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Original Research

Differential Expression of Monocarboxylate Transporter 1 and Ancillary Protein CD147 in Red Blood Cells of Show Jumping Horses

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ABSTRACT

We compare the expression levels of the lactate transporter complex consisting of the lactate transporter, monocarboxylate transporter 1 (MCT1), and its ancillary protein, cluster of differentiation 147 (CD147), in the membranes of red blood cells (RBCs) from two breeds of jumping horses and associate the expression levels of these proteins with their jumping ability. The expression levels of MCT1 and CD147 proteins on the membranes of RBCs collected from 30 show jumping horses of two different breeds were quantified: the Brazilian Sport Horses (n = 17) and the European Warmbloods (n = 13). The levels of MCT1 and CD147 in the RBC membranes were measured by western blot using horse-specific antibodies. Statistical analyses included unpaired Student *t* test and chi-squared test. According to the expression levels of MCT1 and CD147 proteins, 88% of the Brazilian Sport Horses were categorized as high lactate transporters (HTs) and the remaining 12% as low lactate transporters (LTs). The opposite was found for the European Warmbloods, where most animals (77%) were classified as LTs and the remaining animals (23%) were classified as HTs. Brazilian Sport Horses express statistically significantly higher levels of CD147 and MCT1 than European Warmbloods. The classification of horses considering the expression of proteins involved in the ability to transport lactate through the complex MCT1-CD147 seems to be breed dependent, with horses that are able to jump higher obstacles showing lower expression of the MCT1-CD147 complex in their RBCs.

1. Introduction

During intense exercise, glycolysis contributes to energy production with a concomitant increase in the concentration of muscle lactate and H^+ ions. These ions are carried simultaneously from the myofibers onto the circulation (blood/plasma) by monocarboxylate transporter (MCT). This mechanism may create a lactate concentration

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gradient between the muscular compartment and the plasma, favoring the removal of lactate and H^+ ions from the muscle, thereby affecting intramuscular pH during exercise [1]. Among the 14 MCT isoforms identified so far, MCT1 is the best studied [2] and the most abundant in equine red blood cells (RBCs) membranes [1]. This protein requires the ancillary protein cluster of differentiation 147 (CD147) [3] to enable to translocate lactate and H^+ ions from the cytoplasm to the plasma membrane [4]. In horses, up to 50% of these ions can be carried into RBCs [5]. According to Mykkänen et al [1], lactate transport activity can be correlated to the optical density (OD) of the MCT1-CD147 complex in the RBC's membranes of horses.

Studies examining Standardbred horses stated that MCT1 and CD147 expression is distributed bimodally in high lactate transporters (HTs) and low lactate transporters (LTs), lactate transport activity in RBCs [5,6], and extended to Thoroughbred and Finnhorses [1]. In the latter study [1], three different breeds were compared, including specificities (Trotters vs. Racehorses) and different athletic abilities. Furthermore, training has been shown to increase RBC lactate transport activity in reindeer and sled dogs, but not in horses [1,6].

The levels of MCT1 and CD147 mRNA correlated with the expression of CD147 and suggest that the bimodality of their expression is regulated at the transcriptional level [7]. However, little is known about the expression profiles of the MCT1 and CD147 proteins in jumping horses. The present study is the first to investigate the expression profiles of MCT1 and CD147 in jumping horses and to associate the expression patterns with the jumping ability of the animals.

2. Materials and Methods

2.1. Horses

Thirty horses were distributed into two groups according to their breeds: group Brazilian Sports Horses (BH, n = 17; body weight 490 ± 53 kg; age 11 ± 3 years) and group European Warmbloods (EW, n = 13; body weight 550 ± 50 kg; age 10 ± 4 years). The BH horses belong to the Brazilian Army and are stabled in Resende, RJ, Brazil. They were also used in a recently published study [8]. The EW horses belong to the Brazilian elite Equestrian Society (Sociedade Hípica Paulista).

At the time of blood sampling, all horses had participated in jumping competitions with height obstacles no higher than 1.20 m for the BH group and no lower than 1.30 m for the EW group. The horses were clinically healthy and all had participated for at least 10 months. The study followed the Ethical Principles in Animal Experimentation (Jaboticabal, Brazil, Approval number: 019 281/13). The results have been partially presented at the ninth International Conference on Equine Exercise Physiology (ICEEP 9) in June 2014, in Chester, England [9].

