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Phenotypic and cytogenetic characterization of expanded adipose derived stem cells

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ABSTRACT

Introduction: Human adipose derived stem cells (hASCs) have great potential for regenerative medicine. The demand for hASCs, especially in the development of off-the-shelf products, is increasing. Although the initial receipt of hASCs was relatively limited, there is now greater interest and also awareness that *in vitro* expansion of hASCs be further explored. The purpose of this study was to assess the integrity of mesenchymal cell characteristics and the mutant capability of chromosome number on hASCs undergoing *in vitro* expansion.**Methods:** In this study, three hASC samples from three Vietnamese people were collected and proliferated in MSCCult medium (Regemedlab, Ho Chi Minh City, Viet Nam) to the 5^{th} cell passage. Next, hASCs were evaluated for change of mesenchymal stem cells (MSCs) characteristics including shape, immunophenotype (CD14, CD34, CD44, CD73, CD90, and/or CD166), and trilineage differentiation ability. Finally, the number of chromosomes after passages 1, 3, and 5 was evaluated by karyotyping technique.**Results:** The results showed that after five passages of culture, hASCs preserved the characteristic shape of MSCs, high expression of mesenchymal markers (e.g. CD44, CD73, CD90, and CD166). However, the cells also maintained their differentiation capacity to develop into various tissues such as bone, cartilage, and fat. The hASCs showed no mutation in the number of chromosomes. However, markers of hematopoietic cells (such as CD14 and CD34) exhibited heterogeneous changes between the samples during proliferation.**Conclusion:** In conclusion, at passage 5, hASCs retained the integrity of MSC features and there was no mutation discovered in the number of chromosomes. However, further evaluation is needed to conclude that the use of cultured cells in treatment is effective and safe.

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1. Background

The term ”stem cell” was first coined by Ernst Haeckel in 1868 Ramalho-Santos and Willenbring (2007). By 1956, the first marrow transplant was performed by E. Donnall Thomas (Nobel Prize, 1990) 242 (2018b). In the 1970s, Friedenstein discovered mesenchymal stem cells (MSCs) in bone marrow (BM-MSCs) Mafi et al. (2011). Since then, the number of MSC-related studies has increased dramatically due to the superior properties of MSCs, which include high self-renewal, ability to immunomodulate, and ability to differentiate into mesodermal, ectodermal and endodermal lineage Marion and Mao (2006); Gnecchi and Melo (2009); Piryaei et al. (2011); Bossolasco et al. (2005). In 1980, Plaas and Cryer found a new MSC source — adipose-derived stem cells (ASCs)- which also showed great potential Plaas and Cryer (1980).

ASCs are derived from the abundant fatty tissue removed during liposuction. While there are many advantages of BM-MSCs, one major advantage of ASCs (compared to BM-MSCs) is that its acquisition is less invasive and extensive (compared to acquisition from bone marrow). ASCs fully meet the ideal standards of therapeutic applications for regenerative medicine Frese et al. (2016); Gimble (2003). Besides its applications in regenerative medicine, MSCs can also support the delivery of drugs, nanoparticles, and micro-RNA to targets, such as tumors Sherman et al. (2017). Currently, there are approximately 170 clinical studies pertinent to human adipose derived stem cells (hASCs) that are listed on the National Institutes of Health website 242 (2018a).

In Viet Nam, stem cell research is slowly evolving from the bench to the bed. Several MSC transplants, such as the use of hASCs for treatment of knee osteoarthritis, have been performed at Bach Mai Hospital (Hanoi, Viet Nam) and Van Hanh General Hospital (Ho Chi Minh City, Viet Nam) with very optimistic results. The need of hASC for transplant as well as for research has risen. In addition, the next evolution of stem cell therapy is the concept of stem cell drug. Stem cell drugs have high homogeneity but do not depend on people, and is adopted by the National Institutes of Health as a medicine which can be delivered to patients in the form of off-the-shelf products Pham (2016). While the number of primary hASCs is limited Eom et al. (2011), a large-scale proliferation of these cells under *in vitro* conditions is essential to provide the adequate quantity for treatment and stem cell production.

However, as it is currently, stem cell therapies (in general) and off-the-shelf products (in particular) are experiencing some difficulties. Specifically, there is no accurate description of standardization and characterization of pre-transplanted cells OB́rien and Barry (2009). Indeed, researchers are still worried and cautioned about the role of MSCs in tumor formation Frese et al. (2016); Sherman et al. (2017). Many scientific studies have shown that chromosomes of hASCs in long-term *in vitro* culture are not mutated Dominici et al. (2006); Zhu et al. (2010). Meanwhile, some studies have yielded opposite findings Ramalho-Santos and Willenbring (2007); Mafi et al. (2011); Sherman et al. (2017). According to Pan *et al*. (2014), MSCs are likely to undergo transformation when culture is very rare and usually only after a long period of time (5 weeks) Pan et al. (2014). Wang *et al*. (2013) reported that long-term cultures of MSCs led to some abnormalities but did not undergo malignant transformation Wang et al. (2013). In 2016, Lechanteur *et al*. have expanded 68 samples of MSCs from 59 donors over 4 weeks. All samples met the standards of the European Group for Blood and Marrow Transplantation (EBMT) and International Society for Cellular Therapy (ISCT) Lechanteur et al. (2016). However, many countries, including Viet Nam, are still very cautious when applying these potentially beneficial stem cells to treat human diseases. Clinical studies have so far rarely used hASCs in *in vitro* culture due to concerns about safety when transplanted into the human body. Thus, it is important to assess the quality of the hASCs by evaluating

MSC characteristics and the stability of the chromosome number, among other criteria. Characterization of hASCs can indicate the clinical potential and utility of the cells.

2. Methods

2.1 Materials

Three samples of hASCs and MSCCult medium were provided by the Laboratory of Stem Cell Research and Application, VNUHCM University of Science. All the cell samples were the product of a state-level research project "Study stem cell therapies for diabetes mellitus in animal models" (Code: ĐTĐL.2012-G/23).

