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ORIGINAL ARTICLE NON-SURGICAL AESTHETIC

Correction of Tear Trough Deformity Using Autologous Fibroblast Combined with Keratin: New Soft Tissue Filler

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Abstract
Objective To evaluate the effectiveness and safety of autologous fibroblasts combined with keratin gel for tear trough deformity rectification as injectable soft tissue filler.
Materials and Methods The new injectable soft tissue filler was derived from autologous fibroblasts and keratin gel. A total of 35 patients received treatment of this filler injection for tear trough deformity rectification. All the patients were followed up, and the clinical features including photographs and satisfaction were collected and assessed at 1, 3, 6, 12 and 24 months after injection. The efficacy of each patient was evaluated independently by blinded evaluators at different time points. All patients consented to publish identifiable photographs in this study.
Results Tear trough deformity was improved even at 18–24 months post-injection. No severe adverse effects were observed resulting from the filler injection.
Conclusion Combination of autologous fibroblasts and keratin is efficient and safe for correction of the tear trough deformity with long-term satisfaction and desirable result.

Level of Evidence IV This journal requires that authors assign a level of evidence to each article. For a full description of these Evidence-Based Medicine ratings, please refer to the Table of Contents or the online Instructions to Authors www.springer.com/00266.

Keywords Esthetics · Blepharoplasty · Fibroblasts · Keratins

Introduction

Tear trough deformity is an outer appearance of the aging process. It is characterized by a gap or hollow below the medial lower eyelids, which leads to a dark shadow over the lower eyelid and a fatigue appearance of the patient. Loeb [1] defined the concave deformity as a "nasojugal groove," and the term "tear trough deformity" was introduced by Flowers [2]. Orbital septum, orbicularis muscle, suborbicularis fascia and herniated fat pad are all responsible for tear troughs [3]. Tear trough deformity has always been considered a great challenge in facial rejuvenation.

Soft tissue filler injection of tear trough deformity rectification is more popular in clinical application, compared with surgical operations such as blepharoplasty, which was not well accepted because of the following tissue trauma [3–5]. An ideal soft tissue filler needs to be biocompatible, easy to obtain and relatively low cost with long-lasting satisfying results. Autologous-derived fillers came into view and were well accepted gradually because of their advantage of being non-immunogenic, such as autologous fat [6]. In 2011, a new commercial soft tissue filler which contains cultured autologous fibroblasts (LavivTM, Fibrocell Tech- nologies, Inc., Exton, PA) was introduced and approved by the US Food and Drug Administration (FDA)

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[7, 8]. Fibroblast injection might be effective and safe for nasojugal grooves correction, but it is more interesting that when Isolagen was injected with Restylane, the mixture could provide long-lasting effects [9, 10].

Theoretically, a tissue-engineering soft tissue filler that contains cells and scaffold might improve the inherit problem of the traditional tissue filler, such as short-term treatment and endless therapy sessions. Human hair keratin possessed a strong potential for tissue development as a clinically relevant biomaterial and could support fibroblast attachment and proliferation in vitro [11]. Hair keratin with different diameters had been tested in an animal model and showed good biocompatibility and biodegradation [12]. Furthermore, in a study published earlier, a new tissue-engineering soft tissue filler containing cultivated autologous fibroblasts and hair-derived keratin achieved satisfied outcomes in neck wrinkle rejuvenation clinically [13]. In this study, fibroblasts and keratin filler will be first applied to correct tear trough deformity, long-term safety and efficacy will be further evaluated in follow-up observation.

Materials and methods

From January 2015 to January 2017, totally 35 healthy subjects with different grades of tear trough deformity were enrolled in our clinical trial as volunteers. The median age was 40.3 years (range 22–58 years). The tear troughs of all the 35 volunteers were assessed before injection: Three cases were grade I deformity, 13 cases were grade II, and 19 cases were grade III according to the Barton's grading system (listed in Table 1).

