Original Article

Basic and Clinical Evidence of an Alternative Method to Produce Vivo Nanofat

Hong-Sen Bi[1], Chen Zhang[1], Fang-Fei Nie[1], Bo-Lin Pan[1], E Xiao[2]

[1]Department of Plastic Surgery, Peking University Third Hospital, Beijing 100191, China

[2]Department of Oral and Maxillofacial Surgery, Peking University, School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing 100081, China

Abstract

Background: Fat grafting technologies are popularly used in plastic and reconstructive surgery. Due to its size limitation, it is hard to directly inject untreated fat tissue into the dermal layer. Nanofat, which was introduced by Tonnard, solves this problem by mechanically emulsifying fat tissue. However, the viability of the cells was greatly destroyed. In this study, we reported a new method by "gently" digesting the fat tissue to produce viable adipocytes, progenitors, and stromal stem cells using collagenase I digestion and centrifugation. This was named "Vivo nanofat".

Methods: Human liposuction aspirates were obtained from five healthy female donors with mean age of 28.7 ± 5.6 years. Colony-forming assay, flow cytometry analysis, and adipogenic and osteogenic induction of the adherent cells from the Vivo nanofat were used to characterize the adipose mesenchymal stem cells (MSCs). To investigate *in vivo* survival, we respectively injected Vivo nanofat and nanofat subcutaneously to the back of 8-week-old male BALB/c nude mice. Samples were harvested 2 days, 2 weeks, and 4 weeks postinjection for measurement, hematoxylin and eosin staining, and immunostaining.

Results: Our results showed that the Vivo nanofat contained a large number of colony-forming cells. These cells expressed MSC markers and had multi-differentiative potential. *In vivo* transplantation showed that the Vivo nanofat had lower resorption ratio than that of nanofat. The size of the transplanted nanofat was obviously smaller than that of Vivo nanofat 4 weeks postinjection (0.50 ± 0.17 cm vs. 0.81 ± 0.07 cm, $t = -5783$, $P = 0.01$).

Conclusion: Vivo nanofat may serve as a cell fraction injectable through a fine needle; this could be used for cosmetic applications.

Key words: Adipose Tissue; Cell Therapy; Mesenchymal Stromal Cells; Rejuvenation

Introduction

Fat grafting technologies are popularly used in plastic and reconstructive surgery due to their autogenous and completely biocompatible nature, and the low expenses associated with their administration. In the past decade, adipose-derived mesenchymal stem cells (MSCs) were characterized,[1] and the factors affecting the long-term survival of transplanted adipose tissue and the mechanism of improving their interaction with surrounding tissues were greatly elucidated.[2] Cell viability was considered as an important factor affecting the long-term survival and regeneration of the grafted fat tissue and the nourishment of the surrounding tissues.[3,4]

Due to the size limitation, it is hard to directly inject untreated fat tissue into the dermal layer using a very fine sharp needle for rejuvenation purposes aimed at correcting superficial rhytides, wrinkles, and atrophic scars. To overcome this disadvantage, "nanofat" was manufactured by the mechanical emulsification of fat tissues. However, during mechanical emulsification, cell viability was

Address for correspondence: Dr. E Xiao, Department of Oral and Maxillofacial Surgery, Peking University, School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing 100081, China
E-Mail: xiaoe@bjmu.edu.cn

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markedly destroyed, which may contribute to inflammation and increased cell apoptosis.[5]

In this study, we developed a new method of emulsifying fat tissues, which not only reduced the size of the tissue but also maintained the viability of the adipose cells; enzyme digestion was used instead of mechanical emulsification.

Methods

Ethical approval

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the local Ethics Committee of Peking University Health Science Center (No. 2017-303-02). Informed written consent was obtained from all patients before their enrollment in this study. All procedures used in this study were in accordance with our institutional guidelines that complied with the international ethics and humane standards for animal use. Every effort was made to minimize the number of animals and reduce their suffering.

