The Application of Decellularized Adipose Tissue Promotes Wound Healing

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Abstract

BACKGROUND: Due to adipose-derived stem cells (ASCs) being easy to obtain, their rapid proliferation rate, and their multidirectional differentiation capabilities, they have been widely used in the field of regenerative medicine. With the progress of decellularized adipose tissue (DAT) and adipose tissue engineering research, the role of DAT in promoting angiogenesis has gradually been emphasized.

METHODS: We examined the biological characteristics and biosafety of DAT and evaluated the stem cell maintenance ability and promotion of growth factor secretion through conducting *in vitro* and *in vivo* studies.

RESULTS The tested ASCs showed high rates of proliferation and adhered well to DAT. The expression levels of essential genes for cell stem maintenance, including OCT4, SOX2, and Nanog were low at 2–24 h and much higher at 48 and 96 h. The Adipogenic expression level of markers for ASCs proliferation including PPAR γ , C/EPB α , and LPL increased from 2 to 96 h. Co-culture of ASCs and DAT increased the secretion of local growth factors, such as VEGF, PDGF-bb, bFGF, HGF, EGF, and FDGF-bb, and secretion gradually increased from 0 to 48 h. A model of full-thickness skin defects on the back of nude mice was established, and the co-culture of ASCs and DAT showed the best *in vivo* treatment effect.

CONCLUSION: The application of DAT promotes wound healing, and DAT combined with ASCs may be a promising material in adipose tissue engineering and regenerative medicine.

Keywords Decellularized adipose tissue · Adipose-derived stem cells · Nude mouse model · Wound healing

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1 Introduction

Incurable wounds and large-scale skin defects are often caused by trauma, burns, tumor resection, radiation exposure, and certain systemic diseases [1]. Nearly half of these nonhealing chronic wounds do not respond sufficiently to existing treatments [2]. Clinically used repair methods, such as skin flaps and flap transplants, require sacrificing the donor site, causing significant damage and have a weak curative effect. Research and development of stem cells provide new ideas for solving this problem [2]. In 2001, Zuk et al. [3] isolated adipose-derived stem cells (ASCs) for the first time. Due to ASCs being easy to obtain, proliferating rapidly, and having multidirectional

differentiation capabilities, they have been widely used in the field of regenerative medicine [4]. Traditional stem cell transplantation methods have unsatisfactory therapeutic effects, whether they are administered systemically or injected directly into diseased tissue. The cell damage caused by injection, ischemia and inflammatory responses in the microenvironment can lead to decreased cell survival. For example, the post-injection cell survival rate of human bone marrow mesenchymal stem cells is less than 10%, and it is even lower in the long-term [5]. Recently, the combined transplantation of stem cell and scaffold material has been put forward as an effective way to increase cell survival rates and survival time [6]. Therefore, it is necessary to find an effective, easily prepared, and highly injectable scaffold material for transplanting stem cells.

The extracellular matrix (ECM) is a polysaccharide, protein, or proteoglycan macromolecule, which is synthesized and secreted out of the cell and distributed on the cell surface or between cells and plays an important regulatory role in cell adhesion, migration, proliferation, differentiation, and gene expression [7]. Recently, the ECM has been regarded as a regulator of stem cell maintenance and fate, as it is one of the essential extrinsic factors regulating the fate of a stem cell [8]. Decellularized adipose tissue (DAT), which is rich in ECM components, such as type I and type IV collagen and laminin, shows a loose and porous structure after dehydration and drying [9]. Based on the reliable adhesion between DAT and ASCs, DAT can successfully support the three-dimensional culture of ASCs *in vitro*, and it is a scaffold material that has been well studied in the field of adipose tissue engineering [10, 11]. With the progress of DAT and adipose tissue engineering research, the role of DAT in promoting angiogenesis has gradually been emphasized. Multiple studies have shown that the transplantation of a combination of DAT and ASCs promotes fat regeneration and increases the local secretion of vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF)-bb, and b-fibroblast growth factor (FGF) [12–14].

