Thesis for the Degree of Master of Engineering

Development of dermal filler using cartilage extracellular matrix

by

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연골 세포외기질을 이용한 성형용 필러 개발

Advisor: Prof. Sang Hyug Park

by

Nahyeon Kim

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Development of dermal filler using cartilage extracellular matrix

A dissertation

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연골 세포외기질을 이용한 성형용 필러 개발

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요약

피부의 노화로 인해 콜라겐이 감소하여 진피층이 얇아져 탄력이 저하되고 주름이 생성된다. 이러한 주름들을 효과적으로 개선하고자 성형용 필러 연구가 활발하게 진행되어 왔다. 이상적인 필러란 생체적합하고 면역 반응이 일어나지 않으며 오래 지속되고 독성 없이 생체에서 분해되어야 한다. 히알루론산 필러가 가장 널리 사용되지만 세포외기질과 비교하여 지속 기간이 짧고 기계적 강도가 낮다는 단점이 있다. 연골의 세포외기질은 제 2 형 콜라겐과 글리코사미노글리칸, 프로테오글리칸 그리고 당단백질 등으로 이루어져 있어 압축 응력에 대한 높은 탄성을 가질 수 있다.

본 연구에서는 돼지 연골 세포외기질이 기존 히알루론산 필러의 빠른 분해도를 극복하고 생체 이식 후에 인가되는 물리적 자극에 잘 견디는 개선된 성형용 필러로서의 사용 가능 여부를 실험하기 위하여 미세구조, 기계적 성질 그리고 생체 적합성 실험을 수행하였다.

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1. Introduction

1.1. Skin anatomy

The skin is the main protective and immune organ with the largest cross-sectional area in the body. The skin is the body's largest organ, accounting for about 15% of the total adult weight.[1] It performs many vital functions, including protection against external physical, chemical, and biological assailants, as well as prevention of excess water loss from the body and a role in thermoregulation. The integumentary system is formed by the skin and its derivative structures.[2] The skin is composed of three layers as shown in figure 1: the epidermis, the dermis, and subcutaneous tissue. The outer most level, the epidermis, consists of a specific constellation of cells known as keratinocytes, which function to synthesize keratin, a long, threadlike protein with a protective role. The middle layer, the dermis, is fundamentally made up of the fibrillary structural protein known as collagen. The dermis lies on the subcutaneous tissue, or panniculus, which contains small lobes of fat cells known as adipocytes.[3] The thickness of these layers varies considerably, depending on the geographic location on the anatomy of the body. The eyelid, for example, has the thinnest layer of the epidermis, measuring less than 0.1mm, whereas the palms and soles of the feet have thickest epidermal layer, measuring approximately 1.5mm.[4] The dermis is thickest on the back, where it is 30-40 times as thick as the overlying epidermis.[5] Skin is besides protecting the body from water loss and microorganism infection, it has an important cosmetic role. Young

