

Adipose-derived stem cells and hyaluronic acid based gel compatibility, studied *in vitro*

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Abstract. Minimally invasive aesthetic and cosmetic procedures have increased in popularity. Injectable dermal fillers provide soft tissue augmentation, improve facial rejuvenation and wrinkles, and correct tissue defects. To investigate the use of adipose-derived stem cells integrated with a hyaluronic acid based gel as a dermal filler, the present study used cytotoxicity studies, proliferation studies, adipogenic and osteogenic differentiation, apoptosis assays and scanning electron microscopy. Although hyaluronic acid induced low levels of apoptosis in adipose-derived stem cells, its significantly promoted proliferation of adipose-derived stem cells. Hyaluronic acid demonstrates little toxicity against adipose-derived stem cells. Adipose-derived stem cells were able to differentiate into adipocytes and osteoblasts. Furthermore, scanning electron microscopy revealed that adipose-derived stem cells maintained intact structures on the surface of hyaluronic acid as well as in it, and demonstrated abundant cell attachments. The present study demonstrated the compatibility of adipose-derived stem cells and hyaluronic acid based gels *in vitro*.

Introduction

In the last decade, minimally invasive aesthetic and cosmetic procedures have increased in popularity due to their speed and safety. Injectable dermal fillers provide soft tissue augmentation, improve facial rejuvenation and wrinkles and correct tissue defects including scars and lipoatrophy (1). An ideal implant should provide structural support and be safe, effective, long-lasting and biocompatible. Research in the field of tissue engineering has demonstrated that it appears increasingly possible to use multipotent stem cells for injectable

dermal fillers. Adipose-derived stem cells are an attractive cell resource as they have an abundant source, are easy to isolate from fat tissue and inject immediately post-isolation, are able to proliferate at a high rate, and possess multipotent differentiation properties when cultured in lineage-specific induction media. Multiple animal studies and clinical reports have demonstrated that adipose-derived stem cells repair various defects, including adipose tissue, cartilage, bone and nerve defects (2-4). However, adipose-derived stem cells should be integrated into a scaffold to maintain their position. Hyaluronic acid, which exists in the extracellular matrix, is considered to be a suitable injectable material. Hyaluronic acid-based gels may survive *in vivo* for 6 months to maintain tissue augmentation (1,5). They may be the ideal scaffold for adipose-derived stem cells, as they have no risk for allergic reaction and provide a favorable environment for cells.

To investigate the potential for the use of adipose-derived stem cells integrated with hyaluronic acid based gel as a dermal filler, the present study investigated the compatibility of adipose-derived stem cells and hyaluronic acid *in vitro*.

Materials and methods

Cell culture. All animal experimental protocols were approved by the Ethics Committee of China Medical University (Shenyang, China) and performed according to China Medical University guidelines. Five Male Sprague-Dawley rats (age, 8-12 weeks; weight, 200-250 g) were obtained from the experimental animal department of China Medical University (Shenyang, China). Rats were pre-medicated with Ketalar (50 mg/ml, 0.2 ml/100 g body weight; Jiangsu Hengrui Medicine Co. Ltd., Lianyungang, China), and sacrificed to harvest inguinal fat pad and dissect adipose tissue. The adipose tissue was digested using 0.1% collagenase type I (V900891; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at 37°C for 30 min. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added for neutralization, and the stromal vascular fraction was separated from the floating adipocytes by centrifugation at 300-500 x g at room temperature for 10 min. The cells in the stromal vascular fraction were resuspended and cultured in DMEM supplemented with 10% FBS. The adherent cells were reserved by changing the

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culture medium after 24 h, and were passaged 3 times prior to use in further experiments.

