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Biomarkers of Stress and Inflammation in Assessing the Quality of Welfare of Conditioned Vaquejada Horses

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Abstract

In recent decades, the intensity of training and equestrian competitions has significantly increased, thus the assessment of the well-being of the equine athlete has become essential in all equestrian modalities. The aim of this study was to ascertain whether equine athletes submitted to a *vaquejada* simulation test (VqST), comprised of three races, presented changes in blood biomarkers related to stress and health status. Fourteen healthy Quarter Horses, used as pull horses in this equestrian modality, were evaluated. Ten animals were submitted to the VqST and the remaining four were used as a control group. Blood samples were collected pre-test (during fast), immediately after, and at 1, 4 and 24 hours of recovery. The assessed blood biomarkers included cortisol, interleukin (IL)-6, IL-1β, iron, urea, creatinine, and gamma-glutamyl transferase (GGT) concentrations and results were analyzed using One Way ANOVA (time) with the SigmaStat 13.0 software. No differences between sample times were detected in both groups ($p > 0.05$) and no differences were found between groups ($p > 0.05$). The results suggest that all horses were well conditioned for the level of effort imposed by the three vaquejada races. The adaptation to physical exercise may enable the regulation of the acute response to stress in the tissues involved in the exercises, with no differences being observed in stress and health biomarkers, such as IL-6, IL-1β and cortisol. In conclusion, well-conditioned vaquejada horses exhibit a balanced regulation of biological processes, which contributes an increased athletic longevity and better quality of athletic life.

Keywords

Horse Races, Welfare, Hormones, Cortisol, Interleukins, Iron

1. Introduction

The characterization of the well-being of athletic horses has been determined through invasive and non-invasive methodologies. Recent approaches aim to better understand the processes involved in the acute and chronic stress of these animals through the evaluation of different biomarkers [1]-[5]. Equestrian sports that derive from rural activities of different countries, such as those developed in cattle farms, can benefit from such methodologies as they can provide new insights into metabolism during and after the physical activity in this large group of athletes.

In recent years, the frequency and intensity of training and competitions have significantly increased, which may compromise the well-being of athletic horses if the horses are not regularly evaluated. V*aquejada* horses, for example, are trained 4 to 6 days a week for up to 60 minutes in many training centers [6], because the trainers believe that this is the best way to prepare these horses for the championships. Moreover, in a *vaquejada* competition, horses must run at least five times to qualify for the final race, which takes place one or two days after the initial qualifications. Then they will run five or more times until the result is known. In these cases, regular evaluation of different blood biomarkers can serve as an indicator of metabolic and physiologic changes induced by the competitions and training, providing important information about the health and well-being of these animals. Thus, a regular assessment can contribute to athletic longevity and produce a positive impact on legislations and regulations related to equestrian operations and horses' welfare.

Previous studies with *vaquejada* horses have reported that these equine athletes, when competing and training under official rules [7] [8] [9], show transient changes in different blood biomarkers associated with stress, health, and well-being [3] [10] [11]. These variations in properly trained horses occur physiologically, with an acute increase in different biomarkers such as lactate, hematocrit, creatine kinase (CK), and cortisol, among others, with the recovery of baseline values in less than 24 hours. Such studies used several indicators and signals which can included in the different models for assessing equine welfare [4].

More recently, the concomitant assessment of biomarkers of stress and inflammation has been indicated for the characterization of animal welfare, as they provide information regarding the effects of exercise on different tissues [12] [13] [14]. Thus, stress and inflammation indicators, such as cortisol, interleukin (IL)-6 and IL-1β, were proposed as a method to assess the animal behavior and possible impact, positive or negative, on different tissues. Additionally, acute phase proteins (APP) can also be used as inflammatory biomarkers, such as transferrin/iron, since APP are influenced by variations in IL-6 [15] [16]. Ferritin/Iron is a negative acute phase reactant, as its concentration is reduced in the acute phase of inflammation. In horses, the normal concentration for serum iron ranges from 80 to 277 µg/dL, but concentrations below 64 - 85 µg/dL are considered indicative of some degree of systemic inflammation [16] [17] [18] [19]. Another biomarker that can also be used is gamma-glutamyl transferase (GGT). This enzyme has been associated with overtraining and thus can be used to assess the health status of equine athletes [20] [21] [22].

