

Effects of Sleep Deprivation on Acute Skeletal Muscle Recovery after Exercise

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ABSTRACT

DÁTILLO, M., H. K. M. ANTUNES, N. M. N. GALBES, M. MÔNICO-NETO, H. DE SÁ SOUZA, M. V. L. DOS SANTOS QUARESMA, K. S. LEE, C. UGRINOWITSCH, S. TUFIK, AND M. T. DE MELLO. Effects of Sleep Deprivation on Acute Skeletal Muscle Recovery after Exercise. *Med. Sci. Sports Exerc.*, Vol. 52, No. 2, pp. 507–514, 2020. **Purpose:** Sleep is considered essential for muscle recovery, mainly due to its effect on hormone secretion. Total sleep deprivation or restriction is known to alter not only blood hormones but also cytokines that might be related to skeletal muscle recovery. This study aimed to evaluate whether total sleep deprivation after eccentric exercise-induced muscle damage (EEIMD) modifies the profiles of blood hormones and cytokines. **Methods:** In two separate conditions, with a crossover and randomized model, 10 men (age, 24.5 ± 2.9 yr; body mass index, 22.7 ± 2.3 kg·m⁻²) performed a unilateral EEIMD protocol that comprised 240 eccentric contractions of the knee extensor muscles using an isokinetic dynamometer. In one condition, a “muscle damage” protocol was followed by 48 h of total sleep deprivation and 12 h of normal sleep (DEPRIVATION). In the other condition, the same muscle damage protocol was conducted, followed by three nights of regular sleep (SLEEP). Isometric muscle voluntary contraction tests and blood samples were collected serially throughout the protocol and analyzed for creatine kinase, free and total testosterone, IGF-1, cortisol, tumor necrosis factor-alpha, interleukin (IL)-1beta, IL-6, receptor antagonist of IL-1 and IL-10. **Results:** Muscle voluntary contraction and serum creatine kinase increased equally over the study period in both conditions. From the cytokines evaluated, only IL-6 increased in DEPRIVATION. No differences were detected in testosterone levels between conditions, but IGF-1, cortisol, and cortisol/total testosterone ratio were higher in DEPRIVATION. **Conclusions:** Total sleep deprivation after EEIMD does not delay muscle strength recovery but modifies inflammatory and hormonal responses. **Key Words:** ECCENTRIC EXERCISE-INDUCED MUSCLE DAMAGE, SLEEP, MUSCLE STRENGTH, IL-6, TESTOSTERONE, CORTISOL, IGF-1

For several years, it was believed that sleep was of primary importance for the maintenance of brain functions (1). However, a new milestone has been reached in the science of sleep because sleep is now known to impact several physiological factors [e.g., glucose (2) and lipid metabolism (3), blood pressure (4), eating behavior (5), energy metabolism (6), hormonal secretion (7), and the immune system (8)].

A series of reports have also consistently linked sleep to exercise performance [as reviewed by VanHelder and Radomski (9) and Fullagar et al. (10)]. Although lack of sleep seems to

impair physical performance, especially in activities of long durations, and it is popular knowledge that skeletal muscle recovers and grows during sleep, little is known about the role of sleep in muscle recovery. In 2011 (11), our group started to explore this issue and postulated that sleep may also influence skeletal muscle physiology. Later, we showed that sleep deprivation causes a loss of muscle mass (12), mainly due to type IIb muscle fiber atrophy (13), and reduces muscle regeneration after damage induced by cryolesions in rats (14). Some data on humans have been published more recently, which have shown that only one night of sleep partial sleep deprivation impairs recovery from a single exercise session (15,16), but the mechanisms for this phenomenon were not explored.

Various molecules, many of which are influenced by sleep, play a role in the maintenance and recovery of skeletal muscle physiology. If lack of sleep has a negative effect on skeletal muscles, hormones and cytokines may contribute to these effects, especially after the introduction of stimuli that result in greater skeletal muscle damage (e.g., eccentric actions). In both rats (17) and humans (7), sleep debt is associated with

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an increase in catabolic hormones and a reduction in anabolic hormones, leading us to postulate that partial or complete sleep deprivation could, in some way, disrupt skeletal muscle homeostasis and affect its recovery and/or adaptation after exercise (11). Thus, this study aimed to evaluate the recovery kinetics of the peak isometric force and the profiles of hormones and cytokines during the first 60 h (48 h of total sleep deprivation followed by 12 h of sleep) after an eccentric exercise-induced muscle damage (EEIMD) protocol.

