The Efficacy of Using Human Serum Supplemented Medium for Culturing Adipose-derived Stem Cells

ประสิทธิภาพของการใช้ซีรั่มจากมนุษย์ต่อการเพาะเลี้ยงเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมัน

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ABSTRACT

The aim of this study was to compare efficacy of using autologous human serum (AHS) with fetal bovine serum (FBS) as a supplement for medium for culturing adipose-derived stem cells (ADSCs) isolated from buccal fat pads. The results demonstrated that the numbers of CFU-F of the AHS group were significantly greater than those of the FBS group. The cells of the AHS group rapidly grew over 21 days of culture, whereas those of the FBS group gradually grew. No statistical difference was detected between two groups for the expression of the mesenchymal and hematopoietic stem cell markers. Both groups also demonstrated multi-differentiation potential including osteogenicity, adipogenicity and chondrogenicity. It can be concluded that the AHS could support self-renewal capacity and growth of the ADSCs better than the FBS without altering their immunophenotypes and differentiation capacity.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อเปรียบเทียบประสิทธิภาพของการใช้ซีรั่มจากมนุษย์และซีรั่มจากตัวอ่อนวัวในครรภ์ ซึ่งใช้ผสมในอาหารเลี้ยงเซลล์ ต่อการเพาะเลี้ยงเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันที่นำมาจากเนื้อเยื่อไขมันบริเวณกระพุ้งแก้ม จากการทดสอบพบว่ากลุ่มที่มีส่วนผสมของซีรั่มจากมนุษย์มีจำนวนโคโลนีมากกว่ากลุ่มที่มีส่วนผสมของซีรั่มจากตัวอ่อนวัวในครรภ์อย่างมีนัยสำคัญทางสถิติ และจากการเพาะเลี้ยงเซลล์ 21 วัน พบว่ากลุ่มที่มีส่วนผสมของซีรั่มจากมนุษย์มีแนวโน้มแบ่งตัวและเจริญเติบอย่างรวดเร็วเมื่อเปรียบเทียบกับอีกกลุ่มหนึ่ง ผลการแสดงออกของโปรตีนผิวเซลล์ที่จำเพาะต่อเซลล์ต้นกำเนิดมีเซนไคม์และจำเพาะต่อเซลล์ต้นกำเนิดเม็ดเลือดไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างทั้งสองกลุ่ม นอกจากนี้เซลล์ต้นกำเนิดทั้งสองกลุ่มยังมีความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์จำเพาะทั้งเซลล์สร้างกระดูก เซลล์ไขมัน และเซลล์กระดูกอ่อนได้ไม่แตกต่างกัน จึงสรุปได้ว่าอาหารที่มีส่วนผสมของซีรั่มจากมนุษย์สามารถส่งเสริมความสามารถในการแบ่งตัวเองและการเจริญเติบโตของเซลล์ต้นกำเนิดได้ดี โดยที่ไม่ได้ส่งผลให้ลักษณะการแสดงออกของเซลล์ต้นกำเนิดเปลี่ยนแปลงไป

Keywords: Adipose-derived stem cells, Human serum, Fetal bovine serum
คำสำคัญ: เซลล์ต้นกำเนิดจากเนื้อเยื่อไขมัน ซีรั่มจากมนุษย์ ซีรั่มจากตัวอ่อนวัวในครรภ์

