Effect of Stromal Vascular Fractions on Angiogenesis of Injected Diced Cartilage

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Objectives: This study explored the effect of adipose-derived stromal vascular fractions (SVFs) on angiogenesis in injected autologous diced cartilage.

Methods: Stromal vascular fractions were extracted by enzymatic digestion. Cartilage grafts were harvested from 1 side of the auricular cartilage of New Zealand rabbit and then diced to a size of 1.0 mm³. The grafts were divided into 2 groups. The control group was diced cartilage mixed with culture medium, and the experimental group was diced cartilage mixed with SVFs. The 2 groups of composite grafts were subcutaneously implanted on both sides of the back of each rabbit. After 4, 12 and 24 weeks, the volume of tissue was measured. Further, the expression of Hypoxia inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) mRNA and protein were measured by RT-PCR and Western blotting, respectively. The results showed that the mRNA expression of VEGF and HIF-1α in the experimental group was increased. The mRNA level remained higher than that of the control group at 24 weeks (P < 0.05). And the relative expression levels of VEGF and HIF-1α protein in the experimental group were higher than those in the control group at 4, 12 and 24 weeks (P < 0.05).

Conclusion: Autologous diced cartilage mixed with adipose-derived SVFs can promote angiogenesis when transplanted by injection. Further research showed that SVFs could increase the expression levels of VEGF and HIF-1α in the grafts, which may be part of the mechanism that SVFs promoted the angiogenesis of diced cartilage.

Key Words: Adipose stem cells, angiogenesis, diced cartilage, injection transplantation, stromal vascular fractions

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A utologous cartilage graft has become an ideal material for rhinoplasty, especially for revision surgery.1 However, many problems in the clinical application of cartilage remain unresolved. When we need to sculpt the whole cartilage, it depends on the surgeon’ s proficiency to achieve satisfactory results. In addition, sensation, patient site morbidity, and time delayed bending are insurmountable problems.2 The use of the diced cartilage graft in rhinoplasty was firstly proposed by Peer in 1943.3 Subsequently, Erol popularized this technique, which diced cartilage grafts were wrapped in Surgicel.4 Daniel and Calvert modified the original technique and proposed wrapping the diced cartilage in deep temporal fascia.5 Also, some articles reported other transplantation methods of diced cartilage, such as direct injection, flexible packing materials, and application of adhesives.6,7 Diced cartilage grafts have the advantages of easy accessibility, easy molding, and less friability. However, postoperative absorption was always a problem. In clinical research, the blood supply of the graft was very important, which was related to the survival and long-term effect of the graft. However, spontaneous new blood vessels were unable to be established in a timely and effective manner. Therefore, the construction of vascularization of autologous diced cartilage graft was the key to improve the survival and regeneration of cartilage.8

Stromal vascular fractions (SVFs) are a heterogeneous group of cell populations that contain adipose-derived stem cells (ADSCs), endothelial cells (ECs), hematopoietic stem cells, pericytes, and vascular smooth muscle cells.8,9 Stromal vascular fractions can participate in angiogenesis secreting a large number of angiogenic factors, and can also differentiate into ECs to participate in sprouting.11 Adipose-derived stem cells are the main component of SVFs, which has many research in the field of regeneration and tissue

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engineering. Adipose-derived stem cells and ECs can interact to promote angiogenesis, whereas ADSCs have a strong paracrine effect and secrete a variety of growth factors to regulate angiogenesis. Stromal vascular fractions were better than the separate application of ADSCs in promoting the regeneration. (1) ADSCs separation needed a long time and was easy to pollute. However, SVFs obtained relatively simply by enzymatic method, mechanical method, etc. (2) Stromal vascular fractions had similar biological functions to ADSCs, including anti-inflammatory, immunomodulatory, and vascular regeneration. Moreover, ADSCs in the SVFs may interact with other cells contained therein, produced effects that were not available by using ADSCs alone. (3) Stromal vascular fractions could stimulate ADSCs long-term retention and proliferation.

However, it is not clear whether SVFs have a positive effect on the induction of microvascular network formation of diced cartilage grafts. To address this issue, this study was conducted to compare the formation of microvascular network of diced cartilage grafts with mixed SVFs to unmixed.

