



# Effects of increase of overload training on biochemical and hormonal parameters in rats\*

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## ABSTRACT

The training will be efficient if it is intensive enough to promote homeostasis break, adaptation and super compensation consequently. On the other hand, excessive stress conditions induced by exercise may promote undesirable effects. This paper aims to evaluate the effects of the increase in overload training upon some hormonal and biochemical parameters similar to overreaching. The animals were divided in three groups: SED (sedentary animals), MOD (moderate training during six weeks) and EXT (similar training to MOD for four weeks and increase to two and three daily training sessions in the 5<sup>th</sup> and 6<sup>th</sup> weeks, respectively). There was an increase in glutamate in EXT group ( $p < 0.05$ ) in relation to SED and in GLN/GLU ratio in relation to SED and MOD groups ( $p < 0.05$ ). Moreover, the MOD group presented increase in soleum muscle and liver glycogen and GH plasmatic concentration ( $p < 0.05$ ), whereas a testosterone decrease was found ( $p < 0.05$ ) in relation to SED. The EXT group showed similar changes to MOD as to muscle and liver glycogen. The GH concentration in EXT group was smaller than in the MOD group ( $p < 0.05$ ) and urea increased ( $p < 0.05$ ) in relation to SED. Thus, we came to the conclusion that the EXT group protocol was not able to induce signs of overreaching in the animals.

## INTRODUCTION

The training sessions must be sufficiently intense to promote the homeostasis break and consequently promote the adaptation and performance increase to reach the desired effect<sup>(1,2)</sup>. Following such trend, exercise can be considered an excellent means to study the body answers to stress events and also for human beings to adapt to stress<sup>(2)</sup>.

Whenever there is an adequate synchronism between training intensity and volume with the resting time, there is a favorable situation to adaptation and consequently we see the super-compensation. However, when the stress agent is excessive and/or the recovery insufficient, undesirable effects may occur<sup>(3-6)</sup>. If the resting time is inadequate, the capacity to adapt may decrease, preventing the individual from responding to the training in an appropriate manner. The excessive stress that may happen with an odd performance decrease is known as overreaching if transitory or overtraining if chronic<sup>(4)</sup>.

There are many hypotheses to try to explain this excessive stress condition and performance decrease<sup>(4,5,7,8)</sup>, however, the great individual variation in the studied parameters makes the early diagnosis and the understanding of the involved mechanisms in this syndrome progression difficult<sup>(6)</sup>.

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Among the observed variations in such conditions, we can highlight the neuroendocrinal, immunological, biochemical and psychological ones<sup>(2-4,8,9)</sup>. The neuroendocrinal changes that show off the most are the ones related to the smallest activity of the hypothalamus-hypophysis-glands axis, which makes it difficult to answer to stress and adapt to exercise<sup>(1,2,4,5,9)</sup>. The biochemical and metabolic variations that are frequently observed are, among others, the decrease of glycogen supplies<sup>(3)</sup>, changes in amino acids metabolism, mainly in the Skeletal muscle<sup>(3,8)</sup> and increase in cellular lesion due to excessive training<sup>(5)</sup>.

In human beings, two weeks of extensive training have been enough to induce the overreaching and the first signs of the syndrome<sup>(3,4,8)</sup>. However, the study concerning the effects of excessive training in human beings is difficult to happen, once due to ethical issues, it is not allowed to submit human beings to extensive training in a long period nor investigate them by invasive means, such as muscular and hepatic biopsis. Thus, the study on extensive exercise effects in animal models can be of great use not only in the understanding of the involved mechanisms in overreaching and overtraining, but also in the body capacity to respond and adapt to stress.

As a conclusion, this study aims to evaluate whether a protocol of great increase in the training volume, with excessive stress to animals is capable to induce signs of overreaching in rats.

