Rasayely
International Journal of Equine Science
https://rasayely-journals.com/index.php/ijes
Vol 1(2); 36–46, 2022

Original Article Open Access

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.

Received: 18 May 2022; Revised: 14 July 2022; Accepted: 01 August 2022; Published: 15 September 2022

Academic Editor: Carlos Alberto Hussni, São Paulo State University (UNESP), Brazil

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.

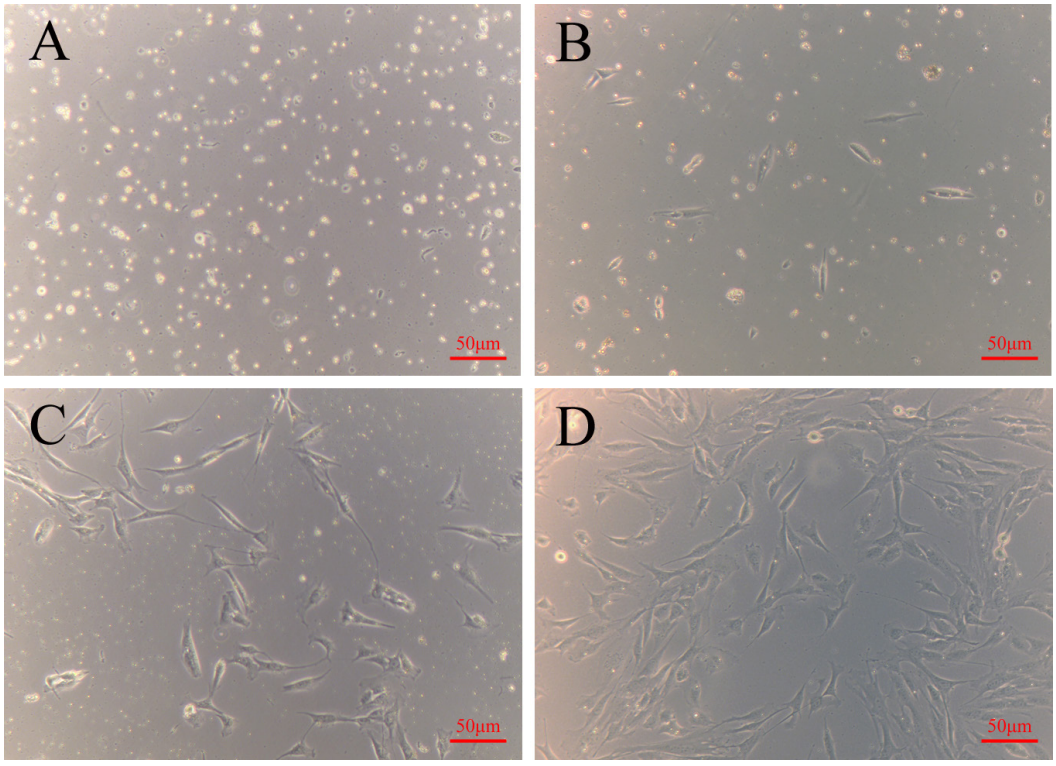

Figure 1: Morphology of in vitro cultured equine muscle satellite cells at different times. (**A:** 1 day; **B:** 3 days; **C:** 5 days; **D:** 7 days).

