New soft tissue filler derived from autologous keratin and fibroblast for neck wrinkles

Yue Wang MD | Bingqing Wang MD | Qingguo Zhang MD | Jiguang Ma MD

Plastic Surgery Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China

Correspondence
Jiguang Ma and Qingguo Zhang, Plastic Surgery Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China.
Emails: joycezhang1978@hotmail.com and plastic135@126.com

Summary
Objective: To assess the effectiveness of injection of autologous keratin gel and fibroblast for neck aging as soft tissue filler.

Materials and methods: Totally 30 volunteers received treatment of autologous keratin and fibroblast for neck wrinkles and 5 of them received hyaluronic acid (HA) treatment as control. Clinical features of the volunteers were collected at 1st, 3rd, 6th, 12th, and 24th months after treatment. The volunteers were independently assessed using Lemperle’s methods at different time points.

Results: The neck wrinkles ameliorated observably and no severe complications were reported after treatment. The effect and maintain time of autologous keratin and fibroblast were better than HA control.

Conclusion: Autologous keratin and fibroblast as soft tissue filler is an effective treatment option for neck rejuvenation with long-term efficacy for reversing of skin aging.

KEYWORDS
fibroblast, keratin gel, neck wrinkle, soft tissue filler

1 | INTRODUCTION

Wrinkle is outer manifestation of skin aging. Neck wrinkles are easy to come out and recognized for neck’s special anatomy and physiology. In 1990, CM Lapiere found that the change in dermal structure is the main cause of skin aging.1 In Scott Shadfar’s researches, there are many reasons causing neck aging, and loss of dermal matrix volume results in neck wrinkles.2 And in Yaar’s study, they found with the increase of age, the number of collagen fibers, and fibroblasts decreased gradually. There are many procedures to neck rejuvenation, such as laser, surgery, and filler injection.4-6 Soft tissue filler injection is easier to be accepted and be easy controlled, so it is widely used in clinical practice.

Injectable soft tissue fillers are used in clinical very early, the ideal material for soft tissue augmentation should be biocompatible, nonimmunogenic, easily obtainable, low cost, nonresorbable, and easily stored.7,8 Autogenous tissues are the most convenient materials without causing biologic reactions and are becoming popular.9 Isolagen (autologous cultured fibroblasts), which were proved by FDA at 2011, is a autogenous issue filler under rigid production procedures, and it can produce collagen all the time, which makes long-lasting effect, and is widely used in clinical.10-12 It is really interesting that when Isolagen was injected with Restylane, the mixture could provide longer-lasting effects.12 Keratin is first used in textile industry, and its biocompatibility is gradually realized with the studies going on. Hong et al implanted sterile hair into face to correct face defect. In our previous study, we derive a new keratin-derived material and after testing its biocompatibility, we used animal model to test their biodegradation. We injected the keratin from hair into mice and rabbits, which turned out it acted well.13 In this research, autologous cultured fibroblasts from retroauricular skin and keratin from hair were mixed, and the mixture was injected into neck wrinkle in healthy volunteers. After the treatment, we made followed up visits to evaluate the effect.14,15

Ma and Zhang are co-corresponding author to this manuscript.

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2  |  MATERIALS AND METHODS

2.1  |  Subjects

Totally 35 healthy female and male volunteers suffering severe neck wrinkle were enrolled in this clinical trial aging from 35 to 50 years old, which including 25 women (mean age is 40) and 10 men (mean age is 42). There are 25 volunteers with severe neck wrinkles, whose average length of neck wrinkles are about 9.6 ± 0.3 cm. Other 10 volunteers had mild and moderate neck wrinkles, whose average length of wrinkles are about 5.0 ± 0.5 cm and 7.0 ± 0.5 cm, respectively. Thirty volunteers were randomly allocated to the filler group, and the remaining 5 volunteers were treated with hyaluronic acid (HA) as parallel control. Written informed consent forms were obtained from all individuals (or their legal guardians) for biological investigations. This research was reviewed and approved by the Ethics Committee of the Plastic Surgery Hospital, Chinese Academy of Medical Sciences.

