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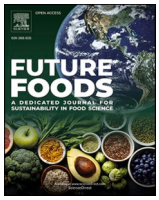

Development of fetal bovine serum substitute derived from egg for muscle satellite cell culture: A preliminary study

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ABSTRACT

This study investigated the effect of extracts from unfertilized eggs and fertilized eggs with different culture periods as FBS substitutes for cell culture. A mixture of 5 % FBS and 20 % egg white extract or 10 % FBS and 10 % egg white extract was found to be the optimal condition for increasing cell proliferation. In particular, egg white extract obtained from unfertilized eggs was more effective in terms of increased cell viability and price competitiveness and showed similar effects to commercial FBS. In addition, when using this extract, successful proliferation of satellite cells was confirmed, as well as expression of Pax7, a protein present in muscle satellite cells. Additionally, OVF and OVA in the extract are believed to have an indirect effect on FBS replacements for chick muscle satellite cells towards cultured meat development. Egg white extract can replace up to 50–75 % of FBS. Further developments of the extracts could result in complete FBS substitution.

1. Introduction

Cultured meat technology is a form of tissue culture technology that extracts muscle satellite cells from animal tissues, artificially cultivates muscle satellite cells into muscle cells, and differentiates them into muscle. The culture media required for cell culture methods is a mixture of basal media and serum, which is added to the media for cell growth. Common basal media include Minimal Essential Medium (MEM), Ham's F-10, Dulbecco's Modified Eagle Medium (DMEM), and RPMI 1640. These basal contain mainly nutrients, such as amino acids, vitamins, lipids, sugars, and minerals, while fetal bovine serum (FBS) is the most commonly used serum additive for cell growth (van der Valk, 2022). FBS is an animal material with components that are necessary for cell attachment, proliferation, and maintenance, such as albumin, globulin, transferrin, and cholesterol (Chelladurai et al., 2021). Since FBS is a blood serum extracted from the calves of slaughtered pregnant cows, its production is limited. Moreover, its process of production causes problems related to animal welfare since it is controversial to slaughter pregnant livestock. In addition, FBS can contain significant differences in components due to geographical and seasonal differences (Versteegen et al., 2019), meaning that consistency is not maintained between components, and these differences affect the growth promotion of certain cell types of FBS, depending on the source of production (Fang et al., 2017). However, FBS is still widely used in most studies because it can provide excellent efficacy in cell adhesion and growth, and there are currently no effective substances to replace it. In particular, FBS is the biggest obstacle to the industrialization of cultured meat because the cost is very high.

Thus, over the past few years, efforts have been made by evaluating substances that can replace FBS for the industrialization of cultured meats, using various materials, such as edible insects, algae, food ingredients, and by- products (Andreassen et al., 2020; Dong et al., 2023; Kim et al., 2023; Okamoto et al., 2020). Fertilized eggs are materials that contain large amounts of nutrients and growth factors required to produce embryos. Moreover, various substances necessary for cell and muscle growth are expressed while the embryos inside the eggs grow into chicks (Jebessa et al., 2018). Eggs are relatively accessible and inexpensive compared to conventional culture additives. Egg extracts can be used as an inexpensive FBS substitute in the production of cultured meat in addition to being a suitable media additive in cultivating various cell lines, even though eggs are an animal resource. Therefore, the purpose of this study is to verify the efficacy of an FBS

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substitute derived from unfertilized and fertilized eggs, with varying incubation periods, on chick muscle satellite cells.

2. Materials and methods

2.1. Materials

Unfertilized eggs were purchased at the local market (Anseong-si, Korea). Fertilized eggs were purchased from Cheongsolwon (Hadong, Korea) and were grown in an egg incubator (ZT020, Zhejiang, China). The bottle top filter was purchased from Nalgene (Rochester, NY, USA). Pierce BCA protein assay kit and protein ladder were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ham's F-10 was purchased from Welgene (Gyeongsan, Korea). DMEM, and 10X Dulbecco's phosphate-buffered saline (DPBS) were purchased from HyClone (Cytiva, Marlborough, MA, USA). FBS was purchased from Corning (New York, NY, USA). Horse serum (HS), 2.5 % trypsin, and penicillin/streptomycin (P/S) were purchased from Gibco (Grand Island, NY, USA). Albumin from chicken egg white, conalbumin from chicken egg white, collagen, and collagenase D were purchased from Sigma-Aldrich (St Louis, MO, USA). Dispase II was purchased from Roche (Basel, Switzerland).

2.2. Preparation of FBS substitutes using egg white extracts from fertilized and unfertilized eggs

Egg white extract was obtained from unfertilized and fertilized eggs and used according to the number of incubated days (0, 5, and 10) (Fig. 1). The 5 mL of egg white was mixed with distilled water (DW) at a ratio of 1:5 (vol/vol). The mixture was homogenized with an electric homogenizer for 5 min and sonicated for 5 min (5-second intervals), following a modified method from a previous study (Sasse et al., 2000). The sample was frozen at −20 °C for 72 h until required. The samples were slowly thawed at an ambient temperature and centrifuged (1977 *g*) for 10 min at −4 °C. The supernatant was filtered using a series of decreasing pore sizes of Whatman paper (11 μm, 6 μm, and 2.5 μm), followed by bottle top filtration with a membrane filter (0.1 μm). Afterward, the samples were stored at −20 °C until further analysis.

