J Anim Reprod Biotechnol 2023;38:10-16
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.38.1.10

Original Article

Development of an effective dissociation protocol for isolating mesenchymal stem cells from bovine intermuscular adipose tissues

Jeong Min Lee[1,2], Hyun Lee[1] and Seung Tae Lee[1,2,*]

[1]Department of Applied Animal Science, Kangwon National University, Chuncheon 24341, Korea
[2]Kustogen, Chuncheon 24341, Korea

Received December 19, 2022
Revised February 1, 2023
Accepted February 1, 2023

***Correspondence**
Seung Tae Lee
E-mail: stlee76@kangwon.ac.kr

Author's Position and Orcid no.
Lee JM, PhD candidate,
https://orcid.org/0000-0002-1894-0629
Lee H, PhD candidate,
https://orcid.org/0000-0002-1090-3479
Lee ST, Professor,
https://orcid.org/0000-0002-8952-3881

ABSTRACT Intermuscular fat is essential for enhancing the flavor and texture of cultured meat. Mesenchymal stem cells derived from intermuscular adipose tissues are a source of intermuscular fat. Therefore, as a step towards developing a platform to derive intermuscular fat from mesenchymal stem cells (MSCs) for insertion between myofibrils in cultured beef, an advanced protocol of intermuscular adipose tissue dissociation effective to the isolation of MSCs from intermuscular adipose tissues was developed in cattle. To accomplish this, physical steps were added to the enzymatic dissociation of intermuscular adipose tissues, and the MSCs were established from primary cells dissociated with physical step-free and step-added enzymatic dissociation protocols. The application of a physical step (intensive shaking up) at 5 minutes intervals during enzymatic dissociation resulted in the greatest number of primary cells derived from intermuscular adipose tissues, showed effective formation of colony forming units-fibroblasts (CFU-Fs) from the retrieved primary cells, and generated MSCs with no increase in doubling time. Thus, this protocol will contribute to the stable supply of good quality adipose-derived mesenchymal stem cells (ADMSCs) as a fat source for the production of marbled cultured beef.

Keywords: bovine adipose tissue-derived stem cells, culture meat, intermuscular adipose tissue-derived stem cells, isolation procedure, physical step

INTRODUCTION

Animal-derived meat is mainly composed of muscle tissue, intermuscular fat tissue, connective tissue, and blood vessels (Warner, 2019; Rubio et al., 2020). The ratio of each tissue plays a pivotal role in determining the flavor and texture of meat (Dekkers et al., 2018; Warner, 2019; Srutee et al., 2021). Intermuscular fat tissue located between and within muscle fibers is directly linked to meat flavor and indirectly linked to meat tenderness (Arshad et al., 2018; Warner, 2019). Therefore, it is important in the production of cultured meat with the same flavor and texture as meat derived from livestock.

Because of the ease of retrieval and manipulation, intermuscular or subcutaneous fat derived directly from slaughtered livestock can be a source of fat incorporated into the muscle fibers of cultured meat. However, large numbers of livestock need to be sacrificed for adequate fat supply, resulting in difficulty producing sacrifice-free and environmentally friendly cultured meat. Alternatives,

such as stem cells with competencies of infinite proliferation and differentiation into specific tissues (Lee et al., 2019; Mastrolia et al., 2019), can be used to produce cultured meat. In addition, intermuscular adipose-derived mesenchymal stem cells (ADMSCs), which produce adipose tissue with the same properties as intermuscular fat with long-term proliferation (Bukowska et al., 2021; Khazaei et al., 2022), can be a source of fat for cultured meat production.

To date, several protocols for isolating intermuscular ADMSCs from intermuscular adipose tissue have been reported in humans and mice (Megaloikonomos et al., 2018; Palumbo et al., 2018; Bukowska et al., 2021). On the other hand, there are few reports of the isolation of intermuscular ADMSCs from intermuscular adipose tissues derived from livestock such as cattle or chicken (Lu et al., 2018; Yue et al., 2018; Bukowska et al., 2021). In those few studies, dissociation of intermuscular adipose tissues, an important step in the establishment of intermuscular ADMSCs, focused on optimization of dissociation enzymes; optimization of physical forces stimulating tissue dissociation has not yet been performed.

