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Research Article

Preparation of Nanofat Concentrate and Its Use for Hypertrophic Scar Treatment

Sheng-Hong Li¹, Yin-Di Wu¹, Ze-Hua Li¹, Xiao Jiang¹, Xuan Liao¹, Guang-Hui Xie¹, Li-Ling Xiao¹, Ting Wan², Hai-Ling Huang¹, Hong-Wei Liu^{1*}

¹Department of Plastic Surgery, the First Affiliated Hospital, Jinan University, Guangzhou, Key Laboratory of Regenerative Medicine, Ministry of Education, Institute for New Technologies Plastic Surgery of Jinan University, No. 613 West, Huangpu Avenue, Guangzhou, Guangdong, 510630, China

***Corresponding Author:** Hong-Wei Liu, MD, PhD, Department of Plastic Surgery, the First Affiliated Hospital, Jinan University, Guangzhou, Key Laboratory of Regenerative Medicine, Ministry of Education, Institute for New Technologies Plastic Surgery of Jinan University, No. 613 West, Huangpu Avenue, Guangzhou, Guangdong, 510630, China; Email: liuhongwei0521@hotmail.com

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Abstract

Objective: Nanofat grafting has been reported to improve tissue repair by the Stromal Vascular Fraction (SVF) of nanofat. However, the oil and liquid portions of fat tissues were released by mechanical emulsification during the nanofat procedure. Here, we reported a simple method to remove the oil and water for concentrating nanofat.

Methods: The concentrate of nanofat was obtained by processing nanofat using negative pressure produced by syringer and two-step centrifugation. The volume of oil and fat tissue as well as morphological changes of nanofat concentrate obtained by different procedures was examined. Meanwhile, the SVF numbers, the proliferation and differentiation capacity of Adipose-Derived Stem Cells (ADSCs) were determined. The clinical outcome of nanofat concentrate in treatment hypertrophic scar was evaluated.

Results: The centrifugation and negative pressure had no significant effect on the proliferation activity of the SVF in the concentrate of nanofat. The concentrated rate after 0, 15, 30 and 60 times of emulsification was 2.08 ± 0.042 , 2.63 ± 0.07 , 3.8 ± 0.01 and 8.2 ± 0.12 times greater than the original, respectively. The SVF number per 10 ml of fat in the above-mentioned groups

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were increased by 1.80 ± 0.15 , 2.68 ± 0.27 , 4.64 ± 0.20 and 3.44 ± 0.27 times, respectively. The capacity of cell proliferation and differentiation decreased significantly after more than 30 times emulsification. The concentrates of nanofat effectively improve the appearance and hardness of hypertrophic scar.

Conclusion: The nanofat concentrate prepared by our method include more SVF cells and less oil and might be more effective during clinical use.

Keywords

Adipose Tissue; Nanofat; Fat Grafting; Stromal Vascular Fraction; Stem Cell

Abbreviations

SVF: Stromal Vascular Fraction; CAL: Cell-Assisted Lipotransfer; ECM: Extracellular Matrix; ADSCs: Adipose-Derived Stem Cells; SEM: Scanning Electron Microscopy; DMEM: Dulbecco's Modification Of Eagle's Medium; CCK8: Cell Counting Kit 8; TGF: Transforming Growth Factor; PDGF: Platelet-Derived Growth Factor; ILGF: Insulin-Like Growth Factor; VEGF: Vascular Endothelial Growth Factor; A-SMA: Alpha-Smooth Muscle Actin

Introduction

Fat grafting has been widely used in clinical practice for numerous purposes, including treating wounds, tissue defects, scars and scleroderma, or for breast augmentation and facial fillings in cosmetics [1]. Fat transplanting has become increasingly popular because of the abundance of mesenchymal stem cells in fat tissues. a variety of methods have been studied to improve the effect of fat grafting, such as Cell-Assisted Lipo-transfer (CAL), which could improve the density of ADSCs of fat grafts [2].

Because Fat grafts have high content of water and oil and these components often cause inflammation, infection, poor graft survival, or absorption after transplantation. Therefore, many efforts have been made to remove these inactive substances from lipoaspirates using physical methods, retaining only the effective components to reduce adverse reactions while enhancing the therapeutic effects after transplantation [1,3]. In 2013, Tonnard, et al., introduced the concept of 'nanofat,' which refers to the substance obtained after several steps of emulsification and filtration of microfat [4]. Three different fat forms were named according to doctor Tonnard in that paper, such as "macrofat". Macrofat is harvested using a cannula with side holes of 2 mm × 7 mm; microfat is then harvested from this substance using a cannula with 1- mm diameter side holes. Subsequently, nanofat is obtained after the process of emulsification and filtration using a Luer Lock connector, which ensures that nanofat can be injected through a

27-gauge needle. Animal studies have shown that the dermis thickness of nude mice increased after the application of nanofat grafts [5]. However, Lo Furno D, et al., suggested that many ADSCs may be damaged during this procedure, thereby affecting the survival rate of nanofat grafts [6]. Additionally, there was no evidence of complications during the fat transplantation process, such as fat necrosis, fat embolism, or sepsis. Such complications are less frequent in the process of nanofat transplantation [7-9].