**METHODS**

**Experimental Animals**

Twenty-four New Zealand rabbits were acquired for these experiments. The rabbits were 4 months old and weighed 2.50 to 2.90 kg. In addition, the rabbits had sufficient cartilage in their ears to harvest graft materials, as well as a wide dorsal region to accommodate the grafts. This study was approved by the Laboratory Animal Management and Ethics Committee of the First Affiliated Hospital, University of South China (Hengyang, Hunan, China). All procedures complied with the People's Republic of China Laboratory Animal Administration rules.

**Acquisition of Rabbit Adipose Tissue**

Each rabbit was given general anesthesia by intramuscular injection of 2% xylazine hydrochloride at 0.05 mL/kg and ear intravenous injection of 3% pentobarbital sodium at 0.5 mL/kg, local anesthesia was induced with 0.5% lidocaine. About 3 cm oblique incisions were made along bilateral groin, and subcutaneous adipose tissue was exposed. Blunt separation of the capsule was to release fat, and 15 ml of fat tissue was acquired (Fig. 1). The wound was cleaned and closed the incision with 4-0 absorbable suture. Then the adipose tissue was rinsed fully, and put in 50 ml centrifuge tube containing normal saline. Finally, the centrifuge tube was sealed and put in ice box immediately.

**Preparation of the Stromal Vascular Fraction**

The enzymatic method was used to aseptically extract primary cells from SVFs, and a superclean table was prepared 30 minutes in advance. Fat from the inguinal region of the New Zealand rabbit was taken bilaterally (adipose tissue, complete culture medium, type I collagenase, etc) should not be irradiated by an ultraviolet lamp. Fifteen milliliters of fresh adipose tissue were repeatedly rinsed with PBS and cut into diced pieces in a beaker. Then, the prepared diced fat was placed into the test tube, and an equal volume of 0.25% type I collagenase was added. The test tube was sealed and placed into a 37°C constant temperature water bath and shaken every 5 minutes, and fat digestion was observed for approximately 30 to 40 minutes. After the fat was fully digested, an equal volume of complete medium was added to stop the digestion. The undigested adipose tissue and fat in the upper layer were removed by centrifugation at 1500 r/minutes for 10 minutes. After filtration with a 200-mesh sieve, the sample was centrifuged again at 1000 r/minutes for 5 minutes. After discarding the supernatant, 5 ml of complete medium was added. The heavy suspension was divided into T25 culture flasks, and 5 ml of complete medium was added to each culture flask. Finally, the culture flask was incubated in a 5% CO₂ and 37°C incubator. The medium was changed for the first time after 24 hours, and the culture medium was changed again 2 to 3 days later (Fig. 2). Stromal vascular fractions cell phenotype was identified by flow cytometry. One group was set of CD90 and CD73 as positive markers and CD45 and CD31 as negative markers. The other group had CD34 and CD31 as positive markers.

**Cartilage Harvest and Graft Preparation**

Each rabbit was given general anesthesia by intramuscular injection of 2% xylazine hydrochloride at 0.05 mL/kg and ear intravenous injection of 3% pentobarbital sodium at 0.5 mL/kg, local anesthesia was induced with 0.5% lidocaine. The ear was debrided of skin, fascia, and perichondrium and soaked in saline containing gentamicin injection for 10 minutes. Ear cartilage was diced to approximately 1.0 mm³ size of particles. Two incisions were made on both sides of the spinal dorsal midline. Dissection down to the superficial fascia of back muscles was performed. A subcutaneous, suprafascial pocket was made along the spinal midline. Two 10 cm oblique incisions were made on both sides of the spinal midline. The wound was closed with 4-0 absorbable suture.
Immunohistochemical Analysis

Groups were set: Group 1 (left side, complete medium was created for graft placement. After the syringe went through the transplantation. Preparation of diced cartilage graft. (A) Diced cartilage; (B) in vivo transplantation.

FIGURE 3. The Journal of Craniofacial Surgery/C15

was created for graft placement. After the syringe went through the subcutaneous tunnel to the given area, the diced cartilage was slowly injected. Remove the syringing to avoid transposition of the transplanted diced cartilage. A 4-0 absorbable suture was used to seal the wound, and the surface was smeared with erythromycin ointment. Two groups were set: Group 1 (left side, complete medium + diced cartilage as control group) and Group 2 (right side, SVF + diced cartilage as experimental group) (Fig. 3).

