Nitrotyrosine, PAR, FasL, Bax, and Bcl-2

Tyrosine nitration and poly (ADPribose) polymerase activation were detected as previously described (Abdelrahman et al., 2004) in pancreas and gut sections using immunohistochemistry. At 18 h after zymosan or saline injection, tissues were fixed in 10% (w/v) PBS-buffered formalin and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (Vector Laboratories, Burlingame, CA). The sections were then incubated overnight with anti-TNF-α antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), anti-iNOS antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), anti-nitrotyrosine antibody (Millipore, 1:500 in PBS, v/v), anti-poly (ADP)-ribose antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), anti-Fas ligand antibody (Abcam, 1:500 in PBS, v/v), anti-Bax rabbit polyclonal antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), or anti-Bcl-2 rabbit polyclonal antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v). Controls included buffer alone or non-specific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated specific secondary anti-IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA). To verify the binding specificity for TNF-α, iNOS, nitrotyrosine, PAR, FasL, Bax, and Bcl-2 some sections were also incubated with primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody). In these situations, no positive staining was found in the sections indicating that the immunoreactions were positive in all the experiments carried out. In order to confirm that the immunoreactions for the nitrotyrosine were specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity.

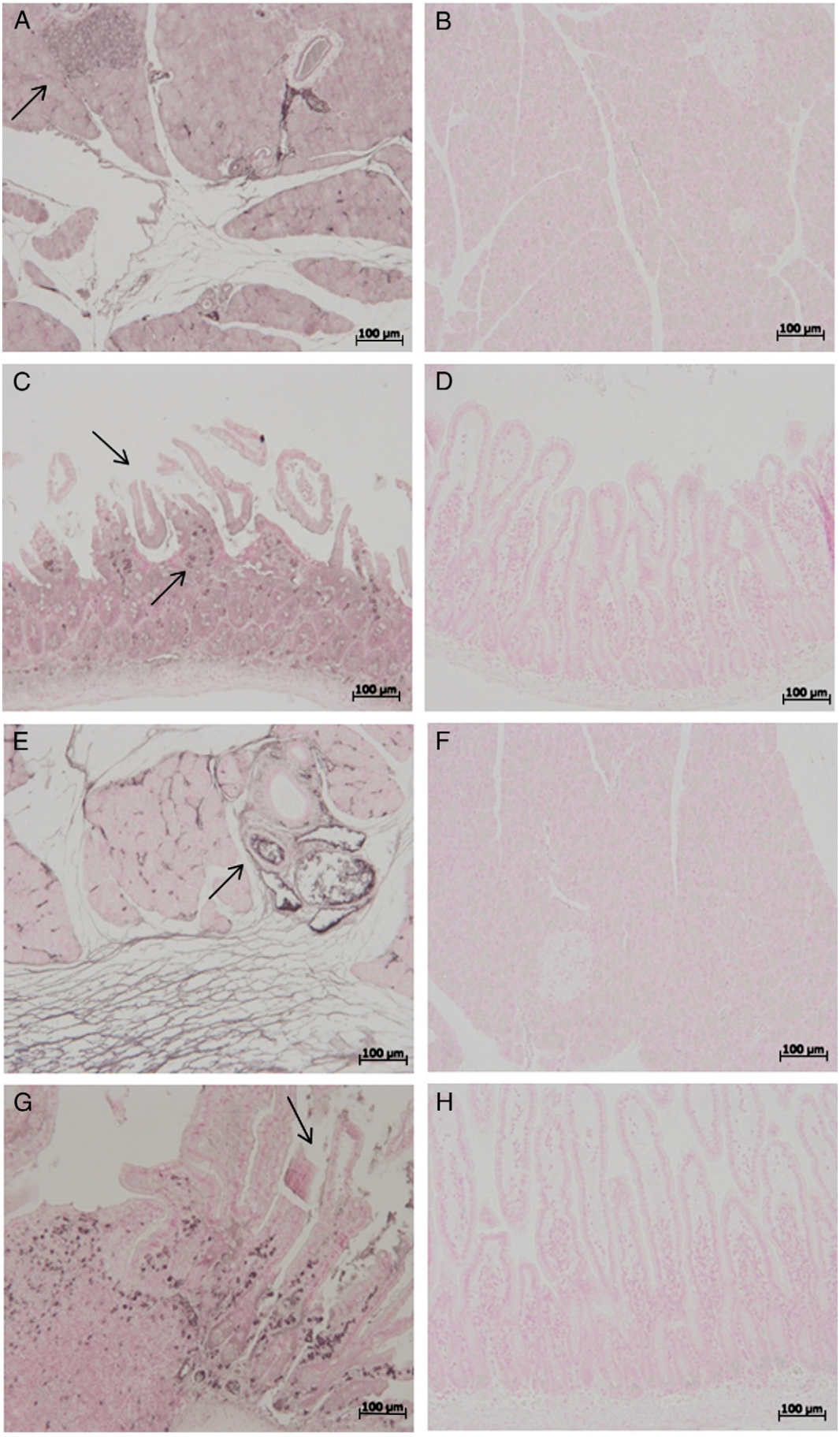
2.9. Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin end Labeling Assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL) assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Apotag horseradish peroxidase kit; DBA, Milan, Italy). Briefly, sections were incubated with 15.2 μg/mL proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyl transferase buffer containing deoxynucleotidyl transferase and biotinylated deoxyuridine 5-triphosphate in TdT buffer, incubated in a humid atmosphere at 37 °C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-fluorescein isothiocyanate horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine.