Nanofat grafting has been approved for treating scars, wrinkles and skin discolorations [10]. In order to achieve better clinical outcomes, Lu, et al., extracted a form of fat in 2017 and called it as adipose extracellular matrix/SVF gel. The Extracellular Matrix (ECM)/SVF gel was prepared by a series of procedure such as the microfat was shifted in a stable speed and the oil was collected, mixed and finally discarded [11]. Their subsequent experiments on animals showed that ECM/SVF gel can repair wounds more rapidly than common fat. However, the method proposed by that study is too complex and it's difficult for us to control the shifting strength, by the way its application scope is limited, due to the small remaining volume of fat.

In the present study, a simple method for concentrating nanofat graft was proposed. Briefly, the obtained microfat was collected after emulsification, using a Luer Lock connector with different apertures and the remaining fat is nanofat concentrate. The present study describes a simple method for concentrating nanofat for fat transplantation surgery.

Material and Methods

Cases Information

The microfat used in this study was collected from clinical liposuction patients, who signed an informed consent form. Between September 2016 and July 2018, twelve patients were recruited. Nanofat concentrate was injected into scar tissue for treating hypertrophic scar and clinical efficacy had been evaluated.

Fat Concentration

After obtaining approval from the Institutional Review Board of Medical Science, Jinan University and a signed consent to participate. The liposuction site was located at the lower abdomen, thighs and knee. Topical infiltration anesthesia was performed using the tumescent anesthesia solution (25 ml of 2% lidocaine + 2 mg of adrenaline + 12.5 ml of 8.4% sodium bicarbonate + 1,000 ml of normal saline). A side-opening liposuction needle with an inner diameter of 1-2 mm was inserted into the subcutaneous fat tissue, a 20 ml syringe was connected and subcutaneous fat was extracted using the syringe liposuction technique.

Microfat is then harvested from this substance using a cannula with 1- mm diameter side holes (Tulip Medical Products, San Diego, CA, USA) was used for aspiration. The microfat were prepared followed by the description of doctor Tonnald.

Then the microfat was centrifuged at 2000× g for 3 min. After centrifugation, the underlying swelling fluid was removed. The nanofat was processed by emulsifying the microfat between two 10-ml syringes connected to each other by a Luer Lock connector (Tulip, San Diego, Calif) with a diameter of 2.4 mm and 1.2 mm. The microfat was divided into four groups: the control group, emulsified 15 times group (Luer Lock connector with diameter of 2.4 mm), emulsified 30 times group (Luer Lock connector with diameter of 2.4 mm) and emulsified 60 times group (Luer Lock connector with diameter of 2.4 mm for 30 times and 1.2 mm or 30 times).

The obtained nanofat of 60 times group was finally filtered by a 500-mesh. Then, 10 ml of nanofat was placed in two 10-ml syringe connectors, while the two syringes were pulled back to 10 ml-scale to create negative pressure and the nanofat was pushed back and forth for 3 times and then mixed once. The fat was centrifuged again at 2000×g for 3 min. The middle layer that formed was referred to as nanofat concentrate. The lower liquid layer was removed and the large amount of oil droplets in the upper layer was discarded. Fat concentration fold = volume of microfat / volume of nanofat concentrate we obtained after centrifugation (n = 6).

SVF Isolation

SVF was extracted by partly referring to the method of Bura et al. and was divided into the following steps: 1% type I collagenase (Gibco, USA) was digested and centrifuged to collect the underlying cells pellet. After being filtered through a 200-mesh filter, the red cells were removed using 0.3% sodium chloride [13]. Finally, the cells pellet was re-suspended in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum and a small portion of the cells were removed for cell counting.

SEM to Observe Fat Tissue

After being fixed with 2.5% glutaraldehyde for 3 hours, the sample was rinsed 2-3 times with 0.1 mol/L sodium cacodylate buffer (pH 7.4) and the tissue was placed in a buffer solution overnight at 4°C. On the second day, the buffer was removed, 1% citric acid was added for 1 h, the fixative was discarded and the buffer was repeatedly rinsed 5 times for 15 min. Gradient ethanol dehydration (alcohol concentration 50%, 70%, 90% and 100%) was repeated for 3 times for 15 min each and isoamyl acetate replacement was repeated 2 times: the first 5 ~ 10 min, the second > 30 min. Vacuum dried IB-3 ion plating was observed by scanning electron microscope (S-3500N, Japan) [14].

