Eccentric Exercise Leads to Glial Activation but not Apoptosis in Mice Spinal Cords

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Abstract
The aim of this investigation was to evaluate the effects of 3 overtraining (OT) protocols on the glial activation and apoptosis in the spinal cords of mice. Rodents were divided into control (C; sedentary mice), overtrained by downhill running (OTR/down), overtrained by uphill running (OTR/up) and overtrained by running without inclination (OTR). The incremental load test, ambulation test, exhaustive test and functional behaviour assessment were used as performance evaluation parameters. 36h after the exhaustive test, the dorsal and ventral parts of the lumbar spinal cord (L4-L6) were dissected for subsequent protein analysis by immunoblotting.

The OT protocols led to similar responses of some performance parameters. The ventral glial fibrillary acidic protein (GFAP) protein levels were diminished in the OTR/up and OTR compared to CT and OTR/down groups. The ventral ionized calcium binding adaptor molecule 1 (Iba-1), and the dorsal GFAP and Iba-1 protein levels were increased in the OTR/down compared to the other groups. The ratio between the cleaved caspase-3/caspase-3 and cleaved caspase-9/caspase-9 measured in the spinal cord were not sensitive to the OT protocols. In summary, the OTR/down activated the glial cells in the motor (i.e. Iba-1) and sensory (i.e. GFAP and Iba-1) neurons without leading to apoptosis.

Introduction
The benefits of regular moderate physical exercise contribute to the reduction and control of inflammatory parameters, as well as to the prevention and treatment of several diseases [17, 42]. However, to achieve the mentioned benefits, the prescription of the exercise sessions should respect the physical capacity of each individual. Otherwise, the use of high-intensity and/or high-volume sessions may cause muscle damage and other negative effects to the organism [31]. The imbalance between training and recovery may lead to non-functional overreaching (NFOR), a performance decrement that can be reversed after weeks or months of recovery and may be associated with psychological and hormonal disturbances [31]. Pereira et al. [33] developed a new overtraining (OT) protocol based on eccentric exercise (EE) sessions that led to NFOR, and were associated with low-grade chronic inflammation [33] and insulin-signalling impairment [34] in the skeletal muscles of mice. Previous reports have shown that skeletal muscle inflammation is able to activate glial cells in the central nervous system (CNS), in particular microglia and astrocytes [9, 38]. Activation of these cells is characterized by marked changes in their number, morphology, gene expression and function that result in the release of trophic factors, cytokines and chemokines [19, 43]. However, glial hyperactivity may have paradoxical effects on the CNS acting in synapse homeostasis maintenance, regulating neuronal signaling and protecting neurons from oxidative damage, or causing central sensitization of sensory neurons [18, 40]. Some studies have demonstrated the effects of physical exercise on glial activity, particularly in the hippocampal formation [29, 36]. However, in the spinal cord, where sensory and motor neurons initiate the control of skeletal muscle fibers, the responses of astrocytes and microglia to exercise are unknown.

Although the OT protocol proposed by Pereira et al. [33] leads to skeletal muscle inflammation, it is not possible to state whether this EE protocol is able to modulate the responses of glial cells in the sensory and motor neurons. Thus, the first aim of the present investigation was to verify the effects of this protocol on the glial fibrillary acidic pro-
tein (GFAP) and ionized calcium-binding adaptor molecule 1 (Iba-1), specific markers of astrocytes and microglia, respectively, in the ventral and dorsal horns of spinal cord. In addition, it is known that EE is characterized by singular features [21] such as the lengthening of the muscle-tendon complex, unique strategies of activation by the nervous system, and the ability to achieve high force levels with reduced oxygen consumption. Once NFOR can also be induced without the predominance of EE sessions [22] in order to discriminate the EE effects on GFAP and Iba-1 contents, our second aim was to compare the responses of these parameters to Pereira’s protocol [33] with their responses to other 2 protocols with same intensity and volume, but performed uphill and without inclination. Finally, based on the relationship between microglia activation and apoptosis [1], we also verified the responses of the caspase-3 and caspase-9 to the studied protocols.