2.3. Isolation of chick muscle satellite cells

Fig. 2 shows the process of obtaining chick muscle satellite cells. Fertilized eggs were obtained from the egg incubator (37 °C and 60 % relative humidity). The embryonated eggs were harvested before 14 embryonic days and were cracked carefully in the first petri dish containing cold 1X DPBS + 2 % P/S, and the head of the chick embryo was immediately severed using a scalpel. The body was transferred to a second petri dish, also containing cold 1X DPBS + 2 % P/S. The excess skin on the legs was removed and the muscles were stripped from the bones using tweezers and a scalpel. These were transferred to a new petri dish containing cold DPBS and minced using the scalpel. The sample was centrifuged at 1977 *g* for 3 min and the supernatant was discarded. Subsequently, the tube containing the sample was weighed, and digestive enzymes, including collagenase D, dispase II, and 100 mM $CaCl_2$ (all pre-warmed at 37 °C), were added to the sample at a ratio of 1:1:0.05, respectively. The sample with the digestive enzymes was placed in the water bath at 37 °C for 30 min and agitated every 10 min. Then, the sample was diluted in cold 1X PBS at four times the volume of the sample. This mixture was strained using 100 μm, 70 μm, and 40 μm cell strainers, and centrifuged at 100 *g* for 5 min. The pellet was diluted and resuspended in 5 mL of Ham's F-10 basal media and transferred to a non-coated cell culture dish, then, incubated for 5 min at 37 °C and 5 % CO_2. The non-coated dish was removed from the incubator very steadily, avoiding the movement of the content in the dish. Only the content on the surface was transferred to collagen-coated dishes, which contained 5 mL of growth media (Ham's F-10 + 20 % FBS + 1 % P/S).

2.4. Chick muscle satellite cell culture

Following isolation, the growth media was used for chick satellite cell culture. The 100 pi cell culture dishes were coated with 0.1 % collagen. Further, the cells were incubated at 37 °C and 5 % CO_2. 0.25 % trypsin was added, and the cells were incubated at 37 °C in a 5 % CO_2 incubator for 3 min. Following this, the content was transferred to the

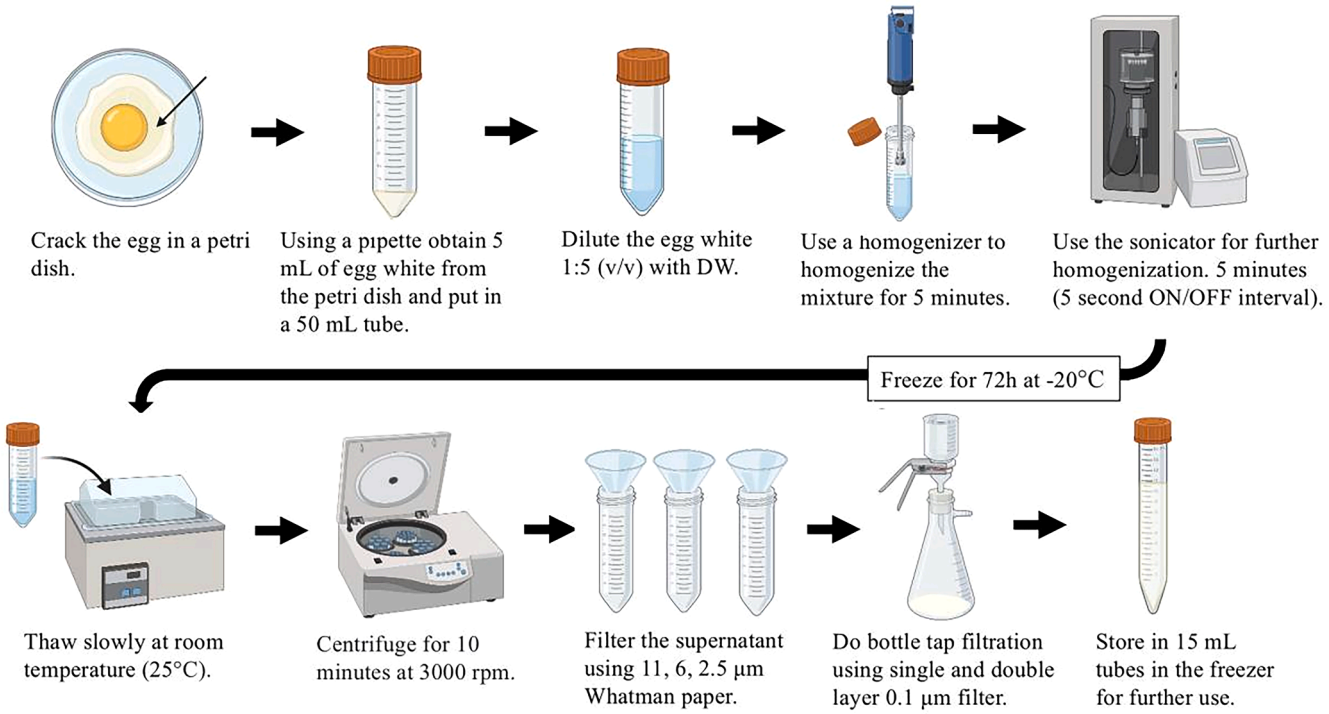

Fig. 1. Preparation of egg white extracts from fertilized and unfertilized eggs.