Accordingly, here, we established a protocol for dissociation of intermuscular adipose tissues for isolation of intermuscular ADMSCs from intermuscular adipose tissues in cattle. For this, physical steps were added to enzymatic dissociation of the tissue derived from cattle, and the effects of these steps on the establishment of intermuscular ADMSCs were investigated.

MATERIALS AND METHODS

Preparation of intermuscular adipose tissues

Intermuscluar adipose tissues were immediately harvested from 29- to 33-month-old castration male or female Hanwoo cattles slaughtered at a local slaughterhouse (Kwell LPC, Hongcheon, Korea). Subsequently, the retrieved adipose tissues were used as donors of ADMSCs. All experimental procedures were conducted to comply with the Animal Care and Use Guidelines of Kangwon National University and approved by the Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval no. KW-220714-1).

Harvest of primary cells from intermuscular adipose tissues

The retrieved intermuscular adipose tissues were sequentially washed one time in 70% ethanol (Samchun Chemical, Seoul, Korea) and two times in Dulbecco's phosphate-buffered saline (DPBS; Welgene, Daegu, Korea) supplemented with 2% (v/v) antibiotic-antimycotic solution (Welgene). The rinsed intermuscular adipose tissues were cut into small pieces using surgical scissors and the small pieces of intermuscular adipose tissues were placed in a tube containing 0.1% (w/v) collagenase (Sigma-Aldrich, Saint Louis, MO, USA) dissolved in 0.25% trypsin-EDTA (Gibco, Grand Island, NY, USA) (herein referred to the adipose tissues + dissociation enzymes-containing tubes). Subsequently, their dispersion was conducted according to the following ways: Method 1 and Method 2. Method 1 was conducted by incubating the adipose tissues + dissociation enzymes-containing tubes at room temperature for 15 minutes and then incubating them in water bath set at 37℃ for 15 minutes. For Method 2, the adipose tissues + dissociation enzymes-containing 50 mL conical tubes (Hyundai Micro co., Seoul, Korea) were incubated in water bath set at 37℃ for 5 minutes and shaken intensively and vertilically thirty times, and this process was repeated six times. Then, primary cells dispersed from intermuscular adipose tissues were re-suspended in low-glucose Dulbecco's modified Eagle's medium (LG-DMEM; Welgene) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Welgene) for dissociation enzyme inactivation. Debris included in the dispersed primary cells were removed by filtering using a 100-μm cell strainer and the filtered debris-free primary cells were centrifuged at 415 × g for 5 minutes. Red blood cells (RBCs) from the debris-free primary cells was eliminated using a RBC lysis buffer (Sigma-Aldrich) for 10 minutes at room temperature. After centrifuging at 415 × g for 5 minutes, the RBC-free primary cells were re-suspended in LG-DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic-antimycotic (herein referred to as ADMSC proliferation medium) and allocated to the following experiments.

Establishment and culture of ADMSCs from intermuscular adipose tissue-derived primary cells

To isolate ADMSCs from intermuscular adipose tissue-derived primary cells, 1×10^4 intermuscular adipose

tissue-derived primary cells were seeded onto a 35 mm culture dish (SPL, Pocheon, Korea) and then cultured for 8 days in ADMSC proliferation medium at 37℃ in a humidified atmosphere of 5% CO_2 in air with the exchange of medium at 2-day intervals. After the formation of colony forming units-fibroblast (CFU-F) derived from intermuscular ADMSCs on the bottom of culture dishes, the formed CFU-F were dispersed using 0.25% trypsin-EDTA and the dispersed cells were re-suspended in fresh ADMSC proliferation medium after centrifuging at 415 × g for 5 minutes. Subsequently, 2 × 10^5 cells were seeded onto a 60 mm culture dish (SPL) and cultured in ADMSC proliferation medium at 37℃ in a humidified atmosphere of 5% CO_2 in air with the exchange of medium at 2-day intervals. Subculture was conducted whenever the cultured cells reached 60% confluency.