METHODS

Ethical approval. Informed consent was obtained in writing from all subjects. This study conformed to the standards set by the Declaration of Helsinki, and the procedures were approved by the ethics committee of the Universidade Federal de São Paulo/Hospital São Paulo (1678/09). This experiment is registered at *Clinical Trials* (NCT02082600).

Subjects and study design. Ten healthy, recreationally active males who did not have any weight-training experience and did not complete more than two exercise sessions per week (height, 1.77 ± 0.03 m; weight, 71.1 ± 8.5 kg; body mass index, 22.7 ± 2.3 kg·m⁻²; age, 24.5 ± 2.9 yr) were recruited to participate in the present study. Subjects were excluded if they were smokers, drank more than two alcoholic drinks per day

or more than four drinks on one occasion, had musculoskeletal limitations, or took any kind of medications and/or nutritional supplements; subjects were included if they reported having 7 to 8 h of sleep per night (awake between 6:00 AM and 7:00 AM and asleep between 10:00 PM and 12:00 midnight), and morning or evening people were not included. Subjects were required to complete a routine medical screening (including a polysomnography test) and health questionnaire. After the physician's evaluation, individuals who were considered healthy started the protocol procedures.

Experimental design. We conducted a randomized, crossover trial with a washout period of at least 4 wk between the experimental conditions. Before initiating the tests (which were conducted at least 7 d apart), subjects attended the laboratory for a familiarization session with the isokinetic dynamometer using the contralateral leg (the nondominant one). A schematic of the experimental protocol is depicted in Figure 1.

In the SLEEP condition, subjects attended the laboratory at approximately 5:00 PM for the first blood draw (see Hormones and cytokines section), followed by a baseline isometric peak torque assessment (see Isometric peak torque section). After these procedures, at approximately 6:00 PM, the subjects started the EEIMD protocol (see the "EEIMD protocol" section). For the next 60 h (three nights), the subjects remained in the laboratory (the opportunity to sleep was provided from 11:00 PM to

