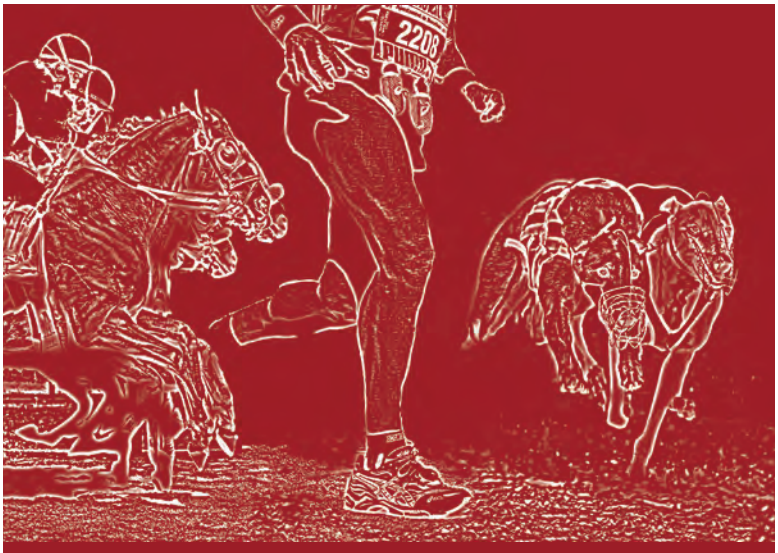


COMPARATIVE
**EXERCISE**
**PHYSIOLOGY**

Publication information
Comparative Exercise Physiology
ISSN 1755-2540 (paper edition)
ISSN 1755-2559 (online edition)

Subscription to 'Comparative Exercise Physiology' (4 issues a year) is either on institutional (campus) basis or on personal basis. Subscriptions can be online only, printed copy, or both. Prices are available upon request from the publisher or from the journal's website (www.wageningenacademic.com/cep). Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Subscriptions will be renewed automatically unless a notification of cancellation has been received before the 1st of December before the start of the new subscription year. Issues are sent by standard mail. Claims for missing issues should be made within six months of the date of dispatch.
Further information about the journal is available through the website www.wageningenacademic.com/cep.

## Paper submission

Manuscripts should be submitted via our online manuscript submission site, www.editorialmanager.com/ecep. Full instructions for electronic submission, as well as the guideline for authors are directly available from this site or from www.wageningenacademic.com/cep.

## Editorial office (including orders, claims and back volumes)


P.O. Box 220
6700 AE Wageningen
The Netherlands
cep_cr@wageningenacademic.com
Tel: +31 317 476516
Fax: +31 317 453417

Comparative Exercise Physiology, 2020; 16 (4): 259-266

Wageningen Academic Publishers

# Glutamine supplementation affects Th1 and Th2 cell populations in endurance horses

**R. Farinelli de Siqueira[1]*, H.C. Manso Filho[2] and W.R. Fernandes[1]**

[1]Departamento de Clínica Médica da FMVZ-USP, Av Dr Orlando Marques de Paiva 87, Cidade Universitária, São Paulo 05580-270, SP, Brazil; [2]Departamento de Zootecnia da Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros, s/n, Dois Irmãos, Recife 52171900, Pernambuco, Brazil; refarinelli@yahoo.com.br

Received: 21 August 2019 / Accepted: 23 February 2020


**RESEARCH ARTICLE**

## Abstract

This study was set out to test the effect of glutamine supplementation on Th1 and Th2 cell populations in endurance horses. The sample was comprised 33 horses competing in four FEI races and successfully completing the final vet check. Horses competed in different distance categories, as follows: 80 km (13 horses, 6 supplemented and 3 controls), 120 km (14 horses; 9 supplemented and 5 controls) and 160 km (6 horses; 3 supplemented and 7 controls). Supplementation consisted of 60 mg/kg bodyweight of oral L-glutamine given for 30 days prior to and 15 days after the race. Blood samples were collected into vacuum tubes with and without EDTA at the following time points: short before race start time, prior to tack fitting (T0); right after and within 3 h of the final vet check (T1 and T2 respectively). Further blood samples were collected at the farm of origin 3, 7 and 14 days after the race (T3, T4 and T5 respectively). Glutamine supplementation affected T-lymphocyte population balance, as shown by increased $CD4^+/CD8^+$ ratio, and increased Th1 and Th2 cytokine production. Glutamine also modulated post-exercise cortisol increased in supplemented horses in this sample. These findings suggest glutamine supplementation attenuates exercise-induced immunosuppression.

**Keywords:** cytokines, endurance, glutamine, horse, lymphocytes

## 1. Introduction

Glutamine is the most abundant free amino acid in the body and is found primarily in the plasma and skeletal muscles. Immune cell replication and growth, acid-base homeostasis, transport of ammonia between tissues and supply of carbon skeletons for gluconeogenesis are some of the functions associated with glutamine (Cruzat *et al.*, 2009).

Exercise modulates glucose and glutamine metabolism and has significant impacts on lymphocyte function, as these cells use both glucose and glutamine for energy production (Newsholme *et al.*, 2003). Insufficient glutamine levels interfere with expression of T lymphocyte surface markers, decrease $CD4^+$ T lymphocyte counts (and hence the $CD4^+/CD8^+$ ratio) and negatively impact interleukin (IL)-2 and interferon (IFN)-γ production (Chang *et al.*, 1999). Different types of exercise affect glutamine production by skeletal muscles in different manners. Extreme endurance exercises have negative impacts on plasma glutamine levels compared to short-term high-intensity exercises due to increased contribution of endurance exercise to processes such as gluconeogenesis and immune cell activation (Kargotich *et al.*, 2007).