The reagents included: S, 0.1 M citric acid, 0.2 M sodium phosphate (Na_2HPO_4), 2-times distilled water (Laboratory of Stem cell Research and Application, VN), 0.25% Trypsin/EDTA (Corning, NY, US), antibodies (CD14-FITC, CD34-FITC, CD44-PE, CD73-FITC, CD90-FITC, and CD166-PE), parafomaldehyde, low glucose DMEM, dexamethasone, glycerophosphate, ascorbic acid, DMSO, Alizarin red, Safranin O, and demecolcine (Sigma-Aldrich, MO, US). Other reagents included StemPro Chondrogenesis Differentiation Kit (Thermo Fisher Scientific, MA, US), 3-Isobutyl-methylxathin-IBMX (I5879, Sigma-Aldrich), Oil red O powder (Merck, NJ, US), and Giemsa (Thermo Fisher Scientific).

2.2 Method of thawing adipose derived stem cells

Cryotubes containing cell samples were thawed at 37^{o}C in a water bath from deep-frozen state in liquid nitrogen (-196^{o}C). The resuspended cells were then cultured in MSCCult medium at 37°C in a humidified atmosphere set at 5% $CO_2$for about 6-10 hours before medium was refreshed to remove the dead cells and the remaining frozen agents. After that, the cells were cultured continuously until confluence.

2.3 Proliferation and passaging

When the cell density reached 80-90%, the culture was passaged at 10^3 cell/cm^2. Briefly, the cells were harvested by 0.25% Trypsin/EDTA, resuspended in MSCCult medium and cultured at 37^{o}C/5% CO_2until cells reached a density of 80-90%. The culture medium was refreshed whenever the medium has turned yellow, usually after 4-5 days. Cells at passages 1, 3, 5 were used to carry out the subsequent experiments.

2.4 Immunophenotyping

The hASCs at passages 1 and 5 were harvested by 0.25% Trypsin/EDTA and subjected to immunophenotyping by flow cytometry (FCM) using the following monoclonal antibodies: CD14-FITC (fluorescein isothiocyanate), CD34-FITC, CD44-PE (phycoerythrin), CD73-FITC, Thy-1 (CD90)-FITC, and CD166-PE. All antibodies were purchased from BD Biosciences except for CD73-FITC and Thy-1 (CD90)-FITC, which were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, TX, US). Briefly, 10^5 cells per an antibody were stained for 30 minutes, washed with FACS buffer, re-suspended in sheath fluid, and analyzed on a FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, US).

2.5 Evaluating trilineage differentiation

To induce the differentiation into osteoblasts, the hASCs were cultured in low glucose DMEM/F12 supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, US), 1% Antibiotic-Antimycotic (Sigma-Aldrich), 50 μg/ml AsAP (apoptosis- and splicing-associated protein, Sigma-Aldrich), 0.1 μM

dexamethasone (Sigma-Aldrich), and 100 mM β- glycerophosphate (Sigma-Aldrich). After 30 days, the cells were stained with Alizarin Red to assess calcium phosphate accumulation.

The hASCs were cultured using the StemPro Chondrogenesis Differentiation Kit (Thermo Fisher Scientific) to induce differentiation into cartilage. After 21 days, the cells were stained with Safranin O to assess the presence of proteoglycans. Finally, hASCs were cultured in low glucose DMEM/F12 supplemented with 10% FBS, 1% antibiotic, 1 mM dexamethasone, 0.5 μM 3-Isobutyl-methylxathin-IBMX, 200 μM Indomethacin, and 10ng/mL insulin. After 7 days, the cells were stained with Oil Red O to detect the presence of fatty tissue products (triglycerides, phospholipids and cholesterol).

2.6 Karyotyping

Initially, hASCs were incubated with Demecolcine (Sigma-Aldrich) at a final concentration of 0.1 μg/mL for 1 hour at 37^{o}C/5% CO_2. Then, the cells were harvested by 0.25% trypsin/EDTA, resuspended in hypotonic solution, and incubated in a water bath set at 37^{o}C. Next, the cells were fixed in Carnoy solution 3 times before dropping on the microscopy slide. The samples were dried naturally at room temperature and then at 60^{o}C for 3 hours. Finally, the sample was immersed in 0.025% Trypsin/EDTA (pe-warmed for 30 seconds), then cold PBS (2-3 times), and then Giemsa dye (5 minutes); the samples were air-dried naturally at room temperature and rinsed with double distilled water.

A chromosomal set of hASCs was chosen randomly, captured, processed and analyzed by IKAROS software on a Metasystem (Carl Zeiss, Germany) with prerequisites of non-overlapping and having clear G-bands.

3. Results

3.1 Proliferation of human adipose derived stem cells

The hASCs were re-activated from the cryopreserved state in liquid nitrogen. After 6 hours, most of the cells spread on the surface of the culture vessel. After 24 hours, the cells returned to their characteristic fibroblast shape of MSCs, with a survival rate of 80-90% (**Figure 1**).

