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Original Research

Correlation Between Serum Activity of Muscle Enzymes and Stage of the Estrous Cycle in Italian Standardbred Horses Susceptible to Exertional Rhabdomyolysis

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ABSTRACT

Equine exertional rhabdomyolysis (ER) is a well-recognized clinical syndrome affecting racehorses. Prevalence analysis of ER showed that female sex was a significant risk factor. The aim of this research was to evaluate the differences and correlations in the serum activity of muscle enzymes and the stage of the estrous cycle in ER-susceptible and control (C) mares. Serum muscle enzyme activity before and after exercise and sex hormones were analyzed in the two groups of mares. Ten cyclic ER and 10 cyclic C mares were examined weekly for 4 weeks. During diestrus, ER horses had significantly higher resting and postexercise aspartate aminotransferase (AST) activity, but not creatine kinase (CK) activity, compared with controls; only postexercise AST activity was significantly higher during estrus compared with activity levels in controls. During estrus, 17β-estradiol and AST activity were significantly negatively correlated in the control but not ER mares. Based on our results, further studies should be performed to characterize the presumptive different roles played by sexual hormones in horses susceptible to ER compared with healthy mares.

1. Introduction

Equine exertional rhabdomyolysis (ER) is a well-recognized myopathy affecting racehorses [1]. It is a clinical syndrome characterized by stiff, painful muscles following exercise, as well as by skeletal muscle fiber necrosis [1]. Although the clinical signs of the syndrome are well known, the etiology, pathogenesis, and treatment are not fully understood [2].

The risk factors for and the prevalence of ER have been investigated in several studies [3–8]. Female sex has been recognized as a significant risk factor in Swedish and Italian Standardbreds (IS) [6,7], as well as in other horse breeds [4,5]. The reason for the higher prevalence remains unclear.

In human medicine and animal models, several studies have demonstrated the influence of estrogen on postexercise muscle damage and repair-related processes [9–13]. In contrast, less is known about the influence of progesterone on postexercise muscle inflammation [14]. A study about the changes in the serum concentrations of muscle enzymes in Thoroughbred racehorses demonstrated marked fluctuations but could not highlight any relationship with the stage of the estrous cycle [15]. However, samples were collected from fillies and colts in training with no history of clinical signs of ER [15]. The relationship between the

Animal welfare/ethical statement: The study hereby presented, titled "Correlation between serum activity of muscle enzymes and stage of the estrous cycle in IS horses susceptible to exertional rhabdomyolysis," was performed with the best standard of veterinary care in accordance with the Guiding Principles in the Care and Use of Animals approved by Italian and European laws. The study used private client-owned animals and required informed client/owner written consent.

Conflict of interest statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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activity of muscle enzymes and the stage of the estrous cycle in mares predisposed to ER has never been investigated to the authors' knowledge. This study aimed to evaluate the changes in muscle enzyme activities in mares affected by ER compared with control horses before and after typical exercise and the correlation of such changes with the stage of the estrous cycle and sex hormone fluctuations.

2. Methods

2.1. Animals

The study used private client-owned animals and required client or owner written informed consent. It was performed with the best standard of veterinary care in accordance with the Guiding Principles in the Care and Use of Animals approved by Italian laws.

Twenty cyclic female IS racehorses belonging to different stables within a single training yard were examined weekly for 1 month from June 1 to July 1. Mares enrolled in the study were provided by the yard veterinary practitioner and were all in good training conditions and race fit.

The horses were divided into two groups, hereinafter referred to as disease groups. The ER group included 10 mares that had experienced at least two ER bouts in the previous training season. No episodes of ER were reported in the last month, and no specific dietary or training practices to prevent recurrence of ER were in place. The ER bouts had always been from mild to moderate, characterized by sweating and signs of discomfort, stiff gait, reluctance to move, and swollen and painful gluteal muscles. None of the mares had ever presented severe signs, that is, prolonged recumbency or inability to stand up. Myoglobinuria was detectable in approximately 50% of all episodes. The clinical diagnosis was always confirmed based on the serum activity of creatine kinase (CK) and aspartate aminotransferase (AST), which were elevated more than twofold of the reference value (CK 135 I.U.; AST 290 I.U.). The control (C) group included 10 mares that had never shown any clinical sign of ER or abnormal increase in the serum activity of muscle enzymes at routine screenings. For each ER horse, the control was chosen from the same stable. Although the statistical analysis was not applied to training and feeding practices, management was similar for all horses.

