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Evaluation of Paste-Type Micronized Acellular Dermal Matrix for Soft Tissue Augmentation: Volumetric and Histological Assessment in a Mouse Model

페이스트형 미세화 무세포 동종 진피의
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의학과 의공학 전공
장 란 숙

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지도교수 최 영 빈

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장 란 숙

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위 원 장 이 정 찬 (인)

부위원장 최 영 빈 (인)

위 원 김 상 화 (인)

위 원 정 기 훈 (인)

위 원 김 연 환 (인)

ABSTRACT

Evaluation of Paste-Type Micronized Acellular Dermal Matrix for Soft Tissue Augmentation: Volumetric and Histological Assessment in a Mouse Model

Lan Sook Chang

College of Medicine, Department of Biomedical Engineering

The Graduate School

Seoul National University

Background: Being noninvasive, injectable materials for soft tissue volume augmentation are gaining tremendous attention**.** Paste-type micronized acellular dermal matrix (ADM), an injectable form of ADM, has been proven effective in wound healing. This study aimed to compare the augmentation effect of paste-type micronized ADM with conventional fillers in animal experiments to predict its potential as a soft tissue filler.

Methods: Two distinct paste-type micronized ADMs, which were mixed with distilled water (mADM) and gelatin (mADM+GEL), respectively, were compared with conventional fillers, hyaluronic acid (HA) and polymethyl methacrylate (COL+PMMA). Micronized ADMs were evaluated for particle size and surface morphology during *in vitro* studies. The rheological parameters of each filler were measured using a rotational rheometer. For animal study, four different types of fillers were injected into the dorsum of nude mice to compare the volume maintenance and biocompatibility. Volumetric analysis was performed using ultrasound and computed tomography (CT) imaging during an 8-week experimental period. Histological evaluation was performed using hematoxylin and eosin and CD 31 staining.

Results: Micronized ADM particles had an average size of 664.2 ± 389.9 μm. Rheologic measurements showed that mADM and mADM+GEL had higher elastic modulus (G') and complex viscosity (η^*) than conventional fillers. According to the CT images at week 8, the mADM and mADM+GEL showed volume persistence rate of 113.54% and 51.12%, compared with 85.09% and 17.65% for the HA and COL+PMMA, respectively. Ultrasound images at the interval of 2-weeks represented the change of shape in each fillers. The ratio of height to width appeared in the order of HA, mADM, mADM+GEL, COL+PMMA at week 8 meaning that the mADM group had stronger tendency to spread in the injection plane than HA. Histological analysis showed marked fibrous invasion and neovascularization with the mADM and mADM+GEL compared to that of the

conventional fillers.

Conclusions: The paste-type micronized ADM exhibited soft tissue augmentation by tissue replacement which efficacy is comparable to that of commercial fillers. Therefore, the paste-type micronized ADM has a potential as an alternative material for a soft tissue filler. Further research, including optimization of particle size and ADM fraction, is warranted for precise clinical application.

Keywords: Skin Filler, Acellular Dermal matrix, Mice, Nude, Hyaluronic acid, Polymethyl methacrylate,

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LIST OF ABBREVIATION

ADM, Acellular dermal matrix

CH, Calcium hydroxyapatite

COL, Collagen

CT, Computed tomography

EPDS, Engineered particulate dermal substitute

GEL, Gelatin

HA, Hyaluronic acid

H&E, Hematoxylin and eosin

MRI, Magnetic resonance imaging

PLLA, Poly-L-lactic acid

PMMA, Polymethyl methacrylate

VWF, VonWillebrand factor

INTRODUCTION

There has been a historical interest in soft tissue augmentation in the aesthetic and reconstruction field. It is essential for alleviating the stigmata of aging, wrinkles and sagging, and for correcting contour defect caused by various diseases and trauma. Due to their noninvasive nature and ease of application, injectable materials for soft tissue volume augmentation are gaining popularity. Various new soft tissue fillers have been developed with their applications according to their composition and characteristics. Biological and synthetic materials are the most used among the widely accepted classification of fillers.

Classification of current soft tissue fillers

Classification of current soft tissue fillers according to biological and synthetic materials is listed in Table 1 (1-13). Biologic materials, derived from organic sources (humans or animals), have advantages of high biocompatibility and biodegradability. Collagen and hyaluronic acid products are major types of biologic tissue fillers. Bovine collagen was the first commercially marketed injectable filler. Despite proven effectiveness and safety in the clinic, its popularity is decreasing due to the short longevity and the need for skin tests due to animal origin (1,2). Human collagen cultured from human dermal fibroblasts are free from the skin test, but short longevity made it no longer available (3).

Table 1. Classification of current soft tissue fillers

Category		Product	Composition	Indication	Duration	Advantage	Disadvantage
Biologic filler	**Collagen**	Zyderm Zyplast	Bovine collagen	Superficial dermis Deep dermis	~3m	Proven effectiveness and safety	Need for skin test Short duration
		Cosmoderm Cosmoplast	From fibroblast	Superficial dermis Deep dermis	4~7m	No skin test	Short duration
	Acellular dermal matrix (ADM)	Cymetra	Micronized acellular dermal tissue	Lip, Nasolabial folds, Acne scar	4~18m	No skin test Versatile application	Needs to be mixed prior to use.
	Hyaluronic acid (HA)	Restylane_fine line	HA (bacterially derived)	Dermoepidermal juction Superficial dermis	~6m	Fine line	Short duration Hypersensitivity Granuloma
		Restylane Juvederm_XC Elevess Hylaform	HA (bacterially derived) HA (avian culture derived, Hylaform)	Mid-to-deep dermis	6~9m	Long lasting Deep tissue Not immunogenic	Swelling Hypersensitivity Granuloma
		Perlane Juvederm_voluma Puragen	HA (bacterially derived)	Deep dermis	~1y	Long lasting Deep tissue Not immunogenic	Swelling Hypersensitivity Granuloma
Synthetic filler	**Polymethylmethacrylate (PMMA)**	Bellafill ArteColl	PMMA + Bovine collagen	Deep dermis or subcutaneous	~5yr	Long lasting Deep folds and acne scars	Bumps, granuloma Collagen reactivity Need for skin test
	Calcium hydroxyapatite (CH)	Radiesse	CH+polysaccharide gel	Deep dermis or subcutaneous	1~2yr	Long lasting Deep tissue Not immunogenic	Swelling Irregularity Granuloma
	Poly-L-lactic acid (PLLA)	Sculptra	PLLA	Deep dermis or subcutaneous	~2yr	Long lasting Deep tissue Not immunogenic HIV-associated lipoatrophy	Erythema, ecchymosis Multiple treatments required Granuloma

Currently, hyaluronic acid products are the most commonly used soft tissue fillers. Its origin from skin component and universality across all animal species and microbes make it highly biocompatible and non-immunogenic. The high capacity for holding water is related to its excellent clinical performance (4,5). However, one of the major drawbacks of hyaluronic acid is its short half-life due to enzymatic hydrolysis, and many efforts, including chemical cross-linking and incorporating other materials, are being made to improve stability (6,7).

Synthetic materials for soft tissue fillers have been developed with the need for permanence. Calcium hydroxyapatite, poly-L-lactic acid, and polymethyl methacrylate are representative synthetic materials for permanent filler, which are usually mixed with transport carrier, including polysaccharide gel, cellulose, bovine collagen, respectively, to increase injectability (8-10). Their non-resorbable or slowly resorbable components give them permanence, and they can persist an average of 1-2 years compared with several months for biologic fillers. Although non-resorbable components may persist indefinitely, the transport carrier occupying most of the injected volume is rapidly absorbed, and much of the perceived augmentative effect may be gone quite quickly (11). Because of the particulate nature, the potential for clumping and aggregation and the granuloma is likely higher than biologic fillers.

Acellular dermal matrix (ADM) for tissue regeneration

Despite the advent of various new materials, the ideal filler has not yet been found.

Acellular dermal matrix (ADM) is a good alternative biologic material for soft tissue fillers in that it can provide permanence through tissue replacement. The ADM is processed by removing the epidermis and dermal cell components from a cadaver or porcine skin. The resultant acellular sheet of dermal proteins exhibits very low antigenicity and excellent stability, while retaining the native dermal structure (14). This product behaves as a scaffold repopulated and revascularized by the host, and its safe and effective tissue integration has been demonstrated in various applications (15). It has been widely used for dermal replacement in various indications such as breast, abdominal wall, extremity, and nasal reconstruction (16-21).

Current status of micronized ADM application

Micronized ADM is a particulate ADM made by freeze-fracturing the acellular dermis with resulting variable particle size. While sheet-type ADM requires an incision to apply and has limitations in shaping, micronized ADM can be injected without an incision, and the indication for graft can be extended. Cymetra (LifeCell, USA) is a commercialized micronized ADM and has been approved for subcutaneous injection to lips and nasolabial folds (22). However, it has not been widely used for filler due to the requirement of hydration before use as processed into powder type and duration is relatively short and unpredictable. Most micronized ADMs have been outstanding in wound healing. Banta et al. reported the successful treatment of the refractory sinus tract with micronized ADM (23). In a randomized controlled multicenter clinical trial, Kim et al. showed that

micronized ADM promotes wound size reduction compared with standard care in chronic wounds (24). Micronized ADM has advantages for deep and narrow wounds that are difficult to treat because they can be injected into nooks and crannies that sheet-type dressings cannot reach (25). Recently, the micronized ADM is processed by compounding other polymers for wound dressing, such as gelatin and hyaluronic acid, to make a paste that improves applicability (26,27).

Micronized ADM for soft tissue augmentation

Based on abundant clinical experience, the author observed that the soft tissue defect was filled by tissue replacement when paste-type micronized ADM was applied to a tunnel-shaped wound. Recent studies have demonstrated that micronized ADM with bioengineered cells has a volume effect by serving as a scaffold for cell growth (28,29). However, only some studies have evaluated the extent of the augmentation effect of paste-type micronized ADM compared to existing fillers. This study aimed to compare the augmentation effect of paste-type micronized ADM with conventional fillers in animal experiments to predict its potential as a soft tissue filler.