Our previous work demonstrated that the co-culture of ASCs with gelatin microcryogels could promote the proliferation of ASCs, enhance anti-apoptotic ability, and promote wound healing [15]. Considering that DAT has a structure and function similar, in many ways, to GMs, we explore the role of DAT in wound healing. We examined the biological characteristics and the biosafety of DAT and evaluated the stem cell maintenance ability and promotion of growth factor secretion of DAT *in vitro* and *in vivo*.

2 Materials and methods

2.1 Preparation of decellularized adipose tissue

Sterile lipoaspirate was obtained from a healthy adult female, and it was centrifuged at 1000 rpm for 5 min. After letting it stand on ice for 30 min, the upper free lipid droplets were discarded. Then, the same volume of phosphate-buffered saline (PBS) solution was added as the lower layer of liquid, and it was shaken for 2 h and then washed. The filtered out adipose tissue was washed with deionized water three times for 3 min each time. Then, 1% sodium dodecyl sulfate solution prepared with the same volume of deionized water was added and shaken together. After 48 h, the adipose tissue was filtered out and washed with deionized water three times for 3 min each time. Then, the same volume of digestion solution (25 mmol/l SD + 5000 U/l porcine lipase) prepared with PBS buffer was added, and this was shaken for 24–48 h in the dark. Shaking was stopped when the tissue color became consistently white. To obtain extracellular matrix (ECM) powder, the cleaned white tissue was placed in a low-temperature freeze dryer for 48 h. Then, the tissue was placed in a homogenizer and crushed at 12,000 rpm for 10 min. Finally, the ECM powder was sterilized using cobalt-60 γ radiation and collected for future use.

2.2 Isolation and cultivation of adipose-derived stem cells

First, we obtained sterile lipoaspirate from a healthy adult female, and it was placed in a centrifuge tube, centrifuged at 1300 rpm for 2 min, and the lower layer liquid was aspirated. An equal volume of PBS buffer was added. This liquid was centrifuged at 1300 rpm for 2 min, and the lower layer liquid was aspirated. Then the tissue was digested thoroughly with 0.2% type I collagenase for 30 min. After natural separation into three layers, the upper layer of oil was collected, shaken thoroughly, and filtered. After centrifuging at 1200 rpm for 15 min, we removed the top layer of liquid, added erythrocyte lysate and antibodies, blown thoroughly, and let them stand at room temperature for 10 min. After centrifuging at 1200 rpm for 5 min, we collected the pellet, and PBS was added for washing. Finally, the ASCs were inoculated at a density of 2×10^6 /ml and incubated at 37 °C, 5% CO₂, and 95% humidity. When fusion of 80–90% of ASCs occurred, 0.25% trypsin-0.02% ethylenediaminetetraacetic acid was used for digestion and subculture.

2.3 Examination of biological characteristics and biosafety

After preparation, a general observation was made of the color and characteristics of the DAT.

For histological examination, paraffin sections were taken for hematoxylin and eosin (H&E) staining, as well as Masson staining, and cell structure was observed under a light microscope. After Sirius Red staining, the collagen structure was observed using a polarized light microscope. Frozen sections were then taken for oil red staining and observed under a light microscope to determine whether lipid droplets were present.

The paraffin sections were also used for 4',6-diamidino-2-phenylindole (DAPI) fluorescence staining and observed using an inverted fluorescence microscope to determine whether DNA residues were present.

For microstructure observation, the lyophilized acellular matrix tissue was taken and observed using a scanning electron microscope.

2.4 Induced adipogenesis and identification of adipose-derived stem cells

The third-generation ASCs that showed good growth were inoculated in a six-well plate at a density of 1×10^5 /ml for routine cultivation. When they grew to 80% of the ASCs touching, the conventional Dulbecco's modified eagle medium was replaced with an adipogenic induction solution to induce adipogenesis. The culture medium was changed every 3 days, and oil red O staining was performed after 14 days.