2.2. Methods

During the study period, owners were asked not to administer any drugs to the mares enrolled in the study. During the first examination, signalment, anamnestic, management, and performance data of each horse were noted. In particular, the name, age best time/km and money earned over the entire career, temperament, feeding practice, raced/unraced status, and training practice were recorded. The examinations were executed at 10:00 AM to 11:00 AM in the morning after a day of rest.

A first blood sample was collected before the examination (T_0). Soon after, transrectal palpation and ultrasound examination of the reproductive system were performed, always by the same operator (MPP), with portable ultrasound equipment using a 6–8 MHz multifrequency linear probe (Sonovet 600; Medison Co, Ltd, Seoul, Korea). The mares were judged in estrus if they presented a doughy uterus, uterine folds, a dominant follicle >30 mm in diameter, and a soft cervix.

Then, the horses underwent work, consisting of jogging at a comfortable and relaxed speed on track for 40 minutes. A second blood sample was collected between 4 and 6 hours after the end of the training (T_1). All samples were collected from the jugular veins into glass vacutainers, one with ethylenediaminetetraacetic acid for complete blood cell count (CBC) and the other plain for serum biochemistry. The samples were sent to the laboratory on ice packs within 2 hours of collection, immediately centrifuged and processed.

Parameters evaluated at T_0 included CBC, CK, AST, alanine aminotransferase (ALT), lactate dehydrogenase (LDH), sorbitol dehydrogenase (SDH), gamma-glutamyl transferase (γGT), sodium, potassium, ionized calcium, chloride, progesterone (P4, ng/mL), and 17β-estradiol (E2, pg/mL). At T_1, only the serum activity of CK and AST was evaluated.

2.3. Hormone Assays

Serum E2 and P4 levels were determined by a chemiluminescence immunoassay using a commercial kit (Medical Systems S.p.a., Genova, Italy). The sensitivity was 10 pg/mL for estradiol and 0.2 ng/mL for progesterone. The intra-assay variations for both were <10%, whereas both interassay variations were <15%.

2.4. Statistical Analysis

All data were managed using a computerized spreadsheet (Microsoft Excel 2011 for Mac, Microsoft Corporation, Redmond, WA) before importing them into a program for statistical analysis (IBM SPSS Statistics v. 26.0, IBM; Prism 8 for macOS v. 8.2.0; GraphPad Software, Inc, CA). Normality was tested with Shapiro–Wilk's *W* test.

The CK and AST serum activities were log-transformed [16], and a mixed model was applied to each of them, including as fixed effects the group, exercise (values detected pre- or post-exercise), and the presence of estrus, and as random effects the subjects and the measurement week. Because no variance of the model was explained by the random effects, the four measurements for each mare were pooled together as single measurements. Then, a general linear model (GLM) procedure was applied to the log-transformed CK and AST serum activities separately, including as effects the group, exercise effect, the presence of estrus, E2 and P4 serum levels, and their interactions (i.e., group $\times$ estrus, group $\times$ E2, group $\times$ P4, group $\times$ exercise, exercise $\times$ estrus, and group $\times$ exercise $\times$ estrus). If the GLM showed that an effect significantly influenced the CK or AST serum activity, further analysis was applied.

Continuous data (the E2 and P4 levels, the CK and AST activities, age, money earned, and best time) from each group were pooled together, after the same procedure described earlier, considering the exercise (when deemed necessary) and estrous cycle phase influence. They were compared between disease groups using a pooled Student's *t* test when normally distributed and a Mann–Whitney *U* test when not normally distributed. Correlations between CK, AST, E2, and P4 levels were studied within disease groups considering the phase of the estrous cycle by using Pearson's *product-moment* (r) or Spearman's *rank-order correlation* (r_s). Statistical significance was set at $P < .05$.

3. Results

During the period of observation, one mare from the ER group was sold before the last evaluation, so clinical, hematological, and biochemical data were compared between 40 samples collected in the C group and 39 samples in the ER group. All the mares had a regular estrous cycle and did not show any evident clinical signs of ER during the period of evaluation.