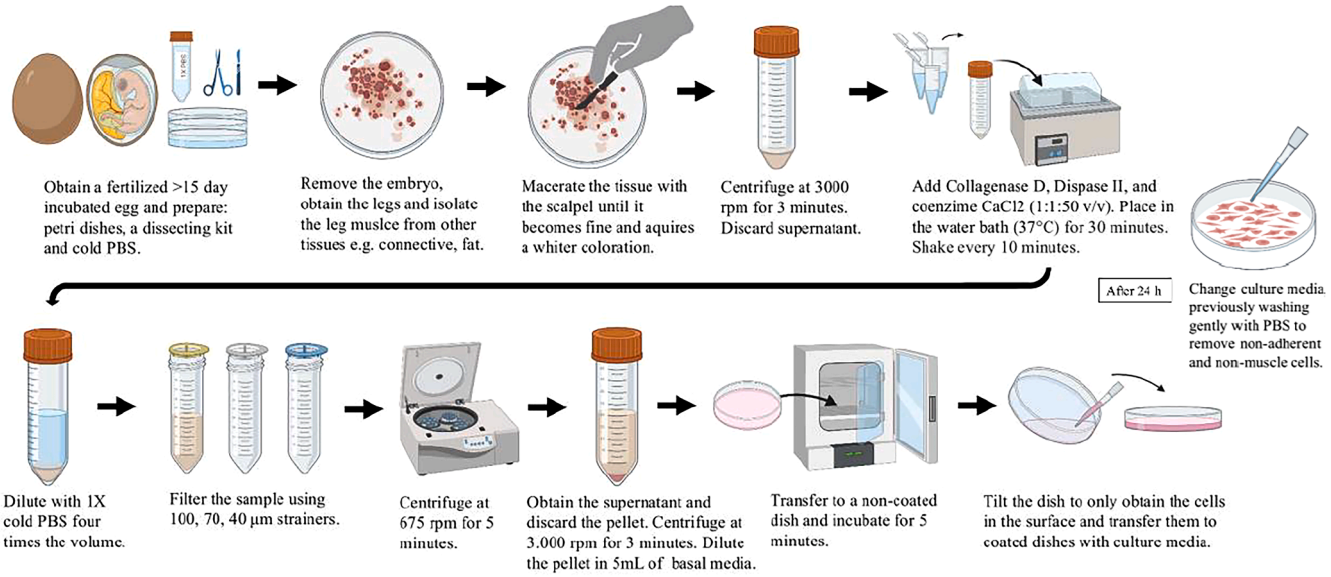

Fig. 2. Preparation of chick satellite cells.

tube, thereby allowing the DPBS to neutralize trypsin. Afterward, the cell solution was centrifuged at 1977 *g* for 3 min to separate the cells. The cells (2.5×10^5 cells/dish) were seeded in 100 pi cell culture dishes containing 5 mL of growth media and incubated at 37 °C in a 5 % CO_2. Cells were passaged 3–4 days after seeding or until the optimal confluency was reached.

Prior to cell seeding for cell proliferation assays, treatments were prepared using Ham's F10 containing varying amounts of FBS (20 %, 10 %, 5 %, 1 % or no FBS) and egg white extracts UFE or 0-,5-, and 10-day-incubated FE). The appropriate number of cells were seeded per treatment then distributed to individual wells according to the assay to be performed.

2.5. Determining cell proliferation in chick muscle satellite cells

2.5.1. Determining cell counts using a counting chamber

Cell counting was performed using cell microscopy after culturing chick muscle satellite cells, as follows: First, the old growth media was removed, and then, the cell was washed with 1X DPBS. The cells were desorbed by treatment with a 0.25 % trypsin solution for 3 min. After that, the desorbed cells were transferred into a 15 mL tube, and centrifuged at 1977 *g* at 4 °C for 3 min. After centrifugation, the supernatant was removed, and the cell pellet was suspended in 1 mL of Ham's F-10. After mixing, 10 µL of the cell suspension was added to 10 µL of trypan blue dye, and 10 µL of the mixture was placed on to the counting chamber, then, cell counting was performed.

2.5.2. Determining cell viability using MTT assay

The MTT solution (10 mg/mL) was prepared by dissolving thiazolyl blue tetrazolium bromide in 1X DPBS. The cells were seeded at 5×10^3 cells/well in 96-well plates and incubated for 24 h at 37 °C in an incubator with 5 % CO_2. After 24 h, the growth media was removed from the plate and the MTT solution was diluted with Ham's F-10 basal media to 0.5 mg/mL. The 96-well plate was covered with aluminum foil and incubated for 4 h at 37 °C in an incubator with 5 % CO_2. After 4 h, the MTT reagent was discarded. Subsequently, 100 µL of dimethyl sulfoxide (DMSO) was added to each sample well and the plate was left to react for 20 min in a dark room. Afterward, the absorbance was measured by a microplate reader at 540 nm.

2.6. Determining egg extract contents using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The contents of ovalbumin (OVA) and ovotransferrin (OVF) in the egg extracts were determined using SDS–PAGE. The BSA served as a stock solution for the BCA standards, for which serial dilutions were prepared using BSA and DW, according to Thermo Fisher's BCA protocol. An equal protein concentration of 10 µg was established for each sample. The electrophoresis chambers were connected to the power supply at an initial voltage of 60 V. When the samples reached the running gel, the voltage was increased to 100 V. After the gels had finished running, the staining buffer was applied to the gels. Subsequently, the distaining buffer was applied to the gels for 2 h, again at 60 rpm on the rocker. Finally, the gels were washed 2–3 times with DW and visualized.

2.7. Immunocytochemical analysis

Chicken muscle satellite cells were fixed with 4 % paraformaldehyde and permeabilized with 0.2 % triton X-100. Blocking solution was 2 % BSA in PBS. Between each process, it was washed using PBS. Primary antibodies was used Pax7 (1:500) and reacted overnight at refrigerated temperature (4 °C). Secondary antibodies were used to fluorochrome, FITC conjugated (1:2000). Afterwards, Hoechst (1:2000) was treated to stain the nucleus of the cell, and was observed after washing once.

2.8. Statistical analysis

The cell cultures experiments were performed three times at each batch (same culture), and analysis performed with 3 to 5 repetitions. Statistical analysis of the experiment were conducted by one-way ANOVA using the SPSS 22.0 program (IBM, Armonk, NY, USA). Statistical significance was assessed using the multi-range test of post hoc Tukey's range test and the significance level for all data was evaluated based on $P < 0.05$.