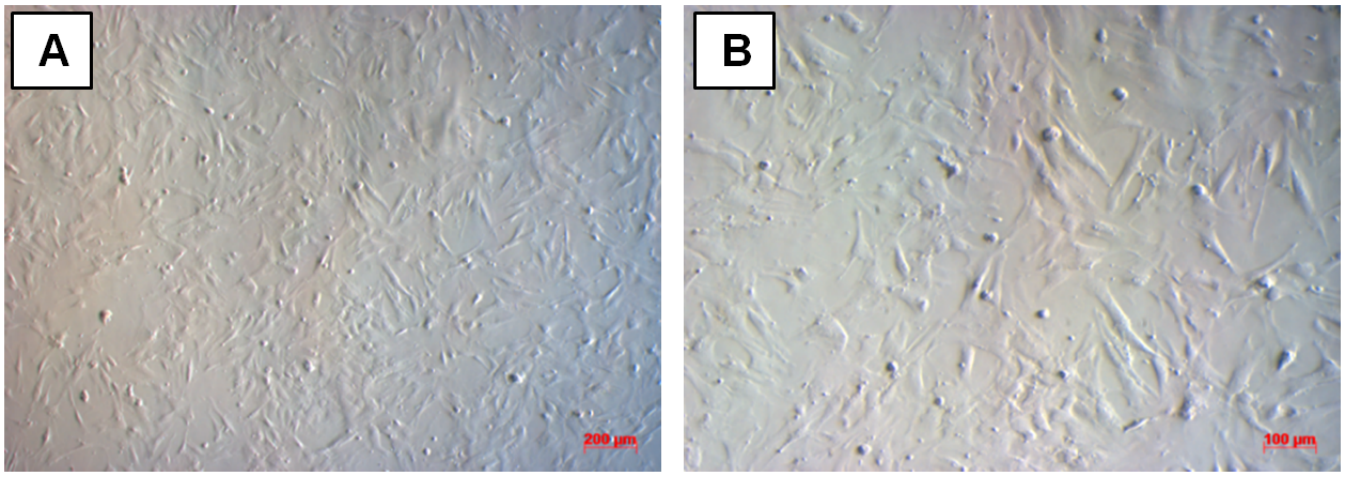

Figure 1. Thawed hASCs after 6 hours. (**A**) 10X, (**B**) 20X; After 6 hours of reactivation, hASCs were spread uniformly on the culture surface with survival rate approximately 80-90%; hASCs: human adipose derived stem cells.

In *in vitro* culture using the MSCCult medium provided by the Laboratory of Stem Cell Research and Application, hASCs proliferated quickly. After 6 hours of culture, the cells adhered to the bottom of the flask. After 2 days, the cells began to grow stronger and spread evenly. Then, 4-5 days after seeding, the cell density in the culture flask reached approximately 80-90%. This time it was suitable for passaging to increase space and provide nutrients for the cells to grow.

At passage 1, hASCs were not uniform in size and shape, unlike passage 5 of culture. Athough the cells were larger in size and longer in shape, they were more uniform and stable (**Figure 2**). As a result, hASCs have been successfully cultured at the Laboratory of Stem cell Research and Application over 5 passages. In *in vitro* culture, hASCs had a slight increase in size but retained the characteristic fibroblast shape of human MSCs. The culture provided a sufficient number of cells for the experiments to access: (1) immunophenotyping; (2) trilineage differentiation to bone, cartilage, and fat; and (3) stability of chromosomal number.

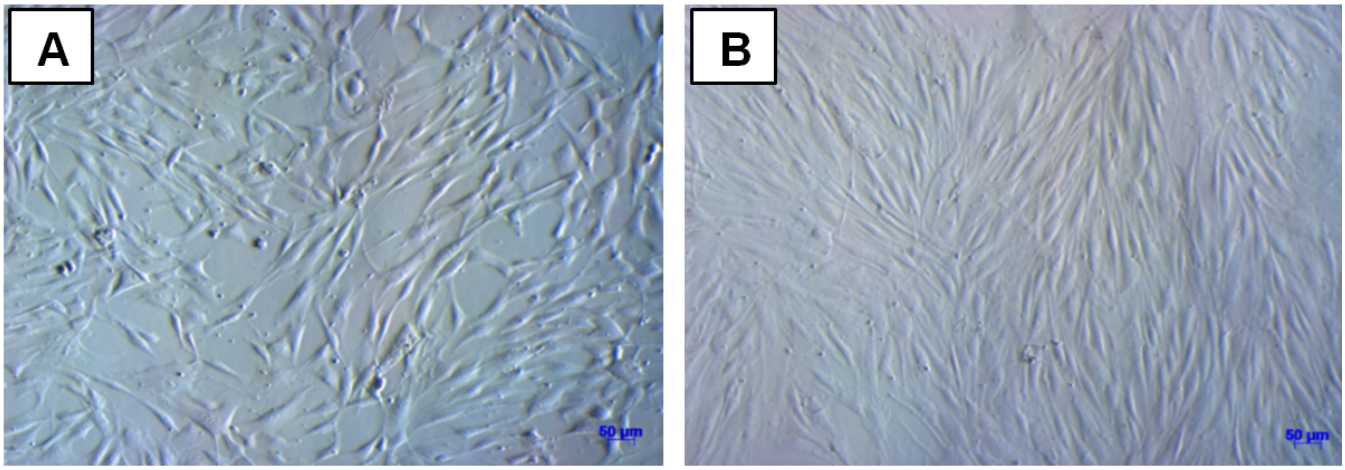

Figure 2. hASCs changed their shape in *in vitro* culture. (**A**) passage 1; (**B**) passage 5; After 5 passages of culture, the cells became more uniform in shape, however, the cell size was larger and longer; hASCs: human adipose derived stem cells.

3.2 Changes in immunophenotype of human adipose derived stem cells

Three hASC samples from passage 1 and 5 were used for immunophenotype analysis with six markers, including CD14, CD34, CD44, CD73, CD90 and CD166. Changes in the expression of markers CD14, CD34 between the first and 5th passage of culture were very different between samples. However, the expression of the remaining markers (CD44, CD73, CD90 and CD166) was quite similar. Specifically, in sample 1, expression of CD14 marker decreased from 2.1% to 0.05%; expression of CD34 marker increased from 0.38% to 40.67%; markers CD44, CD73, CD90, CD166 showed no significant change in expression; and CD73 marker increased the most from 96.33% to 99.55%. In sample 2, CD14 expression decreased from 0.07% to 0.00%, and CD34 decreased from 66.49% to 1.02%; Similar to sample 1, markers CD44, CD73, CD90, and CD166 showed no significant change in expression (CD73 markers decreased the most at 1.25%, from 99.70% to 98.45). In sample 3, markers CD14, CD34 showed weakness in passage 1 (17.72% and 12.37%, respectively) and significantly decreased in passage 5 (0, 51% and 0.20%, respectively); Similarly, CD44, CD73, CD90, CD166 markers showed no significant difference (the highest was a slight decrease of 1.2% on CD166 marker) (**Figure 3A**). Besides, there was no differ-

ence in the mean expression across the 3 samples of all CD14, CD34, CD44, CD73, CD90 and CD166 markers between the passage 1 and passage 5 (**Figure 3B**).