Mares belonging to the ER group were 3.5 $\pm$ 1.1 (3.5; 2–5) years old, and C mares were 3.7 $\pm$ 1.6 (3; 2–7) years old; no difference was detected between the disease groups. There were no

Table 1
Other enzymes serum activities and electrolyte concentrations.

Enzyme (Units)	ER Group, Mean ± SD (Median)	C Group, Mean ± SD (Median)
Estrus		
ALT (U/L)	21 ± 24 (14)	12 ± 5 (12)
LDH (U/L)	386 ± 124 (374)	428 ± 131 (433)
SDH (U/L)	2.6 ± 0.7 (2.1)	2.5 ± 0.6 (2.2)
γGT (U/L)	14 ± 7 (12)	14 ± 5 (12.5)
P4 (ng/mL)	0.6 ± 0.2 (0.5)	0.6 ± 0.2 (0.6)
E2 (pg/mL)	23.2 ± 16.5 (22.0)	12.7 ± 7.3 (11)
Na (mEq/L)	138 ± 2 (138)	139 ± 2 (138)
Cl (mEq/L)	106 ± 4 (106)	103 ± 4 (103)
K (mEq/L)	4 ± 0.3 (4)	4 ± 0.2 (4)
iCa (mg/dL)	6 ± 0.4 (6)	6 ± 0.3 (6)
Diestrus		
ALT (U/L)	23 ± 25 (13.5)	13 ± 5 (11)
LDH (U/L)	409.5 ± 184.5 (366)	419 ± 107 (397.5)
SDH (U/L)	2.4 ± 0.5 (2.2)	2.4 ± 0.5 (2.2)
γGT (U/L)	15 ± 7 (13)	14 ± 7 (13)
P4 (ng/mL)	11.6 ± 9.1 (11.5)	10.1 ± 6.5 (10.8)
E2 (pg/mL)	10.5 ± 14.2 (5.0)	9.1 ± 5.1 (5.0)
Na (mEq/L)	138 ± 3 (138)	137 ± 2 (137)
Cl (mEq/L)	103 ± 4 (102.5)	103.5 ± 3 (103)
K (mEq/L)	4 ± 0.2 (4)	4 ± 0.3 (4)
iCa (mg/dL)	6 ± 0.4 (6)	6 ± 0.2 (6)

Abbreviations: γGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; C, control; Cl, Chloride; E2, 17β-estradiol; ER, exertional rhabdomyolysis; iCa; ionized calcium; K, potassium; LDH, lactate dehydrogenase; Na, sodium; P4, progesterone; SDH, sorbitol dehydrogenase.

significant differences between the two disease groups regarding money earned and the best time/km. All the mares followed a training program consisting of 30–40 minutes of daily jogging (training at slow speed) and working (training at high speed or racing) 2 d/wk. One day per week, the horses rested in a box or a small paddock.

All mares were in estrus only once during the study period, but one mare from the ER group had two estrus periods. The P4 and E2 serum activities did not differ between the disease groups, during any phase of the estrous cycle. The SDH, LDH, ALT, and γGT activities were within the reference ranges in both disease groups, and there was no difference between the groups (Table 1). The mean (±SD, and median) muscle enzyme values for the ER and C groups at T_0 and T_1 and in estrus, and diestrus are reported in Tables 2 and 3, respectively; scatter plots of the log-transformed data are reported in Fig. 1.

The GLM could not identify any effect of the group ($P = .13$) and of the estrous cycle phase ($P = .89$), exercise ($P = .44$), E2 ($P = .35$), or their interaction on CK activity, except for a significant effect of P4 levels ($P = .002$). On the other hand, a significant effect of the group ($P = .007$) on AST activity was detected, as well as an effect of both E2 and P4 levels ($P < .0001$, for both), but no effect of estrous cycle phase ($P = .54$), exercise (0.76), or their interaction could be detected. The ER AST activities were significantly higher than those of the C group at T_0 ($P = .004$) and T_1 ($P = .004$) during diestrus and at T_1 ($P = .04$) during estrus. A graphical representation of T_1 AST activity over the study period in the two disease groups is reported in Fig. 2.

Table 2
Muscle enzymes serum activities during estrus.