Flow cytometry. Rat adipose-derived stem cells from the 3rd passage were lifted with trypsin and washed with phosphate-buffered saline (PBS) 3 times. The cells were incubated with fluorescein isothiocyanate (FITC)-conjugated primary antibodies: Anti-CD29 (SAB4700397, 1:1,000), anti-CD44 (SAB4700189, 1:500), anti-CD45 (SAB4700480, 1:500) and anti-CD11b (SAB4700388, 1:500) (all from Sigma-Aldrich; Merck KGaA), in the dark at room temperature for 20 min. Following this, the cells were washed with PBS and flow cytometry (FACSaria; BD Biosciences, Franklin Lakes, NJ, USA) was performed, the results were analyzed with WinMDI2.9 (developed by Joe Trotter; Purdue University Cytometry Laboratories, West Lafayette, IN, USA).

Cell viability assay. Rat adipose-derived stem cells from the 3rd passage were seeded into 96-well plates at a density of 2×10^4 cells/ml with 100 μ l hyaluronic acid based gel (Shandong Freda Biotechnology Co. Ltd., Linyi, China), and cultured in DMEM supplemented with 10% FBS. Following culture for 1, 2, 3, 4, 5, 6 or 7 days, cells were incubated with 10 μ l Cell Counting kit-8 (CCK-8) solution (Beyotime Institute of Biotechnology, Haimen, China) at 37°C for 3 h. The samples were measured at a wavelength of 450 nm with an Infinite M200PRO Microelisa reader (Tecan Group Ltd., Männedorf, Switzerland). Adipose-derived stem cells were cultured in 100 μ l DMEM supplemented with 10% FBS alone as a control group.

Cytotoxicity study. For the cytotoxicity study, 100 μ l hyaluronic acid-based gel and 0.5×10^4 adipose-derived stem cells were cultured in 100 μ l DMEM supplemented with 10% FBS, and subsequently seeded into 96-well plates. CCK-8 solution (10 μ l) was added following 3 days of culture. Following incubation for 3 h, absorbance values were detected by a spectrophotometer at a wavelength of 450 nm. Adipose-derived stem cells were cultured in 100 μ l DMEM supplemented with 10% FBS alone as a control group.

Proliferation study. For the proliferation study, 100 μ l hyaluronic acid based gels and 0.2×10^4 adipose-derived stem cells were cultured in 100 μ l DMEM supplemented with 10% FBS, and then seeded into 96-well plates. CCK-8 solution (10 μ l) was added 1, 3 or 6 days following this into the co-cultured system. Following incubation for 3 h, absorbance values were detected by a spectrophotometer at a wavelength of 450 nm.

Apoptosis assay. An Annexin V-fluorescein isothiocyanate Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA) was used to evaluate the effect of hyaluronic acid based gels on apoptosis in adipose-derived stem cells, and 100 μ l hyaluronic acid based gel and 1×10^6 adipose-derived stem cells were cultured in 100 μ l DMEM supplemented with 10% FBS, and subsequently seeded into 24-well plates. The cells were lifted with trypsin 1, 3 or 6 days later, and resuspended in binding buffer. Annexin-V FITC and propidium iodide were added and incubated in the dark for 15 min. The FACScan flow cytometer (BD Biosciences) was used to

detect the effect on apoptosis and the results were analyzed using WinMDI2.9. Adipose-derived stem cells cultured in 100 μ l DMEM supplemented with 10% FBS alone served as a control group.

Adipogenic and osteogenic differentiation of rat adipose-derived stem cells. To detect the multipotential differentiation capacity of the adipose-derived stem cells within the hyaluronic acid based gel, rat adipose-derived stem cells from the 3rd passage were used. Once the cells reached 80% confluence, they were cultured in adipogenic induction medium [DMEM supplemented with 10% FBS, isobutyl-methylxanthine (0.5 mM), dexamethasone (1 μ M), insulin (10 μ M) and indomethacin (200 μ M)] for 14 days, osteogenic induction medium [DMEM supplemented with 10% FBS, dexamethasone (0.1 μ M), ascorbate-2-phosphate (50 μ M) and β -glycerophosphate (10 mM)] for 21 days. Oil-Red O and Alizarin Red S staining were subsequently used to confirm adipogenic and osteogenic differentiation of the rat adipose-derived stem cells, respectively.

