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ORIGINAL ARTICLE

# Plasma iron levels appraised 15 days after spinal cord injury in a limb movement animal model

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**Study design:** Experimental, controlled trial.
**Objectives:** The purpose of this study was to evaluate plasma iron and transferrin levels in a limb movement animal model with spinal cord injury (SCI).
**Setting:** Universidade Federal de São Paulo, Departamento de Psicobiologia.
**Methods:** In all, 72 male Wistar rats aged 90 days were divided into four groups: (1) acute SCI (1 day, SCI1), (2) 3 days post-SCI (SCI3), (3) 7 days post-SCI (SCI7) and (4) 15 days post-SCI (SCI15). Each of these groups had corresponding control (CTRL) and SHAM groups. Plasma iron and transferrin levels of the different groups were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test.
**Results:** We found a significant reduction in iron plasma levels after SCI compared with the CTRL group: SCI1 (CTRL: $175 \pm 10.58\,\mu g\,dl^{-1}$; SCI: $108.28 \pm 11.7\,\mu g\,dl^{-1}$), SCI3 (CTRL: $195.5 \pm 11.00\,\mu g\,dl^{-1}$; SCI: $127.88 \pm 12.63\,\mu g\,dl^{-1}$), SCI7 (CTRL: $186 \pm 2.97\,\mu g\,dl^{-1}$; SCI: $89.2 \pm 15.39\,\mu g\,dl^{-1}$) and SCI15 (CTRL: $163 \pm 5.48\,\mu g\,dl^{-1}$; SCI: $124.44 \pm 10.30\,\mu g\,dl^{-1}$) ($P<0.05$; ANOVA). The SHAM1 group demonstrated a reduction in iron plasma after acute SCI (CTRL: $175 \pm 10.58\,\mu g\,dl^{-1}$; SHAM: $114.60 \pm 7.81\,\mu g\,dl^{-1}$) ($P<0.05$; ANOVA).
**Conclusion:** Reduced iron metabolism after SCI may be one of the mechanisms involved in the pathogenesis of sleep-related movement disorders.
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**Keywords:** periodic limb movement; restless legs syndrome; spinal cord injury; plasma iron

## Introduction

Periodic leg movement (PLM) and restless leg syndrome (RLS) are sleep disorders arising from neurological disorders that may involve the dopaminergic and iron systems, among other factors.[1–4]

PLM is also sometimes associated with other conditions such as spinal cord injury (SCI).[5,6] Esteves *et al.*[7] evaluated the occurrence of limb movements during sleep in rats with SCI. In their study, the animals began to present leg movements during sleep at 4 days after SCI. In 2007, Esteves *et al.*[8] analyzed sleep patterns in SCI animals. In addition to experiencing reduced sleep efficiency and an increased number of arousals, the animals presented limb movements that began at 4 days after lesion and remained until the end of the experiment, thus constituting a PLM animal model with SCI. Similar to this SCI animal model, a decreased sleep efficiency, an increase in the number of arousals and in the presence of PLMs have been reported in clinical situations.[9]

Iron concentration is another factor associated with SCI and PLM. Liu *et al.*[10] analyzed the total concentrations of iron, protein carriers and low-weight iron in rats with SCI between the T3 and L1 vertebrae. Their study found a rapid increase in the total extracellular iron levels 20 min after lesion impact that peaked at $1.32 \pm 0.77\,\mu m$ in the first sample. Iron levels had declined by 1 h after lesion, and they remained low for 2 h.

However, long-term variations in iron levels after SCI have not yet been addressed in the literature. Most studies have examined variations in iron levels shortly after SCI but have not monitored long-term developments. Therefore, the aim of this study was to evaluate plasma iron and transferrin levels in a limb movement animal model at longer time periods after SCI.

## Materials and methods

This study was part of the Psychobiology and Exercise Research Center research program at the Psychobiology

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Department, Universidade Federal de São Paulo and was approved by the Research Ethics Committee of Hospital São Paulo, Universidade Federal de São Paulo (0061/04).

Male Wistar rats at 90 days of age were used in this study. The rats were from the breeding facility at the Psychobiology Department, Universidade Federal de São Paulo.

Rats were kept under a 12-h light/dark cycle, and the temperature was maintained at $23 \pm 2\,^{\circ}C$. Chow and water *ad libitum* were supplied to standard cages. Cages were cleaned every day to remove the sawdust used as bedding before the experiment.

