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Non enzymatic isolation of adipose tissue and stromal vascular fraction derived cells

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Abstract: The therapeutic potential of the adipose tissue is also supported by a source of mesenchymal stem cells (MSCs), endothelial progenitor cells, mast cells, T- lymphocyte, B lymphocyte, and adipose-resident macrophages with repairing and regenerative ability. The purpose of this study was to compare adipose tissue derived mesenchymal stem cells (ADSCs) and stromal vascular fraction cells (SVF cells) in terms of the usage of non-enzymatic isolation, detection of cell adhesion, fibroblastoid cell formation, properties of cell population, cell culture duration until 3rd passages under in-vitro culture condition. We used 3 months old, 4 male Sprague Dawley rats (mean of live weight about 250 g) to examined the variation of the cell population, cell properties, phases of cellular formation, cell culturing time, subculturing duration, differences in confluency between SVF cells and ADSCs. In in-vitro expansion, ADSCs displayed higher rate of adhesiveness, homogenous cell population, faster proliferation and formation of fibroblast like cells compared to SVF cells. As a result, we showed that ADSCs have better adhesive ability, higher proliferative capacity in all of the 3 passages and require shorter time to reach confluency compared with SVF cells in vitro. These findings may contribute to future studies that deal with isolation and selection of stem cells from various tissues, as well as design clinical trials based on ADSCs and SVF cells.
Keywords: Adipose tissue derived cells, mesenchymal stem cells, stromal vascular fraction cells.

Adipoz doku ve stromal vasküler fraksiyon kökenli hücrelerin non enzimatik izolasyonu

Özet: Adipoz dokusunun terapötik potansiyeli; mezenkimal kök hücreler (MKH) ile desteklenmesinin yanında endotel progenitor hücreleri, mast hücreleri, T lenfositleri ve adipoz dokusundaki makrofajların etkisi ile tedavi ve rejenerasyon potansiyeli ile de desteklenmektedir. Bu çalışmanın amacı; adipoz doku kökenli mezenkimal kök hücreler (ADMKH) ve stromal vasküler fraksiyon kökenli hücrelerin (SVF hücreleri) non enzimatik hücre izolasyonu yöntemi, hücrelerin plastik yüzeye tutunması, fibroblastoid yapının oluşumu, hücre populasyonunun özellikleri, 3. pasaja kadar in vitro kültür yönünden karşılaştırılmasıdır. Bu çalışmada, SVF hücreleri ve ADMKH'lerin arasındaki; hücre popülasyonu, hücrelerin yapısal özellikleri, hücresel gelişim fazları, hücre kültür süresi, subkültür süresi, konfluensinde bulunan farklılıkları incelemek için 3 aylık yaştaki, 4 erkek *Sprague Dawley* ırkı sıçan (ortalama canlı ağırlıkları yaklaşık 250 g) kullandık. İn vitro ekspansiyonda, SVF hücreleri ile karşılaştırıldıklarında ADMKH'ler; yüksek oranda hücresel tutunma, hızlıca fibrolastoid hücre yapısını oluşturma, homojen hücre populasyonu, daha hızlı proliferasyon gösterdi. Sonuç olarak, ADMKH'lerin her 3 pasajda daha fazla oranda plastik yüzeye tutunduğu, daha yüksek proliferasyon kapasitesine sahip olduğu ve in vitro kültürde SVF hücrelerinden daha kısa sürede konfluense ulaştığını gözlemledik. Bu bulgular, çeşitli dokulardan kök hücrelerin izolasyonu ve seçimi ile ilgili ileriki çalışmalara katkıda bulunmasının yanı sıra, ADMKH'ler ve SVF hücreleriyle yapılan klinik deneylerin tasarlanmasına da katkı sağlayacaktır.
Anahtar sözcükler: Adipoz doku kökenli hücreler, mezenkimal kök hücreler, stromal vasküler fraksiyon hücreleri.