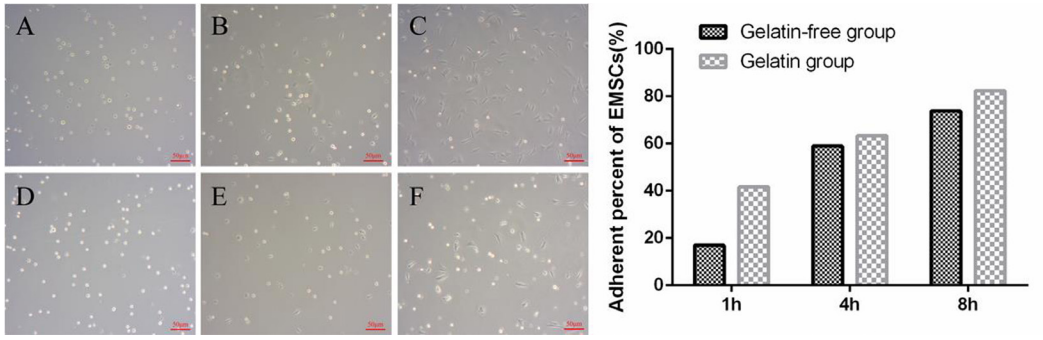

Figure 2: The influence of gelatin on the adherent percent of EMSCs at different cultural times. A-C. EMSCs in the gelatin group, adhering for 1 h **(A)**, 4 h **(B)**, and 8 h **(C)**. D-F. EMSCs in the gelatin-free group, adhering for 1 h **(D)**, 4 h **(E)**, and 8 h **(F)**.

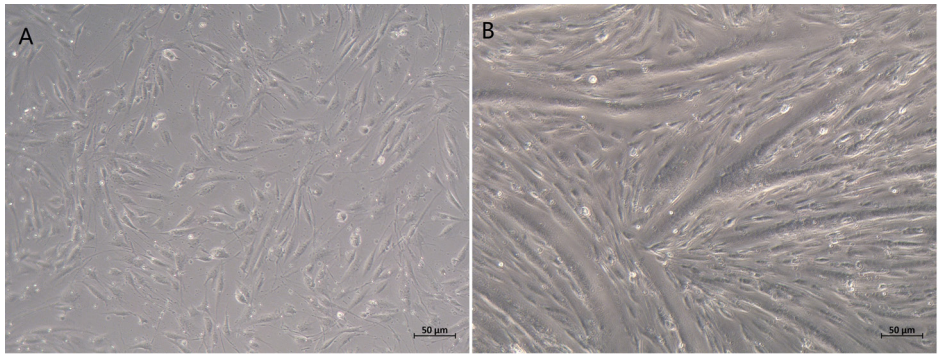

Figure 3: Equine muscle satellite cells before differentiation and after differentiation (**A:** equine muscle satellite cells before differentiation; **B:** differentiation for 24 h of equine muscle satellite cells formed myotubes).

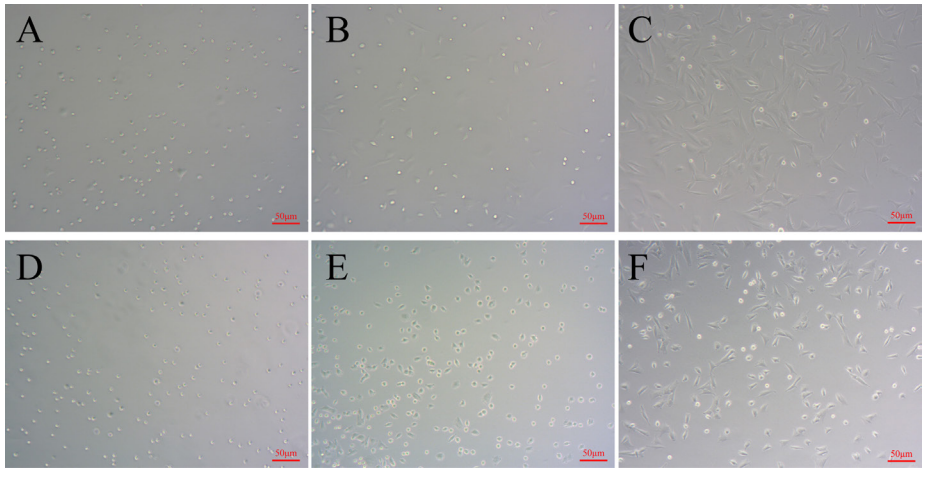

Figure 4: Differential adherence of EMSCs and EFCs at different times. EFCs, adhering at 1 h **(A)**, 4 h **(B)**, and 8 h **(C)**; EMSCs, adhering at 1 h **(D)**, 4 h **(E)**, and 8 h **(F)**.

