CELL BIOLOGY

Isolation, culturing and 3D bioprinting of equine myoblasts

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Abstract

Isolating and culturing myoblasts is essential for techniques such as tissue regeneration and in vitro meat production. This research describes a protocol to isolate primary myoblasts from skeletal muscle of an adult horse. The equine primary myoblasts expressed markers specific to myoblasts and had multipotent potential capabilities with differentiation into chondrocytes, adipocytes and osteoblasts in vitro. The horse myoblasts did not adhere to Cytodex 3 and grew poorly on CultiSpher-S microcarriers during in vitro cultivation. Our studies showed that the use of GelMa bioink and ionic cross-linking did not have negative effects on cell proliferation at the beginning of cultivation. However, cells showed reduced proliferative activity by day 40 following in vitro culturing. The population of primary equine myoblasts obtained from an adult individual, and propagated on microcarriers and bioink, did not meet the requirements of the regenerative veterinary and manufacturing meat in vitro regarding the quantity and quality of the cells required. Nonetheless, further optimization of the cell scaling up process, including both microcarriers and/or the bioreactor program and bioprinting, is still important.

Keywords: myoblasts, bioprinting, microcarriers

Introduction

The creation of functional skeletal muscle via tissue and cell engineering holds great promise for both regenerative veterinary medicine and for manufacturing meat in vitro. A basic method is required for muscle regeneration which includes mesenchymal stem cells and myoblasts. This is necessary because life-long regeneration of damaged muscle via cell transplantation presents an ideal therapy for animals with large volumes of muscle loss and/or muscular diseases (Liu et al., 2018).

In vitro meat has more recently emerged as a new concept in food biotechnology and occupies a special place among many types of alternative protein products (Sharma, Thind and Kaur, 2015). In 2005, the US National Aeronautics and Space Administration (NASA) conducted research on muscle cultures from turkey cells (Edelman, McFarland, Mironov and Matheny, 2005; Webb, 2006). Technological approaches have also been developed to create the first edible cultured fish fillet from goldfish cells.

In 2007, it was shown that culturing stem cells on a collagen matrix stimulated proliferation and induced the formation of muscle fibers (van Eelen, 2021). In 2013, the world's first bovine stem cell cultured meat burger was prepared from livestock (Post, 2014), and was sold for several thousand dollars, showing the demand and interest in such products.

When culturing meat, significant numbers of animal muscle cells must be grown in the laboratory. In order to increase the biomass of these cells, they are

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cultivated on various scaffolds (Kubis et al., 2016). However, it is difficult to reproduce meat tissue due to the complex arrangement of cells, extracellular matrix, proteins, nutrients, and growth factors (Ong, Choudhury and Naing, 2020). Recently, 3D bioprinting technology has been developed and has become an important part of culturing meat. Bioprinting allows the creation of scaffolds in which muscle fibers are formed, which ultimately become meat (Bhat, Kumar and Fayaz, 2015; Sun et al., 2018). This process is quite complex, as it involves the printing of muscle, fat, and other cells supporting the extracellular matrix, but it has proven to be the most suitable method for producing cultured meat on a large scale (Stephens, Sexton and Driessen, 2019).

To obtain large scale cell cultures, bioreactors are most commonly used, whereby cells are grown on microcarriers (Nienow et al., 2006). The advantages of this technology are that the microcarriers can provide more culture surface area per unit volume of medium compared to tissue culture flasks (Nienow et al., 2006). Also, the culture area can be increased further by adding new microcarriers since the cells are able to migrate and colonize new microcarriers (Ohlson, Branscomb and Nillson, 1994). Some of these microcarriers, such as Cytodex 3 (Merck, Germany) and CultiSpher-S (Sigma, USA), were used in our studies because they yield huge amounts of cells and are suitable for cell agitation (Elashry et al., 2016, Storm et al., 2010).

This preliminary study evaluated the possibility of culturing myoblasts from the muscles of an adult horse in monolayer, also using a bioreactor and bioprinting cells with GelMa hydrogel. Further research is expected to optimize the cultivation and bioprinting of myoblasts. The developed technology will be used in regenerative veterinary medicine or in food technologies.