2.2  |  Collection of fibroblasts

After normal disinfection, draping, and local anesthesia, a 3 × 10 mm² skin tissue is collected from retroauricular area; with removed fat and blood, washed with normal saline for repeatedly 3 times, placed into a 35-mm culture dishes, digested with 0.25% Dispase II (Sigma, Cat:D4693) overnight. Tweezers were used to separate the epidermis and dermis; dermis was further digested with 5% collagen II for about 2 h and then centrifuged by 300g for 10 min. After discarded supernatant, fibroblast cells were washed for 3 times with phosphate buffered solution (PBS) and seeded into 60-mm culture dishes, and then cultured and subcultured for 4-6 weeks with DMEM medium (Gibco BRL, Gaithersburg, MD, USA).

2.3  |  Collection of keratin

For the collected keratin, 3-5 g of hair was obtained from volunteers and processed according to our previous research,14 disinfected with iodine (0.2% I, 310101, Shanghai Likang disinfectant Hi-Tech Co., Ltd.), washed by sterile PBS solution for repeatedly 3 times, cut into small pieces with scissors, rinsed with detergent solution, and dried after degreasing and washing. Then, the dried hair was soaked in the working solution A (mixing 30 parts 0.5% H₂SO₄ with 1 part 0.32% degrading and washing. Then, the dried hair was soaked in the pieces with scissors, rinsed with detergent solution, and dried after washed by sterile PBS solution for repeatedly 3 times, cut into small pieces with scissors, rinsed with detergent solution, and dried after degreasing and washing. Then, the dried hair was soaked in the working solution B (mixing 30% H₂O₂, sodium pyrophosphate (8 g/L), potassium persulfate (3 g/L), and aqueous ammonia (14 g/L)) for 3.5 h at 40°C in a water bath until the color whitened, and then rinsed for 3 times to remove the remaining reagent, and the bleached hair was dried at 30°C. Planetary high-energy ball mill (QM-ISP4) and a plurality of planetary mill nylon pots were used for producing hair particles. Hairs were mixed with distilled water at a ratio of 7:1 (v/w) and added to the planetary mill nylon pots. The hair particle size was controlled by the predetermined period of time. This step was setup at 8 h to get the desirable particle size (the mean diameter is about 80 µm). The particles were sterilized by radiation sterilization using 60 Co-radiation (25 kGy). All the products can be lyophilized and stored at 4°C for future use (Patent No: CN101530636, Qingguo Zhang).

2.4  |  Collection of the mixture

From each volunteer, 10 mL of venous blood was collected, and serum was obtained by centrifuging. Every 1 mL of serum was combined with 5 × 10⁷ fibroblasts and 200 µL keratin gel to make injectable soft tissue filler which should be injected as soon as possible.

2.5  |  The treatment of neck wrinkle

Compound lidocaine cream (1501042, Unispharm, Beijing, China) is used for skin topical anesthesia, disinfected with alcohol (75%v/v), and inoculated in neck subcutaneous tissue using a 30-gauge needle (the inner diameter of the 30-g needle is 0.15 mm and 0.31 mm outer diameter) and 1-mL syringes. The mixture is injected in tunnel way that giving bolus injection when retreating the needle. The implant site is between dermal reticular layer and fat layer, and tape is used for fixed for 3 days after treatment. The first injection dose is 1 to 2 mL each volunteer depends on theirs’ local situation of neck wrinkles, and the second injection is after 15 days. In the control group, HA was injected according to the standard procedure⁴ and 2 treatments were provided for each volunteer. After received 2 treatments totally, follow-up started and the volunteers were evaluated at different points, such as 1st, 3rd, and 6th months, until 1–2 years.

3  |  RESULT

3.1  |  Photographic documentation

All the volunteers did not show any severe local reaction and inflammation and systematic immune reaction in 1 to 2 weeks after injection. The follow-up went well at 1st, 3rd, and 6th months, while 10% volunteers lost communication at 12th month after treatment, and only 70% of them followed the 24-month observation at the end of our study.

During the entire 2 years, most of the 30 volunteers that received keratin gel and fibroblast tissue filler injection reached satisfied results, which showed remarkable reduction in the depth and retarded progress of the neck wrinkles without any complaints. But in HA injection group, the effect only last for about 6 months. The photographic documentation was presented for clinical evaluations (Refer to Figs 1-6). Figures 1 to 4 show 4 volunteers who had severe neck wrinkle, and Figure 5 shows 2 mild and moderate wrinkle cases. HA was used as parallel control, and the result was presented in Figure 6.