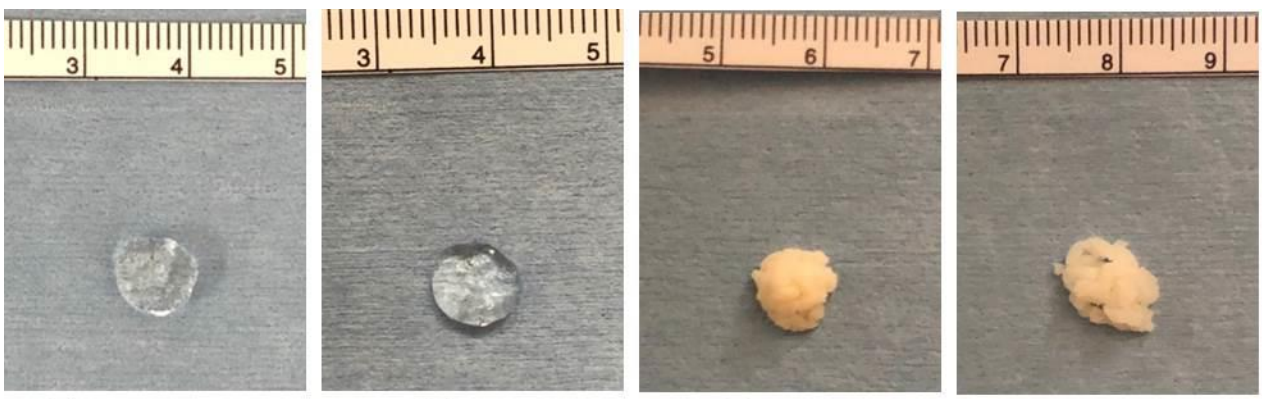
MATERIALS AND METHODS

Materials

The acellular dermal matrix was lyophilized at -80℃ for 48h and micronized into particles to make dermis powder. The dermis powder was made of two types of paste by evenly suspending them in sterile distilled water (CG reallo, **mADM**) or mixed with gelatin (CG paste, **mADM+GEL**). mADM contained 16wt% of dermis powder, and mADM+GEL contained 13wt% of dermis powder with 3wt% of gelatin. To compare the efficacy of current widely-used fillers, cross-linked hyaluronic acid filler (Giselleligne, **HA**), as a representative biologic filler, and bovine collagen plus polymethyl methacrylate (PMMA) microbeads (Artesense, **COL+PMMA**) were chosen as representative synthetic fillers (Fig. 1).

Figure 1. Gross morphology of four types of filler after extrusion.

(A) COL+PMMA appears transparent viscous gel. The contour of the microparticle can be visualized from the surface. (B) HA appears to be transparent viscous gel with smooth surface. (C) mADM has a paste formulation with yellow microparticles. (D) mADM+GEL has a similar formulation to mADM but the color is more white and less viscous.



Characterization of micronized acellular dermal matrix

Particle size and distribution of dermis powder were measured using a particle analyzer (Mastersizer 2000, Malvern Instruments Ltd., UK). Samples were suspended in distilled water and scanned using a particle analyzer for mean particle size and distribution analysis.

The surface morphology of the dermis powder was investigated by scanning electron microscopy. Prior to the observations, the specimens were lyophilized overnight and sputter-coated with gold-palladium (E-1030, Hitachi, Japan). The samples were observed under a scanning electron microscope (S-4700, Hitachi, Japan) at 10 kV.

Rheological measurements

The rheological parameters of each filler were measured using a rotational rheometer (ARES-G2, TA Instruments Ltd., USA) equipped with a parallel plate geometry (plate diameter of 25 mm). Frequency sweep tests were carried out at a constant strain (1%) over a frequency range from 0.1 to 100 Hz. The elastic modulus (G′) and complex viscosity (η^*) were determined and interpolated at an oscillation frequency of 5 Hz. Based on previous analyses, the expected range of physiological stress common to the skin varied between 0.1 and 5 Hz (30,31).

Extrusion force measurements

The injectability of the mADM and mADM+GEL were evaluated by comparing the extrusion force. mADM and mADM+GEL were prepared by filling in 3mL syringes with a 21-gauge needle and the force needed to extrude the material through a needle was measured using a universal testing machine (ME-002, Test one Ltd., Korea) at a rate of 1mm/s.

Experimental Design

Four types of fillers (COL+PMMA, HA, mADM, and mADM+GEL) were injected into the dorsum of nude mice to compare volume retention and biocompatibility. During the 8-week experimental period, computed tomography (CT) scans were taken at weeks 0 and 8, and ultrasound evaluations were performed at 2-week intervals for volumetric analysis. Histological evaluation was performed at weeks 4 and 8 after harvesting the injected filler with the covered skin. All experiments were approved by the Institutional Animal Care and Use Committee of the institution (IACUC No. 18-0268-S1A1), and animals were maintained in the facility accredited by AAALAC International (#001169) in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition, NRC (2010).

Animals

Twenty 7-week-old male BALB/c nude mice were obtained from Orient Bio Inc. (Seongnam, Korea) and fed a standard diet. After 1 week of acclimatization, the mice were injected with fillers under isoflurane ventilatory anesthesia. Isoflurane

(2%) was used for induction, and 1.5% isoflurane was maintained through a nose cone. Depending on the size and skin condition of the mouse, two or four injections of fillers were administered to each mouse. The injections were located symmetrically on the dorsum based on the spinal line (Fig. 2). The type of filler injected was randomly assigned; 200 μL of each filler was injected subcutaneously using a 23-G needle.

Figure 2. BALB/c nude mice with an injection of filler material on the dorsum.

The injections were located symmetrically on the dorsum based on the spinal line. The type of filler injected was randomly assigned; 200 μL of each filler was injected subcutaneously using a 23-G needle.

Volumetric Analysis

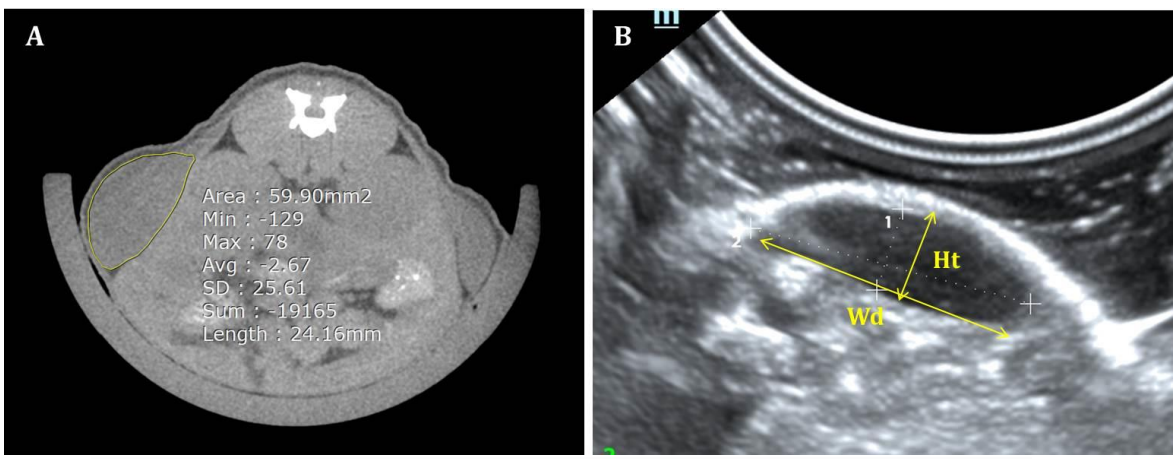
CT and ultrasound scans were used for volumetric analysis. CT scans were performed at weeks 0 and 8 under isoflurane ventilatory anesthesia (Quantum GX II in vivo micro CT, PerkinElmer Inc., USA). The cross-sectional area of filler mass was automatically computed using the INFINITT PACS system (INFINITT Healthcare, Korea), and the object volume was estimated by integrating the areas measured at regular intervals (32) (Fig. 3A). The volume persistence rate was calculated as Vol_{week8}/Vol_{Week0}.

Ultrasound evaluations were performed at 2-week intervals under isoflurane ventilatory anesthesia (DC-80, X-insight, Mindray, China). Measurements were performed by the same author each time. Using a convex probe, the width of the filler mass was measured in the long- and short-axis views, and the height was measured by the maximal vertical projection, which was perpendicular to the width (Fig. 3B). The persistence rate was obtained separately for the height and width and was calculated as the ratio of the measured values of week 8 to week 0. Due to irregular geometric shape of the filler mass, it was not appropriate to calculate volume by substituting the measured value into the formula for an ellipsoid. The ratio of height to width, used to estimate the shape of mass, was calculated at 2-week intervals to evaluate the shape change.

Figure 3. Volume measurements in computed tomography (CT) scans and ultrasound.

(A) In the CT scans, the cross-sectional area of the filler mass was automatically computed using the INFINITT PACS system, and the object volume was determined by integrating the areas measured at regular intervals.

(B) In the ultrasound, the width of the filler mass was measured in the long- and short-axis views, and the height was measured by the maximal vertical projection, which was perpendicular to the width.



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Histologic Analysis

Fillers with overlying skin and underlying subcutaneous tissue were excised under isoflurane ventilatory anesthesia at weeks 4 and 8, and then mice were euthanized. The excised tissues were fixed in 10% formalin and embedded in paraffin. After sectioning at 6 μm, the sections were stained with hematoxylin and eosin (H&E). Immunohistochemical staining for CD 31 was performed to evaluate neovascularization. Microscopic evaluation was performed in a blinded manner by a pathologist. On microscopic examination, inflammatory cell infiltration, fibrous capsule, fibrous invasion, and neovascularization were assessed and scored semiquantitatively using a grading system (−: none, +: mild, ++: moderate, +++: severe) (27,34).

Statistical analysis

Statistical analysis was performed using SPSS version 26 (IBM Corp.), with statistical significance set at $p < 0.05$. The Kruskal–Wallis test was used to compare the filler groups, and post-hoc testing was performed using the Bonferroni method.

RESULTS

Characterization of micronized acellular dermal matrix

Particles of dermis powder had an average size of 664.2 ± 389.9 μm (Fig. 4). The surface morphology on scanning electron microscope demonstrated that the dermis powder had an irregular spiculate shape of non-uniform size (Fig. 5).

Figure 4. Particle size and distribution of dermis powder.

Particles of dermis powder had an average size of 664.2 ± 389.9 μm.

