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Original Article

Modified autologous adipose transplantation in the treatment of depressed scars: an experimental study

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Abstract: Objective: Complex depressed scars can cause tissue adhesion, resulting in serious joint dysfunction. In recent years, autologous adipose and adipose-derived stem cells have been widely used to treat depressed scars, but there are still limitations in these treatment that should be resolved. This study aimed to investigate the therapeutic effects of adipose tissues collected with modified technique on the depressed scars in animals. Methods: The adipose tissues were collected with a forward technique, and tissue viability in vitro and the survival of transplanted tissues in in nude mice were further assessed. Furthermore, the therapeutic effects of adipose tissues collected with new technique and traditional technique on the depressed scars were explored in an animal model of bleomycin induced scar formation. Results: The adipose tissues collected with the new technique had a higher glucose transport ($P<0.01$); after transplantation into the nude mice, the amount of residual tissues and the survival rate in the modified group were higher than in the traditional group ($P<0.05$); electron microscopy showed the intercellular space was covered with reticular structure, in which there was a large amount of microvessel structure in the adipose tissue of the modified group; immunohistochemistry showed that the microvessel density (MVD) in the modified group increased significantly ($P<0.01$). At 28 d after transplantation into the scar animals, the dermal collagen fibers became thicker and showed regular arrangement, the myofibroblasts became regenerative and inflammation was improved as compared to blank control group. In the untreated scar group, the collagen fibers were loose and irregular, and a large amount of inflammatory cells was observed. In addition, the dermal expression of α-SMA and TGF-β_1 in the transplantation group reduced significantly as compared to scar group ($P<0.05$). Conclusion: The autologous adipose tissues collected with the new technique possess higher activity ad contain more. In scar animals, transplantation of these adipose tissues may improve the scar structure and inhibit the scar formation which may be related to the suppressed expression of α-SMA and TGF-β_1.

Keywords: Depressed scar, adipose transplantation, adipose-derived stem cells, adipocyte activity, scar model, nude mice

Introduction

The depressed scar is often caused by the defect of dermis and subcutaneous tissue as a result of surgery, trauma or infection, and often results in pigmentation, hypopigmentation, pigmentation deficiency and texture change [1-4], which significantly affects the cutaneous appearance and functions [5, 6]. In recent years, the autologous adipose transplantation has been widely used in the treatment of depressed scar in the soft tissues [7-16]. Moreover, the adipose stem cells (ASC) and vascular matrix separated with newly developed method have significantly improved the survival rate of transplanted adipose tissues as well as the therapeutic efficacy of adipose transplantation [15-19]. Thus, the adipose transplantation has evolved from simple volume supplementation to multiple-gain treatment. Recently, the survival rate of autologous adipose tissues clinically collected is about 40-60%, and transplantation of these tissues achieves a poor efficacy. This may be partially ascribed to the damage to the adipocytes and tissues during the collection of adipose tissues [20-22]. In our department, a modified technique was employed for the collection of adipose tissues: the opening of the

liposuction needle was pushed forward, which reduces the damage to the adipose tissues collected, thus preserves the adipose structure and increases the viable adipocytes, leading to the improved efficacy of adipose transplantation [23, 24]. On the basis of this technique, we further tested the viability of collected adipose tissues with glucose transport test, and then these tissues were transplanted into the back of healthy nude mice. The general condition was observed after transplantation. Then, the tissues at the transplanted sites were collected for the scanning electron microscopy to assess the microstructure and for immunohistochemistry to assess the microvessel density (MVD). Thereafter, a scar model was established with bleomycin in animals and then adipose transplantation was done. At 28 days after transplantation, the tissues at the transplantation sites were collected for Masson staining, the collagen fibers were observed and the positive cells were counted. Our results showed transplantation of these adipose tissues collected with the new technique improved the therapeutic efficacy, and we further explored the potential mechanism. Our findings may provide a new technique for the treatment of depressed scars.