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Introduction

Bone tissue engineering is an emerging field that aims to overcome the limitation of conventional treatments of bone defects including oral and maxillofacial deformities. This approach is based on the tissue engineering triad, which was derived from the three major components: scaffolds, cells and signaling substances (Scheller et al., 2009). Many previous studies demonstrated that when combine scaffold with cells, including mesenchymal stem cells (MSCs) from bone marrow, dental pulp and adipose tissue could provide better results in promoting new bone formation (Diomede et al., 2016; Wongsupa et al., 2017a; Wongsupa et al., 2017b; Chen et al., 2018). Over decades, stem cell therapy has become more popular alternative method in the fields of reconstructive surgeries and bone tissue engineering. In principle, the stem cells can be harvested from various intra-oral sources including dental pulp tissue, periodontal ligament and buccal fat pad using non-invasive techniques under local anesthesia (Egusa et al., 2012). Buccal fat pad is a fat tissue, from which adipose-derived stem cells (ADSCs) can be obtained. ADSCs were found to have similar expression of immunophenotyping markers to bone marrow mesenchymal stem cells. Furthermore, under proper inductive conditions, they have ability to differentiated to various cell types, especially bone forming cells which can produce bone matrices similar to the autogenous bone cells (Gimbel et al., 2007). Buccal fat pads have been considered to serve as a suitable intra-oral source of the ADSCs which is available in a large amount of fat tissue that is easily to be harvested in routine intraoral surgical fields, with minimal patient discomfort (Farre-Guasch et al., 2010). The processes of culture and induction of the stem cells are routinely performed in laboratory using specific culture mediums which usually contain fetal bovine serum (FBS). FBS plays the important roles for stem cell viability due to containing high levels of growth factors, hormones, and essential nutrients. However, FBS was reported to transfer approximately 7–30mg of xenogenic proteins per 10^8 MSCs in culture. Moreover, FBS supplemented mediums may pose undesirable risks of immunological reactions from the Anti-FBS antibodies and cross-species pathogen infections such as virus, mycoplasma and prions transmittable to patients in clinical use (Tekkatte et al., 2011). In Thailand, FBS is imported, and it cost approximately 2,000 Baht for 100ml. Human serum has been an alternative to FBS, since it contains sufficient serum proteins, growth factors, growth hormones and essential nutrients for cell metabolism, growth, and proliferation (Gstraunthaler, 2003). The serum preparation is practical, and it requires low-cost basic instruments. The major advantages of autologous human serum (AHS) over allogenic human serum (Allo-HS) are biocompatibility and having no risk of blood-transmitted diseases from other donors. Therefore, culturing the stem cells using AHS from individual patients and re-implanting them back into their own hosts for clinical use would be a cost-saving and safer strategy, resulting in more patient acceptability.

Objectives of the study

The objective of this study was to compare the efficacy of the culture mediums supplemented with AHS and FBS for culturing ADSCs isolated from intra-oral buccal fat pad in terms of colony-forming unit-fibroblast (CFU-F), cell proliferation, mesenchymal stem cell CD markers, and multi-differentiation potential.

Methodology

Patient enrollment

Six volunteer patients, undergone either surgical removal of maxillary impacted third molars or orthognathic surgeries for correcting their skeletal discrepancies in Oral & Maxillofacial Surgery Clinic, Dental hospital, Faculty of Dentistry, Prince of Songkla University, were enrolled in this study. Those patients signed informed consent forms for participation prior to the experiments. The protocol of this study was approved by the Ethics Committee of the Faculty of Dentistry, Prince of Songkla University (EC6012-37-P-LR). The inclusion criteria of the participants included ASA class I, age 20-40 years old, weight >50 kg, and hematocrit ≥35 %. The excluded patients were those with systemic diseases, including hereditary blood diseases, blood and blood components disorders, blood transmitted diseases, and diabetes.

Harvesting fat tissue and blood collection

Buccal fat pad was harvested from each donor after usual manuals of surgical removal of maxillary impacted third molar and orthognathic surgeries. The part of fat tissue which exposed intraorally was excised and immediately stored in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) until used. Fifty milliliters (ml) of venous blood were drawn from each patient and collected into the centrifuge tubes with clot activator (VACUETTE® Serum Clot Activator Tubes, Greiner bio-one, USA). After blood clotting, the tubes were centrifuged at 2,000g for 15 min, and the supernatant of serum from each tube was collected and sterilized through a 0.2 µm-pore syringe filter (Minisart® High Flow Syringe Filter, Germany). Total serum of each patient was recorded, and a portion of serum was used as medium supplement for culturing ADSCs. For later usage, aliquots of the sterile AHS were kept at -20°C.