An oil red stock solution was made by adding 1 g of oil red O powder to 100 ml isopropyl alcohol and heating the mixture in a 60 °C water bath for 30 min. The oil red O stock solution was then mixed with deionized water at a 6:4 ratio and let stand for 30 min. After adipogenic induction culture for 14 days, the medium was discarded, and the ASCs were washed three times using PBS solution. A neutral formaldehyde was used added for 10 min. The ASCs were rinsed with distilled water, and oil red O to stain was added for 30 min.

2.5 Co-culture of decellularized adipose tissue and adipose-derived stem cells *in vitro*

Sterile DAT powder was immersed in the ASC culture solution, equilibrated in the incubator for 24 h, and collected into a 15 ml centrifuge tube. After the third generation, ASCs were digested with trypsin, a cell suspension with a concentration of 1×10^7 was prepared and mixed with the above DAT suspension, pipetted evenly, and placed in an incubator at 37 °C and 5% CO₂ for 48 h.

2.6 Examination of proliferation and adhesion

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to detect the proliferation ability of ASCs, and the growth curve of ASCs was plotted according to different cultivation days.

The cell concentration was set to 1×10^6 /ml and mixed with DAT scaffold suspension. After pipetting evenly, the suspension was placed in the incubator and incubated for 4 h. The incubated ASC and DAT suspension was then added to a 12-well plate and incubated in the incubator for 2 h. Then, trypsin was added to each well for digestion, and 100 µl of working solution was added to terminate the digestion.

Attachment rate

$$= (\text{Total cell count} - \text{Non-adherent cell amount}) / \text{Total cell count} \times 100.$$

2.7 Examination of cell stem maintenance ability

After culturing for 2, 12, 24, 48 and 96 h, the reverse transcription-polymerase chain reaction (RT-qPCR) method was used to detect the expression of important genes for stem cell maintenance, including OCT4, SOX2 and Nanog, and the most widely checked adipogenic markers for ASCs proliferation including PPAR γ , C/EPB α , and LPL. In addition, the RT-qPCR method was used to detect the expression of genes of different growth factors, including VEGF, hepatocyte growth factor (HGF), epidermal growth factor (EGF), bFGF, and FDGF-bb.

2.8 Establishment of animal model

Nude mice (BALB/c-nu) of 6–8 weeks of age were anesthetized intraperitoneally with 250 mg/kg tribromoethanol. The diaphragm, with an inner diameter of $1 \times 1 \text{ cm}^2$, was sutured from the back to the muscle layer. For the sarcolemma, a square skin defect with an area of $1 \times 1 \text{ cm}^2$ was prepared. In each group, eight nude mice were injected with four experimental reagents on each wound surface: DAT-ASC, ASC, DAT, and sham. The dressing was changed on the 3rd, 6th, 9th, and 11th days after the operation, and the animals were sacrificed on the 11th day to obtain wound materials.

2.9 Evaluation of *in vivo* effect

On the 3rd, 6th, 9th, and 11th postoperative days, the wounds were photographed, and ImageJ software was used to analyze the residual wound area and draw a healing curve.

Specimens were embedded in paraffin, and H&E staining was used to observe the wound surface. The thickness of the wound granulation tissue was measured. CD31 staining was used to calculate the microvessel density (MVD) of the wound, and proliferating cell nuclear antigen (PCNA) staining was used to assess the proliferation of the basal cells of the wound epidermis. Western blot was used to detect VEGF, bFGF, HGF, EGF, and PDGF-bb levels *in vivo*.

2.10 Statistical analysis

The experimental data are expressed as mean $\pm$ SD. SPSS19.0 software was used to perform paired data line *t* tests and analysis of variance of multiple data lines to determine whether differences were significant. A *p* value < 0.05 was considered statistically significant.

3 Results

3.1 Biological characteristics and biosafety of decellularized adipose tissue-adipose-derived stem cells

The preparation of DAT was successful. As shown in Fig. 1, the DAT prepared from adipose tissue was a milky white gel, and it became a white powder after freezing and drying.