Scanning electron microscopy. To evaluate the cell attachment capacity of hyaluronic acid based gels, scanning electron microscopy was applied. Briefly, 3% glutaraldehyde solution in cacodylate buffer (0.1 M) was used to fix the adipose-derived stem cells with hyaluronic acid for 3 h. The fixed complex was subsequently washed with PBS three times. Graded ethanol solutions (30, 50, 70, 85, 90, 95 and 100%) and hexamethyl disilazane were used for dehydration. The dried complex was coated with Pt/Pd, and scanning electron microscopy was applied.

Statistical analysis. All data were expressed as the mean \pm standard deviation. One-way analysis of variance followed by Tukey's post hoc test was used to analyze the differences between groups. Statistical analyses were conducted with SPSS13.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a significant difference.

Results

Identification of rat adipose-derived stem cells. Rat adipose-derived stem cells isolated from adipose tissue were adhered following 3–4 h culture, and the suspended cells were removed following 24-h culture. The spindle cells demonstrated proliferation from 3 days and colony formation from 5 days, and were passaged after 7 days when they reached 80–90% confluence. Rat adipose-derived stem cells were successfully obtained at the 3rd passage and were used in subsequent experiments (Fig. 1). Flow cytometry demonstrated that rat adipose-derived stem cells from the 3rd passage were CD29 and CD44 positive, but CD45 and CD11 negative (Fig. 2).

Cell viability assay. The results of the cell viability assay indicated that rat adipose-derived stem cells within hyaluronic acid proliferated slowly for the first 48 h, but proliferation accelerated from 3 days onwards and appeared to peak at 6 days, which was similar to the results observed for adipose-derived stem cells alone (Fig. 3).

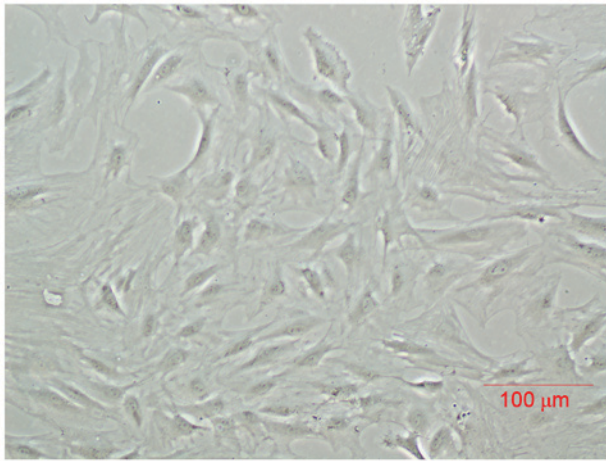


Figure 1. Rat adipose-derived stem cells from the 3rd passage, which formed a large, flat monolayer and were arranged in bundles or whorls. Scale bar=100 μ m.

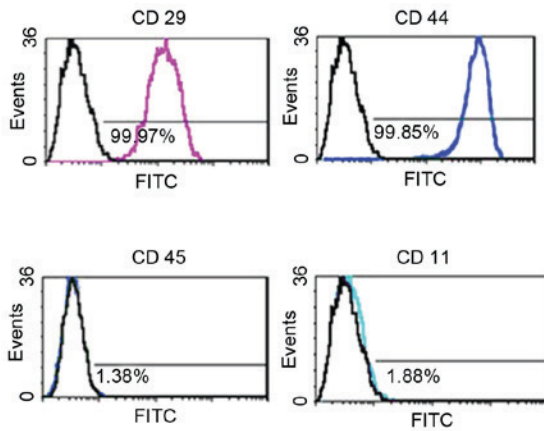


Figure 2. Flow cytometry revealed that rat adipose-derived stem cells from the 3rd passage were CD29 and CD44 positive, but CD45 and CD11 negative. FITC, fluorescein isothiocyanate; CD, cluster of differentiation.