Isolating ADSCs from fat tissue

After washing with sterile phosphate buffer saline (PBS) to remove contaminating debris and red blood cells, the volume of fat tissue was measured using a 5 ml sterile disposable syringe. The fat tissue was then minced into small pieces and enzymatically digested using 3 mg/ml type I collagenase (Gibco, USA) in PBS at 37°C with gentle agitation for 60 min. The cell pellets were obtained by centrifugation at 1,200g for 10 min. The cell pellets were resuspended in DMEM without being supplemented with serum and filtered through a 100 µm cell strainer (Corning, USA) (Phopetch et al., 2018). The cells in the solution that passed the filter were equally plated into 6-well plates (Corning, USA) and divided into FBS and AHS groups. The cells were cultured in 200 µL of DMEM supplemented with 10% FBS (Gibco, USA) or 10% AHS, with 50 µg/ml penicillin-streptomycin. The cells of both groups

were cultured in a humidified atmosphere with 5% CO_2 at 37°C until reaching 70-80% confluence, and then subculture was performed. The cells of passages 1-5 were used for the following experiments (n=4/group/testing) to determine the characteristics of the cells.

Self-renewal capacity: Colony forming unit fibroblast (CFU-F)

The cells of passages 1-2 of each group were plated in 6 well plates (Corning, USA) at a density of 100 cells/well. After 10 days, the cells were fixed in 4% paraformaldehyde and stained using 0.1% toluidine blue (Sigma, USA). Under a light microscope (Nikon, Japan), numbers of colonies were counted if the colonies consisted of at least 50 cells or were >2 mm in diameters (Wongsupa et al., 2017a).

Flow cytometry analysis

The MSC immunophenotypes of the cells of each group were defined following the International Society for Cellular Therapy (ISCT) protocols (Dominici et al., 2006). The analysis was performed using a fluorochrome-conjugated monoclonal antibody cocktail in the MSC Phenotyping Kit human (Miltenyi Biotec, Germany). In brief, 5 x 10^5 cells from each group in passages 2 and 3 were incubated in the antibodies against the surface antigens CD73, CD90, and CD105 as the positive markers and CD14, CD20, CD34, and CD45 as the negative markers. At least 10,000 events were acquired for each sample using a fluorescent-activated cell sorting instrument (CytoFLEX S, Beckman Coulter, Germany), and the data was analyzed using CytExpert Software for CytoFLEX (Beckman Coulter, Germany).

Cell proliferation assay

The cells of each group at passages 2-3 were seeded into 24-well culture plates (Corning, USA) at 5x10^3 cells and cultured in 300 µl of the medium supplemented with 10%FBS or AHS (n=4/group/time point). On days 1, 3, 7, 14 and 21 after seeding, the amount of the viable cells was measured using resazurin-based solution (PrestoBlue® Cell Viability Reagent, Invitrogen, USA). In brief, the medium of each well was removed and washed using PBS. Two hundred µl of fresh culture medium without serum was replaced into each well, and then 20 µl of PrestoBlue reagent was added. The well plates were incubated for 10 min in 5% CO_2 at 37°C protected from direct light. After the incubation, 100 µl of the medium of each well was transferred into 96-well plate (Corning, USA) in duplicate and the absorbance of each well was read at 600 nm using a microplate reader (Multiskan™Go, Thermo Fisher Scientific). The levels of optical density (OD) were compared with a standard curve to infer the amounts of the cells (Wongsupa et al., 2017a).