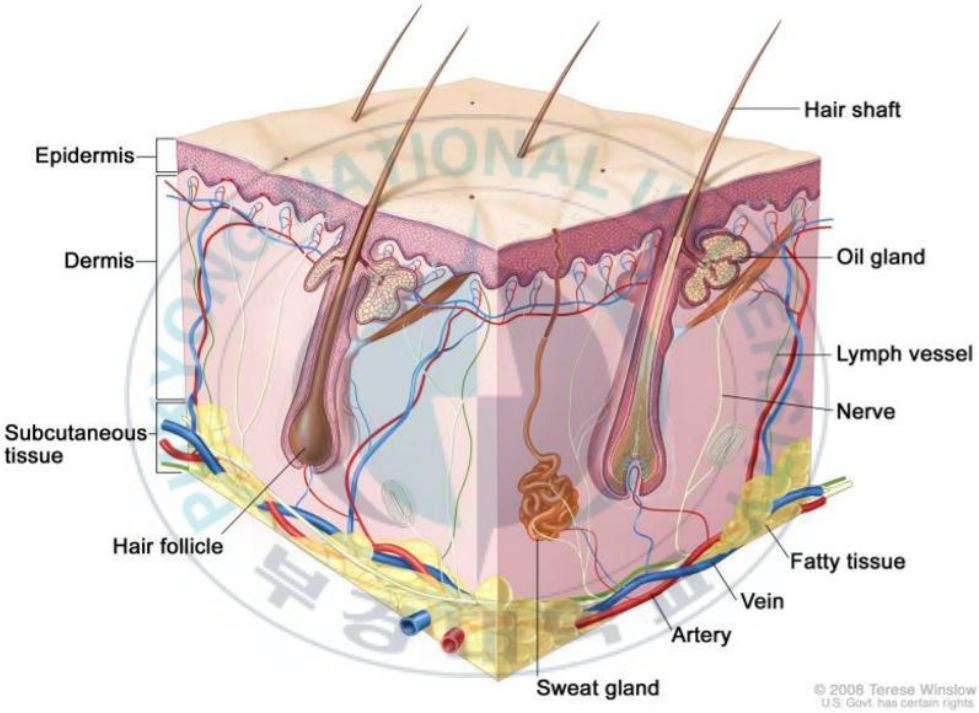

Figure 1. Anatomy of the skin [6]

and beautiful appearance may have a positive influence on people's social behavior and no exception for the skin. As the most voluminous organ of the body, the skin shows clear and visible sign of aging when one becomes older. Cutaneous aging is induced by both intrinsic and extrinsic factors.[7] Intrinsic aging is an inevitable physiological process that results in thin, dry skin, fine wrinkles, and gradual dermal atrophy, while reproductive status. However, aging of organs begins from the time when one is born, and there is extrinsic aging is engendered by external environment factors such as air pollution, smoking, poor nutrition, and sun exposure, resulting in coarse wrinkles, loss of elasticity, laxity, and rough-textured appearance.[8] Notably, long-term exposure to solar ultraviolet (UV) radiation is the primary factor of extrinsic skin aging and is referred to as photoaging.[9]

1.2. Dermal filler

The past decade has seen an evolution in the filler market for meaning full volume restoration in the aging face. There are now 35 major filler product companies worldwide.[10] The days of treating a nasolabial fold with single skin filler injection is gone, and a new time of more sophisticated approach of thoughtful restrained, and effective filler injection has come. Deep-volume increase, combination approaches, natural looking outcomes, and safety measures are the most important considerations for filler use.[11] Skin fillers on the market today are categorized into transitory biodegradable or resorbable within months and years respectively, and permanent or non-resorbable fillers. Biodegradable agents can be divided into two categories: (1) nonpermanent fillers, also named replacement fillers (collagen, hyaluronic acid and biological fillers), which has a short duration with typical lengths of several months to one year and are eventually reabsorbed through macrophage activation; (2) semipermanent fillers, or stimulatory fillers (poly lactic acid and calcium hydroxylapatite), which have a longer duration of aesthetic improvements lasting up to years with minimal side effects.[12] They will typically result in a foreign body reaction that elicits fibroblast activation and collagen synthesis at the site of injection.[13] Permanent implants (polymethyl methacrylate, silicone and hydroxyethylmethacrylate) could provide long-lasting results and could also induce fibrogenesis and collagen production, but with higher potential risk of complications.[14]

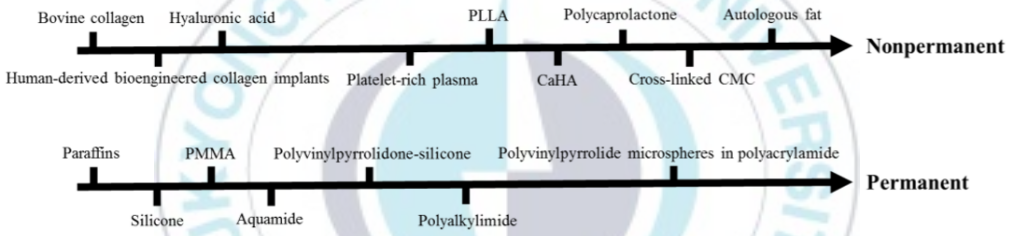

Figure 2. Summary of the dermal filler in the decades [10]

