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Original Article

Biological properties of human adipose-derived stem cells collagen sponge complex in promoting angiogenesis

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Abstract: This study aims to explore the clinical biological properties of human adipose-derived stem cells-collagen sponge complex (ADSCs-CSC) in promoting angiogenesis. The biological properties of ADSCs were determined by flow cytometry, and the porosity and adhesive rate of ADSCs were detected. The proliferation rate of ADSCs in ADSCs-CSC was determined by Cell Counting Kit (CCK) assay. The tubule formation of HUVECs was detected by Matrigel assay. Vascular endothelial growth factor (VEGF) expression was determined by ELISA assay and RT-qPCR assay. The counts of newborn vessels were determined with chorioallantoic membrane (CAM) assay. The surface signs of the P3 generation of ADSCs were CD34-negative and CD90-positive; meanwhile, the lipid droplet secretion and alizarinred assay proved the successful culture of ADSCs. The adhesive rate of ADSCs-CSC reached to 93.04±0.67%. The proliferation and tubule formation of HUVECs were notably enhanced, and content and transcription level of VEGF in ADSCs-CSC group were improved significantly than those by ADSCs alone. The counts of newborn vessels were enhanced by nearly twice in ADSCs-CSC implanted on CAM than that in ADSCs implanted alone. ADSCs could adhere to medical-grade collagen sponge with good biocompatibilities, and further promote angiogenesis by enhancing the proliferation of ADSCs and the secretion of VEGF.

Keywords: ADSCs, collagen sponge, biocompatibility, scaffold, angiogenesis

Introduction

People's pursuit towards beauty explodes the rapid developments of implanted biological tissue engineering materials, while due to the heterogeneity of these biological materials, they would inevitably produce such heterogeneity reactions as fiber-capsular contracture and scar hyperplasia, etc. [1]. Autologous tissue augmentation could eliminate these heterogeneity reactions, such as autologous adipose particle injection, which could be performed simultaneously with liposuction, and the fat sucked out could be used as the filling material. But this method might exist various degrees of liquefaction and absorption, and 10-12 months after the transplantation, 30-60% of the graft would be absorbed [2] because the growth of tissues needed adequate blood supply [3], while the fat cells implanted could not receive adequate nutritional support, thus leading to liquefaction and absorption, as well as nodules in the late period. Adipose-derived stem cells (ADSCs) is a kind of mesenchymal adult stem cells obtained from adipose tissue and derived from the mesoderm, it has high degree of differentiation and could maintain in vitro self-renewal; it could also significantly secrete a lot of angiogenic and anti-apoptotic factors. A number of in vivo and in vitro experiments had confirmed that ADSCs played leading roles in repairing body's refractory damages [4, 5]. It had become one of the main methods to improve the survival of implanted biological tissue engineering materials by directly transplanting ADSCs-degradable biomaterials into the body and inducing the formation of the target tissues [6]. Collagen is the main structural component of the connective tissues, could promote cell adhesion and proliferation, and has good biocompatibility and degradation; meanwhile, its degraded products could be utilized by cells, so it has become one of the ideal materials for scaffold. It was found that [7]

ADSCs could adhere and grow on type I collagen scaffold. Kato et al. [8] used ADSCs-artificial skin complex to effectively promote the repair of chronic diabetic skin defect. Pallua et al. [9, 10] found that ADSCs-collagen scaffold could promote the angiogenesis and survival inside the implanted scaffold, and the effects were better than collagen sponge scaffold alone, so it could be implanted in vivo for soft tissue reconstruction. This study aimed to investigate the relationships between the growth of ADSCs inside ADSCs-CSC and its effects of pro-angiogenesis and pro-secretion of vascular endothelial growth factors.