Written consent forms were received from all individuals, and all procedures involving human participants were approved by the institutional ethics committee and conformed to the ethical standards of the institutional and national research committee and the World Medical Association Declaration of Helsinki (June 1964).

Autologous Fibroblasts Collection

The autologous fibroblasts were harvested from healthy skin of the retroauricular area to avoid visible scar. The donor sites were disinfected, draped and locally anesthetized, and a skin biopsy approximately 3×10 mm^2 was collected. After removing fat and blood vessels, the skin tissue was washed by sterile 0.9% NS solution three times and digested with 0.25% dispase II (Sigma, USA) overnight at 37 °C. The dermis of the skin was separated from the epidermis and further digested with 5% collagenase II for 2 h at 37 °C. The suspension was centrifuged for 10 min at 300 g, and the supernatant was discarded. The extracted fibroblasts were washed by sterile phosphate-buffered solution (PBS) three times and seeded to 10-cm culture plates with DMEM medium (Gibco BRL, Gaithersburg, MD, USA). After culturing and passaged for 4–6 weeks, fibroblasts were resuspended and counted, and then cell viability was tested.

Autologous Keratin Production

Hair mass was collected from volunteers and manipulated according to the procedures reported before to produce autologous keratin [12]. Generally, 3–5 g of hair of each volunteer was collected and disinfected, washed by sterile PBS 3 times and cut into small pieces. The hair fragments were degreased by 95%(v/v) ethanol, washed by distilled water and then dried. Two working solutions were prepared earlier. Working solution A was a mixture of 30 parts of 0.5% H_2SO_4 and 1 part of 0.32% NaClO, and solution B was a mixture of 30% H_2O_2, sodium pyrophosphate (8 g/L), potassium persulfate (3 g/L) and aqueous ammonia (14 g/L). The dried hair was chloridized with working solution A for 20 min at 20 °C and washed with sterile PBS solution three times. The hair mass should be bleached and showed a whitening appearance by immersion in working solution B for 3.5 h at 40 °C in a water bath until the color fades. The bleached hair was further washed three times by sterile distilled water to remove the remains of chemicals and dried at 30 °C. The whitened hair was then

Table 1 Tear trough deformity assessment according to Barton's grading system before operation

Grade	Description	Case number	Percentage
0	Absence of medical or lateral lines demarcating the arcus marginalis or the orbital rim, and a smooth, youthful contour without a transition zone at the orbit–cheek junction	0	0
I	Mild, subtle presence of a medical line or shadow; smooth lateral transition of lid–cheek junction	3	8.6
II	Moderate prominence of a visible demarcation of the lid–cheek junction, extending from medical to lateral	13	37.1
III	Severe demarcation of the orbit–cheek junction, with an obvious step between the orbit and the cheek	19	54.3

mixed with sterile 0.9% normal saline at a ratio of 12–15:100 (w/v) and put into a planetary high-energy ball mill (QM-ISP4) to make the keratin particles. The hair mass was ground for 8–12 h to get the desirable size of an average diameter of 60–80 μm. The keratin particles were finally sterilized by radiation of 60Co-radiation (25 kGy). The keratin products were lyophilized and stored at 4 °C until use (Patent No: CN101530636).

Mixture of Fibroblasts and Keratin Preparation

Before injection, keratin particles should be fully diluted in sterilized saline at a ratio of 8–10:100((w/v) to make injectable keratin gel. Ten milliliters of venous blood were harvested from each volunteer and centrifuged to obtain serum. The fibroblasts were resuspended in the volunteer's serum (5×10^5cells/ml), and 200 μl keratin gel was added into every 1 ml serum. The mixture is injected right after it is ready.