Thus, we hypothesized that *vaquejada* horses would present an increase in blood biomarkers following physical exercise which would return to baseline values after a period of recovery. For this purpose, the concentrations of blood cortisol, IL-6, IL-1β, iron, GGT, urea and creatinine before and sequentially after *vaquejada* simulation tests (VqST) were assessed. The same evaluation protocol was performed in a group of animals that was not submitted to VqST. Such information may contribute to the assessment of the welfare in equine athletes of this equestrian sport, particularly during regular training for *vaquejada* races.

2. Material and Methods

All procedures were approved by the Committee for Ethics in the Use of Animals of Centro Universitário Cesmac (CEUA/CESMAC) under the registration number 01200702819/2016-97.

Animals and breeding system: Fourteen Quarter Horses (age: 4 - 8 years old; weight: ~450 Kg; body score: 5 - 6/9) were housed in individual stalls, measuring 15 m^2 and with good views of other horses, at a training center for *vaquejada* races (−7.973887, −35.021494), located in the Pernambuco's Atlantic Forest zone, Brazil. All male horses used during this study were pull vaquejada horses and had not participated in a vaquejada race in the last 5 days. *Vaquejada* is a high-intensity and short-duration exercise in which helper horses (HH) are responsible to keep a bull running in a line while pull horses (PH) work to put the bull down after 100 m of running.

The 14 animals were randomly distributed into two groups: the exercise group (n = 10) and the control group (n = 4, without exercise). The exercise group underwent the *vaquejada* simulation test (VqST) as indicated in the literature [2] [23], whilst the control group remained stabled and did not perform the exercise test.

Both groups of horse were fed approximately 20.0 kg of fresh chopped elephant grass (*Pennisetum purpureum* Schum.), which was divided into three daily meals (5 a.m., 2 p.m., 6 p.m.). Each animal also received an additional supplementation with 6.0 kg/day of concentrate, divided into three meals of ~2.0 kg/animal (6 a.m., 12 p.m., 5 p.m.) (MaxEquinos Trabalho Mix, CP: 14% (min); EE: 5% (min); FB: 10% (max); MM 10% (max); ADF 15% (max), DuRacho Nutrição Animal, Brazil). Salt and water were offered *ad libitum* to all horses.

All animals had the same level of physical conditioning and a similar training, which comprised of trot and gallop exercises, performed 3 to 4 times a week, for 30 to 40 minutes, on an official track for the sport. This exercise routine could be performed with or without running with cattle. At least twice a week, the animals were ridden for 60 minutes at walk in areas with varied topography.

***Vaquejada* simulation test:**

The VqST was performed according to previous citations and in accordance with the official regulation [2] [7] [23]. In the exercise group, the test comprised of 10 minutes of warm-up, at trot and gallop, followed by three races with cattle on a 130m long sand track, lasting 15 to 20 seconds per race with 1-minute interval between them. In the end, the horses walked during 15 minutes for recovery. The races were held between 7 a.m. and 8 a.m. and the duration of the horse's races was measured with a digital stopwatch. Cattle and horses were evaluated 24 hours before, during, and 24 hours after performing the VqST through the "Five Domains" system [24]. As previously mentioned, the control group animals did not perform the VqST and remained in the stables during this period.