Materials and methods

Preparation, culture, and biological characteristics detection of ADSCs

ADSCs was originally obtained from the adipose tissues discarded after liposuction in the department of orthopedics, Jiangsu Provincial Hospital: repeatedly rinsed the fat tissues with buffer solution under sterile conditions, then washed with Hyclone phosphate buffer saline buffer (PBS, Thermo Scientific, Beijing, China) for 3 times; digested with type I collagenase (Sigma, Saint Louis, USA) for 40 minutes, filtered the undigested tissue mass and cell clumps, centrifuged for 5 min. Discarded the supernatant, prepared the precipitate with Hyclone DMEM medium (Thermo Scientific, Beijing, China) into single cell suspension liquid; culture in one incubator, and changed the medium once every 2-3 days; when the cells reached 70% to 80% of confluence, sucked the old culture medium, rinsed, cultured and digested with collagenase for 5 min. When the cells were observed floating and turning round, terminated the digestion; centrifuged and discarded the supernatant, and prepared the precipitate into the cell suspension; evenly inoculated the cell suspension, and performed the surface antigen identification by flow cytometry, as well as the lipid droplet red O staining (Ziyi Reagent Factory, Shanghai, China) and cell alizarin staining (Sigma, Saint Louis, USA).

Detection of the porosity in collagen sponge, and the composition and adhesion rate of ADSCs-CSC

Weighed the collagen sponge (recorded as ms, Wuxi BIOT Bio-technology Co., Ltd., Wuxi, China); filled one pycnometer with ethanol (recorded as m^1), and then immersed the collagen sponge into the pycnometer and made ethanol sufficiently filling the pores of the collagen sponge (recorded as m^2); removed the collagen sponge, weighed the rest ethanol and pycnometer (recorded as m^3); porosity (%) = (m^2-m^1-ms)/(m^1-m^3) × 100%; under sterile conditions, prepared the collagen sponge into 10 mm × 10 mm × 1 mm blocks, then added 0.2 ml of the cell suspension with P3-generation ADSCs (cell density 2 × 10^5 cells/ml) onto each block of the collagen sponge. After 4 h standing, moved the sponge into incubator with 5% CO_2; determination of the adhesive rate: after 8 h culture, removed the collagen sponge to the plate, washed with the culture medium, and measured the loss of the cells; after 24 h culture, repeated the above steps to obtain the non-adherent cells. Adhesive rate = (inoculated cell number-the number of the cells lost-the number of non-adherent cells)/(inoculated cells-the number of the cells lost) × 100%.

Proliferation of ADSCs (cell counting Kit-8, CCK-8 method)

Experimental grouping: the ADSCs group and the ADSCs-CSC group. Selected the sample, then added into 100 μl of complete medium and cultured in an incubator for 24 h; added 10 μl of CCK-8 (Dojindo, Kumamoto, Japan) into each well and incubated for another two hours; detected the absorbance at 450 mm with a microplate reader on the 2^{nd}, 4^{th} and 6^{th} day. Determination of cell proliferation: the OD value of each test well minus the OD value of the background (the complete medium plus CCK-8, without cell); the OD values of each duplicate wells were averaged and expressed as mean ± standard deviation ($\overline{x}$±s).

Endothelial cell tube formation assay

After grown to approximate 80% confluence, HUVECs were collected and seeded with 75,000 cells on the surface of Matrigel (BD Biosciences, Bedford, MA, USA), 250 μL of which covered per well in 24-well plates. Each sample was periodically observed by microscope in the process of forming capillary-like tubule structures. After 8 h of incubation, samples were photographed, and the average of number of tubules was assessed from observation of five independent microscopic fields.

Table 1. Sequences of primers applied in the qRT-PCR assay

Gene	Primer sequences (5'-3')	Length of PCR product (bp)
VEGF	F: CCCACTGAGGAGTCCAACAT	158
	R: TCCCTTTCCTCGAACTGATT	
GAPDH	F: GAGTCAACGGATTTGGTCGT	185
	R: GACAAGCTTCCCGTTCTCAG	

Detection of vascular endothelial growth factors (VEGFs) secreted by ADSCs and ADSCs-CSC

The grouping was the same as 1.2.3. Sampled the specimens according to each designed test time point and performed 20 min centrifugation at 3000 rpm for the supernatant; removed the suspended solids and sediment, packaged with sterile tubes; analyzed the VEGF levels (ng/ml) in the medium according to the instructions of ELISA assay kit (Millipore-packaged standards) (Mergene Co. Ltd., Nanjing, China).

