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Detection of *Mycoplasma equigenitalium* from Genital Tract of Healthy Domestic Donkeys (*Equus africanus asinus*)

Pablo Tamiozzo[1,*], Erika Sticotti[1], Ana Flores Bragulat[2], Carolina Alonso[2], Mauro Marió[1], and Luis Losinno[2]

[1]*Department of Animal Pathology, Faculty of Agronomy and Veterinary Medicine, National University of Río Cuarto, Argentina*
[2]*Equine Production Laboratory, Department of Animal Production, Faculty of Agronomy and Veterinary Medicine, National University of Río Cuarto, Argentina*

[*]Author to whom any correspondence should be addressed; e-mail: ptamiozzo@ayv.unrc.edu.ar

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Abstract

Mycoplasma equigenitalium (*M. equigenitalium*) has been identified in the reproductive tracts of both fertile and infertile mares and stallions. However, there are scarce antecedents on its detection in the reproductive tract of donkeys. A cross-sectional study was conducted with healthy domestic donkeys. Jennies (n=6) and jacks (n=6) were sampled through the collection of vaginal and preputial swabs. The specimens were plated into a commercial *Mycoplasma* medium and analyzed through the use of a species-specific PCR. *M. equigenitalium* was detected by PCR in four specimens (4/12), one from a jack (1/6) and three from jennies (3/6). Moreover, from one of the PCR positive jennies, *M. equigenitalium* could be isolated. Presumably, this is the first report of *M. equigenitalium* detection from healthy domestic donkeys by both culture and PCR methods despite the small number of analyzed specimens. The obtained results are a starting point for the study of *Mycoplasmas* affecting donkeys.

Keywords

Mycoplasma equigenitalium; reproductive tract; vagina; prepuce; jennies; jacks

1. Introduction

Mycoplasmas are common inhabitants of the respiratory and reproductive tract of humans and animals. Although, in some species, *Mycoplasmas* are recognized as causes of reproductive failures [1,2], in horses, *Mycoplasma equigenitalium* (*M. equigenitalium*) has been identified in both fertile and infertile mares and stallions [3–9].

Seldom do veterinarians take into account diseases caused by *Mycoplasmas* in the differential diagnosis of reproductive disorders from horses. Thus, *Mycoplasma* detection tests are almost never requested. That's why there is a lack of information about the presence of *M. equigenitalium* and other *Mycoplasma* species involved in reproductive failures in horses [3–5]. Even rarer is the detection of *Mycoplasma* species in the reproductive tract of fertile or infertile donkeys [10].

Considering the scarcity of the information about the occurrence of *M. equigenitalium* in donkeys and in order to obtain a first approach on its detection in their reproductive tracts, the present study aimed to detect *M. equigenitalium* from specific areas of reproductive tracts from healthy adult domestic donkeys.

2. Materials and Methods

2.1. Ethics Statement

The study was approved by the Research Ethics Committee of the Universidad Nacional de Río Cuarto, according to

the international guidelines of the Council for International Organizations of Medical Sciences (CIOMS).

2.2. Experimental Design, Sample Collection, Processing, and Testing

A cross-sectional study was conducted with resident donkeys that were used for teaching purposes from the Laboratorio de Reproducción Equina, Facultad de Agronomía y Veterinaria (FAV), Universidad Nacional de Río Cuarto (UNRC).

Donkeys were crossbred native from Argentina, with a wide phenotypic variability. Their weights ranged from 180 to 250 kg, and their body scores ranged from 4 to 5 on the Pearson and Ouassat scale [11,12]. The animals were freely grazing mixed grasses and alfalfa pasture with *ad libitum* water and were clinically healthy. All the animals utilized in the current study had a proven fertility; the jennies had at least one foal, and jacks were utilized for breeding during the reproductive season (two years prior to the study). Although donkeys were located in paddocks, separated from other animals, some horses were hosted on the same premises. Horses were not known to have *M. equigenitalium* and were not sampled. Adult (4 to 18 years old) jennies (n=6) and jacks (n=6) were sampled through the collection of vaginal and preputial swabs through the use of sterile Dacron® swabs (Deltalab®, Spain). In jennies, specimens were obtained by swabbing the vaginal wall at the level of the vestibule (caudal vagina) for 10 s avoiding contact with the vulva. In jacks, specimens were obtained by swabbing the prepuce for 10 s avoiding contact with the skin around.

2.3. Samples Processing and Testing

For *M. equigenitalium* isolation, swabs were plated onto *Mycoplasma* Base Medium with selective *Mycoplasma* supplement (Oxoid, Basingstoke, UK). All plates were incubated at 37ºC under 5% CO2 and examined at 96 hrs. For molecular detection, DNA from collected swabs and *Mycoplasma*-compatible colonies was extracted through the use of Puriprep-S Kit (Inbio Highway, Argentina) following the manufacturer's instructions. For species-specific detection of *M. equigenitalium,* DNA specimens were analyzed through the use of a PCR targeting *rpoB* gene as previously described [9]. DNA extraction, amplification, and visualization were carried out in three different rooms. Negative controls were included every five samples, and filter tips were utilized throughout the process. As positive control, lyophilized *M. equigenitalium* DNA from DNA collection (FAV-UNRC) was used.

3. Results and Discussion

M. equigenitalium was detected by PCR in four specimens (4/12), one from a jack (1/6) and three from jennies (3/6). Furthermore, from one of the PCR positive jennies, *M. equigenitalium* could be isolated. Presumably, this is the first report of *M. equigenitalium* detection from healthy adult domestic donkeys by both culture and PCR methods. *Mycoplasma* isolation is time-consuming and requires complex media and further testing for species identification. However, despite the small number of analyzed specimens, *M. equigenitalium* could be isolated through the use of a commercial culture medium. A higher number of specimens rendered PCR-positive results. The lower sensitivity of isolation of *Mycoplasmas* in comparison with molecular methods might be due to a low number of viable *Mycoplasmas* in clinical specimens and/or special requirements for culture [8].

Although, as previously mentioned, *M. equigenitalium* detection in the reproductive tract of fertile and infertile horses has been already informed [3–7], our results were an interesting starting point for the study of *Mycoplasmas* affecting the reproduction of donkeys. In this case, we cannot conclude that *M. equigenitalium* was exclusively in donkeys due to the contact with horses, and the donkeys sampled did not show fertility problems. It is well known that donkeys have a different susceptibility to certain infectious agents and clinical manifestations in comparison with horses. In this regard, some differences, concerning viral diseases of *equids*, have been thoroughly reviewed and highlighted recently [13]. There are few scientific reports of infectious diseases affecting donkeys in general [13–16] and of reproductive or respiratory diseases caused by *Mycoplasmas* in particular. The information about infectious diseases affecting donkeys is scarce, and there is a tendency to extrapolate the knowledge from experimented horse veterinarians to donkeys' medicine [15], which can be erroneous also in diseases caused by *Mycoplasmas*.

Detection of various *Mycoplasma* species may vary according to different sites from the reproductive tract of mares [7], stallions [8], and along the different phases of the estrous cycle [7]. Moreover, several *Mycoplasma* species might be detected from the reproductive tracts of horses [7,8]. Taking this into consideration, further in-depth studies are required in order to associate the occurrence of *Mycoplasmas* species, particularly *M. equigenitalium,* with fertility disorders of donkeys. Nowadays, we are looking for other *Mycoplasma* species in the reproductive tracts of donkeys.

Authors' Contributions

Luis Losinno and Pablo Tamiozzo planned and designed the study. Ana Flores Bragulat and Carolina Alonso collected the specimens, while Pablo Tamiozzo, Erika Sticotti, and Mauro Marió ran the experiments. All authors read and approved the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest related to this paper.

Data Availability

The data supporting the findings of this study are available within the article.

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Isolation, Culture, Identification, and Bioenergetics Metabolism of Equine Skeletal Muscle Satellite Cells

Xinzhuang Zhang[1,#], Gerelchimeg Bou[1,#], Jingya Xing[1,#], Yali Zhao[1], Caiwendaolima[1], and Manglai Dugarjaviin[1,*]

[1]*Inner Mongolia Key Laboratory of Equine Genetics, Breeding and Reproduction; Scientific Observing and Experimental Station of Equine Genetics, Breeding and Reproduction, Ministry of Agriculture and Rural Affairs; Equine Research Center, College of animal science, Inner Mongolia Agricultural University, Hohhot, 010018, China*

[*]Author to whom any correspondence should be addressed; e-mail: dmanglai@163.com

[#]These authors contributed equally to the study.

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Abstract

Equine skeletal muscle satellite cells (EMSCs) are muscle stem cells in horses, responsible for the postnatal growth, repair, and homeostasis of skeletal muscles. EMSCs are an attractive model for horses to investigate the mechanisms of muscle growth and spontaneously fuse to form differentiated muscle fiber types through activating a battery of muscle-specific genes. Previous reports on the successful isolation and culture of skeletal muscle satellite cells mostly used skeletal muscles of young animals. With the high value of horses, skeletal muscle samples of foals are very difficult to obtain. The present study describes protocols for enriching the satellite cell fraction from the semitendinosus of a 2-year-old Mongolian horse to isolate the EMSCs. Optimized culture conditions with gelatin layering accelerated the adhesion speed of EMSCs. The identification of EMSCs was carried out through multiple dimensions including cell morphology, myogenic induction, differential adhering, and molecular signatures. In particular, the Seahorse Extracellular Flux analyzer was utilized for evaluating the bioenergetics metabolism of EMSCs by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The present study provides reference for the isolation, purification, identification, and bioenergetics metabolism characteristics of EMSCs, which would be useful for studying the molecular mechanisms for muscle development, muscle fiber type differentiation, and recovery from muscle injury in horses.

Keywords

Equine; muscle satellite cells; isolation; bioenergetics metabolism

1. Introduction

Since the domestication, various selection criteria aiming at enhancing the usability of horses in transportation, agriculture, or horsemanship had been applied [1]. Horses are remarkable athletes whose producible muscle force and performance depend upon their powerful musculature, accounting for 55% of their body weights [2]. Skeletal muscle satellite cells are monocytes that lie between the plasmalemma and the overlying basal lamina [3] and are identified as resident stem cells from skeletal muscle [4]. In young mammals, skeletal muscle satellite cells are stimulated by environmental factors in the muscle tissue in order to mediate the continuous proliferation and differentiation, promoting effective muscle development. Skeletal muscle satellite cells are normally mitotically quiescent in adult mammals [5]. Quiescent skeletal muscle satellite cells specifically express Pax-7, Myod, and Desmin, which are considered molecule markers. Horses easily suffer from oxidative stress during vigorous exercise

in speed, endurance, and three-day event competition [6]. Oxidative stress can lead to muscle damage, reflected by a significant increase of aspartate aminotransferase (AST) and creatine kinase (CK) after short and endurance races [7,8]. Recurrent exertional rhabdomyolysis (RER) in horses occurs frequently during intense training and exercise, which influences the performance, health, and welfare of athletic horses. When the skeletal muscles are injured, skeletal muscle satellite cells are activated from state of rest and reenter the cell cycle for self-renewal through downregulating Pax7 and activating myogenin expression [9]. The loss of satellite cells or their function impairs skeletal muscle regeneration capacity resulting in a decrease in skeletal muscle strength [10]. Therefore, skeletal muscle satellite cells play important roles in regulating muscle homeostasis, hypertrophy, and regeneration [11].