CFU-F assay

A colony consisting of more than 50 cells with a typical fibroblastic morphology was regarded as a CFU-F. The formed CFU-Fs were fixed with 4% (v/v) formaldehyde solution (Junsei Chemical, Chuo-ku, Japan) for 15 minutes at room temperature and the fixed CFU-Fs were stained by incubating for 10 minutes at room temperature in 0.5% (w/v) crystal violet (Sigma-Aldrich) dissolved in 20% Methanol (Samchun Chemical). Subsequently, the stained CFU-Fs were washed two times with distilled water for eliminating the remaining crystal violet. Their images in each group were recorded using a digital sight camera (EOS 1000D; Cannon, Tokyo, Japan) equipped to the inverted microscope (CKX-41; Olympus, Tokyo, Japan) and analyzed using an IMT iSOlution Lite (Ver 10.0; Image & Microscope Technology Inc., Vancouver, Canada) for measuring number and size of the formed CFU-F.

Measurement of doubling time

Intermuscular ADMSCs were seeded on 60 mm culture plates at a density of 2 × 10^5 cells and cultured in ADMSC proliferation medium at 37℃ in a humidified atmosphere of 5% CO_2 in air. When intermuscular ADMSCs reached 60% confluency, they were dissociated with 0.25% trypsin-EDTA, and total number of the dissociated intermuscular ADMSCs were counted using a hemocytometer. Subsequently, the doubling time of intermuscular ADMSCs was calculated as $t\log_2/(\log N_t - \log N_0)$, where t is time to confluence, N_t is the number of intermuscular ADMSCs at the end of the growth period, and N_0 is the initial number of intermuscular ADMSCs. This process was equally conducted in each passage until 5th passage

Real-time polymerase chain reaction (PCR)

Total mRNA was extracted using the Dynabeads™ Oligo$(dT)_{25}$ (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions, and then cDNA was synthesized using the ReverTra Ace™ qPCR RT Master Mix with gDNA remover kit (Toyobo, Osaka, Japan). Real-time quantitative of the specific gene expression was performed using Prime Q-Mastermix (GeNet Bio, Daejeon, Korea) with qTOWER^3 Real-time PCR Thermal Cycler (Analytik Jena, Jena, Germany), and identification of PCR specificity was conducted by collecting melting curve data. The relative mRNA level was presented as $2^{-\Delta Ct}$, where Ct = threshold cycle for target amplification, $\Delta Ct = Ct_{\text{target gene}}$ (specific genes for each sample) − $Ct_{\text{internal reference}}$ (*Gapdh* for each sample). Primer3 Plus software (Whitehead Institute/MIT Center for Genome Research) was used for designing primer sequences with the information of bovine cDNA sequence collected from GenBank. Table 1 shows general information and sequences of designed primers in detail.

Statistical analysis

The Statistical Analysis System (SAS) program (SAS institute, Cary, NC, USA) was used for analysis of all numerical data. Comparative analysis among experimental groups was performed using the least-square difference or Duncan's method, and analysis of variance (ANOVA) in the

Table 1. Primer sequences and PCR cycling conditions

Genes	GenBank number	Primer sequence		Size (bp)	Temp (℃)
		Sense	Anti-sense		
CD44	NM_174013.3	5'-CGGAACATAGGGTTTGAGA-3'	5'-GGTTGATGTCTTCTGGGTTA-3'	301	60
CD73	NM_174129.3	5'-CAATGGCACGATTACCTG-3'	5'-GACCTTCAACTGCTGGATA-3'	428	60
CD34	NM_174009.1	5'-CCTCATCAGCTTTGCGACTT-3'	5'-CCAGGAGCAAGGAGCACA-3'	314	60
GAPDH	NM_001034034.2	5'-AAAGTGGACATCGTCGCCAT-3'	5'-CCAGCATCACCCCACTTGAT-3'	194	60

SAS package was used to determine the significance of the main effects. The level of significant differences was set at $p < 0.05$.