A set of four experiments was performed using animals divided into groups:

- SCI1: 1 day after SCI ($n = 7$)
- SCI3: 3 days after SCI ($n = 9$)
- SCI7: 7 days after SCI ($n = 5$)
- SCI15: 15 days after SCI ($n = 9$)

For each group, there was a corresponding control (CTRL) group and a corresponding SHAM group.

At the end of the experiment, the rats were decapitated at the same time (1000 hours) each day for blood collection and subsequent iron and transferrin analysis.

*Spinal cord injury*

The rats were anesthetized with a combination of ketamine hydrochloride (Ketamine, Merck, São Paulo, Brazil) at $140\,mg\,kg^{-1}$, intraperitoneal and diazepam (Valium, Roche, São Paulo, Brazil) at $5.5\,mg\,kg^{-1}$, intraperitoneal Once anesthetized, the thoracic level 9 (T9) spinal process was identified by palpation and the rats were subjected to trichotomy in the dorsal region. The rats were kept in a prone position using a stereotactic apparatus (David Kopf, Tujunga, CA, USA) and a midline incision to expose the back muscles was performed. Muscles and ligaments were carefully dissected to expose the spinal apophyses of the T8–T9 vertebrae. The dorsal portion of the T9 vertebra was removed to expose the dorsal surface of the spinal cord. With the aid of an electronic magnifying glass, the dura mater was opened longitudinally and folded back laterally. The spinal cord was exposed, and 2% lidocaine was administered to produce local anesthesia and to minimize the trauma of acute transection. Once the local anesthesia had set in, the spinal cord was carefully transected using a scalpel. After transection, the surgical opening was sutured in two layers (muscle and skin). After sterilizing the surgical sutures, the rats were given an intramuscular injection of 100 000 IU penicillin and placed in a photo-thermo-stimulation cage. After 12 h, animals were put in individual cages with water and chow *ad libitum*. From the surgery day until the end of the experiment, the rats were examined daily to assess their general condition, and abdominal massage was performed to assist urine excretion and defecation in an attempt to minimize the signs or symptoms of suffering, such as inappetence, dehydration, surgical suture infection, abdominal distension and prostration. If these signs appeared, the experiment was halted and the rat was killed.

In the SHAM group, we performed the skin and muscle incision at T9 without SCI; the CTRL group did not undergo any surgical intervention.

*Iron analysis*

Iron levels were determined using the two-point enzymatic method (Vitro, Johnson & Johnson, Raritan, NJ, USA) with a 600 nm wavelength reading.

Transferrin levels were analyzed by immunoturbidimetry reactions (Advia 1650, Bayer, Holliston, MA, USA).

*Statistical analysis*

Variables related to iron and transferrin for the different groups were analyzed using analysis of variance (ANOVA) for independent measures.

Student's *t*-test was used to analyze weight changes in animals before and after the experimental procedure.

All analyses adopted a significance level of $P \leqslant 0.05$, and the results are shown as the mean ± standard error. Weight values are reported in means ± standard deviation.

## Results

The figures below show the plasma transferrin and iron levels of the CTRL, SHAM and SCI animals from each group.

*Experiment: acute SCI (SCI1)*

Compared with CTRL1, the SCI1 (1 day after SCI) and the SHAM1 group showed significantly reduced levels of plasma iron ($F_{(2,15)} = 12.008$, $P = 0.00077$; ANOVA) and transferrin ($F_{(2,15)} = 25.688$, $P = 0.00001$; ANOVA) (Figure 1a).

*Experiment: SCI3*

The SCI3 group showed a significant difference in levels of plasma iron compared with the CTRL3 group ($F_{(2,17)} = 8.5652$, $P = 0.00267$; ANOVA). Plasma transferrin levels in the SCI3 group were significantly different from the SHAM3 group, and the SHAM3 group was significantly different from the CTRL3 group ($F_{(2,17)} = 10.090$, $P = 0.00129$; ANOVA) (Figure 1b).

*Experiment: SCI7*

The SCI7 showed significantly lower plasma iron ($F_{(2,14)} = 11.540$, $P = 0.00109$; ANOVA) and transferrin ($F_{(2,14)} = 12.364$, $P = 0.00081$; ANOVA) levels relative to the CTRL7 and SHAM7 groups (Figure 2a).

*Experiment: SCI15*

The SCI15 group showed a significant difference in plasma iron levels relative to the CTRL15 and SHAM15 groups ($F_{(2,17)} = 19.244$, $P = 0.00004$; ANOVA).

There were significant differences between the SCI15 and SHAM15 groups in plasma transferrin levels ($F_{(2,17)} = 3.7091$, $P = 0.04605$; ANOVA) (Figure 2b).