Materials and methods

Animals and reagents

Female nude mice (n=20) aged 6-8 weeks and weighing 20 g were purchased from the Experimental Animal Center of Army Medical University (SCXK[YU] 2017-0002), Crossbeam 340 scanning electron microscope (Zeiss, Germany), DMEM high glucose (Thermo Fisher Scientific, CN) glucose detection kit (Yuanyuqi Biotech Co., Ltd, Chongqing), Masson staining kit, diaminobenzidine (DAB) kit, Hematoxylin (Bioswamp, Wuhan, CN), mouse anti-mouse CD29 monoclonal antibody, rabbit anti-mouse CD31 monoclonal antibody, mouse anti-mouse CD44H monoclonal antibody (eBioscience, USA), Bleomycin (Solarbio, Beijing), rabbit anti-mouse α smooth muscle actin (α-SMA) polyclonal antibody, and rabbit anti-mouse transforming growth factor-β1 (TGF-β_1) polyclonal antibody (Abcam, UK) were used in the present study. All the procedures were in accordance with the Helsinki Declaration.

Methods

Examination of adipose viability: Collection of adipose tissues: 10 patients were randomly selected, and adipose tissues were collected from the thigh. In brief, after anesthesia, local anesthesia was done with 800 mL of Lactate Ringer, 20 g/L Lidocaine and adrenaline at 1:1000. A new type needle was inserted to slowly collect the adipose tissues via a syringe. In the control group, the traditional needle was used to collect the adipose tissues. The granular adipose tissues in the middle layer were harvested for use.

Glucose transport test: 5 ml of granular adipose tissues was collected from each patient and placed into an aseptic dish, into which 1 U of insulin and 10 ml of serum free DMEM containing 15 mmol/L glucose were added. In addition, there was a blank control (no granular adipose tissues and addition of insulin and serum free DMEM with glucose). The dish was incubated at 37°C in an environment with 5% CO_2 for 1 h. Then, the lower layer solution was harvested and centrifuged at 2500 rpm/min for 10 min. The supernatant was collected for further analysis. Then, 3 μL of supernatant, 3 μL of distilled water and 3 μL of 5.55 mmol/L adjustment solution were added to blank control well, adjustment well and sample well, respectively, and 300 μL of working solution was added to remaining wells of 96-well plate, followed by incubation at 37°C for 5 min. The absorbance of each well was measured, and the glucose concentration was calculated according to the manufacturer's instructions. The difference in the glucose concentration between sample well and blank control well was used as the amount of glucose transported by adipocytes.

Adipose transplantation and observations: The granular adipose tissues (10 ml) collected with modified method and traditional method were independently injected into 4 sites of the back of nude mice (interval between two adjacent sites was about 3 cm). In the modified group, 200 μl of normal saline and 400 mg of adipose tissues collected with modified method were injected; in traditional group, 200 μl of normal saline and 400 mg of adipose tissues collected with traditional method. There were 10 nude mice in each group. At 4 and 12 weeks after

transplantation, the transplanted adipose tissues were harvested for further observation.

Electron microscopy: The transplanted adipose tissues were collected from the transplantation site at 4 weeks and then fixed in glutaraldehyde. After treatment with ethanol and tert-butanol, tissues were stained with osmium acid and then sectioned. The microstructure was observed by scanning electron microscopy. The adipocyte morphology and MVD were assessed.

Observation of microvascular structure and determination of MVD: The adipose tissues were collected from the transplantation site at 4 and 12 weeks after transplantation. After embedding in paraffin, sectioning, deparaffinization, hydration, antigen retrieval and blocking, sections were treated with rabbit anti-mouse CD31 monoclonal antibody (1:200) at 4°C overnight. After washing in PBS, sections were treated with goat anti-rabbit IgG secondary antibody at room temperature for 30 min. Following washing in PBS, visualization was done with DAB, followed by counterstaining with hematoxylin for 3 min. After drying, sections were transparentized in xylene for 3 min (twice), followed by mounting with neutral gum. The sections were heated at 65°C for 15 min in an oven. The microvessels morphology was assessed, and the number of microvessels was determined (brown staining was indicative of positive staining. Three fields were randomly selected, the MVD was determined with Image-Pro Plus (Media Cybernetics, USA) and a mean was calculated.