RESULTS

Influence of the addition of physical steps to the enzymatic dissociation protocol on the retrieval of primary cells from intermuscular adipose tissues in cattle

To improve retrieval efficiency of MSCs from intermuscular adipose tissues, we attempted to develop an intermuscular adipose tissue dissociation protocol to effectively isolate primary cells such as MSCs. Bovine intermuscular adipose tissues were dispersed using an enzymatic dissociation protocol without (Method 1) or with (Method 2) additional physical steps, and the total number of retrieved primary cells from intermuscular adipose tissue was determined. Subsequently, the number or size of CFU-Fs formed from the retrieved primary cells and the total number of cells retrieved from the formed CFU-Fs were enumerated. As shown in Fig. 1, the addition of physical steps to the enzymatic dissociation protocol (Method 2) resulted in a greater number of primary cells from intermuscular adipose tissues than the physical step-free enzymatic dissociation protocol (Method 1) in all replicates. Entirely, successful formation of CFU-Fs was observed in primary cells retrieved from bovine intermuscular adipose tissues using Method 1 (left panel of Fig. 2A) or Method 2 (right panel of Fig. 2A), and no significant difference in the number of CFU-Fs formed from primary cells retrieved from intermuscular adipose tissues was detected between Method 1 and Method 2 (Fig. 2B). However, in all replicates, the size of the formed CFU-Fs was numerically bigger in intermuscular adipose tissues-derived primary cells produced by Method 2 than those by Method 1 (Fig. 2C), and CFU-Fs formed from intermuscular adipose tissue-derived primary cells produced by Method 2 showed numerically more total cell number than those by Method 1 (Fig. 2D). These results demonstrate that Method 2, which included additional physical steps, is an effective dissociation protocol for the retrieval of primary cells including MSCs from intermuscular adipose tissues in cattle.

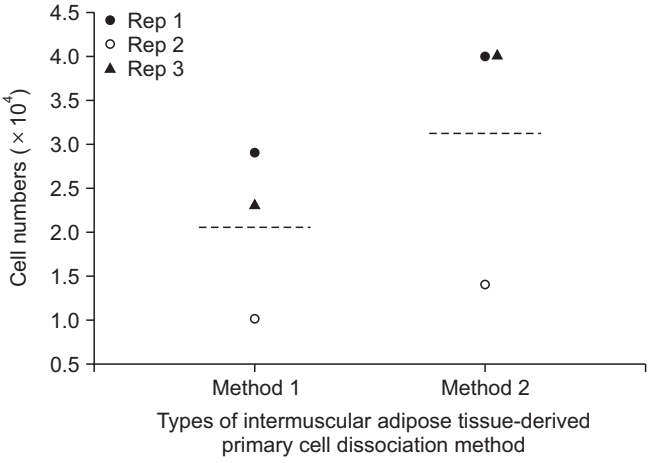

Fig. 1. Number of intermuscular adipose tissue-derived primary cells isolated using different intermuscular adipose tissue dissociation protocols. Each primary cell was derived from intermuscular adipose tissues according to the experimental design. The total number of viable cells not stained with trypan blue dye within intermuscular adipose tissue-derived primary cells was determined by hemocytometry. In all experimental replicates, the total number of live primary cells obtained using Method 2 was greater than Method 1. Dotted lines indicate the mean of three independent experiments. Rep, replicate.

Comparison of marker expression and doubling time of MSCs established from primary cells retrieved from intermuscular adipose tissues using enzymatic dissociation without or with additional physical steps

The CFU-Fs formed via each method were cultured *in vitro* to establish MSCs until passage 5. The identities of intermuscular ADMSCs established via each method were confirmed at passage 5 by investigating transcriptional levels of genes specific to MSCs (CD33 and CD73) and the hematopoietic stem cell-related gene (CD34); the doubling time was measured per passage of intermuscular ADMSCs. The established intermuscular ADMSCs showed high transcriptional expression of CD33 and CD73 but low transcriptional expression of CD34 (Fig. 3). Moreover, as shown in Fig. 4, those established using Method 1 showed a significant increase in doubling time at passage 5, whereas those using Method 2 did not show a significant difference in doubling time in any passage. These results confirm that intermuscular ADMSCs with no alteration of doubling time during *in vitro* culture can be established using Method 2.