2.2. Collection of Blood Samples and Western Blot

Venous jugular blood samples were collected, at rest, in a tube containing ethylenediaminetetraacetic acid, and used to isolate membranes from RBCs according to the method described by Koho et al [5]. Briefly, excess hemoglobin was removed by washing three times with 35 mL of 5 mM sodium phosphate buffer (PBS) pH 8.0 followed by centrifugation at 20,000 × *g* and 4°C for 15 minutes to separate the RBC membranes from the total cell lysate. The samples were then resuspended in 100 mL of the phosphate buffer. The protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA).

For the Western blotting, the membranes containing 40 μg of protein were solubilized in Laemmli sample buffer, separated on a 10% (w/v) SDS-PAGE gel (125 vs. 90 minutes), and electro-transferred (100 V, 60 minutes) onto nitrocellulose membranes (Protran). Nonspecific binding sites were blocked with 5% bovine serum albumin (BSA) in 1× TBST supplemented with 0.1% Tween 20.

The membranes were incubated in 1% BSA in YBST overnight at 4°C with horse anti-CD147 or anti-MCT1 primary antibodies against the C-termini of horse MCT1 and CD147 (sequences CKGTEGDPKEESP and CGHHVNDKDKNVRQRNAS, GenBank accession no. AAR21622.1 and ABQ53583.1, respectively). These antibodies were raised in rabbits and purified by affinity chromatography. They were kindly provided by the Nina Koho of Helsinki University.

The membranes were subsequently incubated with peroxidase-conjugated antirabbit antibody (DAKO). The chemiluminescence capture system (ChemiDoc MP—Bio-Rad) with developer (Bio-Rad) was used for visualization. The reference sample was from a known horse with a high amount of CD147, whose OD value was adjusted to 1.0. Thus, the OD of each experimental immunoblot and, therefore, the average amount of CD147 from each group were expressed as the amount of protein relative to the reference sample [10]. The bands were quantified using the Image Lab (Bio-Rad 4.0) software. The bimodal expression was determined by the CD147 amount according to the method described by Mykkänen et al [1]. The categorization of samples from the horse breeds as LT or HT was based on the ODs obtained in the Western blot experiment.

2.3. Statistics

Differences between breeds were determined using the chi-square test and unpaired Student *t* test. The level of significance was set at $P < .05$. All calculations were performed using the R statistical analysis software.

3. Results

Bands corresponding to the expected molecular weights of MCT1 (44 kDa) and CD147 (46 kDa) were detected in samples from both breeds of jumping horses analyzed (Fig. 1). However, the expression levels of both proteins were lower ($P < .01$) in EW horses than in BH horses (Fig. 2). In the BH breed, 88% and 12% of the horses were classified as HT and LT, respectively. The opposite was seen in the EW breed with most of the horses (77%) being classified as LT and 23% as HT. Fig. 3 shows that the distribution of MCT1 and CD147 in the HT or LT animals is

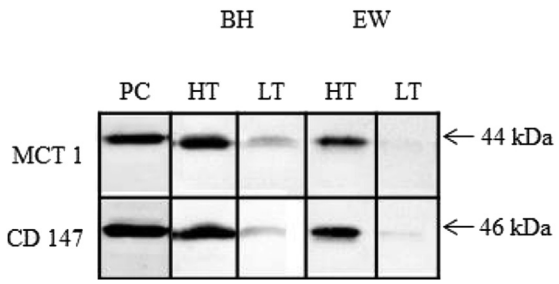

Fig. 1. Western blot detection of CD147 and MCT1 from jumping horses. Note the similar expression levels of the proteins. In most samples of LT horses, the 44 and 46 kDa bands were faint, whereas in most HT horses, the bands showed strong signal at the same molecular weight. BH, Brazilian Sports Horses; CD147, cluster of differentiation 147; EW, European Warmblood horses; HT, high lactate transport; LT, low lactate transporters; MCT1, monocarboxylate transporter 1; PC, positive control.

breed-dependent ($P < .001$). The frequency distribution of the amount of MCT1 and CD147 in the horses quantified by Western blot is shown in Fig. 4.

4. Discussion

The present study investigates the levels of MCT1 and CD147 expression on the plasma membrane of RBCs from BH and EW horse breeds, presenting different jumping abilities. We searched for an association between the HT and LT capacities of these horse breeds and the expression levels of MCT1 and CD147.

The present research was a straightforward breed comparison. First, each horse was identified as having a high or low jumping ability. Although this difference may be due in part to the career stage of the horse and different training levels, our study suggests that the distribution of HT and LT horses is breed-dependent. Indeed, the feature HT or LT is congenital, and horses do not change groups with age or training status [11].

A large proportion of Thoroughbreds, trotters, and even the cold-blooded Finnhorses have high lactate transport activity in their RBCs. However, no correlation between the expression of lactate transporters and jumping ability was not detected herein, and the correlation with racing performance was deemed difficult to establish by Mykkänen et al [1].