**Methods**

**Experimental animals**

Male C57BL/6 mice from the Central Animal Facility of the Ribeirão Preto campus of the University of São Paulo (USP) were kept in individual cages with controlled temperature (22 ± 2°C) on a 12:12-h light-dark inverted cycle (light: 6 p.m. to 6 a.m., dark: 6 a.m. to 6 p.m.) with food (Purina chow) and water ad libitum. The experimental procedures were approved by the Ethics Committee of the University of São Paulo (USP). In addition, the present work adheres to the ethical standards of the IJSM [20]. 8-week-old C57BL/6 mice were divided into 4 groups: control (C; sedentary mice; n = 12), overtrained by downhill running (OTR/down; performed the OT protocol based on downhill running; n = 12), overtrained by uphill running (OTR/up; performed the OT protocol based on uphill running; n = 12) and overtrained by running without inclination (OTR; performed the OT protocol based on running without inclination; n = 12). The C, OTR/down, OTR/up and OTR mice were manipulated and/or trained in a dark room between 6 and 8 a.m. [33].

**Incremental load test (ILT)**

Mice were adapted to treadmill running (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) for 5 days for 10 min.day⁻¹ at 3 m.min⁻¹ [33–35]. As previously described [15], rodents performed the ILT with an initial intensity of 6 m.min⁻¹ at 0% with increasing increments of 3 m.min⁻¹ every 3 min until exhaustion, which was defined when mice touched the end of treadmill 5 times in 1 min. Mice were encouraged using physical prodding. If a mouse became exhausted without completing the stage, the exhaustion velocity (EV; m.min⁻¹) was corrected according to Kuipers et al. [26]: EV = V + (n/b) · a, where V is the velocity (m.min⁻¹) of the last completed stage, a is the test increment (m.min⁻¹), n is the duration (min) maintained in the incomplete stage, and b is the duration (min) of the stage. The EV of mice was used to determine the intensity of the OT protocols.

**Overtraining protocols based on downhill running, uphill running, and running without inclination**

The 8-week OT protocols based on downhill running, uphill running, and running without inclination were adapted from Pereira et al. [33], and each experimental week consisted of 5 days of training followed by 2 days of recovery (Table 1). From the fifth week of the OT protocols, the training volume (min) performed by each experimental group was recorded daily.

**Performance evaluations**

The ILT (i.e. exhaustion velocity), the ambulation test, the exhaustive test (i.e. time to exhaustion) and the functional behavioural assessment were used as performance evaluation parameters. Except for the latter that was performed 24 h after the last OT protocol sessions, the other performance evaluations were performed 48 h after the last OT protocol sessions. The ILT, the ambulation test and the functional behavioural assessment were performed at week 0, and at the end of week 4 and 8. On week 0, all groups (i.e. C, OTR/down, OTR/up and OTR) performed the ILT without inclination. However, at the end of week 4 and 8, the C and OTR performed the ILT without inclination, the OTR/down performed the ILT in downhill running, and the OTR/up performed the ILT in uphill running. As previously described [33–35], due to the high intensity and treadmill inclination, the exhaustive test was performed at the end of week 4 and 8.

**Ambulation test**

As previously described [6], the ambulation test determined the mean length of a step, measured in hind foot ink prints while mice ran freely in a corridor (length = 50 cm, width = 8 cm, height of lateral walls = 20 cm). 4 h after the ambulation test, the experimental groups performed the ILT. The performance was recorded by the ratio between step length and body length.

**Exhaustive test**

As previously described [33–35], 24 h after the ILT, the rodents ran at 36 m.min⁻¹ at 8% treadmill grade until exhaustion which was defined when mice touched the end of treadmill 5 times in 1 min. Mice were encouraged using physical prodding. This value was recorded as the time to exhaustion (s).

**Functional behavioral assessment of the sensory system**

Mechanical hypersensitivity was assessed by the measurement of the paw withdrawal threshold in response to probing calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL). Animals were placed on an elevated meshed grid which allowed full access to the ventral aspect of

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OTR/down: mice performed the OT protocol based on downhill running; OTR/up: mice performed the OT protocol based on uphill running; OTR: mice performed the OT protocol based on running without inclination.
the hindpaws. A logarithmic series of 9 filaments were applied to the left hindpaw to determine the threshold stiffness required for 50% paw withdrawal according to the non-parametric method of Dixon [14] as described by Chaplan et al. [10].

**Metabolic parameters**

The body weight and food intake of the experimental groups were recorded daily. Food intake was determined by subtracting the final food weight (i.e. weight of food put in each individual cage after one day) from the initial food weight (i.e. weight of food put in each individual cage on the previous).

