



Injectable adipose-derived matrix film fragments for soft tissue filling: effects of dilution on retention and vascularization

Shikun Cao^{1,#}, Mengmeng Hou^{2,#}, Yixuan Yu^{2,#}, Yajie Guo², Yiyue Yang^{2,3}, Jun Zhu⁴, Rui Chen⁵, Chenggang Yi⁶, Huichen Li^{1,2}

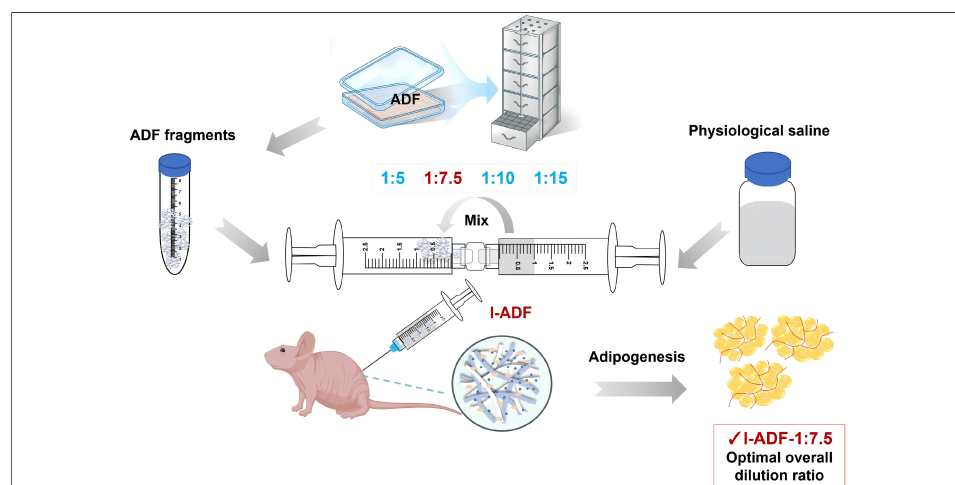
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Abstract

Aim: To compare four dilution ratios of injectable adipose-derived matrix film fragments (I-ADF) and identify the optimal overall dilution ratio based on qualitative extrusion, volume retention, adipose tissue formation, and vascularization.

Methods: Cryopreserved ADF was cut into fragments and mixed with 0.9% saline at 1:5, 1:7.5, 1:10, or 1:15 (w/v). Forty female nude mice were randomly allocated to four formulation groups ($n = 10/\text{group}$), with five animals per group assigned to each terminal time point (weeks 4 and 12). Qualitative extrusion through a 17-gauge needle, implant volume retention, peripheral and central adipose tissue formation, and total and α -smooth muscle actin (α -SMA)-associated vessel densities were evaluated.

¹Department of Plastic Surgery, The Second Affiliated Hospital of Soochow University, Suzhou 215005, Jiangsu, China.

²Department of Plastic Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi, China.

³Department of General Surgery, The Second Affiliated Hospital of Shaanxi University of Chinese Medicine, Xianyang 712000, Shaanxi, China.

⁴Department of General Surgery, The Southern Theater Air Force Hospital, Guangzhou 510010, Guangdong, China.

⁵Health Examination Center, The First Affiliated Hospital of Northwest University, Xi'an 710069, Shaanxi, China.

⁶Department of Plastic Surgery, The Second Affiliated Hospital of Zhejiang University College of Medicine, Hangzhou 310000, Zhejiang, China.

#These authors contributed equally to this work.

Correspondence to: Prof. Chenggang Yi, Department of Plastic Surgery, The Second Affiliated Hospital of Zhejiang University College of Medicine, Hangzhou 310000, Zhejiang, China. E-mail: yichg@zju.edu.cn; Dr. Huichen Li, Department of Plastic Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, Shaanxi, China. E-mail: lihuichen@fmmu.edu.cn

Results: All formulations passed through the needle. The 1:5 and 1:7.5 formulations produced more continuous and visually homogeneous extrudates than the more dilute formulations. At week 12, volume retention was $86.86\% \pm 3.65\%$ for 1:5 and $88.26\% \pm 3.98\%$ for 1:7.5, compared with $50.44\% \pm 6.22\%$ for 1:10 and $41.78\% \pm 10.52\%$ for 1:15. Peripheral adipose tissue formation was similar among groups. The 1:7.5 formulation showed the greatest central adipose-like area on Masson's trichrome staining ($60.50\% \pm 6.31\%$) and the greatest central Perilipin-positive area ($59.94\% \pm 4.28\%$); for both endpoints, $P < 0.001$ vs. the other formulations. The 1:7.5 group also had the highest mean central α -SMA-associated vessel density.

Conclusion: Among the four formulations tested, I-ADF-1:7.5 emerged as the optimal overall dilution ratio under the present experimental conditions in this nude mouse model and warrants further controlled preclinical evaluation.

INTRODUCTION

Soft tissue defects, atrophy, and contour deformities remain common challenges in plastic and reconstructive surgery. Although autologous fat grafting is widely used for aesthetic and reconstructive soft tissue correction, postoperative resorption, technique-dependent outcomes, and local complications remain important clinical concerns^[1-4]. Vascularization is critical for long-term graft survival and volume retention^[5]. Consequently, there is a pressing need for more reliable, minimally invasive soft tissue fillers with regenerative potential.

Extracellular matrix (ECM)-based biomaterials have emerged as promising alternatives for soft tissue filling and regeneration. Decellularized adipose tissue has also been developed into injectable forms^[6], as adipose ECM retains native components, including collagens, glycosaminoglycans, and bioactive proteins, that may support adipogenesis and angiogenesis^[6]. Decellularized ECM derived from various sources, including human dermis^[7], porcine dermis^[8], bovine pericardium^[9], and porcine intestinal submucosa^[10], has been successfully applied in breast reconstruction, wound healing, and volumetric augmentation. More recently, injectable ECM products have gained particular attention due to their minimally invasive delivery and ease of use. For example, injectable acellular dermal matrix formulations have been investigated for breast reconstruction and facial soft-tissue augmentation^[11,12]. Preclinical studies and reviews indicate that injectable adipose ECM scaffolds can support host cell infiltration, adipogenic differentiation, and neovascularization^[13-15]. Manufacturing protocols for injectable human adipose-derived hydrogels have also been described^[16]. However, most of these injectable ECM products are derived from allogeneic or xenogeneic sources, which carry potential risks of immune rejection, pathogen transmission, and batch-to-batch variability^[17-19]. Furthermore, they often require complex decellularization processes that may compromise ECM bioactivity and structural integrity^[17-19].

Patient-derived ECM represents an attractive regenerative material because it may reduce donor-mismatch-related concerns while preserving tissue-specific bioactive components. In our previous study, we developed an autologous adipose-derived matrix film (ADF) using a simple, papermaking-inspired physical method^[20]. Compared with allogeneic or xenogeneic products, ADF is intended to be prepared from patient-derived adipose tissue and may therefore reduce donor-mismatch-related immune concerns in an autologous clinical setting. Notably, ADF retained excellent biological activity and mechanical strength even after prolonged cryopreservation at -80°C , enabling the establishment of an "ECM bank" for personalized medicine. Proteomic analysis further demonstrated that the protein composition of ADF remained remarkably stable after freezing, with no significant degradation of key ECM components such as collagen I, collagen III, and fibronectin. Moreover, ADF showed favorable regenerative performance in wound healing and soft tissue

regeneration, partly through the promotion of vascularization^[20]. These findings provided the basis for further developing ADF as a patient-derived ECM platform^[20].