Real-time quantitative-PCR

Total RNA was isolated using QiagenRNeasy Mini Kit (TaKaRa, Dalian, China). First-strand cDNA synthesis was the performed, in which 20 μL reaction including 100 ng random primer (Invitrogen, Carlsbad, CA), 1.0 μg of total RNA, 10 mM dNTP and 200 units of reverse transcriptase (Invitrogen, Carlsbad, CA) was used. The sequences of primers used in this study were listed in **Table 1**. PCRs were performed in a 25 μL final volume by using SYBR Green master mix from SABiosciences (SABiosciences, Frederick, MD). Relative mRNA expression was calculated by the comparative threshold (Ct) cycle ($2^{-\Delta\Delta Ct}$) method with the expression of GAPDH as an internal control.

Chorioallantoic membrane (CAM) assay

Experimental grouping: the control group, the ADSCs group, the collagen sponge group, and the ADSCs-CSC, with 12 embryos in each group. CAM assay (Jiangsu Academy of Agricultural Sciences, Nanjing, China): using the Ribatti [11] method to prepare the model: disinfected, then cultured the embryo for 7 days; cut the gas chamber of the embryo with one 2 cm-long triangular window; uncovered the eggshell and eggshell membrane attached to it and exposed chorioallantoic membrane. Added 0.2 ml of sterile PBS liquid into the control group, 0.2 ml of the P3 generation of ADSCs suspension (2×10^5/ml) into the ADSCs group, 10 mm × 10 mm × 1 mm of collagen sponge into the collagen sponge group, and 0.2 ml of ADSCs-CSC (2×10^5/ml) into the ADSCs-CSC group. Covered the window with sterile membrane then continued 7-day incubation; measured the microvessels on the CAM around the sample [12]; fixed the CAM sample with methanol: acetone (1:1) for 15 min, cut the CAM at the connection point and spread onto a glass slide; counted the microvessels (diameter between 20~40 μm) with the lattice method in three non-overlapped visions (× 50 magnification) by two independent people, which were then averaged and counted by the bifurcations. The angiogenesis rate of each group = (mean value of each group-mean value of the control group)/mean value of the control group × 100.

Statistical analysis

Statistical analysis was performed using SPSS13.0, One-Way ANOVA for the comparisons among the ADSCs group, the collagen sponge group, and the ADSCs-CSC group.

Results

Appearance and surface antigen characteristics of original ADSCs and the passaged ADSCs

The primary cells adhered to the wall completely after 24 h culture: the cells were round or spindle-shaped, with large nuclear/cytoplasm ratio; the cell volume was then increased, forming clones, protruding processes, and showing the typical shape of fibroblasts. Compared with the original cells, the round passaged cells were less, while the spindle-shaped cells were increased, showing parallel or whirlpool-like growth. Flow cytometry revealed that the surface of the P3 generation of the cells were CD34-negative and CD90-positive; the oil red O staining exhibited the lipid droplets inside the cells after 2-3 weeks of adipogenic induction culture, and the lipid droplets extended with the culture time increasing. The Alizarin Red staining showed positive staining after 3 weeks of osteogenic induction culture. The results showed that these cells extracted from the adipose tissues were ADSCs (**Figure 1A-D**).