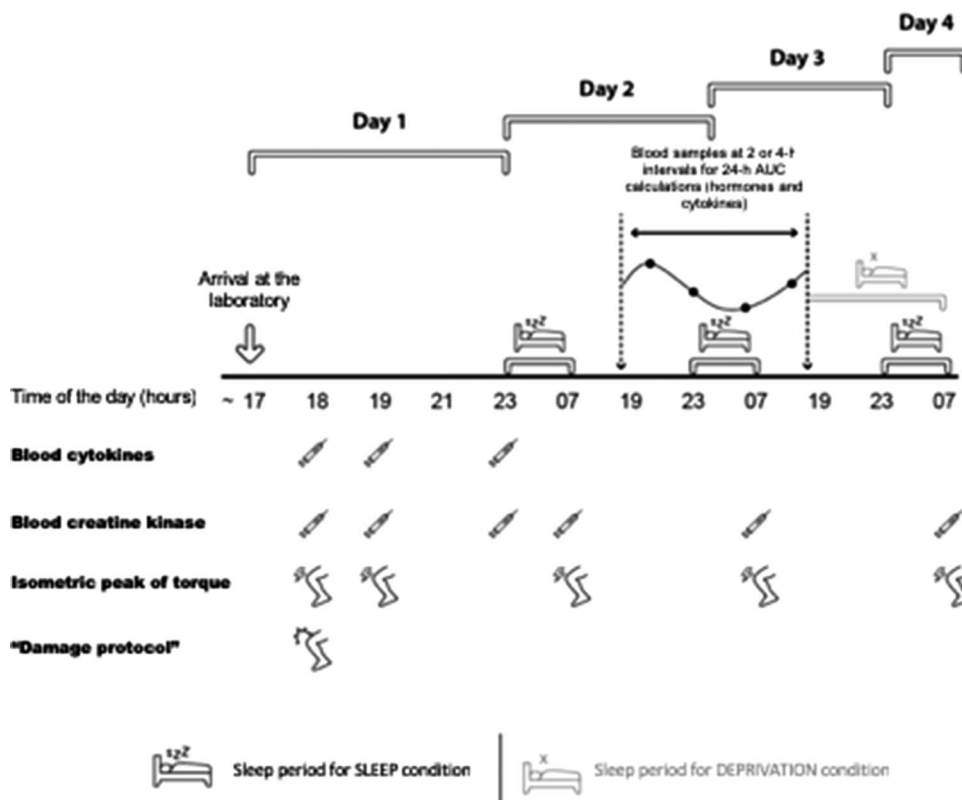


FIGURE 1—Schematic of the experimental protocol. Participants were submitted to a regular sleep routine for three nights (SLEEP condition) and total sleep deprivation for two nights followed by one night of sleep (DEPRIVATION condition). EEIMD consisted of 24 sets (10 repetitions per set) of the knee extensor muscles on the isokinetic. During the second night of the study, blood samples started to be drawn every 2-h (for hormone evaluation) or every 4-h (for cytokine evaluation) throughout the next 24-h period to calculate the AUC for each variable.

7:00 AM) under constant supervision of the researchers to confirm that none of the subjects slept at inappropriate times (e.g., during meals, video games, and bathing). Twenty-four hours after the EEIMD protocol, blood samples were drawn throughout the next 24-h period to calculate the area under the curve (AUC) for each variable (see “Hormones and cytokines” section; Fig. 1).

In the DEPRIVATION condition, the same procedures as those in the SLEEP condition were adopted. However, the subjects were completely sleep deprived for the next 48 h after the EEIMD protocol, and they were only able to sleep from approximately 7:00 PM to 7:00 AM on the third night (sleep rebound).

During the washout period, the volunteers were instructed to maintain their usual eating routine. The wake-sleep cycle was properly maintained because all participants had an established routine of working or studying.

EEIMD protocol. The EEIMD protocol was adapted from the protocol by Beaton et al. (18). The protocol comprised 24 sets (10 repetitions per set) of the knee extensor muscles on the isokinetic dynamometer (Biodex-System 3; Biodex Medical Systems, Inc., NY), with 1-min intervals between sets, at an angular velocity of $0.52 \text{ rad}\cdot\text{s}^{-1}$ (where 1.04 rad was flexion and 3.14 rad was full extension). Subjects remained seated throughout the protocol and had their shoulders strapped to the chair to stabilize their trunk. The subjects were instructed to extend their knee as they raised the Biodex lever arm; there was no resistance, except for the flexion torque produced by gravity on the shank during the concentric phase. Subsequently, subjects were verbally encouraged to maximally resist the lowering of the lever arm of the Biodex during the eccentric phase.

Isometric peak torque. Isometric knee extensor torque was assessed with the subject’s knee at an angle of 1.57 rad, as previously adopted by Beaton et al. (18), using the Biodex at the following times: baseline (pretreatment); immediately before and after the EEIMD protocol; and 12, 36, and 60 h after the skeletal muscle-damaging protocol. Subjects performed three maximal voluntary contractions (MVC, expressed as newton-meters relative to total body mass). Subjects were instructed to build torque for 2 s and then maintain the maximal torque for an additional 3 s. A 60-s rest interval was allowed between attempts. The peak torque value between attempts was used for analysis.

Hormones and cytokines. Before EEIMD, an intravenous catheter was inserted and fixed in the antecubital vein. Blood draws were obtained immediately before, immediately after, and 2 h after the EEIMD protocol (procedures were adopted to ensure that the hormones and cytokines, both before and after the EEIMD protocol, were the same for both experimental conditions). Twenty-four hours after the EEIMD protocol (Fig. 1), blood samples were drawn every 2 h (for hormone evaluations) or every 4 h (for cytokine evaluations) throughout the next 24-h period to calculate the AUC for each variable. For the SLEEP condition, an intravenous catheter was connected via a tube through the wall to an adjacent room, allowing blood samples to be taken during the night without

disturbing the subjects’ sleep. The venous catheter was perfused with 0.9% saline.

Blood samples were either centrifuged immediately at 4°C for 10 min to separate the plasma from the serum or kept at room temperature for 90 min before being centrifuged at 20°C for 10 min to separate the blood serum. Plasma and serum samples were immediately stored at -80°C .