Human fat cell isolation and preparation

Human liposuction aspirates were obtained from five healthy female donors with mean age of 28.7 ± 5.6 years, who underwent abdomen liposuction using a 2.4 mm diameter needle. The nanofat was prepared as described in a previous study.[5] The saline-rinsed fat tissue was mechanically emulsified by shifting the fat between two 10 ml syringes connected by Luer Lock connector. After 30 passages, the effluent was collected.

The Vivo nanofat was prepared by digesting the rinsed fat tissue with 1 ml of 0.2 mg/ml collagenase I (Sigma, Germany) resuspended to a final volume of 20 ml and incubated at 37°C for 15 min (we designed a short-time digestion process to save the viability of the adipocytes). This was then centrifuged at 330 ×*g* for 7 min, and the supernatant fat cell fraction was collected and filtered through a cell strainer, which had a pore with 0.6 mm diameter. The effluent was collected as Vivo nanofat.

Cell viability assay

Since the adipose stem cells in the nanofat were considered trophic factors, they contributed to tissue regeneration in the injected site.[6,7] To analyze the cell viability, trypan blue staining was performed. The total cell number per milliliter and the number of cells appearing blue were calculated. The trypan blue-stained cells were considered dead cells. The positive cell number ratio was calculated by dividing the number of trypan blue-stained cells by the total cell number.

Cell culture and differentiation

Cells collected as Vivo nanofat were seeded into a 100 mm culture dish (Corning, USA) containing Dulbecco's Modified Eagle Medium/F12 (Life Technologies Corporation, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Fetal Bovine, Thermo Fisher Scientific Inc., China), penicillin G, and streptomycin sulfate (Life Technologies Corporation). Following overnight culturing at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, the unattached cells were discarded when the medium was changed. MSCs with a passage number of 3–5 were used for the following experiments. Characterizations of MSCs were also tested by osteogenic and adipogenic differentiation and MSCs-related marker staining, as described in our previous study.[8]

Osteogenic differentiation

Cells were cultured under osteogenic conditions, in the presence of 2 mmol/L β-glycerophosphate (Sigma-Aldrich, Germany), 100 μmol/L L-ascorbic acid 2-phosphate (Wako, Japan), and 10 nmol/L dexamethasone (Sigma-Aldrich) in the growth medium. After a 4-week induction, staining with 1% Alizarin Red S (Sigma-Aldrich) was performed at room temperature, to detect mineralization.

Adipogenic differentiation

For adipogenic induction, 500 nmol/L isobutylmethylxanthine (Sigma-Aldrich), 60 μmol/L indomethacin (Sigma-Aldrich), 500 nmol/L hydrocortisone (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), and 100 nmol/L L-ascorbic acid phosphate were added into the growth medium. After 7 days, the cells were stained with Oil Red-O (Sigma-Aldrich).

Flow cytometry

Cells were harvested at the fourth passage. After three washes with phosphate buffered saline (PBS) 1% FBS, the cells were incubated in the dark for 45 min at 4°C with a fluorescein-conjugated antibody, as per the manufacturer's instructions, using BD Stemflow™ hMSC Analysis Kit (BD, USA). Flow cytometry was performed on a BD Accuri™ C6 (BD, USA).

Animal model

To investigate *in vivo* survival, we injected 300 μl nanofat and 300 μl Vivo nanofat subcutaneously to the left and right side, respectively, of the back of 8-week-old male BALB/c nude mice (Beijing Vital River Laboratory Animal Technology Co., Ltd., China). The grafted tissues were harvested 2 days, 2 weeks, and 4 weeks' postinjection. In each time point, samples from five mice were harvested and then fixed with 10% formalin solution for 0.5–1.0 h and rinsed with PBS, for the following experiments. The sizes of grafted tissues harvested in 4 weeks were measured using liquid overflow method.