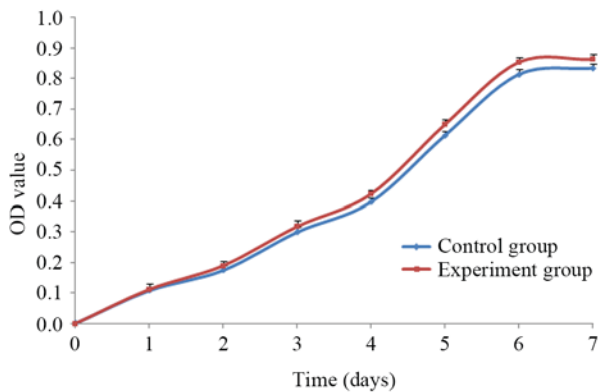


Figure 3. Cell viability assay indicated that there was a lag phase of cell proliferation within the first 48 h, but proliferation increased rapidly from 3 days and appeared to peak at 6 days. Results are presented as the mean \pm standard deviation (n=5). OD, optical density.

Cytotoxicity study. Following 3 days of culture, the mean absorbance value of adipose-derived stem cells within hyaluronic acid was 0.7471 ± 0.0129 , and the control group was

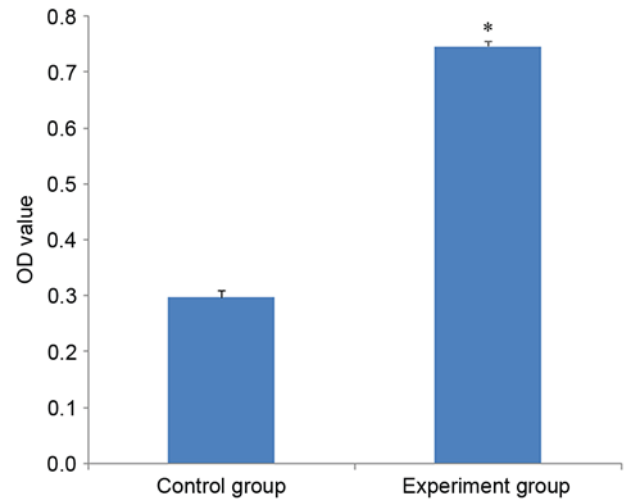


Figure 4. Mean absorbance value of adipose-derived stem cells within the hyaluronic acid based gel experimental group (0.7471 ± 0.0129) was significantly higher than the control group (0.2989 ± 0.0190). Results are presented as mean \pm standard deviation (n=5). *P<0.05 vs. control. OD, optical density.

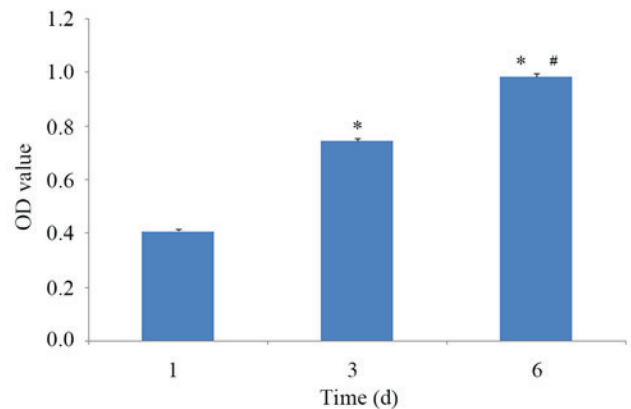


Figure 5. Mean absorbance value of adipose-derived stem cells within the hyaluronic acid based gel at 6 days (0.9829 ± 0.0185) and 3 days (0.7471 ± 0.0129) was significantly higher than at 1 day (0.4089 ± 0.0156). Results are presented as mean \pm standard deviation (n=5). *P<0.05 vs. 1 day, #P<0.05 vs. 3 days. OD, optical density.