In comparing immunophenotype between different samples, we found that in passage 1 of culture, the CD14 marker was weakly expressed in sample 1 (2.1%), sample 2 (0, 07%) and a little stronger in sample 3 (17.72%). CD34 marker was very weak in sample 1 (0.38%), was average in sample 2 (66.49%), and was weak in sample 3 (12.73%). The markers CD44, CD73, CD90 and CD166 were highly expressed on hASCs and there was no significant difference between the different samples (the highest was 3.39%, between CD73 marker of sample 1 and sample 2) (**Figure 4A**).

In passage 5, the expression of CD14 marker was very weak in sample 1 (0.05%), sample 2 (0.00%) and sample 3 (0.51%); CD34 marker in sample 1 was up-regulated (40.67%) and very weak in sample 2 (1.02%) as well as sample 3 (0.2%). Markers CD44, CD90 and CD166 remained strong, and there were no significant differences between the different samples (the highest was 2.18% on CD166 marker) (**Figure 4B**). Thus, the characteristics of hASCs collected from different donors may vary depend on the physiological characteristics of each person. However, the difference was negligible when cultured to the passage 5; except one-third of samples expressed CD34 marker strongly (40.67%). In summary, under our *in vitro* culture conditions, hASCs retained the immunophenotype characteristics of MSCs.

3.3 Expanded human adipose derived stem cells have no change in trilineage differentiation

The hASCs were cultured to passage 5 and reassessed for the ability of osteogenic, chondrogenic, and adipogenic differentiation. First, after 30 days of induction of osteogenic differentiation, passage 5 hASCs gradually shrunk, accumulated calcium and converted to the cube-shaped stack like building block of osteoblasts. These cells, along with the surrounding substrate, were stained bright red by Alizarin Red (**Figure 5A**). Next, after 21 days of culture in chondrogenic induction medium, hASCs accumulated proteoglycans and stained orange-red by Safranin O (**Figure 5B**). Finally, hASCs in passage 5 were tested for the potential of adipogenic differentiation. In adipogenic induction medium, the small lipid droplets formed gradually within the cytoplasm. The cell shape became flattened and stretched. In the process of induction, the droplets accumulated and increased in size. In order to increase reliability, the differentiated cells were stained with Oil Red O, a specialized dye for products of fatty tissues (triglycerides, phospholipids and cholesterol). The result showed that many drops of fat were dyed deep red inside the cell. This demonstrates that hASCs have differentiated into fat cells (**Figure 5C**). Thus, after five passages of culture, hASCs still retained the ability to differentiate into osteogenic, chondrogenic or adipogenic cells in our *in vitro* culture conditions.

The above results demonstrated that hASCs after five passages in our *in vitro* culture conditions maintained the specific morphology and immunophenotype, as well as differentiation potential of MSCs. From there, we conducted the assessment of the stability of the chromosome number of the expanded hASCs.

3.4 The stability of the chromosomal number of human adipose derived stem cells in *in vitro* culture

3.4.1 Cell cycle arrest

When the density of hASCs was approximately 70-80%, we supplemented demecolcine to the culture medium at a final concentration of 0.1 μg/mL. After an hour of treatment, the dividing cells were arrested in the metaphase. The cells tended to shrink and no longer exhibited the characteristic shape of MSCs (**Figure 6**).