To further examine the biological characteristics of DAT and DAT-ASC, H&E staining, Masson staining, and Sirius Red staining were performed (Fig. 2). No nuclear components, only a red-stained extracellular matrix were observed with H&E staining (Fig. 2A). Masson staining showed a blue-stained collagen fiber structure, and the extracellular matrix was preserved intact (Fig. 2B). Oil Red O staining showed no obvious red-stained lipid components (Fig. 2C).

Fluorescence staining was performed using DAPI to observe DNA residues (Fig. 3A). Consistent with the result of H&E staining, DAPI fluorescence staining confirmed that the DAT, which was evidently different from DAT-ASC, contained no cells and nucleic acid components, suggesting that the decellularization process is thorough.

In terms of microstructure research, electron microscopy confirmed that the volume and shape of the DAT powder materials were highly diverse, showing a clear porous structure, and local magnification showed that DAT powder particles had a large surface area (Fig. 3B).

3.2 Adipogenic culture and observation of adipose-derived stem cells

The ASCs grown in the adipogenic induction culture medium presented obvious morphological changes of induced differentiation (Fig. 4). Some cells changed from fusiform to an elliptical shape, and some lipid droplets appeared and aggregated around these cells. Under microscope observation after oil red O staining, the intracytoplasmic lipid droplets appeared orange-red.

3.3 Proliferation and adhesion of adipose-derived stem cells

The cells started adherent growth within 24 h. They showed slow growth in the first 2 days and exponential growth from the third day (Fig. 5A). The third-generation ASCs grew steadily with less cell aging and death. No significant differences were found between both groups.

The adhesion rate of ASCs inoculated in DAT stents was $89.00 \pm 3.97\%$ (Fig. 5B). The ASCs exhibited strong adhesion to the DAT scaffold and achieved good growth on the surface and pores of the DAT scaffold.

3.4 Cell stem maintenance ability of decellularized adipose tissue

The expression levels of important genes for cell stem maintenance, including OCT4, SOX2, and Nanog, are shown in Fig. 5C–E. Consistent with the pattern of cell proliferation, all of the expression levels of all three genes were low at 2–24 h and much higher at 48 h and 96 h with significant differences. Besides, we examined the most widely checked adipogenic markers for ASCs proliferation including PPAR γ , C/EPB α , and LPL, and their expression level increased from 2 to 96 h (Fig. 5F–H).

3.5 Growth factor secretion promoted by decellularized adipose tissue

Co-culture of ASCs and DAT increased the secretion of local growth factors such as VEGF, PDGF-bb, bFGF, HGF, EGF, and FDGF-bb, and their secretion gradually increased with time from 0 to 48 h (Fig. 6). The secretion of these growth factors helps local angiogenesis and promotes wound healing.

3.6 In vivo evaluation of DAT

A model of full-thickness skin defects on the back of nude mice was established (Fig. 7), and in the wound healing after nine days of treatment H&E section staining showed that the wound healing area after co-transplantation of

Fig. 1 Scheme of
A Decellularized adipose tissue
 (DAT) preparation and
B animal experiments

DAT-ASC was significantly larger than that in the sham, DAT, and ASCs groups (Fig. 7A). The subcutaneous granulation tissue in the DAT-ASC group was more abundant, and the thickness of the wound granulation tissue in the DAT-ASC group was higher than that of the sham operation group and the control group. The healing time of the ASC control group was significantly shorter than that of the sham operation group, while the healing time of the co-transplantation of DAT-ASC group was shorter than that of the ASCs group (Fig. 7B). The results of the above-mentioned *in vivo* experiments suggest that co-transplantation of DAT-ASCs can promote wound healing.

The positive expression of CD31 in the treatment group (DAT-ASCs) was significantly higher than that in the sham, DAT, and ASCs groups, suggesting that the

microvessel density in the wounds of the nude mice in the DAT-ASCs group was significantly higher than that in the sham, DAT, and ASCs groups, reflecting the most active tissue angiogenesis (Fig. 8A). Meanwhile, the positive expression of PCNA in the treatment group (DAT-ASCs) was significantly higher than that in the sham, DAT, and ASCs groups, suggesting that the DAT-ASCs group experienced more active basal cell proliferation and a higher ability to repair wound epidermal cells in the nude mice (Fig. 8B).