Myogenic satellite cells were first discovered in the frog anterior tibial muscle [12], and many different methods are subsequently developed to isolate and culture these cells from different livestock, including chicken [13], bovine [14], ovine [15], and Porcine [16]. Skeletal muscle satellite cell proportions decline gradually in rat skeletal muscles with advancing age [17]. Previous reports mostly used skeletal muscles of young animals for isolation and culture of skeletal muscle satellite cells. With the high value of horses, skeletal muscle samples of foals are very difficult to obtain. Equine skeletal muscle satellite cells (EMSCs) were isolated and cultured for the first time from a yearling horse in 1992 [18]. However, the description of isolation procedures and identification from mature horses of EMSCs were not sufficiently known in the previous reports.

Plasticity of bioenergetics metabolism enables stem cells to match the various demands of self-renewal and differentiation to determine cell fate [19]. Satellite cells are more numerous in the predominantly oxidative soleus muscle than in the mixed glycolytic/oxidative extensor digitorum longus muscle [17]. Verdijk *et al.* concluded that exercise training significantly increased satellite cell proportion and type II muscle fiber size in human skeletal muscle [20]. It seems that exercise can increase the oxidative ability of skeletal muscle satellite cells and tends to transform them into type II muscle fibers. A greater understanding of bioenergetics metabolism of satellite cells would provide further insight into how environmental factors govern skeletal muscle remodeling. However, bioenergetics metabolism characteristics of EMSCs were not sufficiently studied in the previous studies.

EMSCs are an attractive model for horses to investigate the mechanisms of muscle growth and spontaneously fuse to form differentiated muscle fiber types through activating a battery of muscle-specific genes. The current study enriches the satellite cell fraction from the semitendinosus of a 2-year-old Mongolian horse to isolate the EMSCs. Optimized culture conditions with gelatin layering accelerated the adhesion speed of EMSCs. The identification of EMSCs was performed through multiple dimensions, including cell morphology, myogenic induction, differential adhering, and molecular signatures. The present study describes protocols for isolating EMSCs from a mature horse, optimizes culture conditions with gelatin layering of EMSCs, and investigates its characteristics of energy metabolism.

2. Materials and Methods

2.1. Reagents and Solution Preparation

The information of reagents involved in the present study is listed in **Table 1**:

Table 1: The involved reagents information.

Reagent	Corporation
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco Life Technologies
Fetal Bovine Serum (FBS)	Gibco Life Technologies
Horse serum (HS)	Gibco Life Technologies
0.25% Trypsin- Ethylene Diamine Tetraacetic Acid (EDTA)	Gibco Life Technologies
100 × Penicillin-Streptomycin (10,000 U/mL)	Gibco Life Technologies
100 × Antibiotic-Antimycotic (10,000 U/mL Penicillin, 10,000 μg/mL Streptomycin, 25 μg/mL Amphotericin B)	Gibco Life Technologies
Dulbecco's Modified Eagle Medium (DMEM)	Gibco Life Technologies
Mouse monoclonal anti-Pax7 primary antibody	LifeSpan Biosciences Technologies
Mouse monoclonal anti-Myod1 primary antibody	Thermo Fisher Scientific Inc.
Mouse monoclonal anti-Desmin primary antibody	Abcam
Secondary antibody with Donkey anti-Mouse	Invitrogen
4', 6-diamidino-2-phenylindole (DAPI)	Solarbio, CHN
100X-Triton	Sigma, CHN

Solutions required for isolation, purification, and culture of EMSCs need to be prepared prior to the experiment: dilute 100× penicillin-streptomycin solution with 98% normal saline (NS) to 2% and prepare 2× penicillin-streptomycin NS (+), filtered by 0.22 μm and stored at 4°C until use. The DPBS (+) consists of 99% DPBS and 1% 100× antibiotic-antimycotic. The DPBS (+) is filtered through 0.22 μm and stored at 4°C until use. The proliferation medium (PM) (+) consists of 20% FBS, 1% 100× antibiotic-antimycotic, and 79% DMEM. The PM (+) is filtered through 0.22 μm, then stored at 4°C, and used within one week. The differentiation medium (DM) (+) supplemented with 2% HS, 1% 100× penicillin-streptomycin, and 97% DMEM is filtered through 0.22 μm and stored at 4°C until application. 5 mg collagenase type IV is diluted into 5 mL DMEM. Next, the solution (collagenase type IV) (+) is filtered through a 0.22 μm filter and prepared freshly.

2.2. Instruments and Consumables

The following instruments are involved in the present study, including centrifuge (TD6A-WS, Hunan Hukang Inc., China), water bath (HWS-26, Shanghai YIheng Inc., China), carbon dioxide incubator (ThermoHERAcell150i/240i CO2, Thermo Fisher Scientific Inc., USA), invert microscope (ZEISS, Germany), real-time PCR (CFX96, Bio-Rad, USA), fluorescence microscope (revolve FL, Echo, USA), and Seahorse XF Analyzers (Agilent, USA). In addition, the consumables including scalpels, razor blades, forceps, scissors, cell strainers (pore size 40 μm, 70 μm), plastic Petri dishes, polypropylene centrifuge tubes (15 mL, 50 mL), and 6-wells Petri dishes also need to be prepared in advance. All the consumables should be sterilized.

2.3. Horse Muscle Sample Collection

The involved animal horse slaughtering procedures and all the sample collections comply specifically with the guidelines approved by the Animal Welfare Committee of Inner Mongolia Agricultural University on experimental animals. After slaughter, muscle samples from the semitendinosus of a 2-year-old Mongolian horse are collected and placed in NS (+) and brought back to the laboratory within two hours using an ice box.

2.4. Isolation, Purification, and Culture of EMSCs

2.4.1. Muscle Treatments

First, the collected muscle samples are quickly soaked and sterilized in a Petri dish with 70% ethanol. The sterilized muscle samples are immediately transferred to a new Petri dish with DPBS (+). The outer alcohol-infested muscle tissues are trimmed, and the remaining muscle blocks are put into a new Petri dish. The muscle masses are rinsed 3 times with DPBS until they are bloodless. Scalpels are utilized for removing visible fat and connective tissue from flushed muscle blocks. The muscle tissue is transferred into a Petri dish containing DMEM and divided into 1 mm3 pieces through the use of ophthalmic scissors in the biological safety cabinet. The ground muscle pieces are transferred into a 15 ml centrifuge tube and centrifuged at 1000 rpm for 5 minutes in order to separate intact myofiber and supernatant.

2.4.2. Enzymatic Tissue Digestion

The intact myofibers are collected in a 15 ml centrifugal tube and added 2–4 times 0.25% Trypsin-EDTA for digestion in the 37°C water bath for 1 h. Meanwhile, a pipet is used to pump the digesting intact myofiber 10–15 times every 10 min in order to gently dissociate the muscle-derived cells. Then, the centrifugal tube is centrifuged at 200 rpm for 5 minutes so as to separate the supernatant and the underlying muscle fragments pellet. In order to enrich the satellite cell fraction, the supernatant and the underlying muscle fragments are simultaneously treated as the following procedures.

On one hand, the supernatant is poured into a 15 ml centrifuge tube, followed by 2000 rpm centrifuge for 5 minutes to collect the cell pellet. The cell pellet is resuspended with 5 ml of PM (+) and then centrifuged at 2000 rpm for 10 minutes to collect the precipitation. The precipitation is suspended with 3 ml of PM (+). Then, the cell suspension is filtered by 70 μm cell strainers and centrifuged at 2000 rpm for 10 minutes to collect precipitation (precipitation A).

On the other hand, the underlying tissue fragment pellet was further digested for 1 h with 2- to 4-fold collagenase type IV (+). Then, the tissue fragments are aspirated for 10–15 times with pipettes to gently detach the cells. After being filtered by a 70 μm filter, the cell suspension is centrifuged for 5 minutes at 2000 rpm in order to separate the supernatant and the cell pellets, and the supernatant is centrifuged for 10 min at 2,000 rpm to collect the residual cell pellet, and the collected cell pellets are mixed. The combined cell pellets are suspended with 5 ml of PM (+). The cell suspension is centrifuged at 2000 rpm for 5 minutes to collect the precipitation (precipitation B). Then, precipitation B is treated the same as precipitation A.

Next, precipitation A and B are suspended with 1 ml PM (+), respectively, and the precipitation suspension A and B are mixed and centrifuged at 2000 rpm for 5 minutes to collect the precipitation again. The precipitation is suspended with 5 ml DPBS and centrifuged at 2000 rpm for 10 minutes again to collect precipitation. The precipitation is suspended with 5 ml of PM (+) and filtered through a 40 μm filter. The cell suspension is adjusted to 10 ml with PM (+) before being centrifuged at 700 rpm for 10 minutes to collect precipitation. The collected precipitation is suspended with 2 ml of 37°C prewarmed PM (+) and then spread in a Petri dish. Then, the cell suspension is cultured for 2 hours at 37°C, in a 5% CO_2 incubator for purification of EMSCs, where the fibroblasts adhere quickly to the bottom of the Petri dish, while the skeletal muscle satellite cells will stay in the supernatant. The purification of EMSCs is frozen with liquid nitrogen for further study.

2.4.3. Optimized Culture Conditions with Gelatin Layering of EMSCs

Two Petri dishes are treated as follows. One dish is paved with 2 ml of gelatin solution (0.0025 g/ml deionized water) and incubated in a 37°C and 5% CO_2 incubator for 2 hours, with the excess gelatin removed, and it is washed twice with DPBS. Then, 1 ml of proliferative culture is added. The same operations are conducted in another Petri dish without gelatin. The same amount of EMSCs is seeded into the two

above Petri dishes and observed using a microscope at 1 h, 4 h, and 8 h of culture.

2.5. Identification of EMSCs

2.5.1. Inducing Differentiation

The isolated EMSCs are inoculated in a new culture dish and incubated at 37°C and 5% CO_2. When the cells confluence reaches up to 70% -80 %, the original PM (+) is discarded. Then, the residual cells are cleaned with DPBS and changed with DM (+) to induce differentiation, and their morphology is observed after 24 h with an inverted microscope.

2.5.2. Differential Adhering Method

The isolated EMSCs are seeded in a new culture dish, while the equine fibroblast cells (EFCs), as controls with an equal number in another Petri dish, are cultured at 37°C under 5% CO_2. Then, they are observed and photographed through the use of an inverted microscope at different time points (1, 4, and 8 hours).