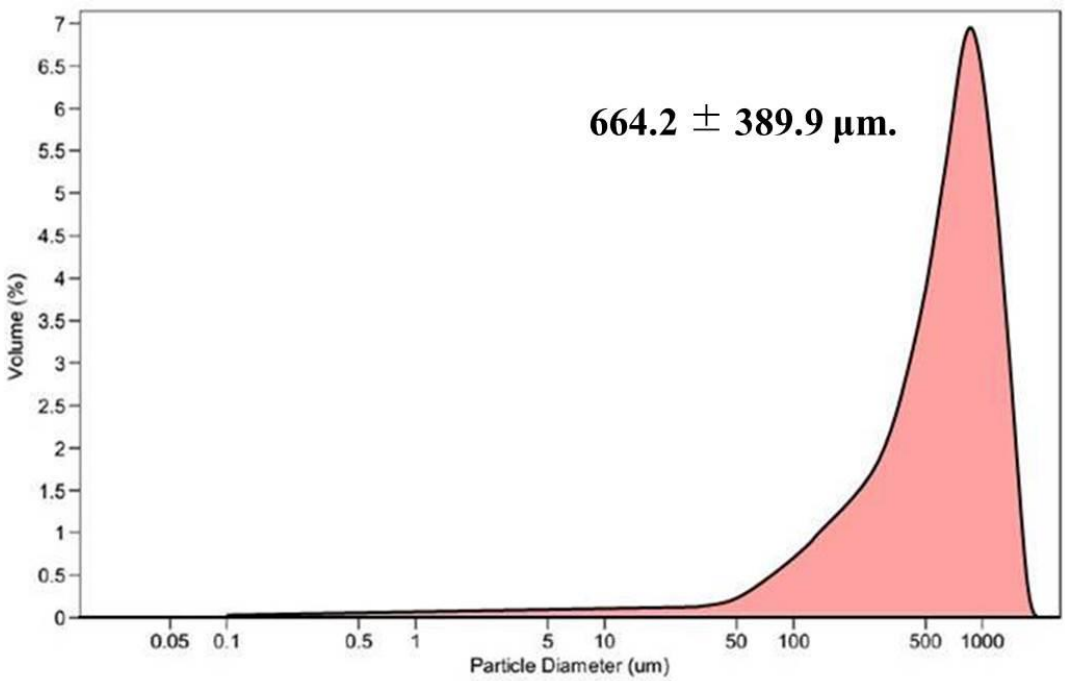
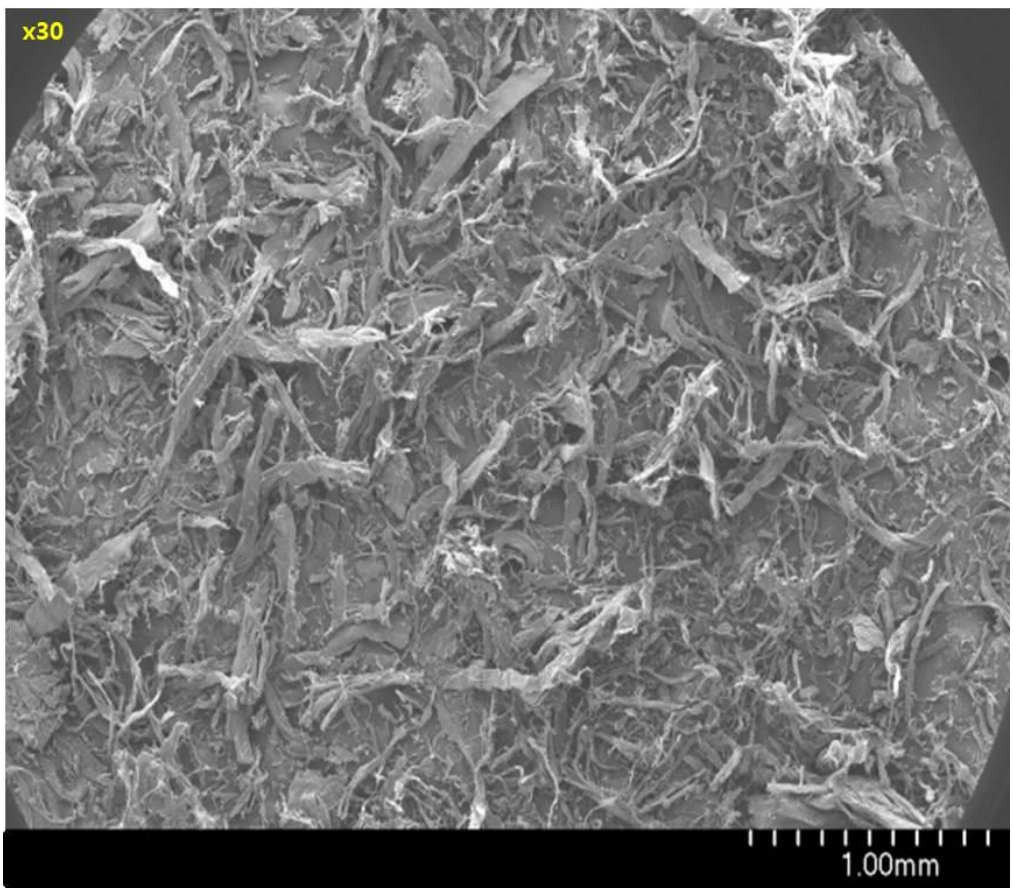


Figure 5. Surface morphology of the dermis powder.

The surface morphology on the scanning electron microscope demonstrated that the dermis powder had an irregular spiculate shape of non-uniform size.



Rheological measurements

The variation of the elastic modulus (G′) and complex viscosity (η*) with the applied force for each of the evaluated fillers are shown in Fig. 6-7. The interpolated values at 5 Hz are summarized in Table 2. The elastic modulus (G′) is a quantitaive measurement of gel stiffness and a product with high G′ has ability to resist deformation under applied pressure. Complex viscosity (η*) measures gel's ability to resist shearing force during and after injection and product with high η* spread less easily (31). All tested fillers showed shear thinning, which is a thin-out phenomenon meaning that η* decreases proportionally to the applied shear force. Paste-type micronized ADM (mADM, mADM+GEL) had higher G′ and η* than conventional fillers (COL+PMMA, HA). Because the vertical axis is scaled logarithmically, absolute numerical differences in value are larger than they appear. Paste-type micronized ADM had 3~5 fold higher G′ and η* than conventional fillers. Among the paste-type micronized ADM, mADM had higher G′ and η* than mADM + GEL, but differences were not significant as with conventional fillers.

Extrusion force measurements

Extrusion force profiles along the length of the syringe are shown in Fig. 8. For mADM+GEL, the extrusion force curve was smooth, suggesting a consistent extrusion force across the distance. By contrast, mADM displayed extrusion force profiles with high variability. The average force needed to extrude the entire sample was higher for mADM (18.65±5.45 N) than for mADM+GEL (11.35±2.13 N).

Figure 6. Variation of elastic modulus with a frequency of applied force for each filler.

The paste-type micronized ADM (mADM, mADM+GEL) had higher G′ than conventional fillers (COL+PMMA, HA) at interpolated values of 5 Hz. Among the paste-type micronized ADM, mADM had higher G′ than mADM + GEL.

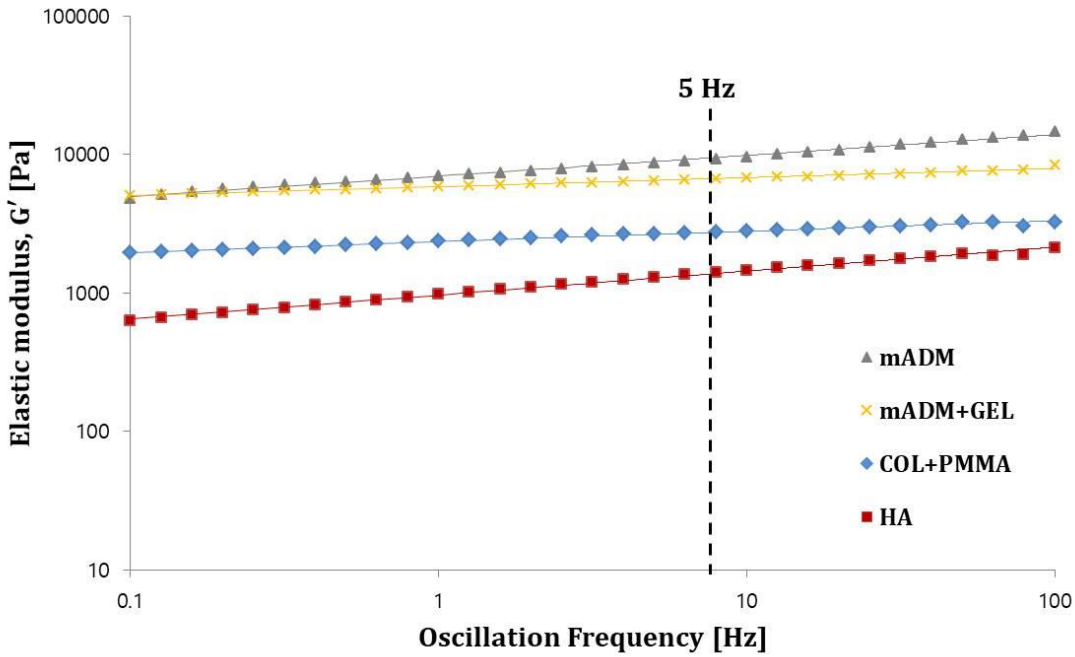


Figure 7. Variation of complex viscosity with a frequency of applied force for each filler.

The paste-type micronized ADM (mADM, mADM+GEL) had higher η* than conventional fillers (COL+PMMA, HA) at interpolated values of 5 Hz. Among the paste-type micronized ADM, mADM had higher η* than mADM + GEL.

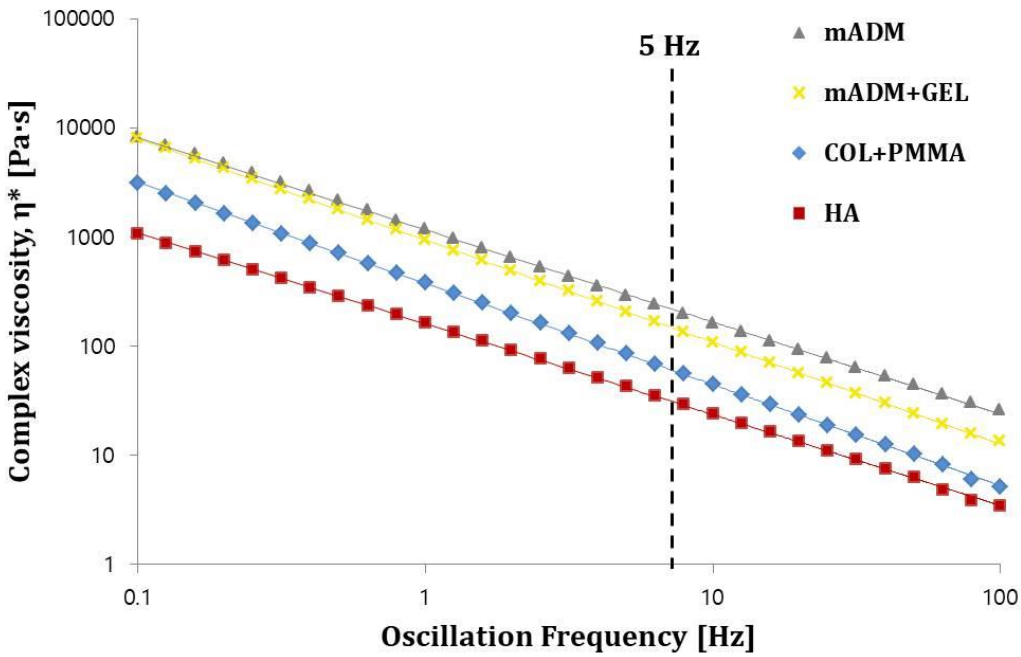
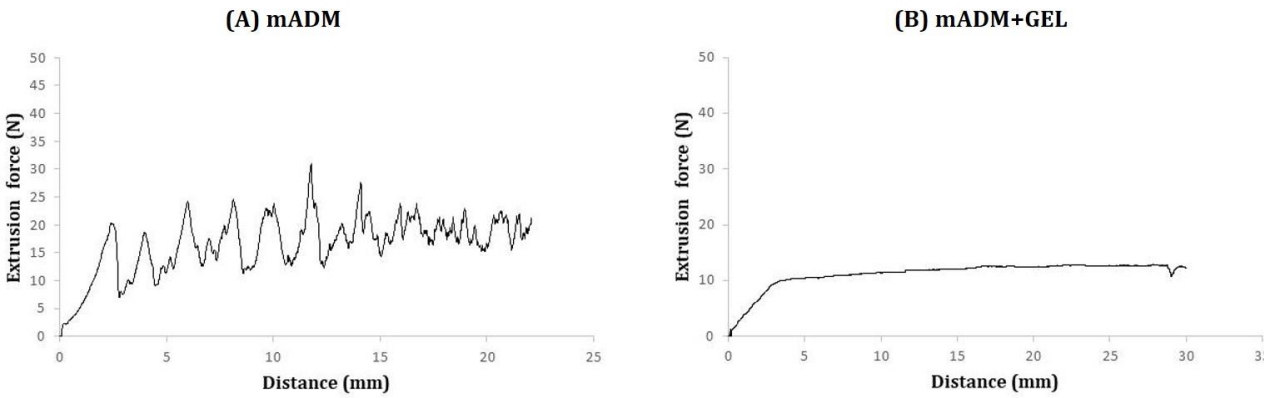