Assessment of therapeutic effect of adipose transplantation on the scar

Establishment of animal model: 18 nude mice were randomly divided into transplantation group, scar group and blank control group (n=6 per group). Animals were anesthetized by intraperitoneal injection with 70 mg/mL chloral hydrate at 5 mL/kg, and then 1 mL of 1 mg/mL bleomycin in PBS was injected to the back to establish scar model. 3 h later, 1×10^6 adipocytes in 0.1 ml of PBS were injected into the back of mice in transplantation group; in scar group, 0.1 ml of PBS was injected. In blank control group, PBS of equal volume was injected at the same time point. After 28-day treatment, the successful establishment of scar animal model was determined by pathological examination. The full-thickness skin was collected, fixed in 40 g/L paraformaldehyde, embedded in paraffin and then sectioned (5 μm) for further use.

Observation of collagen fibers: Sections in different groups were deparaffinized, dehydrated and then subjected to Masson staining. After mounting in neutral gum, sections were observed under a light microscope, and the morphology and arrangement of collagen fibers were assessed.

Detection of α-SMA and TGF-β_1 expression: Sections of different groups were subjected to deparaffinization, hydration, antigen retrieval and blocking, and then treated with rabbit anti-mouse α-SMA polyclonal antibody or rabbit anti-mouse TGF-β_1 polyclonal antibody (1:200) at 4°C overnight. After washing in PBS, sections were incubated with goat anti-mouse IgG at room temperature for 30 min. Visualization was done with DAB, followed by counterstaining with hematoxylin for 3 min. After drying, sections were transparentized in xylene for 3 min (twice), followed by mounting in neutral gum. Sections were dried at 65°C for 15 min in an oven. Sections were observed under a light microscope and the expression of α-SMA and TGF-β_1 was assessed (brown staining was indicative of positive staining). Three fields were randomly selected, the positive cells were calculated with Image-Pro Plus, and a mean was calculated.

Surgical methods

According to the clinical manifestations and the requirements of scarring facial depression and atrophy patients, they were divided into 2 groups: traditional fat transplantation (traditional group) group and modified fat transplantation (modified group). The procedures for anesthesia were identical between two groups, and the fat tissues were collected from the abdomen or the thigh. They received follow up at 3 and 6 months after surgery by hospital visit. The post-operative recovery, complications, satisfaction and requirement for a second surgery were analyzed in these patients. In addition, patients who received breast augmentation by traditional fat transplantation or modified fat transplantation were recruited, and the graft survival was assessed at 6 months after surgery, aiming to investigate whether modified fat transplantation could achieve favorable effectiveness in case of bulk fat transplantation.

Table 1. Amount of glucose transported in each group ($\bar{x}$ ±s, mmol/L)

Group	n	Amount of glucose transported (mmol/L)
Modified group	10	3.081±0.359[a]
Traditional group	10	2.622±0.377

Note: [a]*P*<0.05 vs traditional group.

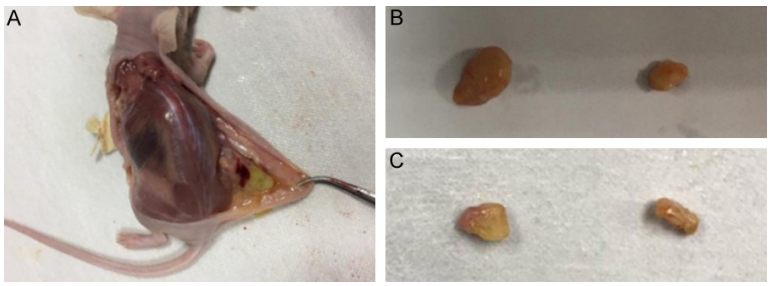

Figure 1. Survival of transplanted adipose tissues at different time points (A: survival of transplanted adipose tissues; B: Modified group; C: Traditional group).

Table 2. Amount of residual tissues and survival rate at different time points

Amount of residual tissues (mg)	Group	n	4 weeks	12 weeks
	Modified group	10	308.5±31.0[a]	191.7±15.0[b]
	Traditional group	10	276.5±32.6	171.1±9.2
Survival rate (%)	Group	n	4 weeks	12 weeks
	Modified group	10	77.1±7.8[a]	47.9±3.8[b]
	Traditional group	10	69.1±8.2	42.8±2.3

Note: [a]*P*<0.05 and [b]*P*<0.05 vs traditional group.