Despite these advantages, the membrane morphology of ADF limits its direct application in injectable procedures, which are preferred in clinical practice. To improve its applicability for minimally invasive delivery, we converted ADF into an injectable form by cutting the membrane into small fragments and mixing them with physiological saline. This formulation, termed injectable ADF fragments (I-ADF), extends the previously established ADF platform to syringe-based delivery while aiming to retain its structural and biological characteristics.

However, the effect of the dilution ratio between ADF fragments and saline on *in vivo* performance remains unknown. Specifically, it remains unclear how different ratios affect qualitative extrusion behavior, volume retention, adipose tissue regeneration, and vascularization after subcutaneous implantation. Identifying the optimal overall dilution ratio is important for subsequent controlled preclinical evaluation. Overly dense formulations may restrict cell infiltration and tissue ingrowth due to limited porosity, while overly dilute formulations may lack sufficient ECM concentration to induce effective regeneration^[21]. Moreover, the relationship between ECM concentration and regenerative outcomes may not be linear, and the optimal balance between structural support, cellular accessibility, and host-material interactions requires systematic investigation^[21,22].

Therefore, the present study aimed to prepare I-ADF at four dilution ratios (1:5, 1:7.5, 1:10, and 1:15, w/v). We evaluated their qualitative extrusion behavior through a 17-gauge needle and compared their volume retention in a subcutaneous injection model in nude mice. Peripheral and central adipose regeneration were assessed separately. Angiogenesis and its correlation with adipose regeneration were also evaluated. We hypothesized that an intermediate dilution ratio would provide a favorable balance among volume retention, tissue formation, and qualitative extrusion behavior, and the resulting findings could provide a basis for further controlled evaluation of I-ADF as an injectable, patient-derived soft tissue filling strategy.

METHODS

Preparation of I-ADF

ADF was prepared according to our previously published protocol^[20]. The precursor material was derived from abdominal adipose tissue obtained by liposuction from multiple female donors aged 25-35 years. Eligible donors were women aged 25-35 years undergoing abdominal liposuction who provided written informed consent for tissue use; donors with positive screening results for infectious pathogens were excluded. Tissue processing began immediately after liposuction. Adipose tissue from eligible donors was pooled to prepare a single precursor ADF batch. The exact number of contributing donors was not retained in the available source records. Human adipose tissue collection was approved by the Ethics Committee of the Fourth Military Medical University (No. KY20243548-1), and all procedures were conducted in accordance with the Declaration of Helsinki.

The pooled adipose tissue underwent sequential crushing, washing, and mechanical pressing to form ADF, which was cryopreserved at -80 °C for 12 months. After thawing, the ADF was manually cut into fragments using sterile surgical scissors. Fragments from the same cryopreserved batch were pooled, thoroughly mixed, and aliquoted before physiological saline was added. The aliquots were mixed with sterile physiological saline at four weight-to-volume ratios: 1:5, 1:7.5, 1:10, and 1:15 (w/v), designated as I-ADF-1:5, I-ADF-1:7.5, I-ADF-1:10, and I-ADF-1:15, respectively. Fragment dimensions were not quantitatively standardized or measured, and the fragment-size range and mean particle size were therefore unavailable. All formulations were prepared within 2 h of use.

Qualitative extrusion assessment

Each freshly prepared formulation was loaded into the same type of syringe and manually extruded through a 17-gauge needle under the qualitative test conditions used in this study. Successful passage through the needle, continuity and visual homogeneity of the extrudate, and visible water exudation or phase separation were documented photographically. Fragment size, injection force, extrusion rate, viscosity, storage modulus, and loss modulus were not quantitatively measured. Therefore, the assessment was restricted to qualitative extrusion behavior under the specified 17-gauge test conditions and should not be interpreted as a quantitative comparison of injectability.

Histological analysis

Implant samples were fixed in 4% paraformaldehyde for 24 h, dehydrated through graded ethanol, cleared in xylene, embedded in paraffin, and sectioned at a thickness of 5 μm .

For hematoxylin and eosin (H&E) staining, the sections were deparaffinized in xylene, rehydrated through graded ethanol, stained with hematoxylin for 8-15 min, differentiated in 1% hydrochloric acid alcohol, blued in running water, and counterstained with eosin for 2-5 min. The sections were then dehydrated, cleared in xylene, and mounted.

For Masson's trichrome staining, deparaffinized sections were treated with potassium dichromate, stained with Weigert's iron hematoxylin for 5 min and acid fuchsin for 10 min, differentiated with phosphomolybdic acid, and stained with aniline blue for 30 s. The sections were subsequently dehydrated, cleared, and mounted.

Histological evaluation included both the peripheral rim and central core of the implant. The archived histopathology records did not contain sufficient information to determine the exact number of sections selected for quantitative analysis, their depth or anatomical level within each paraffin block, or the interval between analyzed sections. The records also did not permit verification of whether section-level sampling was applied consistently across experimental groups. In addition, the archived analysis records did not permit reliable reconstruction of the exact number or selection procedure of microscopic fields used for regional quantitative analysis. H&E staining was used for general morphological assessment, whereas Masson's trichrome staining was used to assess adipose-like areas and collagen-containing tissue within the implants.

Immunofluorescence staining

Paraffin sections were deparaffinized, rehydrated, and subjected to antigen retrieval using 10 mmol/L citrate buffer at pH 6.0. No separate permeabilization step was documented in the archived protocol for these paraffin-embedded implant sections.

Sections were blocked with 5% normal goat serum and incubated overnight at 4 °C with anti-Perilipin-1 (Abcam, Cat. No. ab3526, 1:200) for adipocyte identification, anti-CD31 (Servicebio, Cat. No. GB11063-2, 1:1,000) for total vessel labeling, and anti- α -smooth muscle actin (α -SMA; Servicebio, Cat. No. GB12045, 1:5,000) to identify α -SMA-positive cells associated with vascular structures. After washing, sections were incubated with the corresponding species-specific Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Invitrogen, 1:500) for 1 h at room temperature, followed by 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstaining.

The exact host species and catalog numbers of the secondary antibodies were not retained in the archived service records and could not be reliably reconstructed. The archived records also did not contain sufficient information to verify the exact negative-control configuration used for this specific staining run.

Images were acquired using an LSM 800 confocal microscope (Zeiss, Germany). According to the standardized service workflow, all experimental groups within each staining experiment were imaged using the same acquisition procedure. The exact numerical values for laser power, detector gain, pinhole diameter, scanning speed, and related confocal acquisition parameters were not retained in the archived service records. Images were quantified using ImageJ version 1.53 (National Institutes of Health, USA). The endpoint-specific image-analysis criteria used for adipose regeneration and vascular quantification are described below.

Quantification of adipose regeneration

Newly formed adipose tissue was evaluated separately in the peripheral and central zones of each implant. The peripheral zone was defined as the outer 500- μ m rim of the implant, whereas the central zone was defined as the remaining inner core region. Quantitative measurements were performed separately for the predefined peripheral and central regions of each implant. For the week-12 quantitative analyses reported here, five implants were analyzed per group, with each implant treated as one independent biological replicate. Thus, five independent biological values were obtained per group for each regional endpoint.