**Sample collection and protein analysis by immunoblotting**

Mice were euthanized by cervical dislocation 36h after the exhaustive test (i.e. at the end of week 8). The mice spinal cords were dissected under a Leica KL 200 LED dissecting microscope (Leica Microsystems, Wetzlar, Germany) using a micro-knife (Fine Science Tools). First, the segment between the lumbar spinal cord (i.e. L4 and L6) was removed and kept in PBS at 4°C in a Petri dish for subsequent dissection. Then, the right and left sides were separated using the anterior median fissure as reference. Next, the dorsal and ventral aspects of both hemi-spinal cords were dissected using the central canal fissure as reference. The entire procedure lasted 5–7 min. The dorsal and ventral parts of the lumbar spinal cord (L4–L6) were separately homogenized in lysis buffer containing 137 mM NaCl, 20 mM Tris, 1% Igepal CA-630 (Sigma Aldrich), 10% glycerol, 2 mM sodium orthovanadate, 1% sodium dodecyl sulphate, 50 mM sodium fluoride, 2 mM EDTA and protease inhibitor cocktail (Sigma Aldrich) at pH 7.4. Tissue homogenates were centrifuged at 40000rpm for 10 min at 4°C, and supernatants were collected for analysis. Protein concentration in tissue homogenates was determined by a modified Lowry assay (DC Protein Assay, Bio-Rad, Hercules, CA, USA). These procedures were previously described in part [27,28].

Proteins were denatured by boiling in Laemmli sample buffer containing 100 mM DTT, run on SDS-PAGE gel and transferred to PVDF membranes (Amersham Bioscience, Piscataway, NJ, USA). The transfer efficiency to PVDF membranes was verified by briefly staining the blots with Ponceau red stain. These membranes were then blocked with bovine serum albumin for 1 h at room temperature followed by overnight incubation with primary antibody at 4°C. Antibodies used for immunoblotting overnight at 4°C were anti-GFAP (1:40000), anti-Iba1 (1:1000), caspase-3 (1:1000), caspase-9 (1:1000) and β-actin (1:1000) (Cell Signaling Technology, Beverly, MA, USA). After being washed with TBS containing 0.1% tween-20, all membranes were incubated with secondary antibody (1:2000; ECL anti-rabbit IgG, GE Healthcare Ltd., Buckinghamshire, UK) for 1 h at room temperature. The specific immunoreactive bands were detected overnight at 4 °C were anti-GFAP (1:40000), anti-Iba1 (1:1000), caspase-3 (1:1000), caspase-9 (1:1000) and β-actin (1:1000) (Cell Signaling Technology, Beverly, MA, USA). After being washed with TBS containing 0.1% tween-20, all membranes were incubated with secondary antibody (1:2000; ECL anti-rabbit IgG, GE Healthcare Ltd., Buckinghamshire, UK) for 1 h at room temperature. The specific immunoreactive bands were detected

**Results**

- **Fig. 1a** shows that the training volume of week 6 was higher compared to week 8 – first session for OTR/down (i.e. 74.2%) and OTR/up (53.9%) and to week 8 – second session for the 3 OT protocols (i.e. OTR/down = 203.4% , OTR/up = 154.9% and OTR = 111.8%). In addition, the training volume of week 7 was higher compared to week 8 – second sessions for the 3 OT groups (i.e. OTR/down = 170.8% , OTR/up = 124.3% and OTR = 83.8%). It is important to point out that the training volume of the recorded weeks was not different between the OT groups. **Fig. 1b** shows the EV responses measured at week 0, and at the end of week 4 and 8 among the experimental groups. Week 4 of the OT groups was higher compared to their own week 0 and week 4 of the CT group (i.e. OTR/down = 29.9% and OTR = 33.7% and OTR = 38.2%). In addition, week 8 of the OT groups was lower compared to their respective week 0 and 4 results (i.e. OTR/down = 46.5% and 45.2% , OTR/up = 20.7 and 18.3% and OTR = 14.2 and 11.4%, respectively). According to **Fig. 1d**, the time to exhaustion of the CT group at the end of week 4 was lower compared to that of the other groups (i.e. CT = 45.5% , OTR/down = 33.7% and OTR = 38.2%). In addition, week 8 of the OT groups was lower compared to their own week 4 (i.e. OTR/down = 14.7 and 34.4% , OTR/up = 6.1 and 28.3% and OTR = 5.9 and 26.0%, respectively).