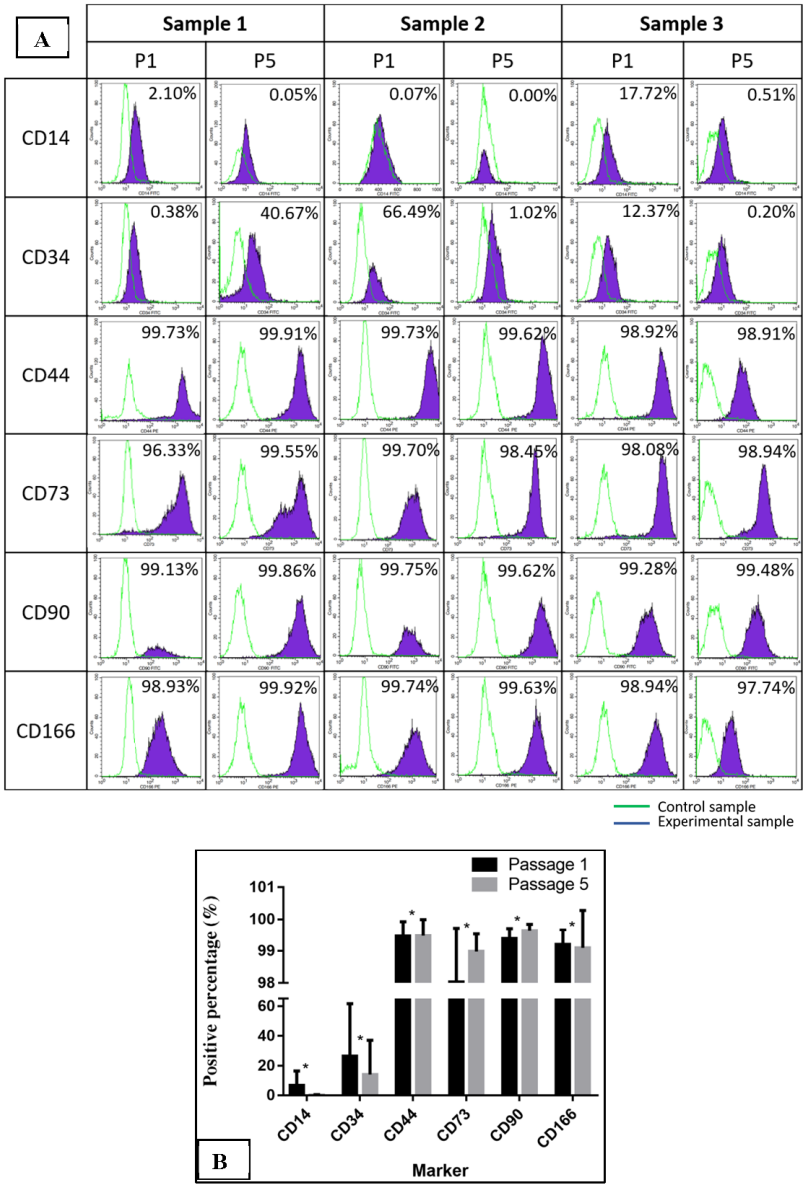

Figure 3. The changes in immunophenotype of expanded hASCs. (**A**): The expression of CD14, CD34, CD44, CD73, CD90 and CD166 of three hASC samples in passage 1 and passage 5; In sample 1, CD14 expression was very weak (2.10% in passage 1, 0.05% in Passage 5), CD34 expression was weak in passage 1 (0.38%) and significantly increased (40.67%) in passage 5. Meanwhile, markers CD44,CD73, CD90, CD166 were very strong (> 96%); In sample 2, CD14 expression was very weak (0.07%) in passage 1, and was no longer detectable in passage 5; However, CD34 expression was quite strong (66.49%) in passage 1 and decreased to 1.02% in passage 5; CD44, CD73, CD90, CD166 was very strong (> 98%); In sample 3, CD14, CD34 expression were weak at passage 1, 17.72% and 12.37%, respectively, and almost no expression at passage 5. In addition, CD44, CD73, CD90, CD166 expression were very strong (> 97.7%); (**B**): The average expression of markers of three hASC samples at passage 1 and passage 5; The difference of all markers between the two passages was not significant; hASCs: human adipose derived stem cells; P1, P5: passage 1, 5, respectively; *: $p>0.05$.

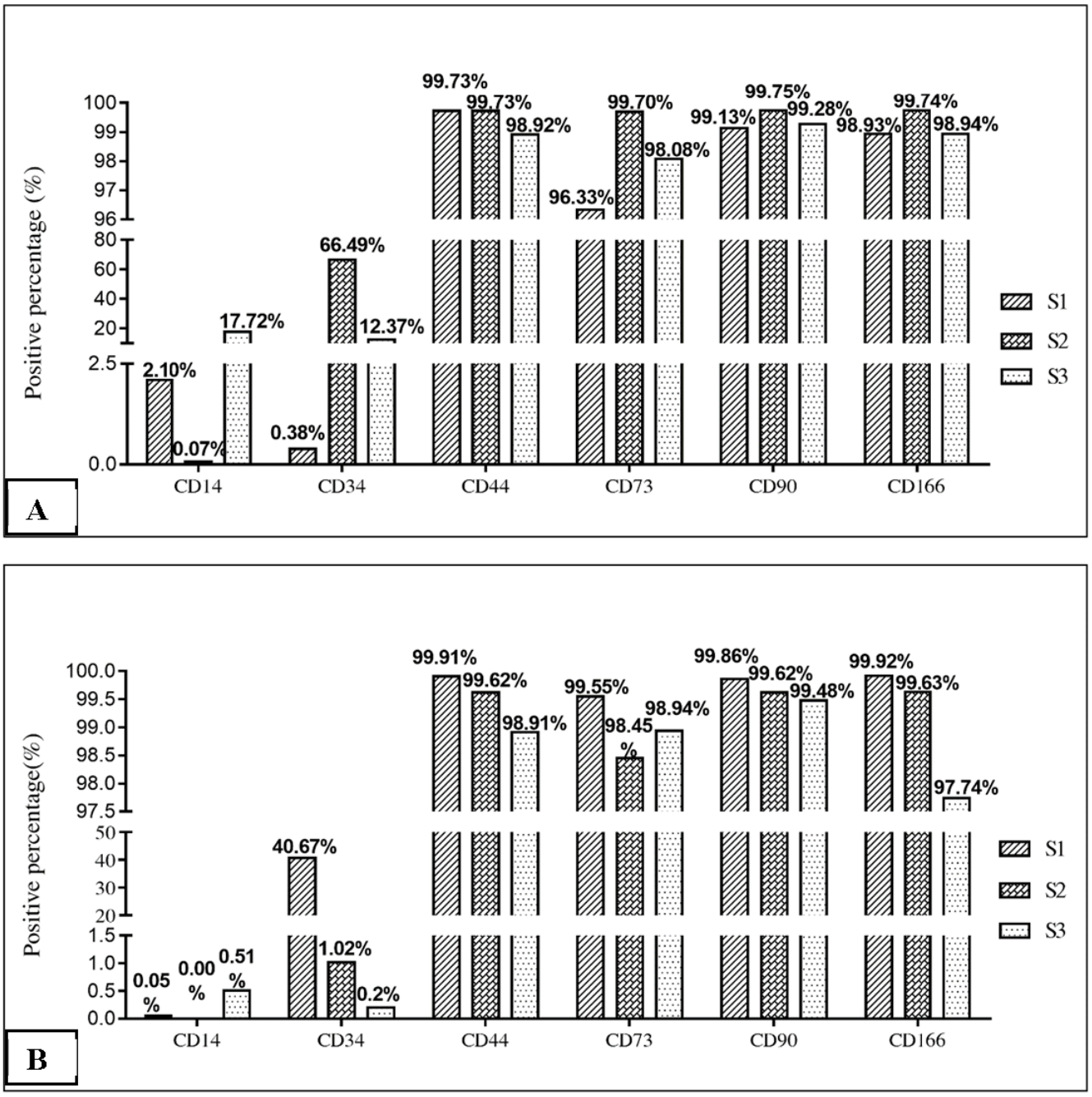

Figure 4. Differences in hASC immunophenotype between different samples. (**A,B**): Histograms comparing the hASC immunophenotype between three samples in passage1 and passage 5, respectively; In passage 1, the expression of CD14 marker was very weak in sample 1 (2.10%), sample 2 (0.07%) and weak in sample 3 (17.72%); CD34 marker was very weak in sample 1 (0.38%), average in sample 2 (66.49%) and weak in sample 3 (12.37%); markers CD44, CD90, and CD166 were highly expressed on hASCs and there was no significant difference between samples. In passage 5, CD14 marker was very weak in all three samples (the highest was 0.51%); CD34 marker in sample 1 was average (40.67%) and weak in sample 2, sample 3 (bellow 1.02%). Similarly, in passage 1 of culture, CD44, CD90, CD166 markers were highly expressed and there was no significant difference between samples; hASCs: human adipose derived stem cells; S1, S2, S3: hASC samples 1, 2, 3, respectively.

