

Good manufacturing practice-compliant isolation and culture of human adipose-derived stem cells

Phuc Van Pham^{1,2,*}, Ngoc Bich Vu¹, Nhan Lu-Chinh Phan¹, Dung Minh Le¹, Nhat Chau Truong¹, Nhung Hai Truong^{1,2}, Khanh Hong-Thien Bui³, Ngoc Kim Phan^{1,2}

¹Laboratory of Stem Cell Research and Application, University of Science, Vietnam National University, Ho Chi Minh city, Viet Nam.

²Laboratory of Animal Physiology and Biotechnology, Biology Faculty, University of Science, Vietnam National University, Ho Chi Minh city, Viet Nam.

³University of Medical Center, Ho Chi Minh University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam.

*Corresponding author: pvphuc@hcmuns.edu.vn

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Abstract— Adipose-derived stem cells (ADSCs) are excellent for regenerative medicine. Like mesenchymal stem cells, ADSCs possess multi-potent differentiation capacity that enables them to differentiate into osteoblasts, chondrocytes and adipocytes, as well as trans-differentiation into other cells. ADSC transplantation has gained attention in recent years, especially *in vitro* expanded ADSC transplantation. This study aimed to provide a new method to *in vitro* primarily culture and secondary culture of ADSCs that were compliant with good manufacturing practice for clinical applications. Stromal vascular fraction (SVF) was extracted from adipose tissue by commercial kits. SVF was expanded *in vitro* in medium with non-allogeneic supplements. Cultured ADSCs maintained immune-phenotype, karyotype, and differentiation potential after ten passages. Moreover, ADSCs at 15th passage could not form tumors in NOD/SCID mice. This research produced a suitable protocol for clinical applications of expanded ADSCs.

Keywords— Adipose derived stem cells; Stem cell transplantation; ADSC transplantation; ADSC application; Stem cell culture

INTRODUCTION

ADSCs have been used widely in many clinical fields. To date, in almost all studies, ADSCs are used as non-expanded MSCs, also called the stromal vascular fraction (SVF), and expanded ADSCs. However, some studies show that the percentage of ADSCs in the SVFs is low (Minonzio et al., 2014; Van Pham et al., 2013b). Therefore, ADSC expansion is essential to enrich and purify the ADSC population. These expanded ADSCs exhibit the MSC phenotype (Bourin et al., 2013; Zimmerlin et al., 2013), as is evidenced by the expression of MSC markers such as CD44, CD73, CD90 and CD105. These cells also successfully differ-

entiate into adipocytes, osteoblasts, and chondrocytes, and could be trans-differentiated into other mesoderm cell types such as insulin producing cells (Karaoz et al., 2013; Moshtagh et al., 2013), hepatocytes (Aurich et al., 2009; Lee et al., 2012; Okura et al., 2010), and neuronal-like cells (Cardozo et al., 2010; Rezanejad et al., 2014).

Recent reports showed that expanded ADSCs gave the better results compared to non-expanded ADSCs. Expanded ADSCs successfully improved in some diseases such as cranio-maxillofacial hard-tissue defects

(Sandor et al., 2014), non-revascularizable critical limb ischemia (Bura et al., 2014), acute myocardial infarction and heart failure (Panfilov et al., 2013), complex perianal fistula in Crohn's disease (de la Portilla et al., 2013; Garcia-Olmo et al., 2009), and chronic myocardial ischemia (Qayyum et al., 2012). Some *in vitro* culture procedures of ADSCs were suggested. ADSCs were first cultured by Zuk *et al.* (2002) in a medium that contained fetal bovine serum (FBS) (Zuk et al., 2002). Other studies successfully isolated multipotent stem cells from adipose tissue (Eom et al., 2011; Tunaitis et al., 2011). However, the FBS used in these procedures represented a significant limitation for the use of these ADSCs in clinical applications.