Table 2. Rheologic values for each fillers interpolated at 5 Hz

	G′ (Pa)	η* (Pa·s)
COL+PMMA	2680	85.7
HA	1300	42.8
mADM	8640	289
mADM+GEL	6460	206

Figure 8. Extrusion force of mADM and mADM+GEL.

(A) mADM displayed extrusion force profiles with high variability. The average force needed to extrude the entire sample was 18.65±5.45 N in mADM. (B) The extrusion force curve for mADM+GEL was smooth and even, suggesting a consistent extrusion force across the distance. The average force needed to extrude the entire sample was 11.35±2.13 N in mADM+GEL.



Animal experiments

The mice tolerated the experiment well, except for two who died during anesthesia. No injection-related complications, including infection or ulceration, were observed. Injections created a subcutaneous mound that was adequately spaced symmetrically on the paraspinal dorsum and initially showed uniform size among the filler types. As the study progressed, flattening of the mound occurred in different patterns depending on the filler type (Fig. 9). COL+PMMA started to decrease remarkably in height 2 weeks after implantation and disappeared by week 8 in all mice; hence, no dome was found externally. For HA, there was a slight decrease in size until week 8, but height was relatively maintained. mADM showed a rather broadened pattern, and mADM+GEL showed a reduction in overall size, decreasing both height and width. All implants, except HA, remained at the injection site, but HA migrated significantly in 40% of the mice. In the mADM group, neovascularization was conspicuous from week 4 and was observed with the naked eye in week 8. Biopsy specimens from week 8 were compared with each other (Fig. 10). Most of the volume of COM+PMMA was absorbed, and the remaining small amount was present in a flat form with an opaque appearance. HA maintained a convex shape wrapped in a transparent membrane. In mADM and mADM+GEL, yellow to white color material existed in a dome shape with prominent blood vessels on the surface.

Figure 9. Comparison of the gross morphology of injected fillers.

(A) In week 0 and week 8 of COL+PMMA, the mass had disappeared, and no dome could be found externally at week 8. (B) Week 0 and week 8 of HA, showed a slight decrease in the size until week 8, but the height was relatively maintained. (C) Week 0 and week 8 of mADM, mADM showed a rather broadened pattern, and neovascularization could be observed with the naked eye. (D) In week 0 and week 8 of mADM+GEL, mADM+GEL appeared to have reduced overall size, decreasing both height and width.

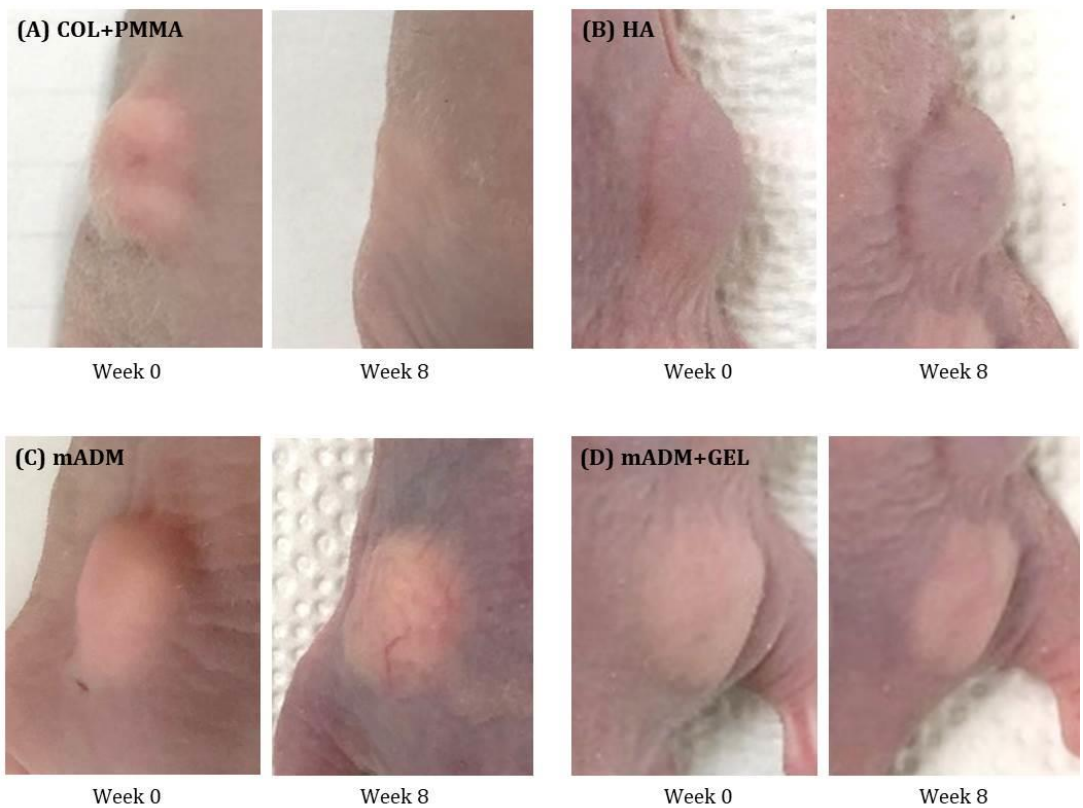


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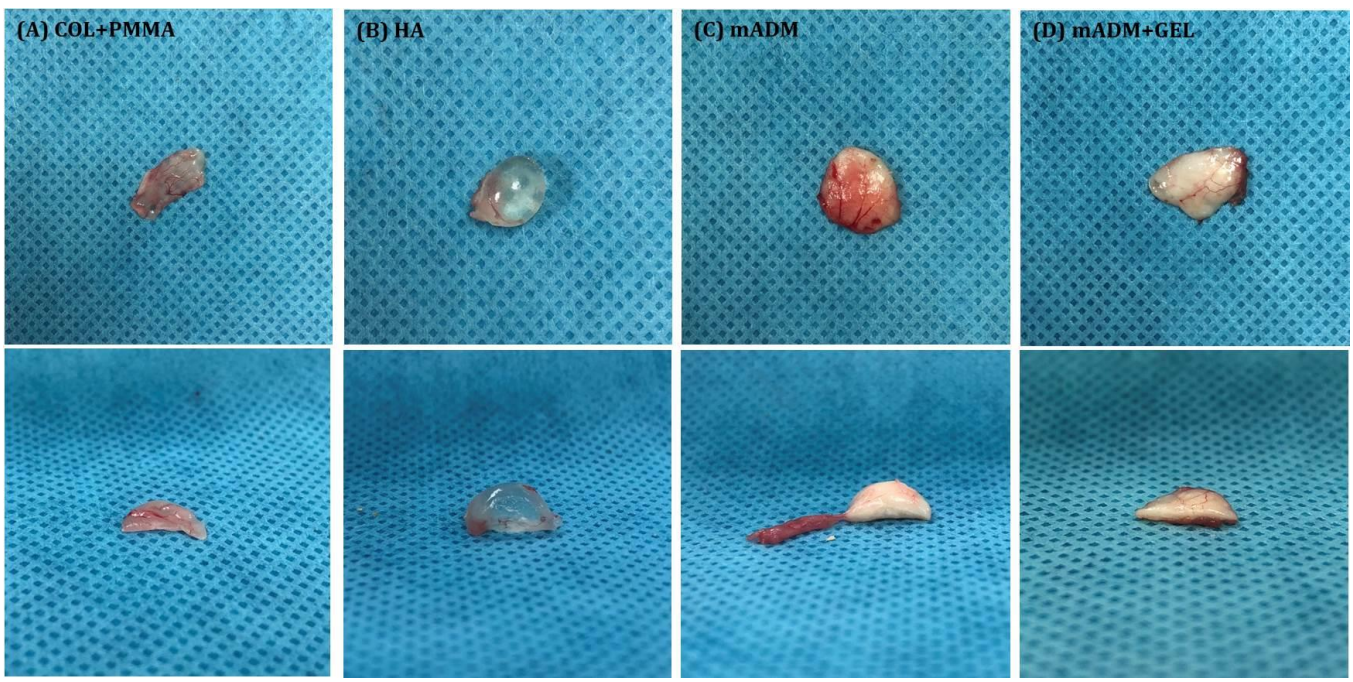
Figure 10. Biopsy specimens of each filler at week 8.

(A) Most of the volume of COM+PMMA was absorbed, and the remaining small amount was present in a flat form with opaque appearance. (B) HA maintained a convex shape wrapped in a transparent membrane. (C,D) In mADM and mADM+GEL, yellow to white color material existed in a dome shape, and prominent blood vessel on the surface was characteristic.



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Volumetric Analysis

A total of 10 masses per filler type were used for analysis, except for mice that died during anesthesia or were sacrificed for biopsy. In the CT scan, all implants showed the same homogenous attenuation regardless of the filler type and had a different attenuation from the surrounding tissue so that the boundary could be traced relatively easily (Fig. 11). The volume persistence rate was calculated by the volume ratio of week 8 and week 0, and there was a significant difference among the filler types ($p=0.016$) (Fig. 12). COL+PMMA appeared to have disappeared in gross observation, but 17.65±2.00% of the original volume was traced in the CT scan; HA showed retention of 85.09±3.35%. Compared with commercial fillers, mADM slightly increased in volume (113.54±3.50%), and mADM+GEL was reduced by 51.12±3.04%.