Cell Proliferation Assay

Cell proliferation assay was assessed by a Cell Counting Kit 8 (CCK8) assay (Dojindo, Japan). SVF of microfat and four groups were plated in 96-well plates at 1×10^4 cells/well (n=6), after

which 10 μ L of CCK8 solution were added to each well for 2 hours. Finally, the absorbance was measured at 450 nm [15].

Cell Differentiation Assay

Four groups of SVF were prepared by plating 1×10^4 cells in a 96-well plate (n=6) and each well was incubated with adipogenic and osteogenic differentiation medium (Gibco, USA) based on the manufacturer's instructions. After an incubation period of 14 days, osteogenic differentiation was prepared through a semi-quantitative analysis method using alizarin red staining, followed by decolorization with 10% cetylpyridinium for 30 min and detection of absorbance at 562 nm using a microplate reader to perform semi-quantitative analysis [16,17]. The semi-quantitative analysis on adipogenic differentiation using the isopropanol decolorization method is briefly summarized as oil red staining, followed by decolorization with 100% isopropanol for 1 hour. The absorbance at 540 nm was measured on a microplate reader [18].

Statistical Analysis

All values are reported as mean $\pm$ SD (n=6). Statistical analysis was performed using SPSS 13.0. The way analysis of variance test was first used, followed by Tukey-Kramer's post hoc test. A P-value less than 0.05 was considered to indicate statistical significance. A P-value of less than 0.01 was considered to indicate a large degree of statistical significance.

Result

SVF proliferation capacity between microfat and control group

The CCK8 assay showed that there was no significant difference between the two groups (Fig. 1).

Fat Concentration Rate

The amount of oil and liquid removed varied between the groups. The concentrated folds of the control group were 2.08 ± 0.042 times of untreated microfat (Fig. 2,3). The concentrated folds of fat gradually increased as the frequency of mechanically-emulsified fat tissue increased. The concentrated rate after 15, 30 and 60 times of emulsification was 2.63 ± 0.07 , 3.8 ± 0.01 and 8.2 ± 0.12 times greater than that of the control group, respectively (Fig. 2,3). The SVF number change per 10 ml of fat ($\times 10^5$) in the above-mentioned groups was 1.80 ± 0.15 , 2.68 ± 0.27 , 4.64 ± 0.20 and 3.44 ± 0.27 , respectively (Fig. 2, 3).

ECM of Nanofat Concentrate

As a whole, the degree of structural damage to fat tissues increased as the times of emulsification increased. Adipocytes were abundant on the surface of the sample, on which cell membrane-like substances were faintly visible. There were slight differences in the morphology and arrangement of adipocytes among these groups, of which the groups of concentrated rates after 15, 30 and 60 times emulsification were relatively different from the control group. In other words, the fat adipocytes were arranged closely and their morphology was destroyed to various degrees, the most obvious of which was the 60 times group. Fat cells were damaged during fat emulsification, resulting in changes in the entire fat structure, with only minor changes observed in the control group (Fig. 4).

SVF proliferation capacity of different groups

The results of the CCK8 assay showed that compared with the control group, the ability to proliferate on the 7th, 9th and 11th day in the lower 15 groups was higher than that in the control group ($p < 0.05$). There was no significant difference after the 3rd day between the 30 times group and the control group ($p > 0.05$) and the proliferation ability was lower in the 60 times group from day 3-11 than in the other three groups ($p < 0.05$) (Fig. 5).

SVF differentiation capacity of different groups

The capacity of cell differentiation was significantly lower after 30 and 60 times emulsification. The adipogenic capacity of the 15 groups under treatment was higher than that of the control group, however, without a significant difference ($p > 0.05$). The next goes the 30 times and 60 times group ($p < 0.05$). The strength of osteogenic differentiation was highest in the control group, followed by the 15, 30 and finally the 60 times groups. There is no difference in the strength of osteogenic differentiation between the control group and the 15 times group. The adipogenic differentiation ability of the 30 times group and the 60 times group were lower than that of the control group (Fig. 6).

Case Reports

Case 1: A 16-year-old woman was worried about the flexibility of her five fingers after skin damage from a hand burn (Figure 7A). Her left hand was injected subdermally using a 26-gauge needle with 12 ml of nanofat concentrate (30 times). Six months after the injection, the treated area appeared looser around the scar and rosier in color (Fig. 7). The result of color Doppler ultrasound showed that the skin thickness of the left hand ulnar wrist was changed from 2.7 mm to 2.1 mm.

Case 2: A 26-year-old woman was examined for treatment of a facial hypertrophic scar and was worried about the itching feeling it brought. A volume limitation was included of traditional fat treating (Fig. 8). Nanofat concentrate (60 times) grafting is the best choice for hypertrophic scars. Thus, 1 ml of nanofat concentrate (60 times) was subdermally injected using a 27-gauge needle into the scar and 0.3 ml of nanofat concentrate was used for intradermal injection into the scar tissue. Fifteen months after injection, the hypertrophic scar was smaller and more flattened and the patient reported the disappearance of the skin-itching symptom (Fig. 8).

