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Growth and Development of Black-Boned Chicken Embryonic Stem Cells for Culture Meat using Different Serums as Medium

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ABSTRACT

This research was conducted to investigate the growth performance of black-boned chicken embryonic stem (ES) cells for the future development of cultured meat. Black-boned chicken ES cells were isolated and cultured from fertilized eggs. The treatments applied were: fetal bovine serum (FBS) (T1), commercial chicken serum (SCK) (T2), Pradu Hang Dam chicken serum (PDC) (T3), and black-boned chicken serum (BBC) (T4). Black-boned chicken ES cells were cultured at 37.0 °C in a humidified environment of 5% CO_2, for 10 days. The growth of black-boned chicken ES cells concentration was measured by the absorbance at 450 nm. A haemocytometer was used to count the number of black-boned chicken ES cells. Comparing the protein content of cultured meat and chicken meat was collected for combustion and proximate analysis. All collected data were analyzed using ANOVA in a completely randomized design. T4 tended to have a higher number and growth rate than the other groups, followed by T3, T2, and T1, respectively. When counted ES cells final, T4 had a significantly higher number and growth rate than the other groups ($p<0.001$). Comparing the protein content, it was found that cultured meat had significantly more protein than the other groups ($p<0.001$), and characteristics (alignment and cross-section of muscle fibers) were not different from chicken cells. Finally, the cultivation of black-boned chicken ES cells necessitates the use of a medium containing black-boned chicken serum in the growth and development of black-boned Chicken ES cells for culture meat.

Keywords: black-boned chicken; cultured meat; development; embryonic stem cell; growth

INTRODUCTION

Black-boned chicken is a native chicken popularly raised and consumed by indigenous peoples. It has an important role in farms and small farmers, and local people. Black-boned chicken is a healthy source of protein; it reduces fatigue and anxiety, stimulates metabolism, controls blood sugar and blood pressure, and helps to strengthen the immune system (Li *et al*., 2012). Currently, the shortage of high-protein food sources, especially chicken, ranks first among land meats (swine and beef providing protein 2nd and 3rd, respectively) and is low in fat. Chicken is one of the fast-yielding economic animals because it takes less time to raise than other economic animals. It also has a high feed conversion ratio (Alexander *et al*., 2017) and good disease resistance, resulting in higher chicken production and consumption than other meat types. The average global chicken consumption rate is 14.80 kg/person/year, followed by pork and beef at 11.10 and 6.40 kg/person/year, respectively. Most chicken is consumed in the form of chilled, frozen, and processed or frozen cooked/seasoned chicken, respectively. Each product has a different production process. Moreover, the global demand for broiler consumption will likely continue to increase (Chaiwat, 2020).

The impact of the poultry farming and raising industry on the community includes air pollution and noise from animal farms, and becoming increasingly severe (Habeeb & El-Tarabany, 2018). Global warming is the result of these impacts, and certain groups of individuals have begun to agitate against eating meat to prevent global warming and the promotion of animal slaughter. However, most people are unable to resist meat-based foods. But there is a drive to cut greenhouse gas emissions while meeting consumer demand for meat. Green meat, created in a laboratory food, has been developed by scientists (Ching *et al*., 2022). Using biotechnology and culinary understanding, laboratory meat has been marketed internationally. This meat is

not the product of processing plant proteins. Rather, it is meat grown from stem cells, specialized cells that can proliferate or morph into any cell. They are then cultivated in labs using a proper culture medium to generate meat for cooking, known as cultured meat. The benefits of synthetic meat include the ability to minimize greenhouse gas emissions while also reducing the usage of soil and water resources. It will also limit future human exposure to antibiotics, stimulants, and certain diseases from meat if the cost of making synthetic meat is reduced further (Mateti *et al*., 2022). Synthetic meat will aid in alleviating food scarcity, especially meat that will soon be difficult to avoid.