3. Results and discussion

3.1. Proliferation of chicken muscle satellite cells

The proliferation effect of egg white extracts in chick muscle satellite cells was measured by counting the cells using a hemocytometer and by

MTT assay. The cell number was significantly higher ($P < 0.05$) in the mixed treatment conditions with FBS than in the pure egg white extracts (Fig. 3a). In addition, the treatment group adding unfertilized egg white-derived extract had a higher cell count than the fertilized egg white extract. The amount of cells significantly increased depending on the culture period of the fertilized egg, but this trend was not confirmed in the absence or very small amount of FBS (Fig. 3a). In treatments where the content of egg white extract was doubled, the cell proliferation effect was the best when mixed with 5 % and 10 % FBS (Fig. 3b). In particular, compared to when 10 % egg white extract was added, the cell proliferation effect of the FE5 treatment group was confirmed to have increased to a level similar to that of CTL (20 % FBS) (Fig. 3).

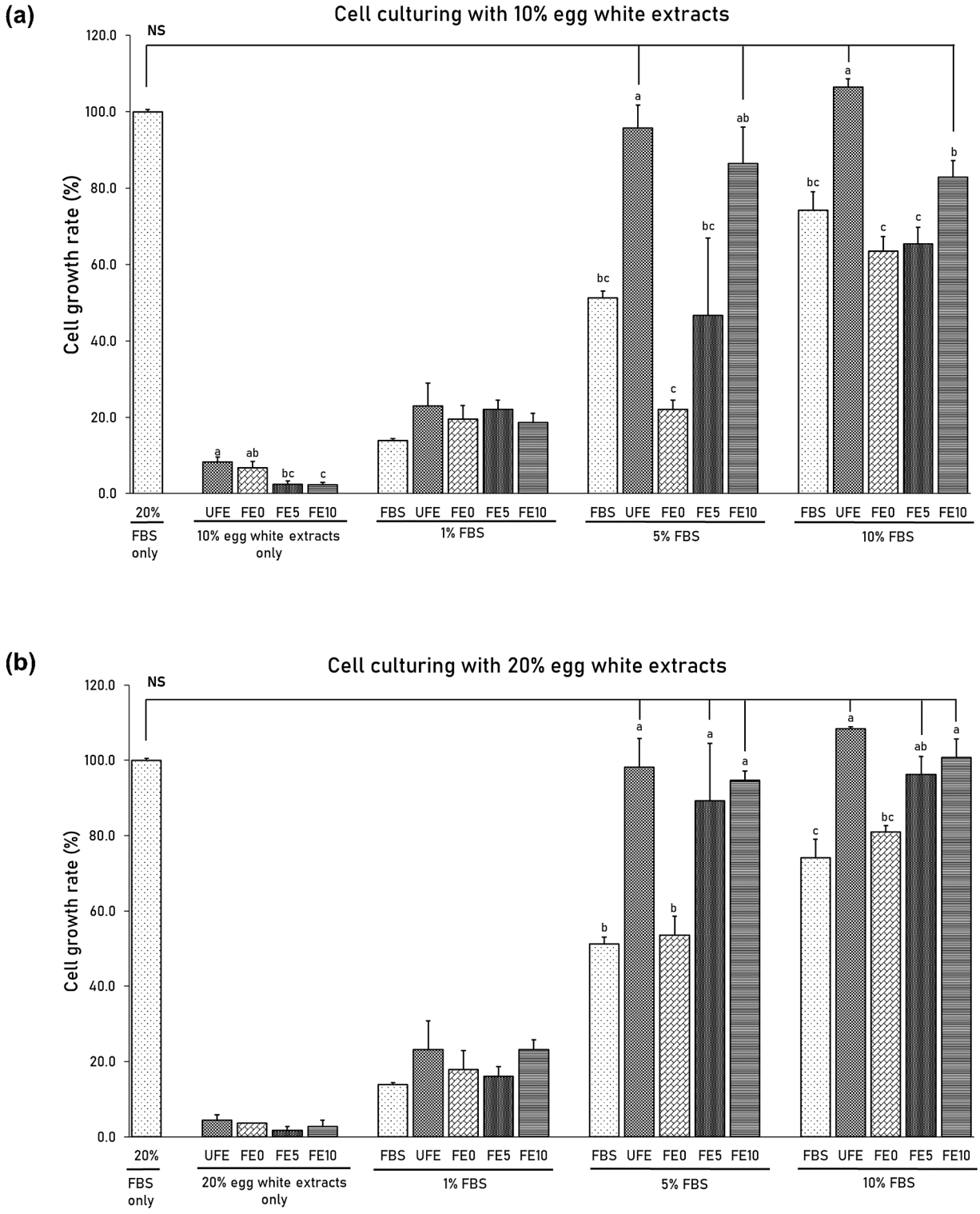

Fig. 3. Effects of egg white extracts on chick muscle satellite cell proliferation via cell counting. Cell culturing with (a) 10 % egg white extracts, (b) 20 % egg white extracts. FBS: fetal bovine serum; UFE: unfertilized egg; FE0: non-incubated fertilized egg; FE5: fertilized egg incubated for 5 days; FE10: fertilized egg incubated for 10 days. Data are presented as mean values ± SD ($n = 3$). $^{a–f}$ $P < 0.05$ depending on egg white extract type in the same FBS concentration. NS: Non-significant ($P > 0.05$).