1.3. Extracellular matrix (ECM)

All tissues and organs contain a mixture of cells and non-cellular components, which form well-organized networks called extracellular matrices (ECM). The ECMs provide not only physical scaffolds into which cells are embedded but also regulate many cellular processes including growth, migration, differentiation, survival, homeostasis, and morphogenesis.[15] The ECMs consist of a large variety of matrix macromolecules whose precise composition and specific structures vary from tissue to tissue. The major constituents of ECMs are fibrous-forming proteins, such as collagens, elastin, fibronectin, laminins, glycoproteins, proteoglycans (PGs), and glycosaminoglycans, which are highly acidic and hydrated molecules.[16] In most tissues, fibril-forming collagen type I and mainly in cartilage collagen type II are the major constituents of ECMs. They are associated with other collagens as well as ECM proteins and PGs to construct large fibrilar structures. These multi-molecular structures are interconnected with ECM molecules, which also associate with each other, building the complex three dimensional matrix network.[17] ECMs can be classified into two major types that vary in composition and structure: the interstitial and pericellular matrices. The interstitial matrices surround cells, whereas the pericellular matrices are in close contact with cells. For example, basement membrane, which is a type of pericellular matrix, is found in the interface between parenchyma and connective tissue providing an anchoring sheet like layer for parenchymal cells in order to be held together preventing them from ripping apart.[18] Basement membranes are composed of collagen type IV, laminins,

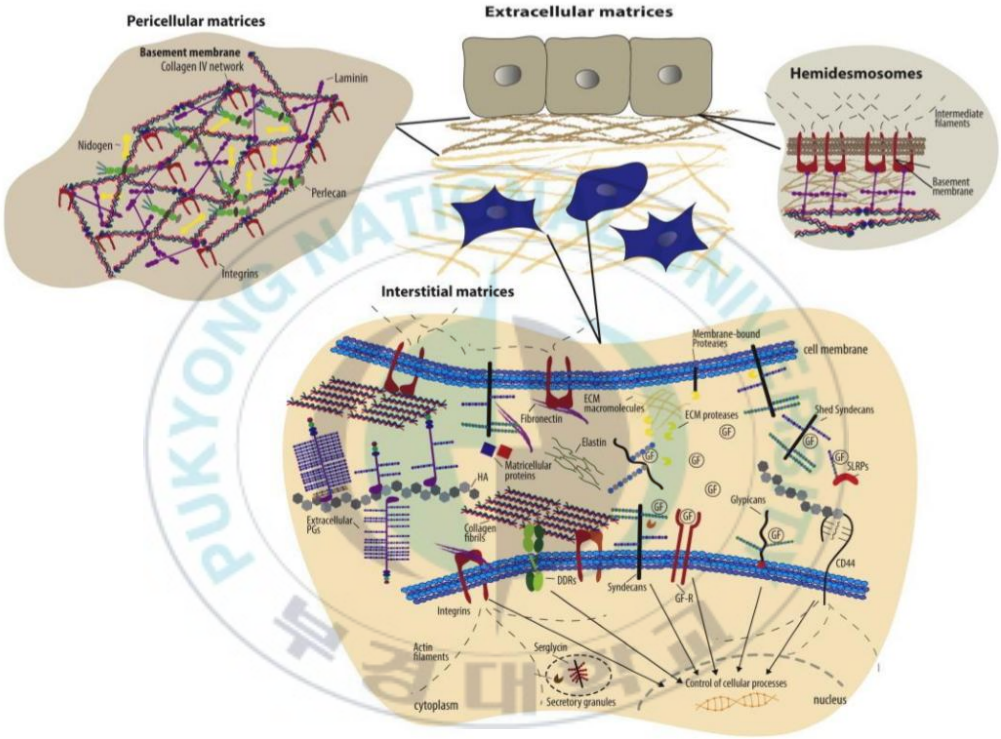

Figure 3. Schematic overview of extracellular matrices [18]