Injection for Tear Trough Deformity

The injection area was cleaned, anesthetized with lidocaine cream and disinfected. The mixture was injected subcutaneously with a 30-gauge needle and 1-ml syringes. The implant site is between the dermal reticular layer and fat layer. The mixture was injected in a tunnel way using a retreating technique along the bilateral nasojugal grooves. The injection dose was about 0.5–3 ml in each side. Generally, 2–3 injections were needed in the treatment. The exact dose of the first injection depends on their local situation and the different grades of deformity in each volunteer. Generally, the second and third injections are performed after 30-day intervals. In this study, we conduct two injections for each case to make the result comparable.

Result

Safety and Adverse Reaction

Follow-up visiting showed good tolerance of this filler during the whole observation term. No severe local reactions, inflammation and systematic immune reactions were observed 1 week postoperatively. No severe redness, bruising or swelling was observed after injection. Figure 1 shows a 45-year-old female with no obvious local bruising or swelling at the 5th day after injection. Treatment-related adverse reactions such as injection-related pain occurred in four cases in the early postoperative period, and all the symptoms recovered spontaneously without any additional treatment.

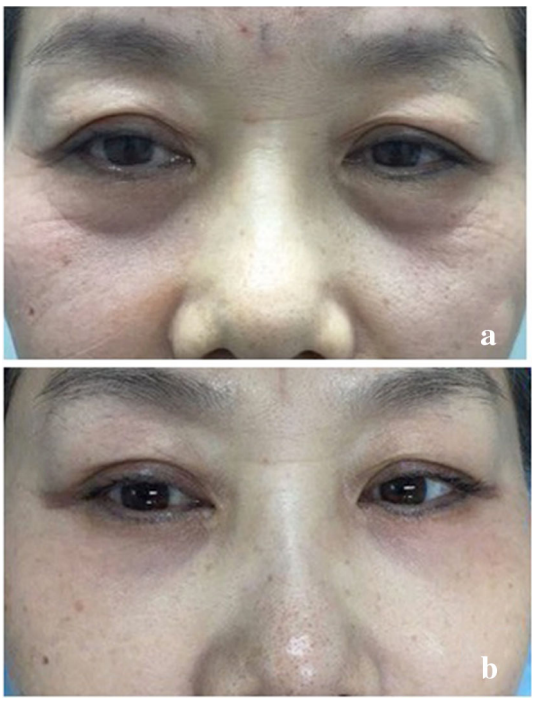

Fig. 1 Case 1, Female, 45 years old, received injection of keratin and fibroblasts as above described, with no severe local bruising or swelling 1 week after injection. **a** Preoperative view of the tear trough deformity. **b** 5th day postoperative view

Photograph Documentation

All the volunteers were followed up from 3rd day, 5th day and 7th day to 1st, 3rd, 6th, 12th and 24th months after injection. Follow-up observations were adopted and photographs were collected to assess the efficacy of the treatment at different preoperative time points based on the outpatient appointments. The filling effect was evaluated blindly to avoid potential bias, that is, the injection and evaluation were administrated by different investigators individually in the whole follow-up procedure. Each investigator used the Global Aesthetic Improvement Scale (GAIS) to assess efficacy, which is listed as follows: "very much improved," "much improved," "improved," "no change" or "worse" [14]. Three independent assessors were invited to do blind ratings for each photograph and case to ensure the result was objective and reliable. Furthermore, participant satisfaction was evaluated by the Freiburg questionnaire. Subjects were interviewed to judge their satisfaction as: "very satisfactory," "satisfactory," "poor satisfactory" or "not at all." Additionally, all the subjects were questioned as to whether they would like to recommend this treatment to others with the same problem at months 12 and 24. The assessment results are presented in Tables 2 and 3.

