0148-6071/05/2901-0036$03.00/0
JOURNAL OF PARENTERAL AND ENTERAL NUTRITION
Vol. 29, No. 1
Printed in U.S.A.

Original Communications

The Effect of Preventive Use of Alanyl-Glutamine on Diaphragm Muscle Function in Cecal Ligation and Puncture–Induced Sepsis Model

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ABSTRACT. *Background:* Low muscle glutamine levels during sepsis are associated with reduced protein synthesis and elevated protein breakdown, in particular myofibrillar protein breakdown. Thus, in a cecal ligation and puncture (CLP)-induced sepsis model in the rat, we hypothesized that glutamine pretreatment would protect the diaphragm muscle function. *Methods:* Eighty-four male Wistar rats weighing between 180 g and 200 g received standard amino acid solution 1.2 g kg^{-1} per day intraperitoneally (IP) or standard amino acid solution 1.2 g kg^{-1} per day plus alanyl-glutamine (GLN) 0.25 g kg^{-1} per day (IP) during the first 6 days of the experiment. On the seventh day, CLP or sham procedures were applied. The sham and CLP groups were equally divided into 3 subgroups according to the termination of the experiment, which took place at either the 24^{th} hour, 48^{th} hour, or 72^{nd} hour. After the compound muscle action potentials (CMAP) were recorded from the diaphragms of the rats at these selected times, they were decapitated under ketamine/xylazine anesthesia, and diaphragms were harvested for biochemical and histopathological examination. *Results:* The mean area and amplitude of CMAP were significantly larger in sham+GLN groups when compared with CLP and CLP+GLN groups at all times ($p < .05$). Diaphragm Ca^{+2}-ATPase levels were found to be significantly decreased in CLP group at all times compared to sham groups ($p < .05$). Diaphragm reduced glutathione levels were significantly higher in sham+GLN groups when compared with CLP and CLP+GLN groups at all times ($p < .05$). In histopathologic assessment, moderate neutrophil infiltration, which was observed in CLP48, was significantly reduced with alanyl-glutamine supplementation in CLP+GLN48 group ($p < .05$). *Conclusions:* This study showed that glutamine pretreatment did not improve diaphragm muscle function, but prevented the biochemical and histopathological changes in diaphragmatic muscle in CLP-induced sepsis. However, further studies are needed to clarify whether a higher dose of glutamine supplementation might protect the diaphragmatic muscle functions. (*Journal of Parenteral and Enteral Nutrition* **29:**36–43, 2005)

Sepsis is the leading cause of mortality in intensive care units. Respiratory muscle dysfunction, especially diaphragmatic dysfunction, plays an important role in respiratory failure, which was demonstrated as a major cause of high mortality rates in sepsis.[1,2] Specifically, recent evidence has shown the reduction of respiratory muscle contractility as a mechanism of diaphragmatic dysfunction during sepsis. Although the role of reactive oxygen/nitrogen species and Ca^{+2}-ATPase was previously mentioned in the mechanism, the pathophysiology of respiratory muscle contractility is not fully understood.

The occurrence of the functional organ insufficiencies in sepsis depends on the prolonged duration of sepsis, which is characterized by wasting of lean body mass.[3–5] The muscle wasting results primarily from accelerated breakdown of the long-living myofibrillar proteins, and the preventive role of nutrition was demonstrated.[6,7] Glutamine, an abundant amino acid, is "conditionally" essential for nutrition in sepsis. Research into the metabolic role of glutamine in sepsis suggests that a conditional deficiency occurs because of increased and altered tissue demands, which exceed endogenous production.[3] Low muscle glutamine levels during sepsis are associated with this reduced protein synthesis and elevated protein breakdown, in particular myofibrillar protein breakdown.[7–9] Besides the pivotal role in muscle pathophysiology, it has been demonstrated that glutamine can significantly attenuate proinflammatory cytokine release, protect against end-organ damage, and decrease mortality rates in sepsis.[10,11]

We hypothesized that GLN pretreatment given to the rat *in vivo* would protect the diaphragmatic muscle function in cecal ligation and puncture (CLP)-induced sepsis model. To measure the glutamine pretreatment effect in this model, diaphragmatic muscle function was determined by compound muscle action potential (CMAP) electrophysiologically and by diaphragm Ca^{+2}-ATPase and reduced glutathione (GSH) levels biochemically. In addition, histopathological findings

Received for publication November 8, 2003.
Accepted for publication October 11, 2004.
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of diaphragm were used as another index of diaphragmatic injury.