Gross View and Weighing of the Graft

The rabbits were randomly killed at 4 weeks, 12 weeks and 24 weeks after the operation (8 rabbits at each time point). The cartilage piece that had fused on each side of the back was removed to observe the general condition of the graft, excess soft tissue was removed, and weighed on an electronic balance. The weights of the experimental and control groups at 4 weeks, 12 weeks and 24 weeks were recorded.

HE Staining

Samples were heated at 60°C for 1 hour and dehydrated in water. The slices were placed in xylene for 10 minutes twice. Then, the sample was placed in 100%, 100%, 95%, 85%, and 75% ethanol for 5 minutes each. The samples were rinsed with distilled water for 5 minutes; incubated in hematoxylin dye for 5 minutes, rinsed with distilled water for 5 minutes, placed in PBS to become blue, stained with eosin for 3 minutes, rinsed with distilled water, and dehydrated in gradient alcohols (95%–100%) for 5 minutes each (or directly dried). After being removed and placed in xylene for 10 minutes, the samples were sealed with neutral gum and observed under a microscope.

Immunohistochemical Analysis

After heating at 60°C for 12 hours, the slices were dehydrated in water. First, they were placed in xylene for 20 minutes 3 times. Then, the slices were treated with 0.5% urea and incubated at 37°C for 30 minutes, washed 3 times with PBS for 3 minutes, incubated in an appropriate amount of trypsin at 37°C for 30 minutes, and washed 3 times with PBS for 3 minutes. Then 1% periodate was added and incubated at room temperature for 10 minutes to inactivate endogenous enzymes. The slices were rinsed 3 times in PBS for 3 minutes and incubated the primary antibody that was properly diluted (CD31) overnight at 4°C. The slices were washed 3 times in PBS for 5 minutes and incubated with 50 to 100 μL of anti-rabbit-IgG-HRP-conjugated secondary antibodies at 37°C for 30 minutes. For DAB coloring, 50 to 100 μL of DAB working solution was added and incubated at room temperature for 1 to 5 minutes. To control the reaction time, the slices were washed with distilled water, stained with hematoxylin for 5 to 10 minutes, rinsed with distilled water, placed in PBS until blue, and dehydrated in alcohol (60%–100%) for 5 minutes each. After being removed and placed in xylene for 10 minutes, the samples were sealed with neutral gum and observed under a microscope.

Measurement of VEGF and HIF-1α mRNA by RT-PCR

The corresponding cDNA information was obtained by reverse transcription and primers were synthesized: Glyceraldehyde phosphate dehydrogenase (GAPDH): upstream 5′-gggccttcgcagacctcagt-3′ downstream 5′-cccaagctcccccttctcc-3′; vascular endothelial growth factor (VEGF): upstream 5′-agcctgagttctggagatg-3′ downstream 5′-ccggcgagccacgacaaga-3′; Hypoxia inducible factor-1α (HIF-1α): upstream 5′-agctgctggagacacaataca-3′ downstream 5′-ctgctccatcgtgcattgaagaatg-3′. The amplification sizes were expected to be 144, 299, and 274 bp, respectively. 20 μL of reaction system was used for PCR: SYBR green mix 10 μL, primer mix 2 μL; nuclease-free water 6 μL; cDNA 2 μL. The PCR was performed according to (30 sec at 95°C, 30 sec at 65°C, 30 sec at 72°C), (30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C), and (30 sec at 95°C, 30 sec at 65°C) for 40 cycles each.

Expression of VEGF and HIF-1α Protein by Weston Bloting

Remove the rabbit tissue sample at 80°C, grind it with liquid nitrogen, add 500 μL of protein lysis solution and lysate it on ice for 30 minutes; centrifuge at 4°C and 12,000 for 10 minutes, carefully aspirate and discard the supernatant with the tip of the gun, transfer the supernatant to a new centrifuge tube; take 1/2 volume of supernatant, add 1/4 volume of loading buffer, leave it at 100°C for 10 minutes, cool it down and you can load the sample or store it at −80°C; store the remaining 1/2 volume of supernatant after BCA measurement of protein concentration at −80°C. BCA method to determine protein concentration, polyacrylamide gel electrophoresis, membrane transfer, and closure. Incubation of primary antibody: remove the closed PVDF membrane, immerse in 1× TBST buffer, and wash slowly on a shaker for 5 minutes. Then transfer into a cassette containing primary antibody (1:1000 for Vascular Endothelial Growth Factor A, 1:1000 for HIF-1α) and shake overnight at 4°C. Finally, membrane washing and ECL chemiluminescence development were performed.