Introduction

Human and animal stem cells have an unrestricted potential for differentiation, self-replication, and self renewal (6, 12, 20). Mesenchymal stem cells (MSCs) are representative of adult MSCs that have capable of self-renewing, self-organizing, and have a multi-lineage differentiation potential (23). Stem cells were isolated from adipose tissue by Rodbell and Zuk et al. (18, 29), which were identified as Adipose-Derived Mesenchymal Stem Cells (ADSCs / AD-MSCs / ASCs). In addition to adipose tissue, MSCs have also been isolated from tendons, the periodontal ligament, synovial membrane, trabecular bone, bone marrow, embryonic tissues and skin (2,10). ADSCs' positive cluster differentiation (CD)

markers are CD90, CD44, CD29, CD105, CD13, CD34, CD73, CD166, CD10, CD49d and CD59 while the most commonly detected negative markers are CD31, CD45, CD14, CD11b, CD19, CD56 and CD146 in ADSCs (12, 17). Stromal Vascular Fraction (SVF) was initially obtained by proteolytic enzymes and centrifugation technique by Rodbell and colleagues (26). MSCs were identified as processed lipoaspirate cells (PLA cells) or SVF (29). SVF has a heterogeneous cell fraction containing stem cells. Different markers CD markers have been reported for ADSCs and SVF cells (5, 11). While SVFs express endothelial (progenitor) stem cell surface markers, including CD31, CD34, CD45, and CD105, ADSCs do not express these markers (15). SVF includes a variety of immunotherapeutic cells and MSCs, which can be used freshly after isolation and immediately transplanted into the degenerative tissues (24). However, SVF cells are cultivated under in-vitro standardization for the transplantation or cryopreservation protocol (1, 25).

The purpose of this study was to compare ADSCs and SVF cells in terms of the usage of non enzymatic cell isolation, detection of cell adhesion, fibroblastoid cell formation, properties of cell population, cell culture duration of 1st, 2nd and 3rd passage confluency under in-vitro culture conditions.

Material and Methods

3 months old, four male Sprague Dawley rats were used (mean of live weight about 250 g). Rats were anesthetized by injection of 10 mg/kg Xylazine and 75 mg/kg Ketamine; adipose tissue samples obtained by subcutaneous and abdominal region. All research activities involving animals were approved by The Uludağ University Local Ethical Committee of Animal Experiments (2019-05/01).

Isolation of MSCs derived from adipose tissue: Adipose tissue (3g) was collected from 2 rats and performed to isolate ADSCs by nonenzymatic isolation technique (10). Adipose tissue was transferred into Dulbecco's Phosphate-Buffered Saline (DPBS) solution (Millipore Cat No: BSS-1006) and rinsed. Then, resected tissue material was first minced into 3-4 mm^3 small fragments using 2 surgical scalpels. The tissue fragments were seeded onto T25 cm^2 tissue culture flasks, and incubated with Rat-MSCs medium (Cat No: RAXMD-03011-440). Cell culture medium was supplemented with 10 % FBS, 100 U/ml penicillin, 100 g/ml streptomycin, (Gibco Cat No: TMS-AB2-C), 2 mM L-glutamine (Gibco Cat No: G7513) and heat-inactivated Fetal Bovine Serum (FBS) 20 % (Gibco Cat No: TMS-013-B). T25 flasks were cultivated under 5 % CO_2 incubator at 37° C in a 96 % humidified atmosphere. ADSCs medium was replaced with fresh medium 3 times a week. Cells at 1st, 2nd and 3rd passages of ADSCs culture were detached with sterile cell scrapers after each of the 70 % cell confluency.

Isolation of SVF derived cells: 3g adipose tissue was collected from two rats and performed to isolate SVF cell population by nonenzymatic isolation technique (21). Adipose tissue was transferred into DPBS solution (Millipore Cat No: BSS-1006) and rinsed. For the non-enzymatic SVF isolation; adipose tissue was minced into 3-4 mm^3 fragments. Then, 3 g of adipose tissue and 3 ml 0.09 % serum isotonic were mixed within a sterile 15 ml centrifuge tube. After this step, SVF content were vortexed in 2400 rpm for 2 minutes and centrifuged in 3500 rpm for 4 minutes. Then, the supernatant from the top layer of the tube was discarded, and the pellet contents were filtered through a 70 µm cell strainer. This freshly isolated SVF pellet form was cultivated in-vitro T25 flasks under 5 % CO_2 incubator at 37° C in a 96 % humidified atmosphere. Rat-MSCs medium (Cat no: RAXMD-03011-440) containing with 10 % FBS, 100 U/ml penicillin, 100 g/ml streptomycin, (Gibco Cat No: TMS-AB2-C), two mM L-glutamine (Gibco Cat No: G7513) and heat-inactivated 20 % Fetal Bovine Serum (FBS) (Gibco Cat No: TMS-013-B). 5 ml cell culture medium was added into each of the flasks and replaced with fresh medium 3 times a week and passaged with cell scraper until 3rd passages.