Muscle Enzyme, Mean ± SD (Median)	ER Group (n = 11), Mean ± SD (Median)	C Group (n = 10), Mean ± SD (Median)
T_0 CK	107.5 ± 39 (101)	107 ± 31 (104.5)
T_0 AST	391 ± 364 (282)	241 ± 46 (239)
T_1 CK	124 ± 43 (119)	110 ± 31.5 (101)
T_1 AST	397 ± 319.5 (280)	242.5 ± 48 (224)

Abbreviations: AST, Aspartate Aminotransferase; C, Control; CK, Creatine Kinase; ER, Exertional Rhabdomyolysis; T_0, pre-exercise; T_1, 4–6 hr postexercise.
The number of measurements per group are reported in brackets.

Table 3
Muscle enzymes serum activities during diestrus.

Muscle Enzyme	ER Group (n = 28), Mean ± SD (Median)	C Group (n = 30), Mean ± SD (Median)
T_0 CK	141 ± 115 (104.5)	105 ± 28 (100.5)
T_0 AST	401 ± 307 (295.5)	247 ± 48 (247.5)
T_1 CK	210.5 ± 368 (107)	108 ± 22 (103.5)
T_1 AST	431 ± 328 (295.5)	256 ± 44 (255)

Abbreviations: AST, Aspartate Aminotransferase; C, Control; CK, Creatine Kinase; ER, Exertional Rhabdomyolysis; T_0, pre-exercise; T_1, 3–6 hr postexercise.
The number of measurements per group are reported in brackets.

In control horses, a significant inverse correlation between AST and E2 levels was detected during estrus both at T_0 ($r_s = -0.69$; $P = .03$; Fig. 3A) and T_1 ($r_s = -0.83$; $P = .003$; Fig. 3B). No other significant correlations were detectable in either disease groups.

4. Discussion

The main results of our study are that both pre- and post-exercise AST activities were significantly higher in susceptible ER mares than in control mares during diestrus and, only after exercise during estrus; that is, AST activities were not significantly different between susceptible ER mares and control mares before exercise during estrus. Exertional rhabdomyolysis is a well-known syndrome with characteristic clinical and serological alterations [17,18]. It has been hypothesized that different underlying pathologic processes may lead to ER clinical syndrome [19,20]. Nonetheless, female sex was identified as a significant risk factor in most epidemiologic studies performed among different horse breeds [4–7] but not in others [19,20]. From this perspective, to the best of our knowledge, our study is the first attempt to elucidate a possible correlation between ER syndrome and the estrous cycle in IS.

In a unique study about the changes in serum muscle enzyme levels associated with the stage of the estrous cycle, Fraunfelder et al studied the changes in CK and AST activities using the plasma progesterone profiles as an indicator of the estrous cycle, without finding an association [15]. However, in that study, horses were categorized based on their CK activity, and mares with increased and markedly increased serum CK activity showed clinical signs in 30%–50% of cases [15]. In our study, we compared mares that had a confirmed diagnosis of rhabdomyolysis based on clinical signs and biochemistry profile and that had at least two episodes in the previous training season with mares that never had clinical symptoms attributable to ER. Our results showed differences in AST activities between the control and ER groups before and after exercise in diestrus but only after exercise in estrus. The different experimental designs might explain the disparities between our results and the previous literature [15]. Moreover, the study by Fraunfelder is centered on recurrent exertional rhabdomyolysis in Thoroughbreds [15], whereas our study investigated ER in IS. It should be noted that ER in IS, as well as in other horse breeds, has not yet been characterized as deeply as in Thoroughbreds and Quarter Horses, and it might be a symptom of different underlying pathologies. Nevertheless, it is intriguing that a sexual predisposition had been found. In our study, the ER group might have included mares showing ER as a common feature of different pathologic entities.