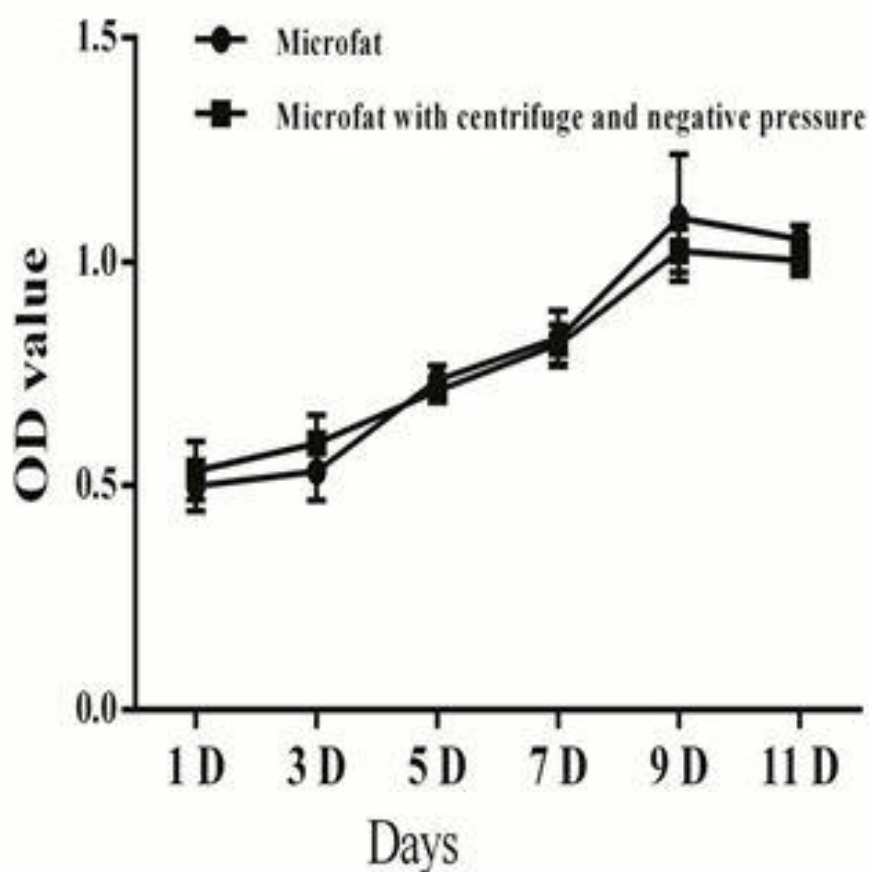


Figure 1: The CCK8 assay detected SVF proliferation capacity in the microfat group and the control group. No significant difference between these two groups was found. * $p < 0.05$.



Figure 2: A: (Above) Oil and liquid removal volume after concentrated procedure in the control group, in which the middle layer (fat layer) is obtained, and the following shows the SVF extracted from 10 ml of fat in the control group. B: (above) Fat remain ratio of the 15 times group. Oil and liquid were removed by centrifugation; the middle layer is the fat layer. (Below) SVF extracted from 10 ml of fat in the 15 times group. C: (Above) Removal of oil and liquid in the 30 times group; the middle layer is the fat layer for actual application. (Below) SVF isolated from 10 ml of fat from the 30 times group. D: (above) 60 times group in which oil and liquid are removed after being concentrated, and the middle layer is the clinical use of the fat layer. (Below) SVF isolated from 10 ml of fat from the 60 times group.

