Cytokine 103 (2018) 69–76

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Positive effects of total recovery period on anti- and pro-inflammatory cytokines are not linked to performance re-establishment in overtrained mice

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ARTICLE INFO

Keywords:
Excessive training
Cytokines
Skeletal muscle
Serum
Mice

ABSTRACT

The association between excessive training sessions (i.e., overtraining/OT) and periods of inadequate recovery is linked to the nonfunctional overreaching (NFOR) state, which is defined as an unexplained decrement or stagnation of performance. The cytokine hypothesis of OT considers that pro-inflammatory cytokines are responsible by the NFOR state-induced performance decrement. Investigations using rodent models of OT verified increased levels of pro-inflammatory cytokines in hypothalamus, liver, serum and skeletal muscle samples. Recently, our research group observed that a 2-week total recovery period was not able to re-establish the NFOR state-induced performance decrement. As the responses of anti- and pro-inflammatory cytokines were not measured, we aimed to investigate the effects of 2-week total recovery period on the protein contents of IL-1beta, IL-6, IL-10, IL-15, TNF-alpha and SOCS-3 in serum and skeletal muscle samples of overtrained mice. Also, a bioinformatics analysis was performed to investigate the correlations of IL-1beta, IL-6, IL-10, IL-15, TNF-alpha and SOCS-3 in skeletal muscle with locomotor activity. In summary, the 2-week total recovery period upregulated the anti-inflammatory cytokines and normalized the pro-inflammatory cytokines without a concomitant re-establishment of performance.

1. Introduction

In order to improve and maximize physical performance, the imbalance between excessive training sessions (i.e., overtraining/OT) and periods of adequate recovery can lead to the nonfunctional overreaching (NFOR) state, which is characterized as an unexplained decrement or stagnation of performance that may be linked to physiological and psychological disturbances [1]. The total recovery of the NFOR state may last weeks or months and depends on the interruption or reduction of the training loads (i.e., the product between intensity and volume of training) [1].

Previously, Smith [2] proposed the cytokine hypothesis of OT to explain the decrement or stagnation of performance, which is the only scientifically recognized marker for the diagnosis of the NFOR state [3–7]. Briefly, this hypothesis considers that the association between high load training sessions and inadequate recovery periods induces musculoskeletal trauma, increasing the production and release of interleukin 1beta (IL-1beta), IL-6 and tumor necrosis factor alpha (TNF-alpha). These elevated serum levels of pro-inflammatory cytokines would act on different organ systems such as the central nervous system, liver, skeletal muscles and white blood cells, initiating most of the signs and symptoms that were previously associated with the NFOR state [2].

In accordance with Smith's theory, our research group verified that overtrained mice exhibited elevated levels of pro-inflammatory cytokines in hypothalamus and liver [8,9]. Recently, using three different OT models, da Rocha and coworkers [10] verified that the NFOR state-induced performance decrement occurred concomitantly with high levels of IL-1beta, IL-6 and TNF-alpha in serum and skeletal muscle samples. Although Pereira et al. [9,11] verified that a 2-week total recovery period was not able to re-establish the NFOR state-induced performance decrement, the responses of anti- and pro-inflammatory

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https://doi.org/10.1016/j.cyto.2018.01.005
Received 10 October 2017; Received in revised form 19 December 2017; Accepted 7 January 2018

cytokines were not evaluated. Therefore, the main aim of the present investigation was to verify the effects of 2-week total recovery period on the protein contents of IL-1beta, IL-6, IL-10, IL-15, TNF-alpha and suppressor of cytokine signaling 3 (SOCS-3) in serum and skeletal muscle samples of overtrained mice. Our hypothesis is that the overtrained mice will present an up- and downregulation of the anti- and pro-inflammatory cytokines, respectively.

2. Methods

2.1. Experimental animals

Eight-week-old male C57BL/6 mice were provided by the Central Animal Facility of the Ribeirão Preto campus and were maintained in individual cages with controlled temperature (22 ± 2 °C) on a 12:12-h light-dark inverted cycle with food (Purina chow) and water *ad libitum*. The experimental procedures were approved by the Ethics Committee of the University of Sao Paulo (ID 14.1.873.53.0) and the National Institutes of Health Guide for care and use of Laboratory Animals (NIH Publications n° 8023, revised 1978) have been followed. Rodents were randomized into control (CT; sedentary mice; n = 6), overtrained by downhill running (OTR/down; n = 6), overtrained by uphill running (OTR/up; n = 6), and overtrained by running without inclination (OTR; n = 6). Mice were manipulated and overtrained in a dark room between 6 and 8 am [12]. After eight weeks of overtraining, the animals were maintained during two weeks of total recovery. Fig. 1 summarizes the experimental procedures that rodents from the CT, OTR/down, OTR/up, and OTR groups were submitted.