2.5.3. Immunofluorescence

With further identification of EMSCs, the 4th passage muscle satellite cells are seeded into a twelve-well plate with glass sheets and cultured to 70%–80% confluence in a 5% CO_2 incubator at 37°C. After washing the cells with PBS, the cells are fixed in 4% paraformaldehyde for 40 min at room temperature and subsequently washed with ice-cold PBS, followed by permeabilizing by PBS penetration for 30 min with 0.1% Triton and incubated overnight at 4°C. Afterward, the cells are washed with cold PBS, and the cells are incubated with blocking solution supplemented with 2% bovine serum albumin (BSA) for 1 h and incubated with 100-fold dilution of mouse monoclonal anti-Pax7 primary antibody, mouse monoclonal anti-Myod1 primary antibody, and mouse monoclonal anti-Desmin primary antibody, respectively, overnight at 4°C. After washing with cold PBS 3 times, cells are incubated with 2000-fold dilution of donkey anti-mouse secondary antibody for 1 h at room temperature under dark conditions and then washed with cold PBS 3 times. The cells nuclei are counterstained with DAPI for 10 min, and then the samples are washed once with PBS before securing to the glass slide and observed under a fluorescence microscope.

2.5.4. Quantitative Real-Time PCR (qRT-PCR)

Fourth-generation EMSCs and EFCs are inoculated into new Petri dishes and cultured to 80% confluence in an incubator at 37°C with 5% CO_2. The TRIzol reagent (Thermofisher, USA) is utilized for extracting total RNA from cells and transcribed into cDNA using a super RT Kit (Takara, CHN). Quantitative real-time PCR (qRT-PCR) is performed to analyze the mRNA expression of Pax7, Myod1, and GAPDH, which is used as a housekeeping gene. Primers used in this study are listed in **Table 2**. Run real-time PCR was performed in a total volume of 20 µl containing 10 µl TB Green Premix Ex Taq II, 6 µl RNase-free water, 0.4 µl ROX Reference Dye, 0.8 µl forward primer, 0.8 µl reverse primer, and 2 µl cDNA template. Quantitative RT-PCR is carried out using the CFX96 (Bio-Rad) with the manufacturer: one step at 95°C for 30 s, 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. Post-PCR melting curves confirm the specificity of the single target amplification with each gene expression being measured in triplicate. The relative expression values are calculated using the formula ($2^{-\Delta\Delta Ct}$) as described by Livak and Schmittgen, where Ct is the cycle threshold **[21]**.

2.6. Bioenergetics Metabolism of EMSCs

The Seahorse Extracellular Flux analyzer (XFp, Agilent Technologies, Santa Clara, CA, USA) is utilized for assessing the bioenergetics metabolism of EMSCs under basal conditions, respiration inhibition, and stressed metabolic state by measuring oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) to reveal the quiescent, glycolytic, and energetic phenotypes of cell energy metabolism, respectively. Briefly, EMSCs are seeded on a Seahorse XF 8 plate at a density of 12500 cells/well and grown overnight in the 37°C CO_2 incubator. After 24 h, the Agilent Seahorse XFp Cell Culture miniplate is removed from the 37°C CO_2 incubator. Then, the PM (+) is changed into pH 7.4 assay medium, which consists of 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose (Gibco) of Seahorse XF Base Medium. Afterward, the Agilent Seahorse XFp Cell Culture miniplate is placed into a 37°C and non-CO_2 incubator for 1 hour prior to the assay. During the assay, EMSCs under basal conditions are measured directly. EMSCs under respiration inhibition are measured and induced by 0.5 µM oligomycin, which is an inhibitor of mitochondrial ATP production. EMSCs under stressed metabolic state are measured and induced by 2 µM FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), which is a mitochondrial membrane depolarizing agent.

2.7. Statistical Analysis

The mRNA expression of Pax7 and Myod1 between equine muscle satellite cells and fibroblasts and OCR and ECAR values under basal conditions, respiration inhibition, or stressed metabolic state were evaluated through the use of ANOVA of the GLM procedures of SAS 9.0 (SAS Institute Inc., 2002). Student's *t*-test was used to analyze differences between mRNA expression, OCR, and ECAR values with least squares means. The difference was significant at $P < 0.05$, $0.05 <$ at $P < 0.1$, and not significant at $P > 0.1$.

Table 2: Primer sequences used in RT-PCR assay.

Primer	Sequence	Genetic ID	Length(bp)
myod1-F	GAACCGCTACGATGGCACCTACTAC	NM_001317253.1	101
myod1-R	CCACGATGCTAGACAGGCAGTCAAG		
pax7-F	GTGCCCTCAGTGAGTTCGATTAGC	XM_014836146.1	108
pax7-R	CTTGGCTTTATTCTCGCCGTCGT		
GAPDH-F	CATCATCCCTGCTTCTACTGG	XM_014866500.1	117
GAPDH-R	TCCACGACTGACACGTTAGG		

3. Results

3.1. Isolation and Culture of Equine Skeletal Muscle Satellite Cells

The morphology of EMSCs was spherical with strong refraction when they were separated. A handful of cells began to attach to the plates within 3 days. The number of adhered cells increased with time (5 d or 7 d), and cells were gradually extended into spindle or polygonal shapes as the normal EMSCs (**Figure 1**).

As the results show in **Figure 2**, the adherent percentage of EMSCs increased over cultural time, and the adherent percentage of EMSCs in the gelatin group was higher than in the gelatin-free group after 1 h of culture. After 8 h of culture, EMSCs extended and hypertrophied obviously in the gelatin group compared to the gelatin-free group.

3.2. Identification of Equine Skeletal Muscle Satellite Cells

80% confluent 4th generation EMSCs before differentiation appeared in spindle or polygonal shapes (**Figure 3A**). After replacing of DM (+) for 24 h, the EMSCs were regularly arranged in parallel and began to fuse formed myotubes (**Figure 3B**).

The EMSCs had no cell adhering at 1 hour (**Figure 4D**), and the equine fibroblast cells (EFCs) had already started to adhere (**Figure 4A**). When the EMSCs had just begun to adhere at 4 h (**Figure 4E**), the EFCs had grown with morphological changes (**Figure 4B**). At 8 hours, numerous EMSCs adhered (**Figure 4F**), whereas EFCs had reached high confluency (**Figure 4C**).

Immunofluorescence analysis of Pax7, Myod1, and Desmin on proliferating EMSCs is shown in **Figure 5**. The results showed that the cells are Pax7, Myod1, and Desmin positive. Both Pax7 and Myod1 were presented in the nucleus, and Desmin was distributed within the cytoplasm adversely.

Both relative mRNA expressions of Pax7 and Myod1 were higher in EMSCs than that of EFCs ($P < 0.05$, **Figure 6**).

3.3. Bioenergetics Metabolism of Equine Skeletal Muscle Satellite Cells

Bioenergetics metabolism of EMSCs under three different conditions is compared and shown in **Figure 7**. The glycolytic EMSCs under respiration inhibition had higher ECAR ($P < 0.05$) compared to the quiescent EMSCs under basal conditions. Energetic EMSCs under stressed metabolic state demonstrated 77.5% increment ($P < 0.05$) in OCR in comparison with the quiescent EMSCs under basal condition.

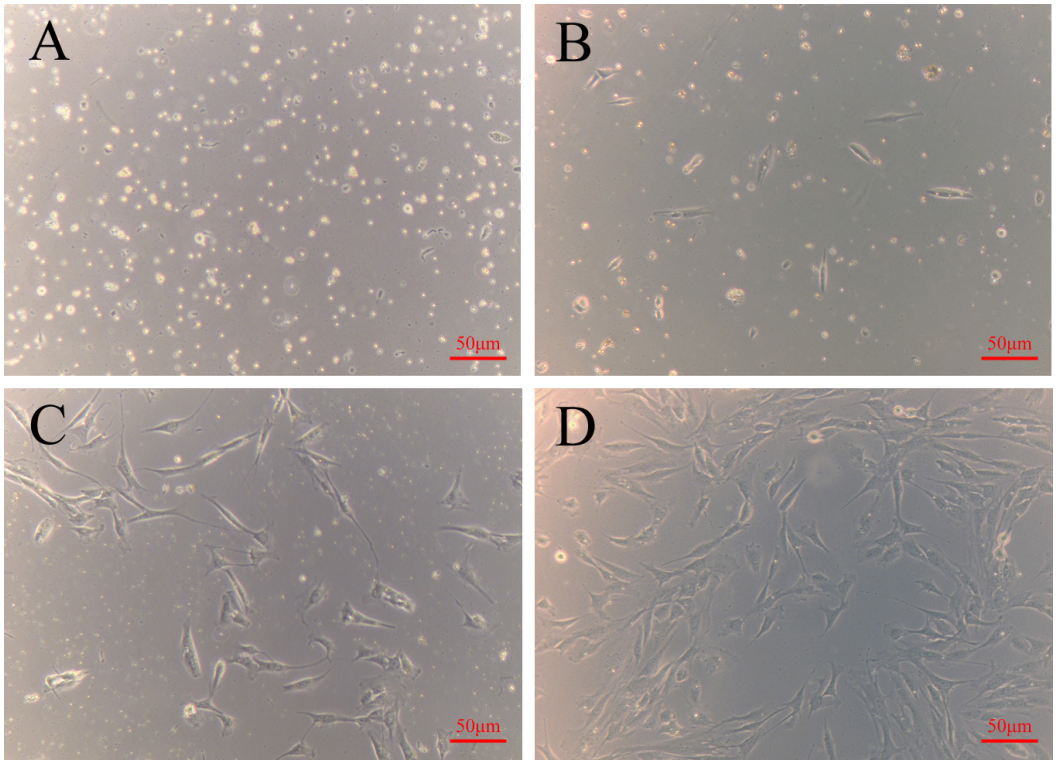

Figure 1: Morphology of in vitro cultured equine muscle satellite cells at different times. (**A:** 1 day; **B:** 3 days; **C:** 5 days; **D:** 7 days).

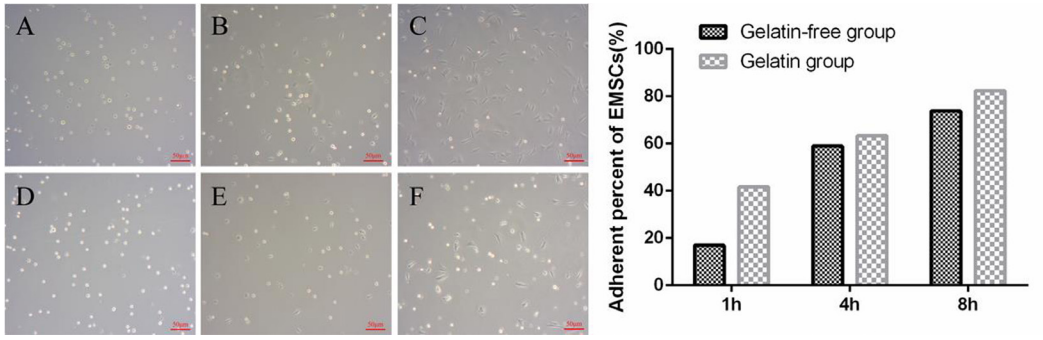

Figure 2: The influence of gelatin on the adherent percent of EMSCs at different cultural times. A-C. EMSCs in the gelatin group, adhering for 1 h **(A)**, 4 h **(B)**, and 8 h **(C)**. D-F. EMSCs in the gelatin-free group, adhering for 1 h **(D)**, 4 h **(E)**, and 8 h **(F)**.