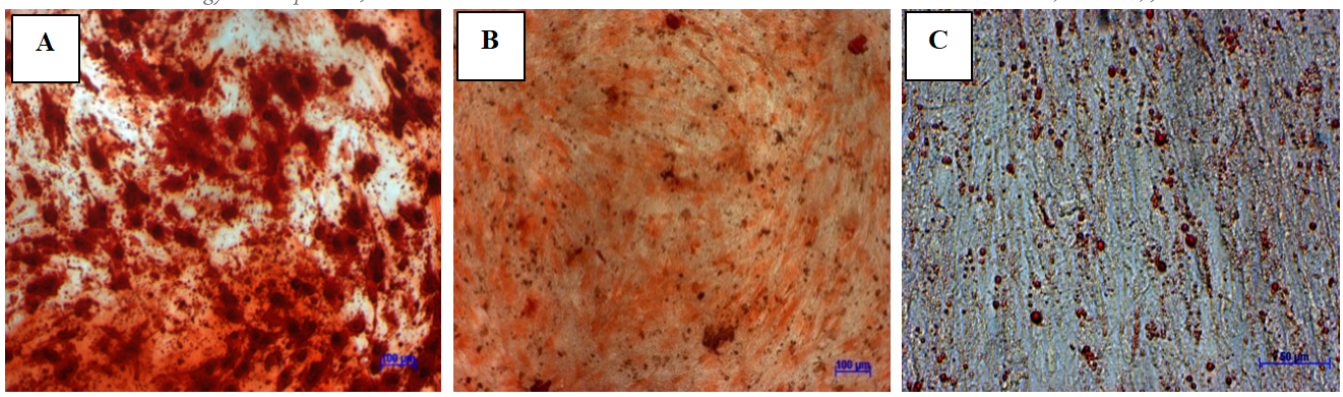

Figure 5. Trilineage differentiation potential of hASCs in passage 5. The osteogenic, chondrogenic, and adipogenic-induced cells were stained with Alizarin Red, Safranin O and Oil Red O, respectively; (**A**) Osteoblasts, (**B**) Chondroblasts, (**C**) Adipocytes

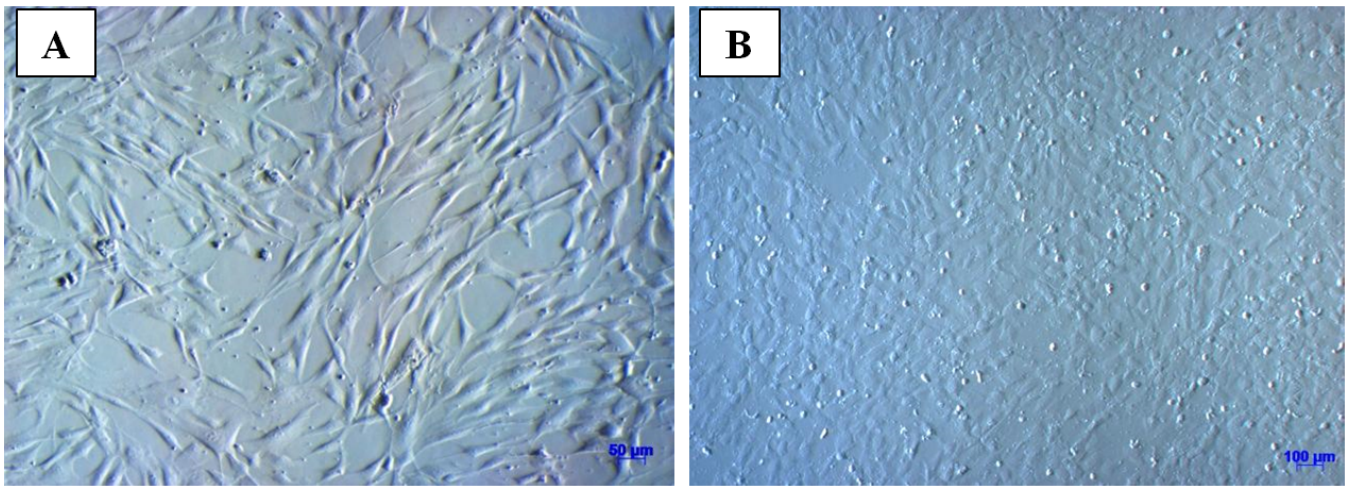

Figure 6. Changes in the shape of hASCs before and after being treated with Demecolcine. (**A**): hASCs were cultured in medium containing no Demecolcine; (**B**): hASCs were cultured one hour in medium containing 0.1 μg/mL Demecolcine; The cells before exposured to Demecolcine had a characterized elongated shape of MSCs; After exposured to Demecolcine for one hour, the cell cycle was arrested in metaphase, the cell shape tended to contract; hASCs: human adipose derived stem cells.

3.4.2 Results of evaluating the stability of the chromosomal number of human adipose derived stem cells

After capturing the cell cycle in metaphase, chromosomal sets of hASCs were spread on the microscopy slide, dyed, and quantified by karyotyping technique. At least, 30 chromosomal sets of each hASC sample in three passages 1, 3, 5 were randomly selected for quantification. The chromosome assessed had to meet the following criteria: spread evenly and stain clearly with Giemsa dye (no overlapping and having clear bands). In our study, the number of hASC chromosomes remained stable from passage 1 to passage 5 of culture (46, XX) in all three samples; No mutations were detected in the number of chromosomes (**Figure 7**).

Thus, hASCs cultured in *in vitro* conditions at the Laboratory of Stem cell Research and Application maintained the stability of the number of chromosomes at least up to passage 5.

