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Evaluation of sepsis treatment with enteral glutamine in rats

Avaliação do tratamento da sepse com glutamina via enteral em ratos

Isadora Moscardini Fabiani[1]; Sérgio Luiz Rocha, TCBC-PR[1].

ABSTRACT

Objective: *to analyze the influence of glutamine on morphological and histological changes observed in the ileum, lung, kidney and liver of Wistar rats subjected to sepsis.* **Methods***: we induced sepsis by cecal ligature and puncture. We divided the animals in two groups: group A, control, with five animals, and group B, experience, with ten animals that received enteral glutamine two days before sepsis induction. We used histological analysis to rank the injury according to a score dependent on the injury severity and the affected organ. The sum of values assigned to each animal resulted in a final grade. We assessed the villi in the ileum, microgoticular steatosis in the liver, interstitial pneumonitis in the lungs, and vacuolation of the proximal convoluted tubules in the kidneys.* **Results***: cell lysis and destruction of the villi of the ileum were more intense in the control group when compared with animals receiving glutamine. In the kidney, we found more pronounced vacuolization in the proximal convoluted tubules in the control group compared with animals receiving glutamine. Both microgoticular steatosis and interstitial pneumonitis were similar between groups.* **Conclusion***: administration of enteral glutamine prior to sepsis preserved the histological structure.*

Keywords: *Sepsis. Glutamine. Peritonitis. Rats.*

INTRODUCTION

Sepsis is the main responsible for death of patients in Intensive Care Units worldwide[1]. The increase in its incidence, morbidity and mortality is due to the advances in the diagnosis and therapeutics of medicine, which allows the treatment of increasingly severe patients[2]. In 1991, the American College of Chest Phisicians and the Society of Critical Care Medicine established definitions for the diagnosis of the inflammatory response to infection. Such concepts, in addition to allowing researchers to compare results, facilitated an early diagnosis, as well as the determination of the appropriate prognosis and treatment for each patient[2,3].

Systemic inflammatory response syndrome (SIRS) is a generalized reaction of the organism to non-necessarily infectious agents, such as trauma, ischemia, burns, hemorrhage, among others. Sepsis, on the other hand, is the SIRS caused by infection, which can only be presumed. A worsening of the sepsis causing organic dysfunction or tissue hypoperfusion is defined as severe sepsis. Septic shock, finally, is sepsis concomitant with systemic arterial hypotension, which remains even after volume resuscitation requiring vasoactive drugs to maintain a Mean Arterial Pressure (MAP) >90mmHg[4].

A series of animal models[5] are used to simulate signs and laboratory findings of sepsis in humans. Induction of sepsis can be done by intravenous or intraperitoneal administration of living bacteria or microbial components, or by models of bowel injury with subsequent release of microbial flora[6]. It is important to note that rodents are, in general, the animals of choice for preclinical evaluations. They, however, have good resistance to endotoxins, limited vascular accesses, blood volume and a cardiocirculatory physiology that is significantly different from that of humans[5].

The systemic response in sepsis is due to the activation of the body's immune system with the release of cytokines, the TNF-alpha being the main proinflammatory mediator[7]. The inflammatory process, in turn, leads to an extensive release of glutamine from its main reservoir, the skeletal muscle. Although its synthesis is enhanced, there is still a depletion in its intracellular concentration. Even with the great release of this amino acid, there is

1 - Pontifical Catholic University of Paraná, Operative Technique, Curitiba, Paraná State, Brazil.

no increase in its serum concentration, demonstrating its high uptake by the other organs, mainly by the liver and cells of the immune system. That is, the availability of glutamine may limit essential cellular activities[8].

Studies have shown that various cell types, such as lymphocytes, macrophages and enterocytes, have increased proliferation as well as improved structural and physiological maintenance when in culture media containing glutamine[9]. It is known to modulate cellular immune function and the production of Cytokines, so that, in critical states, its deficiency is associated with impaired immune response and increased susceptibility to infection, since the exacerbated release of pro-inflammatory molecules results in increased intestinal permeability[10].

The intestinal epithelial cells have their proliferation, migration, differentiation and apoptosis affected by the organism'snutritional state. Glutamine acts as a trophic agent for the enterocytes, maintains the integrity of the mucosa and, therefore, decreases the chance of breakage of the intestinal barrier[10]. Therefore, it prevents bacterial translocation, preventing the spread of microorganisms[7,9]. It is thus evident that administration of glutamine may be useful in the treatment of sepsis, but its dose, means and time of administration are not yet defined.

The objective of this study is to analyze the response of sepsis induced by cecum ligation and puncture in rats previously treated with enteral glutamine, by evaluating intestinal, renal, hepatic and pulmonary histological changes.