0.2989 ± 0.0190 (Fig. 4). This indicated that the cell activity of the group with hyaluronic acid was significantly higher. The results demonstrated that hyaluronic acid was not cytotoxic to adipose-derived stem cells.

Proliferation studies. The mean absorbance value of adipose-derived stem cells within hyaluronic acid at 6 days was significantly higher than that at 3 days (0.9829 ± 0.0185 and 0.7471 ± 0.0129 , respectively; Fig. 5). This indicated that cell activity of adipose-derived stem cells at 6 days was significantly higher than at 3 days. The results suggested that hyaluronic acid may promote adipose-derived stem cell proliferation.

Apoptosis assay. Cell apoptosis rates at 1, 3 and 6 days were 20.34 ± 1.53 , 28.59 ± 0.48 and $37.95 \pm 0.93\%$, respectively. The apoptosis activity of adipose-derived stem cells within hyaluronic acid increased mildly with time, and was higher than the

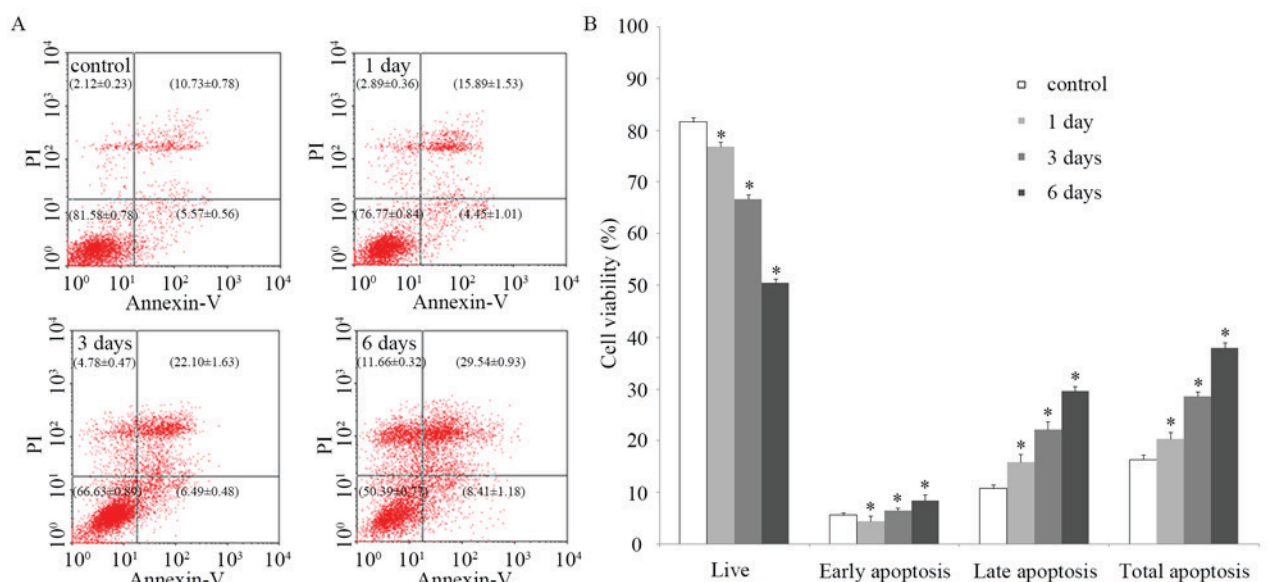


Figure 6. (A) The apoptosis activity of adipose-derived stem cells within the hyaluronic acid gel increased fairly with time, and was higher than control group. (B) Results are presented as mean \pm standard deviation (n=5). *P<0.05 vs. control. PI, propidium iodide.