Recent studies have showed that PRP, which is a pool of growth factors, can replace FBS when culturing MSCs. For instance, umbilical cord blood-derived MSCs were successfully isolated and cultured in the IMDM medium containing 5–10% PRP (Pham et al., 2014; Van Pham and Kim Phan, 2014). Furthermore, PRP was successfully used to culture bone marrow-derived MSCs (Griffiths et al., 2013; Mojica-Henshaw et al., 2013; Pawitan, 2012) as well as ADSCs (Atashi et al., 2014; Pawitan, 2012; Tavakolinejad et al., 2014). In fact, PRP contains a pool of at least seven growth factors (GFs), including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF) and keratinocyte growth factor (KGF). The high concentration of these growth factors gave the therapeutic effects compared to normal plasma.

This study aimed to develop a novel ADSC culture procedure that was free of FBS as well as xenogeneic and allogeneic proteins. Identification of such conditions would satisfy the GMP guideline for clinical use of stem cells.

MATERIAL AND METHODS

Adipose tissue collection

All patients enrolled in this study were required to sign a consent form. All procedures used in the study were approved by the ethical committee of the hospital. Five adipose tissue samples were used in this

study. Fat tissue was collected from the belly by aspiration with a suitable needle. For this, approximately 50–100 mL of lipoaspirate was collected from each patient in two 50 mL sterile syringes. The syringes were stored in a sterile box at 2–8°C and were immediately transferred to the laboratory.

Isolation of the SVF from adipose tissue

The SVF was isolated from adipose tissues using an ADSC Extraction Kit (GeneWorld, Ho Chi Minh City, Vietnam) according to the manufacturer's instructions. Briefly, 50–100 mL of lipoaspirate was placed in a sterile disposable 250 mL conical centrifuge tube (Corning, Tewksbury, MA) and washed in Washing Buffer 1 by centrifugation at 1000 *g* for 5 min at room temperature. Next, the adipose tissue was washed with Washing Buffer 2 as per the procedure for Washing Buffer 1. Then the adipose tissue was digested using SuperExtract Solution containing collagenase at 37°C for 45 min with agitation at 5 min intervals. The cell suspension obtained was centrifuged at 3000 *g* for 10 min, and the SVF was obtained as the pellet. The pellet was washed with Washing Buffer 3 to remove any residual enzymes, and resuspended in PBS for the determination of cell quantity and viability using an automatic cell counter (NucleoCounter; Chemometec, Denmark).

Activated PRP preparation

Activated PRP was derived from the peripheral blood of the same patient as the adipose tissue using a New-PRP Pro Kit (GeneWorld, Ho Chi Minh City, Vietnam) according to the manufacturer's guidelines. Briefly, 20 mL of peripheral blood was collected into three vacuum tubes and centrifuged at 800 *g* for 10 min. The plasma was collected and centrifuged at 1000 *g* for 5 min to obtain a platelet pellet. Plasma in the upper layer was then removed, leaving 3 mL of plasma at the bottom of tube. Finally, PRP was activated using activating tubes containing 100 μ L of 20% CaCl₂.

Primary culture

Five SVF samples were used for primary culture. SVF samples were cultured in MSCCult medium that contained DMEM/F12 supplemented with antibiotic-antimycotic, EGF, basic fibroblast growth factor (bFGF) with 10% aPRP or 10% FBS to act as the control. This chosen concentration was based on the findings of a previous publication. The cells were plated at 5×10^4 cells/mL in T-75 flasks (Corning) and incubated

at 37°C with 5% CO₂. After 3 days of incubation, 6 mL of fresh media were added to each flask. After 7 days, the media were replaced with 12 mL fresh media. The media was subsequently replaced every 3 days until the cells reached 70–80% confluence, where they were subcultured.

Secondary culture

The proliferation rate of SVF secondary cells was evaluated by the eXCELLIGENCE system (Roche Applied Science, Indianapolis, IN, USA). Specifically, cells were seeded at 1×10^3 cells/well into a 96-well E-plate in triplicate. The culture plates were placed into the eXCELLIGENCE system and incubated at 37°C with 5% CO₂. This chosen concentration was like the previous result. In contrast to the primary culture medium, the secondary culture medium was MSCCult supplemented with 5% aPRP. The cell doubling time and slope were calculated using the software associated with the eXCELLIGENCE system.