Table 2 Postoperative assessments according to Global Aesthetic Improvement Scale (GAIS) compared to preoperative baseline

Grade	Case number				
	Month 1	Month 3	Month 6	Month 12	Month 24
Very much improved	29	29	26	23	19
Much improved	3	2	5	3	4
Improved	2	3	3	3	2
No change	1	1	1	2	2
Worse	0	0	0	0	0

Table 3 Participant satisfaction assessments according to Freiburg questionnaire

Grade	Case Number				
	Month 1	Month 3	Month 6	Month 12	Month 24
Very satisfactory	5	5	3	2	2
Satisfactory	26	28	29	25	22
Poor satisfactory	4	2	3	4	3
Not at all	0	0	0	0	0

Data Analysis

All the cases were successfully followed up at the 1st, 3rd and 6th months. However, about 11% of volunteers lost communication at the 12th month and only 77% of them (27 cases) followed the 24-month observation (Table 2). Photographic documentation was presented for clinical evaluations. Figures 1, 2, 3, 4, 5 and 6 show six volunteers' pre- and post-injection views who had different extents of tear troughs and the efficiency of the treatment. Based on the GAIS assessment in Table 2, about 97% of cases showed significant improvement compared to the preoperative baseline at the 1st, 3rd and 6th months. About 93% of cases showed improvement at the 12th month, and still 92% (25 cases) at month 24. Moreover, during the 2-year

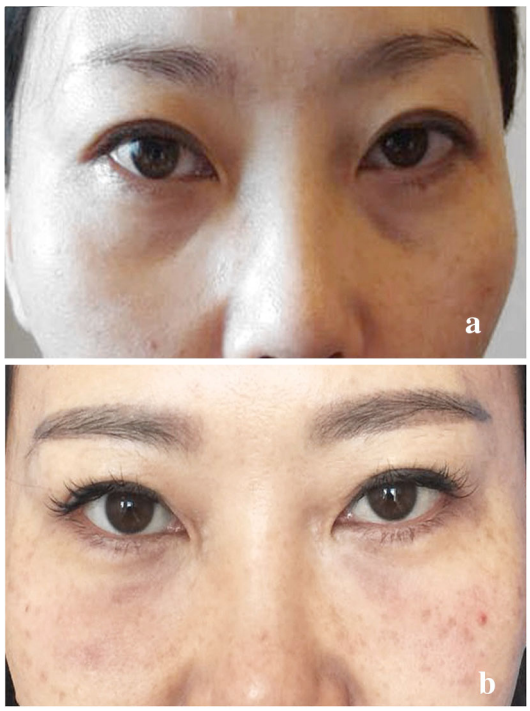

Fig. 2 Case 2. Female, 42 years old, received injection of keratin and fibroblasts as above described. She had no other injection. **a** Preoperative view of the tear trough deformity. **b** 18th month postoperative view with very much improved result and satisfactory

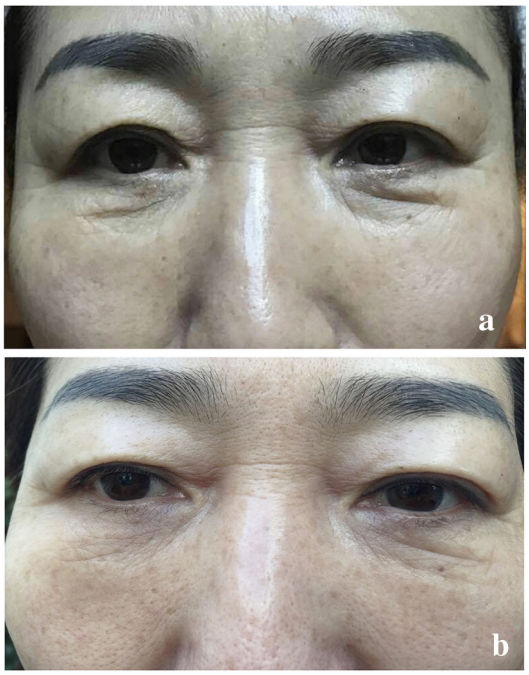

Fig. 3 Case 3. Female, 58 years old, received injection of keratin and fibroblasts as above described. She had no other injection. **a** Preoperative view of the tear trough deformity. **b** 16th month postoperative view with very much improved result and very satisfactory