Newly formed adipose tissue was operationally defined as tissue areas containing morphologically recognizable adipocytes on H&E-stained and Masson's trichrome-stained sections, with adipocyte identity further supported by Perilipin-positive immunofluorescence. The percentage of the analyzed area occupied by adipose-like structures was quantified on Masson's trichrome-stained sections, while the Perilipin-positive area was independently quantified by immunofluorescence.

Image analysis was performed using ImageJ version 1.53 (National Institutes of Health, USA), following the same analytical framework as in our previous ADF study^[20]. For Masson's trichrome-stained sections, unstained or blank spaces were not classified as adipose tissue solely on the basis of staining intensity; adipose-like areas were included only when morphologically recognizable adipocytes were present, according to the operational definition described above. For Perilipin immunofluorescence, the Perilipin-positive area was quantified as the proportion of the predefined region of interest occupied by positive fluorescence. The same documented analytical framework was applied to coded images across all experimental groups within each staining experiment. However, the archived analysis records did not retain the exact numerical fluorescence threshold values, detailed segmentation parameters, or predefined rules for manual correction of technical artifacts; therefore, these pixel-level analysis parameters could not be reliably reconstructed retrospectively. For each implant, the region-specific quantitative value was used as the implant-level value for that region. Each implant was treated as one biological replicate for statistical analysis. Images bearing coded identifiers were analyzed by investigators blinded to group allocation.

Quantification of angiogenesis

Total vessel density was operationally defined as the number of identifiable CD31-positive vascular structures per high-power field (HPF). An α -SMA-associated vessel was defined as a CD31-positive vascular structure surrounded by α -SMA-positive cells, and the number of such structures per HPF was used to calculate α -SMA-associated vessel density. This marker-based classification followed the same analytical framework used in our previous ADF study^[20], in which CD31 and combined CD31/ α -SMA staining were used to assess total and α -SMA-associated vascular structures, respectively. Vessel densities were quantified separately in the predefined peripheral and central regions of each implant. Five implants were analyzed per group at week 12, with each implant treated as one independent biological replicate.

Images were analyzed using ImageJ version 1.53. The same documented vessel-counting definitions were applied to coded images across all experimental groups. However, the archived analysis records did not retain more detailed criteria for resolving ambiguous vascular profiles, nor did they retain the exact

fluorescence threshold or segmentation settings; therefore, these details could not be reliably reconstructed retrospectively. For each implant, the region-specific vessel-density value was used as the implant-level value for that region. Each implant was treated as one biological replicate for statistical analysis. Coded images were analyzed by investigators blinded to group allocation.

Volume retention measurement

Volume retention was assessed at weeks 4 and 12 after injection. The initial implant volume (V_0) was defined as the delivered formulation volume of 0.5 mL. At each prespecified terminal endpoint, the implants were carefully excised with the implant boundary preserved, and macroscopically identifiable surrounding tissues were removed without disrupting the implant structure. Harvested implant volume (V_t) was determined using a physiological saline displacement method. Briefly, the saline volume was recorded before (V_{before}) and after (V_{after}) complete immersion of the implant using a graduated syringe with a resolution of 0.01 mL. Visible air bubbles were removed before the final reading, and harvested implant volume was calculated as $V_t = V_{\text{after}} - V_{\text{before}}$. Volume retention (%) was calculated according to:

$$\text{Volume retention (\%)} = (V_t / 0.5 \text{ mL}) \times 100\%.$$

After harvest, implants were assigned coded identifiers that did not disclose the formulation group. Volume measurements and calculations were performed by investigators blinded to group allocation. Group codes were disclosed only after completion and verification of all volume measurements. Each implant was measured three times, and the mean of the three measurements was used as the implant-level volume value.

Animal model and injection procedure

All animal experiments were approved by the Ethics Committee of the Fourth Military Medical University (No. 20240726). Forty 8-week-old female BALB/c-nu/nu nude mice were allocated to four formulation groups using a random-number table ($n = 10$ per group): I-ADF-1:5, I-ADF-1:7.5, I-ADF-1:10, and I-ADF-1:15. Because sample collection at weeks 4 and 12 required terminal harvest, five mice within each formulation group were prospectively assigned to the week-4 terminal cohort and five to the week-12 terminal cohort. No animal contributed data to more than one terminal time point. Each mouse received a single 0.5-mL dorsal subcutaneous injection of the assigned formulation through a 17-gauge needle.

Blinding was not feasible during injection because differences in formulation consistency were visually apparent. However, all subsequent assessments, including gross volume measurement, histological evaluation, and immunofluorescence analysis, were performed on coded samples by investigators blinded to group allocation.

No formal a priori power calculation was performed, and no numerical effect size was prespecified. Sample-size selection was informed by our previously published study of the ADF platform^[20], which used a closely related dorsal subcutaneous implantation model in nude mice and reported relatively limited within-group variability in week-12 implant-volume measurements. Because that study evaluated membrane-form ADF and cryopreserved ADF rather than injectable formulations with different dilution ratios, its data could not provide a directly transferable numerical effect-size estimate for the present formulation comparison. The present study was designed as an exploratory comparison of four I-ADF formulations. Based on the variability observed in the previous ADF study, the intention to identify relatively large formulation-dependent differences, and the reduction principle of the 3Rs, five mice per formulation at each terminal time point were selected.

Predefined exclusion criteria included postoperative death, severe infection, wound complications, implant loss or displacement, and unsuccessful sample collection.

Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). Before performing the parametric analyses, normality and homogeneity of variance were assessed separately for each endpoint. Normality was evaluated using the Shapiro-Wilk test, and homogeneity of variance among the four formulation groups was evaluated using Levene's test. A *p* value greater than 0.05 was considered to indicate no statistically significant violation of the corresponding assumption. No statistically significant departure from normality or homogeneity of variance was detected for any dataset analyzed using ordinary one-way analysis of variance (ANOVA). Comparisons among multiple groups were therefore performed using ordinary one-way ANOVA followed by Tukey's multiple-comparisons test.

For the volume-retention analysis, the week-4 and week-12 datasets were obtained from independent terminal cohorts and were assessed and analyzed separately within each time point. No repeated-measures analysis or direct statistical comparison between the two time points was performed. Because the two analyses addressed separate, prespecified endpoint-specific questions and no joint or longitudinal inference across time points was made, no additional multiplicity adjustment across time points was applied.

Pearson's correlation analysis was used to evaluate the linear association between central α -SMA-associated vessel density and the central Perilipin-positive area using paired central-zone values from individual biological samples. Before the Pearson correlation and exploratory linear regression analyses, linearity was examined using scatterplots, and the normality and homoscedasticity of the residuals were assessed. No major violation of these assumptions was identified. The correlation coefficient and corresponding *p* value were reported. Exploratory linear regression analysis was used to evaluate the overall association between ADF concentration and week-12 volume retention. A two-tailed *P* value < 0.05 was considered statistically significant. All statistical analyses, including the assumption checks, were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Preparation and characterization of I-ADF

All 40 animals completed the prespecified experimental protocol. No animal met the prespecified animal- or implant-level exclusion criteria. Accordingly, five animals per formulation group were included at each terminal time point. After 12 months of cryopreservation at -80°C , the ADF retained its gross morphology and histological architecture after thawing, indicating preservation of its structural features under the evaluated storage conditions [Figure 1A]. The ADF was then cut into fragments and mixed with physiological saline at four dilution ratios (1:5, 1:7.5, 1:10, and 1:15, w/v), designated as I-ADF-1:5, I-ADF-1:7.5, I-ADF-1:10, and I-ADF-1:15 [Figure 1A and B]. Gross examination showed progressively greater free-water separation as the saline proportion increased. I-ADF-1:5 appeared relatively dry, I-ADF-1:7.5 appeared moister, and visible free water was present in I-ADF-1:10 and I-ADF-1:15 [Figure 1B]. Representative H&E-stained and Masson's trichrome-stained sections showed no obvious qualitative differences in histological architecture among the four formulations before implantation [Figure 1C and D]. These observations indicated broadly comparable baseline histological appearances among the four formulations. We next compared their qualitative extrusion behavior and *in vivo* performance.