The relationship between exercise and susceptibility to infections has long been recognised in humans. Higher incidence of pneumonia in human athletes compared to sedentary individuals and increased risk of progression from upper respiratory tract infection to pneumonia following strenuous exercise have been reported (Cowles, 1918). Profound effects of exercise-related stress on the immune system have been confirmed in later studies investigating associations between exercise, immune responses and disease. Comparatively few data exist regarding the specific effects of exercise on the immune system of domestic animals. Still, this is a topic of interest in veterinary medicine, as horses and other domestic animals are often submitted to strenuous exercise.

CD4+ T helper cells ($T_h$), CD8+ cytotoxic T cells ($T_c$) and regulatory T cells (Treg) orchestrate the adaptive immune response in mammals (Reiner, 2009). Th1 cytokines IFN-γ, tumour necrosis factor (TNF) and IL-2 mediate protection against intracellular viruses, bacteria and protozoa via macrophage activation and cytotoxic T cell induction, or cell-mediated immune response. In contrast, Th2 cytokines IL-4, IL-5 and IL-13 are involved in humoral immune response, mast cell and eosinophil recruitment and activation, immunoglobulin (Ig)E-mediated allergic reactions and elimination of extracellular parasites. CD4+ precursors may also differentiate into IL-10-producing $T_{reg}$ cells associated with immune response suppression (Callard, 2007).

In a study investigating different doses or supplemental oral glutamine in healthy horses (Harris *et al.,* 2006), 60 mg/kg doses were shown to increase serum glutamine levels within a few hours of ingestion, with no concurrent increase in ammonia levels or other side effects. This dose was therefore used in this study. This study set out to investigate the effects of glutamine supplementation on Th1 and Th2 cell populations in endurance horses competing at different distances.

## 2. Materials and methods

This study was approved by the Ethics Committee for the Use of Animals of FMVZ-USP, protocol No. 2606/2012.

### Animals

This study comprised horses (purebred Arabian, Anglo-Arabian or other Arabian crosses) weighing between 400 and 450 kg and trained for endurance riding. Horses were privately-owned and housed at different training centres and horse farms in the state of São Paulo. Horses were turned out in small *Cynodon* grass (tifton or coast cross) paddocks during the day and kept in stalls overnight. Feeding regimen consisted of commercial feed (crude protein 10-11%, crude fat 9-10%) fed at individually adjusted amounts according to nutrient requirements for horses (NRC 2007).

Horses in this sample were selected out of a larger group of 57 horses randomly allocated to receive glutamine supplementation or no supplementation (supplemented and control horses respectively). Supplemented horses were fed 60 mg/kg bodyweight of pure L-glutamine (as per Harris *et al.*, 2006) for 30 days prior to and 15 days after the race. Glutamine palatability was individually tested prior to supplementation. All horses accepted the supplement well; therefore, glutamine was added to daily morning feed, including race day.

### Endurance races

Horses competed in four FEI (Fédération Equestre Internationale) approved races hosted in the cities of Bragança Paulista, Pirassununga, Sorocaba and Campinas, in the state of São Paulo. Horses were fed roughage and commercial feed up to 5 h prior to race start; all horses received supplemental electrolyte-enriched fluids during the race and had free access to water and grass hay during compulsory rest periods.

### Sampling

Horses arrived at the venue the day before the race and were confined to FEI surveyed stalls located in limited access areas overnight. Blood samples were collected into vacuum tubes with and without EDTA (ethylene diamine tetra acetic acid) at the following time points: short before race start time, prior to tack fitting (T0); right after and within 3 h of the final vet check (T1 and T2 respectively).

Only horses successfully completing the final vet check were retained in the sample. The final sample comprised 33 horses competing in different distance categories, as follows: 80 km (13 horses, 6 supplemented and 3 controls), 120 km (14 horses; 9 supplemented and 5 controls) and 160 km (6 horses; 3 supplemented and 7 controls).

Blood samples were also collected at the farm of origin 3, 7 and 14 days after the race (T3, T4 and T5 respectively). All samples were cooled prior to processing. Clotted blood samples were centrifuged at 2,400 rpm for 10 min for serum separation; serum samples were fractioned and stored at -80 °C.

### Laboratory analyses

Laboratory analyses consisted of leukocyte count and differential, T lymphocyte immunophenotyping and cortisol and interleukin (IL-2, IL-4, IL-10 and IFN-γ) level determination. Serum cortisol and interleukins IL-2, IL-4 and IL-10 were measured using the multispecies ADI-901-071 (Enzo Life Sciences, Farmingdale, NY, USA) and the equine Duoset DY 1613, DY1809 and DY1605 (R & D Systems, Minneapolis, MN, USA) ELISA kits respectively. IFN-γ was measured using the horse E-77-806 kit (Bethyl laboratories, Montgomery, TX, USA).