Figure 11. Representative CT images of each type of filler.

In the CT scan, all implants showed the same homogenous attenuation regardless of the filler type and had a different attenuation from the surrounding tissue, so the boundary could be traced relatively easily.

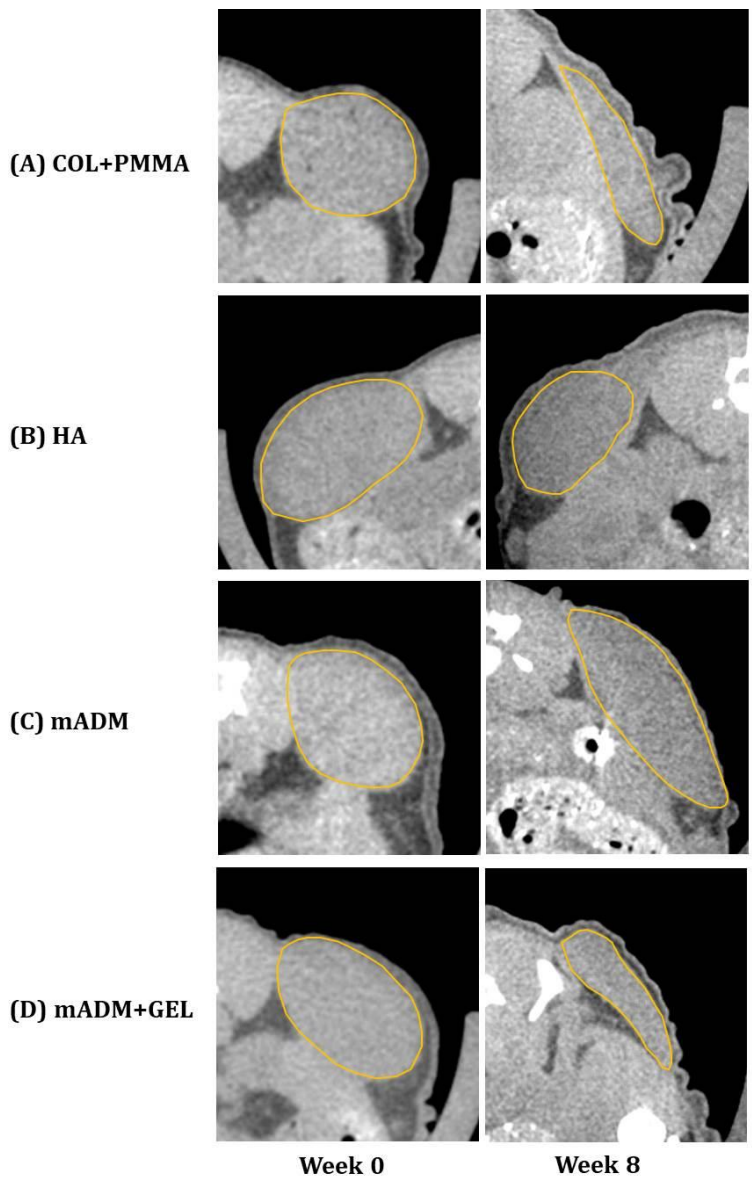
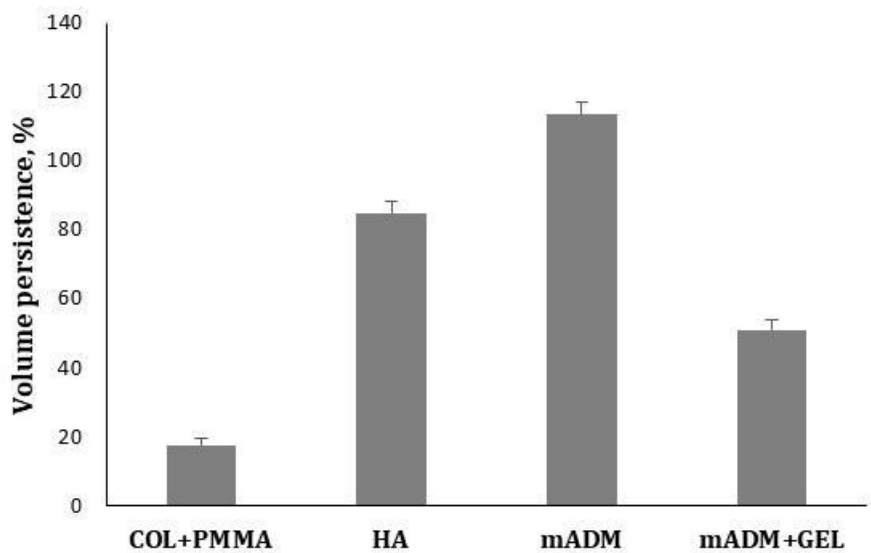


Figure 12. Volume persistence rate of each type of filler.

It was calculated by the volume ratio of week 8 and week 0 in CT scans, and there was a significant difference among the filler types (p=0.016).



Material from: Chang, L.S., Kim, S.H., Kim, H. *et al*. Evaluation of Paste-Type Micronized Acellular Dermal Matrix for Soft Tissue Augmentation: Volumetric and Histological Assessment in a Mouse Model. *Aesth Plast Surg* (2022) (33)

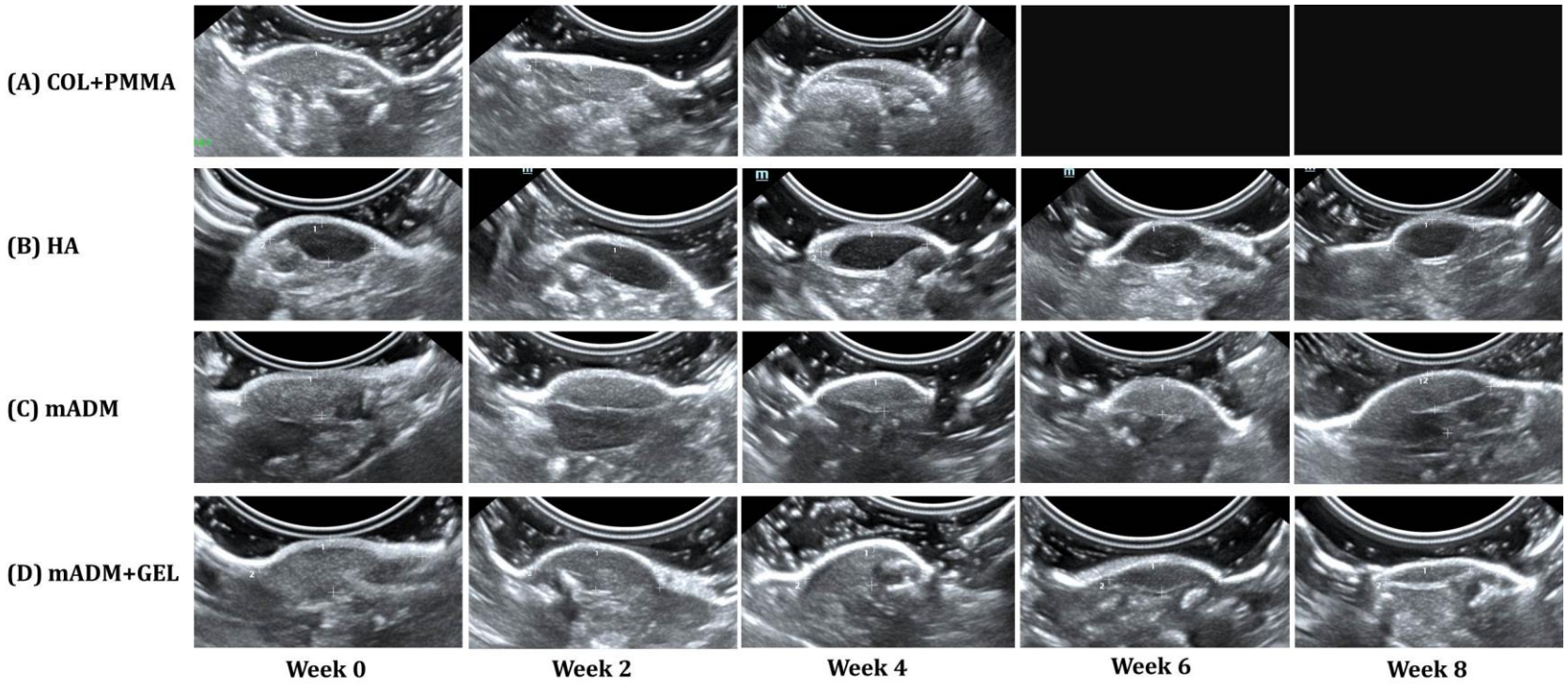
Changes in height and width were analyzed using ultrasound every 2 weeks. All implants showed homogenous echogenicity and were hypoechoic compared with the surrounding tissues in the ultrasound scans. In particular, HA was the most hypoechoic owing to its high water content and was clearly distinguished from other types of fillers. Regardless of the type, they had a similar softness, and care was taken to avoid compressing them by the probe (Fig. 13). The height and width persistence rates were calculated as the ratio of week 8 to week 0 (Fig. 14). The rate of change between height and width differed based on the filler type when the persistence rate was compared. In COL+PMMA, there was no significant difference in the persistence rates of height (5.22%) and width (6.76%). COL+PMMA showed a sharp decrease in both height and width from the 2^{nd} week and disappeared to the extent that it was impossible to detect by ultrasound in the 8^{th} week. HA showed a higher persistence rate in height maintaining almost to the initial value while the width decreased to 79.66% of the baseline. On the other hand, the persistence rate of width was increased to 115.87% in mADM, while the height was preserved at 97.92%. The mADM+GEL showed a steady decreasing trend in both height and width from the beginning, but the final persistence rate differed in height and width, which is much higher in width, 91.03% than 58.98% of the height.

The difference in the persistence rate in height and width reflects the change in the shape of the mass. The height-to-width ratio was used to evaluate the mass shape quantitatively (Fig 15). The higher ratio value means a more convex shape, while the lower ratio value means a more spreading pattern. In the initial phase after

injection, the ratio was high in the order of mADM, mADM+GEL, HA, and COL+PMMA. The ratio value increased gradually in HA while decreased in others. In 8th week, the ratios appeared in the order of HA, mADM, mADM+GEL, and COL+PMMA, consistent with the shape change in gross morphology. Over time, Paste-type micronized ADM (mADM, mADM+GEL) had a stronger tendency to spread in the injection plane than HA.

Figure 13. Representative ultrasound image of each type of filler.