nidogen 1 and 2, and PGs such as perlecan, agrin, collagen type XV, and collagen type XVIII. Basement membranes consist of a laminin polymer that provides mainly epithelial cell adhesion sites and a collagen type IV network that stabilizes the overall structure. Both networks interact with other basement membrane components, which act as molecular linkers interconnecting the two networks.[19] For example, perlecancontaining aggregates are implicated in the tight connection of collagen type IV and laminin networks but also influence the hydration and thus the biomechanical properties of basement membranes.[20] Cells embedded into ECMs interact with this macromolecular network through their surface receptors, such as integrins, discoidin domain receptors (DDRs), cell surface PGs, and the hyaluronan (HA) receptor CD44. In this respect, cells integrate signals from ECMs that dictate their functions and behavior.[21] All cell types (i.e. epithelial, fibroblasts, immune cells, endothelial cells) synthesize and secrete matrix macromolecules under the control of multiple signals thus participating in the formation of ECMs.Variations in the composition and structure of ECMs' components affect both the overall structure and biomechanical properties of the formed network, but also the signals transmitted to cells thus modulating their responses.[22] Various growth factors, cytokines, and chemokines are deposited within ECMs through binding to specific ECM molecules and are able upon well-orchestrated procedures to be liberated and operate at developmentally and physiologically relevant time.[23] ECM remodeling occurred during physiological conditions and as part of disease processes modulates the structure and properties of ECMs in multiple ways. For example, the proteolytic degradation mediated by enzymes, such as matrix

metalloproteases (MMPs), a disintegrin and metalloproteases (ADAMs), ADAMs with thrombospondin motifs (ADAMTSs), plasminogen activators, as well as degradation of heparan sulfate (HS) chains mediated by heparanase, liberate heparin (Hep)-binding growth factors that in turn activate angiogenesis and cell growth during tumorigenesis.[24] Especially, during tumorigenesis, marked alterations in the ECMs take place leading to the formation of fibrotic stroma with increased stiffness, excessive deposition of ECM components, and release of proteolytic enzymes that upon activation result in an abnormal ECM remodeling.[25]

1.4. Articular cartilage extracellular matrix (CAM)

There are three classic types of cartilage: articular, fibrous, and elastic. Articular cartilage, also known as hyaline cartilage, is found in freely movable synovial joints such as the knee. Its function is to provide lubrication, absorb and distribute compressive load, and withstand shear stress during joint movement.[26] Articular cartilage consists of one cell type, articular chondrocytes, and the extracellular matrix provided by these cells.[27] The matrix is comprised primarily of collagen, proteoglycans, and water molecules.[28] Collagens are the most abundant protein family in articular cartilage. Collagen II forms fibrils that provide tensile strength throughout the extracellular matrix. Collagen VI is thought to form hexagonal networks in close proximity to cells where it can be linked to Collagen II fibrils via Matrilin-4 and Biglycan.[29] Proteoglycans are the major non-collagenous proteins found in articular cartilage. The most abundant is Aggrecan, which is modified with chains of chondroitin sulfate and keratan sulfate, and interacts with Hyaluronic acid. The chondroitin sulfate and keratan sulfate chains bind water molecules and the resulting hydrated Aggrecan provides lubrication within the joint and contributes to the loadbearing capacity of articular cartilage.[30] Small leucine-rich proteins/proteoglycans (SLRPs), including Fibromodulin, Decorin, Biglycan, PRELP, and Chondroadherin, are also important for proper articular cartilage function. All SLRP family members are able to bind fibril-forming collagens. Chondroadherin and PRELP are also able to bind members of the Syndecan family of cell surface