## MATERIALS AND METHODS

**Animals:** Male Wistar lab rats weighing 150-200 g were used in the beginning of the experiment. The animals were obtained from the central bioterium of the Instituto de Ciências Biomédicas da Universidade de São Paulo. All the procedures during the study were approved by the Animal Research Ethics Committee of the Institute, according to regulations of the Brazilian School to Animal Experiments. The animals were kept in a room with light/dark cycle of 12:12 hours, with beginning of the light period at 7:00, controlled temperature at  $22 \pm 2^\circ\text{C}$  and relative air humidity constant at  $60 \pm 5\%$ . All animals were kept in collective cages with five animals and had water and food *ad libitum*.

**Experiment groups:** The animals were divided into three groups: the SED group, that was not submitted to any training ( $n = 10$ ), the MOD group: whose animals trained 60 minutes a day, five days a week during six weeks ( $n = 10$ ) and the EXT group ( $n = 10$ ) that trained similarly to MOD group, for four weeks. In the fifth week, they started training two sessions of 60 minutes each, separated by five hours, and in the sixth week, they trained three sessions of 60 minutes with a two and a half hours of break between them.

**Training:** The animals were submitted to swimming training. After the first training week, which had the aim to adapt the animals to the water, they trained with an extra overload equivalent to 5.5% of their body weight attached to their tail, giving them an overload predominantly aerobic<sup>(9)</sup>. The animals were weekly weighed so that the overloads could be readjusted during the study.

The training was have a strict control of water temperature that was kept at  $31 \pm 1^\circ\text{C}$ .

**Sacrifice and sample extraction:** Twenty-four hours after the last training session and six hours of fast, the animals were sacrificed by decapitation for blood collection of the soleo and conducted in an individual swimming system, with a constant water flow in order to gastrocnemii muscles and liver for later measurement.

After the sacrifice, the blood was put in test tubes containing sodium heparin ( $125 \text{ U}\cdot\text{L}^{-1}$ ) and kept on ice until centrifugation. The blood was then centrifuged to  $690 \text{ X g}$  for 15 minutes and  $4^\circ\text{C}$ , the plasma was collected and stored in a  $-80^\circ\text{C}$  freezer until the measurements. The soleo and gastrocnemii muscles were immediately removed after sacrifice, weighed and homogenized in PBS ( $1:9 \text{ p}\cdot\text{v}^{-1}$ ). After that, the homogenized were centrifuged for five minutes to  $5000 \text{ X g}$  and  $4^\circ\text{C}$  and stored in a  $-80^\circ\text{C}$  freezer for later measurements.

### Biochemical and hormonal determinations

**Glutamine:** It was enzymatically determined following methods described by Windmueller and Spaeth<sup>(11)</sup> in a medium with  $\text{KH}_2\text{PO}_4$  (50 mM), glycerol 50%, NADH (4 mM), BSA 10%, GDH (5  $\text{U}\cdot\text{ml}^{-1}$ ),  $\alpha$ -ketoglutarate (4.0 M) e asparaginase (5.0  $\text{U}\cdot\text{ml}^{-1}$ ), pH 8.0.

**Glutamate:** It was enzymatically determined in a medium with glycine (300 mM), hydrazine hydrate (250 mM), ADP (1 mM), NAD (1.6 mM) and GDH (4.5  $\text{U}\cdot\text{ml}^{-1}$ ), pH 9.0, according to method described by Bernt and Bergmeyer<sup>(12)</sup>.

**Glucose:** It was colorimetrically determined using a brandname Sigma kit in a medium with NAD (1.5 mM), ATP (1 mM), hexokinase (1  $\text{U}\cdot\text{ml}^{-1}$ ), glicosid-6-desidrogenasis fosfate (1  $\text{U}\cdot\text{ml}^{-1}$ ) and sodium azide (0.05%).

**Lactate:** It was enzymatically done in a medium with glycine (0.55 mM), EDTA (11.3 mM), hydrazine hydrate (0.7 mM), NAD (0.43 mM) and lactate dehydrogenase (100  $\text{mg}\cdot\text{mL}^{-1}$ ) in pH 9.0, according to method described by Engle and Jones<sup>(13)</sup>.