Immunophenotyping was carried out using a three-tube panel, as follows: one blank tube, one tube containing anti-horse CD2 and CD4 monoclonal antibodies (Serotec IgG2a rat anti-horse CD2 MCA1278 and IgG1mouse anti-horse CD4 MCA1078, respectively; Bio-Rad, Hercules, CA, USA) and one tube containing anti-horse CD2 and CD8 (Serotec IgG1mouse anti-horse CD8 MCA 2385). Tubes were incubated for 30 min at room temperature,

washed and labelled with goat anti-rat IgG RPE and goat anti-mouse IgG FITC secondary antibodies (Serotec 303009 and Serotec 103002, respectively); tubes were then reincubated in the dark for 30 min and washed. Labelled cells were resuspended in 1 ml of phosphate buffered saline and analysed using a flow cytometer (BD FACS Calibur; BD Biosciences, San Jose, CA, USA) for lymphocyte subpopulation identification and count. Flow cytometry data were analysed using software (Flow Jo; BD Biosciences); cell populations detected via immunophenotyping were displayed in histograms and presented in tabular format.

### Statistical analysis

Differences between supplemented and non-supplemented horses were investigated using the Mann-Whitney test at a significance level of 0.05 ($P<0.05$). Intergroup (80, 120 and 160 km) comparisons were based on the non-parametric Kruskal-Wallis test ($\alpha=0.05$). Comparisons of study variables between time points were based on the Friedman test ($\alpha=0.5$). Results were expressed as mean ± standard deviation.

## 3. Results

According to official records, mean race speed of horses competing in 80 km, 120 km or 160 km courses corresponded to 19.04±1.03 km/h, 19.27±2.27 km/h and 17.85±0.69 km/h, respectively. Mean cortisol levels, neutrophil-to-lymphocyte and $CD4^+/CD8^+$ T-lymphocyte ratios, and mean IFN-γ, IL-2, IL-4 and IL-10 levels in supplemented and non-supplemented (control) horses competing in 80, 120 or 160 km races are shown in Tables 1, 2 and 3. Supplemented horses had lower post-race (T1) cortisol levels compared to non-supplemented horses. Cortisol levels remained low for 3 h in horses competing in 80 or 120 km races.

Elevated neutrophil-to-lymphocyte ratio elevation was detected immediately after and 3 h after the race (T1 and T2) in all horses, regardless of supplementation. However, elevation was less pronounced in supplemented horses overall. Increased post-exercise neutrophil counts are to be expected and have been reported elsewhere (Cywinska *et al.*, 2010, 2012; Robson *et al.*, 2003). Elevated $CD4^+/CD8^+$ T-lymphocyte ratio was observed in supplemented horses after the race. Baseline (T0) $CD4^+/CD8^+$ ratio was also higher in supplemented compared to non-supplemented horses competing in 80 or 160 km courses.

Supplemented horses competing in 80 km races had lower IFN-γ levels at time points T1, T2, T3 and T5 and higher IFN-γ levels seven days after the ride (T4) compared to baseline (T0). Supplemented horses competing in 120 km rides had higher IFN-γ levels at time points T0, T1 and T2 and lower levels at time points T3, T4 and T5 compared to

**Table 1. Mean cortisol levels (± standard deviation) in supplemented and control horses competing at different distances.[1]**

| Distance | Sampling time | Supplemented | Control |
|---|---|---|---|
| 80 km | T0 | 1,512.44±459.56$^{A}$ | 1,787.00±1,551.94$^{A}$ |
| | T1 | 4,942.77±2,883.97$^{B}$ * | 7,961.34±3,232.68$^{B}$ * |
| | T2 | 1,056.24±720.32$^{C}$ * | 3,036.86±1,492.01 * |
| | T3 | 1,056.92±1,069.00$^{C}$ | 1,595.89±2,226.63$^{C}$ |
| | T4 | 749.77±342.52$^{D}$ | 848.10±566.28$^{D}$ |
| | T5 | 615.47±318.20$^{D}$ | 593.17±374.91$^{D}$ |
| 120 km | T0 | 1,862.11±953.84$^{A}$ | 1,873.57±753.59$^{A}$ |
| | T1 | 7,736.86±3,067.19$^{B}$ | 6,855.89±2,952.63$^{B}$ |
| | T2 | 2,658.04±2,206.96$^{A}$* | 4,258.84±4,488.96$^{C}$ * |
| | T3 | 1,305.13±1,399.36$^{C}$ | 1,343.72±1,989.62$^{D}$ |
| | T4 | 1,233.90±1,155.60$^{C}$ | 1,204.36±1,103.93$^{D}$ |
| | T5 | 1,092.41±1,140.53$^{C}$ | 732.45±576.95$^{D}$ |
| 160 KM | T0 | 2,844.48±2,123.96$^{A}$ | 4,434.17±1,362.89$^{A}$ |
| | T1 | 5,629.66±976.36$^{B}$ * | 9,140.37±1,911.77$^{B}$ * |
| | T2 | 976.55±511.47$^{C}$ | 976.55±752.82$^{C}$ |
| | T3 | 517.20±283.59$^{D}$ | 659.82±252.08$^{D}$ |
| | T4 | 1,096.71±132.73$^{C}$ | 1,320.59±543.52$^{C}$ |
| | T5 | 1,365.26±728.27$^{C}$ | 929.87±175.53$^{C}$ |

[1] Means followed by different capital letters in the same column differ significantly ($P<0.05$) between time points. Means followed by * in the same line differ significantly ($P<0.05$) between supplemented and control horses.