Statistical analysis

Statistical analysis was performed with SPSS version 22.0. Qualitative data are expressed as frequency or percentage and compared with Chi square test. Quantitative data with normal distribution are expressed as mean ± standard deviation and compared with t test between two groups or one way analysis of variance among groups, followed by LSD test between two groups. A value of *P*<0.05 was considered statistically significant.

Results

Adipose viability

Glucose transport test: The amount of glucose transported in each group is shown in **Table 1**. In the modified group, the amount of glucose transported was 3.081±0.359 mmol/L, which was significantly higher than in the traditional group (2.622±0.377 mmol/L; P=0.001).

Observation of transported adipose tissues: In the traditional group, fat liquefaction was observed at the transplantation sites in 1 mouse at 4 week, but there were no hematoma, ulceration and calcification at the transplantation sites in remaining mice. The transplanted tissues were oval or round and could be easily separated from the capsule (**Figure 1**). At 4 and 12 weeks after transplantation, the amount of residual tissues and the survival rate of transplanted tissues in the modified group were significantly higher than in the traditional group (P<0.05) (**Table 2**).

Electron microscopy of adipocytes: There were normal adipocytes at the transplantation sites at 4 weeks in both groups. However, in the traditional group, adipocyte depression or shriveling was observed with enlarged intercellular space. In the modified group, the adipocytes were full in morphology, there was no adipocyte depression or shriveling and the intercellular space reduced. In the traditional group, the adipocytes had smooth surface, and there were no tube-like or cord like microvessels; in the modified group, tube-like or cord-like microvessels were observed on the adipocytes, reticular structure (microvessels) was widely distributed in the intercellular space and grown into the adipose tissues.

Observation of microvessels and MVD: After immunohistochemistry, the CD31 positive endothelial cells were brown. Under a light microscope, the microvessel was formed with 2 or more brown endothelial cells, and exudation of red blood cells could be observed, and most of microvessels showed irregular morphology.

Table 3. MVD in different groups at 4 and 12 weeks

Group	n	4 weeks	12 weeks
Modified group	10	6.767±0.943[a]	8.03±0.936[b]
Traditional group	10	5.133±0.613	6.57±0.969

Note: [a]*P*<0.05, [b]*P*<0.05 vs traditional group.

At 4 weeks, the MVD was 6.767±0.943 in the modified group and 5.133±0.613 in the traditional group, showing significant difference between them (t=4.592, P=0.000). At 12 weeks, the MVD was 8.03±0.936 in the modified group and 6.57±0.969 in the traditional group, showing marked difference between them (t=3.433, P=0.003). Under a light microscope, the MVD in the modified group increased significantly as compared to the traditional group at the same time point. Moreover, the MVD in each group at 12 weeks was significantly higher than that at 4 weeks (**Table 3**).

Therapeutic effects of adipose tissues on the scar

Collagen fibers: At 4 weeks after transplantation, the dermal structure was complete, the collagen fibers were thin and closely arranged, there was no infiltration of inflammatory cells, and muscle fibers were not observed in the blank control group; in the scar group, the deposition of collagen fibers was noted in the dermis, fibrous bundles became thicker and showed irregular disarrangement, infiltration of inflammatory cells was found among collagen fibers which was more evident around the small vessels, and staggered distribution of muscle fibers was also observed; in transplantation group, the collagen fibers in the dermis became thicker, but thinner than in the scar group, the arrangement of fibrous bundles was similar to that in blank control group, a few muscle fibers were found, and a few inflammatory cells were also noted in the adipose tissues (**Figure 2**).

Expression of α-SMA and TGF-β_1: At 4 weeks after transplantation, the α-SMA expression was mainly found in the microvessels of blank control group; in the scar group, α-SMA expression was also found in the adipose tissues besides the microvessels; in the transplantation group, α-SMA expression reduced as compared to the scar group, but was higher than in the blank control group, and α-SMA expression was also observed in a few tissues surrounding the microvessels (**Figure 3**). At 28 d, the TGF-β_1 expression was comparable between blank control group and transplantation group, but it increased significantly (**Figure 4**). At 28 days after transplantation, the α-SMA and TGF-β_1 expression in the dermis was similar between transplantation group and blank control group (P>0.05), but was significantly lower in both groups than in the scar group (P<0.05) (**Table 4**).