Materials and methods

Cells isolation and characterization

The biopsy of muscle was obtained from a horse aged 2.5 years old. The permission for the operation and tissue extraction was obtained from the owner, and the biopsy was taken in the Kazan Hippodrome veterinary clinic by a veterinary professional. A 1 cm^3 volume biopsy was taken from the gluteus medius muscle under local anesthesia conditions. For this, lidocaine was injected subcutaneously (Organika LLC, Russia) and an incision was made in the croup, 5–7 cm away from the sacral spine. Following collection, the tissue was placed into sterile saline and the sample was transported to the laboratory within two hours to enable myoblast isolation.

The muscle sample was cleaned free of connective and adipose tissue in a laminar flow cabinet under sterile conditions. It was then washed with phosphate-buffered saline (PBS, PanEco, Russia) and mechanically dissociated using sterile scissors. Then the pieces of muscle were transferred into sterile centrifuge tubes and incubated for 1 h in an incubator shaker within trypsin-EDTA solution (PanEco, Russia) at a final concentration of 0.1 %. Thereafter, the cell suspension was filtered through a 40 μm cell filter (SPL, Korea) into a new tube to remove any large undigested fragments of muscle tissue. The resulting suspensions were centrifuged for 10 min at 800 g to pellet the cells. Afterwards the pellet was washed and resuspended in alpha-MEM (PanEco, Russia), containing 20 % fetal bovine serum (FBS, PanEco, Russia), 2.5 ng/ml fibroblast growth factor (FGF2, Santa Cruz, USA), 100 U/ml penicillin (PanEco, Russia) and 100 μg/ml streptomycin (PanEco, Russia), and cultivated in a standard T-flask (75 cm^2). The cells were then incubated for 2 h to separate the satellite cells from the fibroblasts, at 37 °C with a 5 % CO_2 concentration. Then the suspension was moved into a T-flask coated with 0.1 % gelatin (PanEco, Russia), and the cells were grown in the medium at 37 °C with a 5 % CO_2 concentration. The medium was changed every other day. Speed of cell growth was detected as a ratio of the number of cells grown compared to the number of cells seeded, and was measured every 72 h until the 14^{th} passage.

Flow cytofluorometry

In order to identify the cell populations, the following antibodies were used: anti-MYH ½ (SANTA CRUZ, USA, sc53088), anti-myogenin (SANTA CRUZ, USA, sc52903), anti-PAX7 (CUSABIO TECHNOLOGY, USA, CSB-PA891015), anti-desmin (SANTA CRUZ, USA, sc14026) and anti-Ki67 (ABCAM, USA, ab15580). Cells were analyzed using flow cytometry (Guava 8T, Millipore, USA).

Differentiation of horse myoblasts

Differentiation of the cultured cells was carried out in three directions: osteogenic, adipogenic, and chondrogenic. To study the ability of the obtained cell culture to undergo adipo- and osteodifferentiation, cells from the third passage were seeded into 12-well plates, 30,000 cells per well, and incubated in growth medium until a monolayer had been obtained. Thereafter in order to induce differentiation, the cells were cultured in special differentiation media (Gibco, USA). Differentiation in the chondrogenic direction was carried out in accordance with a previously published method (Zakirova, Azizova, Rizvanov and Khafizov, 2015). The medium was changed every 3 days for 21 days. Afterwards the cell cultures were fixed with 4 % paraformaldehyde for 20 min at room temperature and samples for microscopy were prepared.

To detect chondrogenic differentiation, the cells were stained for the presence of acidic mucopolysac-

charides, markers of chondrogenesis, with a solution of Alcian Blue (Sigma, United States) according to the previously described method (Zakirova et al., 2014). Briefly, the fixed cells were washed with PBS three times for 5 min, stained for 1 h with Alcian Blue (1 g Alcian Blue/100 mL 0.1 M HCl) and then washed with PBS. To determine the presence of mineralization, a sign of osteogenic differentiation, Von Kossa staining reactions were used according to previously described methods. Briefly, silver nitrate solution (2 % (w/v)) was applied onto the cells with a subsequent incubation for 10 min under dark conditions, then for 1h under bright illumination (Naumenko, Guryanov, Zakirova and Fakhrullin, 2021). To detect lipid intracellular inclusions, the cells were stained with the lipophilic dye Nile Red according to the previously published method (Zakirova, Aimaletdinov, Tambovsky and Rizvanov, 2021). The Nile Red solution was incubated with the samples for 30 min at a temperature of 37 °C. The nuclei of the cells were dyed with the fluorescent dye Dapi (0.1 μg Dapi/1 ml PBS). Differentiation was assessed using a microscope AxioObserver Z1 (Carl Zeiss, Germany).