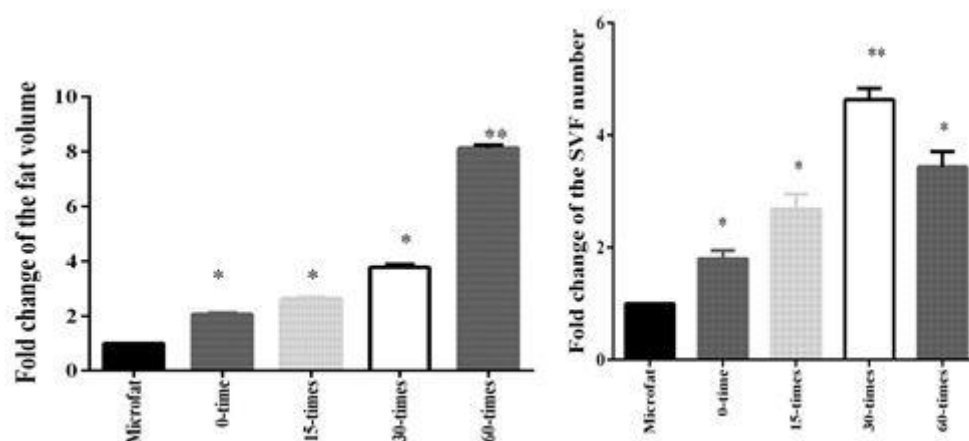


Figure 3: A: (Left Side) The fold change of fat volume of control group, 15, 30, and 60 times of emulsification group was 2.08 ± 0.042 , 2.63 ± 0.07 , 3.8 ± 0.01 and 8.2 ± 0.12 times greater than the microfat; B: (Right Side) Fold change of SVF per 10 ml of fat in the above-mentioned groups was 1.80 ± 0.15 , 2.68 ± 0.27 , 4.64 ± 0.20 , and 3.44 ± 0.27 , respectively.

* $P < 0.05$, ** $P < 0.01$.

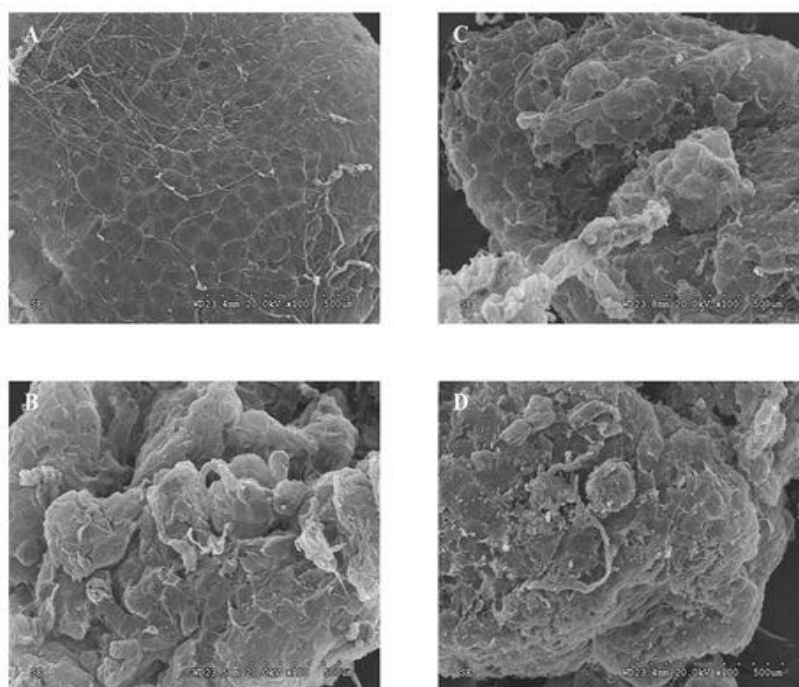


Figure 4: A: Structure of fat tissues in the control group observed using Scanning Electron Microscopy (SEM). B: Structure of fat tissues in the 15 times group observed by SEM. C: Structure of fat tissues in the 30 times group observed under SEM. D: Structure of fat tissues in the 60 times group observed under SEM. The results show that with increasing treatment time, the gross morphology of adipocytes breaks up.