Statistical Analysis

SPSS 20.0 software package was used for statistical analysis. The form of data was expressed as mean ± standard deviation (x ± s), and t test and one-way ANOVA were used for statistical methods. P < 0.05 was considered statistically significant.

RESULTS

Stromal Vascular Fractions Culture

The SVFs were dynamically observed under an inverted microscope (×200). Twenty-four hours later, adherent cells were observed and were round, short, spindle-shaped or polygonal, with few broken cell impurities. On the 4th day, the cells extended, appeared fusiform, and arranged irregularly. On the 10th day, the cells showed long fusiform shapes, most had regular arrangements with approximate swirl shapes. After approximately 10 days, more than 80% of the SVFs could be reached observed and the P1 generation SVFs were obtained by subculture at 1:3 ratio. After that, the P1 generation was subcultured every 5 to 7 days according to the cell growth conditions (Fig. 4).
Phenotypic Results of Stromal Vascular Fractions
The expression of surface antigens of SVFs in the P0 generation was measured by flow cytometry. CD90/CD73 were positive markers, and CD45/CD31 were negative markers. CD90/CD73 were highly expressed, meanwhile CD45/CD31 were expressed at low levels. When added CD34/CD31 positive markers, they were highly expressed. This finding was consistent with the phenotypes of ADSCs and ECs, suggesting that SVFs contain ADSCs and ECs (Fig. 5).

Macroscopic Appearance
No animals were diseased or prematurely died. No infection, seroma, or hematoma was observed in the operation site postoperatively.

For gross analysis of the grafts, the materials were collected at the 4th week after the operation. In the control group, the grafts of diced cartilage were soft and irregular, and scattered in the center after incision. However, in the experimental group, the grafts of diced cartilage were fused and regular, and the texture was slightly hard. At the 12th week, the periphery of materials in the 2 groups were covered with thin film. At the 24th week, the grafts of the 2 groups were blunt, smooth, and covered with obvious capsules (Fig. 6). The weight of the unilateral graft was 2.083 ± 0.186 g before the operation. The wet weight of the bilateral graft was measured at 4, 12, and 24 weeks, and the trend in the wet weight change in each group was observed over time that showed in Supplementary Digital Content, Table 1, http://links.lww.com/SCS/D10.

Histological Observation
As showed in Figure 7 of HE staining, at 4 weeks, a small number of neoformative chondrocytes could be seen in the diced cartilage grafts in the control group. Meanwhile, minorities of apoptosis of chondrocytes and inflammatory reaction could be seen in the peripheral area of grafts. And no obvious vascular growth was observed. At 12 and 24 weeks, lower proliferation of cartilage, fewer layers of chondrocytes and less distribution of blood vessels were observed in the control group than that in the experimental group. Immunohistochemical staining of CD31 was to analysis roughly the neovascularization of diced cartilage grafts that showed in Figure 8. At 4, 12, and 24 weeks after the operation, the number of microvessels in the control group was significantly lower than that in the experimental group [(35.27 ± 1.95)/(55.42 ± 2.15, 41.80 ± 2.51)/67.32 ± 2.21,

FIGURE 4. Adipose stem cell growth under an inverted microscope. Note: (A-C) day 1, 4, and 8 after cell extraction and culture, respectively (×200); (P1-3) 1-3 generations of passaged culture, respectively (×100).

FIGURE 5. Phenotypic results of SVFs analyzed by flow cytometry. Note: Group A: the results of blank control, CD90, CD73, CD45, and CD31, respectively; Group B: the results of blank control, CD34 and CD31, respectively. SVFs, stromal vascular fractions.

FIGURE 6. Macroscopic appearance of the grafts. (A-E) Represent the 4, 12, and 24-week control groups, respectively. (F-L) Represent the 4, 12, and 24 week experimental groups, respectively.
The vascular density of the diced cartilage increased significantly after SVFs treatment. 