Hematoxylin and eosin staining and immunostaining of the grafted tissue

By embedding with optimal cutting temperature, the harvested tissue was frozen at −80°C overnight. Ten micrometers thick tissue sections were cut and placed in distilled water, dipped in alum-hematoxylin solution (for 1–2 min), washed twice with tap water (10 s each), dipped in 1% ammonia solution (for 5–10 s), and then stained with eosin, and dehydrated, cleaned, and mounted. For the immunostaining of the grafted tissue, the slides were hydrated and first stained with the primary antibody against perilipin, which was used at a 1:200 dilution (Cell signaling, USA). Staining with the secondary antibody, which was fluorescein isothiocyanate-conjugated goat anti-rabbit IgG used at a

1:200 dilution (ZSGB-Bio, China), was then performed. Coverslips were mounted with Fluoroshield containing DAPI (Sigma-Aldrich, Germany), to allow the visualization of the cell nuclei.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA, USA). Data are presented as a mean ± standard deviation (SD), and paired *t*-test was performed to test the differences, and the normal distribution of the data was tested. Statistical significance was defined as a two-tailed $P < 0.05$.

Results

Characteristics of Vivo nanofat

Macroscopy and microscopy of fresh isolated fat tissue were shown in Figure 1a and 1c. The enzyme-isolated Vivo nanofat could be smoothly injected using a 27G needle, as shown in the Video 1. The "gently" digested Vivo nanofat was golden sand-like and clearly floated on normal saline [Figure 1b], which was not observed after using the other methods of fat emulsion. Usually, 20 ml fat tissue could produce 10 ml Vivo nanofat. The size of the collected Vivo nanofat cell fraction was distributed from 10 to 70 μm [Figure 1c]. The dead cell ratio was 21% ± 18% for the Vivo nanofat cell fraction, while with that of the nanofat cell fraction was 61% ± 17% ($n = 4$, $t = 7.626$, $P = 0.005$) [Figure 1d].

Vivo nanofat contained more colony-forming cells

To determine which kind of nanofat contained more colony-forming cells, we seeded 0.5 ml nanofat and 0.5 ml Vivo nanofat onto 60 mm dishes. After culturing for 9 days, the number of colonies was counted. As shown in Figure 2a, 1% crystal violet staining showed that more colonies were formed in case of the Vivo nanofat group (38.67 ± 11.59), compared to the nanofat group (6.67 ± 1.53) ($n = 3$, $t = -4.918$, $P = 0.039$). To confirm whether these colony-forming cells were MSCs, we analyzed passage four of these colony-forming cells by flow cytometry. These cells expressed CD90, CD44, CD105, and CD73 but not CD45, CD11b, CD19, HLA-DR, and CD34 [Figure 2b]. Flow cytometry analysis of nanofat was shown in Figure 2e. Oil red O and alizarin red staining showed that these cells also could differentiate into adipocytes and osteoblasts after induction [Figure 2c and 2d].

High adipocyte survival after subcutaneous transplantation

To test whether the Vivo nanofat has an advantage in volumization, we transplanted 300 μl of nanofat or Vivo nanofat under the skin on the back of mice; the samples were harvested 2 days, 2 weeks, and 4 weeks' posttransplantation [Figure 3a and Supplementary Figure 1]. The size of the transplanted nanofat in 4 weeks was obviously smaller than that of Vivo nanofat (0.50 ± 0.17 cm vs. 0.81 ± 0.07 cm) ($n = 4$ in each group, $t = -5.783$, $P = 0.01$). Hematoxylin and eosin (H and E) staining showed that the transplanted nanofat caused tissue fibrosis, and barely any adipocytes were observed even on day 2 posttransplantation. On the other hand, in case of the transplanted Vivo nanofat, a large number of adipocytes were found to be alive on day 2, and the adipocytes on the outer layer survived even 2-week and 4-week posttransplantation [Figure 3b]. The immunofluorescent staining showed that only the adipocytes on the outermost layer of the nanofat graft were perilipin positive, while the number of perilipin-positive adipocytes on the outermost layer of the Vivo nanofat was much higher, and these adipocytes were not limited just to the outermost layer of the graft [Figure 3c].