Flow cytometry

Cell markers were analyzed following a previously published protocol. Briefly, cells were washed twice in PBS containing 1% bovine serum albumin. The cells were then stained with anti-CD14-FITC, anti-CD34-FITC, anti-CD44-PE, anti-CD45-FITC, anti-CD73-FITC, anti-CD90-PE antibodies (all purchased from BD Biosciences, San Jose, CA, USA). Stained cells were analyzed by a FACSCalibur flow cytometer (BD Biosciences). Isotype controls were used in all analyses.

In vitro differentiation

For differentiation into adipogenic cells, ADSCs were treated as described previously. Briefly, cells were plated at 1×10^4 cells/well in 24-well plates. At 70% confluence, the cells were cultured for 21 days in IMDM containing 0.5 mM 3-isobutyl-1-methyl-xanthine, 1 nM dexamethasone, 0.1 mM indomethacin, and 10% FBS (all purchased from Sigma-Aldrich). Adipogenic differentiation was evaluated by observing lipid droplets in cells under a microscope.

For differentiation into osteogenic cells, ADSCs were plated at 1×10^4 cells/well in 24-well plates. At 70% confluence, the cells were cultured for 21 days in IMDM containing 10% FBS, 1×10^{-7} M dexamethasone, 50 μM ascorbic acid-2 phosphate, and 10 mM β-glycerol phosphate (all purchased from Sigma-

Aldrich). Osteogenic differentiation was confirmed by Alizarin red staining.

Karyotyping

Secondary cells at the 15th passage were treated with 0.10 μg/ml colcemid for 24 hours. Cells were harvested by trypsinization, and subsequently incubated in a hypotonic solution for 1 h at 37°C. The cells were then fixed at least 3 times in Carnoy's solution, which included an overnight fixative step. The fixed cell suspension was placed onto prepared slides, and dried at 60°C for 3 h. These slides were used for G-banding staining. Chromosomes were visualized using Ikaros software (MetaSystems, Altusheim, Germany) in the upright microscope (A2 Image, Carl Zeiss, Germany).

Tumorigenicity assay

The tumorigenicity of ADSCs at third and tenth passage was examined in athymic nude mice. All manipulations with the mice were approved by the Local Ethics Committee. Each mouse was injected subcutaneously with 5×10^6 ADSCs (three mice per group). As a positive control, mice were also injected with breast cancer cells at a different site. Tumor formation in mice was followed for one month.

Statistical analysis

Significant differences between mean values were assessed by t-tests and analysis of variance (ANOVA). A P-value of <0.05 was considered statistically significant. All data were analyzed by Prism 6 software.

RESULTS

ADSCs exhibited the MSC phenotypes

ADSCs cultured in MSCCult supplemented with either FBS or PRP adhered to the flask surface after 3 days, and exhibited a stromal phenotype that was similar to fibroblasts. These cells continuously proliferated to reach 70–80% confluence after 10 days when cultured in the MSCCult medium supplemented with PRP (**Fig. 1**), after 15 days when cultured in the MSCCult medium supplemented with FBS. These cells were sub-cultured and passaged five times, after which they were used to determine the presence of MSC surface markers. The results are presented in **Fig.**