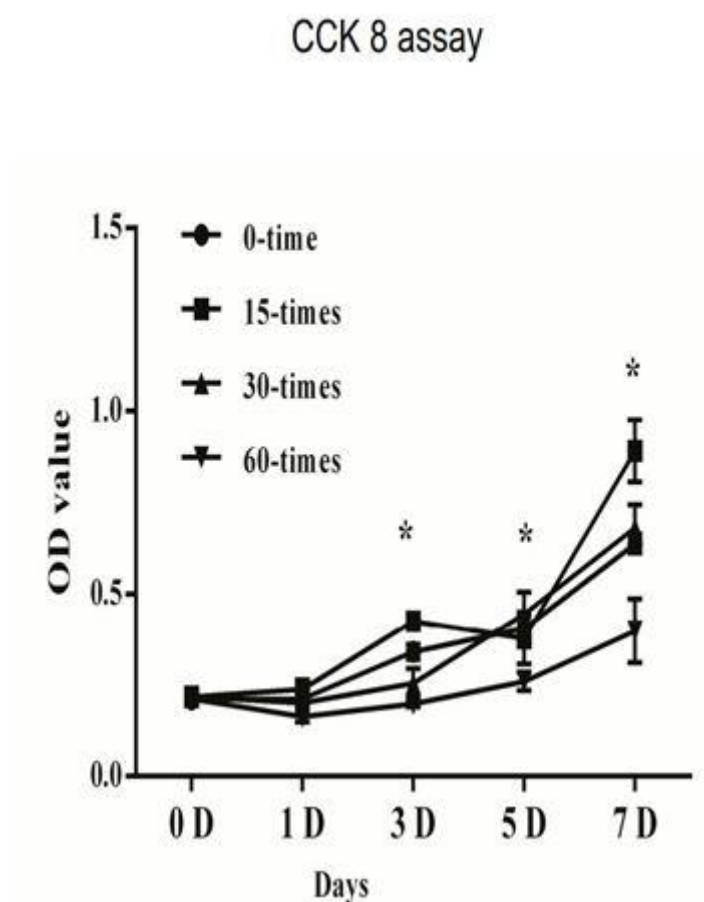


Figure 5: The CCK8 assay detected SVF proliferation capacity in various groups. The results showed that there was no significant difference between the 30 times group and the control group after three days, and the 60 times group showed lower proliferation ability from day 3-11 than the three other groups. * $p < 0.05$.

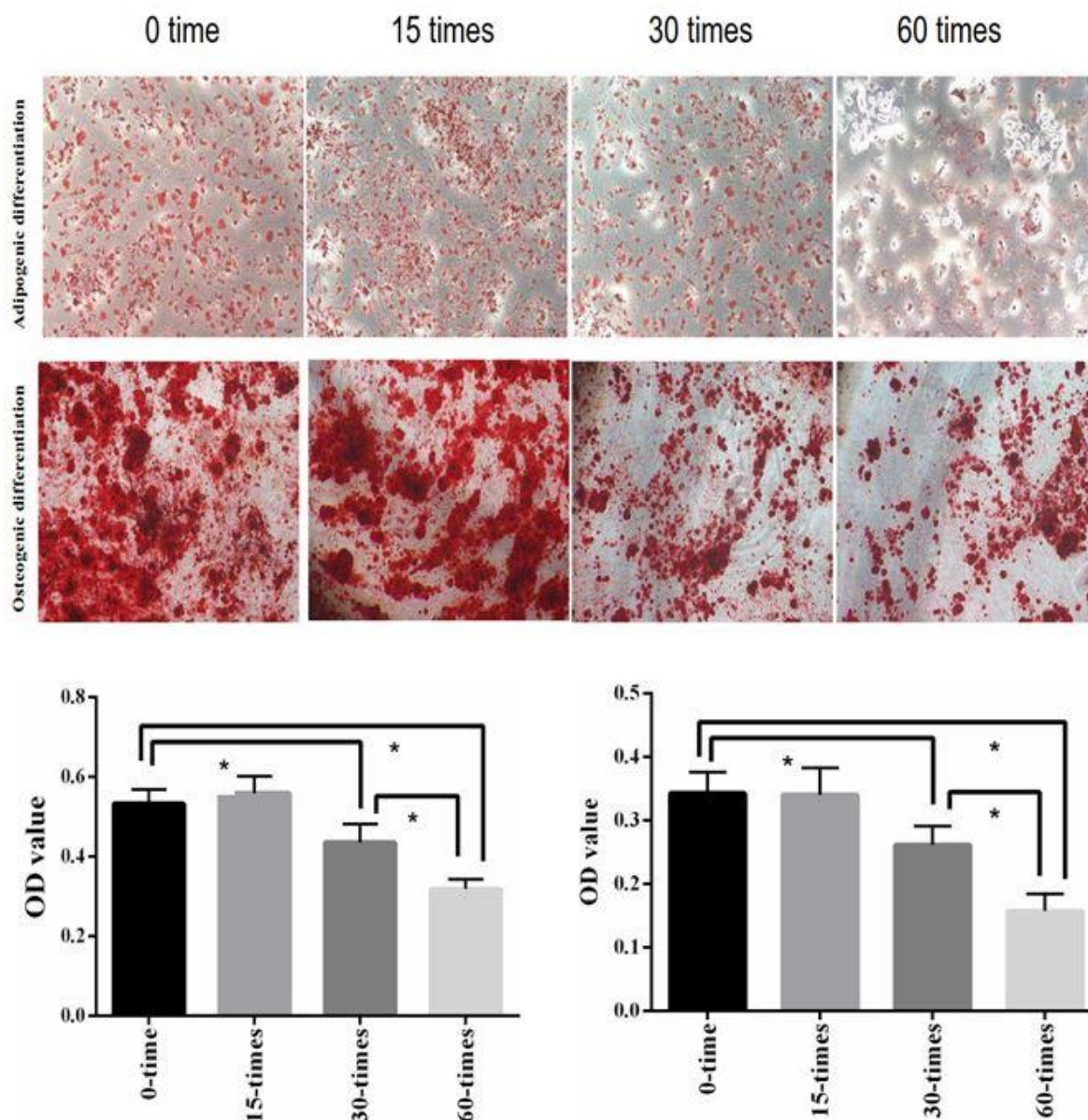


Figure 6: A: The microscopic images of SVF from four groups cultured in adipogenic culture medium or osteogenic differentiation for 14 days, followed by staining using oil red O or alizarin red. B: After staining with oil red O, the cultured plate were decolorized with 100% isopropanol, followed by measurement of the absorbance at 540 nm in the case of four sets of absorbance. C: After staining the induced cells with alizarin red, the cells were decolorized using 10% methylpyridinium, followed by the measurement of absorbance at 562 nm to compare the differences in the ability of each group of cells after osteogenic differentiation. The results showed that there was no significant difference between the 15 times group and the control group, and the ability of adipogenic differentiation in 30 times and 60 times group was weaker than that in control group. The order of osteogenic differentiation ability was as follows: the control group, 15 times group, 30 times group, and