2.10. Cytokine Production

The levels of TNF-α and IL-1β were evaluated in the plasma at 18 h after zymosan or saline administration. The assay was conducted by using a colorimetric commercial kit (Calbiochem-Novabiochem, La Jolla, CA). The ELISA has a lower detection limit of 10 pg/mL.

Fig. 6. 18 h following zymosan injection, a positive nitrotyrosine staining was found in the pancreas (A) and gut (C). There was no detectable immunostaining for nitrotyrosine in the pancreas (B), and gut (D) of zymosan-administered mice when mice were treated with glutamine. Moreover, 18 h following zymosan injection, a positive PAR staining was found in the pancreas (E) and gut (G). There was no detectable immunostaining for PAR in the pancreas (F), and gut (H) of zymosan-administered mice when mice were treated with glutamine. Figures are representative of at least 3 experiments performed on different experimental days.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2.11. Quantification of Organ Function and Injury

Blood samples were taken at 18 h after zymosan or saline injection and centrifuged (1610×*g* for 3 min at room temperature) to separate plasma. Levels of amylase, lipase, creatinine, alanine aminotransferase, aspartate aminotransferase, bilirubin and alkaline phosphatase were measured by a veterinary clinical laboratory using standard laboratory techniques. For the evaluation of acid base balance and blood gas analysis (indicator of lung injury) arterial blood levels of pH, PaO_2 and $PaCO_2$ and bicarbonate ion were determined by pH/blood gases analyzer as previously described (Cuzzocrea et al., 2004b).

2.12. Light Microscopy

Pancreas and gut samples were taken 18 h after zymosan or saline injection. The tissue slices were fixed in Dietric solution [14.25% (v/v) ethanol, 1.85% (w/v) formaldehyde, 1% (v/v) acetic acid] for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, New Jersey, USA). Sections (thickness 7 μm) were deparaffinized with xylene, stained with hematoxylin and eosin and observed in Dialux 22 Leitz microscope.

2.13. Materials

Unless stated otherwise, all reagents and compounds used were obtained from Sigma Chemical Company (Milan, Italy).

2.14. Data Analysis

All values in the figures and text are expressed as mean ± standard error of the mean of *n* observations. For the *in vivo* studies *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissues section collected from all the animals in each group.

The results were analyzed by one-way ANOVA followed by a Bonferroni's *post-hoc* test for multiple comparisons. A *P*-value of less than 0.05 was considered significant.

Statistical analysis for survival data was calculated by Kaplan–Meier survival analysis. For such analyses, $P<0.05$ was considered significant. The Mann–Whitney *U* test (two-tailed, independent) was used to compare medians of between the body weight and the clinical score. When this test was used, $P<0.05$ was considered significant.

3. Results

3.1. Pancreas and Gut Injury (Histological Evaluation) Caused by Zymosan is Reduced in Glutamine-Treated Mice

At 18 h after zymosan administration, histological evaluation of pancreas (Fig. 1A) and gut (Fig. 1C) sections demonstrated several marked pathological changes. In the pancreas there was extravasation of neutrophils (Fig. 1A). In the gut, there was infiltration of inflammatory cells, edema in the space bounded by the villus, and separation of the epithelium from the basement membrane (see arrow, Fig. 1C). Treatment with glutamine markedly reduced the histological damage in the pancreatic (Fig. 1B) and gut (Fig. 1D) tissues. No histological alteration was observed in the pancreas or gut from sham-treated mice (data not shown).

3.2. Effect of Glutamine-Treatment on Zymosan-Induced Body Weight Loss and Mortality

Administration of zymosan caused severe illness in mice, characterized by systemic toxicity (Fig. 2A) and significant loss of body weight (Fig. 2B). At the end of the observation period (7 days), 50% of zymosan-treated mice were dead (Fig. 2C). Treatment with glutamine reduced the development of systemic toxicity (Fig. 2A), loss in body weight (Fig. 2B) and mortality (Fig. 2C), caused by zymosan. Glutamine treatment did not cause any significant changes in these parameters in sham mice (Fig. 2A, B and C).

3.3. Effect of Glutamine-Treatment on Inflammatory Cell Infiltration

The accumulation of neutrophils in the pancreas and in the gut is a hallmark of multiple organ failure induced by zymosan. An indirect assessment of neutrophilic infiltration was carried out by measuring the activity of myeloperoxidase, an enzyme that is contained in (and specific for) PMN lysosomes dysfunction (Cuzzocrea et al., 2004a). MPO activity was significantly increased at 18 h after zymosan administration in the pancreas (Fig. 2D) and gut (Fig. 2D) of zymosan-challenged mice, when compared with sham-operated mice (Fig. 2D). MPO activity was markedly reduced in the pancreas (Fig. 2D) and gut (Fig. 2D) zymosan-challenged mice, which were treated with glutamine.