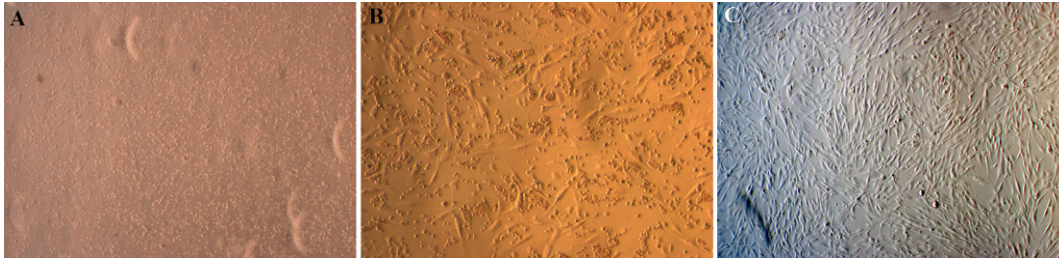


Figure 1. ADSCs appeared in the flask surface and exhibited the fibroblast like cells when cultured in medium plus PRP. SVFs were cultured at Day 0 (A), after 48h (B), and sub-cultured (C). ADSCs with fibroblast like cells adhered to flask surface after 48h. Magnification 100X.

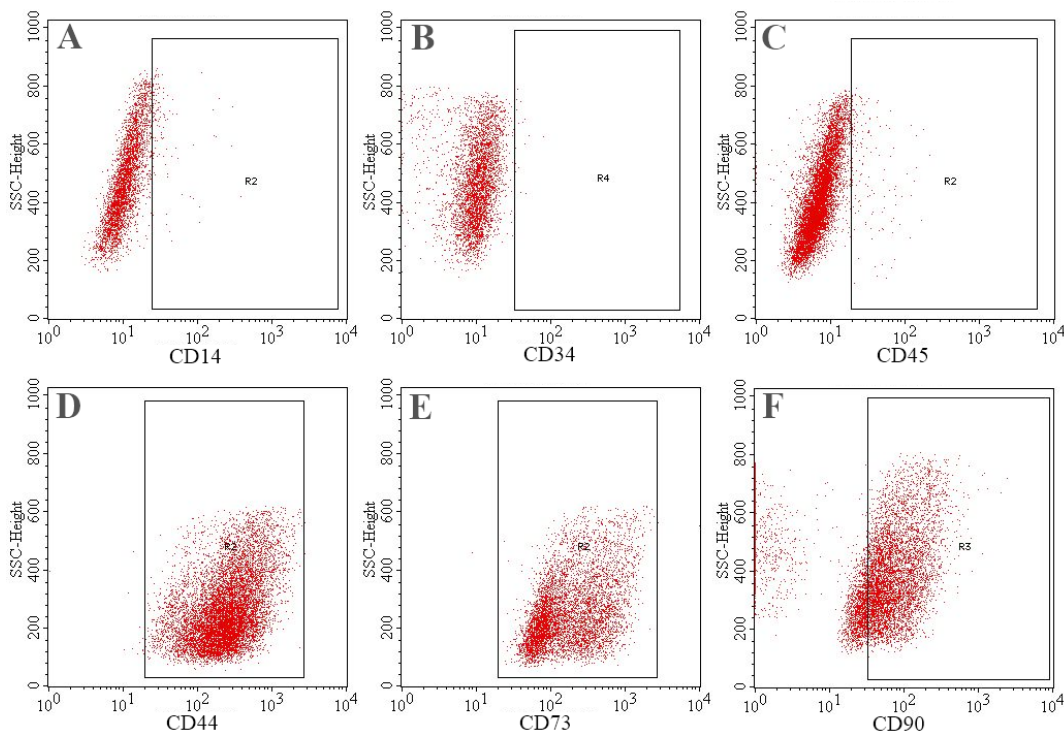


Figure 2. ADSCs exhibited the particular markers of MSCs. They did not express hematopoietic cell markers such as CD14 (A), CD34 (B), and CD45 (C) while they strongly expressed mesenchymal stem cell markers included CD44 (D), CD73 (E), and CD90 (F).

2. Cells cultured in both media exhibited several MSC markers, which included CD44 (99.12%), CD73 (99.81%), CD90 (95.08%) while other markers were not prevalent in the ADSC populations, including CD14 (1.35%), CD34 (1.02%), and CD45 (1.32%) (**Fig. 2**).