Results

ADSCs were observed to adhere to culture flasks within 24 hours and majority of cells were seen to adhere to culture flasks after 48 hours. ADSCs population contained both fibroblast-like cells with spindle shape and migrating cells with round shape, which were prominent on day 3 (Figure 1A). ADSCs reached 70 % confluency on day 8, and also, the cell population had a homogeneous morphology (Table 1). In ADSCs, there were no adipose tissue residues and no dead tissue fragments were observed on the first passage (Figure 2). The second passage of ADSCs reached 70 % confluency faster compared with the first passage of ADSCs. Homogeneous population of cells with fibroblast-like shape observed on the first passage of ADSCs were still apparent on the second (on day 13) and third passages (on day 18) (Figure 3).

In SVF cells, adhesion phase of cells, spindle-like cells, cell migration step, or homogeneous cell population were not detect until day 3. Adherent cells and low cell density at the migrating phase were observed in SVF cells on day 7. Fibroblast-like cells and spindle shape cells were not detected on day 7 but a heterogeneous cell population were observed such as the oval, polygonal and various

small cells (Figure 1B). Heterogeneous cell population, dead adipose tissue residues and adherent cells were observed in SVF cell culture on day 7. Moreover, SVF cells had a long migration phase, and migrating cells significantly increased on day 14 (Figure 4). The small, residual cells, oval, polygonal shaped cells and low density of fibroblast-like cells were observed from 18 to 21 days in SVF cells. The first passage of SVF cells reached 60 % confluency on day 21 (Figure 5). SVF cells were subcultured for the second and third passages at 60 % confluency on days 28 and 35, respectively (Table 1).

Table 1. The differences of in-vitro cultures of ADSCs and SVF cells.

	ADSCs	**SVF cells**
Cell adhesion	24 hours	On day 7
Cell migration	48 hours	On day 14
Fibroblast-like cells	On day 3	On day 18
Properties of cell population	Homogeneous population	Heterogeneous population
1st passage and confluence	On day 8 and 70 % confluence.	On day 21 and 60 % confluence
2nd passage confluence	On day 13 and 70 % confluence	On day 28 and 60 % confluence
3rd passage confluence	On day 18 and 70 % confluence	On day 35 and 60 % confluence
Differences of cell population	Fibroblastoid cells	Fibroblastoid cells, residuel cells, small, oval and polygonal cells

ADSCs: mesenchymal stem cells, SVF cells: stromal vascular fraction cells.