Volume retention after subcutaneous implantation

All four formulations could be manually extruded through a 17-gauge needle under the qualitative test conditions used in this study [Figure 2A]. I-ADF-1:5 and I-ADF-1:7.5 produced relatively continuous and visually homogeneous extrudates. In contrast, I-ADF-1:10 and I-ADF-1:15 showed visible water exudation

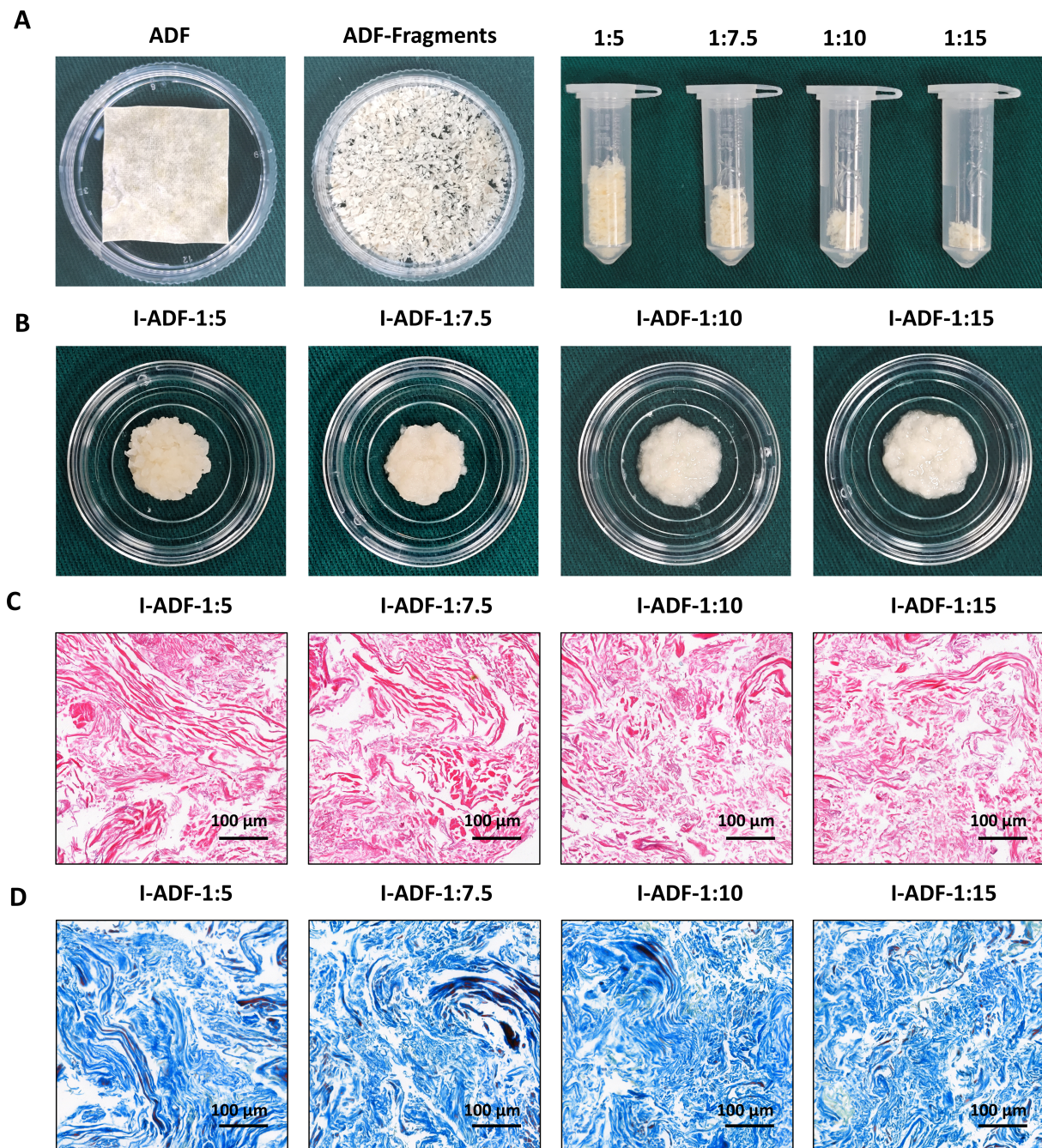


Figure 1. Preparation and characterization of I-ADF at four dilution ratios. (A) ADF was cut into small fragments and mixed with physiological saline at the four dilution ratios; (B) Gross morphology of I-ADF at the four dilution ratios; (C) H&E staining of I-ADF formulations; (D) Masson's trichrome staining of I-ADF formulations. Scale bars are shown in each micrograph in (C and D) and represent 100 μm . ADF: Adipose-derived matrix film; I-ADF: injectable adipose-derived matrix film fragments; H&E: hematoxylin and eosin.

before extrusion of matrix fragments, resulting in less uniform material delivery. However, because fragment size, injection force, extrusion rate, and rheological properties were not quantitatively measured, these observations should not be interpreted as a quantitative comparison of injectability.

At week 4, the I-ADF-1:5 and I-ADF-1:7.5 groups showed similar volume retention rates, with no significant difference between them. Both groups showed higher volume retention than the I-ADF-1:10 group ($P < 0.01$) and the I-ADF-1:15 group ($P < 0.001$; [Figure 2B and C](#)). At week 12, the volume retention rates were $86.86\% \pm 3.65\%$ (I-ADF-1:5), $88.26\% \pm 3.98\%$ (I-ADF-1:7.5), $50.44\% \pm 6.22\%$ (I-ADF-1:10), and $41.78\% \pm 10.52\%$ (I-ADF-1:15), showing a similar intergroup pattern to that observed at week 4. However, because the two time

points were represented by independent terminal cohorts, the present data do not demonstrate within-animal volume stability over time.