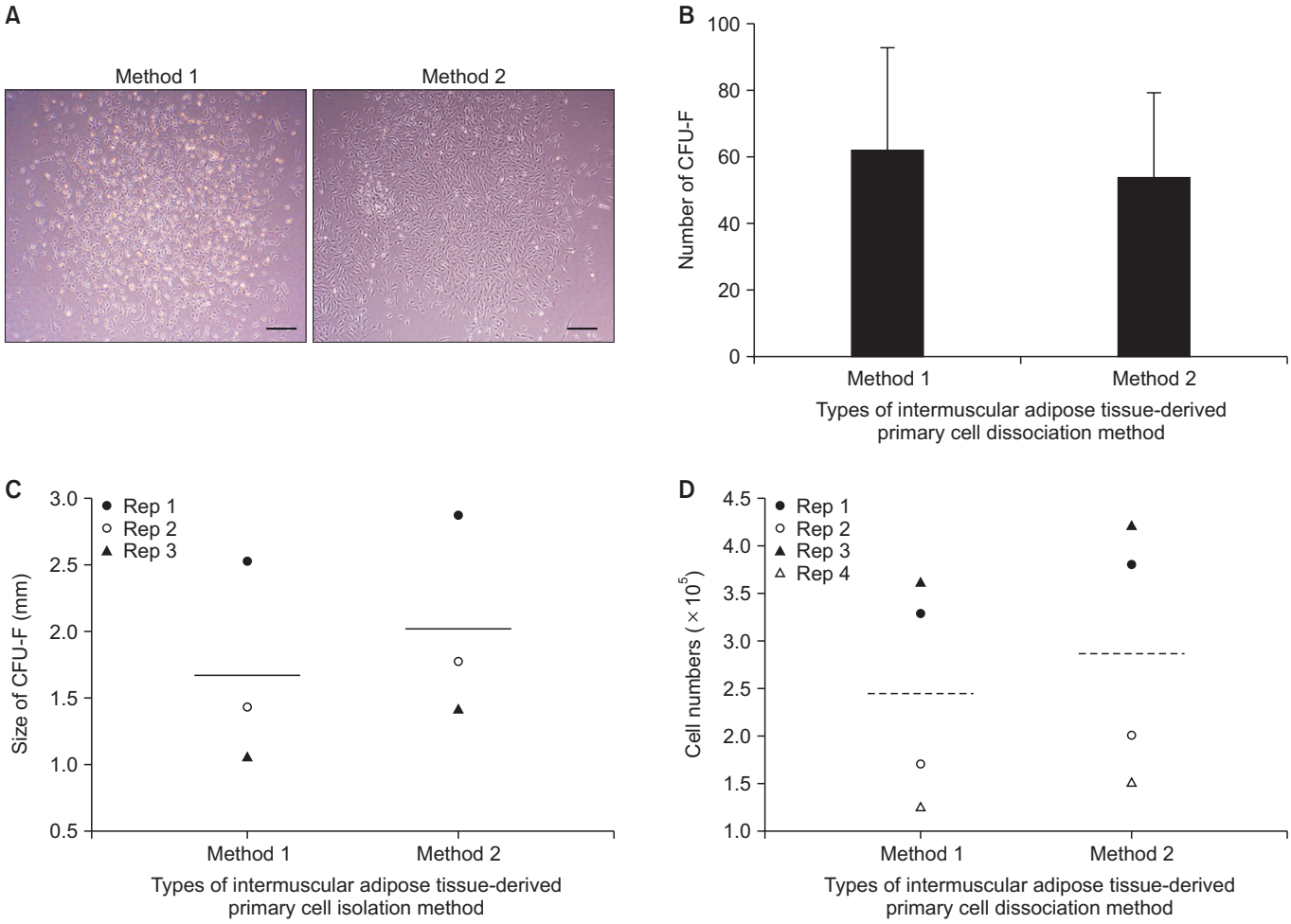

Fig. 2. Number and size of CFU-Fs formed from intermuscular adipose tissue-derived primary cells isolated using different dissociation protocols and the total number of cells retrieved from the formed CFU-Fs. Primary cells retrieved from intermuscular adipose tissues using Method 1 and Method 2 were seeded onto culture plates and cultured for 8 days. Subsequently, the number (B) and size (C) of CFU-Fs consisting of more than 50 cells with typical fibroblast morphology (A) and total number (D) of cells retrieved from the formed CFU-Fs were counted. The methodological difference in the isolation of primary cells from intermuscular adipose tissue did not show any significant difference in the number of CFU-Fs formed from primary cells. In all experimental replicates, primary cells obtained using Method 2 resulted in larger CFU-Fs and a greater total number of cells retrieved from the formed CFU-Fs than Method 1. All data shown in (B) represent mean ± standard error of the mean (SEM) of four independent experiments. Dotted lines in (C) and (D) indicate the mean of three or four independent experiments. Scale bar in (A) is 200 μm. Rep, replicate.

DISCUSSION

Intermuscular fat is often inserted into cultured meat to realistically enhance the flavor. Reliable supply and insertion of such fat can be realized using intermuscular ADMSCs. Therefore, an advanced protocol effectively dissociating intermuscular adipose tissues, an important step in the isolation of MSCs from intermuscular adipose tissues, was developed to reliably obtain high-quality primary cells such as MSCs from intermuscular