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].

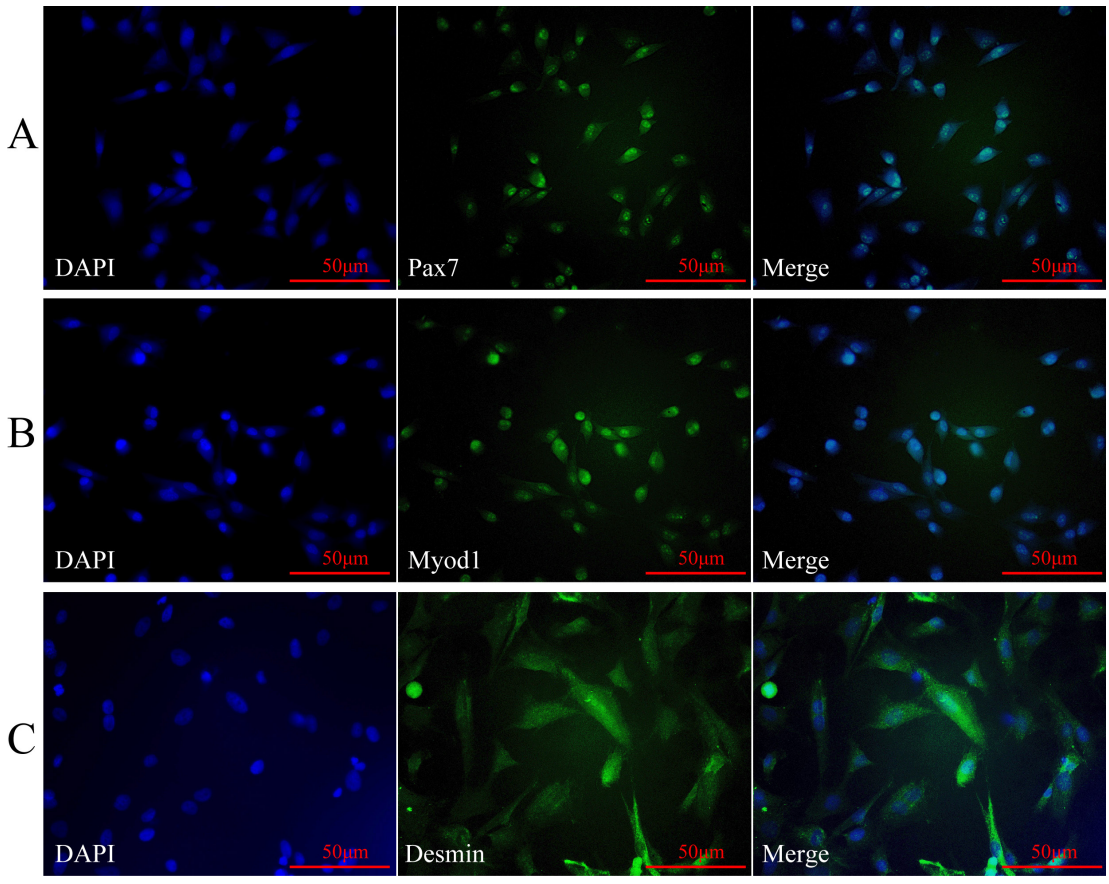

Figure 5: Immunofluorescence analysis of Pax7, Myod1, and Desmin on proliferating equine muscle satellite cells. **(A)** Immunofluorescence analysis of Pax7 on proliferating equine muscle satellite cells. **(B)** Immunofluorescence analysis of Myod1 on proliferating equine muscle satellite cells. **(C)**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