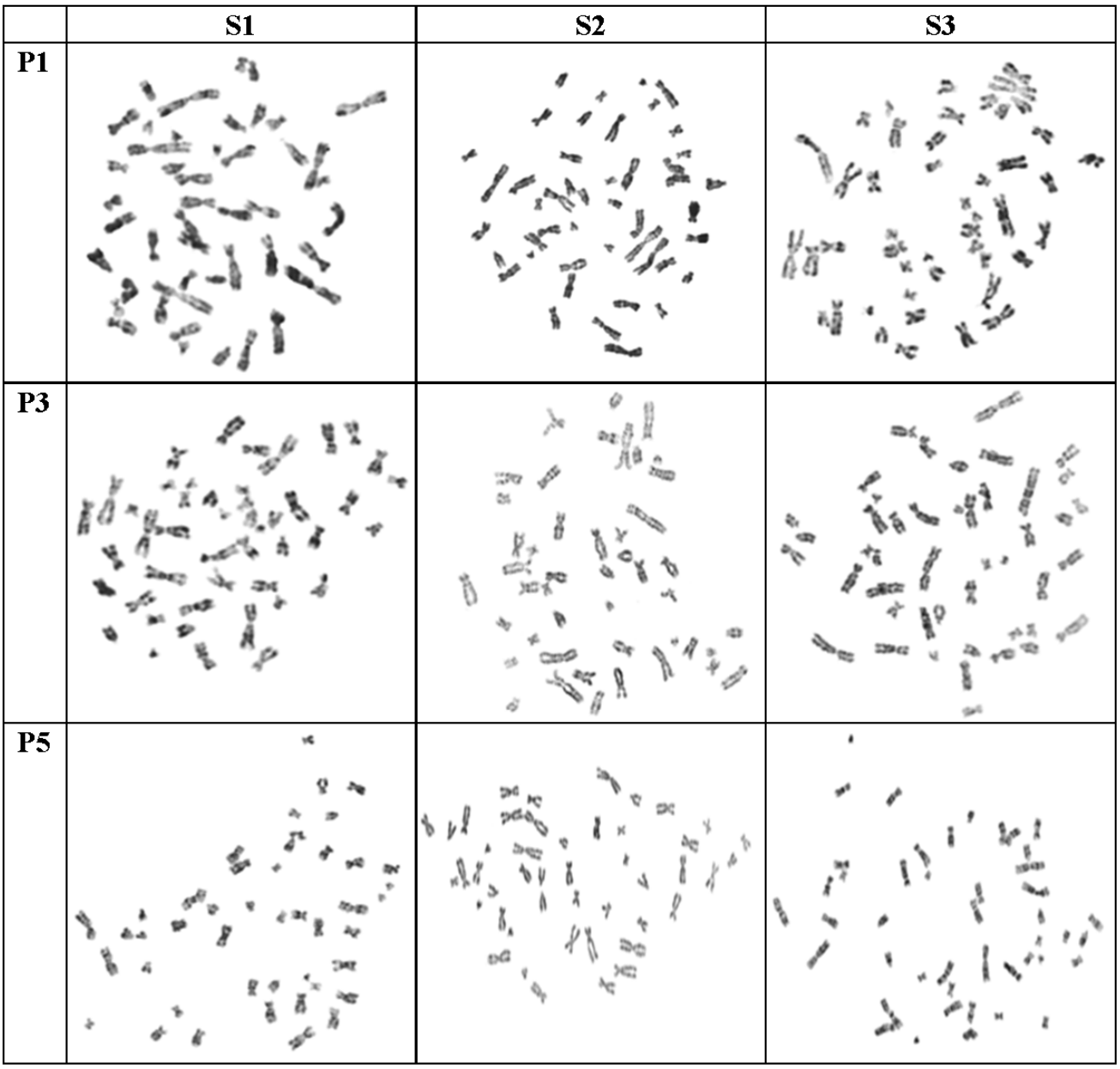

Figure 7. Karyotypes of hASCs in *in vitro* culture. The number of chromosomes of hASCs remained stable from passage 1 to passage 5 (46, XX) in all three samples; hASCs: human adipose derived stem cells; S1, S2, S3: hASC samples 1, 2, 3, respectively; P1, P3, P5: passage 1, 3, 5, respectively.

4. Discussion

To acquire a sufficient number of cells for research and treatment, passaging cells is necessary. However, passaging together with the influence of *in vitro* culture condition might cause hASCs to age and change its characteristics. Our study aimed to re-evaluate the phenotype and karyotype of hASCs after 5 first passages of culture.

According to the ISCT, hASCs must meet three minimum conditions of mesenchymal stem cells Wang et al. (2013). Firstly, hASCs must be plastic-adherent when maintained in standard culture conditions; but

Table 1. The analysis results of the number of hASC chromosomes from three samples in passages 1, 3, 5

Sample	Passage	Number of chromosomes
S1	1	46, XX (n=30)
	3	46, XX (n=30)
	5	46, XX (n=30)
S2	1	46, XX (n=30)
	3	46, XX (n=30)
	5	46, XX (n=30)
S3	1	46, XX (n=30)
	3	46, XX (n=30)
	5	46, XX (n=30)

hASCs_human adipose derivedstem cells

this condition is almost obvious. Instead, we observed the change in the shape of cells. The result showed that after 5 passages, hASCs increased slightly in size but became more homogeneous.

Secondly, hASCs must express markers CD73, CD90, CD105 and not express the hematopoiesis-related markers (CD14, CD34). We substituted CD44 and CD166, the new members in hASC marker profile for CD105 Lechanteur et al. (2016); Mildmay-White and Khan (2017). CD14 and CD34 have been used as markers for hematopoietic cells Sidney et al. (2014); Ziegler-Heitbrock and Ulevitch (1993). Under our *in vitro* culture conditions, CD14 was expressed at very low level and decreased over time. This is consistent with the findings of Zuk *et al*. (2001) Zuk et al. (2001) and Zannettino *et al.* (2008) Zannettino et al. (2008).