METHODS

The present study was based on the project "Therapeutic Interventions in Experimental Sepsis", of Prof. Dr. Sérgio Luiz Rocha. The project was submitted to the Ethics Committee on Animal Use (CEUA) of PUCPR and was approved in 2014 under Protocol 893A.

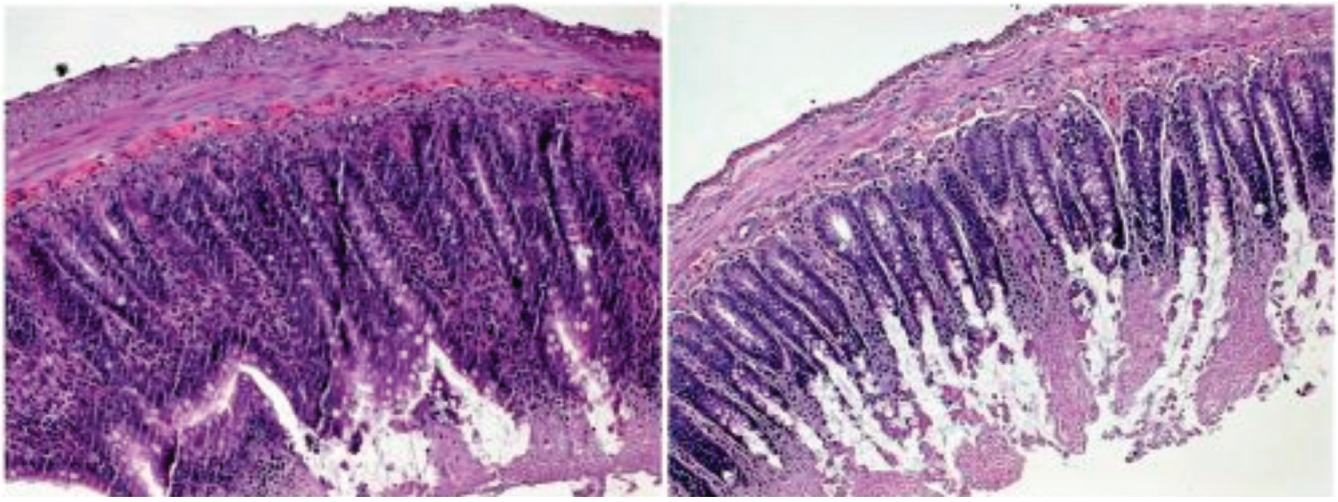
We used 15 Wistar rats, weighing between 250 and 350 grams. We allocated the animals to two groups: Group A – control (n=5), submitted to sepsis by cecalligature and puncture (CLP); and group B – experiment (n=10), which received 0.5g/Kg/day of Resource Glutamine powder, 48 hours prior to sepsis induction also by CLP. Before to the procedures, were anesthetized the animals with intraperitoneally ketamine hydrochloride solutions (80 ml/kg) and 2% xilasin (10ml/kg). After anesthetic induction, we performed tricotomy of the operative region and fixated the animal on the operative tablein supine position. After skin antisepsis with polyvinylpyrrolidone solution (PVPI), we started a 3cm laparotomy at the midline. After exposure of the cecum, we emptied it of feces by external compression, ligated it with 3-0 cotton suture 1cm distal to the ileo-cecal valve, without occluding the intestinal transit, and performed two transfixing punctures with a 40x12mm needle. Then, we re-inserted the cecum into the abdominal cavity and the sutured abdominal incision with 3-0 silk. After 48 hours, we euthanizedthe animals with intraperitoneal Thiopental Sodium at a dose of 180mg/kg, until the animal had cardiorespiratory arrest. Then we harvested the right lung, right kidney, liver and proximal and distal ileum for histological analysis. We used hematoxylin-eosin (HE) staining for the organs' histological analysis.

For subsequent biostatistics, were classified the lesions according to a score in which we attributed 0to absent lesions, 1 to mild lesions and value 2 to severe ones. The lesions in the ileum were multiplied by 3, the ones in the kidneys by 2, and the lesions in the lung and liver, by 1. In the ileum were evaluated hypoxia signs, in the liver, microgoticular steatosis, in the lung, interstitial pneumonitis, and in the kidney, the vacuolization of the proximal convoluted tubules. The sum of all values attributed to each animal resulted in its final grade.

For the total score of each animalwe used the following formula: Organ X Injury Severity = Injury Value; Sum of Injury Values = Animal Score.