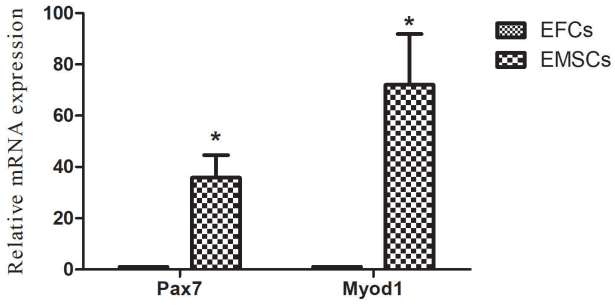

Figure 6: Relative mRNA expression of Pax7 and Myod1 in equine muscle satellite cells (EMSCs) compared to equine fibroblasts cells (EFCs).

* 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.

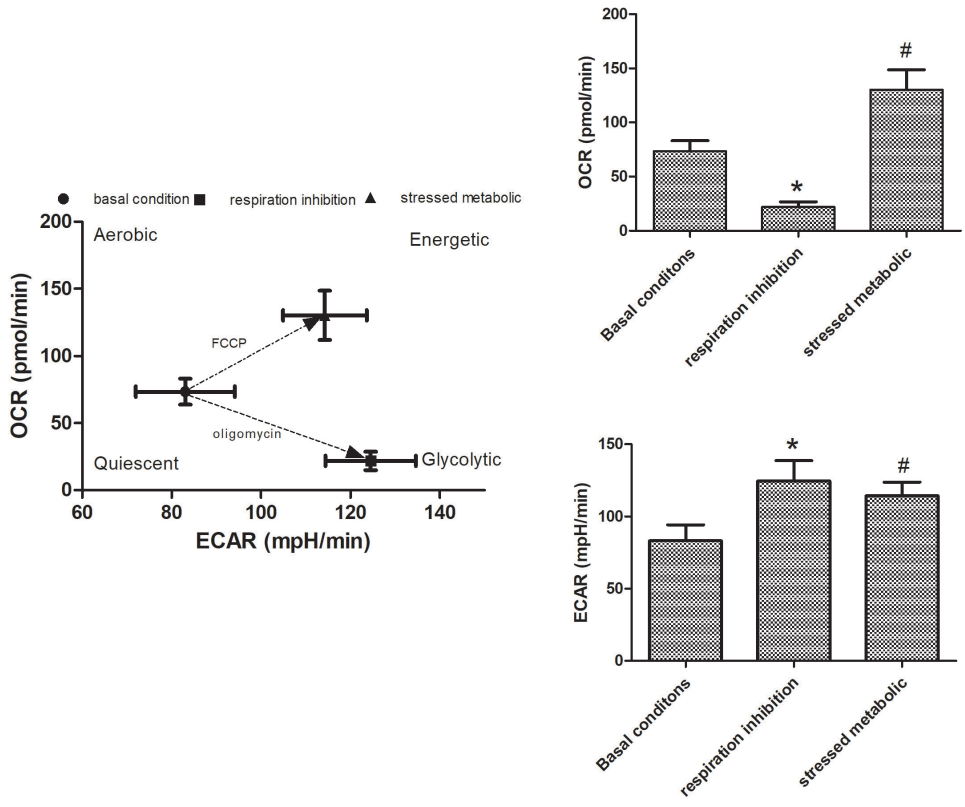

Figure 7: Bioenergetics metabolism of equine skeletal muscle satellite cells.

* 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.

Funding Statement

This work was supported by the National Natural Science Foundation of China [grant numbers 31902188, 3191101008, 31960657], Natural Science Foundation of Inner Mongolia [grant number 2021MS03016], the National Key Research and Development Program of China [grant number 2017YFE0108700], the startup project for High-level Talents of Inner Mongolia Agricultural University [grant number NDGCC2016-01], and Youth Foundation of College of Animal Science, Inner Mongolia Agricultural University [grant number QN201917, QN202030, QN202114].

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.