**Table 2. Mean (± standard deviation) neutrophil-to-lymphocyte and CD4⁺/CD8⁺ T-lymphocyte ratios in supplemented and control horses competing in 80, 120 or 160 km races.[1]**

| Distance | Sampling time | Neutrophil-to-lymphocyte | | CD4⁺/CD8⁺ | |
|---|---|---|---|---|---|
| | | Control | Supplemented | Control | Supplemented |
| 80 km | T0 | 1.19±0.44 $^{A}$ | 1.48±0.35$^{A}$ | 1.75±1.45$^{A*}$ | 2.74±3.15$^{A*}$ |
| | T1 | 4.20±1.61$^{B}$ | 5.4±3.28$^{B}$ | 1.78±1.83$^{A*}$ | 2.77±2.98$^{A*}$ |
| | T2 | 5.66±2.90$^{C}$ * | 3.83±1.70$^{C}$ * | 1.08±0.45$^{B}$ | 0.61±0.46$^{B}$ |
| | T3 | 2.89±1.70$^{A}$ | 1.28±0.85$^{A}$ | 1.20±1.24$^{B}$ | 1.82±1.52$^{C}$ |
| | T4 | 1.50±0.64$^{A}$ | 1.84±0.41$^{A}$ | 0.67±0.38$^{C*}$ | 2.30±1.16$^{C*}$ |
| | T5 | 1.39±0.66$^{A}$ | 1.62±0.35$^{A}$ | 1.05±0.76$^{B}$ | 1.18±0.34$^{A}$ |
| 120 km | T0 | 1.64±0.51$^{A}$ | 1.40±0.53$^{A}$ | 2.10±1.71$^{A}$ | 1.86±0.58$^{A}$ |
| | T1 | 4.87±2.61$^{B}$ | 4.93±2.97$^{B}$ | 1.89±1.30$^{A}$ | 1.61±1.07$^{A}$ |
| | T2 | 4.51±3.40$^{B}$ | 4.79±2.72$^{B}$ | 0.36±0.24$^{B}$ | 0.45±0.40$^{C}$ |
| | T3 | 1.28±0.35$^{A}$ | 1.39±0.65$^{A}$ | 0.89±0.15$^{B}$ * | 1.21±1.04$^{B}$ * |
| | T4 | 1.92±0.87$^{A}$ | 1.40±0.74$^{A}$ | 0.57±0.22$^{B}$ * | 1.22±1.01$^{B}$ * |
| | T5 | 1.71±0.37$^{A}$ | 1.06±0.18$^{A}$ | 0.98±0.43$^{B}$ | 1.09±0.98$^{B}$ |
| 160 km | T0 | 1.21±0.33$^{A}$ | 1.35±0.21$^{A}$ | 0.55±0.22$^{A}$ * | 2.45±0.94$^{A}$ * |
| | T1 | 8.02±4.76$^{B}$ | 7.63±4.49$^{B}$ | 1.52±0.85$^{B}$ | 1.99±0.66$^{A}$ |
| | T2 | 7.78±1.31$^{B*}$ | 4.62±1.94$^{C*}$ | 4.80±1.40$^{C}$ | 3.78±1.40$^{B}$ |
| | T3 | 1.85±1.11$^{A}$ | 1.27±0.73$^{A}$ | 0.39±0.10$^{D*}$ | 1.59±1.20$^{C*}$ |
| | T4 | 1.62±0.04$^{A}$ | 2.08±1.21$^{A}$ | 0.59±0.21$^{A*}$ | 2.07±4.41$^{A*}$ |
| | T5 | 1.76±0.03$^{A}$ | 1.38±0.68$^{A}$ | 1.13±0.14$^{B}$ | 1.85±0.63$^{A}$ |

[1] Means followed by different capital letters in the same column differ significantly ($P$<0.05) between time points. Means followed by * in the same line differ significantly ($P$<0.05) between supplemented and control horses within time points.

non-supplemented horses competing in the same distance category. Supplemented horses competing in 160 km rides had lower IFN-γ levels throughout.

Supplemented horses had lower baseline (T0) IL-2 levels; IL-2 levels remained low up to T4 in horses competing in 120 km races and increased at time points T1, T4 and T5 in horses competing in 80 km races. Supplemented horses competing in 80 km or 160 km rides had lower IL-4 levels at T2, T3, T4 and T5 and T0, T1, T3, T4 and T5, respectively. Horse competing in 120 km rides had higher IL-4 levels throughout. Horses competing in 80 km rides had lower baseline (T0) and higher post-race (T1 to T5) IL-10 levels. Horses competing in longer rides (120 or 160 km) had higher IL-10 levels throughout.

## 4. Discussion

Strenuous exercise has been shown to interfere with innate and acquired immunity in humans, rats and mice by lowering the neutrophil-to-lymphocyte ratio and suppressing immune cell activity. Similar changes are thought to occur in horses; however, data from human and rodent studies cannot be directly extrapolated and applied to the equine species due to differences in physiological responses to exercise (Cywińska *et al.*, 2010). T lymphocyte redistribution and resulting lower CD4+/CD8⁺ ratio (i.e. increased CD8⁺ T lymphocyte count and predominance of Th2 cells) immediately after exercise have been extensively reported in humans. These changes are thought to be associated with immunosuppression and decreased proliferative and cytotoxic responses in the recovery period (Gleeson and Bishop, 2005; Lancaster *et al.*, 2005; Pedersen and Hoffman-Goetz, 2000; Steensberg *et al.*, 2001).