**Urea:** Colorimetrically determined using a namebrand Bioclin kit whose environment contained fosfate buffer (100 mM) pH 7.5, sodium nitroprusside (10 mM), sodium salicylate (60 mM), NaOH (1.5 M), sodium hyporide (10 mM) and urease (10000  $\text{U}\cdot\text{L}^{-1}$ ).

**Creatine kinase:** The maximum activity of the CK was enzymatically determined through a Bioclin kit whose environment consisted of glucose 6-Fosfate creatinine phosphate ADP (2 mM); AMP (5 mM); pentaphosphate diadenosine (10 mM); imidazole acetate (pH 6.7) (100 mM); glucose (20 mM); EDTA (2 mM); NADP+ (2 mM); Hexokinase (3500  $\text{U}\cdot\text{L}^{-1}$ ), magnesium acetate (10 mM) e N-acetylcysteine (20 mM).

**Hepatic and muscular glycogen:** The glycogen in the soleo and gastrocnemii muscles was extracted in KOH (30%) after an hour in boiling water. The precipitate was then washed with ethanol (70%) and centrifugated for 20 minutes to  $243 \text{ X g}$  as described by Sjögren *et al.*<sup>(14)</sup>. In the sequence, the glycogen content was determined in a medium containing sulfuric acid and antrona according to Hassid and Abrahams<sup>(15)</sup>.

**Hormones:** The insulin, testosterone and GH concentrations were determined by radioimmunoassay with DPC commercial kits (Coat-a-coat, DPC, Brazil).

**Statistics analysis:** All the results are expressed as an average  $\pm$  Average Standard Error (ASE). A two-way variation analysis followed by Tukey post-test with significance level of  $p < 0.05$  was used for statistics analysis.

## RESULTS

We evaluated the plasma concentration of various biochemical and hormonal parameters, and also the muscular and hepatic concentration of glycogen in our work. In table 1 we observe the plasma concentration of glutamine and glutamate. We may also experience the interaction between those two aminoacids. No difference

in the plasma concentration of glutamine among the three groups was observed, while the glutamate concentration was significantly higher in the EXT group ( $504.12 \pm 26.12 \text{ nmol}\cdot\text{ml}^{-1}$ ) in relation to the SED groups ( $993.21 \pm 172.01 \text{ nmol}\cdot\text{ml}^{-1}$ ) and MOD ( $1072.01 \pm 98.82 \text{ nmol}\cdot\text{ml}^{-1}$ ). Such change was reflected in the relation GLN/GLU once no difference between the SED and MOD groups was observed. However, in the EXT group ( $1.71 \pm 0.11$ ) this ratio was significantly lower than in the SED and MOD groups ( $3.38 \pm 0.34$  e  $2.89 \pm 0.49$  respectively).

**TABLE 1**  
Plasmatic concentration of glutamine and glutamate (values expressed in  $\text{nmol}\cdot\text{ml}^{-1}$ ) and of the glutamine/glutamate relation (GLN/GLU<sup>-1</sup>) in the sedentary group (SED), moderate (MOD) and exhausting (EXT)

	SED	MOD	EXT
Glutamine	$993.21 \pm 172.01$	$1072.01 \pm 98.82$	$900.40 \pm 31.76$
Glutamate	$293.43 \pm 50.19$	$370.23 \pm 23.8$	$504.12 \pm 26.12^*$
GLN/GLU	$3.38 \pm 0.34$	$2.89 \pm 0.49$	$1.71 \pm 0.11^{* \#}$

\* different in relation to the SED group,  $p < 0.05$ ; # different in relation to the MOD group,  $p < 0.05$ .