Viability of cells in vitro

Analysis was performed using the Annexin V Apoptosis Detection Kit (Santa Cruz, sc4252AK, USA) according to the previously described method (Zakirova et al., 2019). The stained cell suspension was analyzed using flow cytometry (Guava 8T, Millipore, USA).

Formation of myotubes in myoblast cultures in vitro

To obtain myotubes, the isolated cell cultures were incubated until a monolayer formed. After that, the cell culture growth medium was changed weekly (Baquero-Perez, Kuchipudi, Nelly and Chang, 2012). Myotube formation was detected after 10 days. The number of cells which had converted to myotubes was assessed using light microscopy of the unstained cells and expressed as a fraction of the total number. Once myotubes occupied 50–70 % of the area, the cell monolayer was fixed in 4 % formalin for 1 h at room temperature, then washed with PBS. The cells were then stained with hematoxylin-eosin (JSC Lenreaktiv, Russia), Phalloidin Alexa Fluor™ 488 (Invitrogen™, A12379) and Dapi (Dia-M, Russia) as recommended by the manufacturers. Results were assessed using an AxioObserver Z1 microscope.

Cultivation on carriers

Cytodex 3 (Merck, Germany) and CultiSpher-S (Sigma, USA) were used as microcarriers during myoblast cultivation. Cytodex 3 are dextran beads coated with denatured porcine-skin collagen whereas CultiSpher-S are macroporous gelatin-coated microcarrier beads. The microcarriers were sterilized and cell suspensions inoculated onto them in accordance with the manufacturer's instructions. Cell cultivation on microcarriers was carried out in a bioreactor (Synthecon, USA) in the growth media described above.

Myoblast bioprinting

Bioprinting of the samples was carried out on a CELLINK Inkredible bioprinter (CELLINK, Sweden) with the following parameters: temperature 21 °C, pressure 24kPa, speed 60mm/s, and size of nozzles 25G. The composition for bioprinting consisted of 5.5 million myoblasts per 1 ml of GelMa bioink (CELLINK, Sweden). The resulting mixture was printed in 100 μL volumes into culture plates, washed with Crosslinking agent $CaCl_2$ solution (CELLINK, Sweden), followed by a saline wash (PanEco, Russia). Then, the samples containing horse myoblasts were embedded into a culture medium and cultured for 40 days in a CO_2 incubator at 37 °C. Growth medium was changed every 3 days. The proliferative activity of the cells was detected by immunostaining over a 40-day cultivation period. The samples were stained anti-Ki67 (ABCAM, USA, ab15580) and were analyzed using an Axio Observer Z1 microscope.

Statistical analysis

Statistical analyses were conducted on three replicates. The results are presented as mean± SEM. Data were calculated using Microsoft Excel.

Results

The isolated cells presented with a fusiform shape with a centralized nucleus. The viability study showed that 0.2±0.06 % of the cells in the population were undergoing apoptosis. The cell population growth speed was 20±5 by the 2nd passage, 25±4 by the 6th passage, 30±7 by the 10th passage, and 15±5 by the 14th passage in a monolayer. During cultivation in a poor medium, myotube formation occurred on days 7–10 (Fig. 1).

During flow cytometry, the number of cells expressing the studied factors and their levels of fluorescence were evaluated. All cells expressed Ki67, myogenin, Pax7, desmin and MYH1/2 factors, whilst the level of fluorescence were Ki67 — 6.66, myogenin — 116.72, PAX7 — 69.65, desmin — 14.77, and MYH1/2 — 19.50 (Fig. 2).

The equine cells also differentiated into other, more specialized cell types (Fig. 3).