Treatment of horizontal neck wrinkles with Vivo nanofat

The horizontal neck wrinkles are formed by the hyperkinetic activity and atrophy of the platysma muscle. To correct the

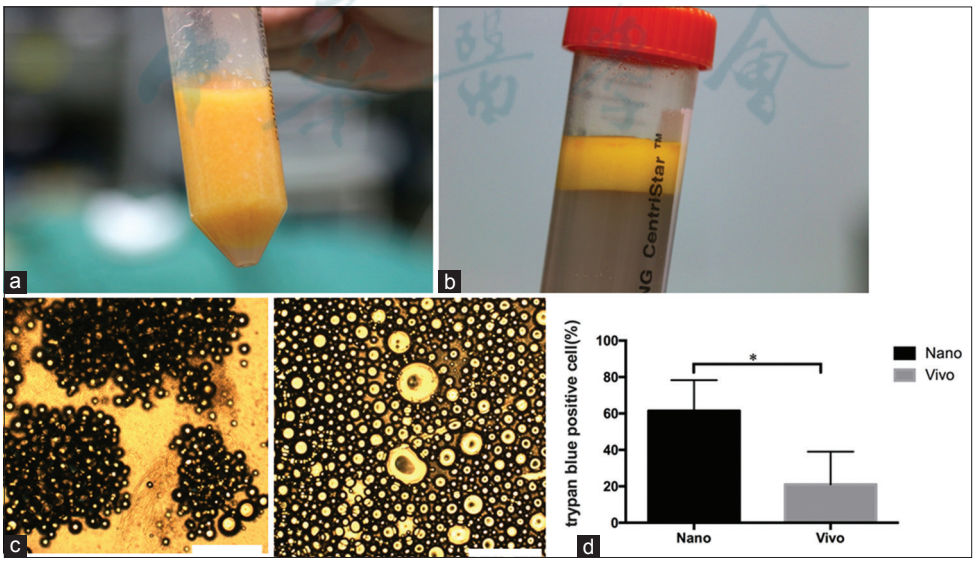

Figure 1: Characters of Vivo nanofat. (a) Macroscopy of the fresh isolated fat tissue. (b) Softly digested "golden sand-like" Vivo nanofat. (c) Microscopy of the fresh fat tissue (left) and Vivo nanofat (right). Bar = 500 μm. (d) Bar graph of trypan blue stain positive cells ($n = 4$, $t = 7.626$. *$P = 0.005$).