References

[1] Gurgul A, Jasielczuk I, Semik-Gurgul E, Pawlina-Tyszko K, Stefaniuk-Szmukier M, Szmatoła T, *et al.* A genome-wide scan for diversifying selection signatures in selected horse breeds. PLoS One 2019;14:e0210751. https://doi.org/10.1371/journal.pone.0210751.

[2] Mickelson JR, Valberg SJ. The genetics of skeletal muscle disorders in horses. Annu Rev Anim Biosci 2015;3:197–217. https://doi.org/10.1146/annurev-animal-022114-110653.

[3] Zammit PS. All muscle satellite cells are equal, but are some more equal than others? J Cell Sci 2008;121:2975–82. https://doi.org/10.1242/jcs.019661.

[4] Di Foggia V, Robson L. Isolation of satellite cells from single muscle fibers from young, aged, or dystrophic muscles. Methods Mol Biol 2012;916:3–14. https://doi.org/10.1007/978-1-61779-980-8_1.

[5] Morgan JE, Partridge TA. Muscle satellite cells. The International Journal of Biochemistry & Cell Biology 2003;35:1151–6. https://doi.org/10.1016/S1357-2725(03)00042-6.

[6] Williams CA. The effect of oxidative stress during exercise in the horse. J Anim Sci 2016;94:4067–75. https://doi.org/10.2527/jas.2015-9988.

[7] Mami S, Khaje G, Shahriari A, Gooraninejad S. Evaluation of biological indicators of fatigue and muscle damage in Arabian horses after race. J Equine Vet Sci 2019;78:74–8. https://doi.org/10.1016/j.jevs.2019.04.007.

[8] Marlin DJ, Fenn K, Smith N, Deaton CD, Roberts CA, Harris PA, *et al.* Changes in circulatory antioxidant status in horses during prolonged exercise. J Nutr 2002;132:1622S-7S. https://doi.org/10.1093/jn/132.6.1622S.

[9] Schienda J, Engleka KA, Jun S, Hansen MS, Epstein JA, Tabin CJ, *et al.* Somitic origin of limb muscle satellite and side population cells. Proc Natl Acad Sci U S A 2006;103:945–50. https://doi.org/10.1073/pnas.0510164103.

[10] Fukada S, Ma Y, Ohtani T, Watanabe Y, Murakami S, Yamaguchi M. Isolation, characterization, and molecular regulation of muscle stem cells. Front Physiol 2013;4:317. https://doi.org/10.3389/fphys.2013.00317.

[11] Rhoads RP, Fernyhough ME, Liu X, McFarland DC, Velleman SG, Hausman GJ, *et al.* Extrinsic regulation of domestic animal-derived myogenic satellite cells II. Domestic Animal Endocrinology 2009;36:111–26. https://doi.org/10.1016/j.domaniend.2008.12.005.

[12] Mauro A. Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 1961;9:493–5. https://doi.org/10.1083/jcb.9.2.493.

[13] Bennett VD, Cowles E, Husic HD, Suelter CH. Muscle cell cultures from chicken breast muscle have increased specific activities of creatine kinase when incubated at 41 degrees C compared with 37 degrees C. Exp Cell Res 1986;164:63–70. https://doi.org/10.1016/0014-4827(86)90454-4.

[14] Dodson MV, Martin EL, Brannon MA, Mathison BA, McFarland DC. Optimization of bovine satellite cell-derived myotube formation in vitro. Tissue Cell 1987;19:159–66. https://doi.org/10.1016/0040-8166(87)90001-2.

[15] Wu H, Ren Y, Li S, Wang W, Yuan J, Guo X, *et al.* In vitro culture and induced differentiation of sheep skeletal muscle satellite cells. Cell Biol Int 2012;36:579–87. https://doi.org/10.1042/CBI20110487.

[16] Li B, Li P, Huang R, Sun W, Wang H, Li Q, *et al.* Isolation, culture and identification of porcine skeletal muscle satellite cells. Asian-Australas J Anim Sci 2015;28:1171–7. https://doi.org/10.5713/ajas.14.0848.