Immune cell metabolism relies on energy derived from glucose; however, glutamine is also an important fuel for lymphocytes, macrophages and neutrophils, with high rates of utilisation even in the quiescent state (Newsholme, 1994). Different from Hiscock and Pedersen (2002), Dos Santos Cunha *et al.* (2004) believe that decreased plasma glutamine levels induce immune cell dysfunction. In this study, glutamine supplementation was associated with lower post-exercise lymphocyte count and higher post-exercise CD4⁺/CD8⁺ T lymphocyte ratio. Supplemented horses competing in 80 or 160 km races also had higher CD4⁺/CD8⁺ T lymphocyte ratio prior to and up to seven days post-exercise, suggesting improved immune response in this groups. These findings may have reflected differences

**Table 3. Mean (± standard deviation) interferon (IFN)-γ, interleukin (IL)-2, IL-4 and IL-10 levels in supplemented (S) and control (C) horses competing in 80, 120 or 160 km races.[1]**

| Distance | Sampling time | IFN-γ (pg/ml) | | IL-2 (pg/ml) | | IL-4 (pg/ml) | | IL-10 (pg/ml) | |
|---|---|---|---|---|---|---|---|---|---|
| | | C | S | C | S | C | S | C | S |
| 80 km | T0 | 0.87±0.80$^{A}$ * | 0.31±0.21$^{A}$ * | 9,069.03±8,158.53$^{A}$ * | 25,000.02±1,633.89$^{A}$ * | 1,166.77±923.18$^{A}$ | 1,089.41±387.47$^{A}$ | 22,689.40±323.21$^{A}$ * | 149,009.70±985.16$^{A}$ * |
| | T1 | 1.67±0.90$^{B}$ * | 1.06±0.66$^{B}$ * | 14,202.55±13,849.22$^{B}$ * | 30,452.34±12,901.52$^{A}$ * | 1,028.99±896.87$^{A}$ | 1,120.47±983.29$^{A}$ | 11,513.75±158.70$^{B}$ * | 593,266.67±909.87$^{B}$ * |
| | T2 | 1.11±0.96$^{B}$ | 0.97±0.47$^{B}$ | 23,563.79±12,359.90$^{C}$ | 22,526.56±14,143.20$^{B}$ | 3,724.97±630.58$^{B}$ * | 733.13±605.93$^{B}$ * | 31,421.76±676.31$^{C}$ * | 478,753.42±748.59$^{B}$ * |
| | T3 | 0.86±0.67$^{A}$ | 0.88±0.72$^{B}$ | 15,086.08±11,307.69$^{B}$ | 17,898.16±11,870.09$^{C}$ | 2,677.07±212.00$^{B}$ * | 678.88±545.57$^{B}$ * | 68,334.64±598.13$^{D}$ * | 166,246.94 450.74$^{C}$ * |
| | T4 | 0.95±0.82$^{A}$ * | 1.49±0.92$^{C}$ * | 15,310.22±11,307.96$^{B}$ * | 27,554.1 2±15,117.94$^{D}$ * | 3,976.48±525.37$^{B}$ * | 836.86±795.02$^{B}$ * | 14,170.78±533.77$^{B}$ * | 517,783.90±639.71$^{B}$ * |
| | T5 | 0.95±0.80$^{A}$ | 0.82±0.56$^{B}$ | 15,546.09±9,894.84$^{B}$ * | 21,112.53±14,631.71$^{D}$ * | 3,111.30±152.26$^{B}$ * | 959.74±576.27$^{A}$ * | 15,881.00±191.06$^{B}$ * | 269,295.52±867.43$^{A}$ * |
| 120 km | T0 | 0.44±0.39$^{A}$ * | 2.39±1.28$^{A}$ * | 11,716.83±8,247.52$^{A}$ * | 7,102.01±661.62$^{A}$ * | 511.56±336.14$^{A}$ * | 1,374.78±349.92$^{A}$ * | 7,516.87±275.47$^{A}$ * | 435,529.68±994.42$^{A}$ * |
| | T1 | 0.30±0.14$^{A}$ * | 3.39±2.01$^{B}$ * | 13,494.12±8,151.57$^{A}$ * | 6,576.15±813.92$^{A}$ * | 567.77±269.40$^{A}$ * | 1,299.88±137.97$^{A}$ * | 8,801.91±668.78$^{A}$ * | 596,731.64±301.56$^{B}$ * |
| | T2 | 2.17±0.47$^{B}$ | 2.37±2.71$^{A}$ | 11,836.67±8,473.48$^{A}$ * | 7,438.07±2,188.78$^{A}$ * | 528.26±303.56$^{A}$ * | 2,134.87±309.30$^{A}$ * | 6,738.56±684.12$^{A}$ * | 581,280.82±410.60$^{B}$ * |
| | T3 | 3.03±2.52$^{B}$ * | 1.70±0.71$^{C}$ * | 11,289.20±5,492.97$^{A}$ * | 5,298.24±314.34$^{A}$ * | 564.94±476.95$^{A}$ * | 1,927.32±190.30$^{A}$ * | 2,935.62±684.18 $^{B}$ * | 487,113.37±296.82$^{A}$ * |
| | T4 | 4.13±2.95$^{C}$ * | 2.02±0.72$^{A}$ * | 15,353.91±8,424.12$^{A}$ * | 6,624.91±324.67$^{A}$ * | 511.57±433.75$^{A}$ * | 2,267.69±179.20$^{A}$ * | 4,372.71±185.96 $^{B}$ * | 628,591.37±510.90$^{C}$ * |
| | T5 | 3.15±2.41$^{B}$ * | 1.94±0.52$^{A}$ * | 24,133.23±7,322.85$^{B}$ * | 5,885.51±9,791.13$^{A}$ * | 554.87±405.19$^{A}$ * | 2,184.45±188.69$^{A}$ * | 18,996.57±199.10$^{C}$ * | 618,012.74±473.79$^{C}$ * |
| 160 km | T0 | 3.16±2.50$^{A}$ * | 2.05±1.46$^{A}$ * | 7,489.78±2,805.38$^{A}$ * | 3,498.45±1,097.65$^{A}$ * | 3,051.22±184.90$^{A}$ * | 1,284.08±195.24$^{A}$ * | 8,871.13±841.27$^{A}$ * | 230,319.78±877.12$^{A}$ * |
| | T1 | 3.04±2.44$^{A}$ * | 1.61±0.46$^{B}$ * | 5,838.50±397.43$^{B}$ | 4,567.58±2,852.11$^{B}$ | 4,398.97±570.94$^{B}$ * | 1,075.26±972.89$^{B}$ * | 42,607.23±940.69 $^{B}$ | 326,815.62±386.31$^{B}$ |
| | T2 | 2.04±0.00$^{B}$ * | 1.48±0.58$^{B}$ * | 4,837.75±2,110.41 $^{C}$ | 6,579.29±4,900.64$^{C}$ | 2,961.66±982.53$^{A}$ | 1,323.69±369.93 $^{A}$ | 383,192.4±857.59$^{B}$ * | 695,361.89±523.21$^{C}$ * |
| | T3 | 1.72±0.10$^{B}$ | 1.53±0.79$^{B}$ | 5,355.57±2,172.36$^{B}$ | 5,107.54±3,688.01$^{C}$ | 3,113.39±391.71$^{A}$ * | 1,354.44±125.33$^{A}$ * | 101,024.2±881.58$^{C}$ * | 144,739.82±805.88$^{D}$ * |
| | T4 | 2.61±1.48$^{B}$ | 2.12±1.72$^{A}$ | 4,973.18±2,611.55$^{C}$ | 5,493.13±4,381.97$^{C}$ | 7,229.99±116.24$^{C}$ * | 1,203.46±960.24$^{A}$ * | 122,497.2±351.21$^{C}$ * | 660,031.43±421.80$^{C}$ * |
| | T5 | 2.33±1.45$^{B}$ * | 1.16±1.27$^{C}$ * | 4,598.43±858.73$^{C}$ | 3,650.26±2,601.73$^{A}$ | 5,350.53±271.35$^{B}$ * | 1,233.83±974.92$^{A}$ * | 158,696.4±640.11$^{D}$ * | 313,913.70±645.18$^{B}$ * |