Collection of blood samples:

Pre-test blood samples were collected with the animals in their stalls, following a fasting period of 8 hours. The remaining samples were collected immediately after the VqST, and at 1, 4, and 24 hours of recovery. For the animals of the control group, blood samples were obtained in the same moments except the phase immediately after the exercises. Blood samples were obtained by jugular venipuncture using negative pressure tubes with EDTA and tubes without anticoagulant, to obtain plasma and serum, respectively. Samples were then stored at −20˚C. The analysis of serum urea, creatinine, iron and GGT were performed with semi-automatic equipment (Doles D250, Doles Equipamentos Laboratoriais, São Paulo, Brazil) using commercial kits of the same brand. Cortisol, IL-6, and IL-1β were measured using ELISA in semi-automatic equipment (Bioclin® Minddray MR-96A, 837, Minas Gerais, Brazil) and the commercially available kits (*Cortisol*: Fine-EH0641-96T, Wuhan Fine Biotech Co., Wuhan, China; *IL-1beta*: BOST-EK0410-96SW, Boster Biological Tech, Pleasanton, California, USA; *IL-6*: BOST-EK0410-96SW, Boster Biological Tech, Pleasanton, California, USA).

Statistical Analysis:

The results were analyzed using One-way ANOVA and the Tukey test, to assess the possible influence of sampling time and to compare groups. In all cases, the significance level was set at 5% and the results are expressed as means ± mean standard error.

3. Results

The analyzed biomarkers did not present significant variations between both groups of horses ($p > 0.05$) (Table 1). Also, no significant differences were found when comparing the two groups regardless of sampling moment ($p > 0.05$) (Table 2).

The speed of the *vaquejada* horses in the exercise group was set between 7.5 and 9.0 m/s and each race lasted on average of 12 to 20 seconds.

All animals of the exercised group were evaluated 24 hours after the races, and they were clinically healthy and showed no signs of lameness. In the animals of the control group, there were also no significant changes during the analyzed period.

Table 1. Biomarkers of health and stress in the exercise group (n = 10), pre- and post-*vaquejada* simulation test, and in the control group (n = 4).

Biomarkers	Experimental phases				
	Pre-race	Immediately after the 3 races	+1 hour recovery	+4 hours recovery	+24 hours recovery
Exercise group					
Cortisol (ng/mL)	50.13 ± 1.90	48.05 ± 3.00	50.10 ± 0.80	51.62 ± 0.60	51.36 ± 0.42
IL-6 (pg/mL)	11.74 ± 1.53	13.47 ± 1.71	12.49 ± 1.89	13.67 ± 1.59	12.65 ± 1.40
IL-1β (pg/mL)	7.51 ± 1.84	5.60 ± 1.59	9.67 ± 2.00	6.94 ± 0.93	9.25 ± 2.49
Iron (μg/dL)	136.7 ± 14.37	158.59 ± 13.54	156.84 ± 15.40	177.63 ± 13.60	169.38 ± 14.73
Urea (mg/dL)	49.63 ± 3.20	41.72 ± 2.92	40.77 ± 2.78	43.35 ± 3.93	44.50 ± 2.57
Creatinine (mg/dL)	2.04 ± 0.06	2.24 ± 0.05	2.28 ± 0.06	2.08 ± 0.12	2.12 ± 0.06
GGT (IU/L)	40.54 ± 1.70	35.74 ± 2.60	32.74 ± 3.15	35.28 ± 2.55	32.50 ± 2.72
Control group					
Cortisol (ng/mL)	51.1 ± 1.51	-	47.85 ± 1.83	51.64 ± 0.12	52.56 ± 0.35
IL-6 (pg/mL)	11.19 ± 3.75	-	13.19 ± 0.47	16.81 ± 1.08	12.52 ± 3.83
IL-1β (pg/mL)	6.77 ± 1.3	-	9.74 ± 1.37	12.84 ± 1.27	6.56 ± 3.11
Iron (μg/dL)	135.54 ± 4.34	-	175.28 ± 25.30	153.90 ± 6.00	176.34 ± 17.00
Urea (mg/dL)	36.93 ± 4.10	-	40.26 ± 3.87	40.7 ± 7.66	45.42 ± 9.66
Creatinine (mg/dL)	1.94 ± 0.13	-	2.13 ± 0.15	2.03 ± 0.13	2.01 ± 0.23
GGT (IU/L)	29.97 ± 5.56	-	41.49 ± 3.49	38.89 ± 1.4	34.85 ± 2.05

Notes. IL-6: interleukin 6; IL-1β: interleukin 1 beta; GGT: gamma-glutamyltransferase; Different letters at same line indicate $p < 0.05$.