MATERIALS AND METHODS

The experiments described in this manuscript were performed in adherence to the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals, and approval of the ethic committee of Mersin University School of Medicine was obtained before study.

Experimental Protocol

Eighty-four male Wistar rats, weighing between 180 and 200 g, were used in the present study. As a standard protocol, all the rats were housed in a quiet, unstressful environment for 1 week before study. All groups took 45-50 kcal/day regular rat chow (23.4% protein, 0.15 g per day of glutamic acid) during the experimental period.

In addition to the regular rat chow, standard amino acid solution (Freeamine III, Eczacıbaşı/Baxter Ltd, Istanbul, Turkey) 1.2 g/kg day intraperitoneally[12] (IP) was given to half of the rats ($n = 42$) during the first 6 days of the experiment. The remaining 42 rats received the regular rat chow, and a standard amino acid solution, 1.2 g kg^{-1} per day plus glutamine 0.25 g kg^{-1} per day as alanyl-glutamine solution (GLN; Dipeptiven, Fresenius Kabi, Austria) during the same period (as shown in Fig. 1).

On the seventh day, CLP or sham procedures were applied. The end of the experiment was chosen as the 24th hour, 48th hour, and 72nd hour for both sham and CLP groups. After the biophysical parameters were recorded at these selected times, rats were decapitated under ketamine/xylazine anesthesia (80 mg/kg, 10 mg/kg IM), and diaphragm was obtained for biochemical and histopathological examination.

CLP and Sham Procedures

In CLP animals, the cecum was ligated tightly at its base, in such a manner that bowel continuity was preserved, and punctured in a single pass through the anterior and posterior walls using an 18-G needle. The punctured cecum was squeezed gently to extrude fecal matter and was then returned to the peritoneal cavity. The laparotomy was repaired with continuous nylon sutures. In sham animals, the cecum was squeezed gently only and then returned to the abdomen, and the laparotomy was closed. All rats were resuscitated with saline solution (5 mL per 100 g body weight) injected subcutaneously at the back during the operation.

Biophysical Procedures

The CMAPs of diaphragm were recorded at the beginning of the study and at the 24th hour, 48th hour, and 72nd hour of CLP and sham groups.

Standardized electromyographic technique was used for recordings of diaphragmatic CMAP. Active and reference surface electrodes (BIOPAC EL200, Santa Barbara, CA) were used for recordings that were made from the diaphragm muscle. The active electrode was