Cultured meat production based on black-boned chicken made in the lab could solve the problem of a lack of meat and keep meat cleaner and free of germs better than that from the market. Cultured meat's nutritional content could also be improved as needed, such as by adding collagen, fat, and omega 3 to provide another choice for healthy people and older people (Moritz, 2017; Mateti *et al*., 2022). It would also help to address food shortages and reduce pollution, space, and resources associated with meat production. So, this study aimed to investigate the growth and development of black-boned chicken embryonic stem cells for culture meat using different serums as a medium.

MATERIALS AND METHODS

A total of 24 fertilized eggs from black-boned chickens (43–45 g weight) were obtained from the poultry farm of the Faculty of Animal Science and Technology, Maejo University. The eggs were cleaned with alcohol and incubated at 38.0 °C at 60% relative humidity (18 h). The treatments applied were fetal bovine serum (FBS) (T1), commercial chicken serum (SCK) (T2), Pradu Hang Dam chicken serum (PDC) (T3), and black-boned chicken serum (BBC) (T4). (Institutional Animal Care and Use Committee (IACUC) approval number: MACUC 043A/2564).

Isolation and Cultivation of Black-Boned Chicken Embryonic Stem (ES) Cells

Black-boned chicken ES cell isolation and culture methods were adopted from Farzaneh *et al*. (2017) and Xiong *et al.* (2020). In brief, blastoderm cells at stage X were collected by syringe in tissue culture dishes and rinsed with phosphate-buffered saline (PBS) to remove the yolks by centrifugation at 1,000 rpm for 10 min and suspension. ES cells were maintained in a 5% CO_2 humidified atmosphere at 37.0 °C with Iscov's Modified Dulbecco's Medium (IMEM; Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS) (T1), 10% commercial chicken serum (SCK) (T2), 10% Pradu Hang Dam chicken serum (PDC) (T3), or 10% black-boned chicken serum (BBC) (T4). The cell colonies were digested at 37.0 °C for 2 to 3 min with 0.025% trypsin. The dissociated ES clusters were suspended with a pipette and subcultured in a 5% CO_2 humidified atmosphere at 37.0 °C in culture flasks containing a feeder cell layer and IMEM. The medium was replaced after 3 days with half of the medium (Ichikawa & Horiuchi, 2023).

Measurement of Black-Boned Chicken ES Cell Growth

The cultivated stem cells were dripped with 0.05% trypsin, put in a cell culture flask at 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 h after incubation time, the medium was withdrawn, and 100 μL of fresh medium was added to 10 μL/well of 12 mM MTT (Component A). The flasks were incubated at 37 °C for 4 h before adding 100 μL/well of SDS (Component B). In a humidity chamber, the contents of the microplate were mixed and incubated for 4 h at 37 °C. The sample was pipetted and the absorbance at 450 nm was measured (UVmini-1240, Shimadzu, Europe); a haemocytometer was used to count the number of black-boned chicken ES cells.

Proving the possibility of stem cells by characteristics under microscope and renewal (Genovese *et al*., 2019), presto blue for cell life, and cell growth in accordance with the cell growth or cell cycle (cell division) theory, which corresponds to the research of Schmitz-Elbers *et al*. (2021), cell development requires the production of enzymes and proteins required for DNA synthesis to 1) begin the cell cycle again (G0) or 2) differentiate to become a specialized cell (G1 phase). 3) DNA synthetic phase (S phase) is the phase in which the quantity of DNA is grown by the replication of the whole set of DNA (DNA replication) and other chemicals are produced required for the division, and 4) pre-mitotic phase (G2 phase) is the stage before splitting. This is the time when the cell checks to see whether the DNA has successfully doubled. The three subphases G1, S, and G2 are interphases before division in the M phase, and 5) the mitotic phase (M phase) is the period during which division occurs and the extra chromosomes are evenly split into two new cells. The cell cycle begins in interphase with cell preparation before division, followed by cell proliferation in the M phase (Alberts, 2017: Pollard *et al*., 2017).