Table 2. Comparison of health and stress biomarkers between exercise group (n = 10) and control group (n = 4).

Biomarkers	Experimental Groups	
	Exercise group	Control group
Cortisol (ng/mL)	50.25 ± 0.73	50.77 ± 0.74
IL-6 (pg/mL)	12.81 ± 0.71	12.25 ± 1.60
IL-1β (pg/mL)	7.90 ± 0.84	9.15 ± 1.13
Iron (μg/dL)	161.00 ± 6.42	162.51 ± 8.82
Urea (mg/dL)	44.00 ± 1.41	40.67 ± 3.03
Creatinine (mg/dL)	2.15 ± 0.03	2.02 ± 0.07
GGT (IU/L)	35.30 ± 1.18	36.30 ± 1.99

Notes: IL-6: interleukin 6; IL-1β: interleukin 1 beta; GGT: gamma-glutamyltransferase; Different letters at same line indicate p < 0.05.

4. Discussion

The hypothesis proposed by the authors was not confirmed, since the imposed *vaquejada* simulation test did not change cortisol concentrations, like previously reported in these equine athletes [10]. The absence of changes in cortisol concentration has also been described in three-barrel horses in the same moments of evaluation [25]. The body stress responses are activated by stimuli present at rest, throughout exercises/training, due to circadian cycle, and inflammatory processes, among others [12] [26] [27]. These stressors stimulate the release of cortisol, IL-1β, IL-6, TNF-alpha, and some other proteins, which form a whole chain of protection for the body of animals in these different conditions [14] [28] [29] [30] [31]. The better the conditioning and the longer the recovery time, the smaller the responses associated with stress and inflammation of the different tissues, activated during physical exertion. This may explain the findings of the present research, which suggest a physiological adaptation to the level of physical exertion imposed during training and physical conditioning throughout the athletic life of the studied horses [6] [21] [22].

The impact of exercise on stress hormones has been described in different species and may vary according to exercise regimes, pre-existing competition stress, as well as physical conditioning level [31] [32]. A regular training regimen adapted to the expected level of physical exercise can modulate the secretion of hormones and inflammatory cytokines, resulting in a reduction of the inflammatory processes by stimulating the intrinsic anti-inflammatory metabolism [12] [22] [29] [32] [33]. The non-alteration of the studied cytokines, associated with the cortisol results, suggest that the *vaquejada* horses, under the conditions imposed during this study, did not present any major disturbances in their homeostasis. This is probably due to the high level of training that these animals possessed, which allowed the practice of this physical activity without it compromising their health and welfare. Thus, the regular assessment of these biomarkers can be used to assess athletic performance and ultimately contribute to longevity in different types of equestrian sports.

IL-6 appears to play a central role in the body's protective processes against stress and inflammation. It reduces the impact of IL-1β and TNF-α on muscles and other tissues during exercise and in the recovery phase [34]. Stimulated by muscle contraction, IL-6 is largely produced at these moments, mainly by fast-twitch fibers that have a higher expression of this cytokine when compared to slow-twitch fibers [35] [36]. An intense activity of fast-twitch fibers is normally observed in horses that practice sprint equestrian sports [37] [38]. Thus, the authors expected a higher production of IL-6 in *vaquejada* Quarter Horses, since these animals can reach peak speeds ≥9.0 m/s during the races [23]. However, the physical activity proposed in the present research was not enough to change these cytokines, even with the horses moving with a maximum speed between 7.5 and 8.0 m/s, which lead to increases in the temperature of the ocular caruncle and some previously described hematological parameters [39].

In 4-beat gaited (*marcha*) horses' simulations events, characterized mainly by resistance exercises in which they work for 40 - 70 minutes at 9 - 12 km/h, animals presented an increase in IL-6 but not in cortisol or IL-1β, having been proposed that these findings are associated with the metabolism of fats and are not result of inflammatory processes [40]. However, in untrained Standardbred horses it has been reported that despite the levels of IL-6 remaining unchanged an increase in IL-1 was observed [41]. Thus, these cytokines appear to behave differently in exercised conditioned and non-conditioned animals. Well-conditioned horses may not present significant variations in the concentrations of IL-6 and IL-1β, corroborating the interpretation made in the present research for serum cortisol.