3.4. Effect of Glutamine-Treatment on Inflammatory Response in the Peritoneal Cavity

The development of acute peritonitis occurred 18 h after zymosan administration was indicated by the production of a large amount of turbid exudate in animals that received only vehicle, while mice treated with glutamine showed a lower and less turbid production of exudates (Fig. 3A). The total number, both live and dead cells, of peritoneal exudate cells (Fig. 3B) was determined by trypan blue staining following intraperitoneal administration of zymosan or saline solution. This demonstrated a significant increase in the polymorphonuclear leukocyte number in zymosan-injected mice when compared with sham mice, which, on the contrary, demonstrated no abnormalities in the peritoneal cavity or fluid.

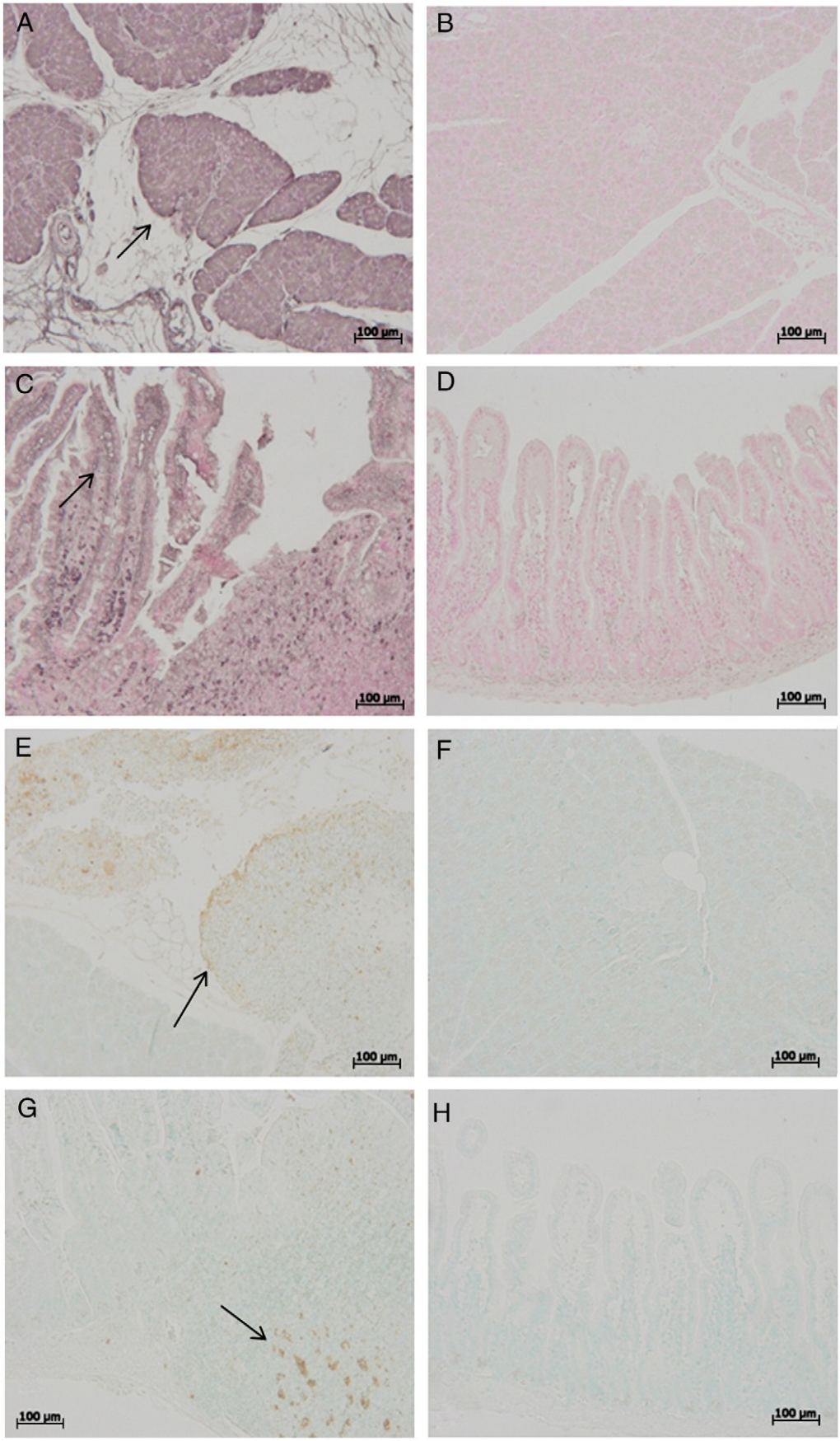
Moreover, since there was a quantitative increase in peritoneal exudate cells following zymosan injection, cytospin preparations were performed of the peritoneal exudate cells for a differential estimation of the types of cells present. Wright–Giemsa stained slides of all controls appeared to contain mostly mononuclear cells including resident macrophages and lymphocytes and very few polymorphonuclear neutrophils, as previously demonstrated (Mahesh et al., 1999).

In sham-operated mice, all cells appeared healthy and intact, while 18 h after zymosan administration, the cells appeared lysed and the nucleus could not be differentiated, so peritoneal exudate cell cytospins were also stained for the differential count.

