

Characterization of Myosatellite Stem Cells from Black Bone Chicken and Effect of Different Animal Serums on Growth Performance

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Abstract

Isolation of muscle stem cells (myosatellite stem cells) of black bone chickens using enzymatic digestion, the pronase, and screening by low-speed centrifugation process combined with 40 µm cell strainer filtration showed that this method was able to separate chicken muscle stem cells efficiently. The results proved that the isolated cells were myosatellite stem cells expressing marker genes, *c-met*, *Pax7*, *MyoD*, and myogenin, by RT-PCR assay. Additionally, immunofluorescence staining with monoclonal antibodies confirmed the isolated cells were MSCs, with positive staining for canonical markers proteins: Pax7, Desmin, and actin. Evaluating the influence of fetal bovine serum (FBS), horse serum (HS), and chicken serum (CKS) supplements on MSC proliferation revealed that FBS emerged as the most effective despite its escalating cost and emerging need for alternatives. Notably, Chicken Serum (CKS) demonstrated potential as an alternative, closely following the cell proliferation rate achieved by FBS. Our findings affirm the efficacy of the devised isolation method and highlight the potential of using chicken serum in cell culture protocols.

Keywords: Stem cell, Isolation, Black bone chicken, Cultured chicken meat, Animal serum

Introduction

Myosatellite stem cells (MSCs), located between the basement membrane and the myofiber sarcolemma within muscle bundles, are pivotal players in muscle development, hypertrophy, and the intricate processes of muscle regeneration [1]. The rising interest in MSCs within the research community can be attributed to their unique properties: They can proliferate *in vitro*, differentiate along multiple lineages, assist hematopoiesis, and produce vital cytokines and growth factors. Their potential in immunomodulation is particularly noteworthy [2,3]. Another notable characteristic of MSCs is their inherent capability to navigate the peripheral circulation and subsequently localize to sites of tissue injury. They multiply and differentiate, catalyzing the healing process by triggering a myriad of mechanisms [4,5]. Furthermore, MSCs have a protective role; they counteract cell damage through a coordinated release of small molecules and extracellular vesicles (EVs). These EVs secreted by MSCs play a vital role in preserving tissue equilibrium. Exploring the multifaceted functions of MSCs can not only refine their role in regenerative medicine but also expand our comprehension of their biological dynamics [6].

While the successful isolation of MSCs from a range of animal species from pigs [7], cattle [8], poultry [9], and fish [10] is well-documented the associated methodologies often present challenges. Conventional techniques, reliant on flow cytometry and magnetic cell sorting underpinned by specific monoclonal antibodies targeting cell surface markers [11,12]. Although these methods are accurate, they can sometimes compromise cell integrity. This can result in decreased survival rates and defects in cell proliferation. Additionally, while enzymatic digestion methods using trypsin and collagenase mixtures have been employed, they often produce heterogeneous cell populations [7]. In our quest for a more uniform MSC yield, we harnessed a synergy of enzymatic digestion, filtration, and strategic low-speed density gradient centrifugation. This approach promises a simple and effective method to isolate MSCs from black-bone chickens, potentially paving the way for future MSC research.

The realm of cell culture further complicates MSC research. Conventionally, cell culture necessitates a milieu rich in nutrients and proliferative factors, predominantly sourced from animal sera like fetal bovine serum [13,14]. However, the potential of alternative serums, particularly the often-discarded chicken serum

from the slaughtering process, remains underexplored in cell culture medium supplementation. While chicken serum is commercially available, its use as a supplement in cell culture mediums is uncommon. Only a few studies on chicken bone marrow-derived DCs (ChBMDC) have indicated that a 5 % concentration of chicken serum supports ChBMDC proliferation [15]. Our study seeks to harness this overlooked resource. We are conducting a comparative analysis of serums sourced from bovines, horses, and chickens to evaluate their efficacy in promoting robust MSC growth.