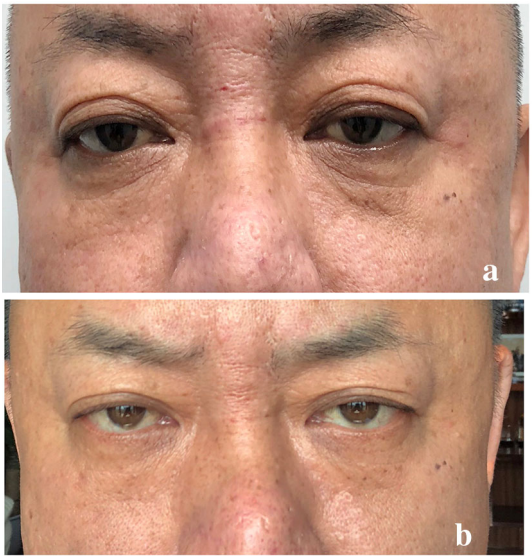

Fig. 4 Case 4, Male, 55 years old, received injection of keratin and fibroblasts as above described. She had no other injection. **a** Preoperative view of the tear trough deformity. **b** 24th month postoperative view with much improved result and satisfactory

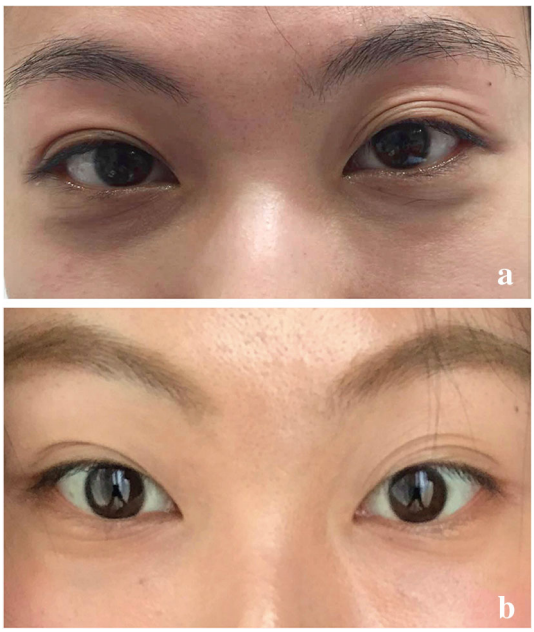

Fig. 5 Case 5, Female, 32 years old, received injection of keratin and fibroblasts as above described. She had no other injection. **a** Preoperative view of the tear trough deformity. **b** 12th month postoperative view with very much improved result and very satisfactory

follow-up, 85% of cases showed remarkable reduction in their tear trough deformity without any complaints. Furthermore, participant-related assessment (Table 3) showed that 88% of cases were satisfied with the results of the treatment. The data showed that the tear trough deformity was improved remarkably without any severe adverse reactions at 18–24 months post-injection (GAIS 92%). At

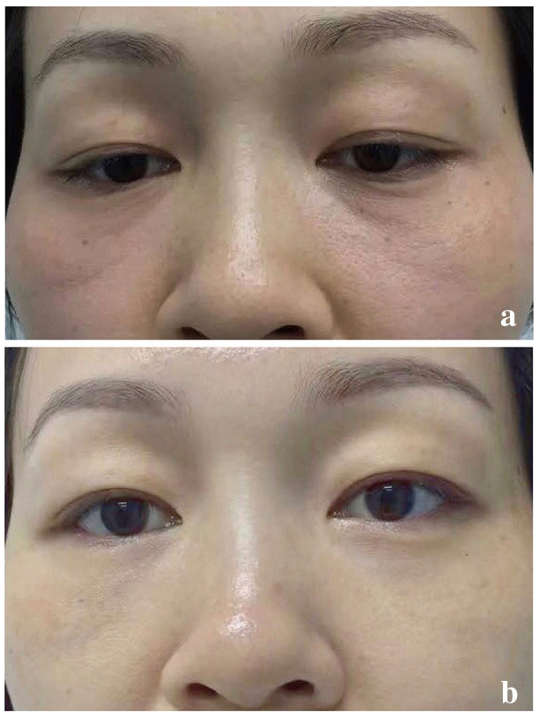

Fig. 6 Case 6, Female, 40 years old, received injection of keratin and fibroblasts as above described. She had no other injection. **a** Preoperative view of the tear trough deformity. **b** 14th month postoperative view with very much improved result and satisfactory

the end of the follow-up, all 27 patients (100%) were satisfied and would like to recommend this method to people that have a similar problem.