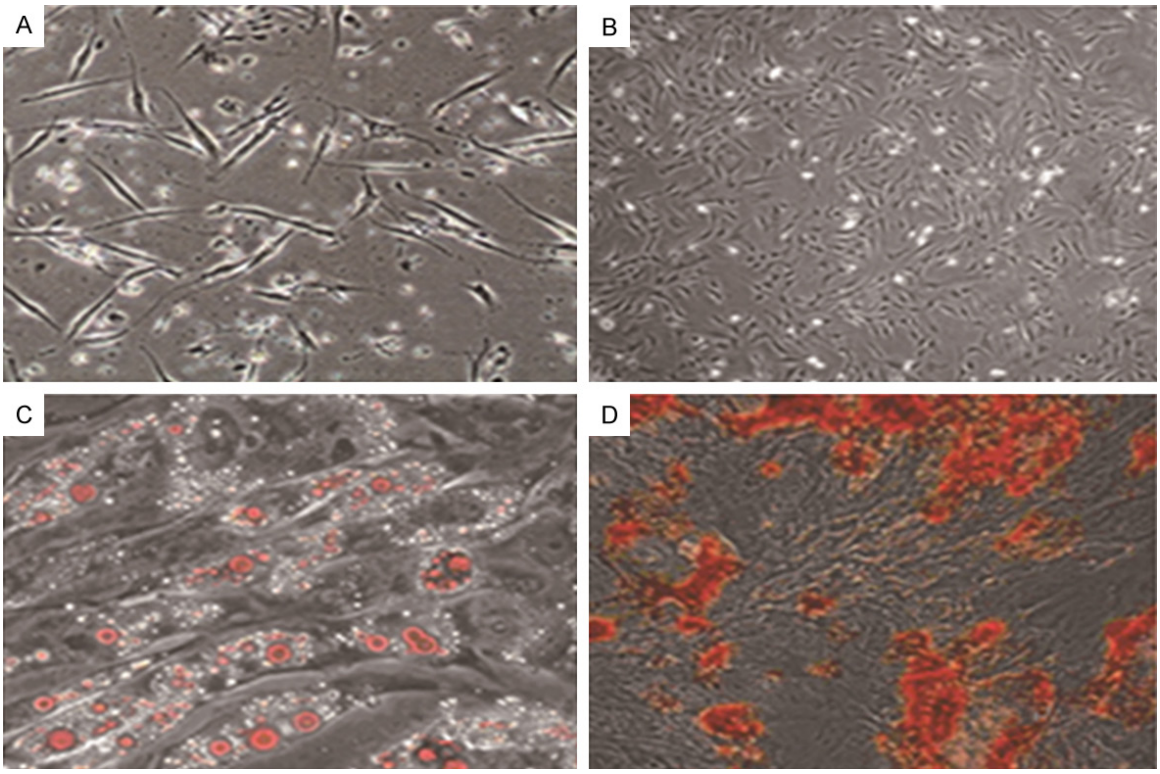

Figure 1. A. The P0 generation of the cells were round or spindle-shaped, with large nuclear/cytoplasm ratio; B. The P3 generation of the cells was arranged parallelly or exhibited whirlpool-like growth; C. Oil Red O staining (2-3 weeks later); D. Alizarin Red staining (3 weeks later).

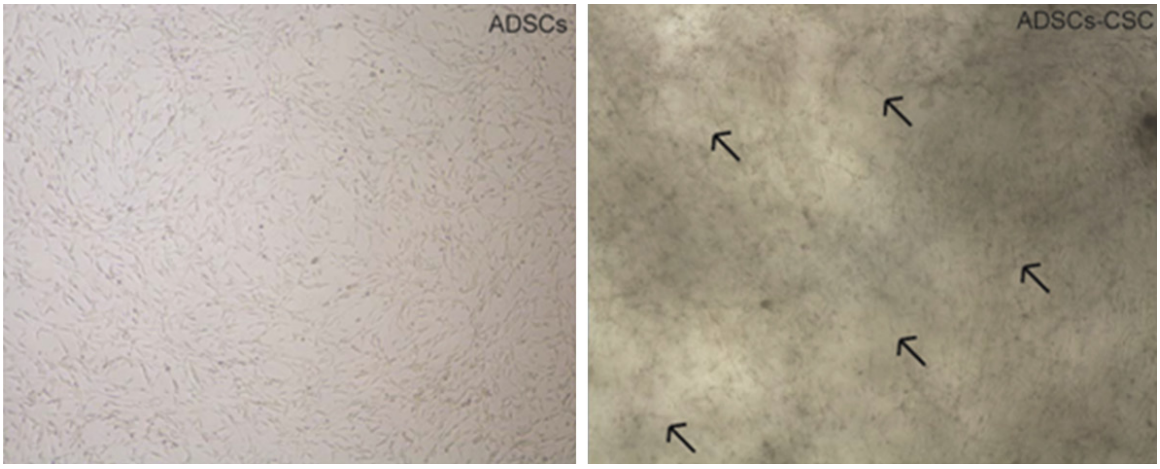

Figure 2. ADSCs-CSC under light microscope (d5) (× 40 times).