We also evaluated the plasma concentration of insulin, testosterone and GH. The insulin plasma concentration was significantly lower in the MOD group D ( $22.67 \pm 1.26 \mu\text{UI}\cdot\text{ml}^{-1}$ ) in relation to the SED group ( $55.53 \pm 10.5 \mu\text{UI}\cdot\text{ml}^{-1}$ ). Likewise, in the EXT group ( $37.92 \pm 7.52 \mu\text{UI}\cdot\text{ml}^{-1}$ ) there was significant decrease of this hormone in relation to the sedentary animals. No difference between the MOD and EXT groups was observed (table 2). The insulin concentration and the plasma concentration of testosterone was significantly lower in the MOD groups ( $4.00 \pm 0.63 \text{ ng}\cdot\text{dl}^{-1}$ ) and EXT ( $2.41 \pm 0.45 \text{ ng}\cdot\text{dl}^{-1}$ ) in relation to the animals of the SED group ( $7.63 \pm 1.07 \text{ ng}\cdot\text{dl}^{-1}$ ). However, no difference between the trained groups was observed. Moreover, the plasma concentration of GH is shown in table 2. We observed that there was significant increase of GH in the MOD group ( $5.42 \pm 0.84 \text{ ng}\cdot\text{ml}^{-1}$ ) in relation to the SED group ( $2.01 \pm 0.48 \text{ ng}\cdot\text{ml}^{-1}$ ). Unlike group MOD, EXT group ( $1.31 \pm 0.10 \text{ ng}\cdot\text{ml}^{-1}$ ) had a GH plasma concentration significantly lower in relation to the MO group and there was not any difference between the EXT and the SED groups (table 2).

**TABLE 2**  
Hepatic concentration and in the soleo muscle and glycogen gastrocnemii (values expressed in  $\text{mg}\cdot 100 \text{ mg tissue}^{-1}$ ) in the sedentary (SED), moderate (MOD) and exhausting groups (EXT)

	SED	MOD	EXT
Soleo	$11.79 \pm 1.53$	$17.3 \pm 3.95^*$	$15.4 \pm 4.01^*$
Gastrocnemii	$5.39 \pm 1.49$	$4.37 \pm 1.13$	$3.48 \pm 0.65$
Liver	$0.97 \pm 0.3$	$2.83 \pm 0.52^*$	$2.07 \pm 0.31^*$

\* different in relation to the SED group,  $p < 0.05$ .

In table 3 the results for the glucose, lactate, urea and CK plasma concentrations are demonstrated. No difference between groups in relation to the glucose, lactate and CK concentrations

**TABLE 3**  
Insulin plasma concentration ( $\text{uUI}\cdot\text{ml}^{-1}$ ), testosterone ( $\text{ng}\cdot\text{dl}^{-1}$ ) and GH ( $\text{ng}\cdot\text{ml}^{-1}$ ) in the sedentary (SED), moderate (MOD) e exhausting groups (EXT)

	SED	MOD	EXT
Insulin	$55.5 \pm 10.5$	$22.67 \pm 1.26^*$	$37.92 \pm 7.54$
Testosterone	$7.63 \pm 1.07$	$4.00 \pm 0.63^*$	$2.41 \pm 0.45^*$
GH	$2.01 \pm 0.48$	$5.42 \pm 0.94^*$	$1.31 \pm 0.10^*$

\* different in relation to the SED group,  $p < 0.05$ ; # different in relation to the MOD group,  $p < 0.05$ .

was found. On the other hand, the urea concentration was significantly higher in the EXT group ( $65.01 \pm 6.21 \text{ mg}\cdot\text{dl}^{-1}$ ) in relation to the SED group ( $52.33 \pm 3.28 \text{ mg}\cdot\text{dl}^{-1}$ ) but there was no difference though, among the trained groups.

Finally, the training protocols did not promote change in the muscular concentration of glycogen in the gastrocnemius or soleus muscles. However, a significant increase of glycogen in the liver was found in the MOD group in relation to the sedentary animals, as demonstrated in table 4.