adipose tissues in cattle. The developed protocol (Method 2) was established by incorporating a physical step into a previous enzymatic tissue dissociation protocol. The retrieval efficiency of intermuscular ADMSCs was further improved by Method 2, and intermuscular ADMSCs retrieved through this method did not show any significant increase in doubling time during *in vitro* culture. These results demonstrate that tissue dissociation combined with physical disruption is effective for isolating intermuscular ADMSCs from intermuscular adipose tissues in cattle.

As shown in Fig. 1 and 2, the incorporation of a physical step during enzymatic dissociation (Method 2) increased the number of primary cells isolated from intermuscular adipose tissues; these primary cells played

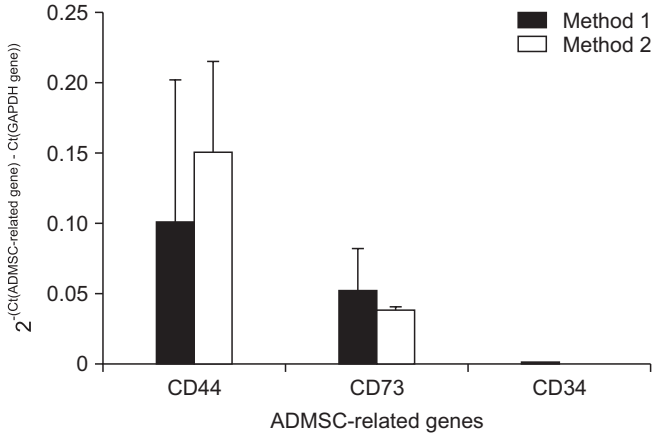

Fig. 3. Transcriptional expression of ADMSC-related genes in intermuscular ADMSCs cultured until passage 5 following isolation from intermuscular adipose tissues using different dissociation protocols. Intermuscular ADMSCs were established by forming CFU-Fs from intermuscular adipose tissue-derived primary cells isolated using Method 1 or Method 2. Quantitative monitoring of ADMSC-related genes CD44 and CD73 and the hematopoietic stem cell-related gene CD34 was conducted in intermuscular ADMSCs cultured until passage 5 using real-time PCR. Regardless of which tissue dissociation protocol was used, increased transcription of CD44 and CD73 genes and decreased transcription of the CD34 gene were observed in intermuscular ADMSCs cultured until passage 5. All data shown represent mean ± SEM of three independent experiments.