Porosity of the collagen sponge and adhesive rate of ADSCs-CSC

Ms = 0.0664 g; m^1 = 124.064 g, m^2 = 125.4 g, m^3 = 123.8082 g. According to the formula, the porosity (%) = (m^2-m^1-ms)/(m^1-m^3) × 100% = 91%; the adhesive rates of 5 collagen at 24 h were 92.24%, 93.25%, 92.66%, 94.02%, and 93.04%, respectively, and the average adhesive rate was 93.04±0.67%. Light microscopy revealed that when just inoculated into the collagen sponge, the ADSCs were round, evenly distributed within the scaffold; and the lucency rate of the collagen was too poor to be observed. After 4 h, the cell adhesion and stretch could be seen indistinctly; after 24 h, his cell adhe-

Table 2. Impacts of ADSCs-CSC on cell proliferation rate of ADSCs (n = 6, $\bar{x}\pm s$)

Group (n = 6)	OD ($\bar{x}\pm s$)		
	2 d	4 d	6 d
ADSCs group (control group)	0.70±0.39	0.72±0.34	0.77±0.09
ADSCs-CSC group	1.03±0.49	1.27±0.57	1.42±0.40*

*Compared with the control group, P<0.01.

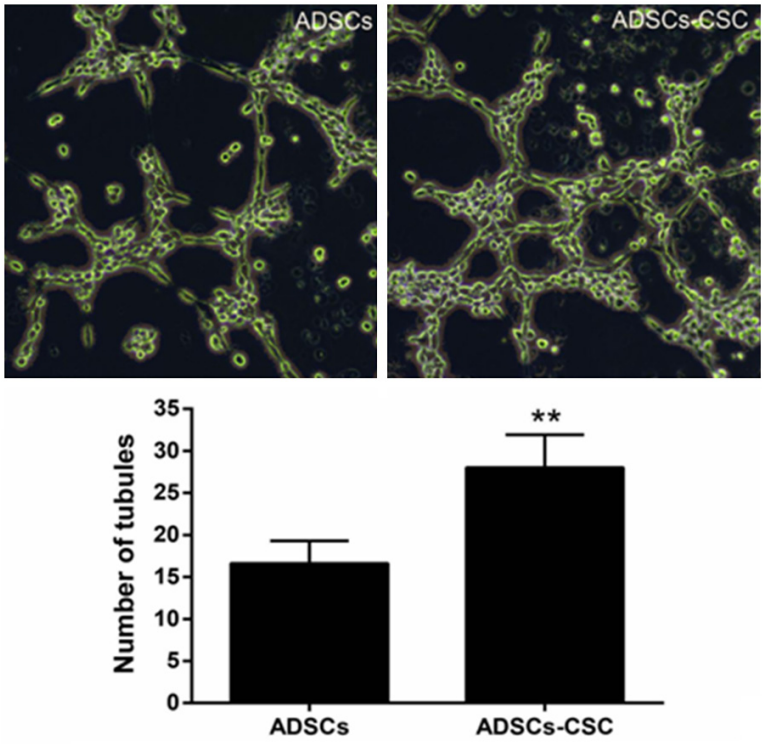

Figure 3. Tube formation of ADSCs.

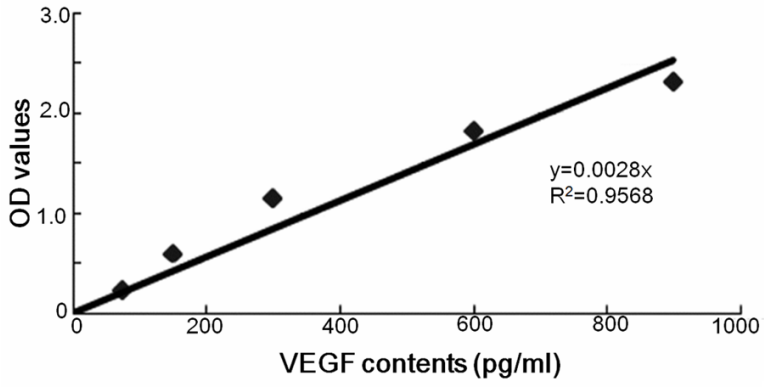

Figure 4. VEGF standard curve.

sion and stretch could be seen clearly. When cultured for 5 days, the cells had already stretched and deformed, with triangular and spindle-shape as the main shapes and mesh arrangement (**Figure 2**).