[17] Gibson MC, Schultz E. Age-related differences in absolute numbers of skeletal muscle satellite cells. Muscle Nerve 1983;6:574–80. https://doi.org/10.1002/mus.880060807.

[18] Greene EA, Raub RH. Procedures for harvesting satelite cells from equine skeletal muscle. Journal of Equine Veterinary Science 1992;12:33–5. https://doi.org/10.1016/S0737-0806(06)81383-3.

[19] Folmes CDL, Dzeja PP, Nelson TJ, Terzic A. Metabolic plasticity in stem cell homeostasis and differentiation. Cell Stem Cell 2012;11:596–606. https://doi.org/10.1016/j.stem.2012.10.002.

[20] Verdijk LB, Snijders T, Drost M, Delhaas T, Kadi F, van Loon LJC. Satellite cells in human skeletal muscle; from birth to old age. Age (Dordr) 2014;36:545–7. https://doi.org/10.1007/s11357-013-9583-2.

[21] Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 2001;25:402–8. https://doi.org/10.1006/meth.2001.1262.

[22] Miersch C, Stange K, Röntgen M. Separation of functionally divergent muscle precursor cell populations from porcine juvenile muscles by discontinuous Percoll density gradient centrifugation. BMC Cell Biology 2018;19:2. https://doi.org/10.1186/s12860-018-0156-1.

[23] Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA. Culturing satellite cells from living single muscle fiber explants. In Vitro Cell Dev Biol Anim 1995;31:773–9. https://doi.org/10.1007/BF02634119.

[24] Mesires NT, Doumit ME. Satellite cell proliferation and differentiation during postnatal growth of porcine skeletal muscle. Am J Physiol Cell Physiol 2002;282:C899-906. https://doi.org/10.1152/ajpcell.00341.2001.

[25] Wilschut KJ, Jaksani S, Van Den Dolder J, Haagsman HP, Roelen BAJ. Isolation and characterization of porcine adult muscle-

derived progenitor cells. J Cell Biochem 2008;105:1228–39. https://doi.org/10.1002/jcb.21921.

[26] Gharaibeh B, Lu A, Tebbets J, Zheng B, Feduska J, Crisan M, *et al.* Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique. Nat Protoc 2008;3:1501–9. https://doi.org/10.1038/nprot.2008.142.

[27] Kim KH, Qiu J, Kuang S. Isolation, Culture, and Differentiation of primary myoblasts derived from muscle satellite cells. Bio Protoc 2020;10:e3686. https://doi.org/10.21769/BioProtoc.3686.

[28] Bai C, Hou L, Li F, He X, Zhang M, Guan W. Isolation and biological characteristics of beijing Fatty chicken skeletal muscle satellite cells. Cell Commun Adhes 2012;19:69–77. https://doi.org/10.3109/15419061.2012.743998.

[29] Bian Y, Mu R, Li X, Li Y, Guan W. Isolation and biological characteristics of skeletal muscle satellite cells of Luxi cattle embryo. Journal of Huazhong Agricultural University 2013;32:90–6.

[30] Siegel AL, Atchison K, Fisher KE, Davis GE, Cornelison DDW. 3D timelapse analysis of muscle satellite cell motility. Stem Cells 2009;27:2527–38. https://doi.org/10.1002/stem.178.

[31] Wang Y, Dai Y, Liu XF, Liu ZW, Li JX, Guo H, *et al.* Isolation, identification and induced differentiation of bovine skeletal muscle satellite cells. Chinese Journal of Animal Science and Veterinary Medicine 2014;41:142–7.

[32] Danoviz ME, Yablonka-Reuveni Z. Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. Methods Mol Biol 2012;798:21–52. https://doi.org/10.1007/978-1-61779-343-1_2.