Multi-differentiate potential

1) Adipogenic differentiation

The cells of each group were induced to differentiate into adipogenic lineage by using MesenCult™ Adipogenic Differentiation Kit (STEMCELL™, Canada). The cells at passages 3-5 were seeded at 3x10^4 cells/well in 6-well culture plates (Corning, USA) and cultured in 2 ml medium supplemented with 10%FBS or AHS. The cells were incubated in 5% CO_2 at 37°C for approximately 7 days to reach 70-80% confluence. The medium from each well was aspirated, rinsed with PBS, and replaced with 2 ml

of complete MesenCult™ Adipogenic Differentiation Medium. The medium was changed every 3 days until lipid vacuoles were observed under low magnification. On day 14, the cells were fixed in 10% formaldehyde for 30 min. and stained with Oil Red O solution (20mg/mL in isopropanol) (Sigma, USA) for 15 min.

2) Chondrogenic differentiation

The cells of each group were induced to differentiate into chondrogenic lineage by using MesenCult™-ACF Chondrogenic Differentiation Kit (STEMCELL™, Canada). The cells of $5x10^5$ in passages 3-5 were resuspended in 0.5 ml of complete MesenCult™-ACF Chondrogenic Differentiation Medium in 15 ml polypropylene tube. The cells suspension was centrifuged at 300g for 5-10 min at room temperature to form cell pellets. The cap of centrifugation tube was left loosened to allow gas exchange and incubate upright in 5% CO_2 at 37°C. The media was aspirated without disturbing the pellet and replaced with 0.5 ml of complete MesenCult™-ACF Chondrogenic Differentiation Medium every 3 days afterward. On day 21, the chondrogenic pellets were fixed in 10% formaldehyde for 30 min and followed by subsequent standard paraffin embedding methods and Alcian Blue staining.

3) Osteogenic differentiation

The cells of each group were induced to differentiate into osteogenic lineage using MesenCult™ Osteogenic Differentiation Kit (STEMCELL™, Canada). The cells from passages 3-5 were seeded in 24-well culture plates (Corning, USA) at density of $2x10^4$ cells/well and cultured in medium supplemented with 10% FBS or AHS. The cells were incubated in 5% CO_2 at 37°C or reaching 70 - 80% confluence. The medium from each well was aspirated, rinsed with PBS and replaced with 300 µl complete MesenCult™ osteogenic differentiation medium. The medium was changed every 3 days. On day 14, osteogenic differentiation of the cells could be visualized by staining with alkaline phosphatase using a protocol of Alkaline Phosphatase Staining Kit (BioVision, USA) and imaged using an inverted microscope (Zeiss, Germany).

Statistical analysis

The measured parameters were analyzed using statistics analysis software (SPSS, version 23, USA). Student t-test was applied to compare the differences between groups. The level of statistical significance was set at a $P<0.05$.

Results

Six subjects were included in this study. Their demographic data was demonstrated in Table 1. Their average age was 25.17±5.64 years old. The average fat volume was 4.17±0.98 milliliters. The average volume of the autologous serum was 20.92±1.50 ml or 41.83±2.99%/50 ml of blood volume.

Table 1 Demographic data of the subjects.

Case	Age	Gender	Fat volume (ml)	Operation	Blood volume (ml)	AHS volume (ml)	AHS/blood volume (%)
1	24	Male	5	BSSRO setback	50	23	46
2	21	Female	3	Surgical removal on 18	50	20	40
3	23	Female	3	Surgical removal on 28	50	21.5	43
4	36	Female	5	BSSRO Advancement	50	22	44
5	21	Male	4	BSSRO setback	50	19	38
6	26	Female	5	BSSRO setback	50	20	40

Colony forming unit fibroblast

Over 10 days of the observation period, the isolated ADSCs of both groups were able to form colonies from the low seeding density that characterized their self-renewal capacity. The CFU-F of the AHS group occurred earlier and resulted in larger and denser colony formation than that of the FBS group. The numbers of the CFU-F of the AHS group were significantly greater than those of the FBS group (P=0.012) (Figure 1-2).