Samples were assayed for the total amount of serum, free testosterone (UniCel Dxl 800®, Beckman Coulter®), and cortisol using an immunochemiluminescent assay (Immulite, DPC Corporation, USA), whereas serum IGF-1 was determined by a radioimmunoassay (DSL-5600; Diagnostics Systems Laboratories - DSL®). Creatine kinase (CK) activity was determined by an immunoinhibition assay. Serum cytokines IL-6 (HS600B), TNF-alpha (HSTA00D), IL-1beta (HSLB00C), IL-1ra (DRA00B), and IL-10 (HS100C) were measured by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Meal schedule during the study. All meals during the protocol were planned based on each participant’s food record. The meals were provided by the researchers and prepared in the laboratory under the supervision of at least one researcher. During each meal, the portions and items consumed were recorded, and these records were used to structure the meals for the subsequent experimental condition. All these procedures were adopted to approximate the feeding routine of each participant to that of his daily life to avoid the possibility of nutrients masking the effect of sleep or sleep deprivation in muscle recovery variables.

Statistical analyses. Statistical software v.12 (StatSoft, Inc., Tulsa, OK) was used to perform the statistical analyses in this study. First, CK was logarithmically transformed. The MVC, CK, and cytokines were analyzed (immediately before, immediately after, and 2 h after the damage protocol) using two-way ANOVA and the *post hoc* Tukey’s multiple comparisons test. The comparisons of the 24-h AUC for cytokines and hormones and the ratio of cortisol to total testosterone between the conditions were performed using paired *t* tests. The effect size was calculated by partial eta-squared (η^2). The following categories for η^2 were used: small = 0.1; medium = 0.3; and large ≥ 0.5 (19). Statistical significance for all analyses was

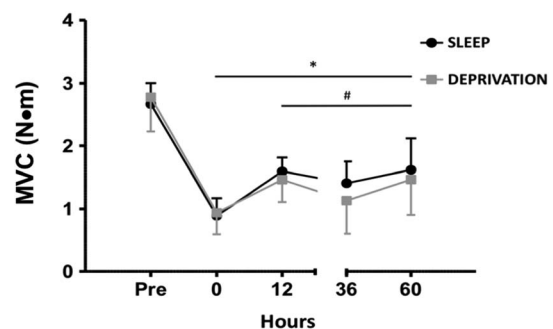


FIGURE 2—Isometric peak torque across the different periods of evaluation. Data are expressed as mean \pm standard deviation. *Different from Pre, in both conditions, $P < 0.01$; #different from hour 0.

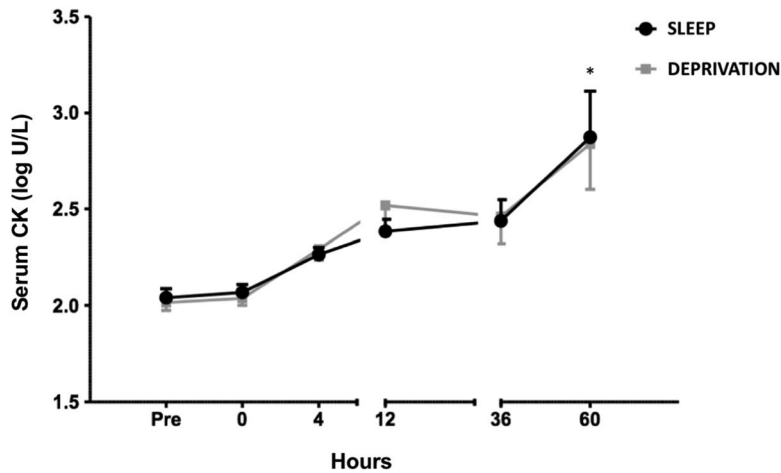


FIGURE 3—Serum CK across the different periods of evaluation. Data are expressed as mean \pm standard deviation. *Different from all times, in both conditions, $P < 0.05$.

accepted as $P \leq 0.05$. All values are expressed as the mean \pm standard deviation unless otherwise stated.