One aspect that should be considered is that EW horses do not belong to a single breed, but are members of a cluster of saddle horses selected in different European countries out of basic native breeding stocks. This fact may be demonstrated through online searches on sites such as HorseTelex [12], where the activity of each stallion is listed in European associations.

Although numerous exchanges of breeding animals have occurred in Europe, the participation of Thoroughbreds in the genetic make-up of EW has diminished. The EW horses have reached the fixed genetic level of Thoroughbred blood, that is, approximately 40%–55%, in most EW, which is the level that works best for current jumping competition. The animals need to be clean, light, have a large gallop stride while being elastic, and have a good mouth and temperament [13]. Indeed, the alleged genetic heterogeneity of Warmbloods may have been compromised by artificial insemination with the widespread use of certain stallions in several countries [14–16].

We found that most of the horses of the EW group were LT (77%). From a historical perspective, this result may be explained by the long selection period endured by the EW

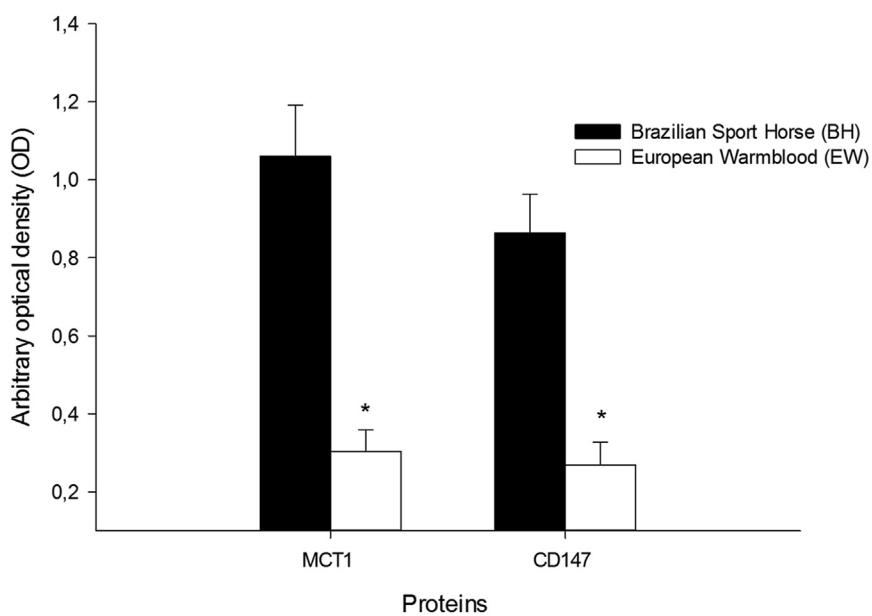

Fig. 2. Mean relative OD values ± standard deviation for MCT1 and CD147 expression on the membrane of red blood cells of show jumping horses. Statistically significant differences are indicated by * according to the Student *t* test analysis ($P < .001$). CD147, cluster of differentiation 147; MCT1, monocarboxylate transporter 1; OD, optical density.

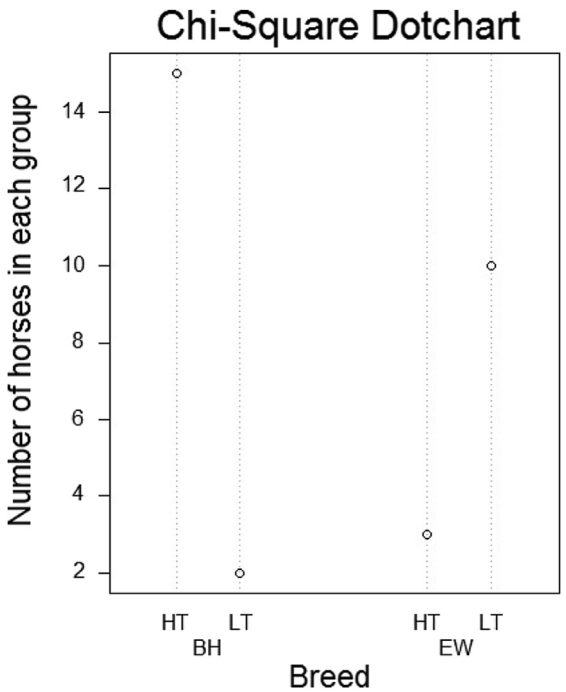

Fig. 3. Dot-chart showing the frequency distribution of horses classified as having HT or LT capacity: BH and EW. There was a difference between the breeds according to the chi-square test ($P < .001$). BH, Brazilian Sports Horses; EW, European Warmblood horses; HT, high lactate transport; LT, low lactate transporters.

breed during which the jumping ability trait was positively selected at the cost of high speed. Today, the level of Thoroughbred blood is high in almost 90% of the world's greatest warmblood stallions (approximately 40%–55%). Mating these stallions with mares with similar levels of the Thoroughbred breed maintains the level of their blood. For example, mating a mare with 48% XX with a stallion with 39% will keep the average at approximately 42%–44% [13].