The possibility of culturing cells on carriers was also studied. Horse myoblasts did not adhere to the surfaces of the Cytodex 3 microcarriers (Fig. 4). When cultured on CultiSpher-S, low adhesion of cells to the surface of the microcarriers, alongside low proliferative

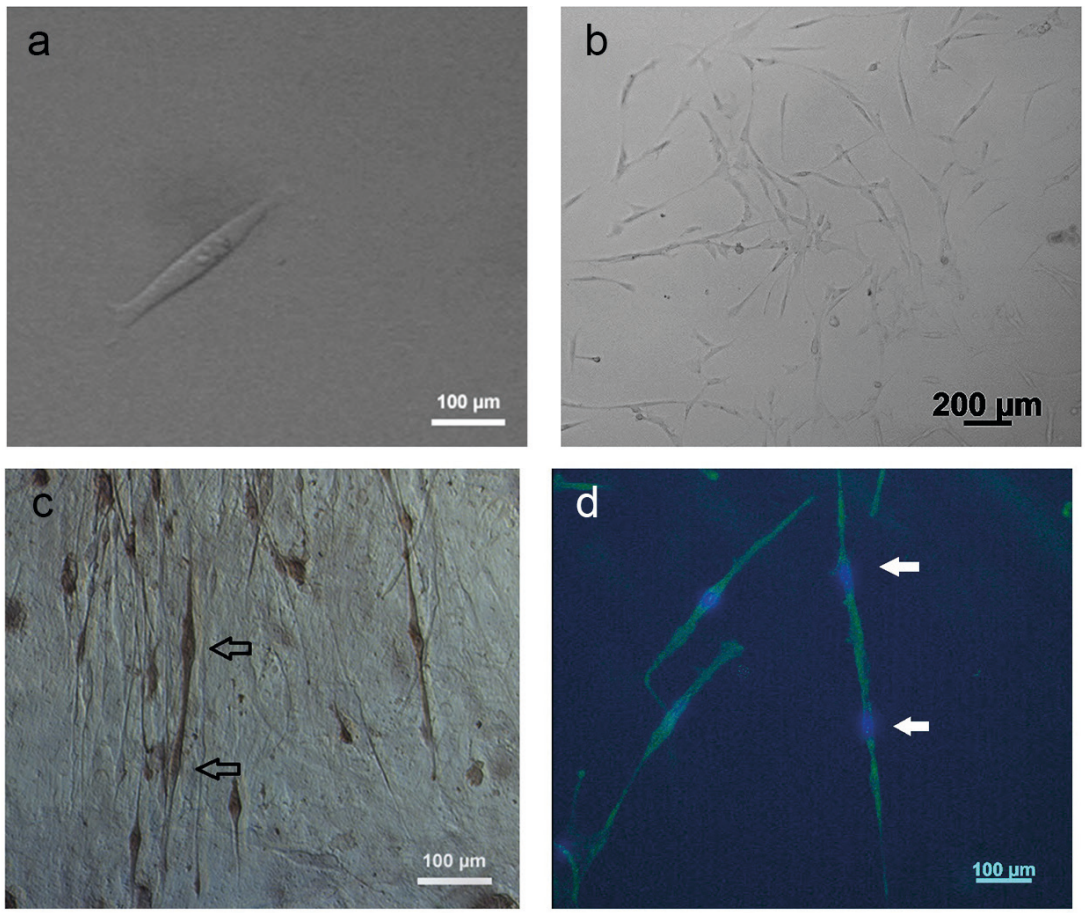

Fig. 1. Primary horse myoblasts. a. 0 passage single myoblast; b. 14th passage myoblasts; c. Staining of the horse myotubes with hematoxylin-eosin demonstrated the multinucleated character of the myotubes (arrows); d. Staining of the cells with Phalloidin (green coloring of actin fibers) and Dapi (blue coloring of cell nuclei) demonstrated the multinucleated character of the myotubes (arrows).