A significant inverse correlation between AST activity, pre- and post-exercise, and estrogen levels was detected in control mares during estrus. This result is consistent with the protective role of estrogens on myofibers demonstrated in previous studies in rats

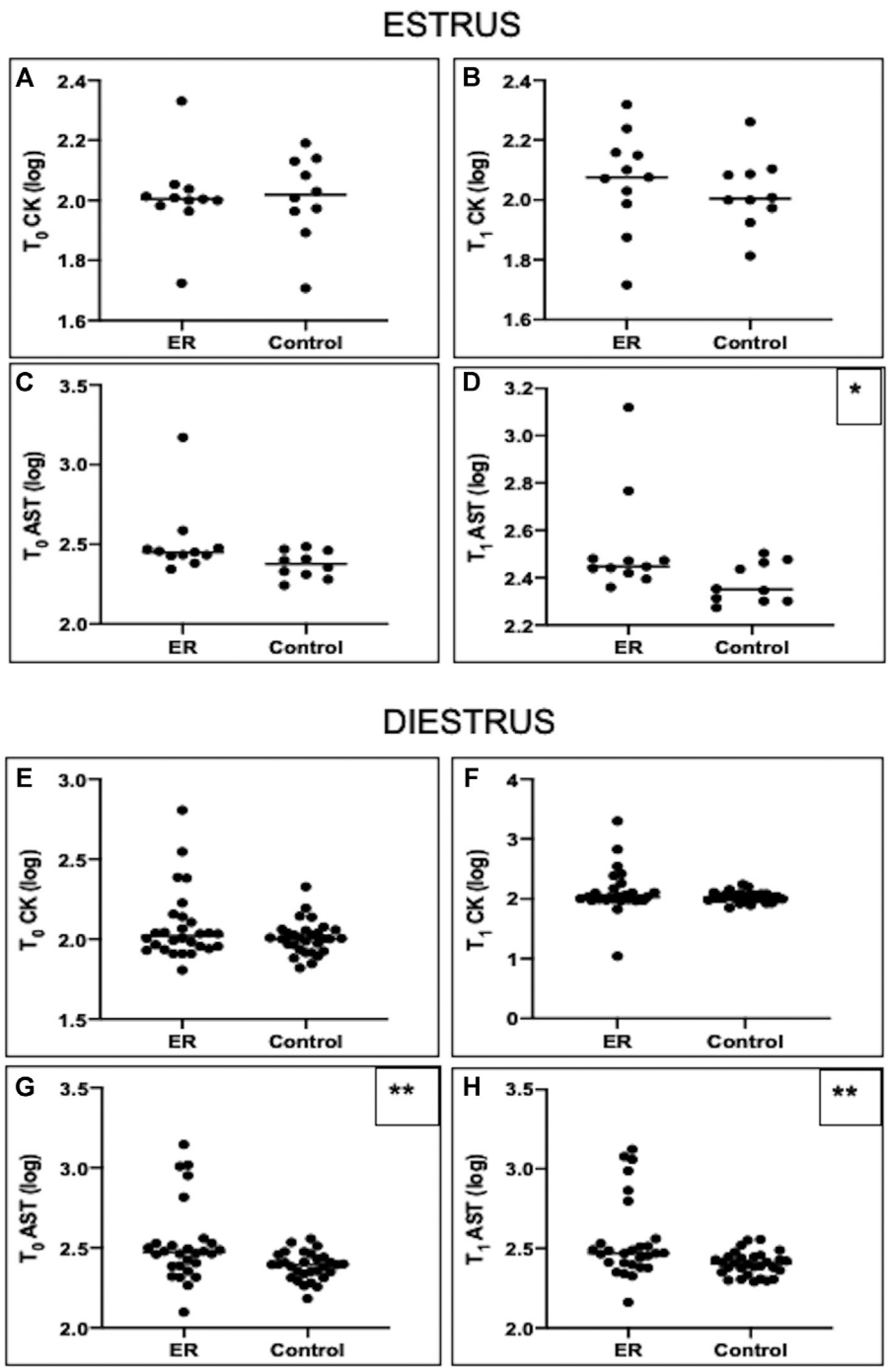

Fig. 1. Graphic representation (scatter plot) of single measurements of log-transformed CK and AST activities in ER and control mares, during estrus and diestrus. Lines within the graphs represent the median. (A) Pre-exercise (T_0) CK activity during estrus. (B) Postexercise (T_1) CK activity during estrus. (C) Pre-exercise (T_0) AST activity during estrus. (D) Postexercise (T_1) AST activity during estrus. (E) Pre-exercise (T_0) CK activity during diestrus. (F) Postexercise (T_1) CK activity during diestrus. (G) Pre-exercise (T_0) AST activity during diestrus. (H) Postexercise (T_1) AST activity during diestrus. *ER*, exertional rhabdomyolysis; C, control; CK, creatine kinase; AST, aspartate aminotransferase. *$P < .05$, **$P < .005$.

and humans [9,12,13,21–24]. In contrast, in the ER group, an inability of estrogens to protect might exist. Further studies should explore by which pathways estrogens might exert their protective role on myofibers in the horse.

