Glutamine Concentration and Immune Response of Spinal Cord-Injured Rats

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Abstract

Background/Objectives: Glutamine plays a key role in immune response. Spinal cord injury (SCI) leads to severe loss of muscle mass and to a high incidence of infections. This study investigated the acute effect of SCI (2 and 5 days) on the plasma glutamine and skeletal muscle concentrations and immune responses in rats.

Methods: A total of 29 adult male Wistar rats were divided as follows: control (C; $n = 5$), sham-operated (S2; $n = 5$) and spinal cord-transected (T2; $n = 7$). They were killed on day 2 after surgery/transection (acute phase). Another set was sham-operated (S5; $n = 5$), spinal cord-transected (T5; $n = 7$), and killed at day 5 after surgery/transection (secondary phase). Blood was collected; the white portion of the epitrochlearis and gastrocnemius muscles and the red portion of soleus muscles were dissected to measure the glutamine concentration. Gut-associated lymphocytes and peritoneal macrophages were obtained for immune parameters measurements.

Results: Glutamine concentration in the plasma, gastrocnemius, and soleus muscles in rats with SCI were significantly reduced but not in the epitrochlearis muscle in the acute (2 days) and secondary (5 days) phases. Phagocytic response was reduced in the acute phase but increased in the secondary phase in rats with SCI. Superoxide production, on the other hand, was significantly increased at days 2 and 5 after SCI, and CD8⁺ lymphocytes subset decreased significantly on days 2 and 5.

Conclusions: Our results showed reduction in plasma glutamine and skeletal muscle concentrations after spinal cord transection. They also suggest that SCI and glutamine reduction contribute to an alteration in immune competence.

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Key Words: Spinal cord injuries; Glutamine; Macrophage response; CD4⁺ and CD8⁺ subsets

INTRODUCTION

Spinal cord injury (SCI) is one of the most incapacitating pathologies, leading to huge rehabilitation challenges (1–4). SCI can cause psychosocial and physiological sequelae, which combined with wheelchair confinement, promote a downward degenerative cycle of inactivity that may result in secondary complications and a decline in quality of life (1–7).

Skeletal muscle paralysis and autonomic dysfunction at the site and below the level of the lesion are two major findings after SCI leading to sensory, motor, and visceral alterations (1). There are 3 phases of SCI response after injury: acute, secondary, and chronic injury process (8,9). The acute phase (spinal shock) extends from the moment of injury through the first week in rats and through the second to third weeks in humans. It can induce a variety of important pathophysiologic processes including abolishment of autonomic activity and flaccid paralysis of the affected muscles of the limbs, which may lead to secondary physiologic disturbances such as autonomic dysreflexia (10,11), loss of sphincter control (12), thermoregulation dysfunction (13), postural hypotension (14), osteoporosis (15), neuro-immune-endocrine distur-

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bances, and severe loss of lean tissue of paralyzed limbs (3,4,16). The secondary phase, which occurs from minutes to weeks after injury (or months in humans), usually involves reduced autonomic activity (instead of abolishment), and the muscle tone gradually increases with the development of spasticity. Finally, the chronic phase occurs from days to months after injury in rats and in years in humans (8,17). However, many age-associated disorders develop prematurely and at a higher prevalence in the population with SCI (18). The upper and/or lower limb impairment usually causes structural, morphologic, and functional alterations in the skeletal muscles (6,16–19). This scenario is accompanied by autonomic disruption and secondary conditions including obesity (18,19), diabetes mellitus (20–22), heart failure (23,24), hypokinetic circulation (14,25), low levels of fitness (24,26,27), limited physical capacity caused by abnormal muscle fatigue enhancement (26–28), a higher incidence of infections (2–4), and an important neuro-inflammatory process at the site of lesion (29,30).

The physiologic characteristics of the paralyzed muscles, such as atrophy, loss of maximal force output, transformation toward fast phenotypic protein expression, metabolic alterations, and decreased resistance to fatigue (31,32), are well documented in the chronic phase, but further studies must be carried out during the early or acute phase when significant reduction of muscle mass occurs (16,18,31).