Cell staining for specific esterases for neutrophil and macrophages was carried out in order to attempt differentiation between cell populations. In agreement with previous observations (Mahesh et al., 1999), we confirmed the presence of 90% mononuclear cells in the peritoneal cavity along with 10% PMNs in all the sham-treated animals.

Exudate formation (Fig. 3A) and the degree of peritoneal exudate cell counts (Fig. 3B) were significantly reduced in mice treated with glutamine.

Fig. 7. 18 h following zymosan injection, positive FasL staining was found in pancreas (A) and gut (C). There was no detectable immunostaining for FasL in pancreas (B) and gut (D) of zymosan-administered mice when mice were treated with glutamine. Moreover, 18 h after zymosan administration, TUNEL-like staining showed a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments in pancreatic (E) and gut (G) tissues. On the contrary, the number of dark brown cells was significantly reduced in pancreas (F) and gut (H) by the treatment with glutamine. Figures are representative of at least 3 experiments performed on different experimental days.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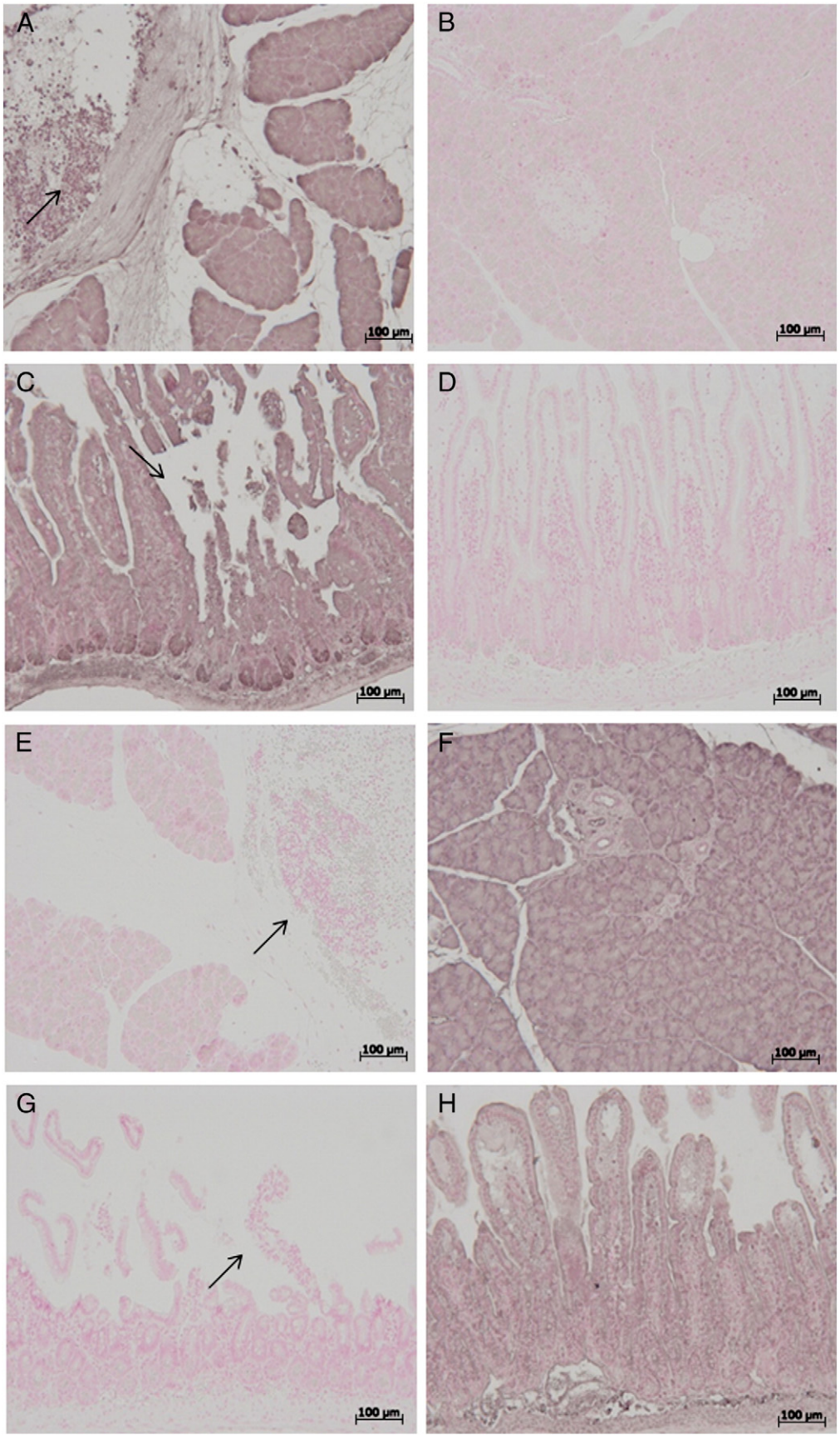

Fig. 8. 18 h following zymosan injection, positive Bax staining was found in pancreas (A) and gut (C). No positive staining for Bax was detected in pancreas (B), and gut (D) of zymosan-treated mice when mice were treated with glutamine. Moreover, 18 h after zymosan administration, no positive staining for Bcl-2 was observed in pancreas (E), and gut (G) from zymosan-treated mice. On the contrary, positive staining for Bcl-2 was observed in pancreas (F) and gut (H) from mice treated with glutamine. Figures are representative of at least 3 experiments performed on different experimental days.