In 3 months after surgery, complications were noted in 2 patients in only traditional group (fat liquefaction at 2 weeks after surgery in 1 patient and subcutaneous nodules at 8 weeks after surgery in 1 patient), and they resolved after corresponding treatments. At 6 months after surgery, complications were not found in both groups. In mixed transplantation group, the satisfaction at 3 and 6 months after surgery was significantly better than in the single transplantation group (Z=-2.566 and -3.084; P=0.010 and 0.002) (**Table 5**). At 6 moths after surgery, a second surgery was needed in 12 patients in the single transplantation group (22.22%), but only 4 patients in the mixed transplantation group needed a second surgery (7.84%), showing significant difference between two groups (χ^2=4.199, P=0.040). At 6 months after surgery, the survival rate of the graft in the modified group was markedly higher than in the traditional group (63.9±4.6% vs 42.8±5.7%; P<0.05).

Discussion

The treatments for depressed scar include physical grinding, laser therapy, soft tissue transplantation, drug injection and surgery [1-3, 22]. These may improve the scar deformity to a certain extent, but may not improve the texture of the scar. In addition, there are still risk for side effects such as pigmentation, scar contraction, adhesion, foreign body rejection and inflammation after these treatment. Currently, there is no effective treatment for complex scar. Thus, it is imperative to develop a breakthrough technique for the treatment of complex scar [7, 12, 19, 25].

The autologous adipose transplantation has been improved with the development of technology and widely used for facial rejuvenation, scar treatment and breast reconstruction [5-8,

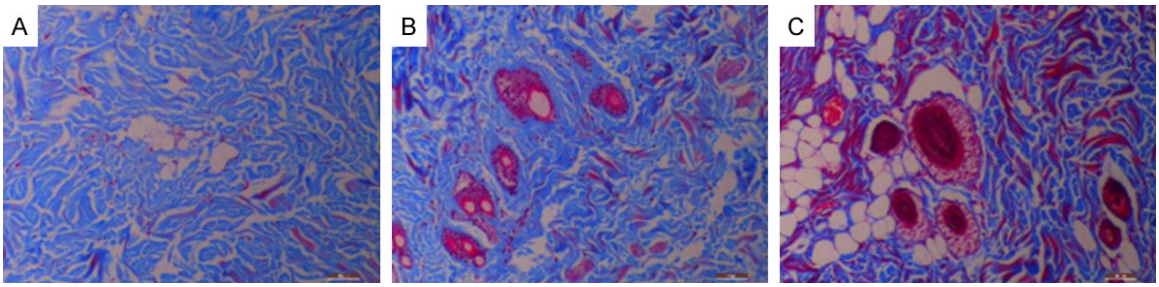

Figure 2. Masson staining of adipose tissues collected at 4 weeks (A. Dense arrangement of collagen fibers in blank control group and no infiltration of inflammatory cells; B. The collagen fibers in the dermis of transplantation group became thicker, but were thinner than in the scar group, and a few muscle fibers and inflammatory cells were noted; C. In the scar group, there was deposition of collagen fibers, the fibrous bundles became thicker and showed irregular arrangement, a large amount of inflammatory cells were observed, and scattered, staggered distribution of big muscle fibers was observed in the adipose tissues. Note: blue, collagen fibers; red, cord like muscle fibers and spotty or granular inflammatory cells, ×200).

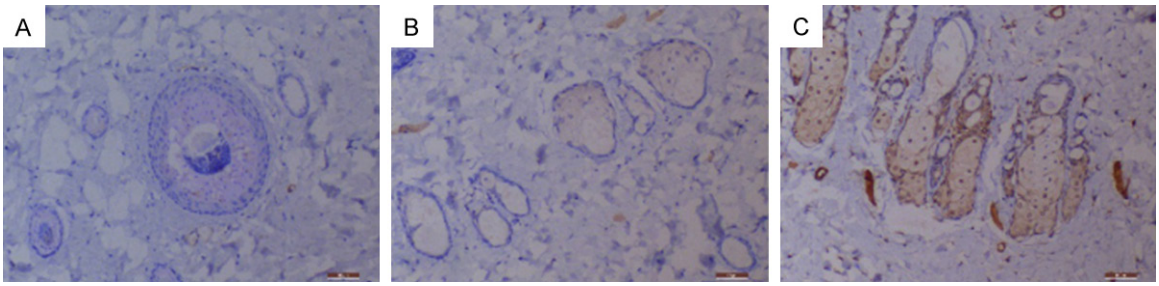

Figure 3. α-SMA expression (brown) in the dermis of different groups (DAB-hematoxylin; ×200. A. α-SMA expression was almost observed in the tube-like microvessels in the blank control group; B. α-SMA expression was observed in the microvessels and surrounding tissues; C. α-SMA expression showed scattered distribution in the scar group).