Actually, little is known regarding the nature and time-course of the effects of complete SCI on skeletal muscle. Cross-sectional studies, in which different groups of a few subjects were examined at different times (mostly years after injury), have already been carried out (33). Therefore, it is important to collect data from the early phase to understand and avoid some deleterious effects of acute SCI.

A high incidence of infections is one of the major causes of morbidity and mortality in subjects with SCI. The most common causes of septicemia after SCI are infection of the urinary and respiratory tracts and infected pressure ulcers. Furthermore, individuals with complete tetraplegia have a twofold higher mortality rate from infections/septicemia compared with those with complete paraplegia. In addition, rehospitalization caused by these infections is frequent, interrupts the rehabilitation process, and contributes to a sedentary lifestyle (2–4). Interestingly, despite all that, the immune system of individuals with SCI has been poorly studied.

Glutamine is the most abundant amino acid in the plasma and is actively produced in different organs such as kidneys, lungs, liver, and skeletal muscles. The skeletal muscles are the major glutamine source and release glutamine into the bloodstream at a high rate (34,35). Several studies have shown that glutamine is used at a high rate by lymphocytes, even in the resting state, and also by macrophages (36,37). Glutamine is required for lymphocyte proliferation in a dependent way. In the

absence of glutamine, T cells do not proliferate, but as the glutamine concentration in the culture medium increases, so does lymphocyte proliferation (36,37).

Glutamine plays many important biochemical roles. Plasma glutamine concentration is lowered in a variety of catabolic states such as surgery, sepsis, burns, trauma, athletic overtraining, and endurance exercise (34–39). All these conditions are associated with increased susceptibility to infections. It has been suggested that this susceptibility might be partly caused by a reduced supply of glutamine to leukocytes (37,40). As a result, the provision of glutamine to subjects in these catabolic states is of great interest (35).

High skeletal muscle proteolysis occurs in individuals with SCI (17) who also have a higher incidence of infections. Therefore, this study aims to investigate the effect of SCI on the plasma and skeletal muscle glutamine concentrations in rats, as well as on immune responses involving lymphocytes and the macrophage immune response during the early phase (2 and 5 days).

METHODS

All studies involving animals were approved by the Local Animal Ethics Committee from the Setor de Ciências Biológicas, Universidade Federal do Paraná.

Animals

A total of 29 adult male Wistar rats (obtained from the animal supply house of Universidade Federal do Paraná) weighing 250 to 300 g each were assigned as follows: controls (C; $n = 5$), one group of sham-operated (S2; $n = 5$), and one group of spinal cord-transected (T2; $n = 7$) rats killed on day 2 after surgery/transection (acute phase). Two other groups of rats, the sham-operated (S5; $n = 5$) and the spinal cord-transected (T5; $n = 7$) were killed on day 5 after surgery/transection (secondary phase). The animals were killed by decapitation using a guillotine. Blood was collected through a funnel into 15-mL tubes and allowed to clot for 30 minutes at room temperature. Serum was prepared by centrifugation and used for the measurement of glutamine concentration. The white portion of the epitrochlearis and gastrocnemius muscles and the red portion of soleus muscles were dissected and immediately frozen to determine glutamine concentration. Gut-associated lymphocytes and peritoneal macrophages were obtained for immune parameter measurements.

SCI Procedure

The animals were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). The midthoracic spinal cord was then exposed surgically through a laminectomy at the T4 vertebra. The cord was completely transected at the T5 spinal segment using a scalpel blade. The laminectomy exposure was then closed (8). After surgery, the animals were kept in individual cages, where they had easy access to food and

water. Manual emptying of bladders was performed 3 or 4 times daily until reflex voiding was established. The Multicenter Animal Spinal Cord Injury Study (MASCIS) (41) protocol for animal care was carried out. Sham-operated rats had the same surgical procedure but no cord transection.