3.5. Effect of Glutamine-Treatment on Cytokine Production

The modulation of glutamine on the inflammatory process through the regulation of cytokine secretion was assessed by determination of plasmatic levels of the pro-inflammatory cytokines TNF-α and IL-1β. A substantial increase in TNF-α and IL-1β formation was observed in zymosan-treated mice when compared to sham mice (Fig. 3C and D, respectively), while a significant inhibition of TNF-α and IL-1β was observed when animals with zymosan-induced injury were treated with glutamine (Fig. 3C and D, respectively).

In addition, tissue sections of pancreas and gut obtained from animals 18 h after zymosan administration, demonstrated positive staining (see arrow) for TNF-α (Fig. 4A and C, respectively). On the contrary, in section of pancreas and gut, the staining for TNF-α was visibly and significantly reduced in zymosan mice treated with glutamine (Fig. 4B and D, respectively). In the pancreas and gut of sham animals no positive staining was observed for TNF-α (data not shown).

3.6. Effect of Glutamine-Treatment on NO Formation and Lipid Peroxidation

The analysis of exudates (Fig. 5A) and plasma (Fig. 5B) levels validates the biochemical and inflammatory changes observed in the peritoneal cavity of zymosan-treated mice, showing a significant increase of nitrite/nitrate concentration (Fig. 5A and B) when compared with sham-operated mice. These values were significantly reduced in mice treated with glutamine (Fig. 5A and B). In addition, zymosan-injection produced a significant increase in MDA levels (Fig. 5C). However, treatment of mice with glutamine after zymosan administration produced a significant attenuation of MDA levels in pancreas and gut (Fig. 5C). The administration of glutamine to sham-operated animals did not have any effect on MDA levels in pancreas and gut (Fig. 5C), which were similar to those obtained from sham-operated animals.

3.7. Effect of Glutamine-Treatment on Nitrosative Stress and Poly (ADPribose) Polymerase Activation

To determine the localization of "peroxynitrite formation" and/or other nitrogen derivatives produced during multiple organ failure, zymosan-induced nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in sections of pancreas and gut tissues, using a specific anti-nitrotyrosine antibody. The samples obtained from sham-operated mice did not stain for nitrotyrosine (data not shown), while sections from zymosan-induced mice exhibited positive staining (see arrow) for nitrotyrosine in pancreas (Fig. 6A) and gut (Fig. 6C) tissues. A marked reduction in nitrotyrosine staining was found in the pancreas (Fig. 6B) and gut (Fig. 6D) of the zymosan-challenged mice treated with glutamine.

Sections of pancreas and gut were taken at 18 h after zymosan administration in order to determine the activation of the nuclear enzyme, poly (ADP-ribose) polymerase, which has been implicated in the pathogenesis of multiple organ failure. Thus, we used an immunohistochemical approach to assess the presence of PAR, an indicator of poly (ADPribose) polymerase activation *in vivo*. There was positive staining for poly (ADPribose) (see arrow) localized in sections of pancreas (Fig. 6E) and gut (Fig. 6G) obtained from zymosan-challenged mice. Treatment with glutamine reduced the degree of positive staining for poly (ADPribose) in the pancreas (Fig. 6F) and gut (Fig. 6H). No positive staining for poly (ADPribose) was identified in tissues from sham-operated mice (data not shown).

3.8. Effect of Glutamine-Treatment on Fas Ligand Expression and Apoptosis

Immunohistological staining for the Fas ligand in the pancreas and gut was determined 18 h after zymosan-induced injury. Tissue sections from the sham-operated mice did not stain for the Fas ligand (data not shown), whereas sections obtained from the zymosan-challenged mice exhibited positive staining (see arrow) for the Fas ligand in the pancreas (Fig. 7A) and gut (Fig. 7C). Treatment with glutamine reduced the degree of positive staining for the Fas ligand in the pancreas (Fig. 7B) and gut (Fig. 7D).

To test whether tissue damage was associated with cell death by apoptosis, we assessed TUNEL-like staining in pancreas and gut. Almost no apoptotic cells were detectable in sections of pancreatic and pulmonary tissues in sham-operated mice (data not shown). At 18 h after zymosan-induced injury, sections of pancreas (Fig. 7E) and gut (Fig. 7G) demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (see arrow). In contrast, pancreatic (Fig. 7F) and gut (Fig. 7H) tissues obtained from zymosan-administered mice treated with glutamine, demonstrated a small number of apoptotic cells or fragments.

3.9. Immunohistochemical Localization of Bax and Bcl-2

To determine the immunohistological staining for Bax and Bcl-2 samples of pancreas and gut were also collected 18 h after zymosan administration. Tissues taken from sham-treated mice did not stain for Bax (data not shown), whereas pancreatic (Fig. 8A) and gut (Fig. 8C) sections obtained from zymosan-treated mice exhibited positive staining for Bax (see arrow). Glutamine treatment reduced the degree of positive staining for Bax in the pancreas (Fig. 8B) and gut (Fig. 8D) of mice subjected to zymosan-induced injury.