Comparing the Protein Content and Muscle Fiber Appearances of Cultured Meat and Chicken Meat

The cell culture flask was removed from the incubator. The outside appearance was observed. A 200× magnification microscope was used to examine the contents in detail. Then, they were centrifuged at 4 °C at 2,000 rpm for 30 min; a 2 mL cryotube was filled halfway with a cell sample and dry at 60 °C in a hot air oven. The samples were collected for protein analysis by nitrogen combustion method (Petracci *et al*., 2014) and nutritional analysis of chicken meat by proximate analysis (AOAC, 2000) in which a piece of meat is digested with a strong acid, releasing nitrogen that is measured by a titration procedure; the nitrogen content in the meat is then used to compute the amount of protein present. The same fundamental method is being used today, albeit with a few enhancements to speed up the procedure and provide more precise results. It is widely accepted as the standard method of detecting protein concentra-

tion (Weng *et al*., 2022). After completely extracting the water from the sample with ethyl alcohol at varying percentages, beginning with a low concentration of the chemical and progressing to a high concentration, the sample was dried using the critical point drying procedure, which allows liquid carbon dioxide to replace the organic matter used to remove water from the sample until it is completely replaced. The sample was then heated to bring the temperature and pressure within the specimen chamber to the critical points for carbon dioxide (31.1 °C and 1.073 psi), resulting in a dry and stable sample. The sample was separated into two sections: one for sectioning (a clinical pathologist approximated the percentage of the tissue structure compositions according to Gibson-Corley *et al*., 2013, as the H&E-stained slides were examined under a light microscope, 10x objective lens magnification, 18 mm ocular lens, observation for 20 fields), and another for attaching the sample to the sample stand with double-sided carbon tape. The sample was then gold-coated and investigated using scanning electron microscopy (SEM) to characterize culture meat muscle fibre compared to a variety of real chicken meat fibre under a scanning microscope (Schaller & Powrie, 1971).

Statistical Analysis

All collected data were subjected to statistical analysis using one-way analysis of variance, and mean differences among treatments were evaluated by Duncan's multiple range and post-hoc tests using the statistical software IBM SPSS Statistics Version 25 (SPSS Inc., Chicago, IL, USA). The significance was considered at a 5% probability.

RESULTS

In the absorbance of black-boned chicken ES cells, T4 grew faster than the other groups after culturing stem cells for 2 h (OD: 0.054) ($p<0.001$). After that, T3 grew faster than any other group between 4, 8, and 12 h (OD: 0.048, 0.052, and 0.046, respectively), and T4 grew more than any other group in 24 h (OD: 0.100). T2 grew more than the other groups between 48 to 192 h (OD: 0.129, 0.163, 0.326, 0.122, 0.353, 1.027, and 1.159, respectively), while T4 had greater growth between 216 and 240 h (OD: 0.179 and 0.179) ($p<0.001$) as shown in Table 1. This corresponds to the ES cell count. At the end of the research, T4 tended to have a higher number and growth rate than the other groups, followed by T3, T2, and T1, respectively (6.23, 5.6, 3.82, 0.4 x 10^7 /mL), as shown in Figure 1.

T4 had significantly more protein than the other groups ($p<0.001$), followed by T2, T3, and T1, respectively (combustion: 99.25%, 91.88%, 84.16%, and 72.81%; CP: 97.71%, 87.08%, 83.13%, and 61.39%), while T1 had significantly more ether extract (EE) and gross energy (GE) than meat from the other groups ($p<0.001$), followed by T3, T2, and T4 (EE: 37.15%, 17.91%, 11.38%, and 0.43%; GE: 685.52, 618.37, 603.25, and 491.44 kcal/100 g). The cultured meat tended to have a similar protein content to chicken meat and is therefore comparable to real chicken meat, as shown in Table 2.