ADSCs maintained the differentiation potential

ADSCs successfully differentiated into three different mesoderm layers, including adipocytes, and osteoblasts. In the adipogenesis medium, ADSCs in both

PRP-medium and FBS-medium groups started to accumulate lipid droplets in the cytoplasm by day 14. These lipid droplets gradually increased in size and were clearly recognized in an inverted microscope at day 21. These drops were confirmed as lipid when they were positive with Oil Red dye (**Fig. 3**). In the osteogenesis medium, ADSCs rapidly changed their shape to become similar in morphology to osteoblasts, and enhanced their matrix protein production. These cells were positive for Alizarin red (**Fig. 3**).

ADSCs rapidly proliferated in the FBS free medium compared to FBS medium

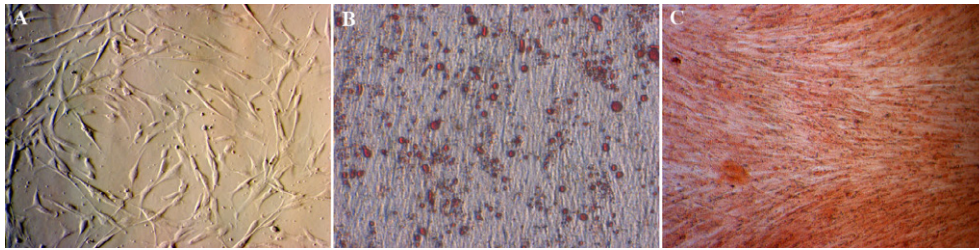


Figure 3. ADSCs cultured in PRP supplemented medium could differentiate into adipocytes and osteoblasts. ADSCs successfully differentiated into adipocytes that positive with Oil red (B); osteoblasts that positive with Alizarin red (C) compared to the control (A). Magnification 200X.

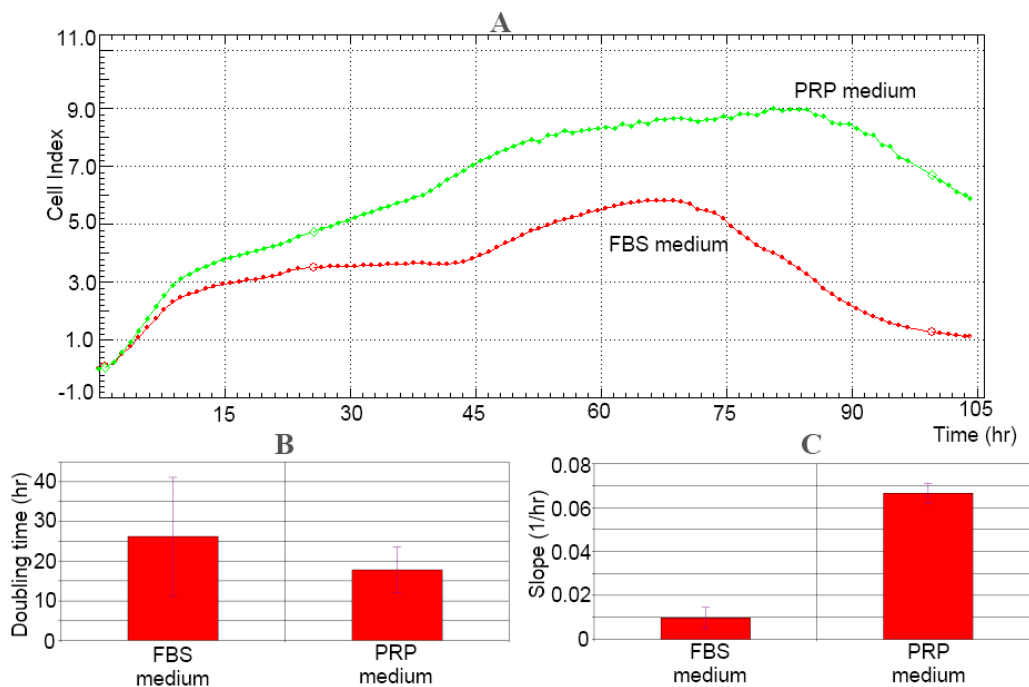


Figure 4. ADSCs rapidly proliferated in the PRP supplemented medium compared to FBS supplemented medium. The cell proliferation rates of both groups were clearly different after 48h (A). Doubling time (B) and slope value (C) were significantly different between two groups.