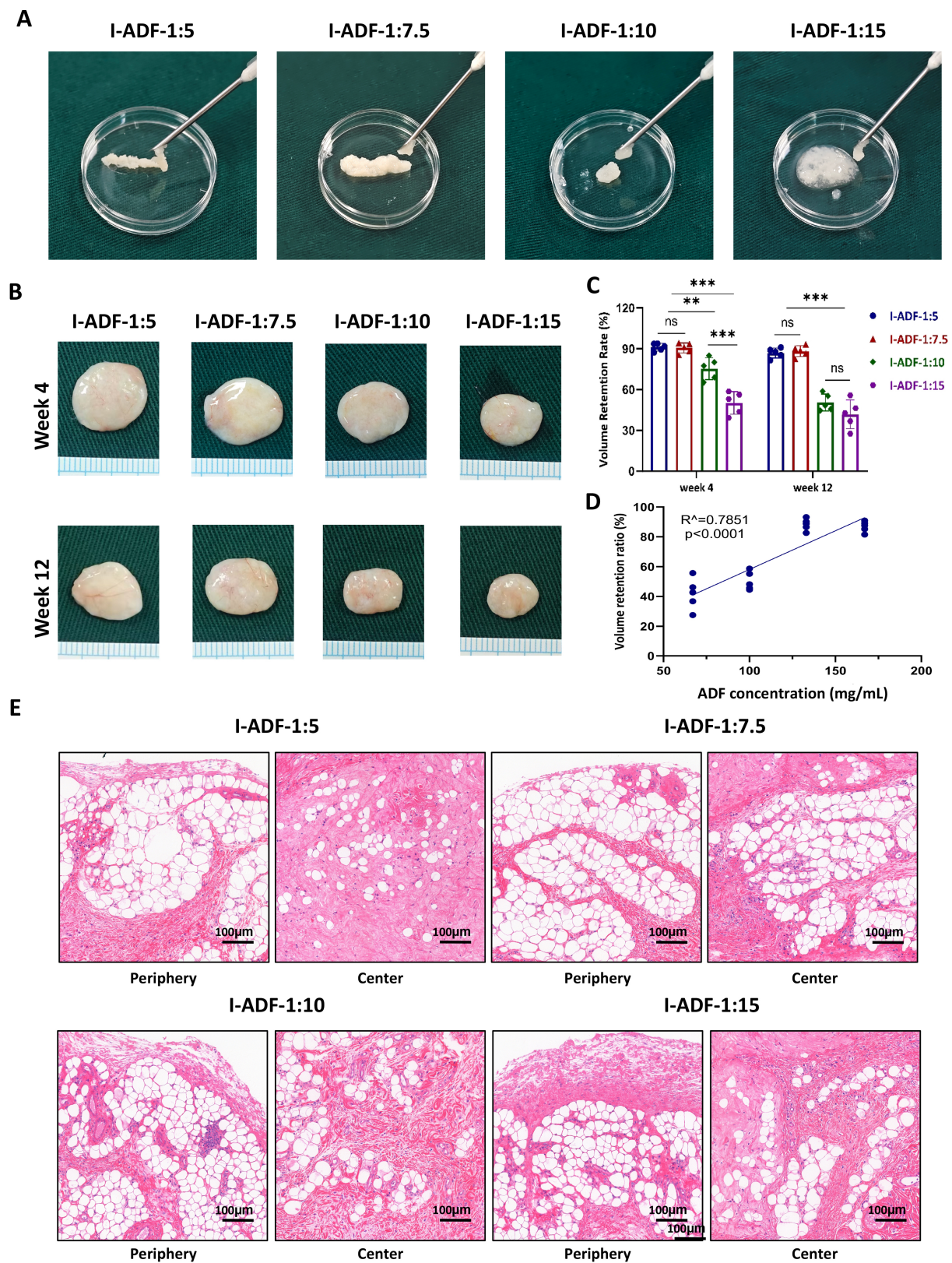


Figure 2. Qualitative extrusion behavior, volume retention, and concentration-retention relationship of I-ADF. (A) Representative images of manual I-ADF extrusion through a 17-gauge needle; (B) Representative gross images of I-ADF implants at weeks 4 and 12; (C) Quantitative volume retention at weeks 4 and 12. Data are presented as mean $\pm$ SD ($n = 5$ biological samples per group at each time point). Statistical comparisons among the four formulations were performed separately at weeks 4 and 12 using ordinary one-way ANOVA followed by Tukey's multiple-comparisons test within each time point. No repeated-measures analysis or direct statistical

comparison between the two time points was performed. $^{**}P < 0.01$ and $^{***}P < 0.001$ for the selected pairwise comparisons indicated by the horizontal lines; (D) Relationship between ADF concentration and volume retention at week 12; (E) H&E staining of I-ADF implants at week 12. Scale bars are shown in each micrograph in (E) and represent 100 μm . ADF: Adipose-derived matrix film; I-ADF: injectable adipose-derived matrix film fragments; SD: standard deviation; ANOVA: analysis of variance; H&E: hematoxylin and eosin; ns: not significant.

At week 12, ADF concentration was positively associated with volume retention across the four tested formulations [Figure 2D]. Volume retention increased from 41.78% in the I-ADF-1:15 group to 88.26% in the I-ADF-1:7.5 group, whereas the I-ADF-1:5 and I-ADF-1:7.5 groups showed comparable retention rates (86.86% and 88.26%, respectively; $P > 0.05$).

H&E staining at week 12 revealed abundant adipocyte-like cells in the peripheral zones of all four groups [Figure 2E], consistent with broadly comparable peripheral adipose-like tissue formation among the tested formulations. In contrast to the peripheral zones, central zone adipose regeneration showed marked differences among groups [Figure 2E]. Central-zone regeneration therefore provided greater discrimination among the four formulations than peripheral regeneration. To systematically evaluate this differential regenerative capacity, we next performed quantitative histomorphometric analysis using Masson's trichrome staining and Perilipin immunofluorescence.

Adipose regeneration within I-ADF implants

Masson's trichrome staining showed that the newly formed adipose structures were surrounded by organized collagen tissue, consistent with local ECM remodeling [Figure 3A]. Consistent with H&E findings, the peripheral zones of all groups showed abundant adipose-like structures. Quantitative analysis showed that adipose-like structures occupied approximately 80% of the analyzed peripheral area across all groups, with no statistically significant differences among the four formulations ($P > 0.05$) [Figure 3B]. These findings indicate broadly comparable peripheral adipose regeneration among the four formulations. At week 12, the I-ADF-1:7.5 group exhibited near-complete filling of the outermost peripheral zone with adipose tissue, closely resembling normal adipose tissue architecture. In the central zone, however, the I-ADF-1:7.5 group displayed large, continuous areas of adipose tissue with mature structural characteristics, whereas the other groups showed scattered or discontinuous adipose distribution [Figure 3A].

Quantitative analysis of the central adipose-like area showed that the I-ADF-1:7.5 group had the highest value ($60.50\% \pm 6.31\%$), significantly exceeding the other groups ($P < 0.001$) [Figure 3B]. The I-ADF-1:10 and I-ADF-1:15 groups showed comparable central adipose-like area values ($30.91\% \pm 5.49\%$ and $26.64\% \pm 7.46\%$, respectively; $P > 0.05$), whereas the I-ADF-1:5 group had the lowest mean value ($19.66\% \pm 3.23\%$). The disparity between peripheral and central regeneration was minimal in the I-ADF-1:7.5 group but most pronounced in the I-ADF-1:5 group, indicating that I-ADF-1:7.5 was associated with the highest central adipose regeneration among the four tested formulations.