The ambulation test data (i.e. step length/ body length ratio) are presented in **Fig. 1c**. Week 8 of OTR/down was lower compared to that of the other groups (i.e. CT = 45.5% , OTR/down = 33.7% and OTR = 38.2%). In addition, week 8 of the OT groups was lower compared to their respective week 0 and 4 results (i.e. OTR/down = 46.5 and OTR/up = 20.7 and 18.3% and OTR = 14.2 and 11.4%, respectively). According to **Fig. 1d**, the time to exhaustion of the CT group at the end of week 4 was lower compared to that of the other groups (i.e. OTR/down = 84.6% , OTR/up = 84.1% and OTR = 83.5%). In addition, week 8 of the OT groups was lower compared to their own week 4 (i.e. OTR/down = 95.6% , OTR/up = 87.9% and OTR = 90.3%).

The functional behavioral assessment of the sensory system data is presented in **Fig. 1e**. Paw withdrawal threshold at week 4 of the CT group was higher compared to the OT groups (i.e. OTR/down = 382.3% , OTR/up = 235.2% and OTR = 136.0%). In addition, at week 8, mice subjected to OTR/down showed higher paw withdrawal threshold compared to week 4 (i.e. 352.5%), and week 4 of the OTR/up was lower compared to week 0 (i.e. 73.6%). **Fig. 2a, c** present the body weight (g) and food intake (g), respectively, responses during the experimental weeks for the experimental groups. The percentage alteration of body weight between week 0 and 8 of CT group (15.5±2.2%) was higher compared to OTR/down (7.5±1.9%) and OTR groups (4.5±1.5%) (**Fig. 2b**). In addition, **Fig. 2d** shows that the percentage alteration of food intake between week 0 and 8 of OTR/up group (29.4±6.3%) was higher compared to CT and OTR/down groups, respectively.
The ventral Iba-1 protein levels were increased by 63.7, 86.1 and 89.8% in the OTR/down compared to CT, OTR/up and OTR groups, respectively (Fig. 3b). Fig. 3c shows that the dorsal GFAP protein levels were increased by 70.3, 175.5 and 133.8% in the OTR/down compared to CT, OTR/up and OTR groups, respectively. In addition, the dorsal Iba-1 protein levels were increased by 214.8, 157.8 and 232.6% in the OTR/down compared to CT, OTR/up and OTR groups, respectively (Fig. 3d). The ratio between the cleaved capase-3/caspase-3 and cleaved caspase-9/caspase-9 measured in the ventral and dorsal horns of spinal cord were not sensitive to the OT protocols (Fig. 4a–e).

Fig. 1 The training volume (min) was measured daily from the fifth week in the overtraining protocols. Once mice from OTR/down, OTR/up and OTR performed the entire training sessions in the fifth week, the figure presents the data from the sixth to the eighth week. a. Responses of the exhaustion velocity (m.min⁻¹) b. step length/body length ratio (i.e. ambulation test) c. time to exhaustion (s) d and 50% threshold (i.e. functional behavioral assessment of the sensory system) e at week 0, and at the end of week 4 and 8 in the experimental groups. Data correspond to means ± SE of n = 12 mice. CT: sedentary mice; OTR/down: mice performed the OT protocol based on downhill running; OTR/up: mice performed the OT protocol based on uphill running; OTR: mice performed the OT protocol based on running without inclination; # Statistical significance (P < 0.05) compared to week 8 – first session; Statistical significance (P < 0.05) compared to week 8 – second session; * Statistical significance (P < 0.05) compared to week 0; † Statistical significance (P < 0.05) compared to week 4; †† Statistical significance (P < 0.05) compared to week 4 of CT group; ** Statistical significance (P < 0.05) compared to week 8 of OTR/down group.

Fig. 2 Body weight (g) and food intake (g) responses during the experimental weeks for the experimental groups a and c. Percentage alteration of body weight b and food intake d between week 0 and week 8 for the experimental groups. Data correspond to means ± SE of n = 12 mice. CT: sedentary mice; OTR/down: mice performed the OT protocol based on downhill running; OTR/up: mice performed the OT protocol based on uphill running; OTR: mice performed the OT protocol based on running without inclination; Statistical significance (P < 0.05) compared to CT group.
Discussion

The main findings of the present investigation are: a) independently from the muscle contraction predominance, the OT protocols led to similar responses of training volume, exhaustion velocity and time to exhaustion; b) the OTR/down-up-modulated the dorsal GFAP protein levels, and the ventral and dorsal Iba-1 protein levels; c) the OT protocols did not modulate the protein levels of cleaved caspase-3/caspase-3 and cleaved caspase-9/caspase-9 in the ventral and dorsal horns of spinal cord. Taken together, our results show that OTR/down presented spinal cord glial activation without leading to apoptosis.