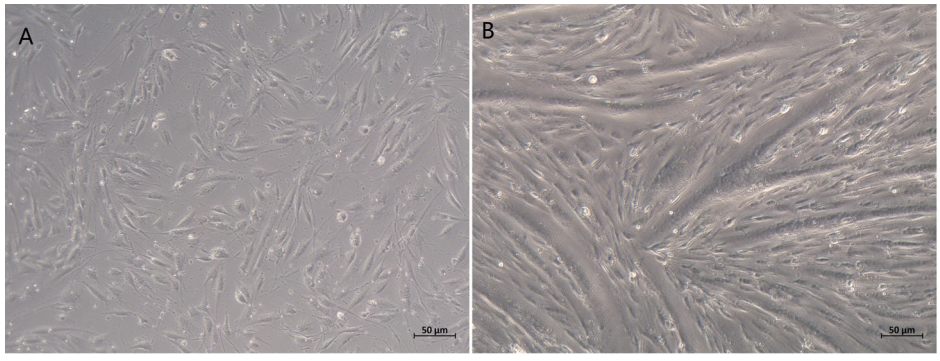

Figure 3: Equine muscle satellite cells before differentiation and after differentiation (**A:** equine muscle satellite cells before differentiation; **B:** differentiation for 24 h of equine muscle satellite cells formed myotubes).

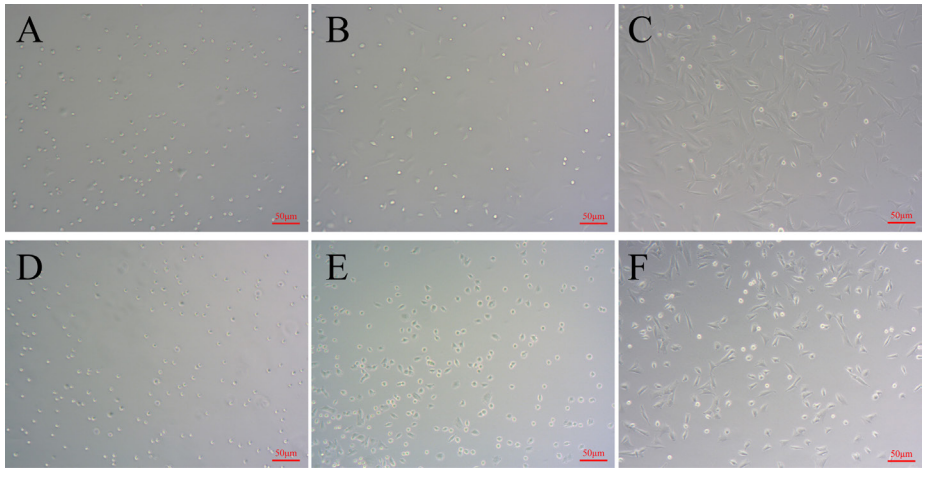

Figure 4: Differential adherence of EMSCs and EFCs at different times. EFCs, adhering at 1 h **(A)**, 4 h **(B)**, and 8 h **(C)**; EMSCs, adhering at 1 h **(D)**, 4 h **(E)**, and 8 h **(F)**.

4. Discussion

4.1. Isolation and Culture of Equine Skeletal Muscle Satellite Cells

Horses easily suffer from muscle damage during high-intense exercise. EMSCs play major roles in muscle development and regeneration, which have greater research value and wide application prospects in muscle regeneration *in vitro* [22]. Isolation of muscle satellite cells from various species requires different protocols. Rosenblatt *et al.* separated muscle satellite cells from rats through tissue culture system [23]. Mesires and Doumit obtained pig skeletal muscle satellite cells through percoll gradient centrifugation [24]. Although many animals have established culture procedures for muscle satellite cells, methods are quietly different between species.

Muscle satellite cells are adult stem cells located between the basal lamina and sarcolemma of the muscle fibers. The number of muscle satellite cells may differ considerably in various muscles and ages of the animals. Di Foggia and Robson studied that *soleus* has a higher number of satellite cells than *extensor digitorum longus* of the mouse [4]. The

semimembranosus, *semitendinosus*, and *longissimus dorsi* muscles are generally utilized for the isolation of skeletal muscle satellite cells. There is no obvious distinction to isolate EMSCs of different muscles [16,25]. Mesires and Doumit found that the relative proportion of porcine skeletal muscle satellite cells gradually declined from 1st to 64th weeks after birth [24]. Previous reports on the successful isolation and culture of skeletal muscle satellite cells mostly used skeletal muscles of young animals. How to isolate muscle satellite cells efficiently from muscles of adult animals is crucial. In the present study, *semitendinosus* from a 2-year-old mature Mongolian horse was selected and successful in isolating EMSCs.

Pronase had been applied to break down the connective tissue and muscle fibers and release the equine muscle satellite cells described by Greene and Raub [18]. Currently, two-step enzyme digestion methods were generally chosen to isolate the muscle satellite cells. Gharaibeh *et al.* utilized 0.2% type IV collagenase to digest for 1 h followed by digesting 0.1% trypsin from murine skeletal muscle for 30 min [26]. Wu *et al.* adopted 0.1% type I collagenase for 50 min followed by digesting 0.25% trypsin from sheep skeletal muscle satellite cells for 10–20 min [15]. Recently, Li *et al.* used 1.5 mg/ml pronase for 1 h followed by 1.5 mg/ml type XI collagenase digestion for 1 h from porcine skeletal muscle satellite cells [16]. According to previous studies, different enzyme combinations could digest muscle and isolate muscle satellite cells. Consistent with previous studies, we also used a two-step enzyme digestion method to isolate EMSCs, adopting 0.25% trypsin digestion for 1 h followed by 1 mg/ml type IV collagenase for 1 h. In addition, the underlying tissue fragment pellet digested by trypsin was collected to enrich the satellite cell fraction and fully isolated during the subsequent centrifugation.

The myoblast enrichment protocol takes an advantage of the fact that myoblasts attach much less to plastic than fibroblasts, so the fibroblasts can be removed from the culture by preabsorption on plastic tissue culture plates. Li *et al.* employed two 30 min differential adhering methods in order to purify the obtained chicken skeletal muscle satellite cells [16]. Kim *et al.* indicated 45 min differential adhering methods in order to purify mouse satellite cells [27].

The newly isolated satellite cells are spherical with strong refraction under a microscope, which is in agreement with Beijing fatty chicken and Luxi cattle by [28] and [29], respectively. The isolated Mongolian skeletal muscle satellite cells had only a small number of cells adhered in the first three days. Wu *et al.* also demonstrated that freshly isolated sheep skeletal muscle satellite cells were difficult to attach to the bottom of culture plates [15]. To solve this problem, this study found that gelatin accelerated the adhering speed of EMSCs, which is similar to that performed in mice [30] and sheep [15].

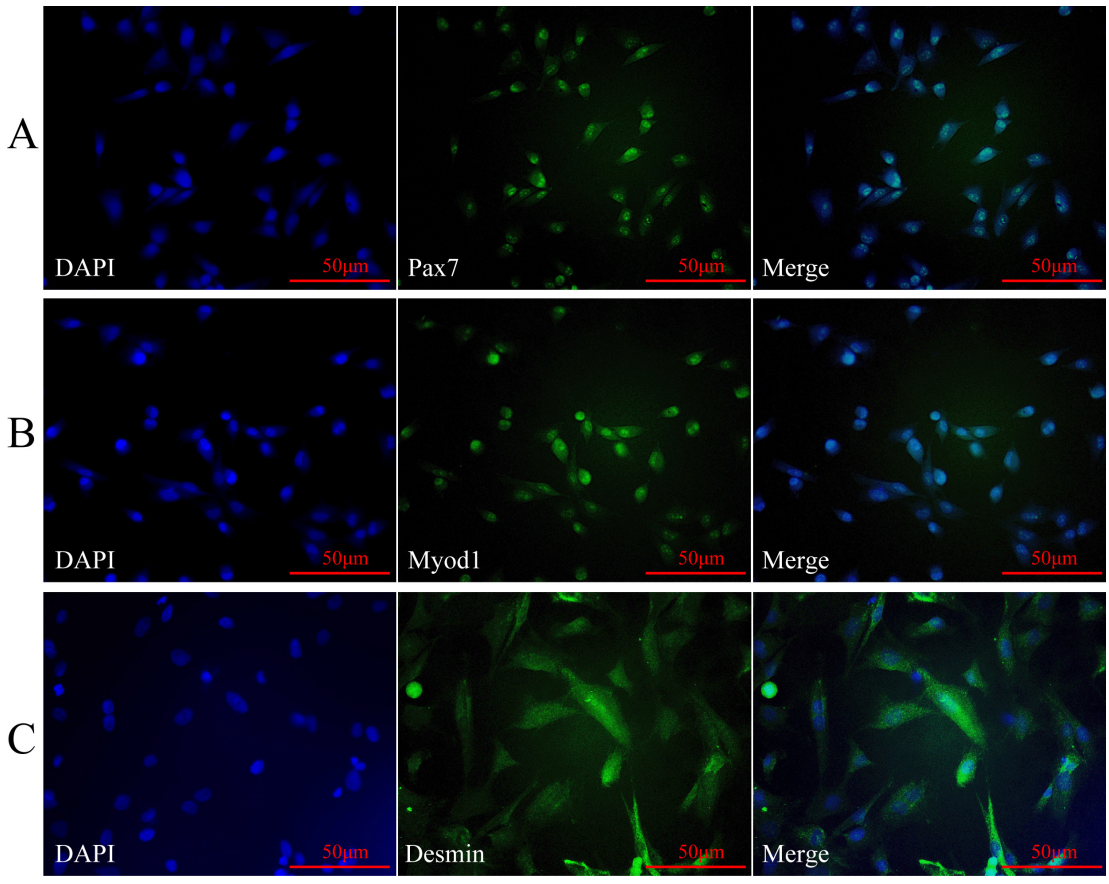

Figure 5: Immunofluorescence analysis of Pax7, Myod1, and Desmin on proliferating equine muscle satellite cells. **(A)** Immunofluorescence analysis of Pax7 on proliferating equine muscle satellite cells. **(B)** Immunofluorescence analysis of Myod1 on proliferating equine muscle satellite cells. **(C)** Immunofluorescence analysis of Desmin on proliferating equine muscle satellite cells.

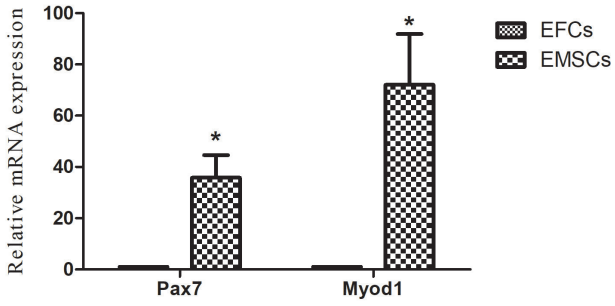

Figure 6: Relative mRNA expression of Pax7 and Myod1 in equine muscle satellite cells (EMSCs) compared to equine fibroblasts cells (EFCs).

* represents significant differences ($P < 0.05$).