In addition, sections of pancreas and gut from sham-treated mice demonstrated positive staining for Bcl-2 (data not shown), whereas in zymosan-administered mice, Bcl-2 staining was significantly reduced (see arrow) in pancreas (Fig. 8E) and gut (Fig. 8G). Glutamine treatment significantly attenuated the loss of positive staining for Bcl-2 in pancreatic (Fig. 8F) and gut (Fig. 8H) samples of mice subjected to zymosan-induced injury.

3.10. Zymosan-Induced Multiple Organ Dysfunction Syndrome is Reduced by Glutamine

3.10.1. Effects on Lung Injury

When compared to sham-operated mice, zymosan-administered mice demonstrated significant alterations in the PaO_2 (Fig. 9A), PCO_2 (Fig. 9B), HCO_3^- (Fig. 9C) and pH arterial blood levels (Fig. 9D), suggesting the development of lung dysfunction. In contrast, treatment with glutamine significantly reduced the lung injury caused by zymosan (Fig. 9A, B, C and D).

3.10.2. Hepatocellular Injury

When compared to sham-operated mice, zymosan-administered mice demonstrated significantly high plasma concentrations of aspartate aminotransferase (Fig. 10A), alanine aminotransferase (Fig. 10B), bilirubin (Fig. 10C) and alkaline phosphatase (Fig. 10D), suggesting the presence of a consistent hepatocellular injury. In contrast, treatment with glutamine significantly reduced the liver injury caused by zymosan (Fig. 10A, B, C and D).

3.10.3. Pancreatic Injury

When compared to sham-operated mice, zymosan-administered mice demonstrated significantly high plasma concentrations of amylase (Fig. 11A) and lipase (Fig. 11B), suggesting the development of pancreatic injury. In contrast, treatment with glutamine significantly reduced the pancreatic injury caused by zymosan (Fig. 11A and B, respectively).

3.10.4. Renal Dysfunction

When compared to sham-operated mice, zymosan-administered mice demonstrated significantly high plasma concentrations of creatinine

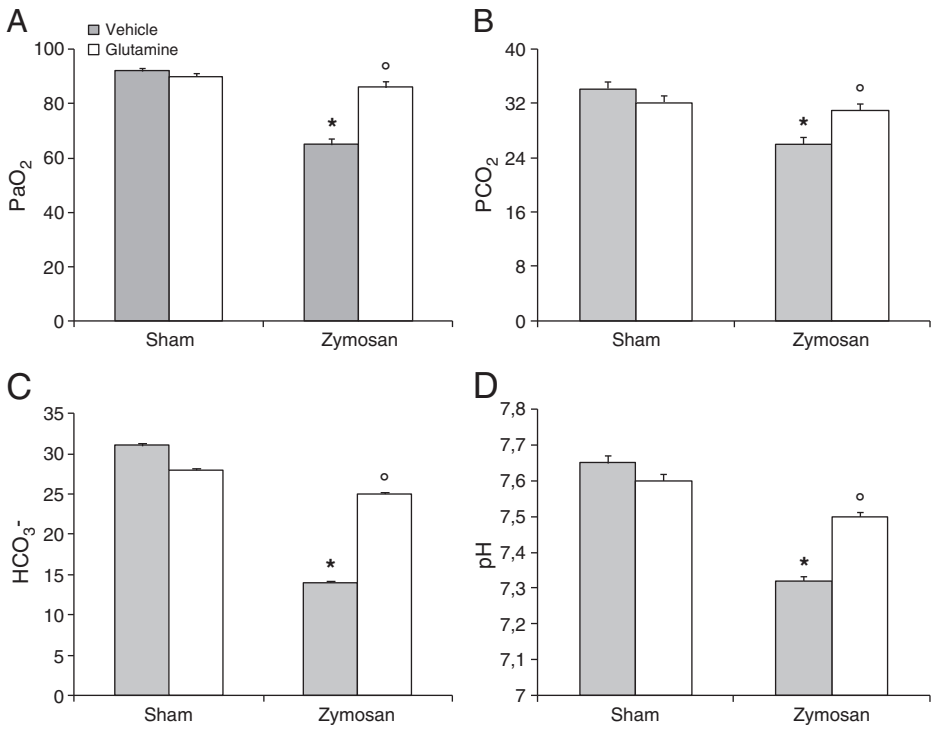

Fig. 9. Effect of glutamine on lung injury. Zymosan administration resulted in significant fall in the arterial levels of PaO_2 (A), PCO_2 (B), pH (C) and HCO_3^- (D). Administration of glutamine prevents lung dysfunctions. Data are means ± S.E.M. of 10 mice for each group. *$P<0.05$ *vs* sham, °$P<0.05$ *vs* zymosan + vehicle.

(Fig. 11C), suggesting the development of renal dysfunction. In contrast, treatment with glutamine significantly reduced the renal dysfunction caused by zymosan (Fig. 11C).

4. Discussion

In a review of 2005, Curi et al. showed that glutamine has a wide range of properties, which may justify its protective action. It plays a regulatory role in several cell specific processes including metabolism (e.g., oxidative fuel, gluconeogenic precursor, and lipogenic precursor), cell integrity (apoptosis and cell proliferation), protein synthesis, and degradation, contractile protein mass, redox potential, respiratory burst, insulin resistance, insulin secretion, and extracellular matrix synthesis (Curi et al., 2005). Moreover, it is well known that glutamine has a role also in the modulation of the immune cell function and in the cytokine production (Curi et al., 2005). Today, the exact molecular mechanism of glutamine action remains to be elucidated but we believe that it involves a complex of factors and not a single pathway, so that the protective power of this molecule ranges from antioxidant proprieties – preserving the metabolism of glutathione – to the activation of defense mechanisms, by the regulation of some anti-inflammatory genes.