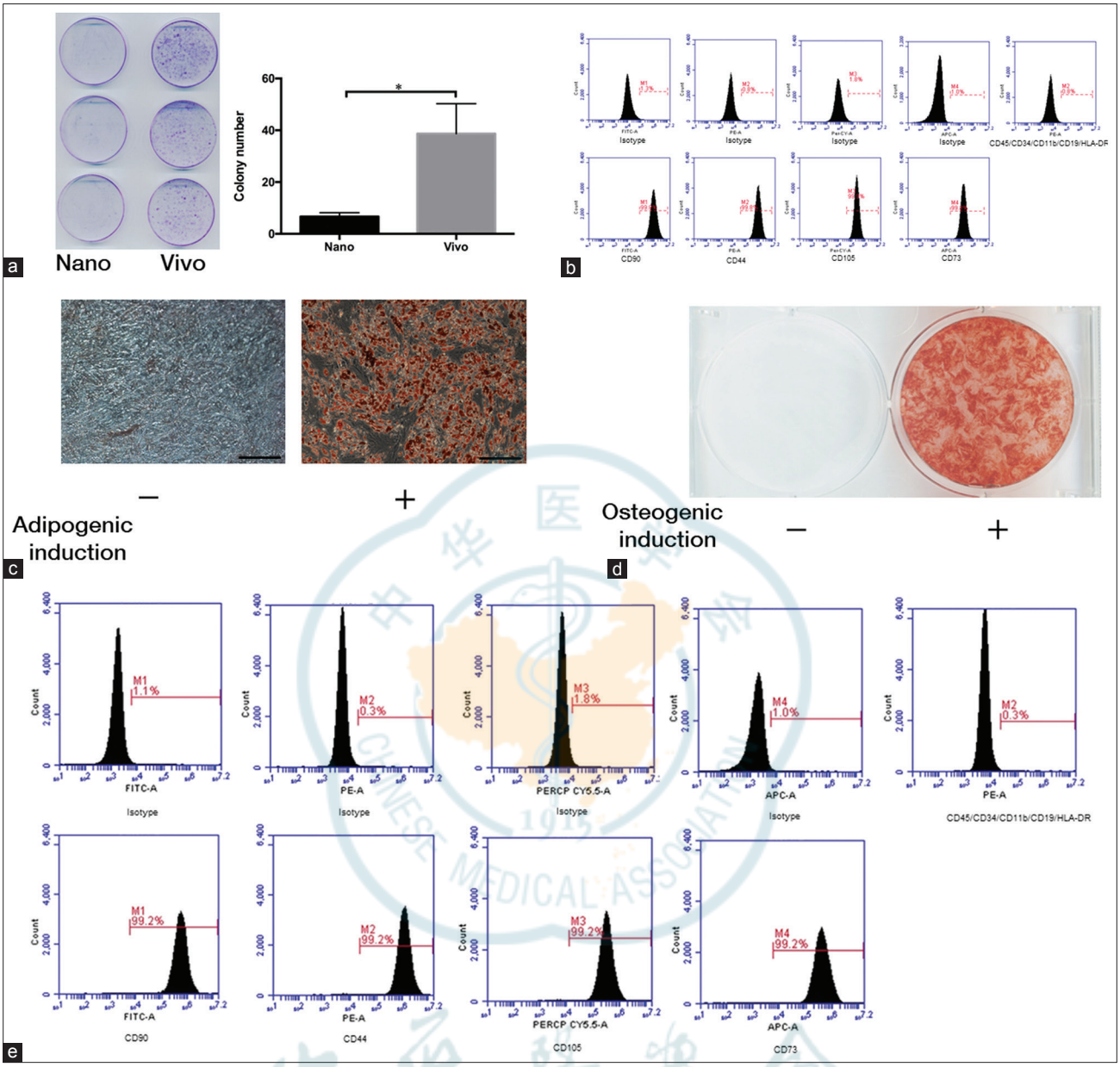

Figure 2: Vivo nanofat containing more multipotential colony-forming stromal cells. (a) Colony forming assay: the Vivo nanofat has more colony number formed ($n = 3$, $t = -4.918$, $*P = 0.039$). (b and e) Flow cytometry analysis showed passage 4 stromal cells in the Vivo nanofat (b) and nanofat (e) expressed the positive markers CD90, CD44, CD105, and CD73 of mesenchymal stem cells while did not express the negative markers such as CD45, CD34 CD11b, CD19, and HLA-DR. (c) Adipogenic induction. Oil red staining showed the adipocyte after induction (Bar = 10 μm). (d) Alizarin red staining showed the calcium node after induction.

neck wrinkles, we collected fat tissue and then softly digested it with collagenase I for 15–20 min at 37°C. This mixture was filtered using a strainer with a diameter of approximately 0.6 mm. A 1 ml syringe and a 27G needle were used to inject the Vivo nanofat into the dermal and subdermal areas of the neck wrinkles [Figure 4a-4d and Video 2]. Three patients had underwent this surgery, and when they were examined 2 weeks and 6 months after the treatment, the correction of the neck wrinkles was found to be stable [Figure 4e].

Discussion

In this study, we introduced a new, alternative method to generate the small viable adipocyte cell fraction, which could be smoothly injected using fine needles, which also contained a high number of colony-forming stromal cells with multi-differentiative potential. Nanofat, which was first reported by Tonnard, is fat tissue produced by mechanical emulsification.[5] It showed promising therapeutic effects in clinical application, due to its high stromal stem cell number and ability to be injected with a fine, sharp needle. However, in the traditional mechanical emulsification procedure, the viability of the adipocytes and the stromal stem cells was greatly destroyed, as seen in the colony-forming experiment and Mashiko *et al.*'s study.[9] In our study, we not only produced the Vivo nanofat fractions that were injectable with