We described theanimals' total scores by averagesand standard deviations, medians, minimum and maximum values. We used the non-parametric Mann-Whitney test to compare the groups in relation to the final score. Values of $p<0.05$ indicated statistical significance.

Figure 1. *Histological analysis of the Ileus (20X magnification).*



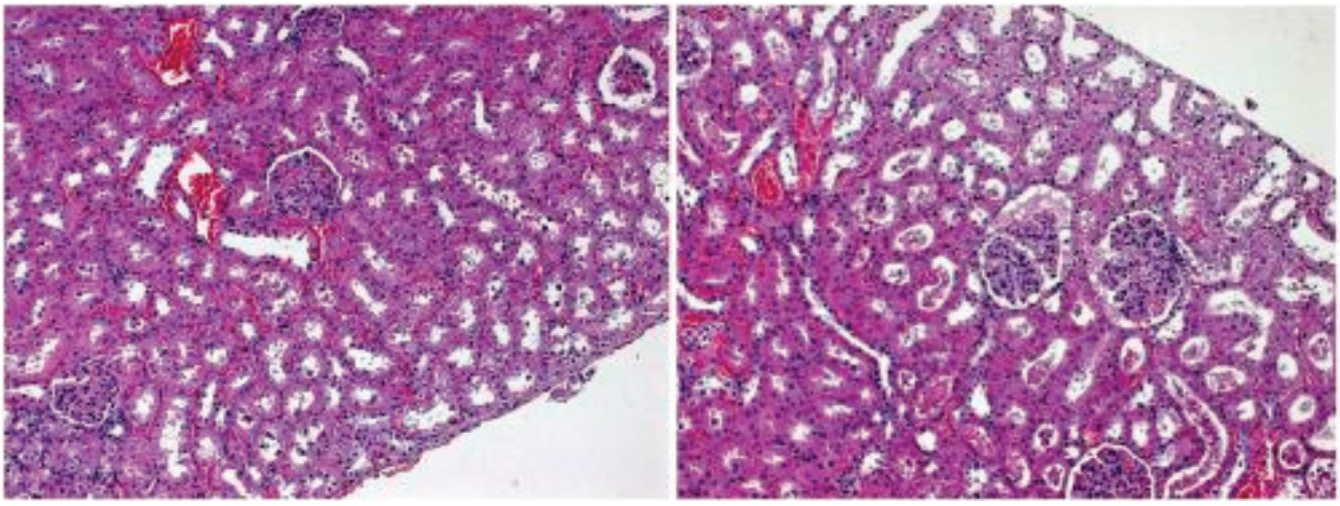
A) with glutamine; *B) without glutamine.*

RESULTS

In the histological evaluation, we found cell lysis and villi destruction in the control group to be significantly more intense compared with animals receiving previousglutamine supplementation (Figure 1). Among the other organs observed, the kidney presented the second greater difference between groups. Proximal convoluted tubule vacuolation was significantly more pronounced in the control group compared with animals receiving glutamine (Figure 2). In the liver, the microgoticular steatosis and, in the lungs, the interstitial pneumonitis observed were similar between the groups. With these observations, it was possible to assign scores to each of the animals taking into account the organs in which there was a greater difference between the groups and the severity of the respective lesions found (Table 1).

Since one animal from the control group and one animal from the experiment group diedduring the research, we reached the final scores presented in table 2, with the experiment group clearly showing lower scores. In the biostatistical analysis by the Mann-Whitney non-parametric test, we tested the null hypothesis that the results of the score are equal in both groups, versus the alternative hypothesis of different results. The result of the statistical test indicated the rejection of the null hypothesis (p=0.008). Thus, we

Figure 2. *Histological analysis of the kidney (20X magnification).*



A) with glutamine; *B) without glutamine.*

Table 1. *Values of lesions according to the affected organ and the degree observed.*

Site of injury/degree of injury	Ileum (3)	Rim (2)	Liver (1)	Lung (1)
Absent (0)	0	0	0	0
Discrete (1)	3	2	1	1
Severe (2)	6	4	2	2

can state that there is a significant difference between the control and experiment groups regarding the score.

DISCUSSION

The model used for the induction of sepsis in the present study was the cecum ligature and puncture, widely used in the study of sepsis, since its main advantage is its rapid performance and low cost. Because sepsis is caused by a surgical procedure, there is no need to culture or count bacteria to cause sepsis. In addition, this model represents the contamination by mixed flora, presenting important similarity with cases of appendicitis and perforated diverticulitis. The main disadvantage of this method is the difficulty of controlling the severity of the induced sepsis. In studies using small rodents, this problem can be solved by searching for a larger sample[5].