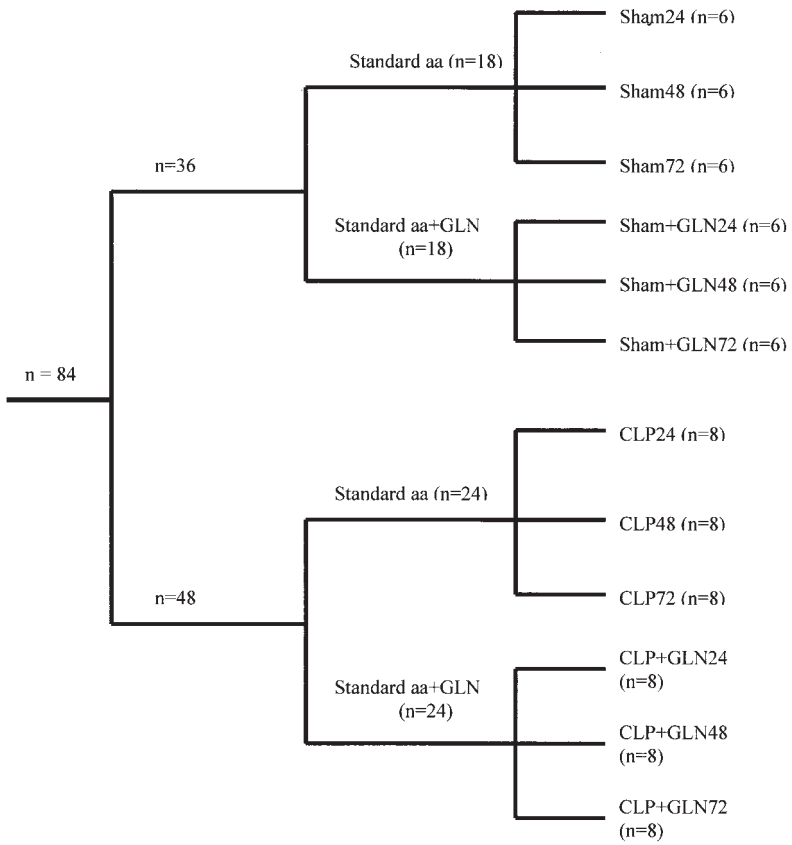

FIG. 1. Scheme shows the groups of rats.

TABLE I
The compound muscle action potentials (area and amplitude) of diaphragm at 24th hour of CLP and sham operation (mean ± SD)

CMAP parameters	Groups			
	Sham24	CLP24	Sham+GLN24	CLP+GLN24
Amplitude (mV)	0.67 ± 0.25	0.43 ± 0.22*	0.73 ± 0.27	0.44 ± 0.09*
Area (mV ms)	0.0049 ± 0.001	0.0022 ± 0.001*	0.0067 ± 0.003	0.0033 ± 0.009*

CLP, cecal ligation and puncture; CMAP, compound muscle action potentials; GLN, alanyl-glutamine.
*$p < .05$ Compared with Sham+GLN24 group.

placed just above the tip of the xiphoid process, and the reference electrode was positioned at the adjacent costal margin. The ground electrode was placed over the lower extremity. The phrenic nerve was stimulated using a standard bipolar surface stimulator (Medelec small bipolar nerve stimulator, number 16894 T, Oxford, UK) by pressing deeply along the lateral edge of the sternocleidomastoid muscle in the supraclavicular region. The supramaximal stimulus consisting of single square pulse (intensity 10 V, duration 0.2 ms) was used.[13]

Data were collected by means of a BIOPAC MP100 Acquisition System, version 3.5.7 (Santa Barbara, USA). BIOPAC Acknowledge Analysis software (ACK 100 W) was used to measure CMAP amplitude and area.

Biochemical Assay

Diaphragmatic Ca^{+2}-ATPase and reduced GSH levels have been calculated biochemically in all groups at the end of the experiment.

Ca^{+2}-ATPase Activity

Measurement of ATPase specific activity is based on the principle of inorganic phosphate release in 1 hour for each milligram protein in the presence of 3 mmol/L disodium ATP, added to the incubation medium. The inorganic phosphate was released from ATP into the incubation medium and then measured according to the method suggested by Reading and Isbir.[14,15] The protein quantity in the sample was determined according to the method developed by Lowry and his colleagues.[16]

The results of the measurements in ATPase enzyme systems were stated in nmol Pi/mg protein/h.

Reduced GSH

After tissue was homogenized in 5% NaCl, reduced GSH was measured according to Srivastava and Beutler.[17] The principle of the test depends on the 5,5′-dithiobis(2-nitro benzoic acid) [DTND], a disulfide compound that is readily reduced by sulfhydryl compounds, forming a highly colored yellow anion. The optical density of this yellow substance is measured at 412 nm.[17]

Histopathologic Examination

The tissues were dehydrated and embedded in paraffin. Sections (5 μm) were cut in a microtome, adhered to glass slides with polylysine. Hematoxylin and eosin–stained specimens were examined by a pathologist (LC) blinded to the sample identity. Diaphragmatic injury was assessed according to the intensity of inflammation, congestion, and changes in the muscle cells.[18] The histologic sections were graded between 0 and 4 according to these histopathologic findings.