Moreover, the cell viability, assessed by the MTT assay, also showed similar data (Fig. 4). The cell viability was significantly greater ($P < 0.05$) in the mixed treatment conditions (5–10 % FBS with 10–20 % egg white extracts) than for the pure egg white extracts. In particular, some treatment, including 5 % FBS and 20 % egg white extract and 10 % FBS and 10 % egg white extract, had a similar effect to the 20 % FBS treatment (Fig. 4). Similar to the previous results, the cell viability of unfertilized egg white-derived extracts was similar or higher than that of most fertilized egg white extracts (Fig. 4). However, there were no significant differences in the cell viability between the incubation periods

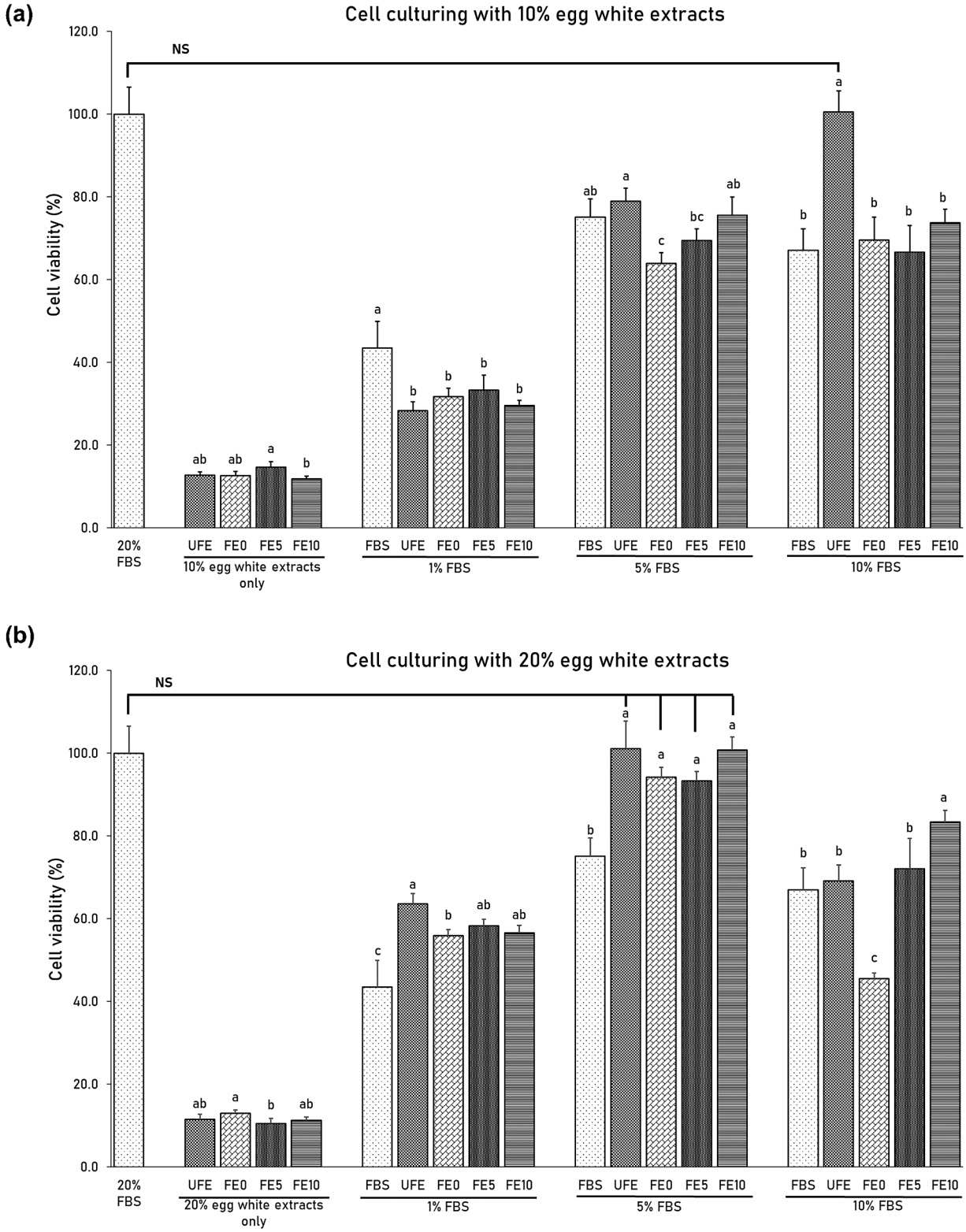

Fig. 4. Effects of egg white extracts on chick muscle satellite cell proliferation via MTT assay. Cell culturing with (a) 10 % egg white extracts, (b) 20 % egg white extracts. FBS: fetal bovine serum; UFE: unfertilized egg; FE0: non-incubated fertilized egg; FE5: fertilized egg incubated for 5 days; FE10: fertilized egg incubated for 10 days. Data are presented as mean values ± SD ($n = 3$). $^{a–c}$ $P < 0.05$ depending on egg white extract type in same FBS concentration. NS: Non-significant ($P > 0.05$).

for the fertilized eggs. Additionally, in the presence of a sufficient amount of FBS (10 %), excessive addition of egg white extract (20 %) resulted in a decrease in cell viability (Fig. 4b).

The distribution of the chick muscle satellite cells, as assessed by microscopy, revealed a similar pattern to the aforementioned results (cell number and cell viability), although it depended on the different treatments (Fig. 5). When combining the results of the three experiments above, these findings suggest that the optimal conditions for replacing FBS based on chick muscle cell proliferation include 5 % FBS with 20 % egg white extract or with 10 % egg white extract.

3.2. Ingredient analysis of FBS and egg extracts

To identify additional factors that affect cell proliferation, this study assessed the main components in the egg white extracts (Fig. 5). SDS–PAGE analysis was performed to determine the protein elements, such as OVA and OVF, which were contained in the crude egg white extracts and were responsible for the effects on the cell survival and proliferation of the chick muscle cells. Since it was anticipated that OVA and OVF in the egg white extracts would form the majority of the crude extracts, they were utilized as controls. OVA is the predominant protein in egg whites and it has a molecular weight of 45 kDa (Zhang et al., 2020), while OVF is the second most abundant, and has a molecular weight of 77 kDa (Abeyrathne et al., 2013). The results revealed that unfertilized and fertilized (incubated at days 0, 5, and 10) egg white extracts had two clear bands at 45 kDa and 70–80 kDa. The standard OVA and OVF proteins showed one prominent band at 45 kDa and 70 kDa, respectively (Fig. 5). Therefore, the unfertilized egg white and fertilized egg white extracts were shown to both contain OVA and OVF; however, the OVA and OVF contents in the fertilized egg white extract increased as the number of incubation days increased. In the study of Campos *et al.* (2003), the incubation of freeze-dried chicken embryo eggs showed an increasing amount of OVA and OVF beyond 5 days of incubation, similar to the result of this study. However, this contrasts with the results reported by Duan et al. (2017) regarding the decreasing abundance of ovalbumin after incubation due to increasing ovalbumin hydrolysis.