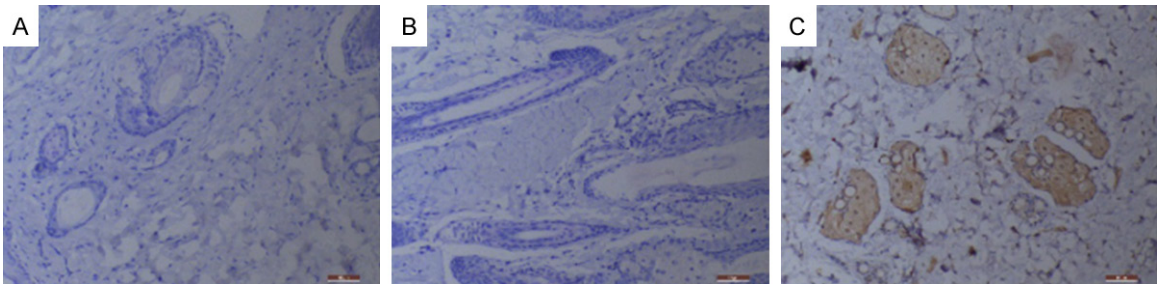

Figure 4. TGF-β_1 expression (brown) in the dermis of different groups (DAB-hematoxylin; ×200. A. Almost no TGF-β_1 expression was observed in the blank control group; B. TGF-β_1 expression was comparable between blank control group and transplantation group; C. TGF-β_1 expression showed wide distribution in the scar group).

10, 26, 27]. Since Zuk et al [7] for the first time reported the use of tissue derived stem cells, studies on adipose transplantation have focused on the matrix and cells rather than mature adipocytes, especially the ADSC, aiming to achieving better efficacy. A variety of studies have shown the good prospect of adipose transplantation. For example, it can improve the survival rate of transplanted tissues, promote the healing of refractory wound, improve the scar hyperplasia, promote hair regeneration and exert anti-aging effect on the skin [5, 15, 22, 28]. In the plastic and cosmetic repair field, studies on ASCs focus on the promotion of survival of transplanted adipose tissues and the improvement of local soft tissues in the breast reconstruction and facial shaping [10, 12, 19]. Although promising results have

Table 4. Expression of α-SMA and TGF-β_1 in the skin of transplantation sites at 4 weeks after transplantation

Group	n	α-SMA	TGF-β_1
Blank control group	6	35±16	44±17
Scar group	6	135±13[a]	121±23[a]
Transplantation group	6	49±12[b]	63±10[b]
F		45.583	16.726
P		0.000	0.004

Note: α-SMA, Alpha smooth muscle actin; TGF-β_1, transforming growth factor β_1; data refer to the number of cells at a magnification of 200; F and *P* values are as the results of comparisons among groups. [a]*P*<0.05 vs blank control group; [b]*P*<0.05 vs scar group.

been achieved in some clinical trials, the specific mechanism is needed to be studied in depth [29-33]. There is evidence showing that adipose transplantation for the treatment of scar may induce the deposition of collagen fibers, angiogenesis and cutaneous regeneration. Autologous adipose transplantation has been used to treat the scar contraction and scar induced pain, and the effectiveness may maintain for 3 months. In the present study, transplantation of granular adipose tissues achieved favorable efficacy in the treatment of depressed scar, which provides a new way for the treatment of depressed scar [14, 34-36]. However, which kind of molecules plays an important role in the therapeutic effects of autologous adipose transplantation is still unknown.