4.2. Identification of Equine Skeletal Muscle Satellite Cells

4.2.1. Myogenic Induction

Greene and Raub had proved that differentiation into myotubes is appropriate for the identification of EMSCs [18]. Wang *et al.* found that the bovine skeletal muscle satellite cells are fused to form multinucleated myofibers [31]. With prolonged cell differentiation culture on the fourth day, cells formed into a large number of muscle tubes and arranged into myofiber bundles. Danoviz and Yablonka showed that round cells were observed at the beginning of culture, while the formation of multi-nuclear muscle tube was observed after 5 days of differentiation [32]. Wu *et al.* also demonstrated that sheep skeletal muscle satellite cells began to fuse and form short myotubes after 5 days of myogenic induction [15]. Consistent with previous reports, equine skeletal muscle satellite cells are regular parallel arrangement, gradual elongation and thinness, the fusion between proliferating cells, and the formation of myotubes after 72 hours of myogenic induction. These studies are consistent with our results.

4.2.2. Differential Adhering Method

As the adhering characteristic of fibroblasts is different from muscle satellite cells, we utilized the different adherence methods to purify EMSCs. The results showed that although fibroblasts adhered within 1 h EMSCs began to adhere to the plate after 4 hours, which made us distinguish between two cell populations.

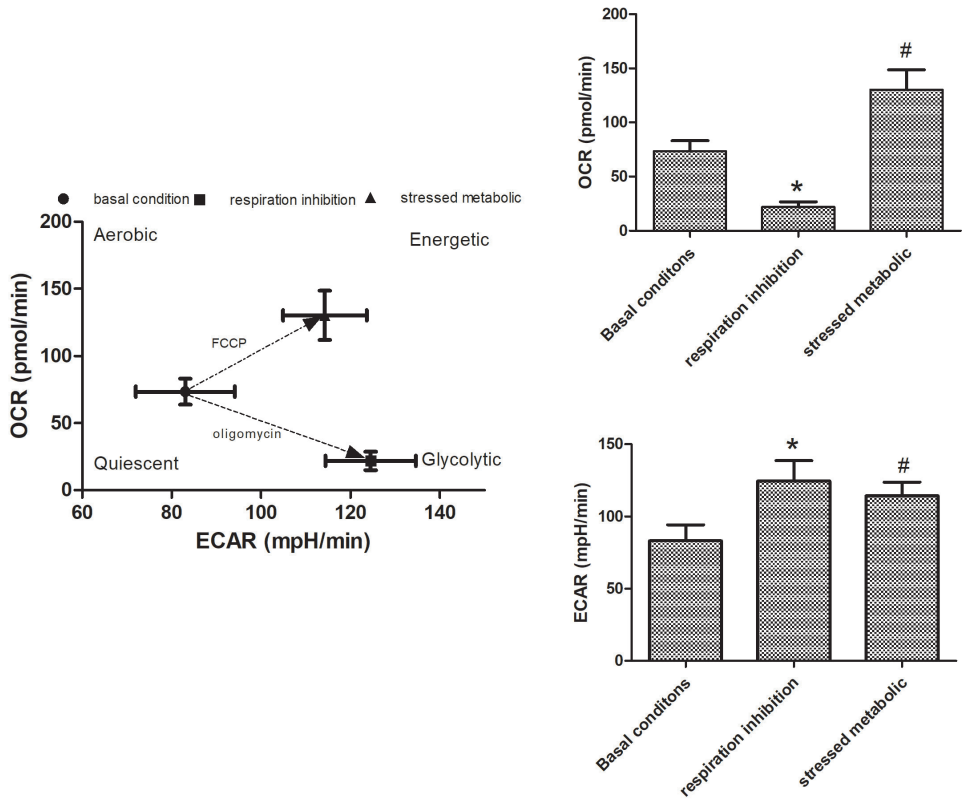

Figure 7: Bioenergetics metabolism of equine skeletal muscle satellite cells.

* represents significant differences between respiration inhibition and basal conditions ($P < 0.05$). # represents significant differences between stressed metabolic and basal conditions ($P < 0.05$).

4.2.3. Molecular Signature of Muscle Satellite Cells

Significantly, several studies have reported that muscle satellite cells could be identified by molecular markers, such as Pax7, M-cadherin, Desmin, and Myod family genes Cxcr4, syndecan3/4, and c-met **[10,33,34]**. Pax7, a major transcription factor to determine quiescent muscle satellite cells, is referred to as a molecular marker to characterize the skeletal muscle satellite cells derived from various species, and the expression of Pax7 is downregulated when satellite cells are committed to differentiation **[35,36]**. Myod gene family members belong to the myogenic basic helix-loop-helix transcription factor family **[37]**. Myod1, which is expressed in proliferating and activating satellite cells, is important during myogenesis **[38]**. Desmin is one of the cytoskeleton components in muscle satellite cells **[39]**. In the present study, Pax7 and Desmin were utilized for characterizing the proliferating muscle satellite cells, while Myod1 was utilized for monitoring the differentiation capability of muscle satellite cells through qRT-PCR and immunofluorescence. Bai *et al*. proved that chicken skeletal muscle satellite cells were positive for four specific genes (c-met, Pax7, Myod, and Desmin) by qRT-PCR **[28]**. The obtained pig skeletal muscle satellite cells were identified, and the immunofluorescence results were positive for Pax7 and Desmin. In our study, the mRNA expressions of Myod1 and Pax7 in EMSCs were greater than in EFCs, and specific proteins of Pax7, Myod1, and Desmin were positively shown by immunofluorescence. Identifying EMSCs in combination with molecular signatures increases the reliability, thus avoiding the controversy of using a single method for identification.

4.2.4. Bioenergetics Metabolism of Equine Skeletal Muscle Satellite Cells

Satellite cells contribute to muscle remodeling and regeneration through migration, DNA synthesis, division, and fusion into existing muscle fibers **[40]**. Equine skeletal muscle myofibers are composed of oxidative and glycolytic myofibers **[41]**. Oxidative myofibers generate energy by mitochondrial respiration for continuous contractions with less fatigue, whereas glycolytic myofibers have a high glycolysis capacity for fast movements **[42]**. The Mongolian horse is famous for endurance with a high proportion of oxidative myofibers than thoroughbred **[41]**. The unique training method, called "hang the horse," combines exercise and feed restricted to enhance the endurance of Mongolian horses. Previous studies had proved that exercise could lead to a switch from glycolytic myofibers to oxidative myofibers **[43]**. White *et al*. found that dietary selenium improves skeletal muscle mitochondrial biogenesis in young equine athletes **[44]**. Moreover, Shamim *et al*. indicated that amino acids improve muscle satellite cell dynamics *in vitro* **[45]**. Whether nutritional factors can stimulate the differentiation of muscle satellite cells into different types of muscle fibers remains to be studied.

The transition between muscle fiber types may depend on the types of bioenergetics metabolism of EMSCs. Bioenergetic metabolisms EMSCs can be used as a biomarker to optimize feeding and training strategies to improve horse performance. The Seahorse Extracellular Flux analyzer has been widely utilized for evaluating the bioenergetics metabolism of cells by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Oligomycin can inhibit the production of ATP by the mitochondria through binding to the proton channel on the Fo component of ATP synthase. As a result, oligomycin blocks oxygen uptake and causes a compensatory increase in the rate of glycolysis. The oligomycin concentrations work on different cells in the region of 0.25–5 μM **[46]**. Here, we showed that 0.5 μM oligomycin successfully induces proton uncoupling after the inhibition of ATP synthesis, as evidenced by lower OCR values and higher ECAR values of glycolytic EMSCs under respiration inhibition in comparison with the quiescent EMSCs under basal conditions. FCCP, a potent uncoupler of oxidative phosphorylation in mitochondria, makes the inner mitochondrial membrane permeable for protons and allows maximum electron flux through the electron transport chain **[47]**. 2 μM FCCP stressed mitochondrial oxidation as evidence of increasing oxygen consumption rate (OCR) in energetic EMSCs in comparison with the quiescent EMSCs. We established a suitable concentration of oligomycin and FCCP to measure the bioenergetics metabolism characteristics of three different EMSCs types.

5. Conclusions

The isolation procedures of equine skeletal muscle satellite cells are elaborated in detail by two-step enzymatic digestion from a two-year-old Mongolian horse. Optimized culture conditions with gelatin layering accelerated the adhesion speed of EMSCs. Furthermore, we identified the equine skeletal muscle satellite cells through a series of methods including cell morphology, myogenic induction, differential adhering, and molecular signatures. In addition, our study investigated the bioenergetics metabolism characteristics of different types of EMSCs induced by 0.5 μM oligomycin and 2 μM FCCP through a Seahorse Extracellular Flux analyzer. These methods in the present study are essential to use the EMSCs *in vitro* for studying the molecular mechanisms of muscle development, regeneration, and muscle fiber differentiation of horses.

Authors' Contributions

Xinzhuang Zhang: Conceptualization, visualization, supervision, and funding acquisition

Gerelchimeg Bou: Methodology, review, and editing

Jingya Xing: Writing the original draft

Yali Zhao: Investigation of resources

Caiwendaolima: Investigation of resources

Manglai Dugarjaviin: Project administration, funding acquisition

Data Availability Statement

The data that support the results of the present study are included in this manuscript.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Animal Welfare

The involving animal horse slaughtered procedures and all the sample collections were specifically in line with the experimental animal guidelines approved by the Animal Welfare Committee of Inner Mongolia Agricultural University.

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Fitness of Eventing Horses Submitted to Interval Training

Juliana A. Gonçalves[1,*], Nelson A. Kretzmann Filho[1], Rafael Rodrigues[2], Willian Jadoski[3], Bruna Beretta[1], Ana Carolina B. Aymone[4], and Adriana P. Neves[1]

[1]*REPROLAB, Faculty of Veterinary, Federal University of Rio Grande do Sul, Brazil*
[2]*Brazilian Army, 3rd Guards Cavalry Regiment, Porto Alegre, Brazil*
[3]*Private Veterinarian, DVM, Porto Alegre, Brazil*
[4]*LACVet, Faculty of Veterinary, Federal University of Rio Grande do Sul, Brazil*

[*]Author to whom any correspondence should be addressed; e-mail: julianaagoncalves@hotmail.com

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Abstract

The fitness of eventing horses was assessed in field tests through the use of heart and respiratory rates and blood biochemical parameters. The first stage (S1) lasted three days and consisted of the following three tests: the incremental speed test (IST), the incremental jumping test (IJT), and the jumping course (JC). Following the first stage, horses were submitted to a six-week interval training program and were then submitted to another evaluation, the second stage (S2), of the same tests as in the first stage. Lower heart rate (HR) values were observed during the incremental speed test in S1 than in S2. The enzymes creatine kinase (CK), aspartate transferase (AST), and lactate dehydrogenase (LDH) showed significant speed-related reductions in the second stage in comparison with the first stage. HR increased significantly during the incremental jumping test in the upper obstacle heights (55 and 70 cm). Respiratory rate showed a significant decrease in the last two laps and at 10 min after exercise in the second stage, compared to the first stage. Values of LDH were lower in the second stage than in the first stage, and AST values were significantly lower at 55 cm and 10 min after exercise in S2. During the jumping course, RR had better recovery in the second stage than in the first stage, and glucose decreased during the course in both stages. The enzymes CK and AST presented higher levels at 10 min after exercise in the second stage than in the first stage. The field tests can be utilized for evaluating the fitness of eventing horses.