Based on previous findings, it has been documented that albumin from the whites of chicken eggs enhances cell adhesion, survival, and proliferation (Kaipparettu et al., 2008). Moreover, the fertilization of the egg induces changes in the OVA molecule, causing it to transition into a thermally stable form, which during the incubation period, means it will be digested and dissociate progressively to provide the necessary nutrients for the chick embryo (Duan et al., 2017; Sugimoto et al., 1999). The latter finding suggests that there can be a variation in the OVA content and an enhancement in the proliferation capacity in the egg white between unfertilized and fertilized eggs, in addition to differences among the different incubation periods. A previous study designed a fertilized egg extraction method to replace FBS, and obtained fertilized egg extracts with different albumin contents depending on the extraction method (Lee et al., 2024). As a result of culturing chick muscle satellite cells by partially replacing FBS with the extracts, the cell proliferation effect of the extract containing the highest amount of a substance presumed to be albumin was superior to that of the extract with less (Lee et al., 2024). In the extract with excellent cell proliferation effect, a protein band of a specific size (12 kDa) found in other extracts was not identified (Lee et al., 2024). This means that substances created (or not removed) during the extraction process may have a greater impact on cell proliferation than the albumin content in the extract. This trend can also be confirmed in OVF. It has been described that OVF significantly decreases the cell proliferation of C2C12 myoblast cells (Mizunoya et al., 2015). However, in the study by Lee et al. (2024), although a protein band that could be assumed to be OVF was identified in only one extract, the extract had the best effect on cell proliferation.

Generally, OVA can contribute to enhancing cell proliferation, whereas OVF has the potential to promote adverse effects on cell proliferation. In addition, this study proposes another hypothesis, whereby the factor of the content of OVF can have a greater effect on the rate of

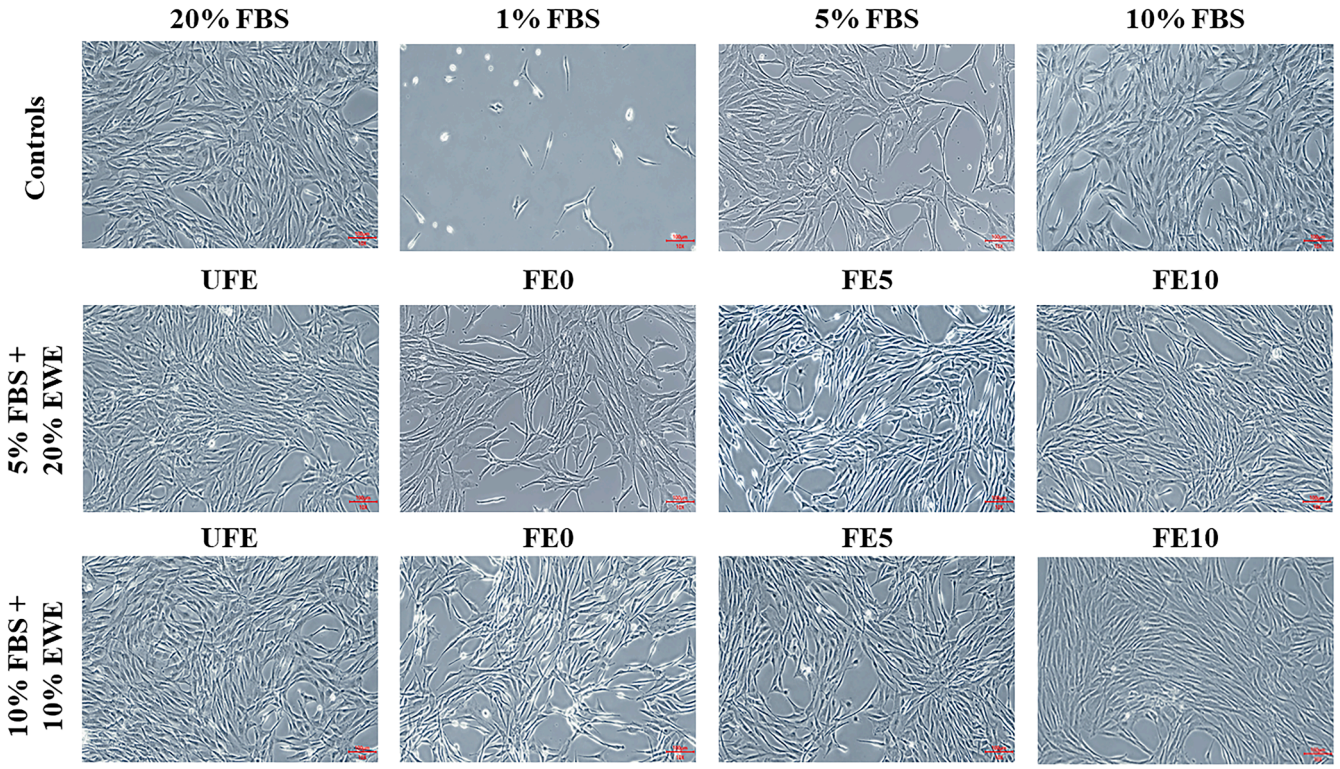

Fig. 5. Morphology of chick muscle satellite cells using light microscopy. FBS: fetal bovine serum; EWE: Egg white extracts; UFE: unfertilized egg; FE0: non-incubated fertilized egg; FE5: fertilized egg incubated for 5 days; FE10: fertilized egg incubated for 10 days. Bar = 200 μm.

cell growth than the OVA content. However, since these may correlate to other complex components in the egg white extract, further studies are needed to confirm the effect of OVA on cell growth. Considering the trend in the cell proliferation results, this study can suggest that OVF and OVA are potentially indirectly affecting the factors involved in cell proliferation (Fig. 6).