60 times group, respectively, but the difference between the control group and cut 15 groups was not statistically significant $*p<0.05$, $**p<0.01$.

Cases 1: Skin thickness



Figure 7: A: Burn-damaged hand and forearm skin in a 16-year-old woman with skin pigmentation and activity limitation. B: Six months after the injection of 12 ml of nanofat concentrate in a subdermal level in the hand. Note the color change of hand skin and flexibility. The skin thickness of the left hand ulnar wrist was changed from 2.7 mm to 2.1 mm.

Cases 2:

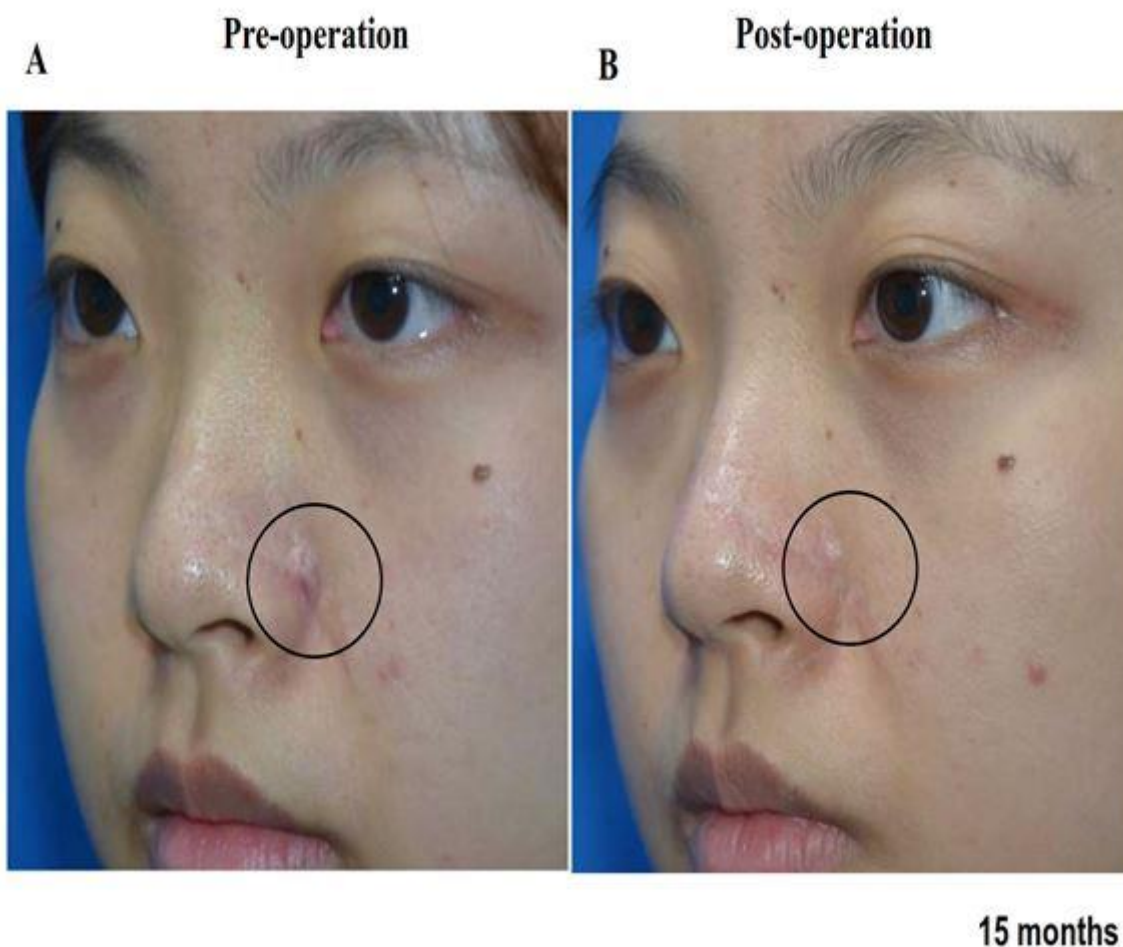


Figure 8: A: 26-year-old woman with a facial hypertrophic scar was not eligible for a traditional fat injection. B: Result 15 months after the subdermal injection of 1 ml of nanofat concentrate (60 times) and 0.3 ml of nanofat concentrate was used for intradermal injection into the scar tissue. Note the smaller and more flattened aspect of the scar, the patient also reported the disappearing of the skin itching symptom.