We observed significant weight loss in the SCI1 group, the SHAM1 group, the SCI3 group and the SCI15 group after the experimental procedures (Student's *t*-test, $P \leqslant 0.05$) (Table 1).

## Discussion

The pathophysiology of movement disorders during sleep is complex and not fully understood. The current status of

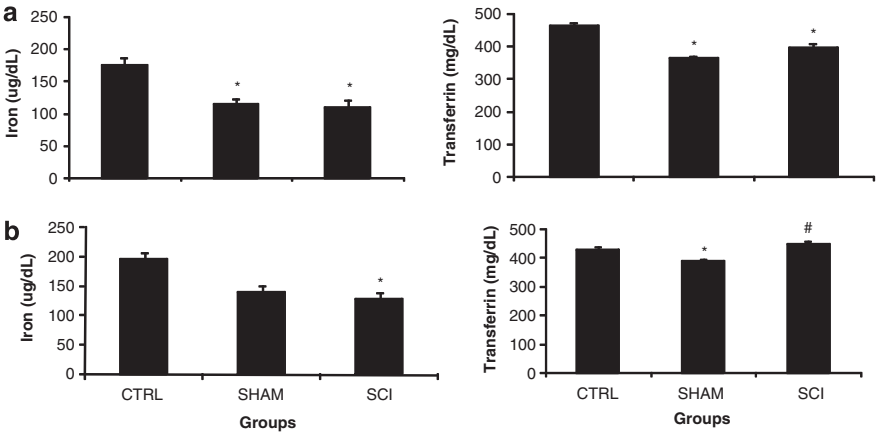


**Figure 1** Evaluation of plasma iron and transferrin levels in CTRL1, SHAM1, SCI1 **(a)** and CTRL3, SHAM3, SCI3 **(b)** groups. *Differs from CTRL group (ANOVA $P<0.05$). #Differs from SHAM group (ANOVA $P<0.05$).

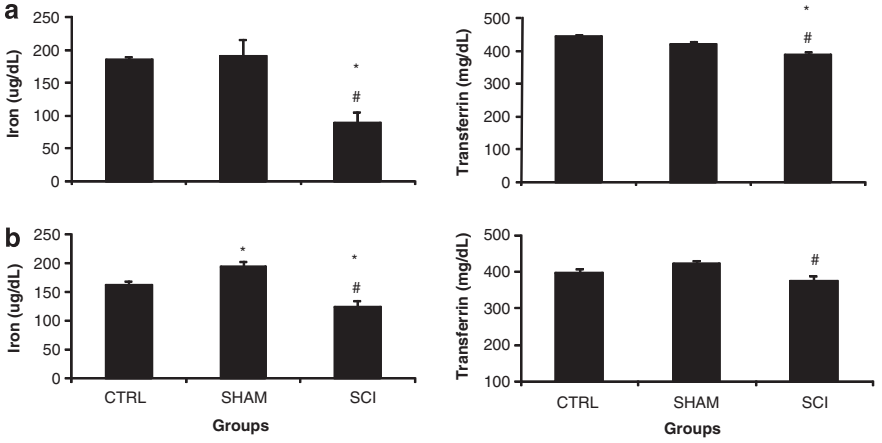


**Figure 2** Evaluation of plasma iron and transferrin levels in CTRL7, SHAM7, SCI7 **(a)** and CTRL15, SHAM15, SCI15 **(b)** groups. *Differs from CTRL group (ANOVA $P<0.05$). #Differs from SHAM group (ANOVA $P<0.05$).

research into these disorders points to depleted iron reserves in the central nervous system (CNS), dysfunctional dopaminergic systems and the likely involvement of the spinal cord.[2]

Our results support the notion that iron may be associated with the development of movement disorders. Conditions associated with secondary PLM, such as pregnancy and terminal kidney disease, are characterized by iron deficiency, which suggests that the latter may lead to the development of RLS and PLM. Although most of the patients with RLS present normal plasma ferritin levels, cerebrospinal fluid ferritin levels are low, suggesting an iron deficiency in the CNS.[1,2,4]

Our results showed significantly reduced plasma iron levels after the first day of SCI that lasted until the fifteenth day after SCI. In the acute (SCI1) experiment, there were reductions in iron in the SCI groups and the SHAM groups relative to the CTRL group. This decrease may be associated with the SCI surgery because surgical intervention leads to the loss of large amounts of blood, and the body may not be producing enough blood to maintain iron homeostasis, thus causing anemia. This hypothesis is supported by the SCI3, SCI7 and SCI15 groups because there was no statistical difference between the corresponding SHAM and CTRL groups.