When studying the appearance of muscle fibre under a scanning microscope (A: 2,000×; B: 10,000×) and compound microscope (C: 200×), we observed that the characteristics of the cultured meat, alignment, and cross-section of muscle fibers were not different from chicken meat cells, as shown in Figure 2. The muscle cells of commercial chicken meat were arranged in bundles separated with loose perimysium. The muscle cells were large and spindle-shaped, with peripherally placed nuclei and dense muscular fibre. Other supportive tissues, such as adipose tissue and vascular structures, were demonstrated and comprised 10%–20% of the submitted samples (Figure 2C1). Pradu Hang Dam chicken meat muscle cells were arranged in small bundles separated by thin perimysium. The muscle cells were large and spindle-shaped, with peripherally placed nuclei and dense muscular fibre. Little adipose tissue (~5%) was observed within the tissues (Figure 2C2). The muscle cells of black-boned chicken meat were arranged in large bundles and fascicles separated with thin perimysium. The muscle cells were large and spindle-shaped, with peripherally placed nuclei and dense muscular fibre. Multifocal pigmentations were noted in the connective tissue and collagenous fibres. The surrounding adipose tissue, approximately 10%–15%, was included within the muscular structures (Figure 2C3). Cultured meat cells were arranged in bundles, fascicles,

Table 1. Growth of black-boned chicken embryonic stem cells on different mediums and incubation times measured at absorbance of OD 450 nm

Treatments	Incubation times (h)													
	2	4	8	12	24	48	72	96	120	144	168	192	216	240
T1	0.019^{d}	0.017^{d}	0.020^{d}	0.019^{d}	0.016^{d}	0.027^{d}	0.007^{d}	0.011^{d}	0.010^{c}	0.033^{c}	0.078^{b}	0.055^{c}	0.017^{d}	0.017^{d}
T2	0.039^{b}	0.030^{c}	0.021^{c}	0.034^{c}	0.052^{b}	0.129^{a}	0.163^{a}	0.326^{a}	0.122^{a}	0.353^{a}	1.027^{a}	1.159^{a}	0.030^{c}	0.030^{c}
T3	0.037^{c}	0.048^{a}	0.052^{a}	0.046^{a}	0.043^{c}	0.045^{c}	0.025^{c}	0.012^{c}	0.052^{b}	0.029^{d}	0.047^{c}	0.035^{d}	0.098^{b}	0.098^{b}
T4	0.054^{a}	0.038^{b}	0.044^{b}	0.035^{b}	0.100^{a}	0.104^{b}	0.059^{b}	0.059^{b}	0.001^{d}	0.074^{b}	0.045^{c}	0.058^{b}	0.179^{a}	0.179^{a}
SEM	0.004	0.003	0.004	0.003	0.009	0.012	0.018	0.039	0.014	0.04	0.126	0.144	0.019	0.019
p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Note: Chicken thigh meat was used for analysis.
T1= fetal bovine serum; T2= commercial chicken serum; T3= Pradu Hang Dam chicken serum; T4= black-boned chicken serum; SEM= standard error of the mean.
[a–d] Means in the same in column with different superscripts differ significantly ($p<0.001$).