Indeed, the degree of myofiber damage and the inflammatory cell infiltration in response to strenuous exercise differed by sex in muscle biopsies collected before and after eccentric exercise in humans and rats, with males showing a higher degree of inflammation and lower plasma estradiol concentrations compared with females [25,26]. It was demonstrated that sex differences in indices of skeletal muscle damage, inflammation, and repair are mostly attributable to estrogen [27]. A critical protective role of estradiol in blunting muscle damage in response to intense eccentric exercise and in preserving muscle function after exercise-induced muscle damage was also suggested [13]. Despite the evidence for the positive effects of estrogens on skeletal muscle, the mechanisms by which it works remain elusive. The primary role that estrogens seem to play is probably because of the mechanisms of action of

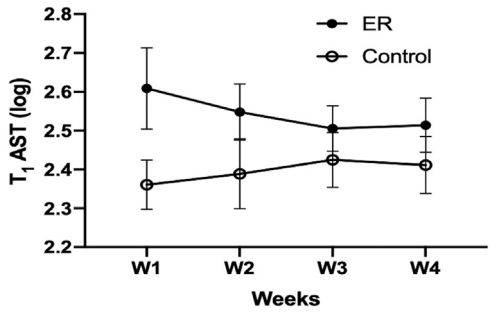

Fig. 2. Graphic representation of the trend over the 4 weeks study period of log-transformed, postexercise (T_1) AST activities (mean ± standard deviation) in ER and control mares. ER, exertional rhabdomyolysis; C, control; AST, aspartate aminotransferase.

these hormones that can act both with receptor-mediated effects and via nonreceptor-mediated events [28,29]. Because of its ability to act as an antioxidant and a membrane stabilizer through its interactions with the phospholipid bilayer, E2 may have a positive effect by reducing muscle membrane damage [22].

The effect of P4, detected by the GLM procedure, on both CK and AST activities, suggests that this sex hormone may also play a role. Little is known about the role played by progesterone in muscle metabolism in either physiological or pathologic conditions. Studies in rats demonstrated that progesterone induces a small but significant attenuation of postexercise muscle leukocyte infiltration [14]. It has been recently demonstrated that performance worsens in Greyhound racing dogs when serum progesterone increases; this has been explained by the altered carbohydrate and fat metabolism in liver and muscles and the reduced glucose uptake by muscles [30]. Hence, the exact role of progesterone in the ER and other muscular conditions needs further investigation.

The importance of applying standardized exercise tests to improve within- and between-studies comparability has been underlined [20,31–33]. In our study, all procedures were performed before and after exercise sessions performed by experienced drivers, all working in the same training yard. Hence, even if not quantified, the training sessions applied were almost identical for all horses included in our study. As reported in other studies [16,18,34], we sampled blood 4–6 hours after the completion of the training session. The timing of blood samples is also crucial when looking for subtle enzyme activity alterations as well as for exercise-induced gene expression [30,34]. Serum AST increases are usually delayed by 36–48 hours after muscle damage when CK levels tend to normalize [1]. Nonetheless, plasma CK and AST activities have been reported to peak 4–6 hours after exercise in healthy horses [16,31]. The plasma half-life of CK in horses is short (108 minutes, 123 ± 28 minutes.), whereas the plasma half-life of AST is 7–10 days [1,34]. Even if AST is not specific for muscle damage, because elevation can also occur with liver necrosis, evaluation of SDH and γGT allows differentiation between muscle and liver necrosis [33–35]. In our cases, SDH and γGT were within the reference range. Thus, AST was considered to result from muscle damage or from an alteration in muscle cell membrane permeability [31]. Moreover, AST was proposed as the best indicator of susceptibility to rhabdomyolysis, even if it does not seem to be predictive of an immediate episode of ER [16]. None of the horses developed clinical signs of myopathy during the study period. Because of the long half-life of AST activity, and the continuous training program over the 4-week study period, we cannot certainly exclude that the higher AST activities recorded in the ER group could be linked to previous-day subclinical ER bouts. Hence, different study designs should be applied to ascertain whether this difference in AST activity between the two disease groups is linked to the presence of a latent condition of muscle distress in horses predisposed to ER or to true subclinical ER bouts.