Discussion

Tissue-engineering soft tissue filler contains living cells and scaffold, which might improve the inherit problem of traditional short-term treatment and endless therapy sessions. Autologous cultured fibroblasts have been proven to be useful to correct nasojugal grooves and injected living fibroblasts are believed to produce collagen to repair a local deformity for a long time [7–10]. Fabrication of keratin scaffolds for long-term cell cultivation was first reported by Tachibana in 2001 [15]. Keratin biomaterials possess many distinct advantages over conventional biomolecules, including a unique chemistry afforded by their high sulfur content, remarkable biocompatibility, propensity for self-assembly, intrinsic cellular recognition and the ability to polymerize into complex 3D structures.

As to the distinct advantages of keratin and the mechanism of promoting cell regeneration, much researches focus on its characteristic and potential value in regenerative medicine, such as neuromuscular recovery, lethal liver injury regeneration and wound site healing gradually,

but not for cosmetic applications [16–18]. In 2006, Zhou et al. [19] reported that keratin could promote proliferation of fibroblast cells and increase type I collagen secretions through subcutaneous transplantation in nude mice. Ma et al. [12] further proved the biocompatibility of keratin through general toxic tests in vitro including the MTT assay, acute toxicity and micronucleus for soft tissue filling. The mouse subcutaneous implant model was employed in their study to investigate the immune reaction, absorption and maintenance of the augmentation effect [12]. Furthermore, more capillaries and fibroblasts were found in the transplant area as well as collagen secretions after the mixture filler injection of keratin and fibroblasts in Bama minipigs [20]. Consequently, keratin was absorbed and replaced by fibroblasts and collagen at about 12 months after injection and the thickness of the local dermis was significantly increased compared with the control area [20]. Autologous fibroblasts and keratin were further used for neck wrinkle rectification in clinical trials, which presented ideal results with long-term efficacy [13]. This evidence indicated the potential usage of this new filler in the cosmetic area.

In this study, fibroblasts and keratin gel were first used for tear trough rectification and 2-year follow-ups were conducted to evaluate the efficacy. Not only the local reaction in the early postoperative period, but also the long-term effect was satisfactory in the follow-up visiting. In this tissue-engineering filler, keratin gel acts as a 3D bio-scaffold and short-term filler for cell growth and tear trough correction. Fibroblasts act as seed cells, which could increase collagen production and maintain the long-term filling result after keratin has been absorbed. Compared with other fillers such as hyaluronic acid or collagen, that are derived from animals or recombination, the fibroblast and keratin bio-system could secrete collagen continuously and increase the thickness of the dermis eventually. This autologous filler could maintain stable and long-term effects with relative lower cost, and without any ethical issues. In the long view, it is safer, cheaper and more acceptable than other fillers, even 2–3 sessions of injection were recommended generally.

Conclusion

The new filler, mixed with autologous fibroblasts and keratin, is safe and effective in tear trough deformity correction and might act as a prospective soft tissue filler in clinical application with great value.

Author Contributions WX, CZ and JZ participated in the clinical application of the technique and the follow-up of the patients and analysis of data for the work. All authors participated drafted the article. QZ contributed the conception and design of the study and revised it critically for important intellectual content. All the authors approved the version to be submitted.

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Compliance with Ethical Standards

Conflict of interest The authors report no financial and personal relationships with other people or organizations that could inappropriately influence the work.

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