Consistent with the *in vitro* experimental results, co-transplantation of DAT-ASCs provided more growth factors, including VEGF, HGF, EGF, bFGF, and PDGF-bb, which are beneficial to wound repair, for skin wounds of nude mice (Fig. 9). The effect of DAT-ASCs group is

Fig. 2 A–C Hematoxylin and eosin (H&E) staining (A), Masson staining (B), and Sirius Red staining (C) of DAT and DAT-ASC. H&E staining showed no nuclear components, only a red-stained extracellular matrix. Masson staining showed a blue-stained collagen fiber structure, and the extracellular matrix was preserved intact. Oil Red O staining showed no obvious red-stained lipid components

better than that of DAT group and ASCs group with statistical significance, indicating the improved wound repairing ability of the combination.

4 Discussion

Stem cell therapy brings new hope for the repair of chronic and refractory wounds and large skin defects. Co-transplantation with scaffold materials could effectively solve this problem, and this study has made progress in the search for an easily accessible and clinically applicable scaffold. Based on our previous studies of ASCs and wound healing, ASCs are easily accessible with abundant reserves in the human body. We obtained fat aspirates from healthy women, isolated DAT and ASCs, co-cultured DAT-ASCs *in vitro*, and determined that the co-culture of ACSs and DAT showed higher proliferation activity and

secreted more growth factors than the single culture medium. Moreover, we also established a wound model of nude mice and found that the wound repair of the DAT-ASCs group was significantly better than that of the sham, ASCs, and DAT groups. Therefore, DAT-ACSs co-transplantation can provide effective support and protection for cell transplantation and a good survival environment for cell adhesion and proliferation, thereby promoting wound healing.

Adipose stem cells are adult stem cells derived from adipose tissue, and the obtained ASCs have been proved to show stable self-replication and proliferative ability and low aging [16, 17]. Studies have confirmed that ASCs not only have specific multidirectional differentiation potential and paracrine functions of bone marrow stem cells, but they also have the advantages of easy material extraction, wide source, and less damage to the body compared with bone marrow stem cells [18, 19]. Xue et al. [20] used

Fig. 3 **A, B** Fluorescence staining with 4',6-diamidino-2-phenylindole (**A**) and scan electron microscopy (**B**) of DAT and DAT-ASC. The DAT was evidently different from DAT-ASC and contained no cells and nucleic acid components. The scan electron microscopy confirmed that the volume and shape of DAT powder materials were highly diverse, showing a clear porous structure, and local magnification showed that DAT powder particles had a large surface area

Fig. 4 **A, B** Observation of morphological changes (**A**) under the microscope and after oil red O staining (**B**). The adipose-derived stem cells grown in the adipogenic induction culture medium presented obvious morphological changes of induced differentiation. Some cells changed from fusiform to elliptical shape, and some lipid droplets appeared and aggregated around the cells. Under the observation of the microscope after oil red O staining, the intracytoplasmic lipid droplets were orange-red

adipose tissue-derived stem cells as an additional new source of seeding cells within a decellularized kidney scaffold, which showed good regeneration ability for further clinical transplantation. Thus, ASCs are suitable for

large-scale culture and considered to be an ideal source of seed cells in adipose tissue engineering [20].

The decellularized adipose tissue matrix is a fat-derived extracellular matrix, and DAT has good biological

Fig. 5 A–H The proliferation ability (**A**) and stem cell adhesion (**B**) of adipose-derived stem cells and cell stem maintenance ability of DAT (**C–H**). The cells started adherent growth within 24 h. They showed slow growth in the first two days and exponential growth from the third day. No significant differences were found between the

two groups. The adhesion rate of ASCs inoculated in DAT stents was $89.00 \pm 3.97\%$. All of the expression levels of the genes were low at 2–24 h and much higher at 48 and 96 h with significance. * $p < 0.05$; ** $p < 0.01$