proteoglycans.[31] Articular cartilage is hyaline cartilage and is 2 to 4 mm thick.[32] Unlike most tissues, articular cartilage does not have blood vessels, nerves, or lymphatics. It is composed of a dense extracellular matrix (ECM) with a sparse distribution of highly specialized cells called chondrocytes. The ECM is principally composed of water, collagen, and proteoglycans, with other noncollagenous proteins and glycoproteins present in lesser amounts. Together, these components help to retain water within the ECM, which is critical to maintain its unique mechanical properties.[33] Along with collagen fiber ultrastructure and ECM, chondrocytes contribute to the various zones of articular cartilage—the superficial zone, the middle zone, the deep zone, and the calcified zone.[34] Within each zone, 3 regions can be identified—the pericellular region, the territorial region, and the interterritorial region. The thin superficial (tangential) zone protects deeper layers from shear stresses and makes up approximately 10% to 20% of articular cartilage thickness.[35] The collagen fibers of this zone (primarily, type II and IX collagen) are packed tightly and aligned parallel to the articular surface. The superficial layer contains a relatively high number of flattened chondrocytes, and the integrity of this layer is imperative in the protection and maintenance of deeper layers. This zone is in contact with synovial fluid and is responsible for most of the tensile properties of cartilage, which enable it to resist the sheer, tensile, and compressive forces imposed by articulation. Immediately deep to the superficial zone is the middle (transitional) zone, which provides an anatomic and functional bridge between the superficial and deep zones.[36] The middle zone represents 40% to 60% of the total cartilage volume, and it contains proteoglycans and

thicker collagen fibrils. In this layer, the collagen is organized obliquely, and the chondrocytes are spherical and at low density. Functionally, the middle zone is the first line of resistance to compressive forces. The deep zone is responsible for providing the greatest resistance to compressive forces, given that collagen fibrils are arranged perpendicular to the articular surface.[37] The deep zone contains the largest diameter collagen fibrils in a radial disposition, the highest proteoglycan content, and the lowest water concentration. The chondrocytes are typically arranged in columnar orientation, parallel to the collagen fibers and perpendicular to the joint line.[38] The deep zone represents approximately 30% of articular cartilage volume. The tide mark distinguishes the deep zone from the calcified cartilage. The deep zone is responsible for providing the greatest amount of resistance to compressive forces, given the high proteoglycan content.[39] Of note, the collagen fibrils are arranged perpendicular to the articular cartilage. The calcified layer plays an integral role in securing the cartilage to bone, by anchoring the collagen fibrils of the deep zone to subchondral bone. In this zone, the cell population is scarce and chondrocytes are hypertrophic.[40]

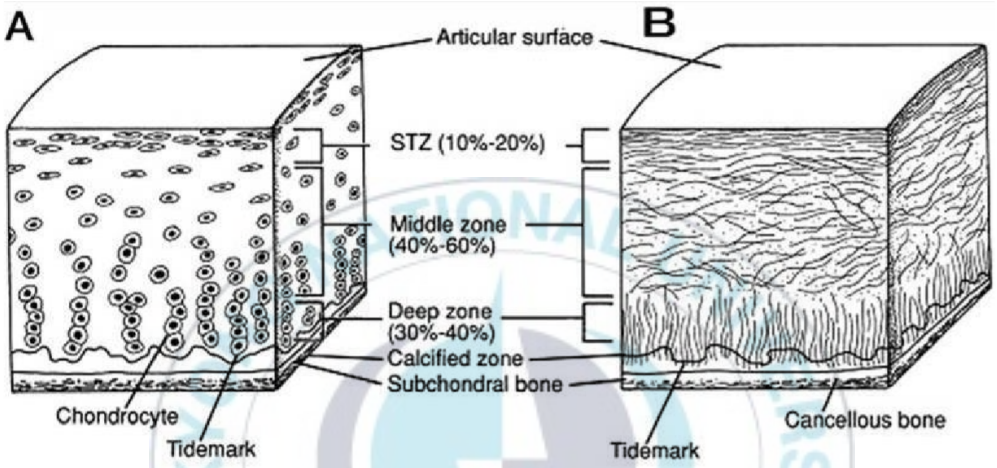

Figure 4. Cross-sectional diagram of healthy articular cartilage [27]