[1] Means followed by different capital letters in the same column differ significantly ($P$<0.05) between time points. Means followed by * in the same line differ significantly ($P$<0.05) between supplemented and control horses within time points.

in training regimens between horses competing in 80 or 160 km races (less and more experienced horses respectively).

Physical effort-related changes in white blood cell count are duration- and intensity-dependent. Higher adrenaline levels during exercise trigger lymphocyte recruitment from the spleen, where these cells are primarily stored; however, this effect is offset by rising cortisol levels at the end of the exercise session. Cortisol stimulates neutrophil release from the bone marrow into the circulation; also, swirling blood flow in response to increased cardiac output increases leukocyte mobilisation from marginal pools such as vessel walls (Robson *et al.*, 2003). These factors culminate in biphasic changes in circulating white cell counts, with lymphocytosis and neutrophilia prevailing during and at the end of the exercise session respectively (Risøy *et al.*, 2003). Exercise-induced neutrophilia is expected and has been widely reported by several investigators (Cywinska *et al.*, 2010, 2012; Krumrych, 2006; Robson *et al.*, 2003). Horses in this study had elevated neutrophil-to-lymphocyte ratio immediately after the race; however, this ratio decreased within 3 h in supplemented horses.

The number of adrenergic receptors in lymphocyte subpopulations may dictate the rate of cell mobilisation in response to catecholamine release. Adrenergic receptor numbers differ between cell subpopulations, i.e. receptor numbers are correlated with responsiveness to exercise. Natural killer (NK) cells have the highest numbers of adrenergic receptors, whereas intermediate and lower numbers are found in $CD8^+$ and $CD4^+$ T lymphocytes respectively. This may explain the higher responsiveness of NK cells to exercise and other stressors compared to less responsive $CD4^+$ T lymphocytes (Pedersen and Hoffman-Goetz, 2000).

Different behaviour of cytokines associated with tissue repair among horses supplemented with glutamine in this study are worthy of deeper investigation. Studies with other species reported conflicting findings. In humans, pro-inflammatory interleukins peak after short bouts of high intensity exercise; these are followed by peak IL-10 levels, with return to baseline in 60 min (Cabral-Santos *et al.*, 2015). Studies with human marathon and ultra-marathon runners revealed that post-race elevation of interleukin levels, IL-10 in particular, is exercise duration-dependent (Cabral-Santos *et al.*, 2018; Nickel *et al.*, 2012).