A great number of the stallions and mares used to breed BH horses were Thoroughbred racehorses [17]. Following the proposed classification based on OD [1,5,18], we found most of the BH horses (88%) were HT, a trait possibly inherited from the Thoroughbreds. The genetic selection of Thoroughbreds caused an increase in the frequency of the C-variant (g.66493737C/T) of the myostatin gene, which contributed to the ability of this breed to race at high speeds [19]. This feature requires a great contribution of muscle anaerobic glycolysis for ATP production, thereby producing substantial amounts of H^+ and lactate, which are carried by the MCT1-CD147 complex. The BHs studied herein presented an HT population ratio similar to the ones found in other breeds by Mykännen et al [1]: 85% of HT for Finnhorses; 82%, for Standardbred; and 88%, for Thoroughbred.

Indeed, it appears that the breeds of horses that run at high speeds have HT capacity. Our group has quantified the lactate transport complex MCT1-CD147 in the RBC membranes of Quarter Horses and Arabian horses by Western blotting. The intensity of the bands of the MCT1 and CD147 proteins were significantly higher in the membranes of RBCs obtained from Quarter Horses than from Arabian horses [20].

The present study shows that the equine breed EW presents lower expression levels of the proteins that compose the lactate transport complex MCT1-CD147 than BH animals. Plasma lactate can reach moderate concentrations (~5.7 mmol/L) after jumping competitions [21]. There are studies reporting higher lactate concentrations (~9.04 mmol/L) [22]. However, these values are still lower than compared with the blood lactate levels found in Thoroughbreds [23].

It has been speculated that the expression intensity of MCT1 and CD147 in the membranes of RBCs may be associated with an increased ability to transport lactate ions from the blood plasma into erythrocytes [5], thereby enhancing the lactate clearing system in horses. The lower blood lactate ion concentration creates a concentration gradient between the intramuscular and plasma lactate ion concentration that stimulates the removal of intramuscular lactate ions [3]. Ultimately, this process may enhance the horse's physical performance due to reduced muscle fatigue [5,24]. Moreover, the bimodal expression of MCT1 and CD147 in the membranes of RBCs is determined genetically, and it is not affected by age, gender, or fitness level [10]. To date, only the expression of muscle MCT1 has been shown to increase its expression levels after a conditioning period [25].

Based on the above information, we hypothesized that horse MCT1 and CD147 expression levels could be directly associated with equine physical performance potential. No such association was observed for the BH horses, which presented the highest expression of these proteins whereas horses able to jump higher (EW) presented lower amounts of MCT1 and CD147 on the surface of their RBCs. The results obtained for the EW horses were particularly surprising. As noted previously, EW horses are better conditioned against moderate lactatemia. Thus, they probably use oxygen more efficiently, a concept known as "running/jumping" economy defined as the amount of oxygen consumed (mL/kg bwt/min) per distance traveled. It is possible that the EW horses consume less oxygen during the execution of jumps at a given speed and height of obstacles, which classifies them as horses with a better jumping economy [26]. In this case, energy metabolism may be strictly aerobic during most of the competition to prevent rapid fatigue due to acidosis. Hence, there is no need to express higher levels of lactate transporters.

This jumping economy may be related to some kinematic variables. The acceleration peaks of the hind limbs at take-off were lower in poor jumpers than in good jumpers. Moreover, the horizontal speed angle was 15° in successful jumps compared with 12° in unsuccessful jumps, whereas the vertical components of the velocity were approximately 0.5 m/second greater in the successfully completed jumps compared with incomplete jumps. Further studies with similar variables, such as age, sex, and kinematic profiles are needed to search for a correlation between lactate transport activity and performance, as well as with the conditioning level [1].