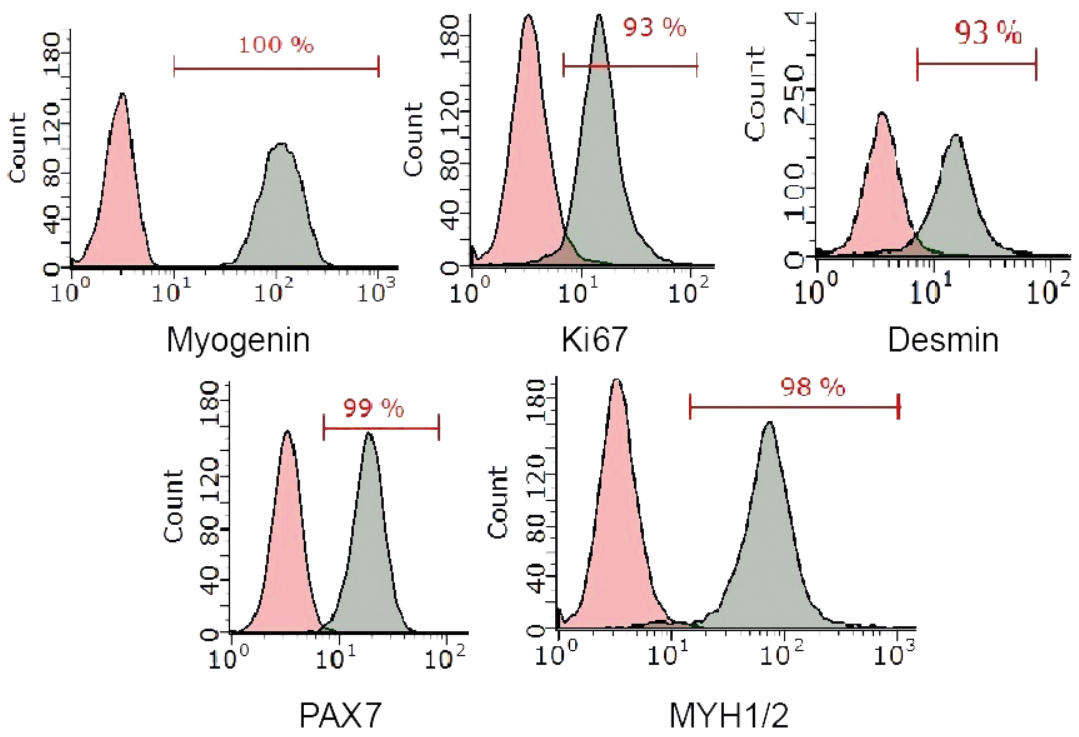

Fig. 2. Flow-cytometric analysis expression of the cell markers myogenin, Ki67, desmin, PAX7, and MYH1/2.