Supplemented horses in this study had low post-race levels of pro-inflammatory Th1 cytokines INF-γ and IL-2, while the behaviour of anti-inflammatory Th2 cytokines was inconsistent. Supplemented horses competing in 120 km rides had higher IL-4 levels whereas those competing in 80 or 160 km had lower IL-4 levels throughout. A study by Ibfelt *et al.* (2002) investigating the effects of strenuous exercise on Th1 and Th2 cytokines in human athletes and mice failed to reveal changes in IL-4 levels following exercise, or impacts of cortisol and epinephrine on levels of this cytokine. IL-4 behaviour in this study seemed to reflect changes in $CD4^+/CD8$ ratio. However, IL-10, a cytokine regulating inflammatory responses primarily via macrophage and monocyte activation (Petersen and Pedersen, 2005), remained elevated in supplemented horses up to 14 days after the race, regardless of distance category. Assuming that muscle inflammation is proportional to glycogen depletion and muscle repair, and that glycogen repletion takes place a few days after competitions, these findings suggest similar inflammation-modulating effects of IL-10 in horses and humans.

Exercise-related elevation of plasma cortisol levels has been widely reported in horses (Ferraz *et al.*, 2010; Nesse *et al.*, 2002) and is supported by findings of this study. All horses in this sample had higher cortisol levels immediately after the race, with return to baseline (T0) or near baseline levels within 3 h. Cortisol level increase was less pronounced right after the ride (T1) and 3 h after the ride (T2) in horses supplemented with glutamine competing at all distances. Findings of this study suggest that glutamine supplementation was associated with lower post-exercise cortisol levels. This was not surprising, as high blood glutamine levels in response to supplementation of this amino acid may decrease the need for glutamine synthase (GS) expression. Cortisol is a major factor behind increased GS expression in glutamine-producing tissues such as muscles and the lungs (Manso Filho *et al.*, 2008); therefore, glutamine supplementation may help spare endogenous glutamine.

Glutamine supplementation attenuated post-race cortisol level increase and was associated with elevated $CD4^+/CD8^+$ T lymphocyte ratio and lower pro-inflammatory cytokine levels, with potential mitigation of the immunosuppresive state induced by long-term exercise.

## 5. Conclusions

Glutamine supplementation affected T-lymphocyte population balance, as shown by increase in the $CD4^+/CD8^+$ T lymphocyte ratio, and interfered with Th1 and Th2 cytokine production. Glutamine also modulated post-exercise cortisol level increase in supplemented horses. These findings suggest that glutamine supplementation attenuates exercise-induced immunosuppression.

## Acknowledgements

To Fundação de Apoio a Pesquisa do Estado de São Paulo (FAPESP) for funding this study.

## References


Cabral-Santos, C., De Lima Junior, E.A., Fernandes, I., Pinto, R.Z., Rosa-Neto, J.C., Bishop, N.C. and Lira, F.S., 2019. Interleukin-10 responses from acute exercise in healthy subjects: a systematic review. Journal of Cellular Physiology 234: 9956-9965. https://doi.org/10.1002/jcp.27920

Cabral-Santos, C., Gerosa-Neto, J., Inoue, D.S., Panissa, V.L., Gobbo, L.A., Zagatto, A.M., Campos, E.Z. and Lira, F.S., 2015. Similar anti-inflammatory acute responses from moderate-intensity continuous and high-intensity intermittent exercise. Journal of Sports Science and Medicine 14: 849-856.

Callard, R.E., 2007. Decision-making by the immune response. Immunology and Cell Biology 85: 300-305. https://doi.org/10.1038/sj.icb.7100060

Chang, W.K., Yang, K.D. and Shaio, M.F., 1999. Lymphocyte proliferation modulated by glutamine: involved in the endogenous redox reaction. Clinical and Experimental Immunology 117: 482-488. https://doi.org/10.1046/j.1365-2249.1999.01009.x

Cowles, W., 1918. Fatigue as a contributory cause of pneumonia. Boston Medicine and Surgery 179: 555-557. https://doi.org/10.1056/NEJM191810311791801

Cruzat, V.F., Petry, É.R. and Tirapegui, J., 2009. Glutamina: aspectos bioquímicos, metabólicos, moleculares e suplementação. Revista Brasileira de Medicina do Esporte 15: 392-397.

Cywinska, A., Szarska, E., Górecka, R., Witkowski, L., Wyszyńska, Z., Hecold, M., Bereznowski, A., Schollenberger, A. and Winnicka, A., 2012. Experimental immunology lymphocyte proliferation activity after limited (Light class) and long (CEI) distance endurance rides in horses. Central European Journal of Immunology 37: 326-331. https://doi.org/10.5114/ceji.2012.32720

Cywinska, A., Wyszynska, Z., Gorecka, R., Szarska, E., Witkowski, L., Dziekan, P., Winnicka, A. and Schollenberger, A., 2010. The effect of the 162 km endurance ride on equine peripheral blood neutrophil and lymphocyte functions. Polish Journal of Veterinary Sciences 13: 279-285.

Dos Santos Cunha, W.D., Giampietro, M.V., De Souza, D.F., Vaisberg, M., Seelaender, M.C. and Rosa, L.F., 2004. Exercise restores immune cell function in energy-restricted rats. Medicine and Science in Sports and Exercise36: 2059-2064. https://doi.org/10.1249/01.mss.0000147626.32295.38

Ferraz, G.C., Teixeira-Neto, A.R., Pereira, M.C., Linardi, R.L., Lacerda-Neto, J.C. and Queiroz-Neto, A., 2010. Influência do treinamento aeróbio sobre o cortisol e glicose plasmáticos em equinos. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 62: 23-29.