Keywords:

Equine; field test; exercise physiology; incremental test

1. Introduction

It is well established that physical exercise induces transient alterations in the horses' homeostasis that need to be restored by cardiovascular, respiratory, metabolic, and musculoskeletal adaptations [1]. In order to introduce horses to the specific procedures needed for clinical evaluation, conditioning must be gradual [2]. Fitness and its evaluation are important parts of all equestrian disciplines, and the standardization of exercise tests is recognized as being valuable for monitoring the training progress [3]. Accordingly, as per Arfuso *et al.*, understanding the physiological adaptations of both athletes that are necessary for the execution of such difficult exercise is crucial for a correct evaluation of athletic performance, as well as for adapting and improving an adequate training program. Field tests simulate speeds, gaits, surfaces, and environments similar to the diverse components of eventing competitions, specifically preserving their individual biomechanics [4,5]. Information obtained by stress tests must provide values which are repeatable, objective, and valid, allowing for a standardization of the results [6].

Physical training promotes physiological changes in the anaerobic energy system, resulting in increased levels of resting anaerobic substrates and the amount and activity of key enzymes that control the anaerobic phase of glycogen

fractionation [7]. Among the parameters considered for the evaluation of athletic performance in horses, cardiovascular parameters are the most studied as they represent good indicators of fitness level and workload effort. Heart rate evaluation is relatively simple, and in the equine athlete, it is essential to be evaluated before, during, and after exercise in order to quantify the intensity of the workload and the horse's fitness, thereby determining the effects of such exercise on its cardiovascular system [4,8–10].

Plasma lactate concentration is the variable which best correlates with the animal's performance, providing additional information on the athlete's actual condition [11]. Lactate production and its association with heart rate and speed represent the main parameters utilized for estimating the efficiency of training protocols in equine athletes, expressed as V4 [12]. The velocity where the blood lactate concentration reaches a certain level is defined as VLa. The quantitative variable determining the speed with which the lactate concentration reaches 4 mmol/l is described as V4 or Vla4. Using this variable offers the investigator a reliable way to determine physical fitness and performance in the equine athlete [11].

Enzymes used as clinical markers of muscle lesions include creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), which leak into the blood circulation due to microtraumas and increases of sarcolemma permeability [13,14].

The current study aims to evaluate the athletic metabolism and fitness of eventing horses, recruited from military use, via an interval training protocol through the use of incremental speed, incremental jumping, and jumping course tests.

2. Materials and Methods

2.1. Location

The experiment was carried out in the 3rd Guard Cavalry Regiment of the Brazilian Army, in Porto Alegre, Rio Grande do Sul, Brazil.

2.2. Animals

Animal Use Ethics Committee approved the work in question. The protocol number is 004/2017 at the Federal University of Pampa, Dom Pedrito, Brazil/RS. Fifteen mixed gender horses of the Brazilian Sport Horse (Brasileiro de Hipismo) and crossbreeds from the Coudelaria do Rincão Stud were used, which were owned by the Brazilian Army. The horses selected for the experiment were destined for Eventing–Level 1, specifications according to the Federation Equestrian International (FEI). All equine training options were at the same performance level. The animals underwent testing and training at the beginning of the competition season.

The average age of the horses was 6.5±1.0 years, and the average weight and height were 508.9±44.9 kg and 1.62±0.48 meters, respectively. Daily nutritional management consisted of supplying 4 kg of alfalfa hay, 4 kg of commercial concentrate (18% protein) offered twice a day, and 2 kg of oat grain once a day. The water was provided ad libitum. The animals were stabled in the 3rd Cavalry Regiment of Guard, and, prior to the experiment, they were submitted to clinical and hematological examination to ensure their health. During the entire period of the experiment, the animals remained healthy without lesions and with daily veterinary monitoring.

2.3. Experimental Design

Parameters were measured at two different times with a temporal interval of six weeks between the first three tests: incremental speed test, incremental jumping test, and jumping course, and the second stage with a repetition of the same three tests. The first test for initial fitness evaluation (stage 1) occurred in the week preceding training. The second evaluation (stage 2) occurred at the end of the sixth week of training. Stage 1 was carried out at the end of May with temperatures varying between 18°C and 28°C, and stage 2 was carried out 6 weeks later, at the end of July, with a temperature variation between 12°C and 22°C.

Animal preparation was conducted prior to all tests, involving the placement and fixation (suture and instant glue) of a flexible intravenous catheter1 14G gauge and a catheter extension with heparinized saline solution (10.000 UI/L). Blood collections were performed before heating the animals (basal) in the intervals between each stage of the tests and 10 minutes after the end of the test. Two tubes were utilized, one containing gel and clot activator and the other sodium fluoride. Immediately after the collections, the blood was centrifuged and separated, plasma and serum, in 2ml tubes for freezing and subsequent analysis. Each rider monitored the speed determined in the tests by the cell phone attached to the forearm, through the KerClockItSport application connected to the Polar Equine H7 heart rate monitor2 fitted to the animals via Bluetooth. The same app stored heart rate, speed, and distance travelled.

2.3.1. Test 1 Protocol: Incremental Speed Test (IST)

Before testing, animals performed a warm-up of 10 min of walking and 10 min of trotting and galloping. The incremental speed test was performed at five different speeds according to **Table 1**. Each lap covered a length of 850 meters in an open circular grass track, with 2 min intervals between laps. Each rider monitored the speed in real time through the use of the KER ClockIt Sport3 app on a smartphone fixed to the rider's forearm, which was also connected to the heart rate frequency meter.

The incremental speed test was used as a test for jumping horses according to the methodology performed by Munk [15]. Blood samples were taken before the animals were warmed (basal) in the intervals between each step of the test and 10 min after exercise.

2.3.2. Test 2 Protocol: Incremental Jumping Test (IJT)

The incremental jumping test was conducted on a 20 x 60 m outdoor sand track, with two rows of five simple vertical obstacles along the lengths spaced at 3 m distances. Three sets of 90 seconds each were performed. The obstacle heights were 40, 55, and 70 cm with elevation between steps. Before commencing the test, horses were warmed up in the same manner as in Test 1, followed by six jumps using the same heights.

1 Disposable intravenous catheter, model 14G, Descarpack, Brazil.
2 Polar Equine H7 Heart Rate Sensor Electrode Base Set, Polar Electro, EUA.
3 KER ClockIt Sport App, Kentucky Equine Research, EUA. Available at ker.com.

Table 1: Incremental Speed Test applied to horses in an open circular track with grass floor.

850m Circular Track					
Lap	1st	2nd	3rd	4th	5th
Speed	240 m/ min	320 m/ min	400 m/ min	480 m/ min	560 m/ min

2.3.3. Test 3 Protocol: Jumping Course (JC)

The jumping course was performed as per **[15]** and was modified to suit the initial sport level of the animals. The height of the course was 1 meter, and the total distance of the course was 380 meters, with 11 jumps, with two of them double and performed at a speed of 325 m/min. All animals were warmed up in the same manner as in Test 2.

2.4. Training Protocol

The horses were trained during a period of 6 weeks, with an average duration of 50 min per day. The interval training was performed three days a week based on the protocol used for jumping horses by Munk **[15]** and modified according to Cavalcanti **[16]** to fit the exercises to the eventing category (**Table 2**).

Training 1 consisted of flat track of 850 m, 90 seconds in V4**, 90 seconds of walking, and 4 repetitions. Training 2 included exercises of jumps with varied combinations, with lines (straight or in curve) of 4 to 8 strides of gallop, and "doubles" combination with 1, 2, or 3 strides of gallop. Combinations used "vertical" (max 1.05 m), "oxer," or parallel type obstacles (maximum 1.05 m x 1.20 m). Training 3 comprised a ring 20 x 60 m, 6 obstacles on the longer sides, 90 seconds of jumping, 90 seconds of walking, speed of 325 m/min, and 4 repetitions. Free training normally consisted of flexing exercises, initially with 10 min of step with free reins, performing figures, changing hands, making circles of 15 and 20 meters, serpentine, and taking advantage of the entire length of the arena to change the direction. These same figures are performed at a trot for 25 minutes and at a gallop for 15 minutes.

2.5. Statistical Analysis

The parameters were measured at two different times with a six-week time interval, between the first 3 tests (TIV, TIS, and PS) and a second stage with the repetition of the same 3 tests (TIV, TIS, and PS). The desired comparison was to confront each of the seven parameters in these two situations. The T-test for paired samples was used because the experimental setup allowed for a "before" and "after" of each animal. Besides the T-test for paired samples, a one-way analysis of variance test (ANOVA) was utilized for evaluating the parameters within each training.

3. Results

3.1. Test 1: Incremental Speed Test

In the incremental speed test, an average increase on HR was found in all stages according to speed increase. The HR values in stage 1 were lower than in stage 2. We obtained significant difference in HR at velocities of 240 m/min, 320 m/min, 480 m/min, and 10 min after exercise ($p < 0.05$; $p < 0.01$; $p < 0.05$; $p < 0.05$, respectively). **Figure 1** shows average results of variables between HR, RR, and speed found in both stages of the test. The RR of the animals tested was higher in stage 1 compared to stage 2. The reduction of this variable proved to be statistically significant in all speeds ($p < 0.01$). The level of plasma lactate had no difference between the two stages. The values of glucose during the test were different only on the 240 m/min speed of stage 2 compared to stage 1 ($p < 0.002$). **Table 3** shows the results obtained in the measurement of lactate and glucose. The measurement of muscle enzymes CK, AST, and LDH showed, in almost totality, a significant decrease at the speeds of stage 2 compared to stage 1 ($p < 0.01$). At the speed of 560 m/min and 10 min after exercise, in stage 2, CK blood level showed no significant decrease. At this same speed, there was no significant decrease in the AST value. These data are presented in **Figure 2.**

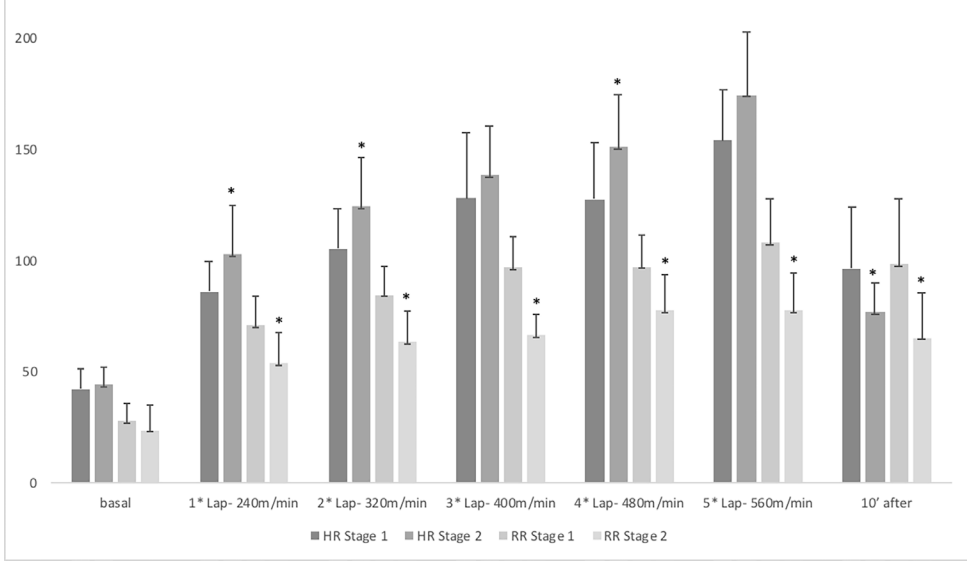

Figure 1: Average Heart Rate (HR) and Respiratory (RR) during IST. * above each bar denotes significant difference ($p < 0.05$) between stages.