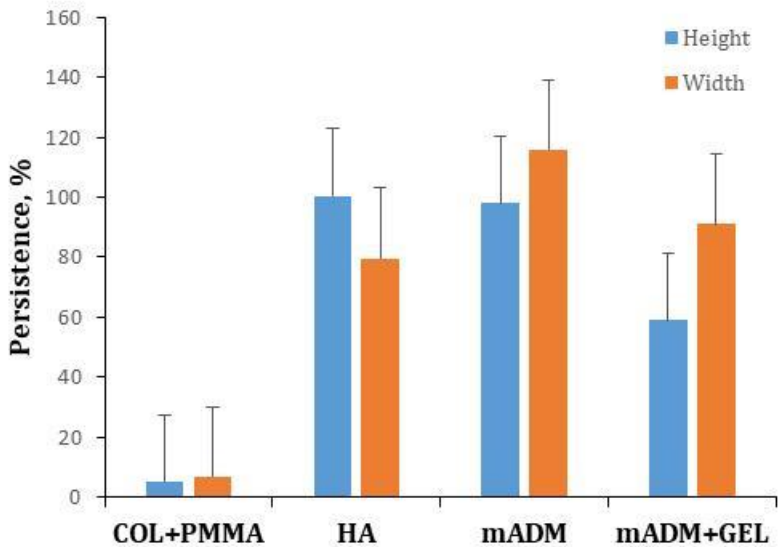
It represented a serial change of height and width of the implant



Material from: Chang, L.S., Kim, S.H., Kim, H. *et al.* Evaluation of Paste-Type Micronized Acellular Dermal Matrix for Soft Tissue Augmentation: Volumetric and Histological Assessment in a Mouse Model. *Aesth Plast Surg* (2022) (33)

Figure 14. Height and width persistence rate compared between week 8 and week 0.

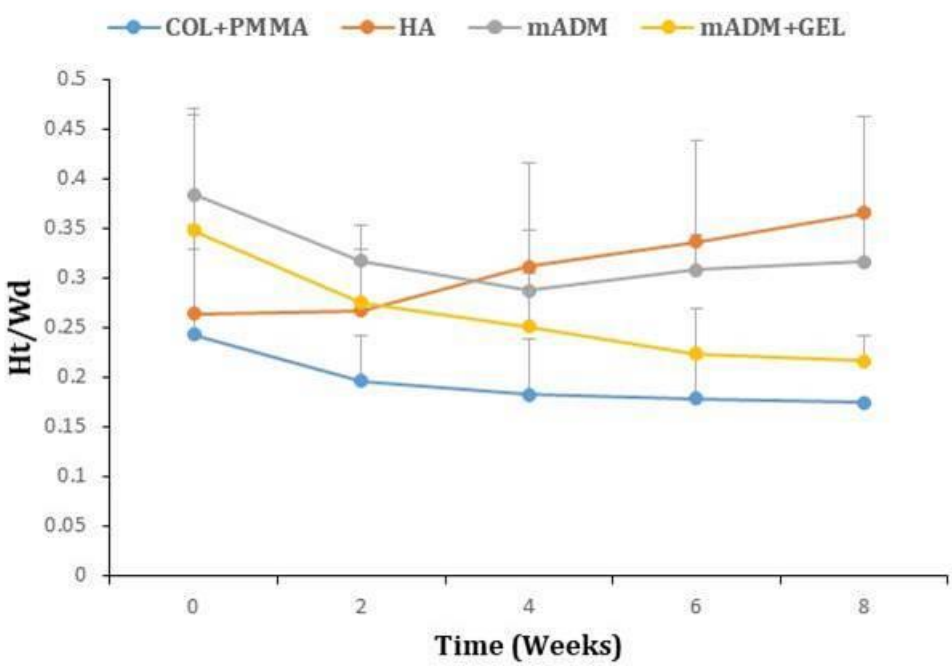
The rate of change between height and width differed based on the filler type.



Material from: Chang, L.S., Kim, S.H., Kim, H. *et al*. Evaluation of Paste-Type Micronized Acellular Dermal Matrix for Soft Tissue Augmentation: Volumetric and Histological Assessment in a Mouse Model. *Aesth Plast Surg* (2022). (33)

Figure 15. The ratio of height to width along the time course.

The ratio of height to width was used to evaluate the shape of mass quantitatively. In the initial phase after injection, the ratio was high in the order of mADM, mADM+GEL, HA, and COL+PMMA. The ratio value increased gradually in HA while decreased in others. In 8th week, the ratios appeared in the order of HA, mADM, mADM+GEL, and COL+PMMA, which was consistent with the change of shape in gross morphology.



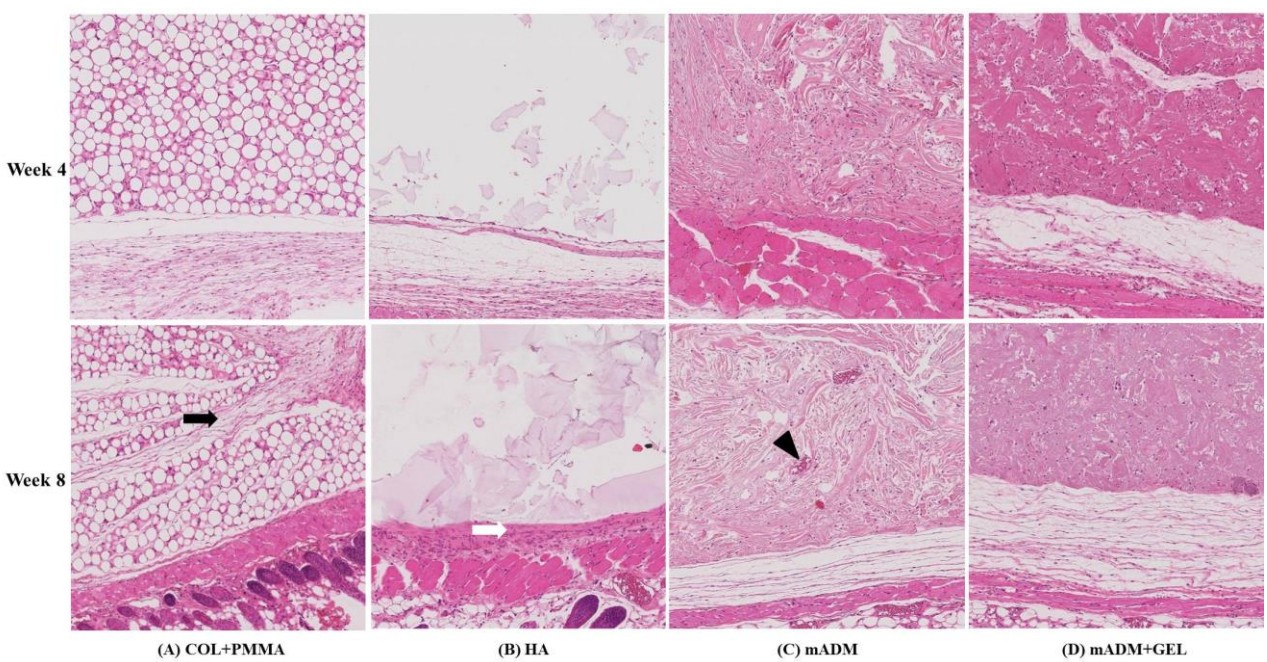
Histologic Analysis

In the case of COL+PMMA, most of the collagen was already absorbed by the 4th week, and PMMA microspheres in the form of translucent round cavities occupied most of the mass (Fig. 16). Inflammatory cells, including polymorphonuclear leukocytes and macrophages, infiltrated the microspheres. In the 8^{th} week, cell infiltration was maintained, and the fibrous septa invaded and split the implant. Fibrous capsules containing two to three layers of fibroblasts and collagen were formed around the implant. HA appeared as an amorphous foreign body, and many parts dropped during the slide production. Inflammatory cell infiltration or fibrous invasion was hardly observed over time, and the uniform fibrous capsule became markedly thicker at week 8. In mADM, micronized ADM particles appeared disarranged with elongated fragments. Inflammatory cell infiltration, including polymorphonuclear leukocytes and macrophages, was moderate at week 4 and was found mainly at the junction of the host tissue and mADM. At week 8, the number of inflammatory cells decreased, and the space between the particles was filled with fibrous tissue as fibroblast invaded the mADM. A thin fibrous capsule around the implant was formed, and newly formed capillaries were observed. mADM+GEL showed a different histomorphology from mADM due to the gelatin content but showed a similar inflammatory reaction. Moderate cell infiltration decreased from week 4 to week 8, indicating invasion of fibrous tissue. Immunohistochemical staining with CD31 showed active neovascularization in mADM and mADM+GEL in a time-dependent manner, which was not observed in HA. $CD31^{+}$ endothelial cells were scattered throughout the mass without luminal

structure in week 4, but luminal structures with the thickened wall were prominent in week 8 (Fig. 17) (Table 3).

Figure 16. Representative microscopic findings of skin and subcutaneous tissues with H&E staining.

(A) COL+PMMA maintained inflammatory cell infiltration, and fibrous septa invaded and split the implant at week 8 (black arrow). (B) In HA, inflammatory cell infiltration or fibrous invasion could hardly be observed over time, and the uniform fibrous capsule became thicker markedly at week 8 (white arrow). (C) In mADM, cell infiltration was moderate at week 4, but inflammatory cells decreased at week 8. The space between the particles was filled with fibrous tissue, and a newly formed capillary was found (arrow head). (D) mADM+GEL showed a similar inflammatory reaction with mADM (H&E staining, original magnification, ×200).