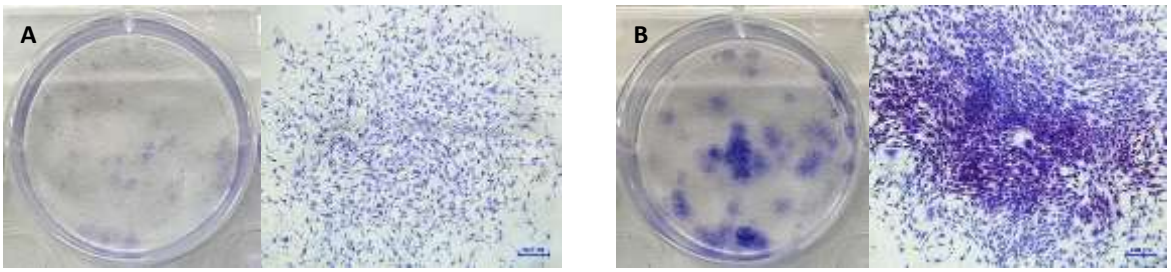

Figure 1 Toluidine blue staining of CFU-F after 10 days of culture (left) and the 5x magnified images (right) demonstrated the morphologies of the CFU-F of Group A: FBS and Group B: AHS.

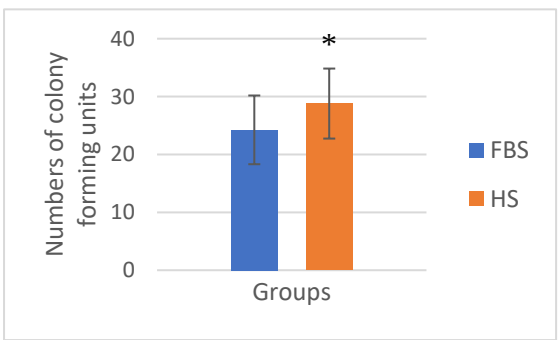

Figure 2 The bar graph demonstrates the numbers of the CFU-F of FBS and AHS groups. (* represents the significant difference between two groups at P<0.05).

Cell proliferation

Profiles of the cell proliferation of both groups were demonstrated in Figure 3. The cells of the AHS group rapidly grew after day 3 and reached the maximum level on day 14, whilst the cells of the FBS group gradually grew and reached the maximum level on day 21. The mean amounts of the cells in AHS group were significantly greater than those of the FBS group on days 14 and 21 (P=0.001, P=0.000 respectively).

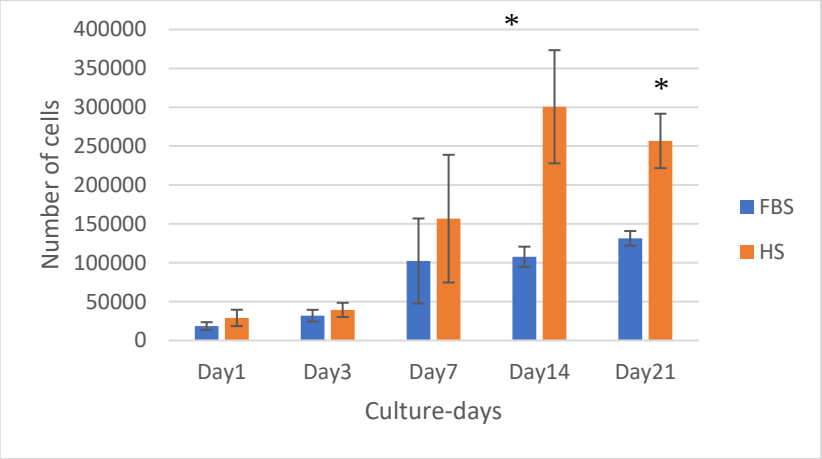

Figure 3 The bar graphs demonstrates the proliferation profiles of FBS and AHS groups over 21 days. (* represents the significant difference between two groups at P<0.05).

Flow cytometry analysis

Expression of the MSC immunophenotypes of the ADSCs was demonstrated in Table 2. and Figure 4. The profiles of the positive markers and negative markers of MSCs between groups were not statistically different (P=0.86, P=0.966, P=0.961, P=0.986, P=0.969 for CD73, CD90, CD105, CD14, 19, 34, 45 and HLA-DR respectively). The cells of both groups expressed CD73 at the highest levels, followed by CD90 and CD105, and they expressed the hematopoietic markers less than 2%.