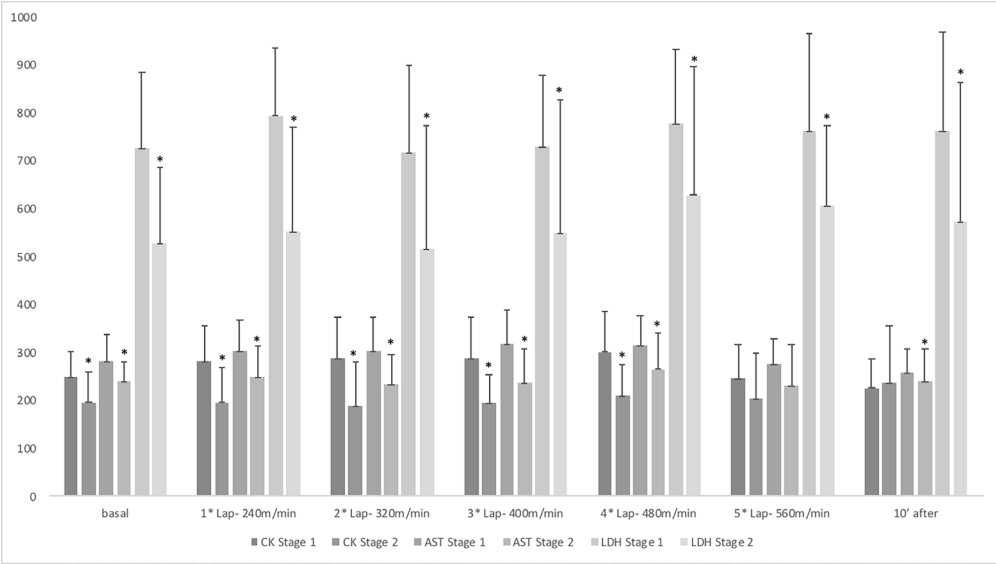

Figure 2: Average values of CK, AST and LDH enzymes during IST. * above each bar denotes significant difference ($p < 0.02$) between stages.

Table 2: Weekly Interval Training Program applied to horses on sand floor, six days a week in the mornings shift.

Weekly Training Program						
Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Training 1	Free	Training 2	Free	Training 3	Jumping Course	Rest

Training 1: Flat track 850m, 90 seconds in V4**, 90 seconds at walk, 4 repetitions.
Training 2: Exercises of jumps with varied combinations, with lines (straight or in curve) of 4 to 8 strides of gallop, and "doubles" combination with 1, 2 or 3 strides of gallop. Combinations using "vertical" (max 1.05m), "oxer" or parallel type obstacles (max 1.05m X 1.20m).
Training 3: Ring 20 x 60m, 6 obstacles on the bigger sides, 90 seconds jumping, 90 seconds walking, speed of 325m/min, 4 repetitions.
Free: Flexion.
* Warm up: 10 min walking, 20 min trotting and galloping. For training 3 plus 8 warm up jumps.
**V4 – corresponds to the speed at which blood lactate reached 4mmol/1 concentration, value determined in the Test 1.

Table 3: Lactate and Glucose values during Test 1 – IST.

	Basal		1st Lap 240m/min		2nd Lap 320m/min		3rd Lap 400m/min		4th Lap 480m/min		5th Lap 560m/min		10 min after exercise	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
Lactate	2.1	1.9*	2.9	2.5	2.9	2.8	3.8	3.2	4.4	4.4	7.2	5.7	4.7	3.2
	±0.3	±0.2	±1	±0.7	±0.8	±1.4	±1.2	±1.0	±1.4	±1.7	±2.4	±1.7	±1.6	±1.2
Glucose	63.6	89.5*	74.3	80.6*	79.3	81.8	84.8	83.6	88.4	86.3	95.7	94.2	85.1	96.2
	±26.6	±9.5	±12	±7.5	±12.6	±6.6	±8.1	±6.7	±10.3	±7.7	±10.9	±7.5	±24.5	±4.8

* demonstrates significant difference ($p < 0.04$) between stages.

3.2. Test 2: Incremental Jumping Test (IJT)

The heart rate in test 2 demonstrated a significant increase in heights of 55 and 70 cm ($p < 0.04$ and $p < 0.02$), respectively. Respiratory rate showed a significant decrease in the last two laps, 55 and 70 cm, and 10 min ($p < 0.001$; $p < 0.001$; $p < 0.002$, respectively) after exercise of stage 2 in comparison with stage 1. **Figure 3** presents the average values obtained for HR and RR.

Lactate and glucose values did not statistically differ between the two stages. However, glucose curve showed similar characteristics between the stages, with a decrease in the first lap, and according to the increment of exercise, there was an increase of blood level.

The CK enzyme showed no significant difference between stages. However, LDH indicated a significant decrease ($p < 0.004$) in all values of the measurements of stage 2 in comparison with stage 1. The AST values decreased significantly at 55 cm and 10 min after exercise from stage 2 ($p = 0.009$; $p = 0.04$) as given in **Table 4**.

3.3. Test 3: Jumping Course

The heart rate showed no difference between the two stages. The respiratory rate had a faster recovery 10 min after exercise,

with a significant decrease (p = 0.02) in stage 2. The average values of HR and RR are given in **Table 5**.

The lactate values did not show the same increase comparing the two stages during the course. According to **Table 6**, a significant difference (p = 0.001) between the two stages was observed. The glucose values decreased during the course but did not show significant variations in any of the stages according to **Table 6**.

Muscle enzymes CK and AST had a significant increase (p = 0.01 and $p < 0.001$, respectively) in basal values and at 10 min/Ae during stage 2 compared to stage 1. The LDH values only showed significant difference in baseline samples. These data are presented in **Table 7**.

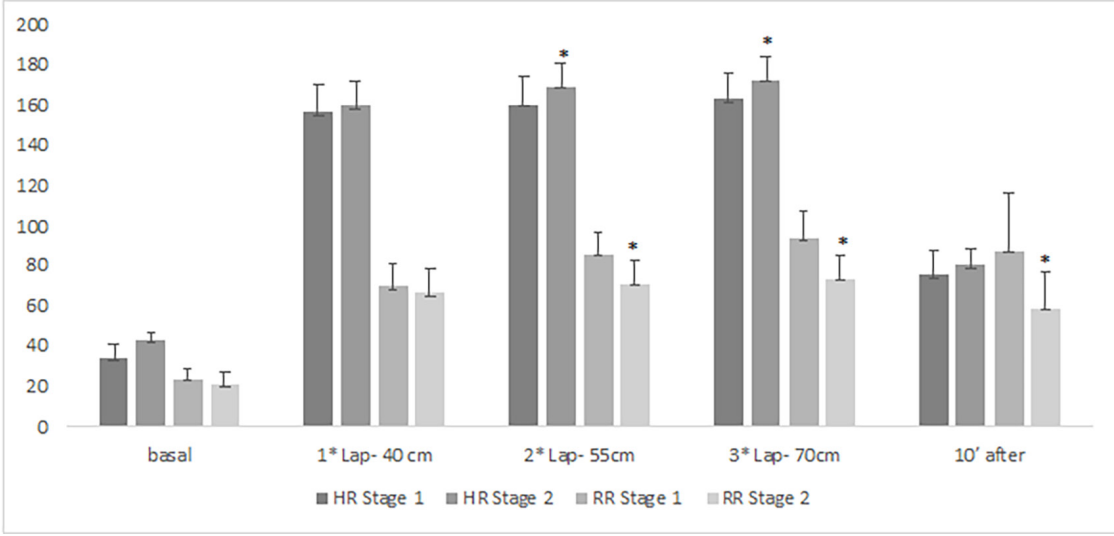

Figure 3: Average values of HR and RR during IJT. * above each bar denotes significant difference ($p < 0.04$) between stages.

Table 4: CK, AST and LDH enzymes values from Test 2 – IJT.

	Basal		1ª Lap 40 cm		2ª Lap 55 cm		3ª Lap 70 cm		10 min after exercise	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
CK	166.8 +37.6	148.7* +42.5	164.6 +34.1	234.1 +189	171.6 +43.7	179.5 +225	171.4 +40.6	166.8 +195.4	177.8 +52	250.3 +222
AST	270.7 +39.9	201.3* +64.4	286.8 +51.2	284.9 +78.8	298.6 +47.5	287.2* +73.5	296.3 +50.4	277.7 +78.6	287.8 +46.2	273.4* +50.9
LDH	645.2 +175.23	468.3* +166.4	674.3 +172	541.0* +195.3	688.2 +205.3	546.2* +116.8	730.2 +239.1	508.8* +163.6	683.4 +231.8	524.4* +136.3

Average values of CK, AST and LDH during IJT, * denotes significant difference ($p < 0.04$) between stages.

Table 5: Average HR and RR values during Test 3 - JC.

	Basal		Jumping Course 1m		10 min after exercise	
	S1	S2	S1	S2	S1	S2
HR	39.0 ±10.4	43.0 ±4.6	178.6 ±17.3	171.4 ±10.8	54.0 ±13.6	59.5 ±13.3
RR	27.0 ±4.6	24.0 ±7.6	58.3 ±9.0	56.1 ±13.4	49.6 ±19.6	36.3* ±13.3

Average HR and RR values during JC, * denotes significant difference ($p < 0.02$) between stages.

Table 6: Average Lactate and Glucose Values evaluated in Test 3.

	Basal		Jumping Course 1m		10 min after exercise	
	S1	S2	S1	S2	S1	S2
Lactate	1.9 ±0.4	1.6 ±0.7	3.4 ±1.2	2.3* ±1.3	2.2 ±0.6	1.9 ±0.8
Glucose	86.1 ±4.7	91.9* ±12.3	83.1 ±10.6	81.2 ±16.1	89.1 ±9.8	91.3 ±13.2

Average lactate and glucose values during JC, * denotes significant difference ($p > 0.02$) between stages.

Table 7: CK, AST, and LDH enzymes Values during Test 3 – Jumping Course

	Basal		Jumping Course 1m		10 min after exercise	
	S1	S2	S1	S2	S1	S2
CK	130.4	242.1*	158.4	210	145.5	195.4*
	±36	±213.9	±58.1	±112.1	±41.5	± 57.4
AST	226.9	268.1*	256.3	289.9	244.6	308.0*
	±37.5	±58.2	±40.6	±75.9	±37.8	±49.9
LDH	485.1	596.4*	567.5	630.1	542.2	561.1
	±148.7	±130.5	±123.2	±244.7	±118.9	±137.1

Average CK, AST and LDH enzymes values during JC, asterisk denote significant difference ($p < 0.01$) between stages.