**TABLE 4**  
Glucose plasma concentration ( $\text{mg}\cdot\text{dl}^{-1}$ ), lactate ( $\text{nmol}\cdot\text{ml}^{-1}$ ), CK ( $\text{U}\cdot\text{ml}^{-1}$ ) and urea ( $\text{mg}\cdot\text{dl}^{-1}$ ) in the sedentary (SED), moderate (MOD) and exhausting groups (EXT)

	SED	MOD	EXT
Glucose	$99.88 \pm 15.65$	$112.42 \pm 18.33$	$118.70 \pm 3.15$
Lactate	$1.25 \pm 0.10$	$1.41 \pm 0.96$	$1.32 \pm 0.09$
CK	$1281.7 \pm 170.21$	$1113.93 \pm 160.06$	$907.17 \pm 152.50$
Urea	$52.33 \pm 3.28$	$57.99 \pm 5.04$	$65.01 \pm 6.21^*$

\* different in relation to the SED group,  $p < 0.05$ .

## DISCUSSION

The performance decrease caused by the training excessive stress is a problem faced by the majority of the high level athletes all over the world. An average of 40% of the athletes of collective sports and about 70% of individual ones, suffer from overreaching/overtraining at least once in their careers<sup>(16)</sup>.

The mechanisms that explain the appearing of the overreaching are many, depending on the relation between the training overload with the inappropriate resting time and also the social and psychological problems over the athlete. Since it is seen as a syndrome, many signs and symptoms should be simultaneously present to classify the athlete suffering from overreaching<sup>(8,17)</sup>.

The involved mechanisms in this situation development are: amino acids imbalance, parasympathetic sympathetic imbalance, glycogen decrease and neuro endocrinal imbalance<sup>(6)</sup>. However, the great individual variation from lab tests results makes early diagnostics difficult and the precise understanding of the physiopathological mechanisms involved in the syndrome. Thus, due to the lack of deeper studies to understand the trigger mechanisms of overreaching, the study with animal use could be of great value.

The animals of the MOD group were submitted to moderate training for 6 weeks, while the animals of the EXT group suffered sharp increase in the training overload in the last two weeks in a trial to induce overreaching.

Various recent studies have shown that the plasma concentrations of glutamine and of glutamate can be used as overreaching and overtraining markers<sup>(18-20)</sup>. Significant increase of the plasma concentration of glutamate in the EXT group was observed, demonstrating that such parameter seems to be sensitive to the training overload increase, influencing directly in the GLN/GLU relation.

Many hypotheses can be used to explain the increase in the glutamate plasma concentration. Rowbottom *et al.*<sup>(18)</sup> suggest a mitochondrial damage of the muscle cells associated to the cell lesion caused by the training increase, as being responsible for the glutamine synthesis impairment. Such fact could as a consequence, cause the glutamate accumulation. However, this hypothesis cannot be used in our study, once no increase in the CK enzyme activity was observed, which would indicate a higher lesion extension. Moreover, an increase in the glycogen concentration in the soleus muscle and maintenance in the gastrocnemius muscle were observed, which are classic alterations due to the training. Besides that, the increase of the hepatic glycogen demonstrated the esquelic muscle integrity in the MOD and EXT groups.

Santos<sup>(18)</sup> demonstrated that the increase in the training overload decreased the glutamine synthesis caused by the partial im-

pairment of the glutamine enzyme synthetase, as suggested by Newsholme<sup>(20)</sup> and Hischock and Pedersen<sup>(21)</sup>. Thus, such evidence, favored the glutamate accumulation, justifying the result found in our study. Beyond that, it cannot be forgotten that the training with very high overload could have increased the protein catabolism. Such fact would increase the glutamate plasma concentration deriving from branched chain amino acids (BCAA), notably the leucine<sup>(22)</sup>. Consequently, we could have an increase of the ammonia plasma concentration that can be changed into urea by the urea cycle in the kidney<sup>(23-25)</sup>. Such hypothesis confirms our results for the EXT group which presented significant urea increase, suggesting that the increase in the training volume can promote glutamate accumulation by the partial impairment of the glutamine synthesis. Moreover, a higher protein catabolism due to the imbalance between training and recovery can be also observed.