Table 2 The percentages of immunophenotyping markers were demonstrated.

CD Markers (%)		FBS Group	AHS Group
MSCs markers	CD73	80.12±8.57%	80.96±7.43%
	CD90	66.26±8.17%	66.03±8.39%
	CD105	41.58±8.11%	41.23±13.1%
Hematopoietic markers	CD14, 19, 34, 45	0.16±0.22%	0.16±0.11%
	HLA-DR	0.42±0.14%	0.42±0.2%

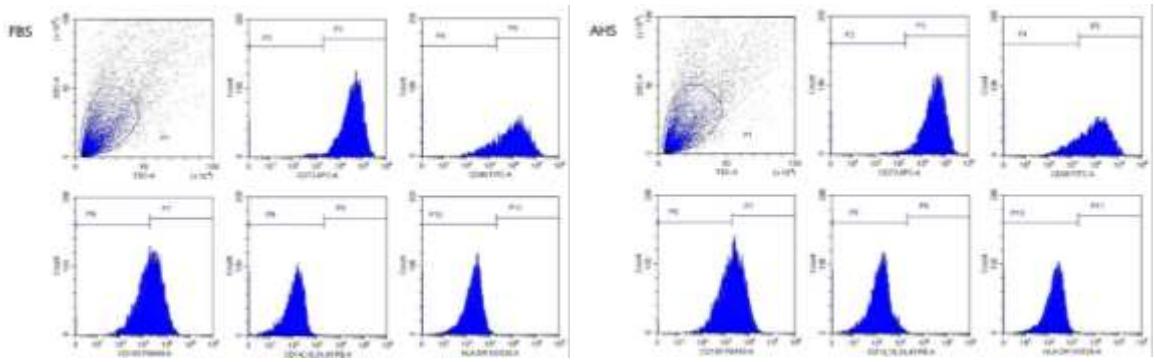

Figure 4 The images of flow cytometry analysis demonstrated the profiles of the MSC markers and the hematopoietic markers of FBS and AHS groups.

Multi-differentiate potential

1) Osteogenic differentiation

After 14 days of cell culturing in osteogenic induction medium, positive alkaline phosphatase staining was detected in the cells of both groups (Figure 5).

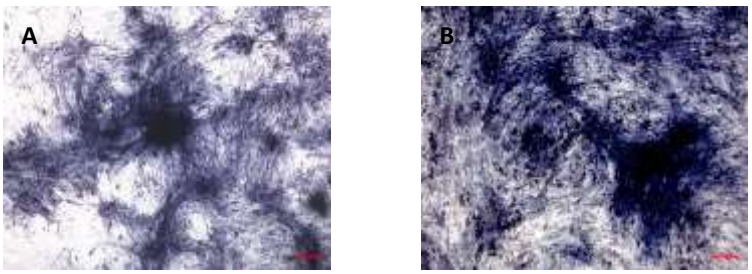

Figure 5 The ALP staining images at 5X magnification of Group A: FBS and Group B: AHS demonstrate in dark bluish-purple precipitation representing increase of alkaline phosphatase activity.

2) Adipogenic differentiation

After 14 days of cell culturing in adipogenic induction medium, Oil red O staining on the lipid droplets in the cytoplasm of the cells revealed well adipogenic differentiation potential of both groups (Figure 6).

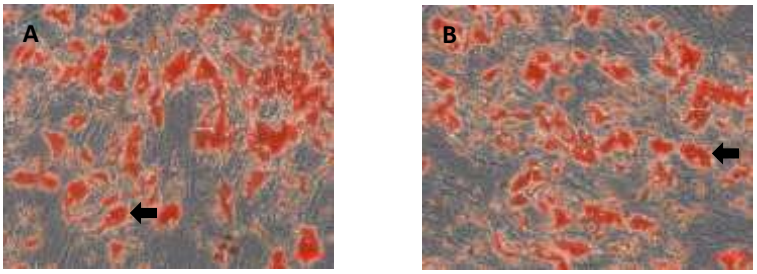

Figure 6 Magnified images at 20X of Oil Red O staining of Group A: FBS and Group B: AHS demonstrate red staining of lipid droplets in cytoplasm of the cells (black arrows).