3.3. *Proliferative ability of chicken muscle satellite cells based on PAX7 expression*

Inactive satellite cells located beneath the basal layer of muscle fibers are activated for growth or damage repair, and Pax7 is a universal marker for identifying satellite cells (Relaix et al., 2006; Olguín and Pisconti, 2012). Pax7 regulates myogenesis in activated satellite cells, and in fact, deletion of Pax7 can result in severe deficiencies in muscle regeneration (Relaix et al., 2006; Zammit et al. 2006; Olguín and Pisconti, 2012). Pax7 deficiency inhibits satellite cell activation and arrests the cell cycle, preventing further proliferation (Zammit et al. 2006; von Maltzahn et al., 2013). Therefore, the expression of Pax7 can be a standard for determining the successful proliferation and maintenance of chicken muscle satellite cells. This is because the expression of a sufficient amount of Pax7 indicates that the muscle cells necessary for the production of cultured meat are actively proliferating. Pax7 expression in chicken muscle satellite cells grown using FBS and the four egg extracts obtained in this study is shown in Fig. 7. Similar to the previous cell proliferation study, cells treated with 20 % FBS had the highest cell number, and different concentrations of UFE and FE10 (10 % and 20 %) added with 5 % FBS or 10 % FBS showed the most similar cell proliferation effects (Fig. 7a,b). The expression rate of Pax7 was expressed by calculating the amount of cells expressing Pax7 compared to the number of cell nuclei, and these results are shown in Fig. 7c. In all selected treatments, more than 93 % of cells expressed Pax7, and insufficient FBS or all types of egg extract had no negative effect on Pax7 expression in chicken muscle satellite cells. These results suggest that four types of egg extract can replace FBS to create an environment in which chicken muscle satellite cells can grow successfully.

Taken together, regardless of whether it is an unfertilized or fertilized egg, egg extracts can replace up to 75 % of FBS when growing chicken muscle satellite cells. Among them, egg white extract obtained from unfertilized eggs and fertilized eggs cultured for 10 days showed the effect of adding 20 % FBS in the presence of an appropriate amount (5–10 %) of FBS. However, when excessive amounts were added, cell proliferation was inhibited. Unfertilized eggs are cheaper than fertilized eggs and do not require artificial culture, so the extract production period is relatively short. The most effective alternative is a mixture of 20 % unfertilized egg white extract and 5 % FBS. This showed a growth rate similar to or better than a mixture of unfertilized/fertilized egg albumen extract and 10 % FBS. Therefore, minimizing the use of FBS and using unfertilized egg white extract, which is the cheapest and fastest extract to produce, can be a way to reduce cost and improve safety, which are major obstacles in the production of cultured meat. In addition, it is predicted that partial replacement of FBS, which is considered the most important element in cell culture, can be applied not only to muscle primary cells for cultured meat, but also to diverse cell cultures.

4. Conclusion

Fetal bovine serum (FBS) is an extremely important growth supplement in cell culture and accounts for approximately 60 % of all cell-culture media costs; therefore, lowering the acquisition costs for FBS is imperative for the industrialization of cultured meat. The results of this study showed that a combination of 5 % FBS and 20 % egg white extract or 10 % FBS and 10 % egg white extract significantly increased cell viability and had a similar effect to adding 20 % commercial FBS. However, these extracts did not show a clear cell proliferation effect when used alone or mixed with a trace amount (1 %) of FBS. The above experimental results indicate that unfertilized egg extract cannot only contribute to the development of FBS as a replacement for cultured chicken, but also have the potential to be used as a medium additive for culturing various experimental cell lines. Nonetheless, since eggs are still an animal-derived material and cannot fully replace FBS, additional research will likely be needed to address this issue.

Ethics statement

The study did not require special ethical consideration as per the guidelines of IACUC involving avian embryo use.