4. Discussion

4.1. Test 1: Incremental Speed Test

The heart rate values contrasted with those found in horses stabled in horse riding schools that showed heart rate of 140 bpm/min at speeds of 270 to 390 m/min [17]. The same authors reported that horses in high levels of conditioning when reached the speeds of 420 to 480 m/min had HR values similar to those found during stage 2 of IST, 138 and 151 bpm/min.

The increase of HR in S2 is justified by training because the cardiovascular system was adjusted to the increased physical activity of skeleton muscle and metabolic demand during the second test, readjusting blood flow with the purpose of increasing the availability of oxygen and energetic substrates for ATP synthesis, transporting the products of elimination, carbon dioxide, hydrogen ions, and lactate, and regulating the homeostasis of the heat generated by muscular work [18]. In the present study, HR difference was observed in stage 2 at three distinct speeds of 240 m/min, 320 m/min, and 480 m/min, as well as at 10 min after exercise. However, in the study with eventing equine training, CCI* category, during progressive treadmill test, no difference was observed in the HR values between the group of trained and untrained equines, only finding difference in the recovering period, two minutes after exercise [19].

Physical activity can be compared to a ventilatory pump, in which the increase of speed would lead to the almost linear increase in ventilation per minute, corresponding to the tidal volume multiplied by the respiratory rate, where both would increase according to the animal's gait [20]. This increase, compatible with gait, was observed in both stages of IST. Studies performed with an incremental speed test reported higher respiratory frequencies at speed of 480m/min in comparison with the two stages of the present study, which were found to be 97 and 98 mov/min, respectively [21]. The significant RR decrease at all speeds in S2 compared to S1 is justified by respiratory dynamics during exercise, which must increase to maintain the pressure of oxygen and blood pH at physiological levels, all while minimizing the metabolic cost of the respiratory muscles [22]. The V4 of the present study was determined individually during S1 for each horse to perform training 1 at this speed. The average speed corresponding to V4 in S1 was 480 m/min, and similar values were found by Azevedo comparing V4 values between field and treadmill tests in progressive stress test [23], Munk evaluating the effect of 3 different interval trainings on jumping horses determined V4 at 490 m/min [15]. Lower values were found by Bitschnau *et al.* [3] and Soares [24], where V4 = 426 ± 54 m/min and V4 = 425.83 ± 59.00 m/min, respectively. One of the expected effects of an equine training protocol is the increase in the aerobic potential of the skeleton muscles, resulting in a greater workload capacity supported until the lactate starts to accumulate (anaerobic threshold); that is, the data curve of lactate by speed moves to the right in well-conditioned animals [12,25,26].

As a consequence of the physiological adaptations, there is a reduction of lactate plasma concentration in horses submitted to interval trainings [27], a parameter that was not observed in the present study possibly due to the short time of the protocol to which they were submitted. The glucose concentration during all laps on S1 showed a significant increase. It is believed that this increase is due to the increase in the glycogenolysis rate, due to the need for glucose. Andrews *et al.* demonstrated that the increase of glucose concentrations in equines submitted to eventing competition was higher in comparison with the group of horses submitted to endurance competition [28]. The response of equines submitted to high intensity and long duration exercises was the increase of glycemia [14].

Glucose values in IST, lower at the onset of progressive gallop and higher during recovery period, may be due to increased

glycogenolysis induced by the tissues' need for glucose and release of catecholamines [29].

It is possible to observe in the present study that despite of CK values between stages 1 and 2, the physiological values described by Pritchard *et al.* [30], which vary between 123 and 358 UI/L for healthy and resting horses, were not exceeded.

In the current study, a significant decrease in CK activity until the fourth lap in S2 was observed in comparison with S1. Training reduces the activity of enzymes resulting from exercise, and resistance training in horses leads to a decrease in CK production [31]. Thoroughbreds horses from 2 to 6 years old presented in the end of a treadmill exercise stress test higher values of CK than basal [31]. This difference was not found in this study.

The activity of the muscle enzyme is usually low in plasma as they are inside the myocyte; however, after exercise or muscle injury, activity increases significantly due to increased cell permeability, cellular necrosis, deficient elimination, or increased synthesis [32].

When evaluating horses of eventing discipline during the progressive stress test, Santiago observed that serum concentrations of AST increased in the progressive gallop, followed by a reduction during the recovery phase, and reported a reduction in serum concentrations of AST in the final phase of the training [33]. Similar data were found in the present study.

LDH concentration in S2 was lower in comparison with S1 with only 6 weeks of training. Divergent data were found in eventing horses submitted to prolonged training where no changes were observed, before and after exercise, in plasma concentrations of LDH [34,35].

4.2. Test 2: Incremental Jumping Test

The principle of sports training is the specificity, where the effect of physical activity exerted is specific for the muscle fibers involved in the exercise [36]. Previous research has indicated that the height of obstacles [37] as well as the height and speed [38] will influence heart rate and blood lactate concentration in horses.

In the current study, an increase of HR was observed when the exercise was started in the two stages of the IJT in the first lap (40 cm). This dynamic was also observed by evaluating jumping horses in a similar incremental test, where an increase in heart rate was observed right after the start of the exercise, attributed to adrenergic discharge [24,29,39].

The HR values found in this study were higher than those reported by Soares [24] where jumping horses with superior and inferior performance and average age of 11 years were compared. A possible justification for these results would be that young or untrained horses presented higher heart rate during exercise compared to older or trained horses [39] and that the maximum heart rate (HR Max) in horses is reduced with age [18]. It should be noted that the average age of the animals in the present study was 6.5 years. Repeating the same evolution of the IST, the IJT also showed a significant difference with a decrease in HR at the second lap, third lap, and 10 min after exercise in S2.

High-intensity exercise is associated with producing energy mainly by anaerobic metabolic pathways (both lactic and alactic) [40]. The higher the intensity of exercise, the higher the amount of lactate and hydrogen ions (H^+) produced. In the study in question, the increase of lactate occurred from the first lap (40 cm), and in the following laps, no a significant increase was observed in its concentration. The average maximum lactate concentration was 3.8 mmol/L before the training period and 2.8 mmol/L after training, as shown in a study conducted by Munk [15]. In the present study, there were no differences in lactatemia in the comparison before and after the 6-week training period, with an average value observed of 3.05 mmol/L between the stages.

Plasma glucose levels also did not show any alteration after the training program conducted by other researches [41]. During S2, the initial reduction of glucose concentration was identified. Ferraz *et al.* [39] reported a decrease in glycemia at the beginning of the incremental test, followed by an increase stimulated by elevated stress intensity, catecholamines, and glucagon action, which act on the liver promoting glycogenolysis.

The CK values found in S2 are in agreement with the physiological values reported by [30] and [41] between 100 and 300 U/L. Training tends to attenuate serum CK and AST alterations over time, although this depends on the intensity and frequency of training. The plasma activity of CK and AST enzymes was also measured in BH crossbred equines submitted to IJT. Baseline values and values after exercise were higher than those found in the present study [24], with the values after exercise being above the physiological reference values.

There is a great variation of the physiological values of LDH. Studies of different equestrian sports have reinforced the importance of establishing reference value for horses of different disciplines and breeds and mainly under tropical climatic conditions [42,43].

Several factors regulate glycolytic pathway activity, including oxygen availability, LDH activity, and the magnitude of the ATP/ADP ratio [44]. LDH is a cytoplasmic enzyme that catalyzes the conversion of pyruvate to lactate at the end of anaerobic glycolysis [45].

A significant decrease in LDH concentration in comparison between the stages was verified in the IJT results. In the same way, in another study evaluating LDH concentration after training, the values presented a significant decrease. According to these authors, the serum LDH values in horses at rest decrease progressively as the animal adapts to training [46]. For example, plasma malate (MDH) and lactate dehydrogenase (LDH) activities change with formation and reflect alterations in oxidative capacity and lipid mobilization [47].

4.3. Test 3: Jumping Course

In jumping competitions, horses are normally in speeds between 300 and 450 m/min with heart rate of 150 bpm/min at the beginning and 190 bpm/min at the end of the course [29]. The cardiac parameters are compatible with those found in the

present study where the speed of 325 m/min was determined during the realization of the course. The metabolic demands of these animals are very different from race and resistance breeds, and undoubtedly anaerobic metabolism plays a major role in a jumping course, although the speed and the duration are lower compared to racehorses [48–51].

However, immediately after the exercise and for a few minutes, although tidal volume progressively approaches resting values, respiratory rate remains high [52,53]. Evaluating the recovery rate in the present study, a significant reduction in S2 is observed, suggesting that the acidemia may continue to influence respiratory activity during these periods. Moreover, it is likely to be an evaporative respiratory contribution to dissipating thermal load induced by exercise [54,55], especially due to the difference in temperature and humidity between the stages.

A significant decrease in the plasma lactate concentration was observed immediately after jumping course at S2. In a study conducted during a jumping course with obstacles with equal height and superior to 100 cm, and also a six-week training, the results obtained were 3.4 mmol/L in stages 1 and 3.1 mmol/L in stage 2 [15].

The average lactate values found 10 min after the course in both stages were approximately 2 mmol/L. Although the speed and duration of this type of event are low, the increase in HR associated with increased plasma lactate and the speed in the approach of jumps [29,55] represents an increase in the exercise intensity, with greater explosion and the use of anaerobic metabolism [48,56,57].

A reduction of blood glucose concentration was observed immediately after the two stages of T3. Depending on the intensity, duration, and type of physical exercise, the principal regulatory systems including nervous, cardiovascular, endocrine, and respiratory systems are called upon to work in concert in order to re-establish homeostatic equilibrium [4,40,58].

The comparison between the two stages allowed the standardization of the tests with repeatability, objectivity, and validity, presenting significant differences in the values of the main markers of the physiological dynamics of the exercise. The differences in the values between the stages were sufficiently significant to show the importance of a specific training for equines destined to eventing discipline.

5. Conclusion

The interval training protocol positively affected the physical conditioning of athlete horses destined to eventing discipline, and the field tests were feasible and compatible with the specificity of the modality. The incremental tests presented important results for the sports evaluation of the animals tested through physiological and biochemical parameters.

Authors' Contributions

Juliana – MSC student and performed most of the field tests and also lab tests (all)

Nelson – laboratory tests advisor, co-advisor

Rafael – Army officer in charge of providing the horses

William – help during field tests

Bruna- laboratory tests (lactate)

Ana Carolina – lab tests biochemistry (CK, AST and LDH)

Adriana – MSC advisor, supervisor at field tests

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Data Availability Statement

The data supporting the findings of this study are available within the article.

Conflicts of Interest

All the authors declare no conflicts of interest.

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Ethical Approval

Animal Use Ethics Committee approved the study. The protocol number is 004/2017 at the Federal University of Pampa - Dom Pedrito - Brazil/RS.

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