**Table 1** Weights of animals before and after SCI

| *Group* | *N°* | *Weight before injury (g)* | *Weight after SCI (g)* | *Weight loss (%)* |
|---|---|---|---|---|
| CTRL1 | 6 | 337.66 ± 21.68 | 339.00 ± 12.12 | |
| SHAM1 | 5 | 420.46 ± 45.64 | 400.63 ± 45.61[a] | 4.71 |
| SCI1 | 7 | 340.14 ± 17.00 | 324.71 ± 17.00[a] | 4.53 |
| CTRL3 | 6 | 396.30 ± 49.83 | 407.07 ± 60.06 | |
| SHAM3 | 5 | 380.06 ± 35.55 | 370.83 ± 33.6 | 2.42 |
| SCI3 | 7 | 325.18 ± 29.88 | 280.66 ± 27.97[a] | 13.69 |
| CTRL7 | 6 | 375.74 ± 53.76 | 421.53 ± 56.48 | |
| SHAM7 | 6 | 315.18 ± 53.76 | 334.35 ± 46.82 | |
| SCI7 | 5 | 378.86 ± 76.36 | 337.74 ± 65.75 | 10.85 |
| CTRL15 | 6 | 404.42 ± 28.71 | 469.11 ± 25.77 | |
| SHAM15 | 5 | 408.32 ± 22.49 | 396.01 ± 21.83 | 3.01 |
| SCI15 | 8 | 340.06 ± 36.79 | 287.34 ± 19.47[a] | 15.50 |

Abbreviations: CTRL, control; SCI1, spinal cord injury at 1 day (acute); SCI3, spinal cord injury at 3 days; SCI7, spinal cord injury at 7 days; SCI15, spinal cord injury at 15 days.
Student's *t*-test; $P<0.05$.
[a]Differs from weight before.

We also analyzed transferrin plasma levels in all groups and found significant reductions in the SCI, SCI3 and SCI7

groups relative to the CTRLs. One hypothesis explaining this observation is that the reduction of transferrin occurs as a protective mechanism, as there was also a reduction in iron during this period.[11]

A number of studies in RLS animal models have suggested that the correlation between iron levels and the presence of the disorder is related to the dopaminergic system. Qu *et al.*[3] found that animals with A11 nucleus lesions contained less iron, especially in the spinal cord, and found reduced iron levels in mice with normal iron ingestion and A11 nucleus lesions. This finding is particularly noteworthy because human RLS patients show reduced brain iron, an observation that is currently unexplained.

The possibility that iron loss causes dopaminergic dysfunction in the CNS has not been addressed and requires further study in humans and in animal models.

Several theories have been proposed to explain the relationship between reduced iron and impaired dopaminergic system functioning in RLS patients. Theoretically, dopaminergic function could be diminished by any (or all) of the following:

- Downregulation of type 2 dopamine receptors.[3]
- Iron-mediated impairment of tyrosine hydroxylase, which produces L-dopa that causes the decarboxylization of dopamine.[12]
- A reduction in the amount of ferritin in some regions of the brain causing a reduction in D1 and D2 dopamine receptors[13] and a subsequent deficiency in extracellular dopamine transport to the striatum.[12]

Our study contradicts other studies such as that of Mizuno *et al.*[14] that describe iron reduction in RLS individuals occurring only in the CNS with plasma iron concentrations remaining at normal values.

Weight loss was also noted in the SCI animals. Skeletal muscle, in which muscle contraction is induced by nerve stimulation, prompts the muscle to work to maintain its structural and functional integrity. However, denervation leads to the non-stimulation of skeletal muscles, muscle atrophy and subsequent weight loss, as observed in the rats in this study. This provides a possible explanation for muscle atrophy in SCI rats because SCI hinders the rat's movements and leads to diminished locomotor activity.[15,16]

A possible limitation of this is that, because of technical problems, ferritin levels were not determined. Such an analysis would be extremely important because ferritin is an endothelial reticulum protein responsible for storing iron, and there is a relationship between serum ferritin levels and the amount of iron stored.[17]

In previous studies,[7,8] we found increased limb movements during sleep in rats from 4 days after SCI onward. Because the present investigation demonstrated reduced levels of plasma iron in rats 1, 3, 7 and 15 days after SCI, our findings suggest a temporal association between increased limb movements induced by SCI and reduced levels of plasma iron.

However, because studies of CNS iron levels have obtained inconclusive results, further research is required.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

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