Expression of VEGF and HIF-1α mRNA and protein

According to the RT-PCR results in Figure 9, the mRNA expression of VEGF and HIF-1α increased in the experimental group at 4 weeks. These genes began to decrease slightly at 12 weeks. However, the mRNA levels remained higher than those in the control group at 24 weeks (P < 0.05). As shown in Figure 10 of the Western Blotting results, the relative expression levels of VEGF and HIF-1α protein were increased in the experimental group compared with the control group at 4, 12 and 24 weeks (P < 0.05).

DISCUSSION

Diced cartilages were easy to shape, have been always used in rhinoplasty. The establishment of blood vessels after transplant that concerned to impact the effect of postoperative. In this research, it was found that adipose-derived SVFs mixed with diced cartilages by injection transplantation, could promote the angiogenesis of diced cartilage grafts. Further study indicated that SVFs increased the expression level of VEGF and HIF-1α, which were proangiogenic factors. This may be part of the mechanism that SVFs promoted the angiogenesis of diced cartilages.

In this experiment, SVFs were prepared from adipose tissue of inguinal region of New Zealand rabbit by enzymatic method. Nunes compared the difference in the proportion of SVF after fresh extraction and culture. The results showed that the EC content of newly isolated SVFs was about 33%, whereas that of cultured SVFs decreased to about 10%. So far, there was no agreed definition to distinguish the exact ratio between these components. But it was clearly that ADSCs and ECs were important components of SVFs. In this study, the phenotype of ADSCs cultured in vitro was CD31−/CD45−/CD73+, announced by the International Association of Applied Adipose Technology in 2013. Endothelial cells used classical CD31+CD34+ positive markers. The results of flow cytometry were consistent with ADSCs (CD90+CD73+CD45−/CD31−) and EC (CD34+CD31+), indicating that ADSCs and ECs were contained in the extracted SVFs.

The formation of stable blood vessels was a complex process, which required the interaction and coordination between ECs, parietal cells and the surrounding environment. In previous studies, the vascularizing effect of ADSCs was positive. In studies on cartilage regeneration, SVFs could well promote cartilage regeneration and repair. This experiment suggested that SVFs could promote angiogenesis by secreting VEGF and HIF-1α. Studies had shown that VEGF was the most thoroughly studied specific regulator of angiogenesis. VEGF activated downstream PKC mainly through PLCγ1 or through the classical MAPK pathway of Ras-Raf-MEK-ERK and the PI3K/Akt signaling pathway, which stimulated the multiplication and survival of ECs. And VEGF activated to upregulate the expression of VE-cadherin, which could be reformed by binding with β-catenin complex leading...
to form stably amicrovessel network. HIF-1α modulated the expression and activity of several histone deacetylases, which were other epigenetic modifications that changed under hypoxic conditions. Over-expression of histone deacetylases 1 enhanced angiogenesis in human ECs by downregulating tumor suppressor genes that responded to hypoxia, including p53 and VHL. HIF-1α was the key upstream regulator of VEGF. At normal oxygen concentration, HIF-1α was in stability to easy to decompose. Although underhypoxia, it increased VEGF expression and induce dangiogenesis after binding to HIF-1β.21

Stromal vascular fractions and ADSCs had great potential in the field of regenerative therapy, and SVFs and ADSCs overlap to a great extent in clinical research. Adipose-derived stem cells and ECs were 2 important components of the SVFs, in addition, included abundant endothelial and mesenchymal progenitor cells. Compared with other individual stem cells, this specific proportion of the cell population of SVFs had strong vascularization.22 Since the diversity of SVFs and its composition would also change during the culture process. It was not clear, which proportion of SVFs had the best vascularization effect, and difficult to elucidate the specific mechanism of promoting vascularization. So, our further researches will be to clarify the phenotypes of various cells and understand the physiological function of each cell component of SVFs in vivo and in vitro, to observe the effect of these cells on tissue vascularization by mixing them in different proportions.

CONCLUSIONS
1. Autologous adipose tissue vascular matrix components, compounded with diced cartilage injected grafts, can promote diced cartilage vascularization.
2. Stromal vascular fractions can increase the expression levels of VEGF and HIF-1α in the composite graft, which may be part of the mechanism by which vascular matrix components promote diced cartilage vascularization.

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