Different acute phase proteins (APP) are produced in the liver tissue in response to several nonspecific factors, including physical activity [16] [18] [42] [43]. They are classified as positive APPs (serum amyloid A and fibrinogen) or negative APPs (albumin and transferrin/iron as an acute phase reactant) according to their increase/decrease in response to the inflammatory stimulus. Recently, another classification has been proposed dividing these biomarkers in positive (C-reactive protein), moderate (haptoglobin and ferritin/iron), and negative (albumin and paraoxonase-1) [19]. These APP classification systems may be useful for a better understanding the physiological impact of exercise on health and welfare. Different studies evaluated supplementation and the impact of physical effort on iron in athletic horses [43] [44] [45].

Assessments of iron concentration were done in two groups of pull *vaquejada* horses, one that regularly performed with professional cowboys and in another group with amateur cowboys, and no variations in iron concentration were observed in each group or between groups after a *vaquejada* simulation test with three races [46]. On the other hand, in another study with three-barrel horses, an increase in serum iron was observed after a race but retuned to baseline values at 30 minutes of recovery [47]. When comparing exercises of different intensities, iron concentrations were found to vary in response to intense and moderate workouts, but no differences between the two intensity levels were found when samples were collected 12- and 24 hours post-exercise [43]. However, analyzing the results of these authors, changes in iron concentrations did not exceed 25%, even with significant differences. In the present studies, no differences were observed in iron concentrations during recovery. These results reinforce that the horses were well conditioned and maintained homeostasis for this biomarker of inflammation during recovery, indicating that the current experimental animals' health and well-being were not compromised.

There are reports in the literature that the liver enzyme GGT is associated with excessive training (overtraining), and therefore the concentration of this enzyme has been regularly measured to assess the horse's performance [20] [48] [49]. It was demonstrated that well-trained horses had a higher concentration of GGT than non-habitual exercised horses, and this enzyme also seems to be related to an increase in training effort, including intensity and duration [50]. GGT concentrations in *vaquejada* horses did not change, again suggesting good athletic conditioning, similarly to findings reported in another study with *vaquejada* horses, both in pull and helper horses [10] and in Quarter Horses of three-barrel trials [25]. However, elevations in GGT concentration are not yet clearly associated with overtraining in athletic horses, but some authors indicate that this enzyme may act as a pro-oxidative enzyme and affect iron availability for some anti-oxidative processes [51]. Therefore, more studies should be carried out to better understand the relationships between GGT, iron, and other biomarkers to assess the health of athletic horses and thus their well-being.

Few studies report variations in urea and creatinine concentrations that may occur according to the degree of physical exertion or overtraining [48]. Initial studies did not indicate variations in these two biomarkers of protein metabolism in horses [52], corroborating the findings of the present research. However, elevation in creatinine, but not in urea concentration immediately after exercise was described in Standardbreds horses [53]. Also, different intensities and durations of exercises led to increases of these biomarkers particularly in response to a more demanding training effort. These processes may also occur during intense exercise, due to the degradation of muscle tissue proteins, or during illness. Nonetheless these variations should not be expected in a well-conditioned horse [52].

5. Conclusion

The current results allow us to conclude that the Quarter Horses used in the present research submitted to the *vaquejada* simulation test, did not present significant variations in the studied stress and inflammatory biomarkers (IL-6, IL-1β, cortisol and iron) suggesting that they were well conditioned for the level of imposed physical exertion. Well-conditioned horses exhibit a balanced regulation of biological processes, which contributes an increased athletic longevity and better quality of athletic life.

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Authors' Contribution

CJFLS, KLGT, RKSC, HECCCM and HCMF, contributed to the data collection and execution. RKSC, HCMF, JDRF, JSAS, CSC and HECCCM contributed to the data interpretation. CJFLS, HCMF, HECCCM, JSAS, CSC and JDRF contributed to the study design, data interpretation, and manuscript preparation. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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