Determination of Glutamine Concentration

Serum was obtained by centrifugation (800g) after deproteinization with perchloric acid (2%) and neutralized with KOH/Tris (2 mol/L/0.5 mol/L). Frozen skeletal muscles samples (gastrocnemius, soleus, and epitrochlearis) were immediately weighed and homogenized in icy perchloric acid (0.5 mol/L). Samples were centrifuged (Eppendorf–Centrifuge 5810 R, Hamburg, Germany) at 3,000 rpm at 4°C for 15 minutes. Glutamine content in the serum and muscle samples was quantified by measuring the absorbance at 340 nm (42).

Analysis of Lymphocyte Subsets

Flow cytometry was used to measure the presence of CD4⁺ and CD8⁺, markers of helper and cytotoxic T lymphocytes, respectively, on the surface of freshly prepared gut-associated lymphocytes. Approximately 10⁶ cells/mL in phosphate-buffered solution (PBS) supplemented with 0.1% serum fetal bovine (wt/vol) and 0.05% sodium azide (wt/vol) were incubated for 20 minutes at 4°C in the dark with fluorescent-labeled monoclonal antibodies to CD4⁺ (clone OX-38) and CD8⁺ (clone OX-8). After staining, the cells were washed twice with PBS and immediately examined for fluorescence using a Becton Dickinson FACScalibur (Becton Dickinson, Lincoln Park, NJ) (43).

Macrophage Isolation

Resident macrophages were obtained by intraperitoneal lavage with 10 mL of sterile PBS. The peritoneal cells were collected by centrifugation (290g at 4°C for 5 minutes), washed, and resuspended in PBS or Roswell Park Memorial Institute (RPMI) medium after counting in a Neubauer chamber by optical microscopy, using a Trypan blue solution (1%); viability was 96%. Peritoneal cells were characterized by flow cytometry; purity was about 50%. Macrophages were further purified by incubating peritoneal cells in tissue culture plates for 2 hours and washing 3 times with PBS to remove the nonadherent cells (44).

Phagocytosis

Aliquots (0.1 mL) of macrophage suspension were added to the wells of a 96-well flat-bottomed tissue culture plate and left to adhere for 40 minutes. Then, 10 µL of neutral-red stained zymosan (1 × 10⁸ particles/mL) was added to each well. After incubation for 30 minutes, the cells were fixed with Baker's formol-calcium (4% formaldehyde, 2% sodium chloride, 1% calcium acetate) for 30 minutes. The cells were washed 2 times by centrifugation (453g for 5

minutes). The neutral-red stain was solubilized by adding 0.1 mL of acidified alcohol (10% acetic acid, 40% ethanol in distilled water) to each well. After 30 minutes, the absorbance at 550 nm of each well was read on a plate reader. Phagocytosis was calculated from a standard curve constructed from known amounts of stained zymosan, and results are expressed per milligram of protein (45).

Lysosomal Volume

The uptake of the cationic dye neutral-red, which concentrates in cell lysosomes, was used to assess the volume of the macrophage lysosomal system. Twenty microliters of 3% neutral-red in PBS was added to 0.1 mL of macrophages suspension per microplate well for 30 minutes. The cells were washed twice with PBS by centrifugation (453g for 5 minutes). Neutral-red was solubilized by a 30-minute incubation, adding 10% acetic acid plus 40% ethanol solution. The absorbance was read at 550 nm, and neutral-red uptake was calculated per milligram of protein (45).

H₂O₂ Production

H₂O₂ production by peritoneal macrophages was measured as described by Pick and Mizel (46). This assay is based on the horseradish peroxidase (HRPO)-dependent conversion of phenol red into a colored compound by H₂O₂. Macrophages (final volume, 0.1 mL) were incubated in the presence of glucose (5 mmol/L), phenol red solution (0.56 mmol/L), and HRPO (8.5 U/mL) in the dark for 1 hour at 20°C. After this period, the absorbance was measured at 620 nm on a plate reader. The H₂O₂ concentration was determined from a standard curve prepared in parallel. H₂O₂ production is expressed as micro mol per milligram of protein.