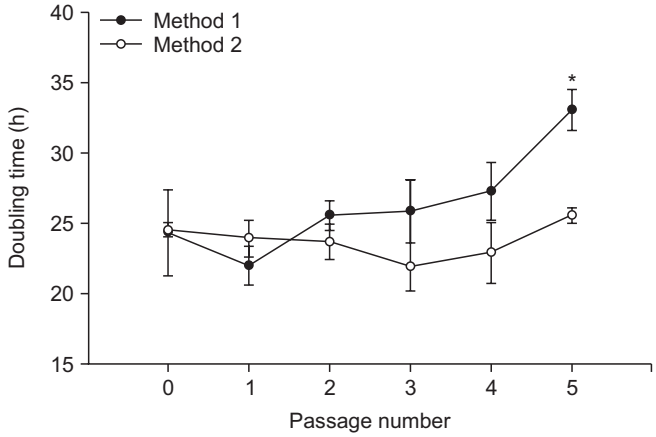

Fig. 4. Comparison of doubling time per passage of intermuscular ADMSCs isolated using different intermuscular adipose tissue dissociation protocols. Method 1 or Method 2 was used to retrieve primary cells from intermuscular adipose tissues and intermuscular ADMSCs were selected from intermuscular adipose tissue-derived primary cells through CFU-F formation. Subsequently, 2×10^5 intermuscular ADMSCs were seeded on 60 mm culture dishes at each passage and, when 60% confluency was reached, doubling time was analyzed. According to the increase in passage number, intermuscular ADMSCs isolated using Method 1 showed a significant increase in doubling time at passage 5, whereas those isolated using Method 2 did not show a significant difference in doubling time. All data shown represent mean ± SEM of three independent experiments. *$p < 0.05$.

an important role in the increased production of MSCs compared to Method 1. In general, intermuscular adipose tissues floats on water, so partial enzymatic digestion of intermuscular adipose tissue can only be triggered on the surface in contact with the dissociation solution. A physical step such as shaking may increase the surface area in contact with dissociation solution and may increase the exposure of intermuscular adipose tissues to the dissociation enzymes, resulting in the release of more MSCs from the intermuscular adipose tissues.

Fig. 4 shows that the doubling time was maintained in intermuscular ADMSCs isolated using Method 2. Intermuscular adipose tissues contain macrophages, pericytes, and adipocytes in addition to MSCs (Russo and Lumeng, 2018; Khazaei et al., 2022), and these cells were isolated in greater numbers by Method 2. Among them, macrophages and adipocytes can produce nitric oxide (NO), which inhibits differentiation of MSCs (Yang et al., 2018; Midgley et al., 2020), and the overall increase in NO during the establishment of intermuscular ADMSCs will reduce the proportion of nonproliferative and differentiated MSCs in intermuscular adipose tissue-derived primary cells retrieved by Method 2. Accordingly, the maintenance of doubling time of intermuscular ADMSCs isolated by Method 2 may be due to the decrease in the number of differentiated intermuscular ADMSCs without proliferative competence by the increased levels of NO, which is supported by the fact that MSCs can differentiate immediately following a changed in the cellular niche (Mor-Yossef Moldovan et al., 2019; Zhang et al., 2019).

CONCLUSION

In conclusion, an advanced intermuscular adipose tissue dissociation protocol for isolation of MSCs from intermuscular adipose tissues in cattle was developed by applying a physical step (intensive shaking up) at 5 minutes intervals during enzymatic dissociation. This technique will contribute to the establishment of good-quality MSCs with no change in doubling time from bovine intermuscular adipose tissues.

Author Contributions: Conceptualization, S.T.L.;

methodology, J.M.L., H.L. and S.T.L.; investigation, J.M.L.; data curation, J.M.L., H.L. and S.T.L.; writing—original draft preparation, J.M.L.; writing—review and editing, S.T.L.; supervision, S.T.L.; project administration, S.T.L.; funding acquisition, S.T.L.

Funding: This work was supported by the Technology Innovation Program (Alchemist Project, 20012384) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea), NRF (National Research Foundation of Korea) grant funded by the Korean Government (NRF-2016-Global Ph.D. Fellowship Program).

Ethical Approval: This study was approved by the Animal Care and Use Committee (IACUC) of Kangwon National University according to the guidelines of the Kangwon National University IACUC committee (IACUC approval no. KW-220714-1).

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

Acknowledgements: None.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

REFERENCES

Arshad MS, Sohaib M, Ahmad RS, Nadeem MT, Imran A, Arshad MU, Kwon JH, Amjad Z. 2018. Ruminant meat flavor influenced by different factors with special reference to fatty acids. Lipids Health Dis. 17:223.