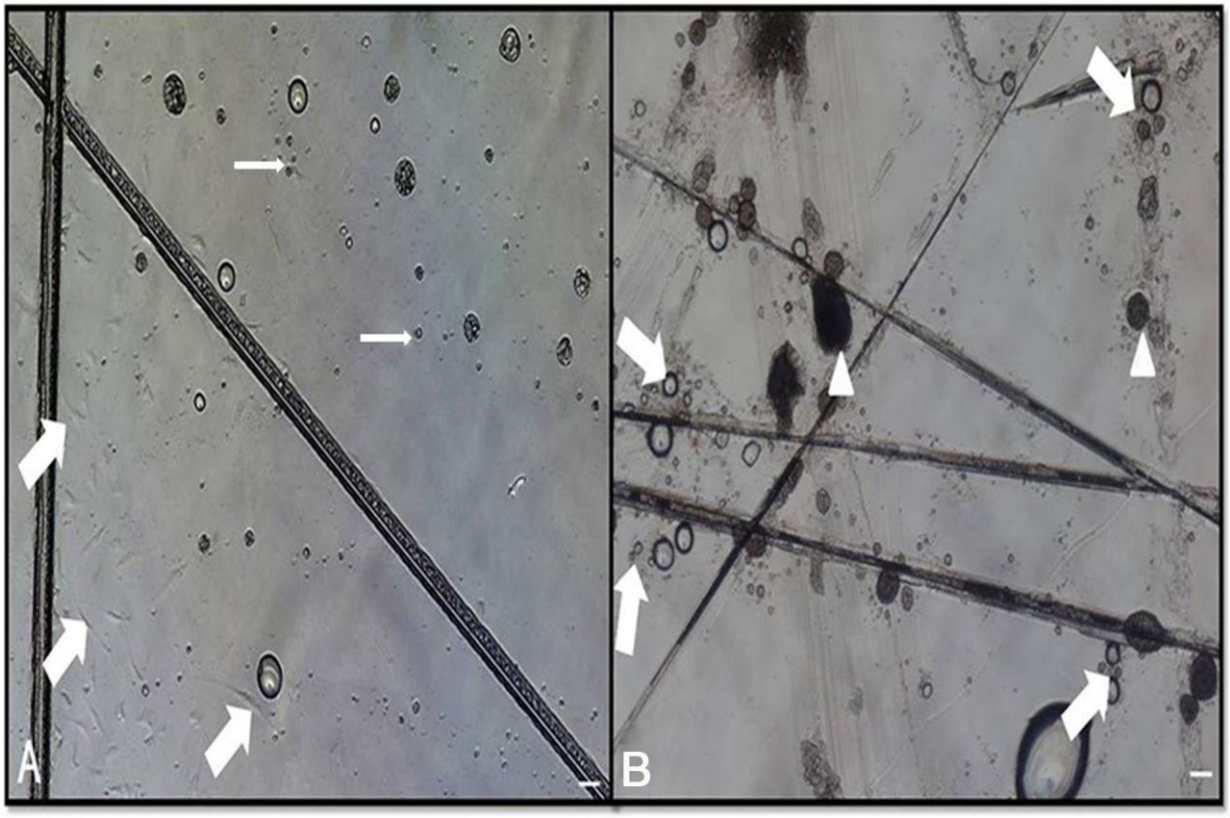

Figure 1. A) Fibroblast-like cells (thick arrows) and round shape of migratory cells in ADSCs (thin arrows); on day 3; B) Low density of cell migrating phase on day 7; heterogeneous cells, oval, polygonal shape and variety form of small cells (thick arrows); dead adipose residues in SVF cell culture (arrowheads) (Bar 100µ).

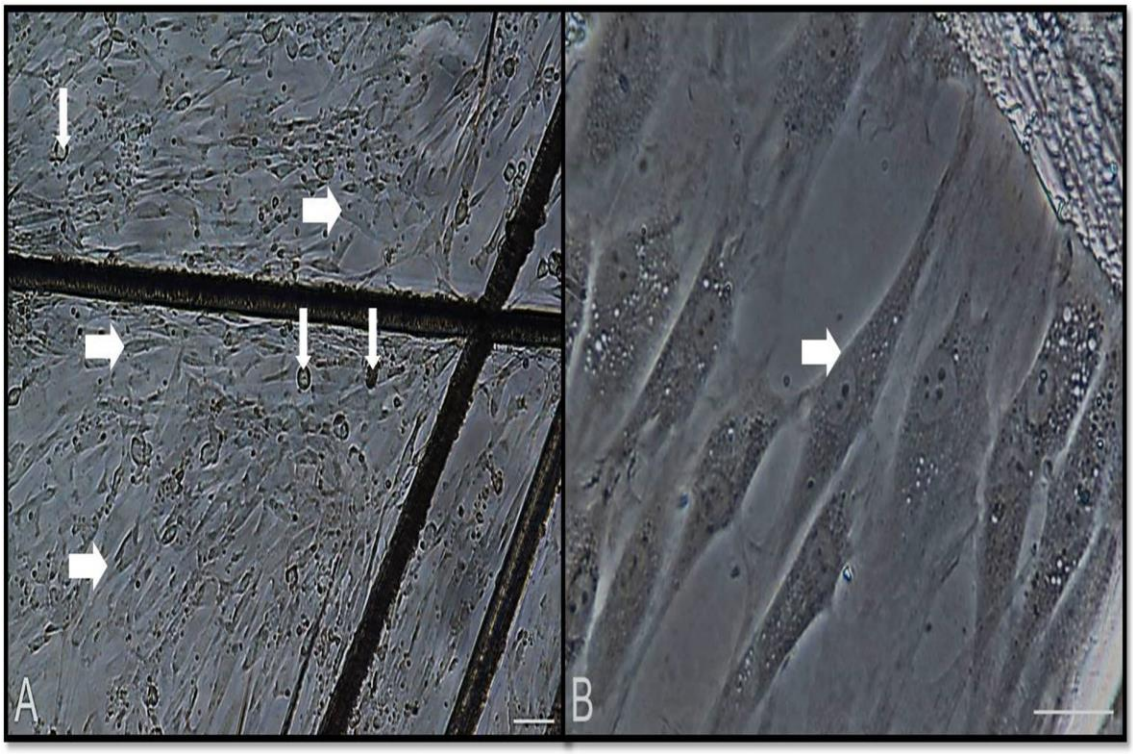

Figure 2. A) First passage 70 % confluency on day 8; migratory cells from adipose tissue (thin arrows); homogeneous and spindle shape of cells in ADSCs (thick arrows); B) thick arrows: fibroblastoid cells (Bar 25µ).