3) Chondrogenic differentiation

After 21 days of cell culturing in chondrogenic induction medium, the cell pellets of both groups exhibited blue staining of an Alcian blue on the glycosaminoglycans (GAGs) in the extracellular matrix (ECM) (Figure 7).

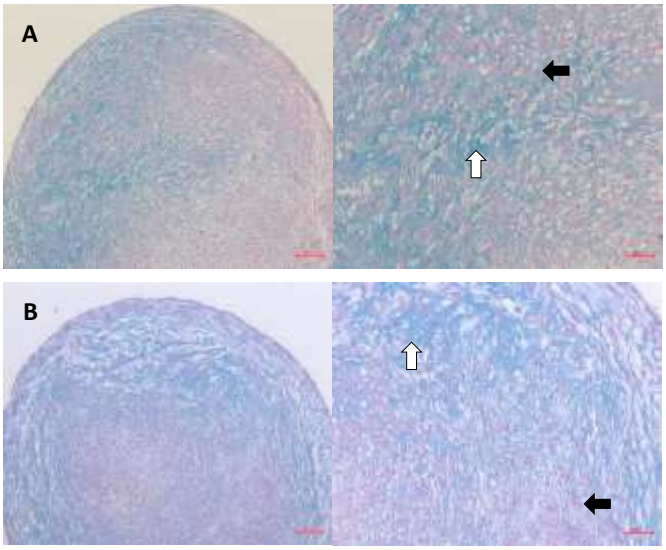

Figure 7 The magnified histological images of the Alcian blue stained pellets at 10X (left) and 20X (right) of Group A: FBS and Group B: AHS demonstrate the blue staining of GAGs deposition in the ECM (white arrows) and the chondroblast-like cells with large nuclei in vacuoles were seen (black arrows).

Discussion and Conclusions

To optimize cell-based therapies, small amount of intially isolated stem cells from original tissue sources usually requires appropriate culture and expansion condition to achieve enough cells. With this regard, a suitable culture medium which can substantially promote cellular proliferation while provide safety in clinical application must be concerned. The processes of culture and induction of the stem cells are routinely performed in laboratory using conventional culture mediums which usually contain FBS. However, FBS has been reported being prone to transmit some diseases and possible to give rise to immunologic reactions due to contamination with bovine proteins (Tekkatte et al., 2011). To avoid exposing animal xenogenic components to the cells, using alternative non-xenogeneic serums have been investigated. Currently, there are several literatures that have investigated efficacy of the non-xenogeneic serums from various sources on culturing MSCs including human serum, human platelet derivatives, and serum-free media (Agata et al., 2009; Shanbhag et al., 2017). Among those, either human serum or platelet derivatives seem to be superior to the serum-free medium because of presence of more exogenous growth factors which are neccessary for growth and differentiation of cells. In addition, biocompatibility of individual growth factors, proteins and hormones on human cells is considered to be the major advantage of AHS (Fani et al., 2016; Shahdadfar et al., 2005). So far, the effects of AHS on stem cells isolated from various tissue sources have been investigated. Regarding the sources of ADSCs,