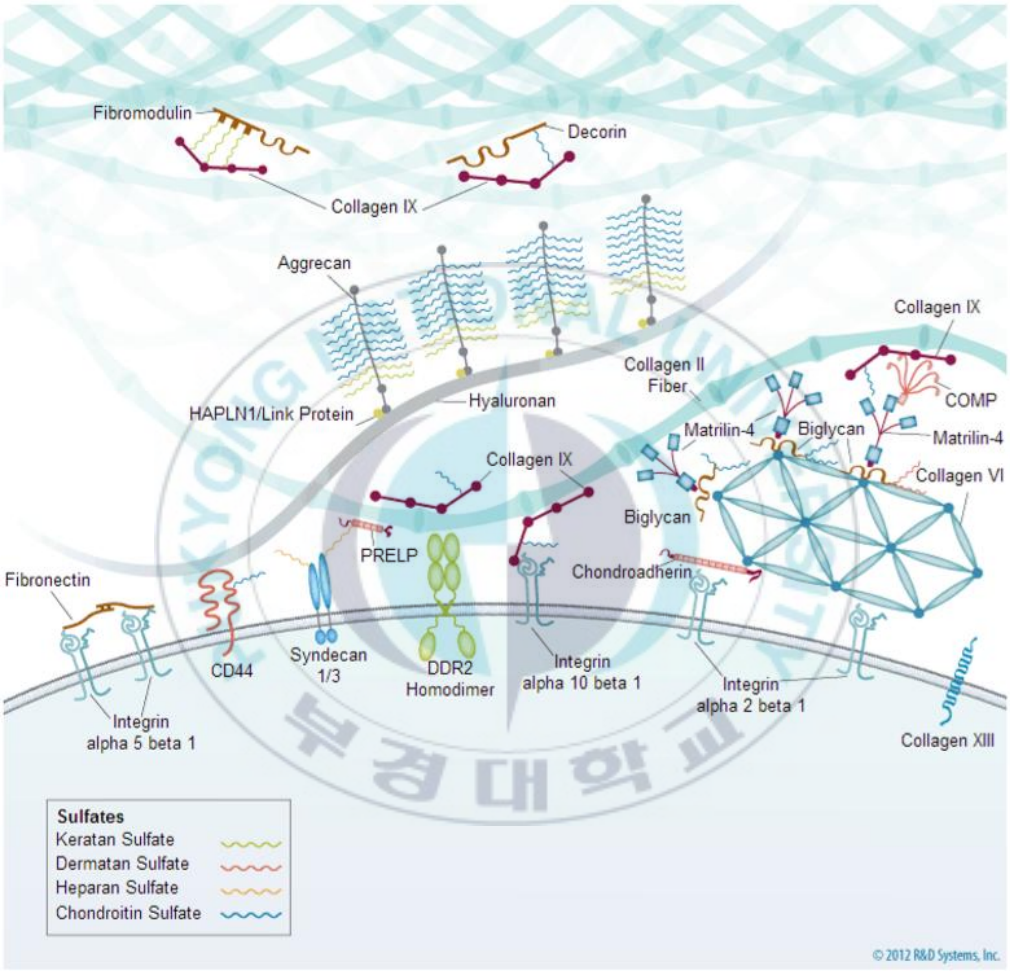

Figure 5. Articular Cartilage Extracellular Matrix [28]