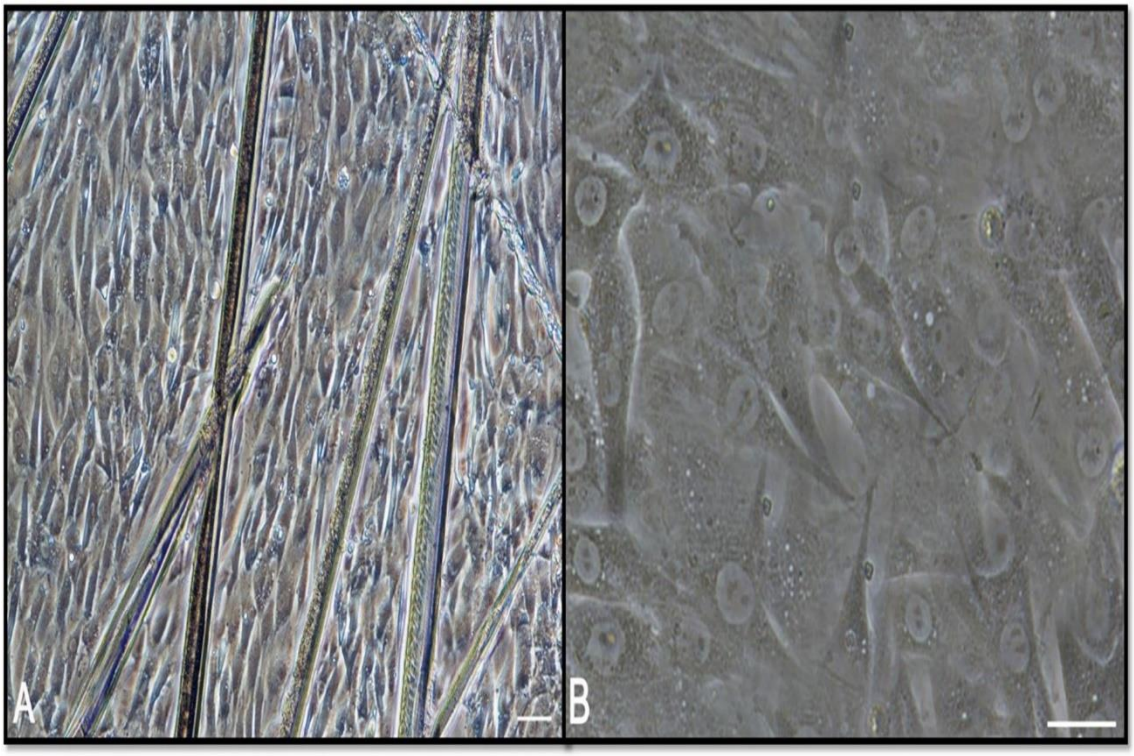

Figure 3. Homogeneous fibroblastoid cells were subcultured for second and third passages in ADSCs on day 13 (A) and 18 (B), respectively (Bar 25µ).