RESULTS

Maximal voluntary contraction. Maximal voluntary contraction demonstrated only a main effect of time ($P < 0.01$, $\eta p^2 = 0.8$) (Fig. 2). Maximal voluntary contraction was reduced immediately after the EEIMD protocol compared with before the protocol ($P < 0.01$, $\eta p^2 = 0.7$). Maximal voluntary contraction was partially recovered 12, 36, and 60 h after the EEIMD protocol compared with before the protocol ($P < 0.05$, $\eta p^2 = 0.6$).

Creatine kinase. Serum CK (Fig. 3) showed a main effect of time ($P < 0.01$, $\eta p^2 = 0.5$), with the serum CK levels being higher at 60 h than at all other time points ($P < 0.05$, $\eta p^2 = 0.3$).

Cytokines. No effects of time or condition were observed for IL-1beta ($P = 0.36$, $\eta p^2 = 0.07$) and TNF-alpha ($P = 0.9$, $\eta p^2 = 0.005$; Table 1). IL-6 increased immediately after the EEIMD protocol compared to before the protocol (SLEEP: $P < 0.01$, $\eta p^2 = 0.45$; DEPRIVATION: $P = 0.02$, $\eta p^2 = 0.74$), with no differences between conditions (main effect for time, $P < 0.01$, $\eta p^2 = 0.45$). A large effect size was identified 2 h after the EEIMD protocol in relation to the preassessment for both conditions (SLEEP: $P = 0.15$, $\eta p^2 = 0.8$; DEPRIVATION: $P = 0.12$, $\eta p^2 = 0.5$). The 24-h AUC for IL-6 showed a tendency to increase in DEPRIVATION with a medium effect size ($P = 0.08$, $\eta p^2 = 0.3$). IL-1ra was higher ($P < 0.01$, $\eta p^2 = 0.49$) at 2 h than before and immediately after the EEIMD protocol in SLEEP ($P < 0.01$, $\eta p^2 = 0.47$ and

$P = 0.11$, $\eta p^2 = 0.36$, respectively) and DEPRIVATION ($P < 0.01$, $\eta p^2 = 0.6$). No differences in IL-1ra were observed by the 24-h AUC between conditions ($P = 0.5$, $\eta p^2 = 0.06$).

IL-10 data were not shown because all measurements were lower than the detection limit of the kits used (assay range 0.8–50 pg·mL⁻¹).

Hormones. Figure 4 depicts all 2-h blood samples performed during the 24-h and the 24-h AUC of total testosterone (Fig. 4A), free testosterone (Fig. 4B), IGF-1 (Fig. 4C), and cortisol (Fig. 4D). No differences were identified between conditions in the 24-h AUC measurements for total ($P = 0.91$, $\eta p^2 = 0.002$) and free testosterone ($P = 0.7$, $\eta p^2 = 0.02$). However, the 24-h AUC for IGF-1 ($P = 0.02$, $\eta p^2 = 0.49$), cortisol ($P < 0.01$, $\eta p^2 = 0.9$), and the cortisol/total testosterone ratio ($P < 0.01$, $\eta p^2 = 0.59$; Fig. 5) were higher in DEPRIVATION than in SLEEP.

DISCUSSION

In this study, we focused on muscle recovery during sleep deprivation by evaluating muscle strength, blood cytokines, and hormonal profiles. The main findings are that acute total sleep deprivation (i) does not affect muscle strength recovery; (ii) increases blood IL-6 levels; and (iii) modifies the blood hormone balance by increasing IGF-1, cortisol, and the cortisol/total testosterone ratio.

Given that eccentric muscle actions (20) lead to severe muscle damage (e.g., damage to myofibrils and the muscle cell membranes), direct and indirect indicators were used to estimate the extent and severity of this damage (20,21). One of

TABLE 1. Blood cytokines levels in SLEEP and DEPRIVATION conditions.