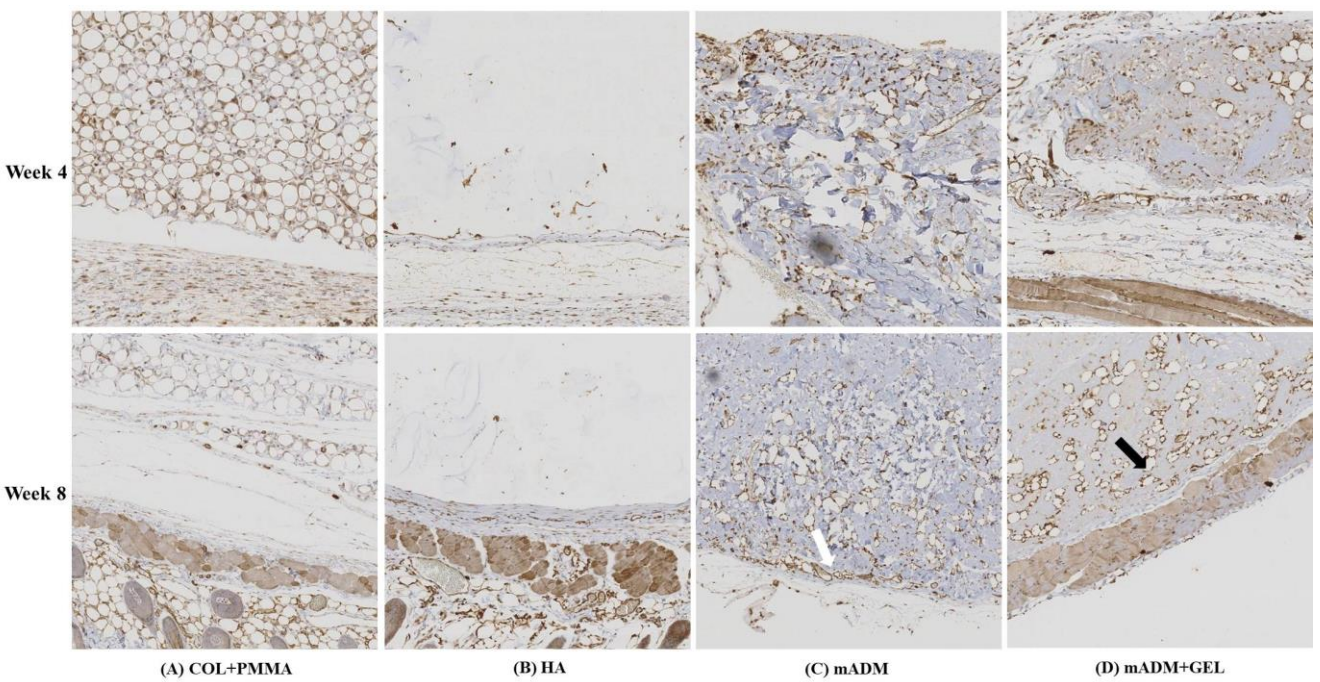


Material from: Chang, L.S., Kim, S.H., Kim, H. *et al.* Evaluation of Paste-Type Micronized Acellular Dermal Matrix for Soft Tissue Augmentation: Volumetric and Histological Assessment in a Mouse Model. *Aesth Plast Surg* (2022) (33)

Figure 17. Representative microscopic findings of skin and subcutaneous tissues with CD31 staining.

(A) COL+PMMA showed minimal capillary proliferation at week 8. (B) In HA, any neovascularization was found at weeks 4 and 8. (C) mADM showed marked capillary proliferation in a time-dependent manner (white arrow).

(D) mADM+GEL showed marked capillary proliferation in a time-dependent manner (black arrow). (CD31, immunohistochemical staining, original magnification, ×200).



Material from: Chang, L.S., Kim, S.H., Kim, H. *et al.* Evaluation of Paste-Type Micronized Acellular Dermal Matrix for Soft Tissue Augmentation: Volumetric and Histological Assessment in a Mouse Model. *Aesth Plast Surg* (2022) (33)

Table 3. Histological analysis using semi-quantitative soring

	Inflammatory cell infiltration		Fibrous capsule		Fibrous invasion		Neovascularization	
	4week	8week	4week	8week	4week	8week	4week	8week
COL+PMMA	++	++	-	+	-	+	-	+
HA	-	-	+	++	-	-	-	-
mADM	++	+	-	+	+	++	++	+++
mADM+GEL	++	+	-	+	+	++	++	+++

-: none, +: mild, ++: moderate, +++: severe

Material from: Chang, L.S., Kim, S.H., Kim, H. *et al.* Evaluation of Paste-Type Micronized Acellular Dermal Matrix for Soft Tissue Augmentation: Volumetric and Histological Assessment in a Mouse Model. *Aesth Plast Surg* (2022) (33)

DISCUSSION

ADM is a powerful scaffold material in the reconstructive and aesthetic surgery because of its versatile use. The conversion of these materials into an injectable form is a logical extension to extend its indication. The injectable form has the advantages of adaption to irregularly shaped defects via minimally invasive procedures, thus minimizing the risk of infection and scarring (35). Injectable materials may be in the form of solutions, pastes, gels, or microparticles and efforts have been made to find effective formulation in the tissue engineering part (36). However, the powder-type formulation of ADM has not gained popularity as soft tissue filler due to the inconvenience of use and unpredictable duration. In this study, micronized ADM was suspended in sterile distilled water or mixed with gelatin to increase convenience and injectability by making it paste. Paste formulation provides convenience to apply and can reduce the risk of material leakage unlike liquid or powder type (37).

Gelatin is a protein derived from collagen and is the major constituent of the extracellular matrix in skin, bone, and connective tissue. It can exhibit *in vivo* resorbability and easily modulate physicochemical properties. These characteristics make it an ideal polymer matrix of composite materials for tissue regeneration. It is widely used as an injectable carrier by developing a gel with suitable injectability and providing biochemical cues for cell adhesion and proliferation. The

hydroxyapatite-gelatin composite was developed as synthetic filler to regenerate bone and cartilage defects (38). The injectable hydrogel of novel agar and gelatin has been demonstrated to be effective as a filler of nerve guidance channels to improve the nerve regeneration process (37). Although gelatin adding micronized ADM has been proven to be effective in wound healing as an effective dressing material (26), this study is the first to evaluate its effect on soft tissue augmentation.

The volume persistence rate in CT evaluation was found to be in the order of mADM, HA, mADM+GEL, and COL+PMMA. Although mADM+GEL had lower volume retention than HA, it showed much higher volume maintenance than COL+PMMA. The volume of mADM increased by 15%, which might be due to the active invasion of fibrous tissue, as supported by the histology results. It is well known that ADM promotes the ingrowth of native cellular elements and neovascularization by providing an extracellular matrix scaffold in studies on the wound-healing effect of ADM (39-41). Even when injected as a bolus in this study, tissue replacement without center necrosis resulted in volume maintenance comparable to commercial fillers. This indicates that paste-type micronized ADM (mADM, mADM+GEL) has the potential for soft tissue augmentation.

It is important to evaluate the change in shape as well as volume maintenance to predict clinical performance as a filler. Fillers with greater projection may be more useful as volumizers in a deeper plane, and those with less lift are more suitable for superficial applications, including the filling of wrinkles (42). Previous studies

have reported that rheological properties are clinically relevant to filler behavior during and after injection (43,44). Firm gels with high G´ and η* provide better resistance to deformation under skin tension resulting in greater lifting capacity (45). In an attempt to account for any relationship between rheological properties and the clinical performance of filler, the correlation between the rheologic value of G′, η*and the ratio of height to width, volume persistence rate was analyzed (Fig. 18-19). The ratio of height to width is a measure of the clinical behavior representing the lifting capacity, whereas volume persistence rate represents the duration of the filler. There is a linear correlation between the value of G′, η*, and the ratio of height to width in week 0 while rheologic data does not seem to correlate with the ratio of height to width in week 8 and volume persistence rate. This result indicates that the ratio of height to width in week 8 and volume persistence rate are affected by other combined factors, including polymer concentration and fibrous invasion. Rheological properties can be helpful in predicting clinical behavior in the early phase of injection. However, this interpretation should consider the fact that the direct comparison of rheological data may not be accurate due to the large difference in particle size between the mADM group and the conventional fillers, and that the comparison between mADM and mADM+GEL, which have similar properties, showed a high correlation even at the 8^{th} week.

Figure 18. Relationship between elastic modulus G′ and the ratio of height to width, volume persistence rate.

There is a linear correlation between the value of G′ and the ratio of height to width in week 0 while G′ does not seem to correlate with ratio of height to width in week 8 and volume persistence rate.

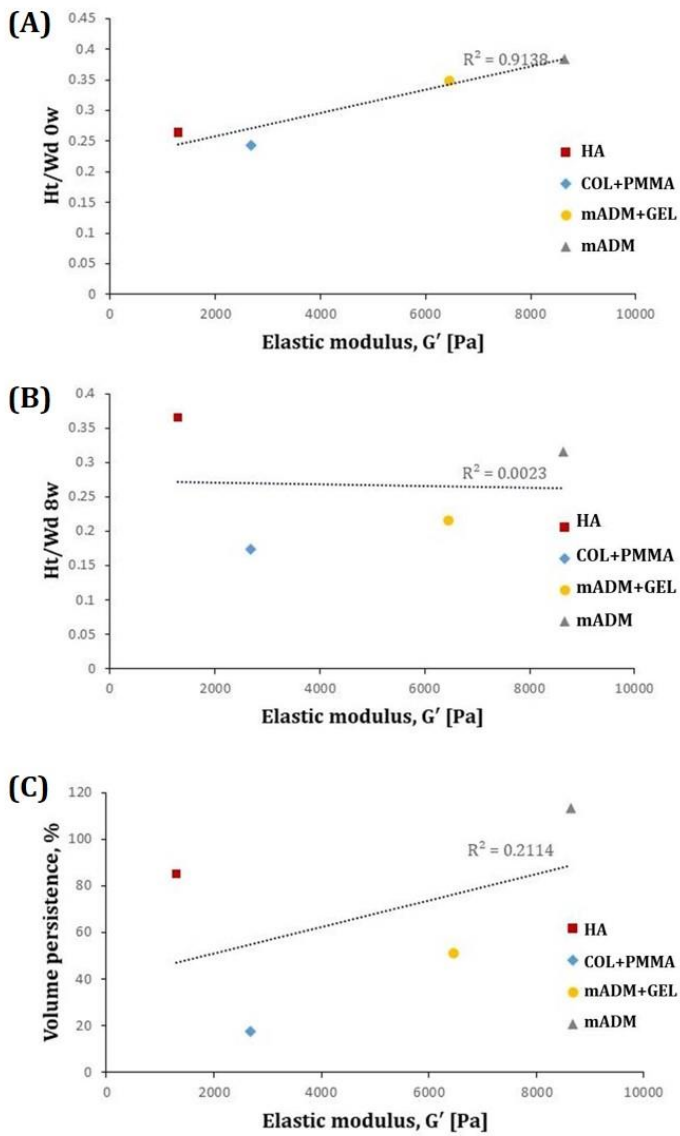
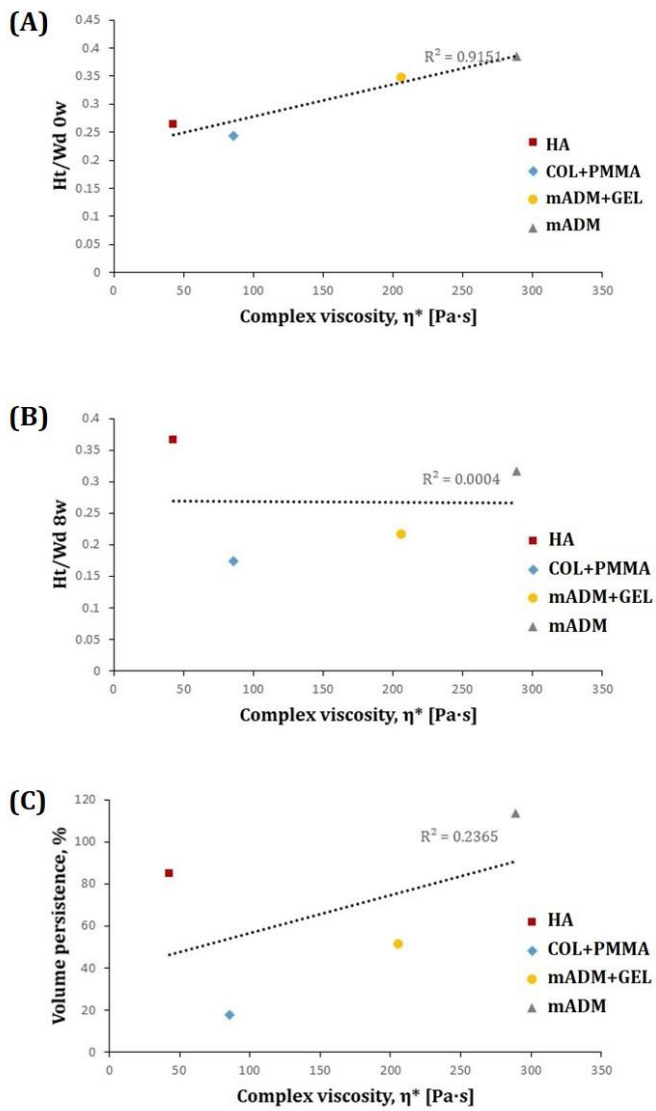


Figure 19. Relationship between complex viscosity η* and the ratio of height to width, volume persistence rate.