CD34 marker, in two-thirds of the samples studied, was expressed weakly in passage 1, which is similar to that reported by Dominici *et al*. (2006) Wang et al. (2013). For the remaining one-third of the samples, CD34 was expressed moderately (66.49%) in passage 1, quite similar to reports by Maikel *et al*. (2006) Varma et al. (2007) and Gronthos *et al*. (2001) Gronthos et al. (2001). In passage 5, two-thirds of the samples showed a decrease in CD34 expression (**Figure 3A**). This result is similar to previous findings of Mitchell *et al*. (2006) Mitchell et al. (2006), Zuk *et al*. (2001) Zuk et al. (2001), and Ngoc Kim Phan *et al.* (2010) Ngọc et al. (2006). According to Hiroe *et al*. (2013), CD34 is associated with cell adhesion; the stronger expression the expression, the lower the cell adhesion Ohnishi et al. (2013). During the subculture process, cells with little or no adhesion were eliminated Mitchell et al. (2006), resulting in reduced expression of CD34. In contrast, the expression of CD34 marker in the remaining one-third of our samples increased. Although this result was different from the above mentioned publications Zuk et al. (2001); Ngọc et al. (2006); Ohnishi et al. (2013), according to Gurudutta *et al*. (2006), CD34 may be an adhesion molecule Gangenahalli et al. (2006) and retained during the process of culture. In other words, CD34 has two phenotypes of adhesion and non-adhesion. Depending on the conditions and duration of culture, CD34 will convert to one of these two forms and affect cell adhesion. hASC populations are, therefore, likely to increase the expression of CD34 in *in vitro* cultures Lin et al. (2012).

Markers CD44, CD73, CD90,and CD166 are important markers of MSCs (in general) and hASCs (in particular) Goodison et al. (1999); Chen et al. (2016); Kisselbach et al. (2009); Hagood et al. (2005); Chappell et al. (2015); Ohneda et al. (2001); Fujiwara et al. (2003). In contrast to the hematopoietic markers, markers CD44, CD73, CD90, and CD166 were strongly expressed (>96%) during culture and there was no significant change between passages in all samples. This result is consistent with the results of Zannettino *et al*. (2008) Zannettino et al. (2008), Mitchell *et al*. (2006) Mitchell et al. (2006).

Thirdly, we evaluated the differentiation potential of *in vitro* expanded hASCs. After 5 passages of culture, hASCs retained the trilineage differentiation ability. Our results are consistent with the results of Kawagishi-Hotta *et al.* (2016) Kawagishi-Hotta et al. (2017). Specifically, when cultured in osteogenic induction medium, the cells were mineralized with the expression of alkaline phosphatase Golub and Boesze-Battaglia (2007), osteocalcin secretion, and calcium phosphate deposition Lee et al. (2013). Thus, the osteogenic-differentiated cells that have the presence of these molecules will conjugate with Alizarin Red dye to appear bright red (**Figure 5A**). When cultured in chondrogenic induction medium, hASCs synthesize proteoglycans, which are well-stained (as orange-red) with Safranin O Munirah et al. (2010) (**Figure 5B**). Next, when culturing hASCs in adipogenic induction medium, the cells accumulated lipid droplets in the cytoplasm, and were stained deep red with Oil red O (**Figure 5C**).

Finally, we analyzed the effect of culture conditions on the number of chromosomes of the hASCs. Our chromosomal analysis results are consistent with studies of Reza Izadpanah *et al.* (2008) Izadpanah et al. (2008), Xu Fang Yang *et al.* (2011) Yang et al. (2011) Ra *et al.* (2011) Ra et al. (2011), Hye Suk Kang *et al.* (2015) Kang et al. (2015), Bellotti *et al.* (2013) Bellotti et al. (2013), and/or other studies on BM-MSCs Sensebé et al. (2012); Cornélio and de Medeiros (2014); Bernardo et al. (2007). However, this latter finding was in contrast to that of Estrada *et al.* (2013), Bellotti *et al.* (2013), Pan *et al.* (2014), Borgonovo *et al.* (2015), Brian *et al.* (2016). Indeed, there are several mutants of the number of chromosomes in some passages of culture Dominici et al. (2006); Bellotti et al. (2013); Estrada et al. (2013); Stultz et al. (2016); Borgonovo et al. (2015).

Studies show that the ability of chromosomal number mutation of hASCs in *in vitro* culture was somewhat different from our own. This difference may be related to the donors (ethnicity, gender, age, medical condition, *etc.*), culture process or culture medium, etc. Depending on the source of the hASCs, the cells from "healthy" donors are less likely to mutate than those from "old age" individuals after undergoing *in vitro* culture. The ability to repair errors in the division of the old cells weakens and the cells accumulate more and more defects. Therefore, it is impossible to exclude the possibility of mutation in the early cultures Bellotti et al. (2013). The differences in these publications may be also due to the fact that hASCs cultured in early passages (under 5 passages) were less affected by endogenous factors such as telomeres' shortening, destruction of reactive oxygen species, or exogenous factors (e.g. chemicals, isolation and sub-culture manipulation) led to the accumulation of more and more mutations. In other words, the higher the number of passages, the higher the likelihood of chromosomal mutations.

In summary, our hASCs still maintained the normal phenotype of MSC lineage as well as chromosomal set (2n = 46) at least to passage 5. Due to the certain limitations, we can only assess the phenotypic and genotypic changes of hASCs to passage 5. Thus, this initial research will be the premise for further exploring and optimizing the culture technology using MSCCult medium. From that, we will continue to expand hASCs in larger passages and conduct further assessments.

5. Conclusions

Interestingly, hASCs (after five passages) still maintained the specific characteristics of MSCs. The number of normal chromosomes in this culture conditions was 2n=46. The cells showed changes in shape and immunophenotype; however, this change was negligible and consistent with many other studies in the world.

6. List of abbreviations

ASCs: adipose derived stem cells; **BM-MSCs**: bone marrow mesenchymal stem cells; **DMEM**: Dulbecco's Modified Eagle's medium; **DMSO**: dimethyl sulfoxide; **FBS**: fetal bovine serum; **hASCs**: Human adipose derived stem cells; **ISCT**: International Society for Cellular Therapy; **MSCs**: mesenchymal stem cells; : phosphate-buffered saline; **VNUHCM**: Viet Nam National University Ho Chi Minh City

7. Competing interests

The authors declare that they have no conflicts of interest.

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9. Authors' contributions

NCT, ATVV, VMP designed the study, read and corrected the manuscript. NCT wrote the manuscript, proliferated hASC samples, evaluated the chromosomal number. NCT, ATVV, VMP evaluated mesenchymal characteristics. All authors read and approved the final manuscript.

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