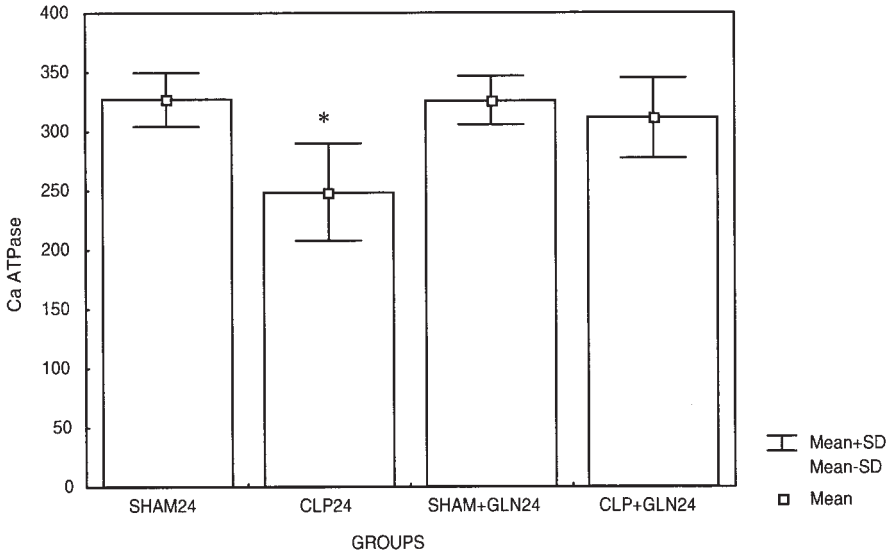

FIG. 2. Comparison of Ca^{+2}-ATPase levels between groups at the 24th hour. Ca^{+2}-ATPase levels were statistically lower in CLP group than other groups (*$p < .05$).

TABLE II
Reduced glutathione levels (μmol GSH/g wet tissue) of diaphragm at 24th, 48th, and 72nd hours of CLP and sham operation (mean ± SD)

GSH levels	Groups			
	SHAM	CLP	SHAM+GLN	CLP+GLN
24th	3.73 ± 0.60	2.88 ± 0.04*	5.03 ± 0.25	3.19 ± 0.04*
48th	3.78 ± 0.09	2.28 ± 0.09*	5.47 ± 0.40	2.71 ± 0.70*
72nd	4.17 ± 0.07*	2.19 ± 0.02*†‡	4.81 ± 0.04	2.74 ± 0.17*†

CLP, cecal ligation and puncture; GSH, glutathione.
Diaphragm GSH levels were significantly higher in sham+GLN group when compared to CLP and CLP+GLN groups (*$p < .05$). However, GSH levels were significantly higher in sham+GLN when compared to sham group at 72nd hour (*$p < .05$). GSH levels were significantly higher in sham group when compared to CLP and CLP+GLN groups at 72nd hour (†$p < .05$). GSH levels were significantly higher in CLP+GLN group when compared to CLP group at 72nd hour (‡$p < .05$).

Grade 0 represented normal histopathology. Grade 1 represented minimal capillary congestion and minimal edema in the interstitium without neutrophil infiltration. Grade 2 represented mild neutrophil infiltration (1–3 neutrophils per high-power field), intense capillary congestion, and edema. Grade 3 represented a moderate degree of neutrophils infiltration (4–10 neutrophils per HPW). Grade 4 represented focal abscess formation (clusters of neutrophils).

Statistical Analysis

Statistical analysis was performed with using SPSS for Windows 9.0 program. Comparisons of the Ca^{+2}-ATPase levels, GSH levels, and CMAP among the groups were made with one-way ANOVA combined with Bonferroni procedure used for *post hoc* comparison of data sets. Comparison of H and E staining score was analyzed using Kruskal-Wallis variance analysis. The results were considered statistically significant when $p < .05$.

RESULTS

Only 5 of 84 rats died at the 48th hour throughout the study.

The mean area and amplitudes in CMAP evaluation at the 24th hour were significantly larger in sham+GLN24 group when compared with that of CLP+GLN24 group and CLP24 ($p < .05$) (Table I). Ca^{+2}-ATPase levels were found significantly lower in CLP24 group when compared with the others ($p < .05$) (Fig. 2). Diaphragm GSH levels were significantly higher in sham+GLN24 group when compared with CLP24 and CLP+GLN24 groups ($p < .05$) (Table II). At the 24th hour, histopathologic examination revealed normal histopathology and minimal capillary congestion and minimal interstitial edema. The diaphragmatic muscle cells were normal in appearance. There was no statistically significant difference between the groups at the 24th hour histopathologic evaluation ($p > .05$) (Fig. 5*A*).