Gleeson, M. and Bishop, N.C., 2005. The T cell and NK cell immune response to exercise. Annals of Transplantation 10: 43-48.

Harris, R.C., Harris, P.A., Routledge, N.B.H., Naylor, J.R.J. and Wilson, A.M., 2006. Plasma glutamine concentrations in the horse following feeding and oral glutamine supplementation. Equine Veterinary Journal 38: 637-642. https://doi.org/10.1111/j.2042-3306.2006.tb05618.x

Hiscock, N. and Pedersen, B.K., 2002. Exercise-induced immunodepression – plasma glutamine is not the link. Journal of Applied Physiology 93: 813-822. https://doi.org/10.1152/japplphysiol.00048.2002

Ibfelt, T., Petersen, E.W., Bruunsgaard, H., Sandmand, M. and Pedersen, B.K., 2002. Exercise-induced change in type 1 cytokine-producing CD8+ T cells is related to a decrease in memory T cells. Journal of Applied Physiology 93: 645-648. https://doi.org/10.1152/japplphysiol.01214.2001

Kargotich, S., Keast, D., Goodman, C., Bhagat, C.I., Joske, D.J.L., Dawson, B. and Morton, A.R., 2007. Monitoring 6 weeks of progressive endurance training with plasma glutamine. International Journal of Sports Medicine 28: 211-216. https://doi.org/10.1055/s-2006-924218

Krumrych, W., 2006. Variability of clinical and haematological indices in the course of training exercise in jumping horses. Bulletin Veterinary Institute in Pulawy 50: 391-396.

Lancaster, G.I., Khan, Q., Drysdale, P.T., Wallace, F., Jeukendrup, A.E., Drayson, M.T. and Gleeson, M., 2005. Effect of prolonged exercise and carbohydrate ingestion on type 1 and type 2 T lymphocyte distribution and intracellular cytokine production in humans. Journal of Applied Physiology 98: 565-571. https://doi.org/10.1152/japplphysiol.00754.2004

Manso Filho, H.C., Costa, H.E.C., Wang, Y., McKeever, K.H. and Watford, M., 2008. Distribution of glutamine synthetase and an inverse relationship between glutamine synthetase expression and intramuscular glutamine concentration in the horse. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 150: 326-330. https://doi.org/10.1016/j.cbpb.2008.03.015

National research council (NRC), 2007. Nutrient requirements of horses. 6th ed. National Academies Press, Washington, DC, USA.

Nesse, L.L., Johansen, G.I. and Blom, A.K., 2002. Effects of racing on lymphocyte proliferation in horses. American Journal of Veterinary Research 63: 528-530. https://doi.org/10.2460/ajvr.2002.63.528

Newsholme, E.A., 1994. Biochemical mechanisms to explain immunosuppression in well-trained and overtrained athletes. International Journal of Sports Medicine 15 Suppl. 3: S142-S147. https://doi.org/10.1055/s-2007-1021129

Newsholme, P., Lima, M.M.R., Procopio, J., Pithon-Curi, T.C., Doi, S.Q., Bazotte, R.B. and Curi, R., 2003. Glutamine and glutamate as vital metabolites. Brazilian Journal of Medical and Biological Research 36: 153-163.

Nickel, T., Emslander, I., Sisic, Z., David, R., Schmaderer, C., Marx, N., Schmidt-Trucksäss, A., Hoster, E., Halle, M., Weis, M. and Hanssen, H., 2012. Modulation of dendritic cells and toll-like receptors by marathon running. European Journal of Applied Physiology 112: 1699-1708. https://doi.org/10.1007/s00421-011-2140-8

Pedersen, B.K. and Hoffman-Goetz, L., 2000. Exercise and the immune system: regulation, integration, and adaptation. Physiological Reviews 80: 1055-1081. https://doi.org/10.1152/physrev.2000.80.3.1055

Petersen, A.M.W. and Pedersen, B.K., 2005. The anti-inflammatory effect of exercise. Journal of Applied Physiology 98: 1154-1162. https://doi.org/10.1152/japplphysiol.00164.2004

Reiner, S.L., 2009. Decision making during the conception and career of CD4+ T cells. Nature Reviews Immunology 9: 81-82. https://doi.org/10.1038/nri2490

Risøy, B.A., Raastad, T., Hallén, J., Lappegård, K.T., Baeverfjord, K., Kravdal, A., Siebke, E.M. and Benestad, H.B., 2003. Delayed leukocytosis after hard strength and endurance exercise: aspects of regulatory mechanisms. BMC Physiology 3: 14. https://doi.org/10.1186/1472-6793-3-14

Robson, P.J., Alston, T.D. and Myburgh, K.H., 2003. Prolonged suppression of the innate immune system in the horse following an 80 km endurance race. Equine Veterinary Journal 35: 133-137. https://doi.org/10.2746/042516403776114144

Steensberg, A., Toft, A.D., Bruunsgaard, H., Sandmand, M., Halkjær-Kristensen, J. and Pedersen, B.K., 2001. Strenuous exercise decreases the percentage of type 1 T cells in the circulation. Journal of Applied Physiology 91: 1708-1712. https://doi.org/10.1152/jappl.2001.91.4.1708