Materials and methods

Isolation of myosatellite stem cells

Myosatellite stem cells were isolated from the muscles of a 4-month-old adult black bone chicken obtained directly from the slaughterhouse in October 2021. Muscle samples, specifically from the biceps femoris, pectoralis major, and pectoralis minor, were aseptically excised and placed in a sterile Petri dish. Approximately 1 g of the tissue samples was minced finely and transferred to a 50 mL centrifuge tube. After washing thrice with phosphate-buffered saline (PBS, pH 7.4) at 800 g for 5 min, tissues were subjected to enzymatic digestion. A medium comprising of Dulbecco's Modified Eagle Media (DMEM-Glutamax-I; Invitrogen), supplemented with penicillin G (100 U/mL), streptomycin (100 µg/mL), amphotericin B (0.25 µg/mL), and pronase (1.4 mg/mL), was added. This mixture was incubated in a water bath at 37 °C for an hour to break down the tissues into single cells.

The cell suspension was then layered over Ficoll-Paque (HiSep™: Himedia India) and centrifuged at a low speed of 300 g for 5 min. The supernatant was carefully collected and passed through a 40 µm cell strainer to remove any tissue debris. This filtered cell suspension was centrifuged again at 800 g for 5 min to pellet the cells. To wash away any residual media and contaminants, the cells were resuspended in PBS and centrifuged at 800 g for 5 min 3 times.

The resulting cell pellet was resuspended and cultured in a T25 tissue culture flask using DMEM-Glutamax-I (Invitrogen). This media was fortified with 10 % Fetal Bovine Serum (FBS), 0.25 % collagen type IV (Himedia: India), penicillin G (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL). Cultured cells were maintained in a 5 % CO₂ incubator at 37 °C, and the media was refreshed every 3 days to ensure optimal growth conditions.

Immunofluorescent technique for protein marker expression

To elucidate the expression profile of marker proteins in myosatellite stem cells, an immunofluorescence assay was performed targeting Pax-7 (Pax-7-PE), Desmin (DES-FITC), and Actin (1A4/asm-PerCP). Myosatellite stem cells, alongside primary chicken cells, were seeded at a density of approximately 10⁶ cells/well on 8-well chamber slides. These were cultured for 5 days, post which cells were washed twice using phosphate-buffered saline (PBS) to remove any residual media.

Cells were fixed in a 4 % paraformaldehyde solution (pH-neutralized with PBS) for 10 min at ambient conditions. After 2 PBS washes, the cells were treated with 0.5 % Triton X-100 (400 µL per well) for a 5-min incubation at room temperature. Cells underwent a blocking step with 400 µL of 5 % bovine serum albumin (BSA) to minimize non-specific antibody binding. This was maintained for 1 h at room temperature. Post-blocking, cells were washed twice using PBS (pH 7.4).

Cells were incubated with specific monoclonal antibodies at a concentration of 1 µg/mL in PBS (pH 7.4). This staining procedure was carried out for 3 h in a 4 °C environment, protected from light. Subsequent to antibody treatment, samples were washed twice with PBS (pH 7.4). Samples were treated with SlowFade Gold antifade reagent (Thermo Fisher Scientific, USA) enriched with DAPI to visualize nuclei. Following the mounting process, cells were rinsed twice using PBS (pH 7.4). Fluorescently labeled proteins were visualized and documented using an Olympus BX52 fluorescent microscope.

Reverse transcriptase (RT) PCR

To determine the expression levels of the myosatellite marker gene in chicken stem cells, a semi-quantitative RT-PCR technique was employed. Chicken stem cells were propagated in DMEM-Glutamax-I medium supplemented with 10 % FBS until they reached confluence. For RNA extraction, cells at a concentration of 10⁸ cells/mL were trypsinized and processed using the QIAGEN RNA extraction kit (QIAGEN, Hilden, Germany) as per the manufacturer's guidelines. Post-extraction, RNA was eluted in RNase-free water. Concentration and purity were ascertained using a NanoDrop™ Lite spectrophotometer (Thermo Fisher, USA).