The first innovation of the present study was the use of 3 OT protocols with similar external load (i.e. product between intensity and volume training), but performed downhill, uphill and without inclination. Interestingly, the responses of training vol-
treadmill running training decreased the hypoxia-induced astrocyte activation (i.e. GFAP protein levels). Under neuronal stress, astrocytes protect neurons from energy depletion by releasing lactate from glycogen stores when glucose expenditure exceeds availability [5]. However, these cells may produce and secrete factors that inhibit the axonal growth and are a major component of the glial scar [24]. In addition, the prolonged activation of astrocytes can cause neuronal apoptosis by activating the p75 neurotrophin receptor [32]. However, in the current study, the OT protocols did not alter the protein levels of molecules involved in the apoptotic pathway (i.e. caspase-3 and caspase-9).

Regarding the Iba-1 protein levels in the ventral horn of spinal cord, OTR/down presented higher values compared to the other groups. It is well established that disturbances or threats to the CNS lead to microglial activation that may induce neurotoxicity or neuroprotection [25]. The beneficial effects of microglial activation on the motor neurons include the decrease of the neuronal electrical activity or the facilitation of the exchange of the neurotrophic activity between neurons and microglia, aiding neuron injury recovery [3]. Conversely, microglial activation may release a number of cytokotic agents, including the cluster of differentiation 95 ligand (CD95L) and TNF-alpha [1]. These agents may upregulate the cluster of differentiation 95 (CD95) receptor and/or the TNF receptor 1 (TNFR1), leading to caspase activation, cytochrome c release and terminal apoptosis [1,2]. However, as previously stated, we did not observe significant alterations of caspase-3 and caspase-9 in response to the OT protocols. It is probable that OTR/down-induced microglia activation is lower compared to that occasioned by spinal cord injury that is associated with CD95L and TNF-alpha release.

On the dorsal horn of the spinal cord, OTR/down presented higher protein levels of GFAP (Fig. 3c) and Iba-1 (Fig. 3d) compared to CT, OTR/up and OTR groups. It is known that neurons and glial cells in the dorsal horn are primarily associated with sensory processing, and some studies have shown the importance of astrocyte and microglia activations to the initiation and maintenance of persisted pain [11,41]. The activation of these glial cells sensitizes dorsal horn neurons through a number of mechanisms including the releasing of pro-nociceptive molecules such as IL-1beta, IL-6, TNF-alpha, nitric oxide, prostaglandin, endocannabinoids, chemokine (C-C motif) ligand 2 (CCL-2) and/or brain-derived neurotrophic factor (BDNF) [11,39]. While microglia activation initiates pain in the spinal dorsal horn [4], astrocyte hyperactivity is associated with the maintenance of long-term central sensitization [16]. Interestingly, the OTR/down did not present significant differences in functional behavioral assessment of the sensory system compared to the other groups at week 8 (Fig. 1e). Due to the discrepancies between the molecular parameters and the 50% threshold data, we hypothesize that the OTR/down group presented an up-regulation of the endogenous analgesic system to counteract the hyperalgesic effect induced by glial hyperactivity. In fact, regular moderate physical exercise is associated antinociceptive effects through increased release of endogenous opioids in the CNS [37]. This effect probably explains the lower results observed in the first 4 weeks of the OT protocols for the 50% threshold compared to CT group. Based on our previous results about OTR/down group [35], skeletal muscle inflammation may be considered to be a peripheral stimulus responsible for the hyperactivation of the glial cells [9,38].
In conclusion, our study demonstrated that the responses of training volume, exhaustion velocity and time to exhaustion were not dependent on the muscle contraction predominance used in the OT protocols. About the molecular data, the OTR/ down activated the glial cells in the motor (i.e. Iba-1) and sensory (i.e. GFAP and Iba-1) neurons without leading to apoptosis.

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Conflicts of interest: The author have no conflict of interest to declare.

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