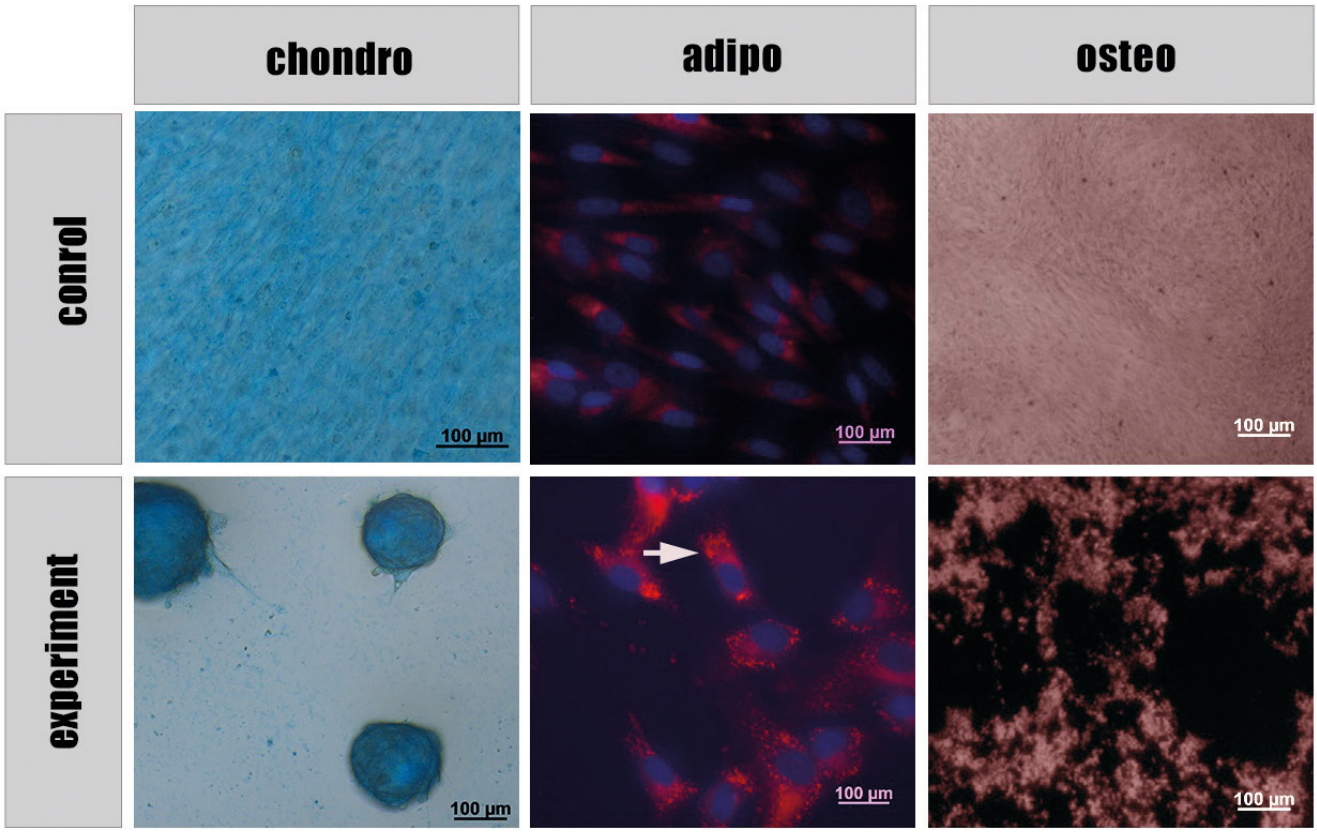

Fig. 3. Myoblasts were cultured under appropriate differentiation media and assessed for differentiation using specific staining methods. Chondrocytic differentiation, the blue color resulted from Alcian blue staining. Adipocytic differentiation, the red color, indicated lipid vacuoles stained by Nile Red (arrow). Von-Kossa staining of osteoclasts differentiated from equine myoblasts.

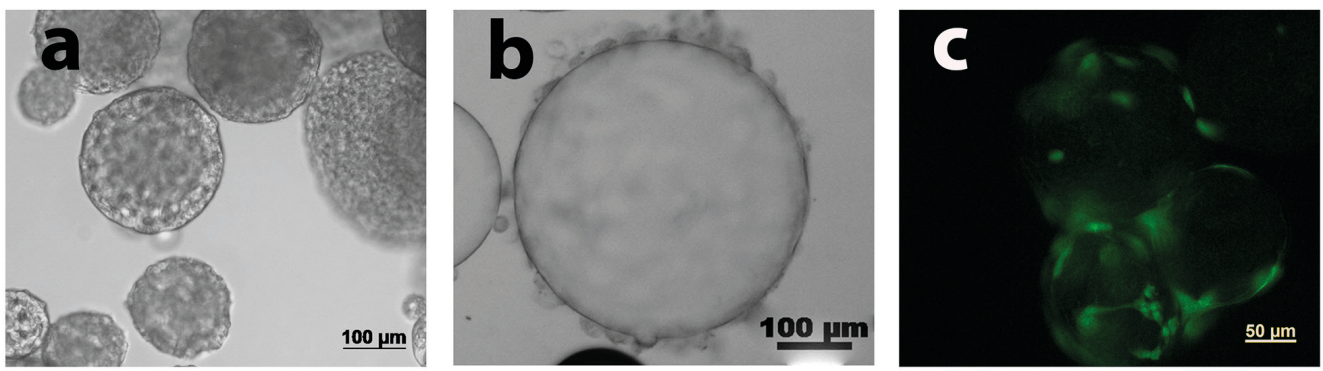

Fig. 4. Equine primary myoblasts grown on Cytodex 3 microcarriers.

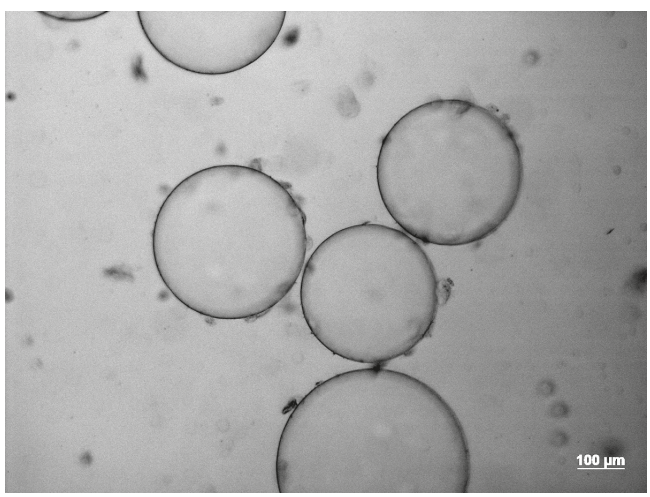

Fig. 5. Equine primary myoblasts grown on CultiSpher-S microcarriers. a. CultiSpher-S microcarriers after sterilization without any myoblasts; b. Equine myoblast adhesion on CultiSpher-S microcarriers after 2 h of co-cultivation; c. Ki67 cell staining demonstrated myoblast proliferative activity after 14 d of cultivation.