Our workgroup and other authors have already showed a role of the glutamine in the decreasing gut ischemia/reperfusion injury (Ban and Kozar, 2008; Demirkan et al., 2010; Mondello et al., 2010), so in the present study we want to assess if glutamine has also a beneficial role in a mice multiple organ dysfunction syndrome model of injury.

Zymosan-administration induces non-septic shock in mice. A first consequence of this event is the increase of blood parameters – such as PCO_2, PaO_2, HCO_3, and pH —, renal dysfunction, neutrophil infiltration (as detected by MPO assay) and the following exudate formation, high level of nitrite/nitrate and cytokine release (revealed by biochemical assays and tissue staining). Animals show a decrease in body weight, increased mortality and heavy signs of acute pancreatitis and gut injury. Otherwise, this study has demonstrated that glutamine can reduce the zymosan-induced damage and the resulting inflammatory and apoptotic cascade.

Also an analysis of our histological data supports the view that glutamine attenuates the degree of non septic shock induced by zymosan in the mice. What, then, is the mechanism by which glutamine reduces non septic shock inflammation?

An enzyme that plays an important role in causing tissue damage is inducible nitric oxide synthase. It generates a reactive nitrogen species, the nitric oxide, an important messenger molecule involved in many physiological and pathological processes. Appropriate levels of nitric oxide production are important in protecting an organ such as the liver from ischemic damage, but high levels of nitric oxide may induce, directly and indirectly, tissue damage and toxicity and contribute to the vascular collapse associated with septic shock (Cuzzocrea et al., 2000, 2001).

In this study we have shown that the treatment with glutamine reduces the circulating NO levels, and, as consequence of a less ROS production, it also decreases the degree of lipid peroxidation.

Moreover, several studies have shown the presence of nitrotyrosine, a biomarker of the reactive nitrogen species peroxynitrite, in the course of the so-called "ZIGI" model (zymosan induced generalized inflammation) (Cuzzocrea et al., 2001) and an increased nitrotyrosine staining is considered as an indicator of increased nitrosative stress (Eiserich et al., 1998).

We have confirmed that nitrotyrosine was present in pancreas and gut taken after zymosan administration and that glutamine reduced the staining in these tissues.

The release of oxidant species causes severe cell damage. It is related to DNA single strand breakage and with the subsequent activation of the nuclear enzyme poly (ADPribose) synthetase (Cochrane, 1991). Massive ADP ribosylation of nuclear proteins by poly (ADPribose) synthetase can result in cellular energy depletion and injury (Szabo et al., 1996) and, ultimately organ damage. Moreover, several studies have shown that inhibition of poly (ADPribose) expression reduces organ injury elicited by intraperitoneal injection of zymosan (Szabo et al., 1997).

We demonstrate here that glutamine attenuates the increase of the poly (ADPribose) activity in the pancreas and gut from zymosan-treated mice.

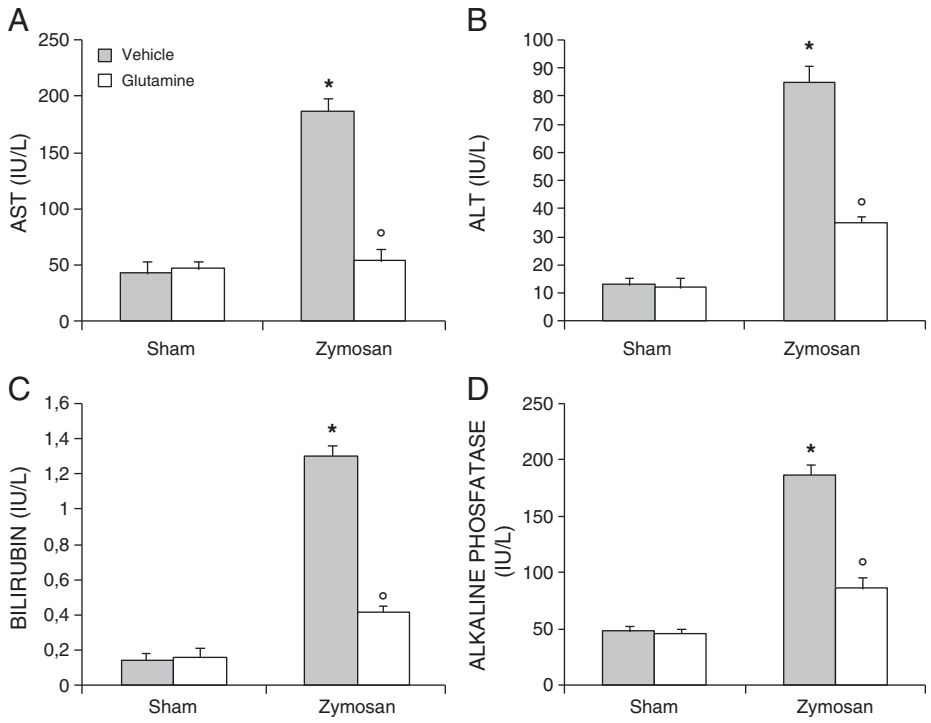

Fig. 10. Effect of glutamine on liver injury. Administration of zymosan resulted in significantly increased levels of plasma aspartate aminotransferase (A), alanine aminotransferase ALT (B), bilirubin (C) and alkaline phosphatase (D). Glutamine treatment significantly reduced all these parameters in zymosan treated mice. Data are mean ± standard deviation from $n = 10$ mice for each group. $^{*}P<0.05$ *vs* sham, $^{\circ}P<0.05$ *vs* zymosan + vehicle.