For RT-PCR, the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase was utilized. The reaction mixture incorporated 2X reaction buffer, equilibrated RNA template, forward and

reverse primers, and the SuperScript III RT/Platinum Taq enzyme mix. The RT-PCR thermocycling conditions were as follows: Reverse transcription at 60 °C for 30 min, activation of the polymerase at 94 °C for 2 min, and 40 cycles of denaturation at 94 °C for 20 s, annealing at 52 °C for 20 s, and extension at 68 °C for 50 s. The final extension was performed at 68 °C for 5 min. The GAPDH gene was employed as a reference control and was co-amplified in the reaction. Specific primers and expected PCR product sizes are provided in **Table 1** [16].

Chicken serum preparation

To obtain and process chicken serum for cell culture applications. Chicken blood was procured aseptically from slaughterhouses located in Phatthalung Province, Thailand. Blood samples were carefully transferred to sterile 500 mL containers. To promote clotting, blood samples were refrigerated at 4 °C overnight. The next day, clotted blood was centrifuged at 5,000 rpm at 4 °C for a duration of 10 min, facilitating the separation of the serum. The supernatant, representing the serum, was cautiously transferred to sterile 50 mL conical tubes. The obtained serum samples were pooled into a sterile bottle. To inactivate complement proteins, the pooled serum was heated to 56 °C for a span of 30 min. Following the inactivation process, the serum was aliquoted and stored in a deep freezer set at -80 °C for subsequent applications.

Table 1 Specific primers to marker gene of myosatellite stem cells [16].

Gene	Sequence (5'-3')	Product	Tm (°C)	cycle
<i>c-met</i>	F: TCGGATGGTGATTACTGT R: CAAAGGGTGGTTGAAGAT	128	49	30
<i>Pax7</i>	F: GAGGAATACAAGAGGGAGAAC R: ATGCCATCTATGCTGTGCTT	212	53.6	30
<i>MyoD</i>	F: GCTACTACACGGAATCACCA R: GGGCTCCACTGTCACTCA	198	53.3	30
<i>myogenin</i>	F: GCTCTGAAACGCAGCACTCT R: CTTCCAGCATCACCATCCC	436	58.6	30
<i>Runx2</i>	F: GCTGGGAACGACGAGAAC R: AGTGAATGGACGGCGAAG	478	55	30
<i>GAPDH</i>	F: TAAAGGCGAGATGGTGAAAG R: ACGCTCCTGGAAGATAGTGAT	242	53.2	30

MTT assay for cell growth in different serum

The MTT assay, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, is a common method used to assess cell viability or cell proliferation. It is based on the ability of viable cells to reduce the yellow MTT to purple formazan crystals by mitochondrial dehydrogenases. For this investigation, around 10⁶ myosatellite stem cells were allocated into a 48-well plate, followed by a 24-h incubation to facilitate cellular adhesion. Subsequently, these cells were grouped into 5 distinct sets and exposed to DMEM-Glutamax-I (Invitrogen) enriched with various 20 % serum concentrations. Group 1 was supplemented with FBS (Gibco: USA), group 2 with chicken serum (Gibco: USA), group 3 with chicken serum prepared in the laboratory, group 4 with horse serum (Gibco: USA), and the control group was cultured with serum-free DMEM-Glutamax-I. Growth observation occurred at 24-h intervals over a week, with the entire procedure replicated thrice.

After the 24-h incubation in the specified medium, the exhausted medium was replaced with 250 µL of fresh medium in each well. To this, 10 µL of MTT solution (5 mg/mL in PBS) was introduced, followed by a 4-h incubation at 37 °C inside a CO₂ incubator. Subsequently, the medium was discarded, revealing the formed formazan crystals, which were dissolved using 50 µL of DMSO in each well. An additional 30-min incubation at 37 °C in a CO₂ incubator was executed. The resultant formazan solution's absorbance was ascertained using an ELISA plate reader (LUMistar Omega, Germany) set to 570 nm. Control cells of a known count were concurrently evaluated using MTT to produce a reference standard curve, facilitating the interpretation of cell proliferation. Absorbance values from each trial were documented for cell growth determination, utilizing the standard curve as a reference.