O₂⁻ Production

Superoxide production was estimated by the nitroblue tetrazolium (NBT) reduction assay. Macrophages (0.45 mL) suspended in PBS were incubated for 1 hour at 37°C in the presence of 0.03 mL phorbol myristyl acetate (PMA; 5 µmol/L final concentration) and 0.1% NBT. The reaction was stopped by adding 0.45 mL of acetic acid. The mixture was centrifuged for 30 seconds at 2,500g. Reduction of NBT results in the formation of blue formazan, which was detected spectrophotometrically (560 nm). The results are expressed as absorbance per milligram of protein (47).

Statistical Analysis

Statistical analysis was performed by 1-way ANOVA followed by a post hoc Tukey test. A value of *P* < 0.05 was taken to indicate statistical significance.

RESULTS

Body Weight

Body weight of all groups on the presurgery day was expressed as 100%. The C group increased their body

Figure 1. Body weight variation for 5 days (%) in the T, S and C rats. Values are given as means $\pm$ SE of 5 (C) and 7 rats (S and T). * ($p \leq 0.05$) when compared with control group (C). † ($p \leq 0.05$) when compared with sham-operated group (S).

weight by 2% and 5% on days 2 and 5, respectively (Figure 1). The S group reduced their body weight by 4% on day 2 ($P < 0.05$ vs C), and on day 5, the body weight recovered to values before surgery. T rats had their body weight reduced by 10% ($P < 0.05$ vs C) on day 2 (T2) and by 12% ($P < 0.05$ vs C) on day 5 (T5).

Glutamine Concentration

Glutamine concentration (Table 1) in the plasma was significantly reduced in the T group after 2 and 5 days of SCI compared with C rats. In the S group, only on day 2 there was a significant reduction in the plasma glutamine concentration ($P < 0.05$ vs C). There was no significant difference in the glutamine concentration in the epitrochlearis muscle between the groups ($P > 0.05$). However, in the white portion of the gastrocnemius and soleus muscles, there was a remarkable reduction of glutamine concentration in the T group on day 2 (T2; $P < 0.05$ vs C and S) and on day 5 ($P < 0.05$ vs C, S, and T2).

Phagocytosis

Peritoneal macrophages (Figure 2) from S2 and T2 rats on day 2 exhibited reduced phagocytosis that was about 4- and 2.8-fold lower compared with C rats ($P < 0.001$), respectively. In contrast, on day 5, there was a significant

Figure 2. Phagocytosis of zymosan by macrophages from C, S, and T rats at days 2 and 5. Data are presented as means $\pm$ SE. * ($p \leq 0.001$) when compared with control group (C). † ($p \leq 0.001$) when compared with 5 day group: sham-operated (S5) and spinal cord-transected (T5).

increase in phagocytosis by tenfold in the S5 and T5 groups compared with S2, T2, and C rats ($P < 0.001$).

Lysosomal Volume

The uptake of neutral red (Figure 3) on day 2 by peritoneal macrophages from S2 and T2 rats was 10-fold lower compared with that of C rats ($P < 0.001$). On day 5, the uptake of neutral-red by peritoneal macrophages from S5 and T5 rats was 1.7- and 1.4-fold higher ($P < 0.05$) compared with that of C rats, respectively.

H₂O₂ Production

H₂O₂ production by peritoneal macrophages on days 2 and 5 after SCI was not different between the groups (Table 2).