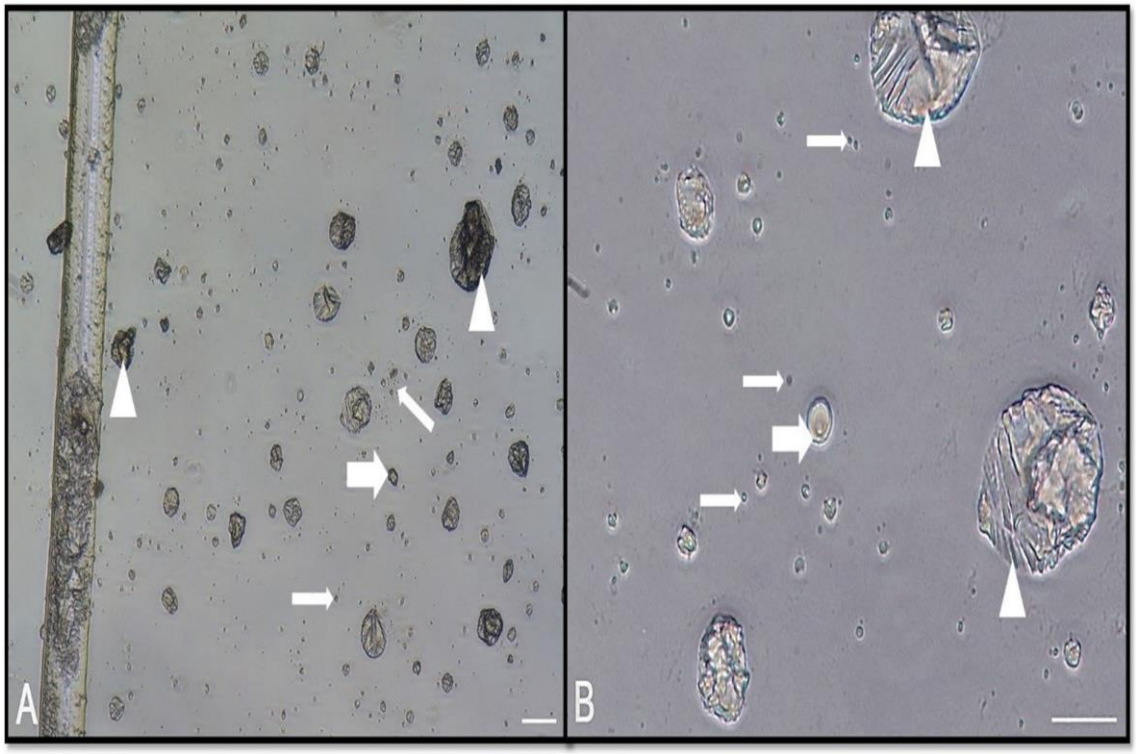

Figure 4. (A and B) Heterogeneous cells (thin arrows), migratory cells (thick arrows) on day 14 and dead adipose tissue residues (arrowheads) in SVF cells (Bar 25µ).