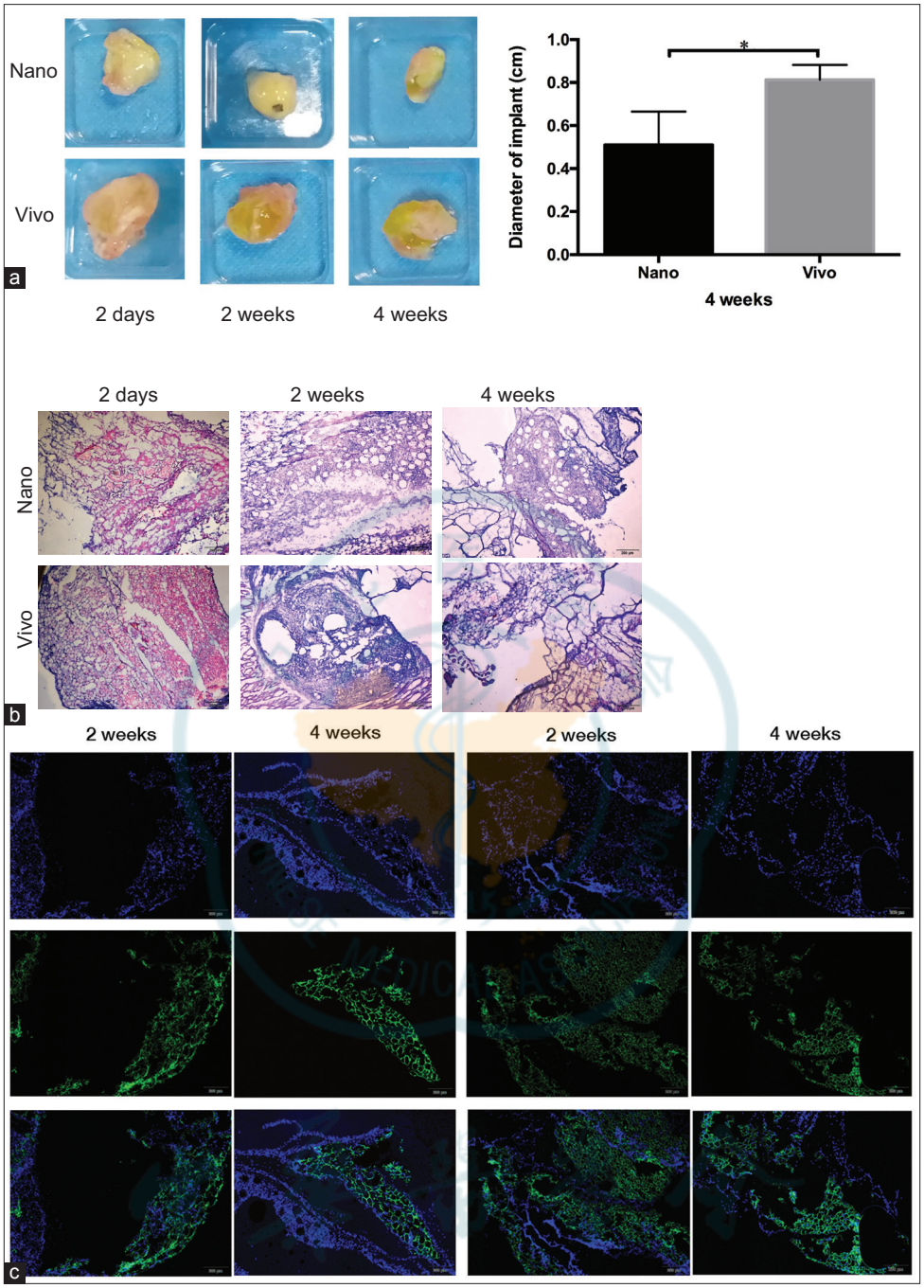

Figure 3: Less resorption in the Vivo nanofat transplantation. (a) Size changes of nanofat and Vivo nanofat after transplantation ($n = 4$ in each group, $t = -5.783$, $^*P = 0.01$). (b) Hematoxylin and eosin staining showed that the fibrosis changes of the nanofat after transplantation and more adipocyte survival in the implant (Bar = 200 μm). (c) Perilipin immunofluorescences staining showed that more viable adipocyte in the Vivo nanofat graft (Bar = 200 μm).

a fine needle but also greatly preserved the viability of both the adipocytes and the stromal stem cells. The MSC surface marker immunostaining and multi-differentiation results proved that these colony-forming cells in the produced fat fraction were multipotent stem cells.