Table 1. Glutamine Plasma Concentration and Glutamine Concentrations of Epitrochlearis, Gastrocnemius, and Soleus Muscles of C, S, and T Rats at Days 2 and 5

	C (n = 5)	S2 (n = 5)	T2 (n = 7)	S5 (n = 5)	T5 (n = 7)
Plasma (mmol/L)	0.56 $\pm$ 0.006	0.53 $\pm$ 0.007 [†]	0.51 $\pm$ 0.002 [†]	0.56 $\pm$ 0.002	0.52 $\pm$ 0.006 [†]
Epitrochlearis (mmol/kg wet weight g)	3.15 $\pm$ 0.08	3.15 $\pm$ 0.02	3.12 $\pm$ 0.02	3.08 $\pm$ 0.07	2.97 $\pm$ 0.04
Gastrocnemius (mmol/kg wet weight g)	3.12 $\pm$ 0.02	3.06 $\pm$ 0.05	2.82 $\pm$ 0.01 [‡]	3.25 $\pm$ 0.06	2.53 $\pm$ 0.10 [‡]
Soleus (mmol/kg wet weight g)	6.22 $\pm$ 0.18	6.29 $\pm$ 0.20	5.04 $\pm$ 0.12*	6.21 $\pm$ 0.28	4.39 $\pm$ 0.25 [†]

Data are presented as means $\pm$ SE.

* $P < 0.05$; [†] $P < 0.01$; [‡] $P < 0.001$

Figure 3. Neutral red uptake by peritoneal macrophages from C, S, and T rats at days 2 and 5. Data are presented as means $\pm$ SE. * ($p \leq 0.001$) when compared with control group (C). † ($p \leq 0.001$) when compared with 5 day group: sham-operated (S5) and spinal cord-transected (T5).

O₂⁻ Production

Superoxide production (Table 2) on day 2 by peritoneal macrophages from S2 and T2 rats was 4.1- and 2.6-fold higher compared with that of C rats ($P < 0.05$), respectively. On day 5, the superoxide production by peritoneal macrophages from S5 and T5 rats was lower than that found on day 2 ($P < 0.05$), but compared with C rats, it was 1.8-fold higher ($P < 0.05$).

CD4⁺ and CD8⁺ Lymphocyte Subsets

SCI did not alter the percentage of CD4⁺ lymphocytes (Table 3) on days 2 and 5 ($P > 0.05$). However, the percentage of CD8⁺ lymphocytes in the T2 group was reduced by 1.3-fold compared with C and S2 groups ($P < 0.05$). On day 5, the percentage of CD8⁺ lymphocytes in the S5 group was not different from the presurgery day. On day 5, the reduction was 1.3-fold in the T5 rats compared with the C and S2 groups ($P < 0.05$).

DISCUSSION

Complete SCI leads to muscle inactivation and profound muscle unloading (6). The muscles of patients with SCI are characterized by severe atrophy and extensive altered metabolic and protein profiles (6,16,17). Indeed, rats

submitted to surgery on day 2 (S2 and T2) presented a significant loss of body weight (Figure 1). This corroborates the findings that major surgery causes body wasting (6,7,16,18). On day 5, because of the healing process, the body weight of animals that were sham-operated returned to presurgery values. On the other hand, in the spinal cord-transected rats, the loss of body weight continued on day 5. It has been shown that, during the early phase of SCI, cord transection leads to flaccid paralysis in affected hind limbs muscles, which is generally maximal up to 7 days (6,16).

There are 2 major findings in our study. One is the reduction of glutamine concentration in the plasma and skeletal muscles (Table 1). Generally, this feature is also reported in special conditions such as sepsis and prolonged exercise, both related to immune suppression (36,38). The plasma glutamine levels dropped immediately after SCI. It has been also reported that subjects with SCI injured for 6 months have plasma glutamine concentration 54% lower compared with able-bodied subjects (48). An interesting finding was the maintenance of glutamine concentration in the epitrochlearis muscle, which is a forelimb muscle. The explanation for this finding might be because of a neurologically preserved muscle. In contrast, glutamine concentration in the white portion of the gastrocnemius and soleus muscles, which were affected by SCI, dropped on days 2 and 5 ($P < 0.001$). Furthermore, on day 5, the reduction was higher than that found on day 2 ($P < 0.05$). The soleus muscle (type 1 fiber) presented glutamine levels 2-fold higher ($P < 0.01$) compared with the white portion of the gastrocnemius and epitrochlearis muscles (type 2 fiber). These results support other findings (49). Our results show that SCI affects glutamine concentration in skeletal muscles. Therefore, SCI induced a lower glutamine concentration in the affected muscles, which contributes to metabolic and structural progressive degeneration as reported (6,16,17).