	SLEEP				DEPRIVATION			
	Pre	Post	2-h Post	24-h AUC	Pre	Post	2-h Post	24-h AUC
IL-1beta (pg·mL ⁻¹)	0.007 \pm 0.004	0.01 \pm 0.01	0.01 \pm 0.06	23.0 \pm 14.5	0.01 \pm 0.01	0.001 \pm 0.05	0.01 \pm 0.01	26.6 \pm 28.5
TNF-alpha (pg·mL ⁻¹)	1.4 \pm 0.5	1.6 \pm 0.4	1.5 \pm 0.3	20.3 \pm 5.5	1.1 \pm 0.4	1.2 \pm 0.4	1.2 \pm 0.4	20.9 \pm 13.6
IL-6 (pg·mL ⁻¹)	0.4 \pm 0.2	1.8 \pm 1.7*	1.3 \pm 0.5	26.9 \pm 9.4	0.5 \pm 0.2	1.6 \pm 0.8*	1.3 \pm 1.0	44.9 \pm 29.7***
IL-1ra (pg·mL ⁻¹)	565.8 \pm 220.5	506.3 \pm 235.4	726.9 \pm 336.4**	3436.5 \pm 1359.6	427.5 \pm 155.4	436.8 \pm 144.0	674.5 \pm 289.8***	3701.1 \pm 1422.3

*Different from Pre, $P < 0.05$.

**Different from Post, $P < 0.05$.

*** $P = 0.08$ compared with SLEEP.

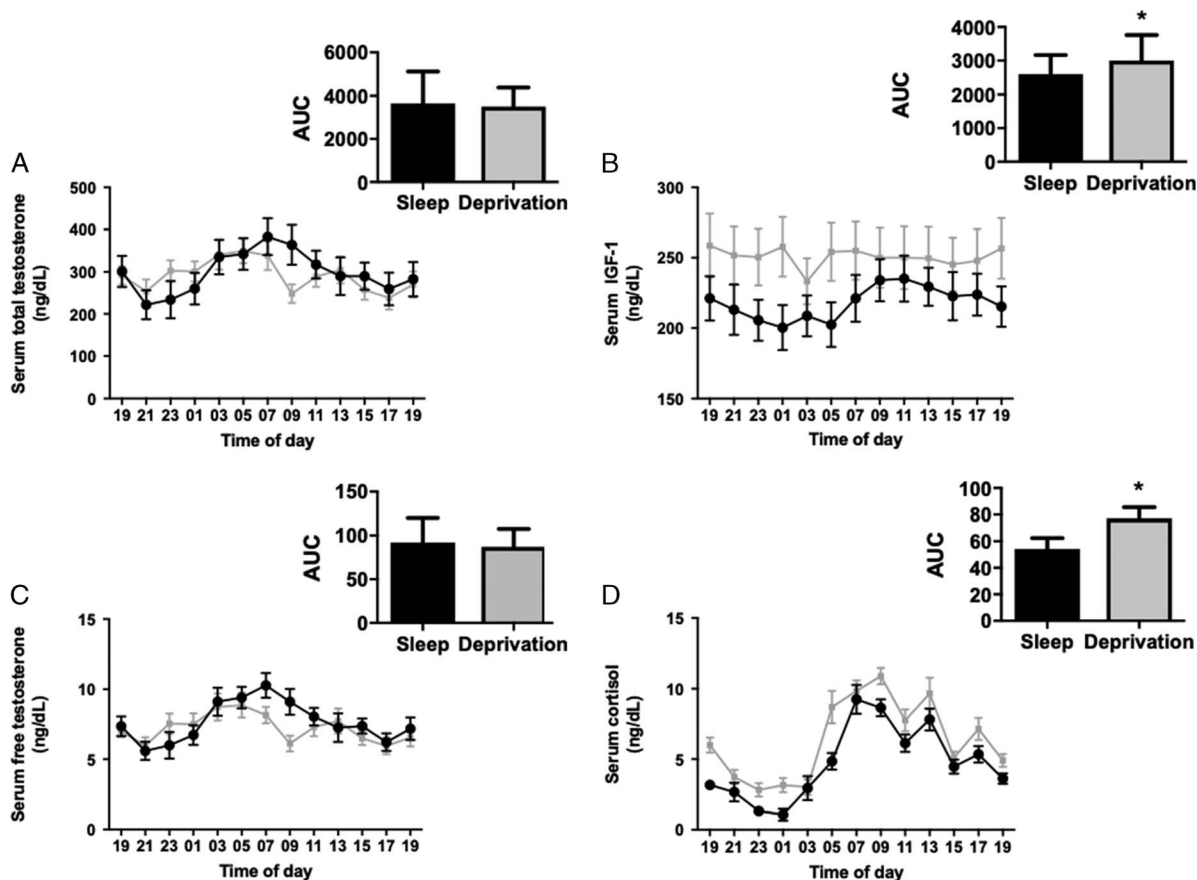


FIGURE 4—Blood variables across 24 h and their AUC. Total testosterone (A), free testosterone (B), IGF-1 (C), and cortisol (D). *Black markers: SLEEP condition; Gray markers: DEPRIVATION condition.* Data are expressed as mean \pm standard error, whereas AUC graphs are expressed as mean \pm standard deviation. *Different from SLEEP condition, $P = 0.02$, $\eta^2_{\text{partial}} = 0.49$.