Another issue to be raised regarding sepsis induction models is that the natural history observed in laboratory animals will often be distinct from that of humans. In humans, the main cause of death from sepsis is multiple organ dysfunction, whereas in animal models death may occur within the first 6-12 hours and may not correspond to clinically observed outcomes. In addition, the agents to be researched are usually administered before or shortly after the occurrence of sepsis induction, a condition that is difficult to apply in the daily clinic[5].

Glutamine is a non-essential amino acid that plays a key role in the biosynthesis of cellular metabolites (nucleotides, glutathione and NAD+), which stimulate cell proliferation[11]. It is a limiting factor for the production of proteins necessary for the inflammatory response and is essential for the synthesis of glutathione (antioxidant molecule)[12]. When sepsis affectsan organism, the metabolism of glutamine in each organ is altered, so that intense release of glutamine from skeletal muscle and lungs takes place. The intestine, strangely enough, absorbs it less and less, while the liver and the immune system become its main consumers[13].

Glutamine plays a central role in the immune system cells, being a precursor of nucleotides and other molecules and contributing to the modulation of the function of macrophages and leukocytes, by reducing the concentration of cytokines[13].

This amino acid is a key element for the proliferation of intestinal epithelial cells and may be an essential substance for intestinal homeostasis during catabolic states. Glutamine-depleted enterocytes have been shown to gradually atrophy and sustain decrease in epithelial proliferation. If these enterocytes have their glutamine levels reestablished, signaling of the mammalian target of rapamycin (mTOR) protein occurs, in order to regain the proliferation and regeneration of the affected mucosa[14].

mTOR is a key mediator in the union of glutamine metabolism and intestinal cell activation. It ensures cell growth only at the right moment, ie when the cell has all the necessary nutrients[15]. It promotes growth in response to the presence of nutrients (amino acids and nitrogen), which leads to the activation of mTOR on the surface of lysosomes. Recently, it has been discovered that the communication between amino acid levels and mTOR is done by means of a complex called Npr2Complex. When nutrients are available, mTOR is activated so that glutamine can be used for biosynthesis. In cases of nutrient depletion, mTOR remains inactive[11].

In addition to its immune function, glutamine is the preferred energetic source of enterocytes and its depletion eventually affects the cellularity of the intestinal mucosa, contributing to alterations in the barrier function

Table 2. *Final scores of each animal.*

Control Group		Group Experiment	
Number of mouse	Score	Number of mouse	Score
10	12	3	4
11	9	4	7
12	12	5	7
16	Died	6	10
17	12	7	7
		8	Died
		9	7
		13	7
		14	9
		15	7

of the digestive epithelium, resulting in predisposition to bacterial translocation and, consequently, to sepsis. Thus, the supplementation of this amino acid can interfere in the following processes: (I) increasing the synthesis of glutathione, potentiating the antioxidant defenses; (II) maintaining the integrity of the intestinal mucosa, since it is an important energy source for enterocytes; (III) enhancing the synthesis of inflammatory responseprotein, attenuating the inflammatory process; and (IV) preserving the immune function, serving as an energy source for lymphocytes and cytokineprecursors[12].

Findings about regulatory functions and the contribution of glutamine to the body in stressful situations become more and more frequent. For septic patients, most studies involve the use of parenteral glutamine in the form of a dipeptide (because it is more soluble and more stable). When glutamine is administered enterally, the observed benefits are not the same. This is due to the first-pass metabolism in the enterocyte and liver, in which it serves as an energy source or precursor to other amino acids. Therefore, the mode of administration may be decisive in obtaining systemic effects. In the present study, we used the enteral route precisely because it had a more targeted action on the intestinal mucosa, allowing histological evaluation of its action and efficacy in relation to bacterial translocation[16].

Although the antioxidant effects observed by the parenteral route are not clearly demonstrated in the enteral use, its intestinal trophic effects justify the use of glutamine in severely burned and polytraumatized patients, since it reduces the incidence of bacteremia, but its use for septic patients is still subject to debate. The recommended dose of glutamine for such patients by the Federal Council of Medicine is 0.3 to 0.5 g/kg/day of supplemental glutamine divided into two or three doses. Recent studies, however, suggest that better effects can be achieved at higher doses, from 0.5 to 0.7 g/kg/day[17]. As enteral glutamine is safe and improves gastrointestinal tolerance, we opted for using 0.5 g/kg/day, a dose that is safe and shows greater efficacy for intestinal trophic actions, an evaluation that our study aimed at.