1.5. Goal of this study

Extracellular matrix (ECM) stores and supplies simple structural linkages between cells, that is, the physical environment as well as the biochemical factors necessary for cell growth and differentiation.[18,41] In particular, extracellular matrix of cartilage is composed mostly of type 2 collagen, glycosaminoglycans (GAGs), proteoglycans, and glycoproteins, and thus can have high elasticity against compressive stress. The extracellular matrix is a natural biomaterial extracted from humans and animals, and has very good biocompatibility and physiological function, so it can provide less biologic function and biodegradation. Because of the decomposition, the long-term effect can be expected from the HA filler which is decomposed by the hydration.[42,43] In this study, we investigated whether porcine cartilage extracellular matrix (CAM) biomaterials can be used as an improved filler to overcome the rapid degradation of existing HA fillers and to withstand the physical stimuli applied after biotransplantation. Microstructure, mechanical properties and biocompatibility experiments were performed.

2. Materials and methods

2.1. Materials

2.1.1. Fabrication of HA hydrogel

10% (wt/vol) HA (Lifecore, USA) with a molecular weight 800kDa was dissolved in 9.8mL of a 0.2M NaOH solution at room temperature. Then, 200uL of the cross-linker, 1,4-butanediol diglycidyl ether (BDDE; Sigma-Aldrich USA), was mixed with HA solution at a final concentration of 2% (vol/vol). The mixture was then distributed into the molds, which were then sealed and incubated at 37 ° C for gelation.[44]

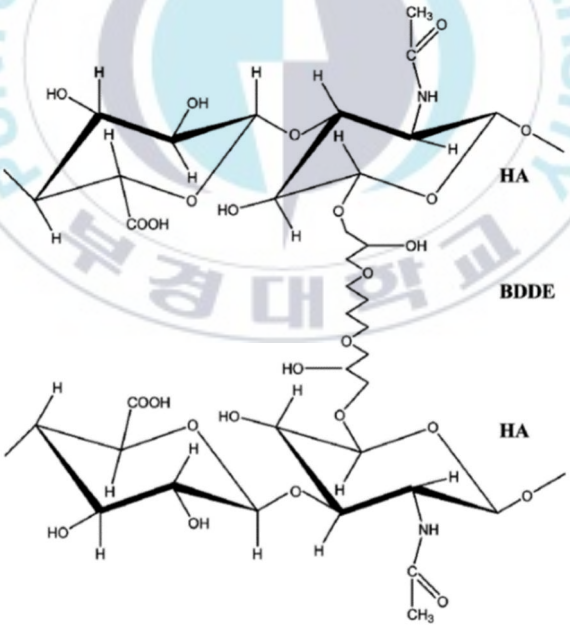

Figure 6. Chemical structure of crosslinked HA with BDDE [45]

Figure 7. Crosslinked HA with BDDE

2.1.2. Fabrication of CAM hydrogel

For the preparation of CAM, the pig knee joint was separated, and only the cartilage was cut out, washed with tertiary distilled water, lyophilized, and then lyophilized. For decellularization, it was treated with storage buffer (10 mM Tris-HCl, pH 8.0) and stirred at 400 rpm for 4 hours. Next, Tris-buffered saline (10 mM NaCl, pH 7.6) containing 1% sodium dodecyl sulfate was added thereto, stirred at 400 rpm for 2 hours, and washed with distilled water. After washing 2-3 times, DNase (100 U / μm, Elpis Biotech, Daejeon, Korea) was added, stirred at 400 rpm for 12 hours, and centrifuged and lyophilized for at least 4 days. The powder obtained by lyophilization was put into a solution containing pepsin (1600 U / mL) in 500 mL of 0.5 M HCL aqueous solution, stirred at 4 ° C. for 24 hours, and aliquoted with 10 M NaOH solution to neutralize to pH 7.7.