the best indirect indicators is isometric peak torque (22). The eccentric exercise protocol used here aimed to induce muscle damage that would require more than 2 d for a total recovery (23) because we wanted to assess the initial phase of muscle recovery. No differences between the normal sleep and sleep deprivation conditions were observed in muscle peak torque over an ~48-h period or after a 12-h sleep rebound. In the study conducted by Chase et al. (15), the authors also did not observe changes in peak torque after a single night of sleep restriction after heavy exercise (60 min of high-intensity cycling intervals and resistance exercises); however, the performance in a cycling time trial test was decreased by $4.0\% \pm 3.0\%$. In another study, Rae et al. (16) found that one night of partial sleep deprivation, after a high-intensity interval training session, attenuated the peak power output on a cycle ergometer test, which lasted 15 min. It seems that sleep debt, at least that caused by acute total sleep deprivation, may not negatively impact strength but may be deleterious to long-duration, energy-demanding exercises, such as time trial performance tests. Among the possible hypotheses for such deleterious effects, we suggest sleeplessness, fatigue (16,24,25), and less motivation to train (15) due to a lack of sleep.

Both anti- and pro-inflammatory cytokines have been found to be elevated after an exercise session, but the blood IL-6

levels have typically remained more consistent (26–28), even after EEIMD involving quadriceps (29–32). In our study, serum IL-6 levels were increased for at least 2 h in both conditions, as expected. However, the main finding was the increased blood IL-6 levels when the 24-h AUC was considered for individuals who were sleep deprived. Sleep deprivation alone is shown to increase blood IL-6 levels (33). A connection between peripheral tissues and the central nervous system may exist because IL-6 induces fatigue and sleeplessness (34). Moreover, IL-6 is important for

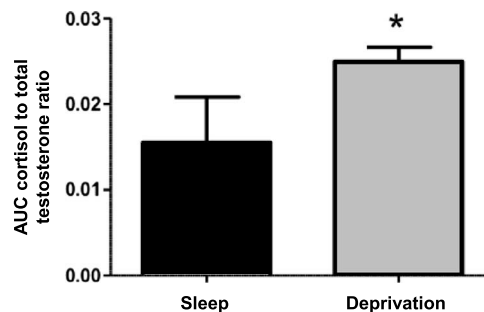


FIGURE 5—Cortisol/total testosterone ratio calculated from 24-h cortisol and total testosterone AUC. Data are expressed as mean \pm standard deviation. *Different from SLEEP condition, $P < 0.01$, $\eta^2_{\text{partial}} = 0.59$.

skeletal muscle. For example, IL-6 stimulates hypertrophic signaling and myogenesis through regulation of the proliferative capacity of muscle stem cells (35). However, chronically elevated IL-6 levels (e.g., for weeks) can be paradoxically deleterious, contributing to skeletal muscle atrophy, such as that found during cachexia (36). Thus, evidence of elevated levels of IL-6 in response to sleep deprivation may warrant future investigations conducted under conditions of physical training and shortened sleep durations over several days. Associations have previously been found between sleep loss and exercise-induced injuries (37). Conversely, good sleeping habits can be beneficial for those who are physically active. Thus, it is not unexpected that an injury that, to some extent, involves muscle plasticity or other physiological systems (e.g., the immune system), occurs when there is a mismatch between training load and duration and quality of sleep.

We evaluated the main hormones in blood that could link sleep to skeletal muscle. Growth hormone is popularly known to be associated with muscle recovery and hypertrophy. However, we do not support this hypothesis because sleep deprivation diminishes nocturnal rises in blood growth hormone (38) but does not alter its 24-h secretion (39). For this reason, we only measured the 24-h AUC for IGF-1 in blood. The increase in IGF-1 in blood after sleep debt has been reported by others (40), but the precise explanation is unknown.