A randomized study published in the New England Journal of Medicine, in 2013, has raised a number of doubts in the use of glutamine and has further associated it with an increased mortality of critically ill patients with multiple organ failure. The authors themselves pointed out as possible cause of these results the use of a high dosage of glutamine, 30 grams per day, higher than the maximum already used in studies up to that time[18].

In our histological analysis, we found different lesion degrees between the glutamine group and the control group. Sepsis is associated with tissue oxygenation

abnormalities, which may arise due to inadequate oxygen distribution at microregional levels. This tends to cause tissue damage and, later, multiple organ dysfunction[19].

We observed a morphology suggestive of early ischemic lesions in the animals'small intestines, which were more severe in the ones that did not receive glutamine. Sepsis and septic shock cause a significant decrease in systemic vascular resistance and a redistribution of blood flow out of the splanchnic circulation, compromising the mucosa, which, in association with the hypermetabolic state characteristic of sepsis, makes small intestine villi the main target of the ischemic lesion. Due to hypoperfusion of the region, there is also depletion in the extraction and utilization of nutrients, mainly amino acids such as glutamine. The latter is decreased in sepsis or endotoxemia, and may cause local metabolic changes and contribute to mucosal atrophy. There are also changes in the red blood cell density, as well as in its average passage speed through the capillaries, increasing the development of areas of hypoxia and cellular injury[19].

The kidneys were the organs in which we observed the second largest variation of tissue involvement between the groups. In them, we found vacuolation of the proximal tubules. Although numerous studies have already been proposed to try to elucidate the induction of acute kidney injury by sepsis, the exact mechanism of sepsis has not yet been elucidated. Among the possibilities are vasodilatation that induces glomerular hypoperfusion, inflammation, oxidative lesions and tubular dysfunction. What is known is that renal tissue ischemia and reperfusion represent the main cause of acute renal injury in sepsis, which results from a decrease in oxygen levels, inducing apoptosis, damage to tubular epithelial cells, and tubular necrosis if the condition is prolonged. Pinto *et al.*[20], when histologically evaluating kidneys of adult rats submitted to sepsis induction by cecum ligation and puncture, observed edema, diffuse interstitial inflammatory infiltrate, flattened tubular cells with lumen dilation and bare basement membrane in the cortical region. They concluded that acute renal injury caused by sepsis results from an association of renal vasoconstriction of hemodynamic and inflammatory origin, characterized by endothelium damage and hemodynamic dysfunction, increased inflammatory mediators and generation of reactive oxygen species by tubular cells.

We did not assess vital signs and other dynamic patterns in the present study. However, previous enteral glutamine supplementation ameliorates the histologically observed lesions in villi of the small intestine and renal cortex in sepsis-induced animals.

Further studies are needed to define the best dose to be used enterally and whether this is in fact beneficial in terms of mortality, since the animals were sacrificed, not allowing such a comparison. There is still much to elucidate about sepsis'causative mechanisms and possible treatments[12,18,19].

We conclude that rats receiving enteral glutamine for 48 hours and then submitted to sepsis by ligature and cecal puncture presented significant preservation of the intestinal wall and kidney structure. In the lungs and liver, there was no significant difference.

R E S U M O

Objetivo: analisar a influência da glutamina nas alterações morfo-histológicas observadas em íleo, pulmão, rim e fígado de ratos *Wistar* submetidos à sepse. **Métodos**: a sepse foi induzida por meio de ligadura e punção do ceco. Os animais foram divididos em dois grupos: grupo A, controle, com cinco animais, e grupo B, experimento, com dez animais que utilizaram previamente glutamina por dois dias por via enteral. Na análise histológica, classificou-se as lesões de acordo com um escore cujo valor atribuído dependia da gravidade da lesão e do órgão acometido. A somatória dos valores atribuídos a cada animal resultou em sua nota final. No íleo, avaliaram-se as vilosidades; no fígado, esteatose microgoticular; no pulmão, pneumonite intersticial; e no rim, vacuolização dos túbulos contorcidos proximais. **Resultados**: a lise celular e a destruição das vilosidades no íleo do grupo controle foram mais intensas em relação aos animais que receberam glutamina. No rim, verificou-se vacuolização mais acentuada dos túbulos contorcidos proximais no grupo controle em relação aos animais que receberam glutamina. Tanto a esteatose microgoticular como a pneumonite intersticial mostraram-se semelhantes em ambos os grupos. **Conclusão**: o uso de glutamina via enteral previamente à sepse na dose de 0,5 g/kg/dia preservou de maneira significativa a estrutura histológica do intestino delgado e os rins em ratos.

Descritores: Sepse. Glutamina. Peritonite. Ratos.

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Mailing address:
Isadora Moscardini Fabiani
E-mail: isadoramf94@gmail.com