Statistical analysis

Statistical analyses were conducted by comparing the mean values across samples treated with different types of serum in cell culture. The t-test was employed to determine the significance of these differences. All analyses were executed using GraphPad Prism version 9.5.1.

Results and discussion

Given the localization of MSCs within the basement membrane and the sarcolemma of muscle bundles, their isolation is inherently challenging due to their proximity to muscle fibroblasts and epithelial cells. Notably, the MSC tissue is microscopic, suggesting that an initial enzymatic digestion of the chicken muscle bundles, followed by cell separation based on size and weight, could yield a pure population of target cells.

Adopting this strategy, we achieved mononucleated cell isolation through a combination of enzymatic digestion, filtration, and low-speed density gradient centrifugation. This method effectively eliminated muscle tissue debris and fragments, resulting in a predominantly viable cell population when cultured in DMEM medium. The yield from this procedure approximated 2×10^5 cells/mL per gram of muscle tissue.

Upon culture in DMEM-Glutamax-I (Invitrogen) medium, supplemented with 10 % Fetal Bovine Serum (FBS), 100 U/mL of penicillin G, 100 mg/mL of streptomycin, and 0.25 mg/mL of amphotericin B, the isolated cells showed robust growth over a 3-day period. Subsequent to this, half the spent medium was refreshed every 3 days. When cellular confluence reached approximately 80 % of the flask's surface, the cells were passage at a 1:2 ratio. Remarkably, after 20 passages, the cells displayed consistent growth, maintained homogeneity, and presented a stable phenotype. Morphologically, the majority resembled epithelial cells with a fusiform shape, and MSC fusion events were discernible (**Figure 1**).

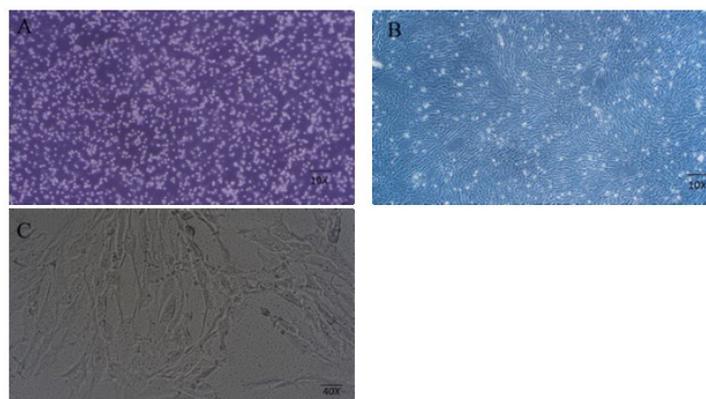


Figure 1 A) Characteristics of the Freshly MSCs isolated on the first day were round and uniform in size, B) Homogeneous confluent cell after 5 passages, and C) MSCs, cells showing fusion characteristics.

The identity of the isolated cells as MSCs was corroborated through immunofluorescence analysis, where the cells positively stained for canonical markers: Pax7, desmin, and actin (**Figure 2**).

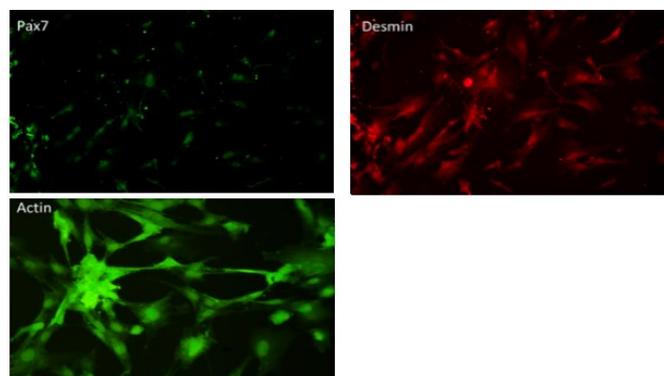


Figure 2 Study on protein marker expression of myosatellite stem cells from isolated chicken venous cells by staining for Pax7, desmin and actin proteins.