Inflammatory mediators, such as TNF-α, are also crucially involved and closely associated to apoptotic processes, that occur in FasL expression (Sharma et al., 2000). Fas forms the Death Inducing Signaling Complex upon ligand binding, a multi-protein complex formed by members of the death receptor family of apoptosis-inducing cellular receptors (Scott et al., 2008).

In this work, immunohistochemical localization of FasL and TUNEL staining, which highlights the presence of apoptotic cell bodies, showed

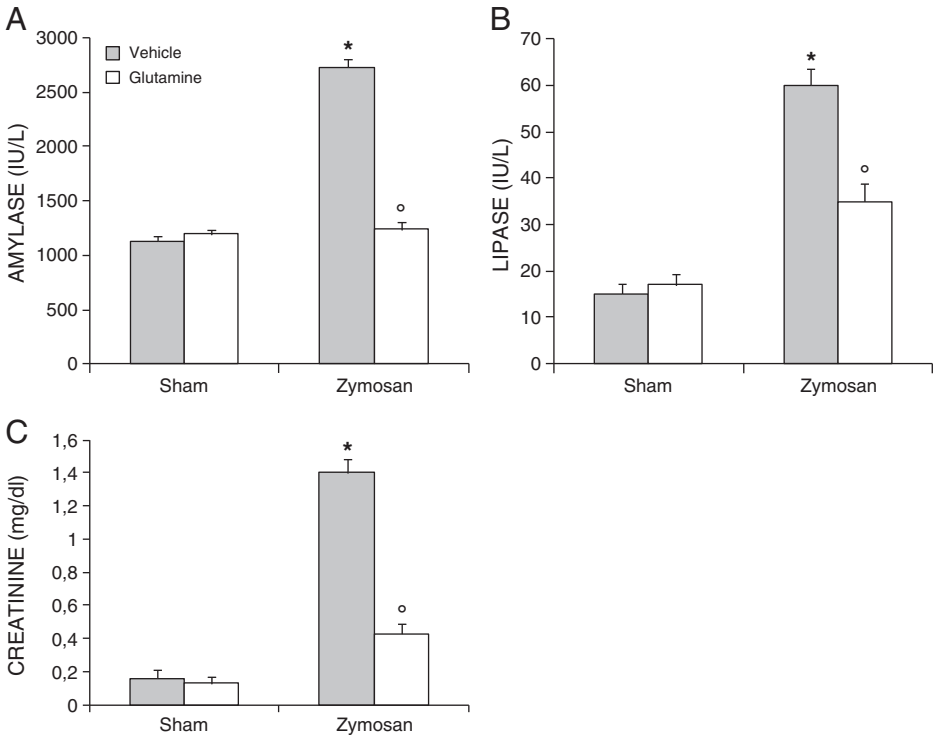

Fig. 11. Effect of glutamine on pancreatic and renal injury. Administration of zymosan resulted in significantly increased levels of plasma amylase (A), lipase (B) and creatinine (C). Glutamine treatment significantly decreases all these parameters in zymosan treated mice. Data are mean ± standard deviation from $n = 10$ mice for each group. $^{*}P<0.05$ *vs* sham, $^{\circ}P<0.05$ *vs* zymosan + vehicle.

that the zymosan-induced increase of FasL expression as well as the TUNEL-positive staining with a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments in pancreas and gut tissues are clearly reduced by the glutamine-mediated inhibition of the apoptotic processes.

Moreover, physiologically a balance between the members of pro-apoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (Bcl-Xl and Bcl-2) family is established. The ratio between these two subsets helps determine, in part, the susceptibility of cells to a death signal (Oltvai et al., 1993). Upon zymosan-injection this balance is lost and the pro-apoptotic homodimers that form in the outer-membrane of the mitochondria, make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c and SMAC, a mitochondrial protein that promotes cytochrome c-dependent caspase activation.

So, our data supported the idea that glutamine treatment reduces the increase of mitochondrial permeability and severe cellular injury, which leads to a higher expression of Bax than Bcl-2 production, as the immunohistochemical localization of Bax and Bcl-2 on sections of pancreatic and gut tissues has revealed.

5. Conclusions

At the end of this study we have clearly showed that glutamine treatment acts in decreasing histological injury, cytokine production, nitrosative stress and apoptosis and, more generally, the physiopathological alteration due to the zymosan injection.

Thus, glutamine treatment might be a therapeutic target for intervention finalized to reduce inflammatory processes and the injury consequent to non-septic shock.

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