Biophysical evaluations at the 48th hour revealed that the mean area and amplitudes in sham+GLN48 group were significantly larger than that of the CLP+GLN48 and CLP48 groups. In addition, sham+GLN48 group was significantly larger than the sham48 group ($p < .05$) (Table III). Ca^{+2}-ATPase levels were significantly lower in CLP48 group when compared with the others ($p < .05$) (Fig. 3). Diaphragm GSH levels were significantly higher in sham+GLN48 group when compared with CLP48 and CLP+GLN48 groups ($p < .05$) (Table II). In the histopathologic examination, moderate neutrophil infiltration was observed in CLP48 group (Fig. 5C). Significantly reduced neutrophil infiltration was seen with glutamine supplementation in CLP+GLN48 group when compared with CLP48 ($p < .05$). The histopathological grades of CLP48 and CLP+GLN48 groups were significantly higher than that of sham48 and sham+GLN48 groups ($p < .05$).

At the 72nd hour biophysical evaluations, the mean area and amplitudes in CMAP in sham72 and sham+GLN72 groups were significantly larger than those of CLP72 and CLP+GLN72 groups ($p < .05$). Additionally, sham+GLN72 group had significantly larger CMAP values than the sham72 group ($p < .05$) (Table IV). Ca^{+2}-ATPase levels were significantly lower in CLP72 group when compared with the others ($p < .05$) (Fig. 4). Statistically significant difference in diaphragmatic GSH levels was detected among all the groups ($p < .05$) (Table II). At the 72nd hour, histopathologic findings were classified as grade 3 in the CLP72 group (Fig. 5*C*). There were significant differences in histopathologic examination when CLP72 and CLP+GLN72 groups were compared with the sham72 and sham+GLN72 groups ($p < .05$).

A normal histopathological appearance (grade 1) of diaphragm muscle was observed in sham and sham+GLN groups at all times.

DISCUSSION

The pathophysiology of the diaphragmatic insult in sepsis without direct diaphragmatic injury is complex and may involve secondary damage from activated inflammatory cells and mediators.[19,20] Glutamine may

TABLE III
The compound muscle action potentials (area and amplitude) of diaphragm at 48th hour of CLP and sham operation (mean ± SD)

CMAP parameters	Groups			
	Sham48	CLP48	Sham+GLN48	CLP+GLN48
Amplitude (mV)	0.66 ± 0.22*	0.28 ± 0.30*	0.95 ± 0.51	0.61 ± 0.38*
Area (mV ms)	0.0031 ± 0.001*	0.0021 ± 0.001*	0.0050 ± 0.003	0.0031 ± 0.002*

CLP, cecal ligation and puncture; CMAP, compound muscle action potentials; GLN, alanyl-glutamine.
*$p < .05$ Compared with Sham+GLN48 group.

TABLE IV
The compound muscle action potentials (area and amplitude) of diaphragm at 72nd hour of CLP and sham operation (mean ± SD)

CMAP parameters	Groups			
	Sham72	CLP72	Sham+GLN72	CLP+GLN72
Amplitude (mV)	1.10 ± 0.43*	0.44 ± 0.28*†	1.71 ± 0.54	0.51 ± 0.30*†
Area (mV ms)	0.0055 ± 0.008*	0.0023 ± 0.001*†	0.0068 ± 0.002	0.0032 ± 0.002*†

CLP, cecal ligation and puncture; CMAP, compound muscle action potentials; GLN, alanyl-glutamine.
*$p < .05$ Compared with Sham+GLN72 group.
†$p < .05$ Compared with Sham72 group.

play a role in mediating the response of the organism at the cellular level, especially in the diaphragmatic muscle. In the present study, we demonstrated that glutamine pretreatment may ameliorate diaphragmatic dysfunction in the CLP-induced sepsis model. Glutamine pretreatment, in addition to inhibition of the decrease in Ca^{+2}-ATPase levels of diaphragm, protected the structural integrity of the diaphragmatic muscle in this experimental model of sepsis.