Perilipin immunofluorescence supported the histological findings [Figure 3C]. In the peripheral zones, all groups showed extensive peripheral Perilipin-positive areas, with no significant differences among groups [Figure 3D]. In the central zone, the I-ADF-1:7.5 group had the largest Perilipin-positive area ($59.94\% \pm 4.28\%$), significantly greater than those in the I-ADF-1:10 ($29.86\% \pm 4.10\%$), I-ADF-1:15 ($27.94\% \pm 3.01\%$), and I-ADF-1:5 ($20.18\% \pm 3.19\%$) groups ($P < 0.001$) [Figure 3D]. The I-ADF-1:7.5 group showed large, continuous Perilipin-positive areas, while the I-ADF-1:10 and I-ADF-1:15 groups showed a mixture of continuous and scattered Perilipin-positive areas, and the I-ADF-1:5 group showed a predominantly scattered distribution. The concordance among H&E, Masson's trichrome, and Perilipin staining further suggests that the 1:7.5 formulation was associated with more uniform adipose tissue formation within the analyzed areas.

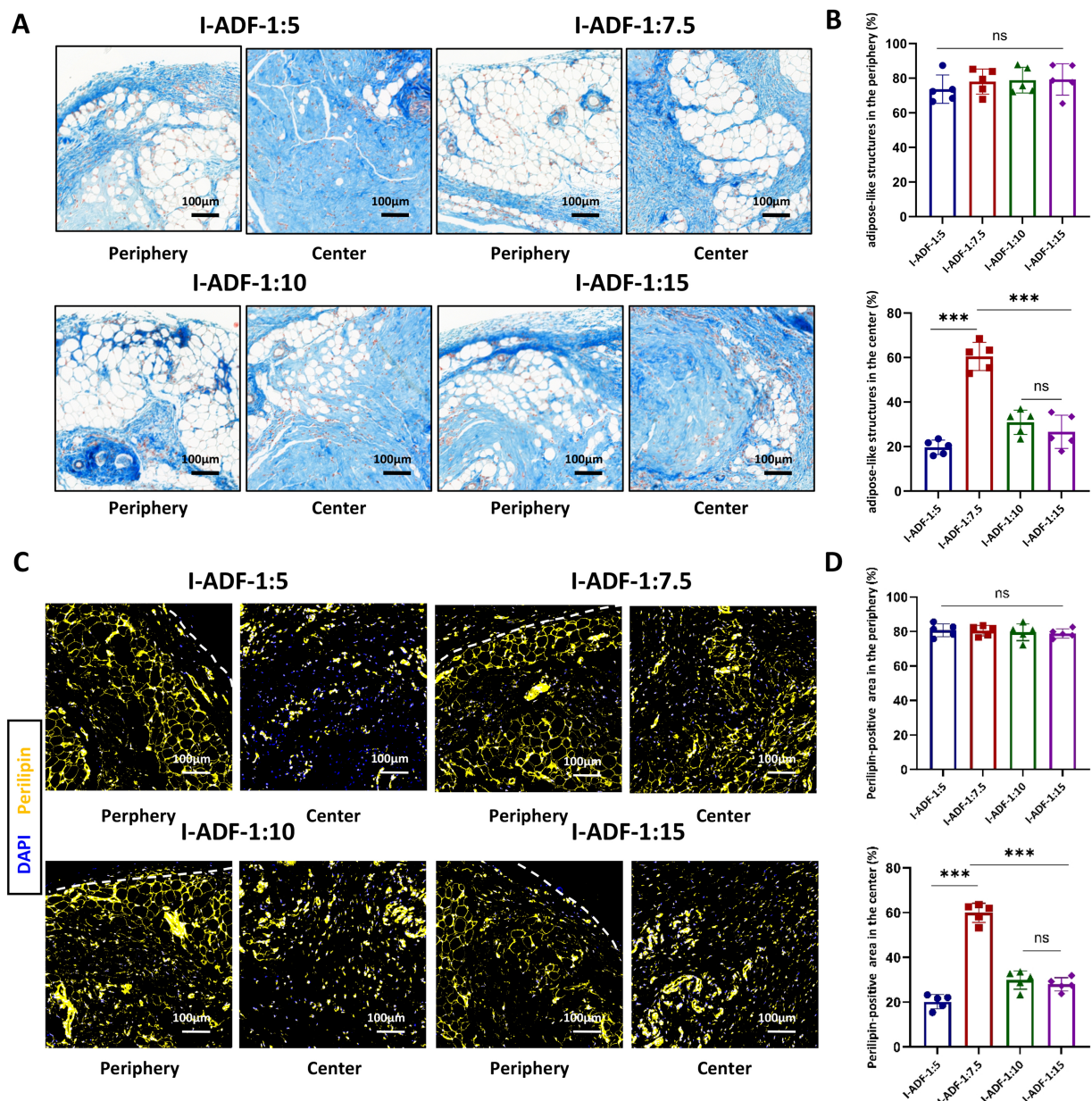


Figure 3. Masson's trichrome staining and Perilipin immunofluorescence. (A) Representative Masson's trichrome-stained images of the peripheral and central zones at week 12. Scale bars are shown in each micrograph and represent 100 μ m; (B) Percentage of the analyzed area occupied by adipose-like structures in the peripheral and central zones, quantified from Masson's trichrome-stained sections. The y-axis label "adipose-like structures (%)" denotes the percentage of the analyzed area rather than the number of individual structures; (C) Representative Perilipin immunofluorescence images of the peripheral and central zones at week 12. Scale bars are shown in each micrograph and represent 100 μ m; (D) Quantification of the Perilipin-positive area in the peripheral and central zones. Data are presented as mean $\pm$ SD ($n = 5$ independent biological samples per group at week 12). The peripheral and central regions were quantified separately for each implant. Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple-comparisons test. *** $P < 0.001$ vs. the I-ADF-1:7.5 group. ADF: Adipose-derived matrix film; I-ADF: injectable adipose-derived matrix film fragments; SD: standard deviation; ANOVA: analysis of variance; ns: not significant.

Given that the 1:7.5 formulation showed the highest central adipose regeneration among the tested ratios, we next investigated whether this advantage was associated with enhanced angiogenesis, particularly in the central zone.