2.2. Incremental load test

Once being adapted to treadmill running (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) for 5 days, 10 min·day^{-1} at 3 m·min^{-1}, rodents achieved the incremental load test 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 treadmill end 5 times in 1 min. Mice were stimulated using physical prodding and, when they became exhausted without completing the stage, the exhaustion velocity (EV; m·min^{-1}) was corrected according to Kuipers et al. [13]. The EV of each mouse was used to prescribe the intensity of the OT protocols [12,14,15].

2.3. Exhaustive test

After 24 h incremental load test was performed, mice ran at 36 m·min^{-1} with an inclination of 8% until voluntary exhaustion, which occurred when the animals touched five times at the end of the treadmill in the interval of 1 min [12].

2.4. OT protocols and performance evaluations

Each week of the downhill, uphill and without inclination OT running protocols consisted of 5 days of training followed by two days of recovery. The incremental load test and the exhaustive test [9,16–19] were applied on week 0 and 48 h after the last sessions of the OT protocols at the end of weeks 4, 8, and 10 (i.e., after the 2-week total recovery period). On week 0, all the experimental groups performed the incremental load test without inclination. At the end of weeks 4, 8 and 10, the CT and OTR groups performed the incremental load test without inclination, the OTR/down group performed the incremental load test in downhill running, and the OTR/up group performed the incremental load test in the uphill running.

2.5. Skeletal muscle and total blood collections

Rodents were anesthetized 40 h after the exhaustive test, which was performed at the end of week 10 (Fig. 1). After a fast period of 12 h, mice were anesthetized with an intraperitoneal (i.p.) injection of 2-2-2 tribromoethanol 2.5% (10–20 μL·g^{-1}). When anesthesia was confirmed by the loss of pedal reflexes, because of their different fiber type composition [20], extensor digitorum longus (EDL) and soleus muscles of both hindlimbs were removed and used for immunoblotting analysis. Afterward, total blood was collected by decapitation, and serum was separated by centrifuging (1100*g*) for 15 min at 4 °C and stored at −80 °C for subsequent determination of anti- and pro-inflammatory cytokines.

2.6. Immunoblotting analysis

The immunoblotting analyses of EDL and soleus muscle samples were performed as previously described [10]. Antibodies used for immunoblotting overnight at 4 °C were IL-10 (SC52561), IL-15 (SC7889), SOCS-3 (SC9023), and Beta-actin (SC69879) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); IL-1beta (AB9722) and IL-6 (AB6672) from Abcam (Cambridge, UK).

2.7. Serum levels of cytokines

Serum levels (pg/mL) of IL-1beta, IL-6, IL-10, IL-15, and TNF-alpha were assessed using Luminex™ multiplex reagents as previously described [10].

2.8. Bioinformatics analysis

Bioinformatics analysis was carried out using a dataset from skeletal muscle of genetically-diverse BXD mice [EPFL/LISP BXD CD Muscle Affy Mouse Gene 1.0 ST (Dec11) RMA] [21], and the locomotor activity values (km) were acquired using a dataset as formerly published [22].

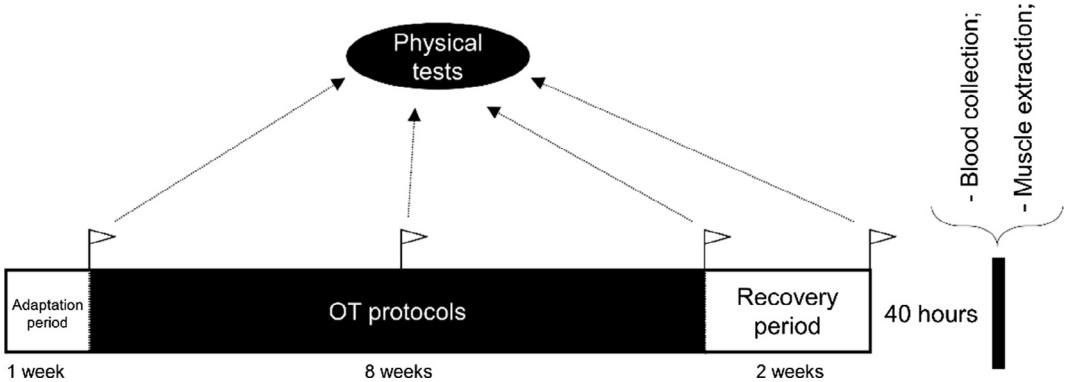

Fig. 1. Schematic representation summarizing the experimental procedures that rodents from the CT, OTR/down, OTR/up, and OTR groups were submitted.