Critical illness is characterized by the presence of several factors, including sepsis and starvation, that can cause marked alterations in the structure and functions of organ systems.[21] Damaged diaphragm function related to these factors has gained more attention recently.[18,22,23] The degree of muscle dysfunction in sepsis appears to be related to protein metabolism, free radicals and Ca^{+2}-ATPase.[22,24,25] In various muscle types, oxygen-derived free radicals have been implicated in alteration of normal Ca^{2+} homeostasis via disruption of normal sarcoplasmic reticulum function. This may be mediated by inhibiting the Ca^{2+}-ATPase pump or by activating the Ca^{2+} release channel.[26,27] Ca^{2+}-ATPase is found in the membrane of the sarcoplasmic reticulum and the plasma membrane, and drives Ca^{+2} from cytosol into intracellular depots such as endoplasmic and sarcoplasmic reticuli. Ca^{+2}-ATPase is an efficient protein reacting with myosin for contractions and relaxations of the diaphragmatic muscle.[18,28] The relationship between the pumping activity and Ca^{+2}-ATPase levels has been shown clinically and experimentally.[26,27,29] It is demonstrated that free radical scavengers might prevent dysfunction of the contractile proteins or altered sarcolemmal function after CLP.[20]

In our study, we found that glutamine supplementation prevented the decrease in Ca^{+2}-ATPase levels in CLP-induced sepsis. This is the first study in the literature measuring the effects of preventive usage of glutamine on Ca^{+2}-ATPase levels of the diaphragm in sepsis.

Endotoxin plays a role in the development of sepsis in CLP.[19] Endotoxin activates macrophages, which produce cytokines such as interleukin-1 and tumor necrosis factor-α (TNF-α). TNF-α is known as a potent activator of polymorphonuclear leukocytes, which triggers the production of oxygen-derived free radicals,[20] which in turn cause cellular damage. The histopathologic changes, namely, the interstitial edema formation and neutrophil infiltration, were observed at 48th hour and 72nd hour in CLP groups.

Glutamine, a constitutionally essential amino acid in sepsis, has several important roles such as being a precursor of antioxidant enzymes and protein synthesis.[6,9,10,30,31] As accumulated data showed that glutamine-enriched parenteral, intraperitoneal, and enteral nutrition enhances protein synthesis in the

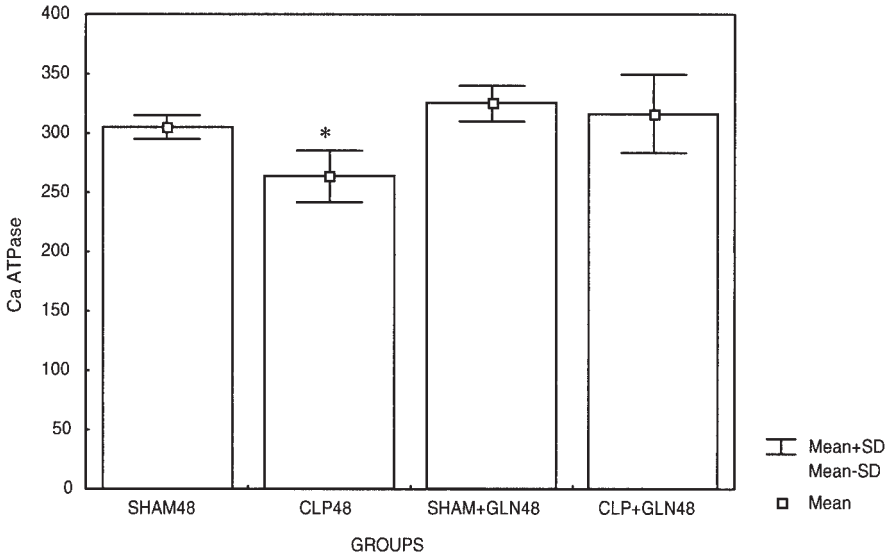

FIG. 3. Comparison of Ca^{+2}-ATPase levels between groups at the 48th hour. Ca^{+2}-ATPase levels were statistically lower in CLP group than other groups (*$p < .05$).