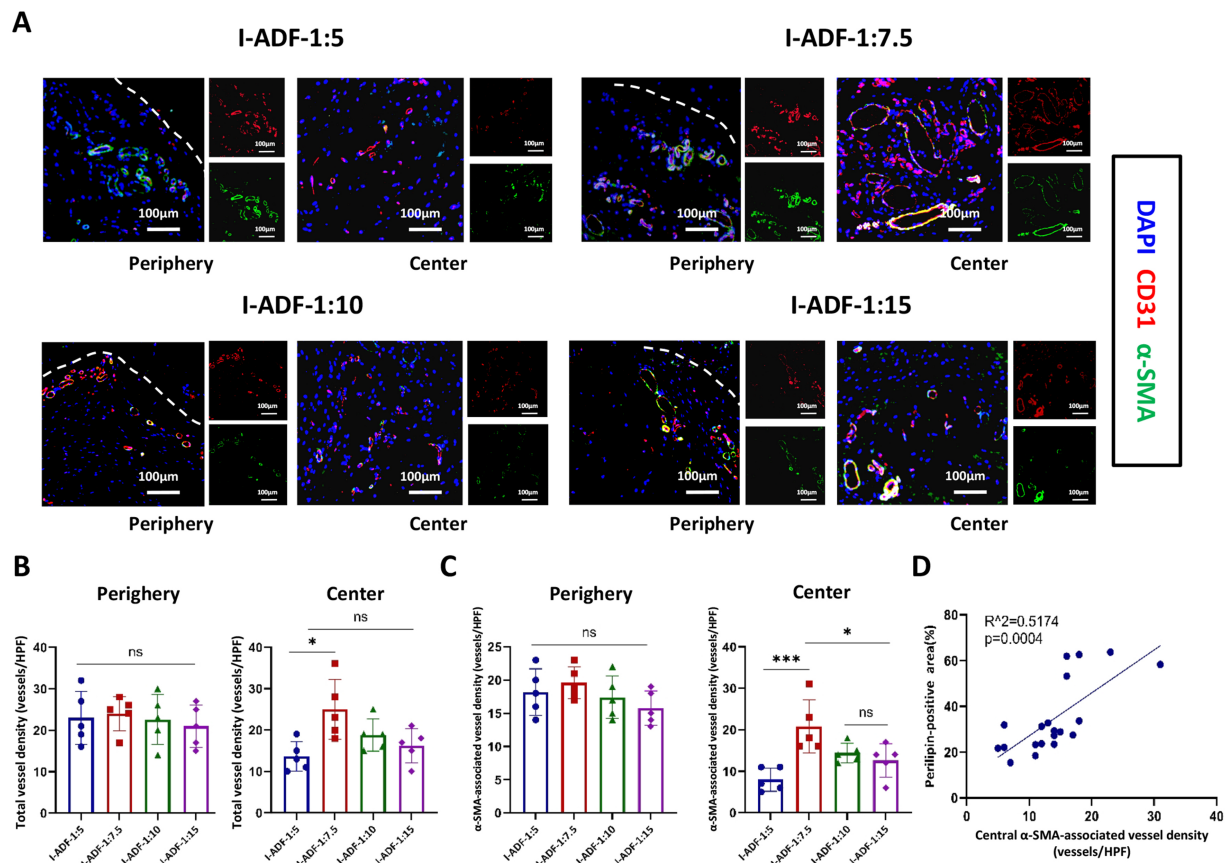


Figure 4. Evaluation of total and α -SMA-associated vessel densities in implanted I-ADF. (A) Representative CD31 and α -SMA immunofluorescence staining in the peripheral and central zones. Scale bars are shown in each micrograph in (A) and represent 100 μ m; (B) Total vessel density, defined as the number of CD31-positive vascular structures per HPF; (C) α -SMA-associated vessel density, defined as the number of CD31-positive vascular structures surrounded by α -SMA-positive cells per HPF; (D) Association between central α -SMA-associated vessel density and the central Periphrin-positive area. Data in (B and C) are presented as mean $\pm$ SD ($n = 5$ biological samples per group at week 12) and were analyzed using one-way ANOVA followed by Tukey's multiple-comparisons test. Pearson's correlation analysis was used for (D). (D) includes 20 biological samples pooled across the four formulation groups. Statistical significance for the selected pairwise comparisons is indicated by horizontal lines: * $P < 0.05$ and *** $P < 0.001$. ADF: Adipose-derived matrix film; I-ADF: injectable adipose-derived matrix film fragments; DAPI: 4',6-diamidino-2-phenylindole; CD31: cluster of differentiation 31; α -SMA: alpha-smooth muscle actin; HPF: high-power field; SD: standard deviation; ANOVA: analysis of variance; ns: not significant.

Angiogenesis assessment

In the peripheral zone, CD31 immunofluorescence staining revealed no significant differences in total vessel density among the four groups ($P > 0.05$). Similarly, CD31/ α -SMA co-staining showed no significant differences in α -SMA-associated vessel density among groups ($P > 0.05$) [Figure 4A-C]. The comparable peripheral vascularization among groups was consistent with similar levels of peripheral adipose regeneration.

By contrast, vascularization in the central zone differed among the four formulations. The I-ADF-1:7.5 group had the highest mean total vessel density (25.00 ± 7.21 vessels/HPF). This value was significantly higher than that in the I-ADF-1:5 group (13.60 ± 3.58 vessels/HPF; $P < 0.05$), whereas the differences from the I-ADF-1:10 (18.80 ± 3.89 vessels/HPF) and I-ADF-1:15 (16.20 ± 4.09 vessels/HPF) groups were not statistically significant [Figure 4B]. The I-ADF-1:7.5 group also had the highest mean central α -SMA-associated vessel density (20.80 ± 6.38 vessels/HPF). It was higher than that in the I-ADF-1:5 group (8.00 ± 2.83 vessels/HPF; $P < 0.001$) and the I-ADF-1:15 group (12.60 ± 4.19 vessels/HPF; $P < 0.05$), but did not differ significantly from that in the I-ADF-1:10 group (14.40 ± 2.30 vessels/HPF) [Figure 4C]. Thus, among the four formulations tested, I-ADF-1:7.5 had the highest mean central total and α -SMA-associated vessel densities.

In an exploratory pooled analysis across all 20 biological samples, Pearson's correlation analysis showed a positive association between central α -SMA-associated vessel density and the central Perilipin-positive area at week 12 ($r = 0.719$, $R^2 = 0.5174$, $P = 0.0004$; [Figure 4D](#)). Because samples from four formulation groups were combined, this association may partly reflect between-formulation differences and does not establish causality.

DISCUSSION

Injectable ECM-based materials have been extensively studied for soft tissue augmentation^[11,12,23,24]. Previous studies have demonstrated that processing techniques, ECM concentration, scaffold architecture, degradation behavior, and biomaterial composition can significantly affect implant performance and regenerative outcomes^[17-22]. The host response to ECM scaffolds, including macrophage polarization, scaffold degradation, vascularization, and host tissue deposition, is also critical for successful remodeling^[21,22,25,26]. Studies using adipose-derived ECM in scaffold, foam, particulate, and hydrogel formats further indicate that matrix architecture, particle size, and formulation characteristics can influence adipogenic induction, host-tissue integration, and implant remodeling^[27-32]. Collectively, these studies indicate that ECM concentration, processing method, scaffold microstructure, and host immune response are key determinants of regenerative efficacy. However, most existing injectable ECM products are derived from allogeneic or xenogeneic sources, and the relationship between ECM concentration and *in vivo* performance, particularly for autologous adipose-derived ECM, remains incompletely understood.

In this study, we systematically evaluated four dilution ratios (1:5, 1:7.5, 1:10, and 1:15, w/v) of I-ADF derived from our previously established autologous/patient-derived ADF platform for soft tissue filling. The present study was designed as a formulation-optimization study of four I-ADF dilution ratios. Among the four formulations tested, I-ADF-1:7.5 emerged as the optimal overall dilution ratio under the present experimental conditions. Volume retention generally increased with ADF concentration across the tested formulations. Although the I-ADF-1:5 and I-ADF-1:7.5 groups showed comparable retention rates, this finding alone does not establish a plateau or a nonlinear concentration-response relationship. Additional concentrations and appropriate nonlinear modeling would be required to characterize the precise relationship between ADF concentration and volume retention.

An important finding was the divergence between peripheral and central regeneration across formulations. Peripheral adipose regeneration was comparable among groups, whereas central regeneration was highest in the I-ADF-1:7.5 group. The lower central regeneration observed with I-ADF-1:5 may reflect reduced physical accessibility within the denser matrix, whereas the lower-concentration formulations may provide insufficient structural and biochemical support. This interpretation is consistent with previous studies showing that adipose-derived ECM particle size and hydrogel formulation can influence cell-matrix interactions, adipogenic differentiation, and *in vivo* compatibility^[31-34]. Thus, the regenerative profile of I-ADF-1:7.5 is consistent with a favorable balance between matrix support and host-tissue accessibility. However, scaffold porosity, nutrient diffusion, degradation behavior, and matrix-bound signaling were not directly measured and require further validation.