Pro-proliferation and pro-tube formation effects of ADSCs by ADSCs-CSC

With time increasing, ADSCs in the two groups remained incremental proliferation state on D2, D4, and D6, and no significant difference existed in the OD values of the two groups on D2 and D4 by One-Way ANOVA (P>0.05). In the early stage of cell proliferation, type I collagen showed no obvious promotion towards the cell proliferation, while on D6, there was statistically significant difference between the ADSCs group and the ADSCs-CSC group (P = 0.006, **Table 2**). Moreover, ADSCs-CSC remarkably preferred to promote angiogenesis than ADSCs (P = 0.001, **Figure 3**).

Detection of VEGFs

The VEGF standard curve was shown in **Figure 4**, and the VEGF content of each group was shown in **Table 3**. With time increasing, ADSCs-secreted VEGF kept an increasing state. There was statistically significant difference in the VEGF content of the ADSCs-CSC group on D4 and D6 by One-Way ANOVA (P = 0.007, P = 0.002). Then, RT-qPCR assay was performed to examine the mRNA level of cells in two groups to determine the effect of ADSCs-CSC on VEGF transcription. Parallelly with Elisa assay, the mRNA level of VEGF was notably improved by ADSCs-CSC compared to ADSCs group (**Figure 5**).

Table 3. Impacts of ADSCs-CSC on secretion of VEGF (n = 3, $\bar{x}$ ±s)

Group (n = 3)	VEGF content pg/mL ($\bar{x}$±s)		
	2 d	4 d	6 d
ADSCs group (control group)	44.30±2.41	48.05±1.25	50.25±1.70
ADSCs-CSC group	47.04±0.36	52.99±1.08*	58.94±1.27*
P	0.325	0.007	0.002

*Compared with the control group, P<0.01.

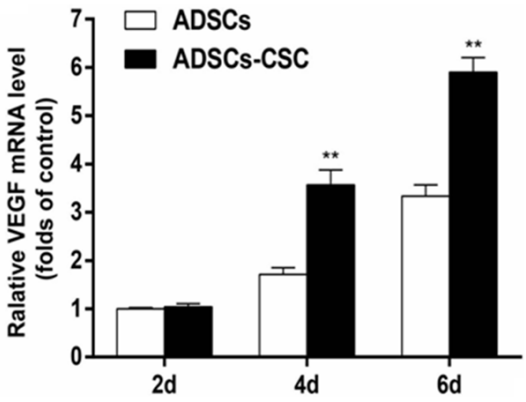

Figure 5. VEGF transcription level.

Table 4. Counts of newborn vessels by CAM assay (n = 12)

Group	Counts of newborn vessels ($\bar{x}$±s)
Control group	88.75±6.5
ADSCs group	109.21±22.83*
Collagen sponge group	119±14.26*
ADSCs-CSC group	169.64±46.03*,#

*Compared with the control group, P<0.01, #Compared with the ADSCs-CSC group, P<0.01.

CAM assay

The vessels at the CAM inoculation site exhibited spoke-like distribution with the inoculation site as the center. On the 3rd day of inoculation, middle and crude vessels could be seen, microvessels were increased, and the new vascular network gradually formed. When sampling, the embryo was still alive and growing, the CAM inoculation site exhibited rich blood vessels and full lumens. The counts of microvessels in each group were more than the control group, among which the ADSCs-CSC group exhibited the most obvious increasing. Compared with the control group, the differences were statistically significant (P<0.05). The ADSCs-CSC group exhibited the maximum number of newborn vessels, while no statistically significant difference could be found between the ADSCs group and the ADSCs-CSC group, P = 0.274>0.05, indicating these two showed little difference in promoting angiogenesis (**Table 4**; **Figure 6A-D** were enlarged by 22.5 times).