[33] Stratos I, Rotter R, Eipel C, Mittlmeier T, Vollmar B. Granulocyte-colony stimulating factor enhances muscle proliferation and strength following skeletal muscle injury in rats. J Appl Physiol (1985) 2007;103:1857–63. https://doi.org/10.1152/japplphysiol.00066.2007.

[34] Kanisicak O, Mendez JJ, Yamamoto S, Yamamoto M, Goldhamer DJ. Progenitors of skeletal muscle satellite cells express the muscle determination gene, MyoD. Dev Biol 2009;332:131–41. https://doi.org/10.1016/j.ydbio.2009.05.554.

[35] von Maltzahn J, Jones AE, Parks RJ, Rudnicki MA. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proceedings of the National Academy of Sciences 2013;110:16474–9. https://doi.org/10.1073/pnas.1307680110.

[36] Tedesco FS, Dellavalle A, Diaz-Manera J, Messina G, Cossu G. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. J Clin Invest 2010;120:11–9. https://doi.org/10.1172/JCI40373.

[37] Hernández-Hernández JM, García-González EG, Brun CE, Rudnicki MA. The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. Semin Cell Dev Biol 2017;72:10–8. https://doi.org/10.1016/j.semcdb.2017.11.010.

[38] Jeong J, Choi K-H, Kim S-H, Lee D-K, Oh J-N, Lee M, et al. Combination of cell signaling molecules can facilitate MYOD1-mediated myogenic transdifferentiation of pig fibroblasts. J Anim Sci Biotechnol 2021;12:64. https://doi.org/10.1186/s40104-021-00583-1.

[39] Yang J, Liu H, Wang K, Li L, Yuan H, Liu X, et al. Isolation, culture and biological characteristics of multipotent porcine skeletal muscle satellite cells. Cell Tissue Bank 2017;18:513–25. https://doi.org/10.1007/s10561-017-9614-9.

[40] Allen RE. Muscle cell growth and development. Designing Foods, Washington, DC: National Research Council National Academy Press; 1988, p. 142–62.

[41] Bao T, Han H, Li B, Zhao Y, Bou G, Zhang X, *et al.* The distinct transcriptomes of fast-twitch and slow-twitch muscles in Mongolian horses. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 2020;33:100649. https://doi.org/10.1016/j.cbd.2019.100649.

[42] Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. Physiol Rev 2011;91:1447–531. https://doi.org/10.1152/physrev.00031.2010.

[43] Seene T, Kaasik P, Alev K, Pehme A, Riso EM. Composition and turnover of contractile proteins in volume-overtrained skeletal muscle. Int J Sports Med 2004;25:438–45. https://doi.org/10.1055/s-2004-820935.

[44] White SH, Wohlgemuth S, Li C, Warren LK. Rapid Communication: Dietary selenium improves skeletal muscle mitochondrial biogenesis in young equine athletes. J Anim Sci 2017;95:4078–84. https://doi.org/10.2527/jas2017.1919.

[45] Shamim B, Hawley JA, Camera DM. Protein availability and satellite cell dynamics in skeletal muscle. Sports Med 2018;48:1329–43. https://doi.org/10.1007/s40279-018-0883-7.

[46] Hearne A, Chen H, Monarchino A, Wiseman JS. Oligomycin-induced proton uncoupling. Toxicology in Vitro 2020;67:104907. https://doi.org/10.1016/j.tiv.2020.104907.

[47] Smolina N, Bruton J, Kostareva A, Sejersen T. Assaying mitochondrial respiration as an indicator of cellular metabolism and fitness. Methods Mol Biol 2017;1601:79–87. https://doi.org/10.1007/978-1-4939-6960-9_7.

How to Cite

Zhang X, Bou G, Xing J, Zhao Y, Caiwendaolima, Dugarjaviin M. Isolation, Culture, Identification, and Bioenergetics Metabolism of Equine Skeletal Muscle Satellite Cells. Int J Equine Sci 2022;1(2):36–46.