The other major finding concerns immune function. Phagocytosis (Figure 2) and lysosome volume (Figure 3) were reduced on day 2 in the rats submitted to surgery (S2 and T2). Interestingly, on day 5, there was a remarkable response leading to values well above those recorded before surgery ($P < 0.001$ vs C) in both groups (S5 and T5). Experimental and clinical studies have shown that surgical trauma markedly affects the immune

Table 2. H₂O₂ and O₂⁻ Macrophage Production of C, S, and T Rats at Days 2 and 5

	C (n = 5)	S2 (n = 5)	T2 (n = 7)	S5 (n = 5)	T5 (n = 7)
H ₂ O ₂ μ mol (mg ⁻¹ protein)	100 $\pm$ 2.92	102 $\pm$ 2.16	101 $\pm$ 1.69	103 $\pm$ 1.88	102 $\pm$ 1.02
O ₂ ⁻ OD (mg ⁻¹ protein)	0.12 $\pm$ 0.02	0.49 $\pm$ 0.07*	0.31 $\pm$ 0.02*	0.22 $\pm$ 0.02*	0.21 $\pm$ 0.02*

Data are presented as means $\pm$ SE.

* $P < 0.05$.

Table 3. CD4⁺ and CD8⁺ Lymphocyte Expression of C, S, and T Rats at Days 2 and 5

	C (n = 5)	S2 (n = 5)	T2 (n = 7)	S5 (n = 5)	T5 (n = 7)
CD4 (% 10 ⁴ events)	49.51 ± 0.77	51.63 ± 0.44	46.88 ± 0.84	43.96 ± 0.10	47.31 ± 0.33
CD8 (% 10 ⁴ events)	34.87 ± 0.91	34.40 ± 0.26	26.65 ± 1.10*	36.34 ± 0.41	25.95 ± 0.84*

Data are presented as means ± SE.

* $P < 0.001$.

system, including both specific and nonspecific immune responses. While it is suggested that minor surgery stimulates individual components of the immune system, it is generally agreed that major surgery, although associated with a local acute phase response, causes immunosuppression (50–52). During the early postoperative course, this may define a state of impaired defense and increased susceptibility to infection and septic complications, particularly in patients with polytrauma, burn injury, and cancer. In addition, the hyperinflammatory response will occur, which is characterized by macrophage oxidative burst among other immune responses. This suggests that the surgery itself induced these responses and not cord transection per se. The H₂O₂ and O₂[−] production (Table 2) are important macrophage-derived cytotoxic agents (43). The O₂[−] production was significantly higher in the S and T groups on day 2 ($P < 0.05$) but was reduced markedly on day 5 compared with C rats ($P < 0.05$). These results suggest that SCI activates the innate immune system and that the mechanisms should be studied in future research.

CONCLUSION

Regulatory T cells are vital to orchestrate the elaborate immune system, and the cytotoxic T cells directly attack body cells that are infected or malignant (51). T-helper cells carry a CD4⁺ marker, a molecule that recognizes class II MHC antigens. A molecule known as CD8⁺, which recognizes class I MHC antigens, is found in many suppressor/cytotoxic T cells. In addition, different T cells have different kinds of antigen receptors—either alpha/beta or gamma/delta (50–52). SCI did not alter the CD4⁺ lymphocyte subset but reduced the CD8⁺ subset markedly on days 2 and 5 (Table 3). Because CD8⁺ cells can act as suppressors of the immune response, the decrease in their numbers may improve T-cell proliferative responses.

Our results showed a reduction in the plasma glutamine and skeletal muscle concentration by spinal cord transection. It also suggested that SCI and glutamine reduction contribute to immune competence alteration.

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