Discussion

Any tissue (except for cartilage) must have blood supply for survival if with a distance of more than 1 mm. Therefore, searching the method that could allow rapid in-scaffold vascularization had always been one of the most challenging areas in the tissue engineering research [13]. Our previous studies had shown that the VEGF-implanted collagen material could promote the proliferation of endothelial cells, and could stimulate the neovasculization in the surrounding tissues and promote the blood vessels to grow inside the biological material from the surrounding tissues in vivo experiments [14-16]. However, various VEGFs have short half-life, would be readily biodegraded, and their costs are expensive, so, looking for one cell-scaffold complex with such characteristics as self-renewal and could secrete pro-angiogenic growth factors would be one feasible implant material of tissue engineering. Human autologous fat cells have rich sources, and could be used as an ideal tissue engineering scaffold-loading cells. But the survival rate of the fat tissue transplanted was low, thus limiting its clinical promotion and application. ADSCs is a kind of multi-potent stem cells with multi-directional differentiation potentials, and it has such advantages as rich sources, easy sampling, small injury towards the body, rapid in vitro proliferation, and stable biological characters, etc., so it could be used as a kind of ideal seed cells for tissue engineering and regenerative therapy [17]. Modern research had shown that human ADSCS-type I collagen complex had good histocompatibility and pro-angiogenic effects [18, 19], and could effectively repair tissue defects [20]. This study showed that the adhesive rate of ADSCs-CSC was more than 90%, indicating that ADSCs and collagen sponge had good tissue compatibility; meanwhile, the secretion of VEGFs were increased in ADSCs-CSC, and the newborn vessels were also increased, consistent with the