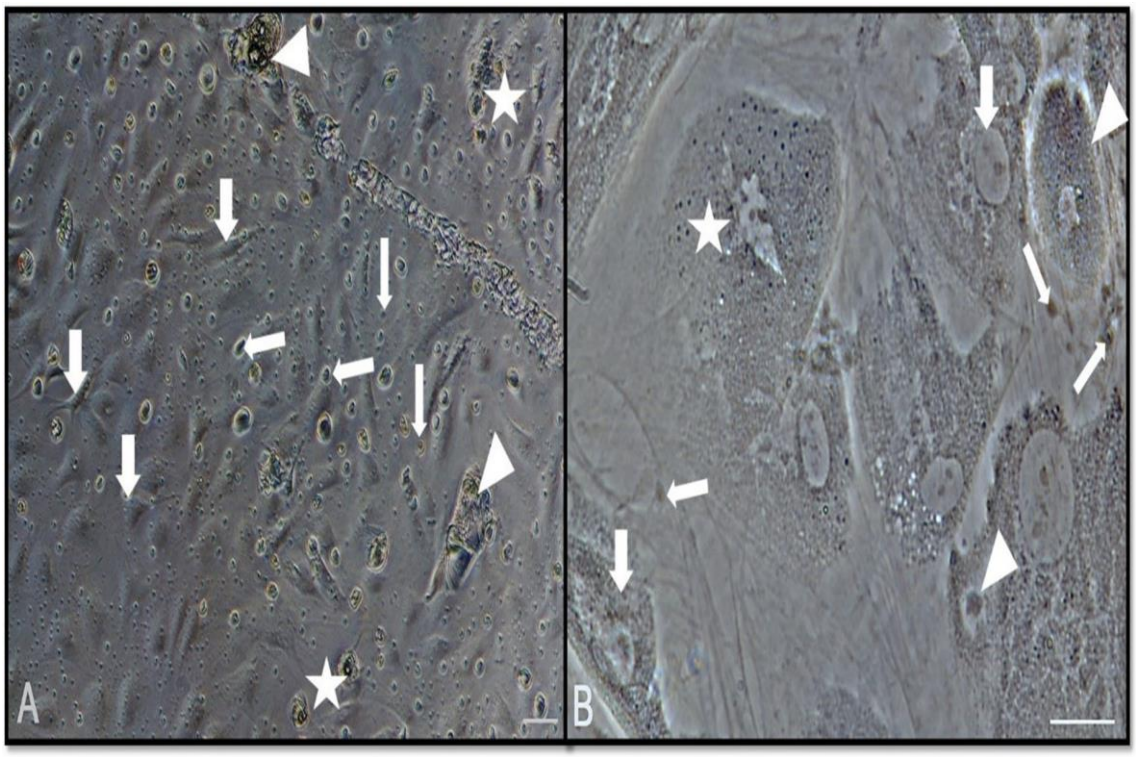

Figure 5. (A and B) SVF cells, approximately 60 % confluency on day 21 and still heterogenous cell population; residual cells of adipose tissue (arrowheads); lower density of fibroblast-like cells (thick arrows); variety form of small, oval and polygonal shaped of heterogeneous cell population (thin arrows); differentiated into heterogeneous cells (asterisks); second and third passages on day 28 (A) and 35 (B), respectively (Bar 25µ).

Discussion and Conclusion

Aronowitz et al. (3) reported that both enzymatic and non-enzymatic isolation methods for SVF cells were equally effective and safe. Zhu et al. (28) reported that non- enzymatic isolation process is less costly than enzymatic isolation process. Additionally, Varma et al. (26) reported that enzymatically isolated SVF cells reached first passage only in a week. However, López et al. (14) reported that enzymatic isolation process adversely affected cell viability and differentiation capacity of SVF cells. In our study SVF cells reached the first passage within three weeks. Katsara et al. (13) demonstrated that (5-12-week-old mice) adolescent mice have higher proliferation capacity than adults (13–34-week-old mice) and they noted more cells obtained from male mice at 1st, 2nd, and 3rd passages compared to female mice. In the present study, 12-week-old adolescent male Sprague Dawley rats were chosen for the cell isolation and ADSCs had higher proliferative capacity as compared to SVF cells. We speculate that the differences in rats' sex properties and ages may have affected ADSCs and SVF cells during the cell culture process. High proliferative capacity of ADSCs after 24 hours of culture were also reported by other studies (9, 16, 19, 21, 30). In accordance with our study, Nadri et al. (16) reported that spindle-shaped ADSCs at the 1st passage reached complete confluency after one week of culture. In the present study, the higher density of adherent cells and earlier 1st confluency were observed in ADSCs compared with SVF cells. Thus, our study suggests that ADSCs have a better cell proliferation capacity compared with SVF cells. Zhu et al. (29) reported that ADSCs have extensive confluency propensity compared with bone marrow-derived stem cells (BMSCs) (29). Condé Green et al. (8) noted that BMSCs and SVF cells consisted of the heterogeneous cell population (such as endothelial cells, leukocytes, vascular smooth muscle cells, ADSCs and pericytes adhered), which may have affected in-vitro culture duration phase. Furthermore, the present study showed that compared with SVF cells, ADSCs exhibited a higher density of adherent cells, an earlier migratory phase and faster growth. We speculate that a more homogenous cell population and better survival capacity may have contributed to enhanced proliferative capacity of ADSCs, as compared with SVF cells. Asumda et al. (4), Sun et al. (23) and Zhu et al. (29) studies suggest that the cross-interaction between endothelial cells and stem cells may have been responsible for diminished proliferative capacity of SVF cells. Chazenbalk et al. (7) study reported that SVF cells expressed a lower density of progenitor stem cells than ADSCs. And also, Sancak (19) and Peng et al. (17) study reported that in ADSCs have more cell adhesion and cell-forming ability. Furthermore, Shah et al. (22), Van et al. (26), Varma et al. (27) and You et al. (28) reported that SVF cells present a hematopoietic cell population on advanced passages and have a heterogeneous cellular composition, secrete different cytokine and growth factors. Also, in our study, the population of SVF cells showed a heterogeneous cell population such as spindle, polygonal or oval shaped cells. As a result of heterogeneity in cell population, SVF cells required longer cultivation phase as compared with ADSCs.

In conclusion; in present study, we showed that ADSCs have a better adhesive ability, higher proliferative capacity in all passages and require shorter time to reach confluency compared with SVF cells in vitro. These findings may contribute to future studies that deal with isolation and selection of stem cells from various tissues, as well as design clinical trials based on ADSCs and SVF.

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Ethical Statement

This study was approved by the Uludağ University Local Ethical Committee of Animal Experiments (2019-05/01).

Conflict of Interest

The authors declared that there is no conflict of interest.

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