Bukowska J, Szóstek-Mioduchowska AZ, Kopcewicz M, Walendzik K, Machcińska S, Gawrońska-Kozak B. 2021. Adipose-derived stromal/stem cells from large animal models: from basic to applied science. Stem Cell Rev. Rep. 17:719-738.

Dekkers BL, Boom RM, van der Goot AJ. 2018. Structuring processes for meat analogues. Trends Food Sci. Technol. 81:25-36.

Khazaei S, Keshavarz G, Bozorgi A, Nazari H, Khazaei M. 2022. Adipose tissue-derived stem cells: a comparative review on isolation, culture, and differentiation methods. Cell Tissue Bank. 23:1-16. (Erratum published 2021, Cell Tissue Bank. 22:737)

Lee HJ, Jeon RH, Park BJ, Jang SJ, Lee SL, Rho GJ, Kim SJ, Lee WJ. 2019. Differentiation inductions altered telomere length and telomerase activity in human dental pulp-derived mesenchymal stem cell. J. Anim. Reprod. Biotechnol. 34:93-99.

Lu T, Pei W, Wang K, Zhang S, Chen F, Wu Y, Guan W. 2018. In vitro culture and biological properties of broiler adipose-derived stem cells. Exp. Ther. Med. 16:2399-2407.

Mastrolia I, Foppiani EM, Murgia A, Candini O, Samarelli AV, Grisendi G, Veronesi E, Horwitz EM, Dominici M. 2019. Challenges in clinical development of mesenchymal stromal/stem cells: concise review. Stem Cells Transl. Med. 8:1135-1148.

Megaloikonomos PD, Panagopoulos GN, Bami M, Igoumenou VG, Dimopoulos L, Milonaki A, Kyriakidou M, Mitsiokapa E, Anastassopoulou J, Mavrogenis AF. 2018. Harvesting, isolation and differentiation of rat adipose-derived stem cells. Curr. Pharm. Biotechnol. 19:19-29.

Midgley AC, Wei Y, Li Z, Kong D, Zhao Q. 2020. Nitric-oxide-releasing biomaterial regulation of the stem cell microenvironment in regenerative medicine. Adv. Mater. 32:e1805818.

Mor-Yossef Moldovan L, Lustig M, Naftaly A, Mardamshina M, Geiger T, Gefen A, Benayahu D. 2019. Cell shape alteration during adipogenesis is associated with coordinated matrix cues. J. Cell. Physiol. 234:3850-3863.

Palumbo P, Lombardi F, Siragusa G, Cifone MG, Cinque B, Giuliani M. 2018. Methods of isolation, characterization and expansion of human adipose-derived stem cells (ASCs): an overview. Int. J. Mol. Sci. 19:1897.

Rubio NR, Xiang N, Kaplan DL. 2020. Plant-based and cell-based approaches to meat production. Nat. Commun. 11: 6276.

Russo L and Lumeng CN. 2018. Properties and functions of adipose tissue macrophages in obesity. Immunology 155:407-417.

Srutee R, Sowmya RS, Annapure US. 2022. Clean meat: techniques for meat production and its upcoming challenges. Anim. Biotechnol. 33:1721-1729.

Warner RD. 2019. Review: analysis of the process and drivers for cellular meat production. Animal 13:3041-3058.

Yang S, Guo L, Su Y, Wen J, Du J, Li X, Liu Y, Feng J, Xie Y, Bai Y, Wang H, Liu Y. 2018. Nitric oxide balances osteoblast and adipocyte lineage differentiation via the JNK/MAPK signaling pathway in periodontal ligament stem cells. Stem Cell Res. Ther. 9:118.

Yue Y, Zhang L, Zhang X, Li X, Yu H. 2018. De novo lipogenesis and desaturation of fatty acids during adipogenesis in bovine adipose-derived mesenchymal stem cells. In Vitro Cell. Dev. Biol. Anim. 54:23-31.

Zhang Z, Qu R, Fan T, Ouyang J, Lu F, Dai J. 2019. Stepwise adipogenesis of decellularized cellular extracellular matrix regulates adipose tissue-derived stem cell migration and differentiation. Stem Cells Int. 2019:1845926.