Many hormones have their concentrations evaluated in athletes suffering from overtraining and in chronic and excessive stress conditions<sup>(4,26)</sup>. The testosterone plasma concentration has been shown sensitive to the training volume increase, once the exhausting training has been associated to its concentration<sup>(25,27)</sup>. Marathon runners and also athletes in overtraining can present lower testosterone plasma concentration showing that this hormone can be associated to severe exercise stress or fatigue caused by exercising<sup>(25,27)</sup>. Thus, our results confirm previous studies, once the found testosterone decrease was proportional to the training overload. The chronic stress and the overtraining may promote lower activation of the hypothalamus-hypophysis-adrenal axis, leading to cortisol liberation decrease<sup>(1,2,5,19)</sup>. Our result suggests that the increase in the training volume can harm the hypothalamus-hypophysis-gonads axis and can somehow be associated to the reproductive function worsening, in men and women, described in those situations<sup>(9)</sup>.

Concerning the insulin, we observed that the MOD group presented lower concentration of this hormone in relation to the SED animals, confirming many studies that observed decrease in the insulin plasma concentration due to the training<sup>(25)</sup>. However, it is remarkable that the EXT group, despite the training, presented insulinemia similar to the sedentary animals, and consequently, higher than the MOD group, opposing to the studies that observed insulin plasma concentration decrease due to the training<sup>(3,28,29)</sup>.

Such results clearly demonstrate that 6 weeks of training are effective in the decrease of insulinemia, probably due to the increase of insulin sensitivity in rest and higher glucose assimilation by the peripheral tissue during exercise. However, when the training overload was suddenly increased for two weeks, this effect seemed to disappear, since there was no difference between the SED and EXT groups. This evidence allows us to speculate that high physical training overloads without suitable recovery time, for a long period of a life time, could lead to the development of peripheral resistance to insulin.

Actually, former studies have suggested that exhausting training for an extensive time may increase the peripheral resistance to insulin in events where the cell lesion, inflammatory answer and pro-inflammatory cytokines concentration increase such as IL-6 and TNF- $\alpha$  are observed and may increase in up to a 100 times due to exercising<sup>(5,19,29,30)</sup>.

The glycogen muscular concentration may be decreased when rest is insufficient<sup>(3,8)</sup>. In our study no decrease of glycogen muscular concentration was observed in any of the three evaluated sites. Such results are parallel to the glycemia maintenance in the three groups in our research work. The decrease of the glycogen muscular concentration has been pointed as the main cause for the glycemia decrease when there is increase in the training overload or overtraining<sup>(30)</sup>. Although our results confirm such hypothesis, they oppose to classical studies, demonstrating that the increase in the training overload without suitable resting time, chronically decreases the muscular storage of glycogen<sup>(3)</sup>. On the other hand,

when training presents suitable recovery time, increase in the glycogen endogenous concentrations are expected, suggesting that in our study the EXT group animals adapted to the training volume increase did not induce consequently, the classical alterations of training excess that were expected under such circumstances. Perhaps, the proportional increase in the training overload in the last two weeks may have contributed to the adaptation and super compensation, which would justify the lack of overreaching and overtraining signs in the EXT group, consequently limiting the use of this protocol for the overreaching study. Moreover, although the metabolic and physiological similarities between humans and rodents and that such animals are excellent models to studies in exercise physiology and biochemistry, the exaggeration of results in animals to humans should always be cared of.

Thus, we may reach the conclusion that the training protocol used for the EXT group, with sharp increase in the training volume in the last two weeks, despite the glutamate plasma concentration increase and decrease of the GLN/GLU relation, was not capable of promoting similar alterations to the EXT group overreaching. As a conclusion, it was not a suitable protocol for the excessive training study, overreaching or overtraining.

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