Discussion

Nanofat grafting is a promising new treatment method, due to its regenerative function and the smaller diameter of the fat particles, which can be injected with a thinner needle. The methods to improve the lifespan of fat transplantation mentioned above remain highly controversial. In general, CAL technology is widely applied, but the extraction of ADSCs with a relatively long lifespan, without fully confirmation of the use of collagenase, etc. Then, so many scholars began to focus on fat concentration. In 1994, Coleman, et al., performed fat-free decontamination after centrifugation at $3400\times g$ for 3 min to achieve the purification of fat [19].

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In 2017, Lu Feng, et al., also extracted a form of fat, which they called ECM/SVF-gel [10]. Animal experiments have found that SVF-gel glue can heal wounds more rapidly than common fat; however, this is a complicated process which takes a long period.

Based on the negative pressure method, we obtained the nanofat concentrate by removing most of the liquid and oil. The nanofat concentrate showed a homogeneous and stable morphology, hence it may be satisfactory in short-term clinical results. Compared with other methods of oil and liquid removal, this method has numerous advantages, such as the simplicity of the procedure and the reduced risk of pollution. The nanofat concentrate folds change varies from group to group, which enables us to select different treatments according to different symptoms and injuries. For instance, microfat could be injected by a 23-gauge sharp needle and subsequently, fat tissue in the 15 times group could be injected by a 24-gauge sharp needle [2]. Additionally, fat in the 30 times group could be injected via a 26-gauge sharp needle. The adipose tissue of the 60 times group could easily be injected through a 27-gauge sharp needle. For example, fat of the 15 times group had higher volume retention and more SVF compared with the microfat and could be injected using a smaller needle, which is generally acknowledged an advantage among doctors and patients. Although the fold change of fat concentration of the 30 times group is lower than that of the 60 times group, the proliferation of SVF is higher, which may be because the emulsification process under 60 times destroys the integrity of SVF, thus reducing the number of cells. However, the specific mechanisms behind this change need to be further studied. However, the biggest advantage of the 60 times group is that when treating hypertrophic scars, due to its large tension, a small volume of concentrated fat and multi-SVF may be more effective.

During our clinical application process, we were surprised to find that nanofat concentrate could be used for scars, improvement of skin color, flexibility of joints in the hand and decreasing scar area, which aided in improving the patients' skin appearance and functional problems [20]. However, the exact mechanisms behind these therapeutic results remain unknown. Chong Hyun Won, et al., suggest that ADSCs might promote the release of multiple growth factors, such as insulin-like growth factor-1 and Vascular Endothelial Growth Factor D (VEGF-D), which could promote angiogenesis of tissue [21]. It is now common knowledge that nanofat concentrate contains a high number of SVF. Qi Zhang, et al., found that ADSCs could improve the appearance of rabbit ear hypertrophic scars by decreasing the Alpha-Smooth Muscle Actin (α -SMA) and collagen type I gene expression [22]. Further research is needed to confirm our findings; for example, there was not enough data to estimate the optimum amount of fat cells to be obtained. Animal experiments have not been conducted to verify the effect of different treatment times on long-term fat survival and there are not enough clinical cases to confirm these findings.

Conclusion

Nanofat concentrate is based on the removal of excess liquid and oil and SVF in nanofat concentrate is also increased compared with nanofat. Different dispose times, different remain

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volume and SVF number, in which the 60 times group has the largest SVF number, but the SVF activity was slightly poorer than that of the control group and the 15 times group. SVF isolated from the 60 times group was poor, but its fat could easily pass through a 27-gauge needle (i.e., the needle of a 1 ml syringe), which can easily achieve an accurate injection. This provides a new direction for fat transplantation in the future. Furthermore, specific laboratory research and tracking of the clinical applications of this method are also very important.

Authors' Contributions

Sheng-Hong Li and Yin-Di Wu: provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing; Ze-Hua Li, Xiao Jiang, Xuan Liao, Guang-Hui Xie, Ting Wan: collection and/or assembly of data; Li-Ling Xiao and Hai-Ling Huang: financial support, administrative support; Hong-Wei Liu: conception and design, data analysis and interpretation, manuscript writing, and final approval of the manuscript.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The procedure was approved by the Institutional Review Board of Medical Science, Jinan University (Approval Number: 2017-018). Registered 04 September 2017, <https://lckj.jnu.edu.cn/pages/llwyh/default.aspx>.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for Publication

All consent for publication files was included in its supplementary information files.

Competing Interests

The authors declare that they have no conflicts of interest to disclose.

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