In the present study, a new pre-position liposuction needle was used for the collection of adipose tissue in the modified technique. This technique may cause little damage to the tissues, and the viability of mature adipocytes and adipose stem cells is significantly improved. The activity test showed the viability of adipocytes collected with new technique increased significantly as compared to that of cells collected with traditional technique. At 4 weeks after transplantation, there was no cell shriveling, cells showed close arrangement, and microvessels were found among adipocytes in the modified group. Moreover, the microvessel reconstruction and MVD in the modified group were also better than in the traditional group. This may be mainly ascribed to the preservation of active ADSCs with multidirectional differentiation potential in the adipose tissues collected with modified technique, which improves the survival of transplanted tissues. It has been confirmed that ADSCs can differentiate into endothelial cells to form microvessels, which may improve the early blood supply [37-44]. In addition, ADSCs can also promote the angiogenesis via paracrine [8, 42]. This is in accordance with the widely accepted mechanism underlying the survival of transplanted adipose tissues: the improved revascularization and differentiation of ADSCs into mature adipocytes are the keys for the improvement of survival of transplanted tissues [44]. Thus, the adipose tissues collected with the new technique are less damaged during the collection, and the relative amount of ADSCs in the collected tissues also increase, both of which are beneficial for the post-transplantation survival of adipose tissues.

In the scar animal model, the adipocytes harvested with modified technique were transplanted into the scars. Pathological examination showed the dermal structure in the modified group was more close to that of normal skin as compared to the untreated group. This suggests that the transplanted adipocytes improve the cutaneous structural remodeling in case of scar. α-SMA is mainly expressed in the activated myofibroblasts, and TGF-β_1 is a key cytokine in the fibrosis. Both are involved in the scar formation, and thus their expression was detected in the present study to reflect the scar formation. Immunohistochemistry showed the expression of α-SMA and TGF-β_1 reduced significantly in the dermis at the transplantation sites after adipose transplantation. Taken together, the adipose tissues collected with modified technique actually inhibit the scar formation, which may be related to the reduced expression of TGF-β_1 [14, 15, 45] and α-SMA in the skin, reduced secretion of collagen protein, inhibition of collagen proliferation and irregular arrangement, suppression of inflammatory cell infiltration and the subsequent improvement of scars. These consequences are found to be related to the more ADSCs with multidirectional differentiation potential and improved secretion, which change the microenvironment in the scar [46-48].

These findings indicated that modified fat transplantation was effective to enhance the survival of fat graft, which may be ascribed to

Table 5. Satisfaction of patients with scarring facial depression and atrophy in 2 groups

Group/time point	n	highly satisfactory	partly satisfactory	Satisfactory	Dissatisfactory	highly dissatisfactory
Single transplantation						
3 months after surgery	54	10	20	13	9	2
6 months after surgery	54	9	16	13	14	2
Mixed transplantation						
3 months after surgery	51	17	23	7	3	1
6 months after surgery	51	15	24	8	3	1

less damage to the fat tissues during the fat collection. In addition, there are more ADSC with multi-directional differentiation potential in the fat tissues collected with modified technique, and thus these ADSCs may differentiate into mature adipocytes to fill in the defect due to adipocyte necrosis. Moreover, ADSCs can also differentiate into endothelial cells to for microvessels, which is helpful for the early blood supply to the transplanted tissues [28]. The paracrine of these cells may also promote the angiogenesis [29, 49]. These finally minimize the ischemia induced necrosis of transplanted tissues. These findings are consistent with widely accepted mechanism of graft survival: the improvement of vascular reconstruction and the differentiation of ADSCs into mature adipocytes are crucial to improve the survival of transplanted fat tissues [30].

We speculate that transplantation of adipose tissues collected with modified technique may not only correct the depression deformity, but also improve the scar texture, prevent the adhesion between the scar and the skin, avoid the tension at the transplantation site, reduces the necrosis of adipose tissues at the transplantation due to the compression, leading to the improved survival of transplanted adipose tissues. In this study, the therapeutic effects were investigated in animal model, and only pathological examination was performed to assess the therapeutic effects. Thus, there were still limitations in the elucidation of therapeutic effects of adipose transplantation. More studies are needed to investigate the specific molecular mechanism underlying the ADSC induced inhibition of α-SMA and TGF-β_1 in the treatment of depressed scar by adipose transplantation.

Disclosure of conflict of interest

None.

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