properties since the decellularization process can effectively remove immunogenicity and cellular and nucleic acid components while retaining relatively complete collagen and glycosaminoglycan components [21–23]. In addition, DAT can simulate the structure of the natural extracellular matrix and provide a three-dimensional environment for the adhesion, growth, and proliferation of seed cells, which effectively promote fat regeneration and angiogenesis [24, 25]. Zhao et al. [26] developed a human DAT and found it as a potential choice for soft tissue enlargement and cosmetic fillers. Mohiuddin et al. [26] found that DAT could promote bone regeneration, and the effect was further enhanced in the presence of osteo-induced ASCs. Moreover, DAT can promote the secretion of multiple growth factors and induce the differentiation of fat stem cells into mature adipose tissue [27]. Zhang et al. [28] directly loaded basic fibroblast growth factor into a heparinized DAT matrix and demonstrated its high efficiency of promoting adipose neotissue formation and neovascularization. Therefore, DAT has been suggested to be an

ideal scaffold material in tissue engineering in the field of regenerative medicine to repair or reconstruct various defects or damaged human tissues [14].

Our study provides novel insights into stem cell therapy of refractory wounds. Yu et al. [29] focused on the function of porous DAT foams for soft tissue regeneration. Turner et al. [30] showed a good performance of DAT microcarriers as an inductive substrate for ASCs by rat animal. Our results were consistent with their finding and showed the combination of ASCs and DAT could exploit the advantage of each and exhibit an improved wound repairing ability by mouse model. Stem cells combined with scaffold material transplantation can effectively solve the complications of fat liquefaction and infection caused by autologous fat transplantation, and effectively reduce the rate of secondary surgery. However, further study is needed to solve the problems of completely removing immunogenic cell components [31, 32]. With increasing research on DAT and the optimization of preparation methods, the DAT can be used as a promising cell delivery carrier and

Fig. 6 A–F The expression of growth factor proteins after 0, 2, 12, 24, and 48 h of adipose-derived stem cell culture *in vitro* (A), and quantification (B–F). The co-culture of ASCs and DAT increased the secretion of local growth factors VEGF, PDGF-bb, bFGF, HGF, EGF,

and FDGF-bb, and the secretion gradually increased with time, reaching a relative maximum after 48 h. Each set of data came from three independently repeated experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Fig. 7 A, B Wound healing after nine days of treatment in a model of full-thickness skin defects on the back of nude mice and the corresponding Hematoxylin and eosin staining (A), and the time required for the complete process of wound healing in different

groups (B). The wound healing area after the co-transplantation of DAT-ASC was significantly larger than that in the sham, DAT, and ASCs groups. The healing time of the co-transplantation of DAT-ASC group was the shortest among all the groups

scaffold material, and it has broad application prospects in the field of regenerative medicine.

In conclusion, the co-culture of DAT and ASC showed a better *in vitro* and *in vivo* treatment effect than independent application. The application of DAT promotes wound healing, and DAT combined with ASCs may be a

promising material in adipose tissue engineering and regenerative medicine.

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Fig. 8 **A, B** Immunohistochemical staining of CD31 (**A**) and PCNA (**B**). The positive expression of CD31 in the DAT-ASCs group was significantly higher than that in the sham, DAT, and ASCs groups, suggesting a higher microvessel density in the wounds of the nude mice. The positive expression of PCNA in the DAT-ASCs group was

significantly higher than that in the sham, DAT, and ASCs groups, suggesting more active basal cell proliferation and a higher ability to repair wound epidermal cells in the nude mice. * $p < 0.05$; ** $p < 0.01$

Fig. 9 **A–F** The *in vivo* secretion ability of different kinds of growth factors (**A**), and their quantification (**B–F**). The co-transplantation group of DAT-ASCs provided more growth factors, including VEGF,

HGF, EGF, bFGF, and PDGF-bb than the sham, DAT, and ASC groups for skin wounds of nude mice. * $p < 0.05$; ** $p < 0.01$. NS, no significance

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical statement All animal care and experiments were performed in accordance with the guidelines of Institutional Animal Care and Use Committee of Chinese Academy of Medical Sciences.

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