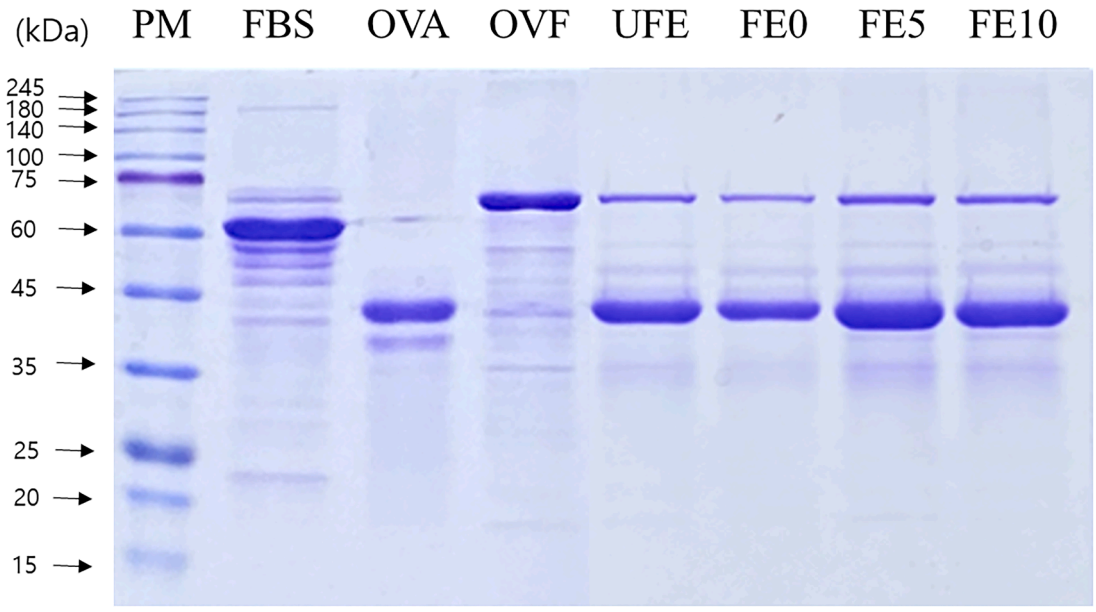

Fig. 6. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) patterns for proteins in egg white extracts. P.M: protein maker; FBS: fetal bovine serum; OVA: ovalbumin (45 kDa, 1 mg/mL); OVF: ovotransferrin (77 kDa, 1 mg/mL); UFE: unfertilized egg (1 mg/mL); FE0: non-incubated fertilized egg (1 mg/ mL); FE5: fertilized egg incubated for 5 days (1 mg/mL); FE10: fertilized egg incubated for 10 days (1 mg/mL).

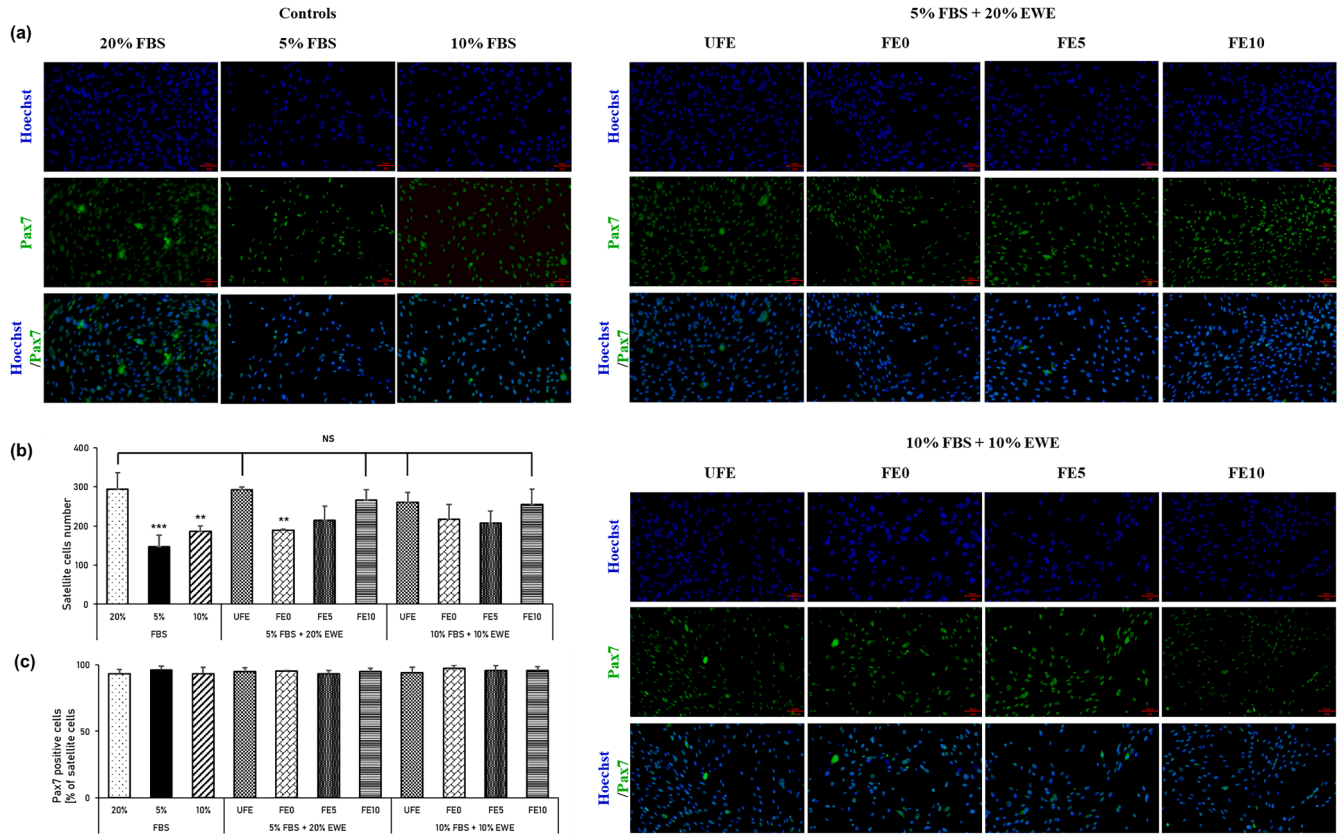

Fig. 7. Pax7 expression in chicken satellite cell with egg extracts. (a) Immunocytochemistry on chicken muscle satellite cell using Hoechst (blue) and Pax7 (green). (b) Number of chicken muscle satellite cells; $n = 4$, $^{***}P < 0.05$, $^{**}P < 0.01$. (c) Percentage (%) of Pax7 positive cell of chicken muscle satellite cell. NS: Non-significant ($P > 0.05$).

CRediT authorship contribution statement

Da Young Lee: Methodology, Investigation, Formal analysis, Writing – original draft. **Monica Piñero Majó:** Data curation, Methodology, Investigation, Writing – original draft. **Dahee Han:** Investigation, Writing – review & editing. **Yeongwoo Choi:** Investigation, Writing – review & editing. **Jin Soo Kim:** Investigation, Writing – review & editing. **Jinmo Park:** Investigation, Writing – review & editing. **Seung Hyeon Yun:** Investigation, Writing – review & editing. **Ermie Mariano:** Investigation, Writing – review & editing. **Juhyun Lee:** Investigation, Writing – review & editing. **Sun Jin Hur:** Funding acquisition, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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