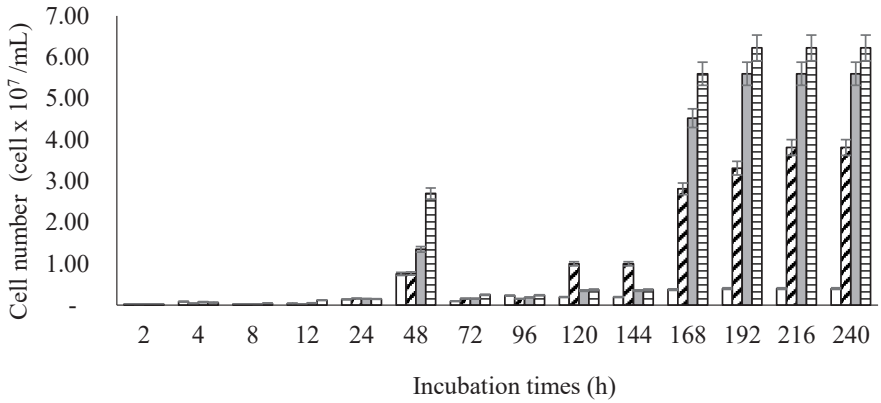

Figure 1. Number of black-boned chicken ES cells at different incubation times.
T1 (□)= fetal bovine serum; T2 (▨)= commercial chicken serum; T3 (■)= pradu hang dam chicken serum; T4 (▤)= black-boned chicken serum.

Table 2. Chemical composition of culture meat compared to a variety of real chicken meat (% of DM basis)

Variables	AD	DM	DM basis			
			Combustion	CP	EE	GE (kcal/100 g)
Commercial chicken meat	31.98[a]	90.84	72.81[d]	61.39[d]	37.15[a]	685.52[a]
Pradu Hang Dam chicken meat	26.08[c]	90.66	91.88[b]	87.08[b]	11.38[c]	603.25[c]
Black-boned chicken meat	27.34[b]	91.77	84.16[c]	83.13[c]	17.91[b]	618.37[b]
Culture meat	12.17[d]	89.94	99.25[a]	97.71[a]	0.43[d]	491.44[d]
SEM	1.910	0.317	2.953	3.986	4.026	21.056
p-value	<0.001	0.252	<0.001	<0.001	<0.001	<0.001

Note: Chicken thigh meat was used for analysis.
DM= dry matter basis; AD= air dry basis; CP= crude protein; EE= ether extract; GE= gross energy; SEM= standard error of the mean.
[a–d] Means in the same in column with different superscripts differ significantly (p<0.001).