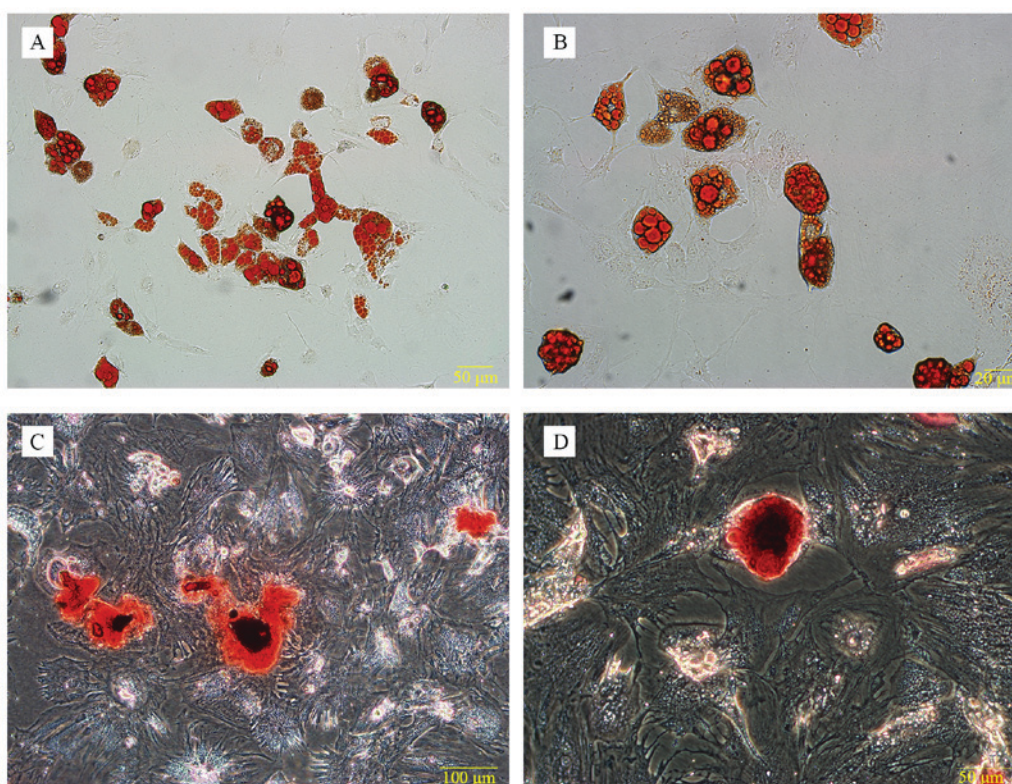


Figure 7. When treated with the appropriate lineage-specific induction medium, rat adipose-derived stem cells within the hyaluronic acid based gel were successfully differentiated into adipocytes and osteoblasts, as confirmed by (A and B) Oil-Red O staining and (C and D) Alizarin Red S staining.

control group, which indicated that hyaluronic acid may induce a fair level of apoptosis in adipose-derived stem cells (Fig. 6).

Adipogenic and osteogenic differentiation of rat adipose-derived stem cells. To verify the maintenance of multipotent differentiation in the adipose-derived stem cells within the hyaluroinc acid gel, adipogenic differentiation

was verified by Oil-Red O staining, which demonstrated the presence of intracellular lipid droplets, while osteogenic differentiation was confirmed by Alizarin Red S staining, which demonstrated calcium deposits (Fig. 7).

Scanning electron microscopy. Scanning electron microscopy indicated that adipose-derived stem cells maintained an intact

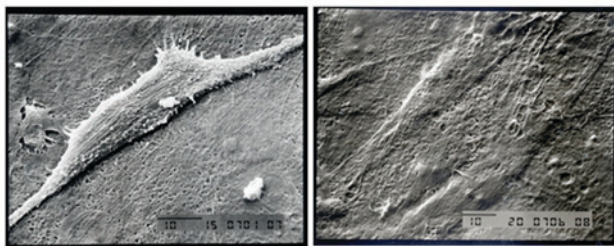


Figure 8. Adipose-derived stem cells maintained an intact structure on the surface of the hyaluronic acid based gel as well as inside it. Images acquired by scanning electron microscopy. Magnification, $\times 1,500$.

structure on the surface of hyaluronic acid gel as well as inside it, and demonstrated abundant cell attachments (Fig. 8).