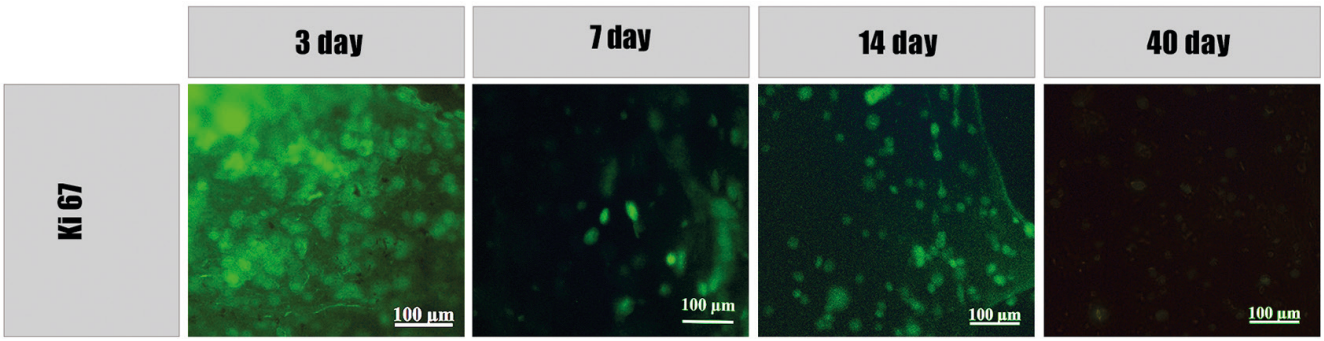

Fig. 6. Proliferative activity of horse myoblasts following 3D bioprinting of GelMA. Cells stained with antibody Ki67 (green).

activity, were observed when compared to culturing in a monolayer, even with prolonged cultivation (Fig. 5). The growth speed of the cell population at 18 days on CultiSpher-S was 13 ± 2 whereas after a 3–4 d cultivation of cells in a monolayer it was 20 ± 5.

Using a bioprinter, a square shape was printed with a height of 5 mm and an area of 1 cm^2. The samples obtained retained their three-dimensional shape well and were attached to the plastic substrate. After 7–14 d, the cultured samples had detached from the plastic and floated in the growth medium. Immunostaining using Ki67 showed that myoblasts did not undergo mitotic activity by the 40 d of cultivation (Fig. 6).

Discussion

We successfully isolated and cultivated cells from horse skeletal muscle. They presented with a myoblast shape, high proliferative activity and viability, and formed myotubes. In total, 93±0.57 % of the cells expressed Ki67, a marker found in cell nuclei during all active phases of the cell cycle: G1, S, G2, and M (Sun and Kaufman, 2018). The cells also expressed the myoblast markers desmin, PAX7, myogenin, and MYH1/2. Desmin is the major subunit of muscle-type intermediate filaments and is expressed by skeletal, cardiac and most types of smooth muscle cells in both embryonic and adult tissues (Shahini et al., 2018). In mature muscle, satellite cells are mitotically inactive and express PAX7, c-met, M-cadherin adhesion molecules (Cdh15), sialomucin CD34, syndecan 3 (Sdc3), syndecan 4 (Sdc4), Foxk1, Sox8 and Sox15. Inactive satellite cells do not express myogenic regulatory factors, including proteins within the MyoD family. Upon activation, satellite cells express specific muscle transcription factors Myf5 and MyoD, followed by myogenin expression and differentiation. PAX7 is expressed in both inactive and proliferating satellites, and is presently the most important marker for identifying myosatellite cells (Shurygin, Bolbat and Shurygina, 2015). The cell population in the present study had a large percentage of cells expressing PAX7, myogenin, and MYH1/2 markers, but these cell markers had different expression levels. The high-level expression of myogenin simultaneously with the lower level of PAX7 in the cells demonstrated they were beginning the differentiation process from myoblasts to adult muscle cells. The marker MYH1/2 had low-level expression in the cells, however it is known it must gradually increase along the muscle differentiation process and reach its peak levels during the later stage of myogenesis (Choi et al., 2020). This is due to the fact that all of the procedures which are used to extract the myosatellites from the sample muscle tissue, inevitably lead to their activation in vitro (Danoviz and Yablonka-Reuveni, 2012). One of the most important markers of activated myosatellitocytes is myogenin, which stimulates myoblast proliferation and subsequent differentiation. It is a late marker of myogenic differentiation that accompanies the exit of myoblasts from the cell cycle and their terminal differentiation. The period of myogenin detection in the nucleus coincides with the moment of terminal differentiation of myosatellites, the synthesis of proteins in the muscle fiber contractile apparatus (MYHs, desmin), and the further formation of muscle tubules and young muscle fibers (Furuichi et al., 2012).