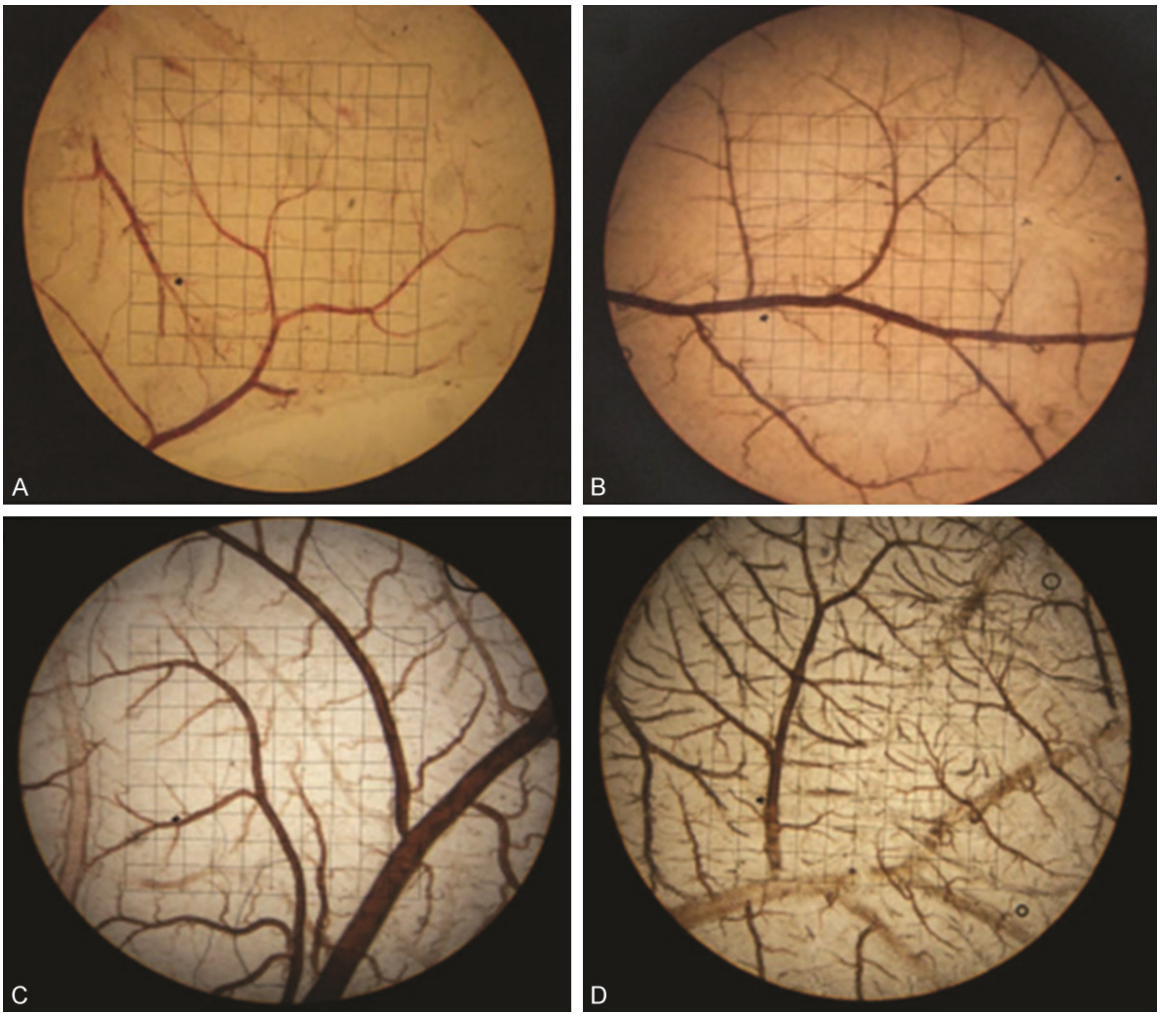

Figure 6. A. Control group; B. ADSCs group; C. Collagen sponge group; D. ADSCs-CSC group.

above researches. This study used the already-clinically-applied collagen sponge produced by Wuxi BIOT Bio-technology Co., Ltd. instead of analytical grade pure collagen sponge, so the results were be more close to clinical use. The collagen production process used in this study was cavernous high-purity type I lyophilized collagen, which had complete triple helix structure, and the molecular weight was about 300,000 Daltons; the aperture of the collagen sponge was uniform (70-100 μm), so it had good restoration ability, and could be used for clinic wound healing [18, 21] and surgical defect filling [22, 23]. The results of this study showed that the porosity of the collagen sponge was 91%, and the adhesive rate of ADSCs was 93.04%; certain study showed that when the porosity and adhesive rate were more than 90%, better growth and tissue compatibility of ADSCs could be obtained [24], therefore, the collagen sponge used in this study could combine with ADSCs well.

Currently, there is no specific surface marker of ADSCs, so its characteristics are mainly determined by directing its directional differentiation abilities. Studies had shown that ADSCs had high expressions of antigen CD29, CD44, and CD90; at the same time, CD31, CD34, and CD45 were negatively expressed. Therefore, flow cytometry could be used for the sorting [25, 26]. In this study, the cells with high expression of CD90 while negative expression of CD34 were sorted by flow cytometry, and these cells could be induced lipid droplets and exhibited positive Alizarin Red staining, indicating that these seed cells were ADSCs. When co-cultured with the collagen sponge, this study

showed that the co-culture could significantly promote the ADSCs seeds 4-6 days later, while when cultured for 2-4 days, although certain proliferation effects appeared, the effects were less significant. Therefore, we could presume that ADSCs could enter rapid growth period after 4-6 days of culture, while 2-4 days culture was still in the early stage of proliferation [27]. Meanwhile, a certain degree of degradation of the collagen sponge could provide its degradation products as the nutrients for the cells, thus contributing to the proliferation of ADSCs [28]. When ADSCs proliferated, the secretion of VEGF would be increased, so that the VEGF level inside ADSCs-CSC was increased, thus stimulating angiogenesis. Our previous studies had shown that the collagen itself could promote angiogenesis [29, 30], and this study showed that ADSCs-CSC could improve angiogenesis by 1-fold; our previous researches showed that when VEGF was loaded into the collagen, its role of angiogenesis was not significant [14, 15], while ADSCs exhibited significant pro-angiogenic effects [19, 26]; therefore, the significant pro-angiogenic effects should be the superimposed results of ADSCs and CS.

The results of this study showed the currently clinically applied collagen sponge had better adhesive rate and compatibility towards ADSCs, and its co-culture with ADSCs could promote the proliferation and tube fomationof ADSCs, improve the transcription level of ADSCs and stimulate their VEGF secretion, thus further promoting angiogenesis. Therefore, it would be conducive to the survival and promoting defect repair when implanted into the body, so this study provided some experimental basis for the clinical applications of collagen sponge as one vascularized tissue engineering material.

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Disclosure of conflict of interest

None.

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