Complementary RT-PCR assays further revealed distinct gene expression profiles. Specifically, these isolated MSCs exhibited elevated expression of *c-met*, *Pax7*, *MyoD*, and myogenin genes, distinguishing them from primary chicken cells utilized as controls. Notably, only a trace expression of the *Runx2* gene was detected (Table 2).

Table 2 Expression of marker genes in MSCs by RT-PCR.

Cell types	<i>c-met</i>	<i>Pax7</i>	<i>MyoD</i>	<i>myogenin</i>	<i>Runx2</i>	<i>GAPDH</i>
MSCs	+++	+++	+++	++	+	++++
Chicken primary cell	-	+	+	-	-	++++

To evaluate the impact of different sera supplements on MSC proliferation, an MTT assay was employed across various mediums: DMEM supplemented with Fetal Bovine Serum (FBS), Chicken Serum (CKS), and Horse Serum (HS). The most pronounced proliferation was observed in MSCs cultured in the FBS-supplemented medium, registering an increase from 5×10^5 cells to 1.7×10^7 cells by day 7. This was closely followed by the CKS-supplemented medium, which achieved a count of 1.2×10^7 cells by day 7. In stark contrast, MSCs in both HS-supplemented and serum-free control mediums manifested comparatively subdued growth, culminating in cell counts of 7.47×10^5 and 4.41×10^5 respectively by day 7.

In the examination of cellular growth between days 1 to 5, we discerned a statistically significant enhancement in cell proliferation when the culture media was augmented with FBS, as opposed to other serum supplements. During the evaluation of growth rates from days 6 to 7, the results indicated negligible differentiation in growth outcomes between commercially-sourced CKS or slaughterhouse-derived CKS and FBS ($p = 0.1559$). Notably, on day 6, the medium enriched with FBS exhibited superior cell proliferation than the medium complemented with HS ($p = 0.0241$). Importantly, media supplemented with FBS, CKS, or HS consistently outperformed the control group, which was devoid of any sera enhancement ($p = 0.0005$) (Figure 3).

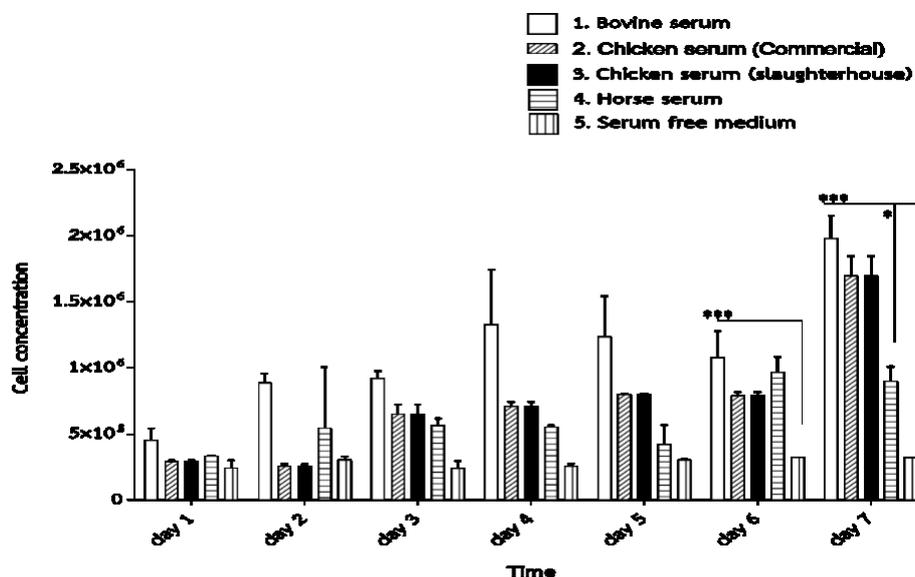


Figure 3 Proliferation of black chicken muscle stem cells in medium supplemented with serum from 3 animal species during 7 days. (** $p = 0.0005$; * $p = 0.0241$).