The angiogenesis findings were consistent with the regional pattern of adipose regeneration. Peripheral vessel densities were comparable among formulations, whereas the I-ADF-1:7.5 group had the highest mean central total and α -SMA-associated vessel densities. The positive association between central α -SMA-associated vessel density and the central Perilipin-positive area suggests that vascular structures with α -SMA-positive cell coverage may be related to deep-tissue remodeling. Previous studies using adipose-derived ECM alone or in combination with cells or proangiogenic factors have reported vascular regeneration and

regenerative tissue formation^[35-37]. However, the correlation observed in the present study does not establish causality, and the possible effects of matrix density, porosity, degradation, and bioactive signaling require direct mechanistic validation. Because the pooled analysis combined samples from four formulation groups, the observed association may partly reflect between-formulation differences.

In the intended patient-derived workflow, I-ADF may reduce donor-mismatch-related concerns relative to allogeneic or xenogeneic injectable ECM products. However, this potential advantage was not directly evaluated in the present human-to-mouse xenogeneic implantation model, and donor- and batch-related variability in patient-specific production remains unknown. The physical fabrication approach may avoid some of the biochemical alterations associated with extensive chemical decellularization^[19-21]; nevertheless, direct comparative studies are required to substantiate this advantage. Independent studies of injectable allograft or acellular adipose matrices have reported host-tissue integration, adipogenic remodeling, vascularization, and soft-tissue volume support, while also indicating that biological performance depends on material source, processing method, and formulation^[38-41]. The ability to cryopreserve ADF while preserving bioactivity, as demonstrated in our previous study^[20], supports the concept of an individualized ECM bank for future patient-derived applications. Notably, among the four formulations tested, I-ADF-1:7.5 emerged as the optimal overall dilution ratio under the present experimental conditions, based on its overall balance of volume retention, central adipose regeneration, angiogenesis, and qualitative extrusion behavior. Our findings further suggest that optimizing ADF content may improve central regeneration of I-ADF, although direct comparisons with commercial ECM materials, standard filling treatments, and established fat-grafting approaches remain necessary^[1-5,11,12,23,24].

Several limitations should be acknowledged. First, although the nude mouse model maintained methodological continuity with our previous ADF platform^[20] and minimized T-cell-mediated rejection, it cannot recapitulate the immune dynamics of an immunocompetent autologous setting. Moreover, because our previous long-term data were obtained using membrane-form ADF, the long-term persistence and safety of injectable I-ADF require independent evaluation. Second, the absence of relevant comparators, such as sham controls, autologous fat grafting, or commercial ECM products, limits the conclusions to relative differences among the tested formulations rather than superiority over existing treatments. The relatively small sample size may also have limited the power to detect modest between-group differences, particularly for vascular outcomes with greater variability. In addition, because the exact number of donors contributing to the pooled precursor ADF batch was unavailable, donor-specific variability could not be evaluated independently. A further limitation is that the exact number, depth, and interval of sections analyzed per implant, as well as the consistency of section-level sampling across experimental groups, could not be retrospectively verified from the archived records. Given the potential spatial heterogeneity within individual implants, this limitation may have introduced section-level sampling bias between groups and may limit the extent to which the histological and immunofluorescence measurements represent the entire implant. In addition, the exact number and selection procedure of microscopic fields used for regional quantitative analysis could not be reliably reconstructed from the archived records. Therefore, the extent to which within-section spatial heterogeneity was captured cannot be fully assessed. The exact ImageJ fluorescence threshold values, detailed segmentation parameters, predefined manual-correction rules, and more detailed criteria for resolving ambiguous vascular profiles were also not preserved in the archived analysis records, thereby limiting the independent reproducibility of the image-based quantitative analyses. Third, the 12-week observation period and qualitative extrusion assessment using a 17-gauge needle represent additional constraints. Fragment size and injection force were not quantitatively measured, and differences in fragment-size distribution among aliquots cannot be excluded despite pooling, mixing, and aliquoting fragments from the same cryopreserved ADF batch. Such heterogeneity may have affected formulation consistency, extrusion behavior, host-cell infiltration, and tissue remodeling; therefore, the observed extrusion differences

cannot be attributed exclusively to the dilution ratio. The compatibility of I-ADF-1:7.5 with 25-27-gauge needles or fine blunt cannulas also remains unknown. Fragment-size standardization, micronization, or enzymatic softening may improve delivery through finer devices, but these procedures may alter ECM composition, structural integrity, mechanical properties, and biological activity and therefore require systematic validation. Future studies should evaluate I-ADF-1:7.5 in species-matched autologous large-animal models using clinically relevant delivery devices, longer follow-up periods, and comprehensive safety assessments before clinical translation.

In summary, this study compared four dilution ratios of I-ADF in a nude mouse model. Among the four formulations tested, I-ADF-1:7.5 emerged as the optimal overall dilution ratio under the present experimental conditions, combining high volume retention with greater central adipose regeneration, the highest mean central α -SMA-associated vessel density, and relatively uniform qualitative extrusion through a 17-gauge needle. Volume retention was comparable between the 1:5 and 1:7.5 formulations, whereas central adipose regeneration was highest with the 1:7.5 formulation. I-ADF-1:7.5 therefore warrants further controlled preclinical evaluation. Species-matched autologous large-animal studies using clinically relevant delivery devices are required to evaluate long-term safety, injection performance, and regenerative outcomes before clinical investigation.

DECLARATIONS

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Authors' contributions

Conceptualization: Cao S, Hou M, Yu Y, Yi C, Li H

Methodology: Cao S, Guo Y, Chen R

Investigation: Cao S, Hou M, Yu Y, Yang Y

Formal analysis: Hou M, Yu Y, Guo Y, Yang Y, Zhu J

Validation: Guo Y, Chen R

Data curation: Guo Y

Writing - original draft: Cao S, Hou M, Yu Y

Writing - review and editing: Cao S, Hou M, Yu Y, Li H

Supervision: Yi C, Li H

Project administration: Yi C, Li H

Funding acquisition: Li H

All authors read and approved the final manuscript.

Availability of data and materials

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AI and AI-assisted tools statement

During the preparation of this manuscript, the AI tool Google Gemini (version 1.5, released 2024-02-15) was used for language polishing and editorial refinement during manuscript revision. In addition, Google Gemini was used for the initial generation and refinement of the graphical elements in the graphical abstract and the mouse illustration based on prompts prepared by the authors. The final layout, text labels, arrows, and scientific annotations of the graphical abstract were completed using Microsoft PowerPoint. The AI-generated graphical elements were not directly copied or imported from published articles, commercial image libraries, websites, or other identifiable third-party sources. The tool did not influence the study design, data collection, analysis, interpretation, or the scientific content of the work. All authors take full responsibility for the accuracy, integrity, and final content of the manuscript.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All animal experiments were approved by the Ethics Committee of the Fourth Military Medical University (No. 20240726) and were conducted in accordance with the approved institutional guidelines for the care and use of laboratory animals. Human adipose tissue collection was approved by the Ethics Committee of the Fourth Military Medical University (No. KY20243548-1). All donors provided written informed consent, and all procedures involving human tissue were conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

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