According to the literature, myoblasts can undergo trans-differentiation into other cell types because they are muscle progenitor cells (Qi et al., 2019; Lin, Carnagarin, Dharmarajan and Dass, 2017). Kindler and colleagues concluded that human myoblasts displayed multipotent potential capabilities with differentiation into chondrocytes, adipocytes and osteoblasts in vitro (Kindler et al., 2021). Our studies demonstrated that equine myoblasts can trans-differentiate into osteoblasts, adipocytes and chondrocytes in vitro, similar to human myoblasts. This research demonstrates that the ability of equine myoblasts to differentiate to adipose cells could be used to create meat in vitro which has natural meat qualities.

We chose carriers with a gelatinous or collagen coating on the surface of the beads to cultivate equine myoblasts in a bioreactor. These types of coatings are recommended for in vitro myoblast cultivation because they contain an Arg-Gly-Asp amino acid sequence which promotes cell adhesion and migration (Enrione et al., 2017). According to the literature, myoblasts from

skeletal muscles of newborn rabbits not only actively proliferated when cultured in bioreactors on gelatin carriers but also formed myotubes (Kubis et al., 2016). Synthemax, CellBIND, and Cytodex carriers have also been recommended for growing bovine myoblasts in bioreactors (Verbruggen, Luining, van Essen and Post, 2018). However, the carriers used by us are not suitable for the cultivation of myoblasts from all animals. For example, myoblasts isolated from adult human muscle cultured on Cytodex 3 showed a lower proliferative potential. The main reason for the low proliferative activity of microcarriers, apparently, is the high sensitivity of adult cells to the action of the shear force during stirring in the bioreactor (Rozwadowska et al., 2016). According to our investigation microcarriers Cytodex 3 and CultiSpher-S were unsuitable for cultivating horse myocytes.

It is known that the most useful materials for bioprinting with living cells are water-based materials called hydrogels (Augst, Kong and Mooney, 2006). We used GelMa hydrogel in our experiments and many researchers have used GelMa bioink for bioprinting various cell types (Kirsch et al., 2019; Zhu et al., 2019; Lev et al., 2018; Seyedmahmoud et al., 2019; Liu et al., 2020). These studies demonstrated that GelMa bioink performed well in experiments, including those aimed at the construction of muscle tissue. This material provides significant biological activity due to the presence of a large number of integrin-binding groups which are sensitive to matrix metalloproteinases (Aubin et al., 2010). In addition, GelMa can mimic the environment of native muscle tissue. However, our studies have shown that mitotically active cell counts decreased at all cultivation time points. The cells did not proliferate by day 40 of the experiment. Therefore, we cannot recommend this bioink for bioprinting equine myoblasts. In addition, myoblasts in GelMa hydrogel did not form myotubes, this process apparently requires a physical impact (mechanical or electrical) (Hosseini et al., 2012).

In this study, the optimal conditions were selected to isolate and cultivate equine myoblasts on a monolayer in vitro. The population of primary equine myoblasts obtained from an adult individual, and propagated on microcarriers and bioink, did not meet the requirements for regenerative veterinary medicine and for manufacturing meat in vitro regarding the quantity and quality of the cells required. Nonetheless, further optimization of the cell scaling up process, including both microcarriers and/or the bioreactor program and bioprinting, is still important.

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