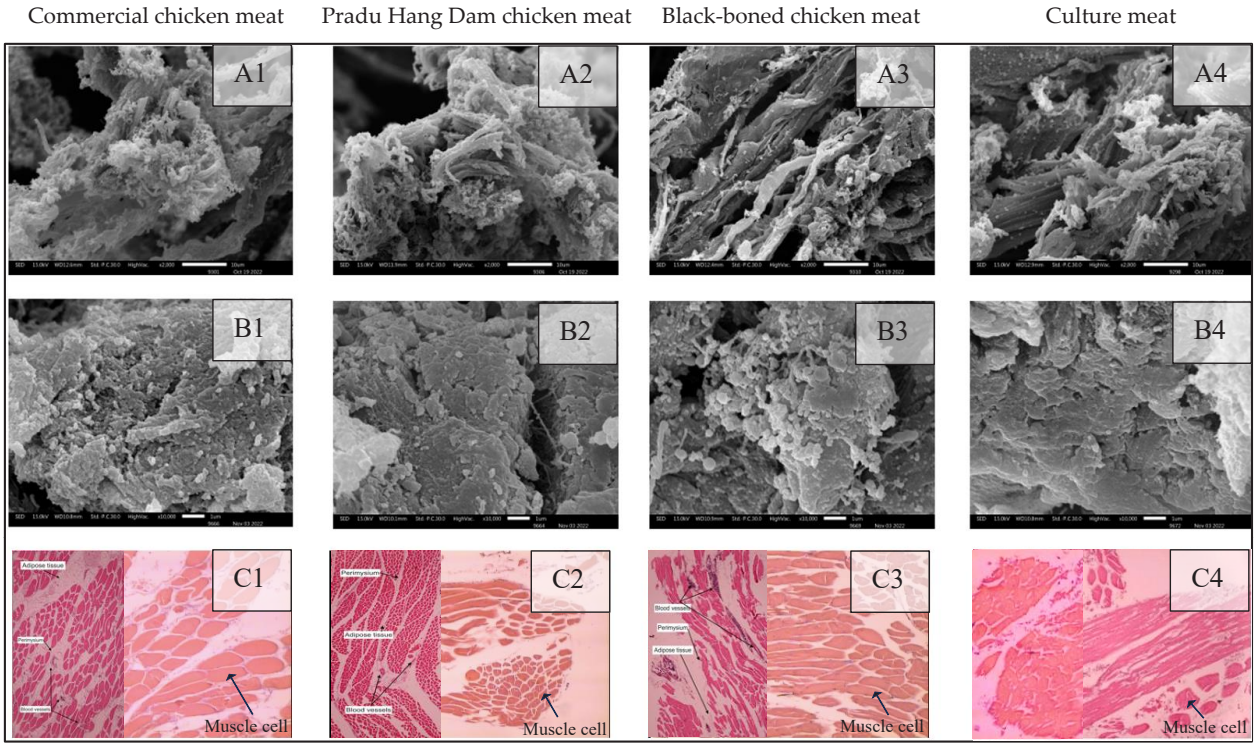

Figure 2. Characterization of culture meat muscle fibre compared to a variety of real chicken meat fibre under scanning microscope (A: 2,000×; B: 10,000×) and compound microscope (C: 200×). Source: lab tests at The Veterinary Diagnostic Centre (VDC), Chiang Mai University Animal Hospital, Chiang Mai University.

reticulated, and occasionally separated individual cells. The muscle cells were spindle-shaped, with peripherally placed nuclei and dense muscular fibre. Variably sized myocytes were noted (Figure 2C4).

DISCUSSION

The absorbance of black-boned chicken ES cells of T4 showed greater growth than the other groups. This is in accordance with the cell growth or cell cycle (cell division) theory, which corresponds to the research of Schmitz-Elbers *et al.* (2021), who used the EC culture method and fluorescence imaging to visualize gastrulation movements as they occurred, electroporated stage X chick embryos with a green fluorescent protein (GFP) reporter gene, and followed the behaviour of electroporated cells at stage 3 (Firmino *et al*., 2016). As gastrulation movements occur, most cells rapidly separate from each other in places distant from the primitive streak. This finding contrasts sharply with previous findings in other epithelia (e.g., *Xenopus* embryos, *C. elegans*, *Drosophila*, and zebrafish), where cells almost always remain in contact (Campinho *et al*., 2013; Muhr & Hagey, 2021). G1 and G2 are two phases in the interphase of the cell cycle. The duration of the cell cycle varies according to the type of organism. G1 is the first substage of interphase and G2 is the final substage of interphase. Significant development processes occur within the cell during G1 (Pollard *et al*., 2017). Compared with G1, G2 is a shorter phase. Proteins synthesized during the G1 phase include mainly histone proteins, and most RNA synthesized is mRNA. Entry of a cell into the G2 phase confirms that the cell has completed the S phase, where DNA replication has taken place. Cell cycle regulatory mechanisms will control both phases (Alberts, 2017). The cell cycle begins in interphase with cell preparation before division, followed by cell proliferation in the M phase. As a result, cell development requires the production of enzymes and proteins required for DNA synthesis to 1) begin the cell cycle again (G0) or 2) differentiate to become a specialized cell (G1 phase). G1 cyclin-dependent kinase–cyclin complexes control and stimulate cells into the following S phase via CdkC (G1CdkC). The S phase (DNA synthesis) is the sole phase in which CdkC exclusively regulates DNA proliferation. In this stage, each chromosome is replicated. The G2 phase is responsible for producing extra proteins, and RNA required for continued cell division. The G1, S, and G2 phases are referred to as interphase (Alberts *et al.*, 2015; Muhr & Hagey, 2021). Counted ES cells at the end of the process, T4 had a significantly higher number and growth rate than the other groups.