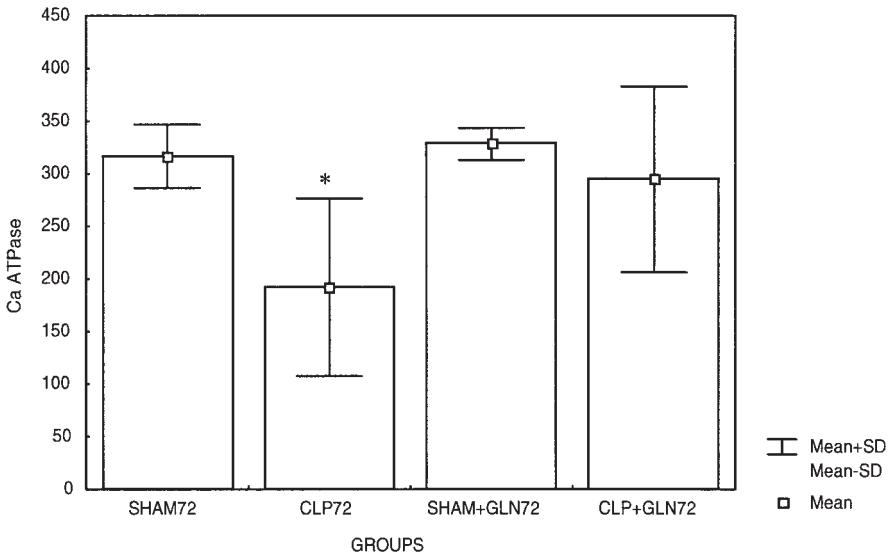

FIG. 4. Comparison of Ca^{+2}-ATPase levels between groups at the 72nd hour. Ca^{+2}-ATPase levels were statistically lower in CLP group than other groups (*$p < .05$).

muscle and plasma GSH stores, the beneficial effect of glutamine on critically ill patients may have been partly caused by this increase in GSH levels.[8–10,32–34] In the present study, although the GSH levels were preserved in the sham groups, decreases in diaphragmatic GSH levels were noticed in the CLP groups. The GSH levels increased more in the glutamine-supported CLP groups in the 72nd hour compared with the CLP-only