Testosterone is one of the most anabolic hormones involved in skeletal muscle hypertrophy (41). No differences were observed in the 24-h AUC for total and free testosterone, despite some authors having described contrary results; without the use of physical exercise, Jauch-Chara et al. (42) observed that one night of total sleep deprivation decreased blood testosterone levels in the morning, but a single blood sample did not represent the 24-h blood hormone profile. In a more comprehensive assessment, Leproult and Van Cauter (43) obtained a 24-h blood testosterone profile in men. After 8 d with 5 h of sleep per night, the study showed that the men had lower testosterone levels than they did on a normal sleep schedule. It is not possible to determine whether skeletal muscle affected by the reduced blood testosterone levels found by Leproult and Van Cauter (43), even under food consumption. It is well known that acute increases in anabolic hormones do not affect muscle protein synthesis (44). However, chronic low testosterone levels could contribute to muscle decrements. For instance, our acute sleep deprivation protocol does not support a testosterone-induced interference on skeletal muscle function. On the other hand, the increased blood cortisol levels in the sleep deprivation condition corroborate some previous data (45–47) but not all previous data (43,48). In addition to the use of different sleep deprivation protocols, it is possible that the form of nutrient delivery during the protocol might influence these results (e.g., meals with or without carbohydrates, fasting, constant glucose infusion) because exogenous glucose controls blood cortisol levels. For example, sleep debt seems to increase cortisol only if meals are not provided (45–47). The fact that we observed increased 24-h AUC of cortisol in blood after sleep deprivation supports the

hypothesis that sleep, *per se*, is important for the hormone profile. It is also possible that increased IL-6 concentrations contributed to increased cortisol levels (49). Moreover, the increase in cortisol, even without modification of blood testosterone levels, increases the cortisol/testosterone ratio, which is widely associated with impairment in physical recovery and physical performance (50). If these increases in blood cortisol levels (as well as the cortisol/testosterone ratio) were observed in people who were chronically sleep restricted, it is plausible to expect that muscle physiology was altered, hampering exercise-induced muscle adaptations (e.g., muscle hypertrophy). However, this assumption must be further investigated.

To the best of our knowledge, no work has traced the kinetics of hormones during a recovery period after physical exercise, in a controlled environment, with a total absence of sleep and without food deprivation. These findings enable the investigation of the effects of chronic sleep restriction under conditions of physical training and suggest a possible intrinsic relationship among sleep, the immune system, and hormone profiles. Moreover, such reciprocity can also be investigated by identifying the role of each stage of sleep. As sleep restriction is a common occurrence for many people, sleeping is the correct “medicine” to counteract any losses that result from sleep deprivation. However, this is not always completely possible due to the routines of work, study, social events, stress, mood disorders, and so on. Therefore, the investigation of nonpharmacological strategies (e.g., mainly sleep hygiene and naps, as well as nutrients, bioactive compounds, melatonin) and/or pharmacological strategies that can at least minimize the damages caused by a lack of sleep are important.

It is important to state that total sleep deprivation and the EEIMD protocol adopted in this study are not real-life conditions. We chose them because (i) sleep is composed of phases; (ii) it is not clear how, or if, total sleep, as well as each of its phases, influences muscle physiology; (iii) the EEIMD protocol was designed to decrease skeletal muscle function (e.g., strength loss) in a severe manner that is not found in most types of physical training. Utilizing these two extreme situations (total sleep deprivation and EEIMD), it was possible to verify that sleep deprivation did not modify the kinetics of muscle strength recovery during the first 60 h, whereas in physical exercise protocols that are similar to physical training situations, a single day of sleep deprivation impaired recovery performance in a subsequent test (15,16). On the other hand, the changes in the inflammatory and hormonal profiles found in our study enable the investigation of individuals who are exposed to short durations of physical training, because damages in musculoskeletal recovery and adaptation, as well as immunocompetence (as a function of the change in the cortisol/testosterone ratio), can be found. In conclusion, total sleep deprivation after EEIMD does not delay strength recovery in the first 60 h but modifies inflammatory and hormonal responses.

The authors report that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation, and state that results of this study do not constitute

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None of the authors declare any conflict of interest.

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