As shown in the Fig. 4A, ADSCs in the PRP-supplemented medium significantly increased compared to in FBS-supplemented medium. After early 24 h, the proliferation curve was non-significantly different in both groups. However, ADSCs in medium supplemented with PRP began to proliferate faster after 24 h when compared to those cells grown with FBS supplementation. The differences in cell proliferation between the two groups were more distinct after 72 h. These results were confirmed in doubling time (Fig.

4B) and slope values (Fig. 4C) analysis. The doubling time of ADSCs supplemented with PRP was significantly reduced as compared to that of those cells sup-

plemented with FBS. Conversely, the slope value for those cells supplemented with PRP was significantly higher than those cells treated with media plus FBS.

ADSCs maintained the karyotypes after 15th passages

ADSCs cultured in PRP-supplemented medium at 15th passage were used to analyze karyotypes. The results showed that cells at 15th passages exhibited normal karyotypes ($2n = 46$) (Fig. 5).

ADSCs could not cause tumors in the mouse

Cultured ADSCs at 15th passage were injected to NOD/SCID mice to determine their tumorigenicity. The results indicated that these ADSCs do not cause tumors at high doses (10^6 – 10^7 cells). While injection of an equal amount of breast cancer cells formed tumors in the NOD/SCID mice. These results were similar to ADSCs cultured in the FBS-supplemented medium.

factor, basic fibroblast growth factor, platelet derived growth factor... These growth factors were demonstrated as stem cell factors that efficiently stimulate stem cell proliferation. In addition to the use of PRP-supplemented medium, xeno- and allogenic protein contamination was reduced by replacing trypsin (used to harvest adherent cells) that originated from bovine or porcine pancreas by Tryp-LE solution.

By applying the above changes to ADSC culture conditions, we successfully produced ADSCs that would

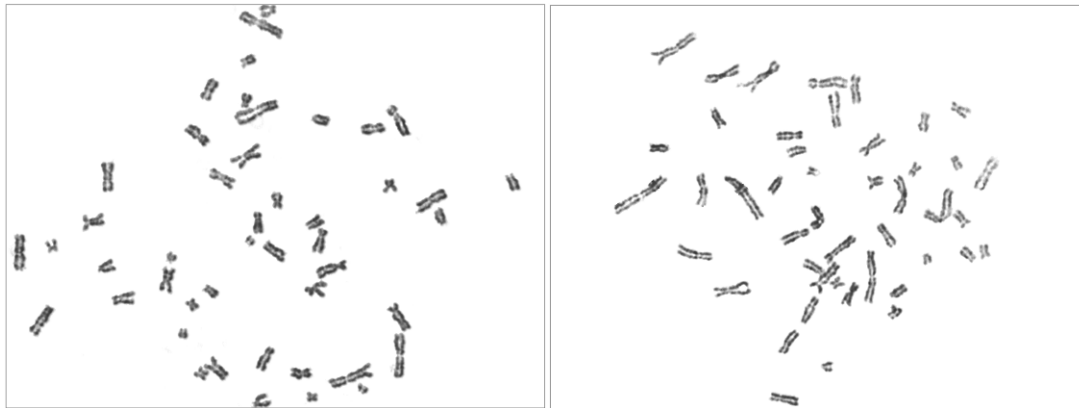


Figure 5. ADSCs exhibited the normal karyotypes at 15th passage.

DISCUSSION

ADSCs have been used in clinical applications since 2010, including their use in the treatment of at least ten diseases (Van Pham, 2014). More importantly, ADSC transplantation exhibited the clinical improvements in almost treated diseases. However, to facilitate clinical applications of ADSCs, the procedure for culturing ADSCs should satisfy the clinical standard, and comply with GMP. This article provided a procedure to isolate and culture ADSCs for clinical use.