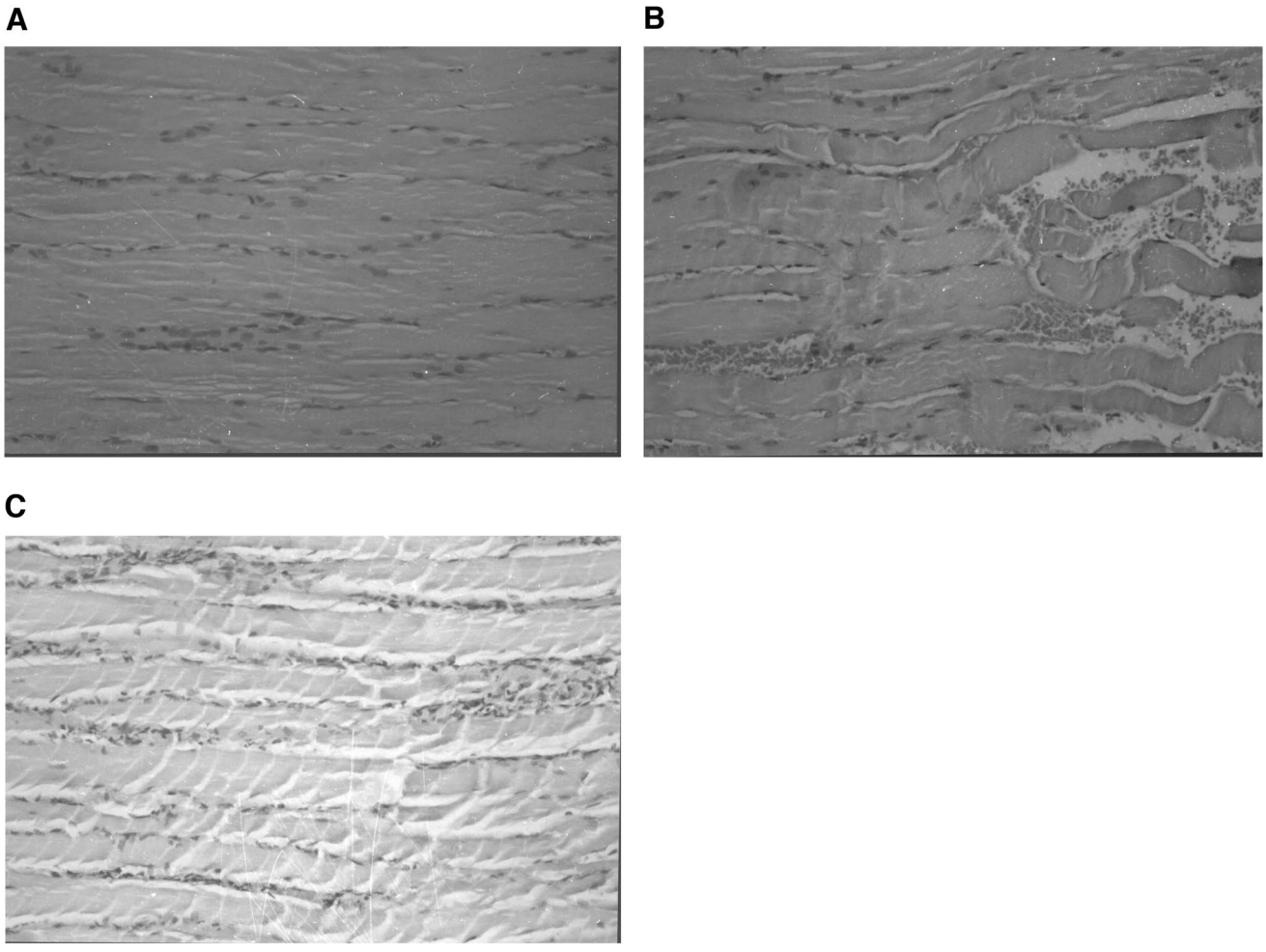

FIG. 5. Hematoxyline-eosin–stained sections of diaphragm showing histopathologic grades. A, grade 0; B, grade 2; C, grade 3 (HE ×200).

group. Besides, histopathologic findings were preserved at the 48th hour in both glutamine supported and nonsupported CLP groups, but at the 72nd hour, the findings between the groups did not differ significantly. This makes us think that the applied glutamine dose may have been too late to produce an effect.

Respiratory muscle dysfunction is described as a contributor to the morbidity of critically ill patients with a variety of illnesses. The difficulties related to ventilatory "weaning" in these patients are caused not only by intrinsic lung disease but also to reductions in respiratory muscle force-generating capacity. A number of previous *in vitro* studies have reported reductions in both respiratory and limb muscle force-generating capacity in response to sepsis and endotoxin administration. Van Surell et al[35] found that injection of *E. coli* lipopolysaccharide was followed by significant reductions in the pressures generated by the *in situ* diaphragm in response to both low (1–20 Hz) and high (50–100 Hz) frequencies of electrical stimulation. In another study, Supinski G et al[2] observed significant decreases in *in vitro* intact muscle fiber force production in both diaphragm and leg muscles of endotoxemic rats. In our study, we assessed the muscle function of the diaphragm with CMAP, which is the summated activity of the synchronously activated muscle fibers innervated by the axons and motor units represented in that muscle. Measurements of action potential area and amplitude are important because they inform us about the number of activated muscle filaments.[36] We found decreased amplitude and area in the CLP-induced sepsis group. Although amplitude and area did not differ between the CLP and CLP+glutamine groups, there was a significant difference between the sham and sham+glutamine groups. We believe that this significant difference is related to the metabolism of the rats. In the sepsis group, they have hypercatabolism; the dose of glutamine might have been insufficient because of this increased catabolic state. In the sham+glutamine group, the metabolism was normal and our glutamine dose was sufficient to play a role. Supporting this hypothesis, some studies have shown that hypercatabolic conditions required higher doses of glutamine. In 1 such study, Weingartmann et al[36] investigated the safety and efficacy of the dipeptide glycyl-glutamine in a dose-finding study in polytraumatized patients when compared with a control group. Nine polytraumatized patients received the dipeptide in 3 different doses: 280 (14 g), 450 (21 g) and 570 (28 g) mg per kilogram of body per day, indicating that the highest dose was necessary to induce a sustained effect on plasma glutamine levels.

In conclusion, this study showed that glutamine pretreatment did not improve diaphragm muscle function but prevented the biochemical and histopathological changes of diaphragm in CLP-induced sepsis. Although further studies are needed to confirm, we suggest that supplementation of glutamine at higher doses may also ameliorate the depression in the diaphragmatic muscle function, considering the relation between muscle fatigue and depressed Ca^{+2}-ATPase activity in sepsis.

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