adipose tissue from lipoaspirates and discarded fat tissue during plastic surgery, but not yet buccal fat pads are examined. Therefore, in this study, effect of AHS supplemented medium on characteristics of the ADSCs isolated from the buccal fat pads were assessed. Regarding our previous study, the result demonstrated that expression of the MSC immunophenotypes and osteogenic differentiation capacity of the ADSCs isolated using CD271+ Magnetic-activated cell sorting technique and plastic adherence method were not statistically different (Phopetch et al., 2018). Therefore, the plastic adherence, which is a standard method for isolating the MSCs from human tissue (Bruder et al., 1997), was used for extracting the ADSCs from the buccal fat tissue in this study. The average volume of AHS in this study was 20.92±1.50 ml from 50 ml of blood volume. It is possible that serum from different donors has various amounts of growth factors, hormones, proteins, and nutrients which are essential for proliferation and differentiation of ADSCs. Therefore, these variations can be minimized by collecting serum from a large pool of donors. Despite improvement in the serum quality, the allo-HS still has risk of sensitization from blood group substances, necessitating the recruitment of multiple AB blood type donors (Tekkatte et al., 2011; Kocaoemer et al., 2007). Thus, AHS from healthy individuals can be promptly used for cell culture expansion with ensured safety when considering re-implanting cells back to host. CFU-F is one of the essential properties of the MSCs, representing the self-renewal capacity of the cells. Regarding the results of this study, the cells which cultured in the AHS mediums demonstrated better self-renewal capacity in terms of the numbers of CFU-F number when compared with those cultured in the FBS medium. By observation, the colonies of AHS group were larger and denser than those of the FBS group. This implied that types of serum might relate to the number of cells forming per colony. This result corresponded to a previous study that bone marrow was collected from the iliac by aspiration and cultured in medium supplemented with 10% AHS or 10% FBS. The authors found that the number and area of the colonies of the fibroblast-like cells in the AHS medium were larger than those in the FBS medium (Yamamoto et al., 2003). The cell proliferation profiles revealed that the growth of the cells of the AHS group was significantly better than that of the FBS group. The highest number of cells proliferating in the AHS group reached 300,617±72,774 cells on day14, whereas the cells of FBS group reached 131,350±9,446 cells on day 21. This meant that AHS could accelerate the cell proliferation and reduce the amount of time spent on culturing the cells. Nimura et al., (2008) indicated that increase of cell proliferation when cultured in AHS medium was due to greater sizes of colony-forming. Regarding the cell differentiation, the results demonstrated that ADSCs could differentiate into three lineages of osteogenesis, adipogenesis and chondrogenesis. These results were consistent with several previous studies, which found that AHS is superior to FBS in terms of promoting proliferation of MSCs while retaining their osteogenic, adipogenic and chondrogenic differentiation potential. Some studies that assessed the effect of AHS on human bone marrow mesenchymal stem cells demonstrated that the culture medium containing 10% AHS (Stute et al., 2004; Kobayashi et al., 2005) 20% AHS (Shahdadfar et al., 2005) enhanced proliferation of the cells better than the FBS medium. Likewise, similar results had

been found in studies of MSCs from synovial membrane (Nimura et al., 2008), dental pulp (Ferro et al., 2012) and subcutaneous fat tissue (Josh et al., 2012). In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) had defined human MSCs to universally standardize the MSC characterization by proposing 3 minimal criteria: the cells must be plastic-adherent to culture flasks, display positive surface antigen CD73, CD90 and CD105 ≥95% and express negative surface molecules CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR ≤2%, and able to differentiate to osteoblasts, adipocytes and chondroblasts in vitro (Dominici et al., 2006). Our results demonstrated that the cells of both groups positively expressed CD73, CD90, and CD105, while the negatively expressed hematopoietic markers were less than 2%. It was noted that the positive cell numbers for MSCs were less than the ISCT criteria. This could be explained that the plastic adherence method utilized for isolating ADSCs from buccal fat tissue might not provide a homogeneous mesenchymal population (Mitchell et al., 2006; Oberbauer et al., 2015). However, such amount of stem and progenitor cells of both FBS and AHS groups obtained through this method did not limit the ability to self-renew, proliferate or multi-differentiate. It can be concluded that the AHS medium could support self-renewal capacity and growth of the ADSCs better than the FBS medium without altering their immunophenotypes and still retain their multipotentiality for differentiation. Therefore, it is possible to use AHS supplemented medium as a standard protocol for culturing stem cells in clinical practice.

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