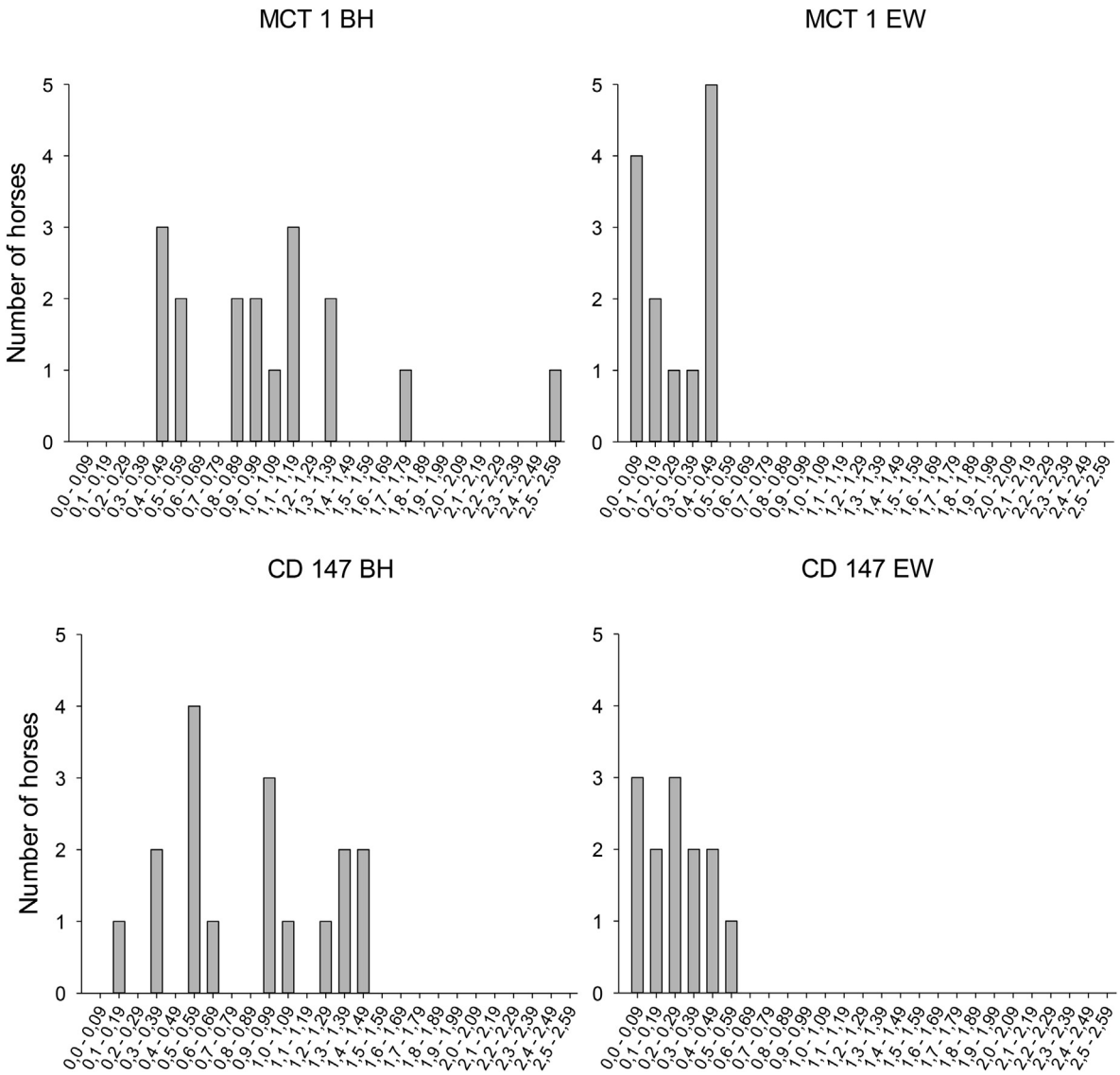

Fig. 4. Frequency distributions of the relative amounts of MCT1 and CD147 as determined by OD evaluation. Western blot samples consisted of erythrocyte (RBC) membranes from two horse breeds: BH and EW. X-axis: relative optical density groups expressed as relative ODs relative to the reference sample, which was arbitrarily set at 1.0; Y-axis: absolute frequency (number) of horses belonging to each OD interval. BH, Brazilian Sports Horses; CD147, cluster of differentiation 147; EW, European Warmblood horses; MCT1, monocarboxylate transporter 1; OD, optical density; RBC, red blood cell.

One limitation of this study is the fact that only protein expression was evaluated. Indeed, high levels of protein expression do not necessarily mean that the transporter is actually functioning more efficiently, in terms of the maximum capacity or affinity of the transporter for its substrate.

This study investigated a subject related to the acid-base homeostasis of show jumping horses [27]. Our results indicated the presence of MCT1 and CD147 in the RBC membranes of the jumping BH and EW horse breeds. However, horses of a breed with greater jump ability (EW horses) displayed low lactate transport capacity. The highest lactate carrying capacity was observed in the BH breed; however, this higher transport capacity does not necessarily translate into a higher jumping ability.

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