Another intriguing aspect, although not explored in our study, is the hypothesized heritability of ER. Indeed, this condition has been reported as moderately heritable, between 39% and 49% in Standardbreds by single nucleotide polymorphism analysis [36]. As discussed hereafter, the absence of a histologic diagnosis, which might lead to different stratifications of ER cases, may further elucidate the different effects of sex hormones on different myopathologies and hence the heritability of such a condition(s).

The main limitations of our study are the low number of mares and the brevity of the study period, which was only 1 month long. It would give more valuable information to have a higher number of horses included in the study for a more extended period, but further blood sampling requires a high level of compliance by the owners. By selecting controls from the same stable, shared feeding routine, similar exercise regimes, and the same track, we attempted to standardize management factors and to reduce the potential influence of factors that can hardly be controlled through statistical analysis in small groups [33]. Moreover, even if pooling repeated measures from a single subject might be seen as a violation of independency of measurements [37], it has been demonstrated to be as reliable as aggregating single measurements from different subjects, as long as the same number of measurements have been recorded from each subject [38]. Another limitation might be the absence of muscle biopsies; such a diagnostic aid should be included in a standard algorithm when dealing with suspected chronic or recurrent muscle pathologies, but again, a high level of compliance from owners would be needed since even when performed on a standing horse, this procedure is seen as too invasive. Nonetheless, histopathological features may allow further

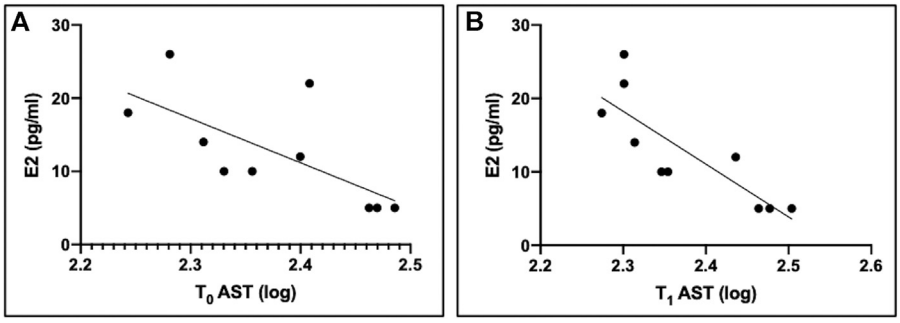

Fig. 3. Graphic representation of the significant inverse correlation between pre- (T_0) and post-exercise (T_1) log-transformed AST and E2 activities, during estrus, in control mares. (A) T_0 correlation ($r_s = -0.69$; $P = .03$). (B) T_1 correlation ($r_s = -0.83$; $P = .003$). ER, exertional rhabdomyolysis; C, control; AST, aspartate aminotransferase; E2, 17β-estradiol.

stratification of the clinical cases and perhaps a better understanding of the different pathologic identities.

5. Conclusions

In conclusion, our results showed that AST activities in ER mares are always higher than those in control mares, except for pre-exercise activities during estrus. Hence, far from being a conclusive study, our results suggest that sex hormones may play a different role in horses predisposed to ER.

Our results suggest that estrogen may not have the capacity to play a protective role in ER horses during estrus, whereas in healthy mares, a negative correlation was found between estrogen and AST activity. Because it is highly improbable that there is a direct causal effect of sex hormones on ER, further studies with a different design, that is, longer study periods as well as a larger sample size and muscle histopathology stratification, should be performed to further explore the effect of sex hormones during ER. Clinical and hormonal evaluations of the estrous cycle stage during the episodes of ER could be useful to correctly verify if a correlation between the recurrence of symptoms and estrous cycle exists. The potential influence of progesterone on skeletal muscles deserves further investigations.

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