In our study, we also showed that the viable nanofat cell fractions had good potential for volumization. The terminally differentiated adipocytes (big size) were fragile and easily destroyed by our enzyme digestion, while the small size adipocyte progenitors and stromal stem cells (small size) were able to survive for the use in the following experiments. In addition, the *in vivo* transplantation showed that the Vivo nanofat was not greatly absorbed after being subcutaneously transplanted into the back of the mice. H and E and perilipin immunofluorescences staining also showed that more viable adipocytes survived when the Vivo nanofat was used than when the nanofat was used. In our method, the concentration of collagenase I used was lower than that used for adipose stem cell isolation (normally

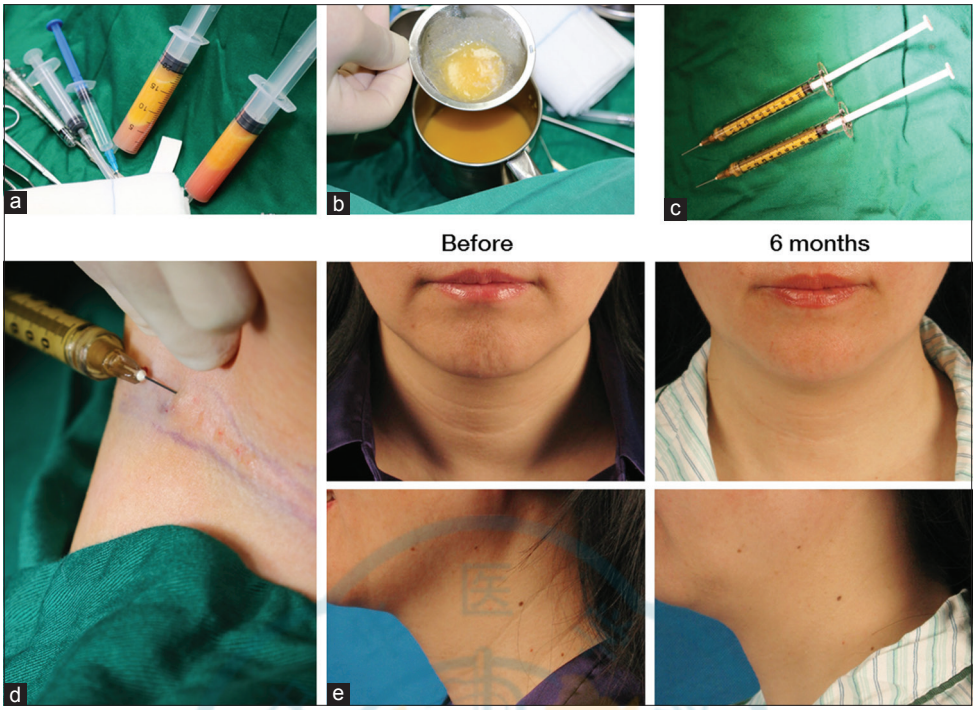

Figure 4: Vivo nanofat correction of the neck wrinkles. (a) Fat tissue after liposuctions. (b) Filtration of the "soft" digested Vivo nanofat. (c and d) Injection of the Vivo nanofat into the dermal and subdermal. (e) Six-month follow-up after Vivo nanofat treatment.

0.075%).[10] Besides, the digestion was done for a 15 min duration, which is only half of that used for adipose stem cells' isolation.

Neck wrinkles are caused by intradermal tissue defects, which could be improved by stromal stem cell fertilization or collagen re-volumization. The Vivo nanofat, containing both the viable stromal stem cells and the viable small size adipocytes, could correct the volume defect and stimulate soft-tissue regeneration at the same time. In our clinical cases, the correction of the neck wrinkles was found to be stable after a 6-month follow-up.

Our study highlights the importance of the Vivo nanofat in cosmetic surgery. However, the survival mechanism of the Vivo adipocytes and the stability of clinical correction after a longer follow-up period still need to be investigated in future research.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

Statement

We declare that our study results are repeatable.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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