Discussion

During the last decade, injectable dermal fillers have become a popular treatment to improve facial rejuvenation and wrinkles, correct facial lipoatrophy and provide soft tissue augmentation. Increasing numbers of patient select this method over traditional surgery (1). The ideal injectable dermal filler for soft tissue augmentation should provide structural support, and be safe, effective, long-lasting, biocompatible and not elicit adverse reactions. However, no injectable dermal fillers currently meet this standard, as they give rise to certain adverse reactions (6,7). Therefore, it may hypothesized that integrating two or more known injectable dermal fillers may maximize the strengths whilst minimizing the weaknesses.

The use of adipose-derived stem cells has emerged for plastic surgery, and they are an attractive source for adipose tissue engineering (8-10). Adipose-derived stem cells have an abundant source, are easy to isolate from fat tissue and inject immediately post-isolation, are able to self-renew with a high growth rate, leave limited donor site morbidity and possess multipotent differentiation properties when grown in lineage-specific induction medium. Multiple animal studies and clinical reports have demonstrated that adipose-derived stem cells may repair various defects (2-4). However, adipose-derived stem cells have some limitations. For example, they must be integrated into a scaffold to maintain their position for a short time. The scaffold must be used as a cell carrier upon which cells are seeded, otherwise the injected adipose-derived stem cells disappear and fail to be detected by the Alu gene (11), indicating the adipose-derived stem cells were not able to remain in the injected position without a scaffold. Thus, an absorbable scaffold integrated with adipose-derived stem cells maybe the ideal choice for adipose tissue engineering to augment soft tissue.

The essential properties of ideal cell carrier scaffolds are managed degradation and immunodeficiency (12). At present, hyaluronic acid based gels are the most attractive injectable dermal fillers (13). Hyaluronic acid is one of the main polysaccharides of the extracellular matrix of the dermis, which adds to volume of the skin by promoting keratinocyte proliferation and binding water in the soft tissue, providing support. Experimental studies have demonstrated that hyaluronic acid

induces synthesis of type I collagen, which may contribute to providing a preferable microenvironment for adipose-derived stem cells (14-16). Evidence suggests that hyaluronic acid does not stimulate the immune response and is completely degraded by 6 months in patients, leaving no serious adverse effects (17).

Relative to its advantage, hyaluronic acid is usually degraded and reabsorbed after 6 to 9 months (17-19). Therefore, to maintain its effect, hyaluronic acid must be injected repeatedly, which may increase pain and economic burden, in particular in developing countries and less developed areas (20-22).

In the present study, the compatibility of hyaluronic acid based gels with adipose-derived stem cells has been demonstrated *in vitro*, and they may be integrated with each other as a potential dermal filler. Although our experiment revealed that hyaluronic acid induced a fair increase in adipose-derived stem cell apoptosis, it promoted proliferation of adipose-derived stem cells significantly, indicating that hyaluronic acid may provide a preferable microenvironment for adipose-derived stem cells. Future studies should further investigate the apoptotic rate of adipose-derived stem cells treated with hyaluronic acid, and how this may be controlled.

The present study revealed that adipose-derived stem cells were able to differentiate into adipocytes and osteoblasts. Furthermore, the data demonstrated that adipose-derived stem cells maintained an intact structure on the surface of hyaluronic acid as well as inside it, and demonstrated abundant cell attachment using scanning electron microscopy. This indicated hyaluronic acid may provide a microenvironment suitable for maintaining cell homeostasis and for differentiating tissue properties.

In conclusion, the present study demonstrated the compatibility of adipose-derived stem cells and hyaluronic acid based gels *in vitro*, with the successful culture of adipose-derived stem cells on hyaluronic acid based gel, cell proliferation, cell toxicity and differentiation assays. Further studies may focus on the combined application of adipose-derived stem cells and hyaluronic acid based gels *in vivo*.

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