Most chicken ES cells are isolated from stage X blastoderm cells in preparation for ES cell cultivation with various feeder cells. Growth factors and cytokines are also present (Aubel & Pain, 2013; Llames *et al*., 2015). Similarly, mouse embryonic stem cells (mESC) may maintain ES cells. Current ES cell isolation methods isolate pluripotent embryonic cells from undifferentiated pluripotent embryonic stages using leukemia inhibitory factor (LIF) as a feeder cell (Azizi *et al*., 2019). *In vitro*, both primordial germ cells (PGCs) and spermatogonial stem cells (SSCs) may develop into neuroblast-like adipocytes and osteoblasts and display comparable gene markers (Kang *et al*., 2015; Ichikawa & Horiuchi, 2023). Consequently, using black-boned chicken serum as a feeder cell in a cell culture medium is a feasible alternative for clearly observing growth results. Thus, chicken ES cells are derived from embryos and cultivated in the laboratory. These are collected from diverse sources at various stages of embryonic development; a test for pluripotent embryonic cells is the ability to form an embryo and split into a single cell, making them equivalent to mammalian stem cells. This serves as a model for research into stem cell biology, etc. (Zakrzewski *et al*., 2019). Long-term continuous culture of stem cells in co-culture with peer cells is effective in cell growth and preservation. Several types of steer cells have been used in poultry stem cell cultures, including Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) and chicken embryonic fibroblasts (CEF) (Whyte *et al*., 2015; Farzaneh *et al*., 2018; Van der Weele *et al*., 2019; Wang *et al*., 2020). However, utilizing different animal cells as feeder cells increases the chance of progenitor cells becoming contaminated with secretions from the different feeder cells, and the activation of those molecules may change the characteristics of stem cells. The use of the appropriate feeder cell type decreases the likelihood of stem cell contamination (Naito *et al*., 2015; Zhang *et al*., 2018). The chicken ES cell culture medium was mixed with black-boned chicken serum in this study. Consequently, the embryos grew faster than with other serums.

The chemical composition of the meat analyzed was not different from chicken meat, in accordance with Infante-Rodríguez *et al*. (2016) research. They studied the effect of diets with different energy concentrations on growth performance, carcass characteristics, and meat chemical composition of broiler chickens in dry tropics; for comparison, the chemical composition of thigh muscles (wet basis) computed on a dry matter basis was given. Similar to the chemical composition, the CP and EE values were comparable to this research. However, since the meat culture was cultivated in a lab, the analyzed product had a high CP and a low EE, which was also observed in the characterization of muscle fiber under a scanning microscope.

The appearance of muscle fibers under a microscope confirmed the experimental results that the characteristics of cultured meat are not different from those of other chicken cells. According to the science of plant-based foods and research constructing next-generation meat, fish, milk, and egg analogs, researchers use soft matter physics approaches to create meat-like structures (McClements & Grossmann, 2021). Culture meat cells were arranged in bundles, fascicles, reticulated, and occasionally separated individual cells. The muscle cells were spindle-shaped, periphery placed nuclei, a dense muscular fiber. Variably size of the myocytes was noted. Similar to Zhu *et al*. (2021), the characterization of muscle development and gene expression in early embryos of chicken, quail, and their hybrids was studied. The characterization of muscle was similar to this study.

Therefore, ES cells can be used to make therapeutic

proteins, monoclonal antibodies, vaccines, and transgenic chickens. They can also be used for basic research, preserving breeds, and making cloned animals. ES cells, the most primitive in domestic poultry, can differentiate into a wide range of cells and may be forced to replicate the crucial phases of normal early embryonic development, making them suitable experimental models for investigating avian developmental biology (Lin *et al.*, 2015).

CONCLUSION

The fact that cultured meat can be developed from black-boned chicken ES cells partly based on their growth performance emphasizes that cultivating black-boned chicken ES cells for cultured meat necessitates using a medium containing black-boned chicken. Furthermore, based on protein analysis by the combustion method, cultured meat is comparable to chicken meat, and cultured meat's characteristics are not different from those of chicken cells.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the manuscript's material.

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