There is a linear correlation between the value of η* and the ratio of height to width in week 0 while η* does not seem to correlate with ratio of height to width in week 8 and volume persistence rate.



In this study, it was expected that adding gelatin would improve the volume maintenance and lifting capacity by acting as a biodegradable scaffold and increasing the viscosity. However, contrary to expectations, mADM+GEL showed lower volume and height maintenance than mADM in the volumetric analysis. Although gelatin did not significantly contribute to volume maintenance through tissue replacement, it should also be considered that the reduced fraction of mADM could be responsible. The only difference between the two products was the inclusion of gelatin and the reduced fraction of mADM. Histologically, the degree of inflammation between the two groups did not differ significantly, suggesting that the fraction of mADM affects the volume maintenance rate. According to the rheologic measurement results, the addition of gelatin resulted in lower elasticity and viscosity to make the material more spreadable. In addition, gelatin can also lower the extrusion force meaning that it can be injected smoothly, resulting in reduced pain and blood vessel damage during injection. Consequently, adding gelatin can be used to modify the gel's softness or decrease extrusion force. As a result, paste-type micronized ADM is considered suitable for filling the depressions or fine lines rather than volumization considering its tendency to disperse and less likely to create visible edges and bumps.

In histological analysis, the inflammatory response in mADM and mADM+GEL resembled the wound healing process in which the initial inflammatory burst was replaced by a later proliferative phase with predominant fibroblast and endothelial cells. This was different from COL+PMMA, which maintained moderate

inflammation with polymorphonuclear leukocytes and macrophages throughout the experimental period, and HA that had no inflammatory cell infiltration.

Revascularization is a crucial factor conferring tissue regeneration ability to ADM. Neovascularization in the mADM and mADM+GEL was confirmed by CD 31 immunohistochemical staining, which is a marker for endothelial cells. The density of endothelial cells increased, and luminal structure with thickened wall was found in week 8. In addition to mature vascular network, the identification of mature and functional endothelial cells is required for microcirculation establishment. The tube formation or sprouting assays can be used for assessing the functionality of endothelial cells and staining with VE-cadherin and vonWillebrand factor (VWF) for assessing the maturity (46).

HA showed a thick and smooth capsule surrounding the implant, which seemed to contribute to significant migration and a well-disclosed HA boundary. In contrast, different degrees of fibrous invasion and neovascularization were found in COL+PMMA and paste-type mADM. Although this has a positive effect on tissue replacement, it can also be a disadvantage because it is difficult to remove when necessary.

Accurate volume estimation is essential to evaluate the effect of filler on soft tissue augmentation, and several methods have been used to measure the volume of injected materials. Calipers or rulers are used to measure the height, width, and length of the mass at the skin surface, and the volume is calculated mathematically using a formula for hemi-ellipse or ellipsoid (47,48). This method is an indirect

measurement of material, and skin thickness may affect the result. In particular, the accuracy can be lowered at low height mass. Ultrasound is a reliable imaging modality for skin science and can obtain high-resolution images of skin and the underlying tissues (49). The length information of the internal mass can be measured relatively accurately from parallel cross-sectional areas. However, the volume estimation by calculating the formula can be less accurate due to the irregular geometry of the mass. CT and magnetic resonance imaging (MRI) can offer the most accurate means of estimating *in vivo* volumes and previous study have proven the accuracy of the simple technique, summation of cross sectional areas (32). CT was chosen for volume estimation in this study due to the precise definition of mass contour, short imaging time, and cost. However, frequent scans were avoided because CT scans required deep and continuous anesthesia. Serial changes in width and height were measured using ultrasound at short intervals.

Recent studies have utilized micronized ADM as a carrier for cellular components to increase graft stability and longevity. Lee et al. cultured preadipocytes in a acellular allogenic dermis and confirmed that three-dimensional proliferation of progenitor cells formed a greater amount of fat tissue (50). Park et al. showed that survival of grafted fat increased when injected with micronized ADM (29). Zhang et al. fabricated engineered particulate dermal substitute (EPDS) using the micronized ADM and human fibroblast, and EPDS demonstrated improved wound healing and potential for soft tissue augmentation (51). Although effective, adding cellular components requires strict conditions and limitations in manufacturing and

clinical use. Studies on synthesizing biodegradable materials with micronized ADM are ongoing, and Kim et al. showed superior volume maintenance of HA-cross-linked micronized ADM compared to fat grafts (27).

Cymetra showed superior results for greater persistence than Zyplast (bovine collagen, Allergan, USA) in the intradermal and subdermal planes of injection in a short-term study (52). However, it is characterized by a more rapid loss in the early phase, which generally stabilizes with some degree of partial permanence due to the mechanism of its action. This inability to predict treatment response, the need for serial injection, and the suboptimal immediate result have been major impediments to the widespread use of Cymetra (53). When Cymetra was injected into the pharyngeal wall of pigs for augmentation, the result showing no augmentation demonstrated that it was not a durable implant at this site (54). The particle size of the micronized ADM used in this study was 664.2 ± 389.9 μm, which is quite large compared to 200 μm of Cymetra. Considering the small particle size of Cymetra, the relatively high-volume retention of the current micronized ADM compared to other commercial fillers might be related to the particle size. Further studies are needed to optimize particle sizes that are small enough for needle injection but large enough to avoid host phagocytosis.

This study has several limitations. Although COL+PMMA is a typical synthetic filler characterized by high durability, it showed very low volume retention in this study compared to that reported in the literature (55-57). COL+PMMA consists of

PMMA microspheres suspended in 3.5% bovine collagen. Dissolution of the collagen carrier, consisting of 80% of the total volume, occurs in the initial phase, causing an abrupt size decrease. New collagen encapsulated surrounding the PMMA sphere, followed by renewed defect correction (58). The short period of this experiment did not reflect the gradual correction of the COL+PMMA. In addition, longer follow-up is required for HA to show a sudden disappearance after isovolemic degradation and for mADM to further ensure volume maintenance through tissue replacement (58). Although many filler studies have been conducted within a short period, between 5 and 9 weeks, a long-term study can provide a more accurate evaluation (28,36,45).

Limitations of the mouse model may also cause the difference in the durability of COL+PMMA. The rodents' skin has higher elasticity than human (57.1±5.1% vs 37.2±4.1%) and is easily stretched in the tissue expansion study (59). In addition, the scant subcutaneous fat layer and loose space over the panniculus carnosus disperse the PMMA microsphere, preventing it from functioning as a nucleus for collagen encapsulation. In order to overcome the differences caused by the experimental model and increase feasibility, large animal studies or human clinical studies are needed.

Further studies should include an optimization process involving particle size of micronized ADM and ADM:gelatin ratio. A large animal model, including dog and pig can implement soft tissue depression to evaluate the effect of not only

augmentation but also depression correction. Finally, the effect as an actual soft tissue filler can be predicted through human clinical research.

CONCLUSIONS

The effectiveness of paste-type micronized ADM on soft tissue augmentation was demonstrated in this study. The paste-type micronized ADM exhibited volume maintenance by tissue replacement whose efficacy is comparable to that of commercial fillers. Further research should be conducted to determine the optimal particle size, the fraction of ADM, and carrier type for precise clinical application.

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국문 초록

서론: 페이스트형 미세화 무세포 동종진피는 주사 가능한 생물학적 조직 대체제 중의 하나로, 기존 조직의 재생을 촉진하여 결손부위를 복구함으로써 창상 치유에 효과가 있음이 입증되었다. 이 연구는 페이스트형 미세화 무세포 동종진피가 창상 치유 뿐 아니라 연조직 보강에도 효과가 있을 것으로 예상하고 이를 상용 필러와 비교 평가하는 실험을 마우스 모델에서 진행하였다.

방법: 증류수 혹은 젤라틴과 혼합한 페이스트형 미세화 무세포 동종진피 (mADM, mADM+GEL) 각각을 히알루론산 필러 (HA), 폴리메틸메타크릴레이트 필러 (COL+PMMA)와 비교하기 위해 상기 4가지 다른 종류의 필러를 누드 마우스의 등에 주입하고 체적의 유지 정도 및 생체 적합성을 평가 하였다. 8주의 실험 기간 동안 초음파와 컴퓨터 단층촬영을 사용하여 체적을 분석하였고 헤마톡실린과 에오신 및 CD31 염색 방법으로 조직학적 분석을 시행하였다.

결과: 8주간의 CT 분석으로부터 mADM과 mADM+GEL의 체적 유지율이 각각 113.54%와 51.12%로, 85.09%와 17.65%인 HA와 COL+PMMA에 비해 높게 유지됨을 확인하였다. 2주 간격의 초음파

결과에서는, mADM과 mADM+GEL이 너비보다 높이의 감소를 더 크게 보이면서 HA에 비해 주입 후 모양이 퍼지는 경향이 강하게 나타났다. 조직학적 평가에서는 mADM 및 mADM+GEL에서 상용 필러에 비해 월등한 기질의 침윤 및 신생혈관 형성을 보였다.

결론: 상기 실험 결과로부터 페이스트형 미세화 무세포 동종 진피가 상용 필러와 견줄 만한 연조직 보강 효과가 있음을 확인하였다. 이는 조직의 대체를 기전으로 하기 때문에 생체 적합성이 뛰어나고 장기간 체적을 유지할 수 있다는 점에서 페이스트형 미세화 무세포 동종진피는 고기능의 필러로써 개발될 수 있는 잠재력이 있다.

주요어: 피부 필러, 무세포 동종 진피, 마우스, 누드, 히알루론산, 폴리메틸메타크릴레이트

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