The present limitation for the use of ADSCs in clinical applications is the fact that cultured cells contain FBS. To overcome this obstacle, this study replaced the FBS by autologous PRP in the culture medium. Therefore, it holds some limitations for clinical usages such as bovine protein contamination, viral transfection... and quality of FBS changing every batch. Activated PRP is an autologous product from the same patient that contains an important pool of growth factors with more than seven different kinds included epidermal growth

be suitable for clinical use. The ADSCs obtained from these procedures maintained the ADSC phenotype after the 13th subculture. Furthermore, we recorded that ADSCs expressed the MSC markers CD44, CD73, and CD90; and did not express markers for other lineages such as CD14 (monocytes), CD34 (hematopoietic stem cells), CD45 (leukocytes). These cultured ADSCs also maintained their differentiation potential, and were able to differentiate into adipocytes and osteoblasts... With these phenotypes, obtained ADSCs satisfied the minimal standard depicted by Dominici *et al.* (Dominici *et al.*, 2006). Similar studies have been successful in replacing FBS with PRP in MSC culture conditions, including those from as bone marrow (Amable *et al.*, 2014; Zou *et al.*, 2014), and umbilical cord blood (Pham *et al.*, 2014; Van Pham and Kim Phan, 2014).

The greatest risk of using cultured MSCs for the treatment of disease is related to tumorigenesis that may occur after long-term culture. This procedure proved that ADSCs cultured after 15th passage main-

tained the normal karyotype under these culturing conditions. Furthermore, these ADSCs did not cause tumor growth in NOD/SCID mice. This *in vivo* assay is a gold standard to evaluate stem cell tumorigenesis in preclinical trials. These results are also supported by several previous studies where ADSCs cultured in FBS showed that they were safe and did not cause tumorigenesis (MacIsaac et al., 2012; Zaman et al., 2014). Moreover, umbilical cord blood-derived MSCs cultured in PRP also failed to cause tumors (Pham et al., 2014).

Finally, ADSCs that were cultured in medium plus aPRP, not only maintained the ADSC phenotype, but also stimulated ADSC proliferation. By xCelligence analysis, ADSCs in the PRP-supplemented media possessed a significantly higher proliferation rate as compared to those cells grown in media supplemented with FBS. Moreover, PRP reduced the ADSCs doubling time, and increased the slope value for cell proliferation. Similar effects were also observed in previous studies on ADSC culture (Kocaoemer et al., 2007; Koellensperger et al., 2014; Van Pham et al., 2013a) and in other MSC culture (Iudicone et al., 2014; Pham et al., 2014). The increased proliferation rate is thought to originate from possible combinatorial effects of the growth factor pool within PRP.

CONCLUSION

ADSCs are important adult stem cells. Cultured ADSC transplantation has gradually increased over the past five years. This article provided a reproducible protocol for the production ADSCs compliant with GMP. ADSCs were successfully cultured in DMEM/F12 supplemented with 5% aPRP, 1% antibiotic-antimycotic and subcultured by Tryp-LE for 15 passages. ADSCs at the 15th passage showed the MSC phenotype, and expressed important MSC markers such as CD44, CD73, and CD90. Non-expression of CD14, CD34, and CD45. The cultured ADSCs were also successful in differentiating into adipocytes and osteoblasts. Moreover, these ADSCs maintained the normal karyotype and did not cause tumors in the NOD/SCID mice. These findings illustrated that this procedure can be applied to produce ADSCs for clinical application or future studies.

ACKNOWLEDGMENT

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ABBREVIATIONS

ADSCs: Adipose-derived stem cells; EGF: epidermal growth factor; FBS: fetal bovine serum; FGF: fibroblast growth factor; GFs: growth factors; IGF: insulin-like growth factor; KGF: keratinocyte growth factor; NOD/SCID: non-obese/ severe combined immunodeficiency; PDGF: platelet-derived growth factor; PRP: platelet rich plasma, SVF: stromal vascular fraction; TGF- β : transforming growth factor beta; VEGF: vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

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