3.2  |  Date analysis

The neck wrinkles of all the volunteers were assessed at pre-injection and 1st, 3rd, 6th and 12th months after injection,
respectively, using Lemperle’s methods,15 through which their wrinkles were graded by 6 levels according to the objective measurement (listed in Table 1). Table 2, 3, and 4 showed the follow-up studies that were made at 1st, 3rd, 6th, and 12th months after

**FIGURE 1** Female, 38 years old, received injection of keratin and fibroblasts as above described. She had none other injection. Figure 1A and C was taken before injection (side and front view, respectively). Figure 1B and D was taken 12 months after operation (side and front view; Figure 1A and C)

**FIGURE 2** Female, 42 years old, received injection of keratin and fibroblasts as above described. She had none other injection. Figure 2A and C was taken before injection (front and side view, respectively). Figure 2B and D was taken 18 months after operation (front and side view; Figure 2A and C)

**FIGURE 3** Male, 50 years old, received injection of keratin and fibroblasts as above described. He had none other injection. Figure 3A was taken before injection (side view). Figure 3B was taken 18 months after operation and corresponding to Figure 3A
injection in each group, including severe, mild, and moderate wrinkles and HA control group. All the results in 1st, 3rd, 6th, and 12th months were compared and calculated with the pre-injection measurement.

Table 2 showed results of keratin gel and fibroblast injection comparing with pre-injection in the severe wrinkle cases. These data showed that at 1st month after the injection, about 95% wrinkles ameliorated by at least 1 level, and at 3 to 6 months, 80-90%
In conclusion, these results indicated that the effects of our filler were generally satisfied after injection. Although the filler were absorbed gradually, it still maintains 70-90% filling effect even 12 months after injection. Relatively, HA can only last for about 6 months after injection.

4 | DISCUSSION

With being aging, transverse wrinkles and platysma muscle bundle shaped belt gradually appeared in the neck, because of relaxation and contraction of the neck skin, elasticity reducing, gravity.18 Transverse wrinkles in neck cause by loss of dermal matrix volume, in which collagen is in the majority, so injecting soft tissue fillers is the main idea to ameliorate the neck wrinkles.19,20 Because of special anatomy, it is difficult to cure the neck wrinkles.

The nonoperative treatment of neck wrinkles mainly includes laser, injection of botulinum toxin, hyaluronic acid, and so on. Laser stimulates local tissue with high-energy laser, which made collagen remodeling and other subtle structural changes, then reducing wrinkles.21 Laser may cause scar and pigmentation; also the recovery time is long, which limits its application in the treatment of neck wrinkles.22 Injecting soft tissue fillers to cure facial and neck wrinkles starts early, and hyaluronic acid and collagen are used to cure neck wrinkles in clinical.7,19,23-26 The neck skin is thin, and it always affect by movement, so the injecting drug is easy to accumulate in local site.4,27-29 Because of the short absorption time of hyaluronic acid, collagen, and the accumulating of the injecting drug, the effect is not very well.30

Autologous keratin is well biocompatible, and its molecular structure is stable, which made it being a good extracellular supporting materials.31 Our previous study turned out hair-derived material after processing showed an intrinsic capacity with preferable fluidity and glutinousness for injection. General toxic evaluation revealed a good biocompatibility and absence of cytotoxicity or mutagenicity. Animal subcutaneous implant models did not show any local or systemic adverse reactions in the observation period. In the absorbance model, hair fiber particle showed preferable augmentation effect which preserved at least 50% of original volume during the observation.14 As to fibroblast cell, it is all known that Isolagen (autologous cultured fibroblasts) is a autogenous tissue filler which received FDA approval in 2011, which produce collagen all the time and make long-lasting effect.10,11 Our new filler consists of autologous keratin and fibroblasts, in which the keratin acts as tissue scaffolds and fibroblasts act as a sustainable remediation system that continuously secreting collagen. In this clinical trial, we injected this new filler to neck wrinkle subcutaneously to resist the neck wrinkles, and the injection treatment effects were very well during the entire follow-up.

These results indicated that the mixture of keratin gel and fibroblast cell in the dermal tissue showed a long-time repair effect because of supporting network and the continuously secreting collagen. This brand new tissue filler with autologous keratin and fibroblasts can effectively reduce neck wrinkles with long-term and stable effect, which might be act as prospective soft tissue filler in clinical application.
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