Our results demonstrate that pronase hydrolysis combined with low-speed centrifugation and a $40 \mu\text{m}$ cell strainer effectively isolates MSCs from chicken backbones. We employed pronase for its non-specific proteolytic activity, encompassing both exo- and endopeptidase properties, affording superior peptide bond degradation compared to trypsin and other proteases [17]. Given the smaller and lighter properties of myosatellite stem cells relative to myoblasts, the employed density gradient separation techniques facilitated efficient MSC isolation. The density gradient centrifugation method is commonly employed to

isolate cell populations based on their size and density. Different cells have different buoyant densities, and when placed in a gradient solution and centrifuged, they will settle at the point in the gradient where their density matches that of the surrounding solution [18]. Previous research has shown that using percoll improved the purity of satellite cells from young rats to nearly 90 %, a rate comparable to that achieved with the conventional method in middle-aged rat [19].

Characterization of the isolated cells affirmed their identity as myosatellite stem cells based on the expression of hallmark genes and proteins. Pax7, a transcription factor, was a vital marker protein. Pax7 is universally identified in myosatellite stem cells across animal species [20]. Meanwhile, desmin protein and the *MyoD* gene are hallmarks of proliferating satellite stem cells, with desmin expression signifying the fusion of myosatellites into muscle bundles [21].

FBS, despite its high cost, remains a staple in cell culture due to its abundant growth-stimulating factors and scarcity of growth inhibitors [22]. The surge in FBS prices by 300 % in recent years underscores the urgency for alternative serum sources [23]. While HS might seem a viable option since it is sourced from domestic horses without culling, its efficacy in supporting chicken MSC proliferation was inferior in our study. Despite HS's protein-rich profile, it lacks essential trace elements for cell growth. Yet, HS possesses growth factors favorable for certain cell types, like hematopoietic cells [24].

Interestingly, CKS showed promising results in myosatellite stem cell proliferation, closely matching FBS by days 6 to 7. While FBS and other animal sera are commonly preferred in cell culture, there is an abundance of commercial chicken serum on the market. However, its application in cell culture is not widespread. Some research indicates that CKS can act as a beneficial supplement for cell growth, especially when using RPMI1640 medium enriched with 5 % CKS for culturing chicken bone marrow-derived DCs (chBMDCs). However, a caveat emerged; chBMDCs cultured with CKS displayed a reduced MHC-II molecule expression compared to those grown in FBS [15]. To provide clarity on this matter, CKS, sharing similarities in mineral salts and proteins with FBS, might stimulate cell proliferation due to its species-specific origin [25]. However, FBS, derived from fetal calves, offers a unique set of growth factors expressed during gestation, which potentially accounts for its superior cell proliferation and differentiation properties [26].

Conclusions

Results from the study on isolating myosatellite stem cells from back bone chickens revealed that cell separation via pronase digestion and selection through low-speed centrifugation, combined with a 40 µm cell strainer, was effective. Consequently, this approach allows for precise cell isolation, resulting in a predominantly single-cell type. Identification of myosatellite stem cell types is further corroborated by the expression of marker genes and proteins. A critical indicator is Pax7, a transcription factor found in the nucleus. In alignment with our findings, *Pax7* gene expression was predominant in myosatellite stem cells, with the protein localized in the nuclear region. This study also evaluated the impact of different serums on cell proliferation, with an eye towards potential future applications. Given the high cost of bovine and horse serums, alternatives are being explored. While fetal bovine serum remains a staple in cell cultures due to its efficacy, its steep price calls for alternatives. Our results demonstrated that chicken serum yielded comparable cell culture outcomes. This suggests that future lab-based chicken meat production might leverage discarded chicken serum, eliminating the need for disease-related culling. This could elevate the value of chicken serum in the market.

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