Finally, the final solution was dialyzed with tertiary distilled water for 24 hours (molecular weight cutoff: 3.5 kDa; Spectrum Laboratories, Calif., USA) and lyophilized. The lyophilized CAM was dissolved in tertiary distilled water, and Sigma-Aldrich (30 mM N- (3-Dimethylaminopropyl) -N'ethylcarbodiimide hydrochloride (EDC) and 30 mM N-hydroxysuccinimide (NHS) purchased from St. Louis, MO, USA) at a 10: 1 (v / v) ratio The concentration (w / v) of was prepared to be 1%, 3%, 5%, 7%.

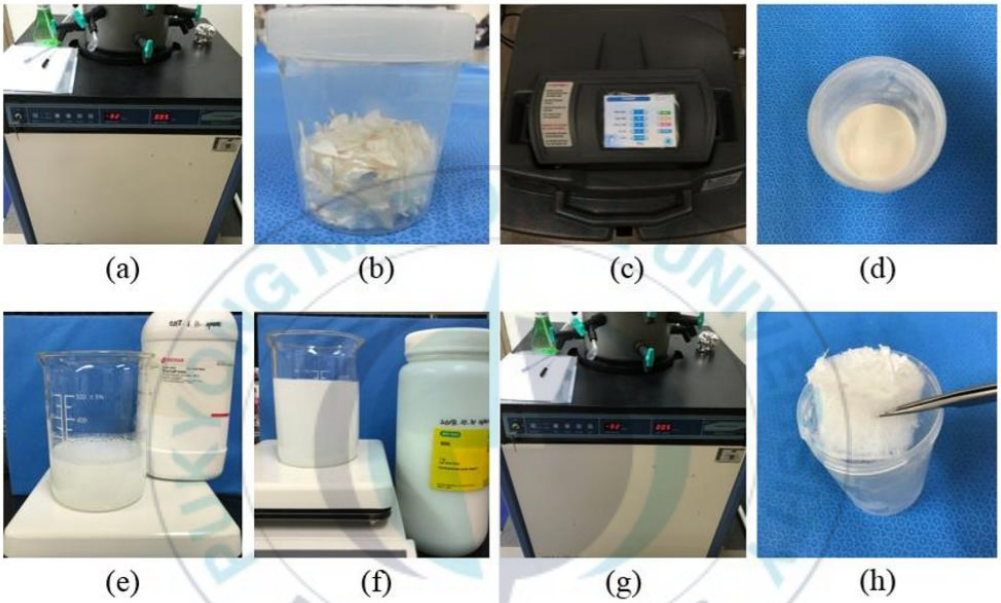

Figure 8. CAM manufacturing process. (a), (b) Lyophilize cartilages, (c), (d) Make cartilage powder using Freeze & mill, (e), (f), Process of decellularization, (g), (h), Lyophilize cartilage ECM

2.2. Morphological analysis of hydrogel

To analyze the microstructure of HA hydrogel and CAM hydrogel, low vacuum scanning electron microscopy (JSM-6490LV, JEOL, Japan: MONO CL3 +, GATAN, UK) was used. In order to remove the remaining crosslinking agent without participating in the crosslinking reaction, the resultant was washed with third distilled water, frozen in a refrigerator for 24 hours, and dried using a lyophilizer. The dried sample was confirmed by using a 15k electron beam with a platinum coating for 1 minute using an ion sputter coater (E-1010, Hitachi, Japan) to obtain a high resolution image.

2.3. Rheometer measurement

In order to compare the storage modulus of HA hydrogel and CAM hydrogel by shear deformation, the analysis was performed using Discovery HR-2wk (TA instrument, Leatherhead, Surrey, UK). In this study, an 8mm plate was used. The hydrogel was placed on a Peltier that was adjusted to 37 similar to the body temperature, and the upper plate was approached to 1000um. Next, the storage modulus G' was measured and compared at a frequency of 1 Hz (6.28 rad/s). All measurements were repeated three times for each sample.

Figure 9. The experimental setup of HR-2 TA Instruments rheometer

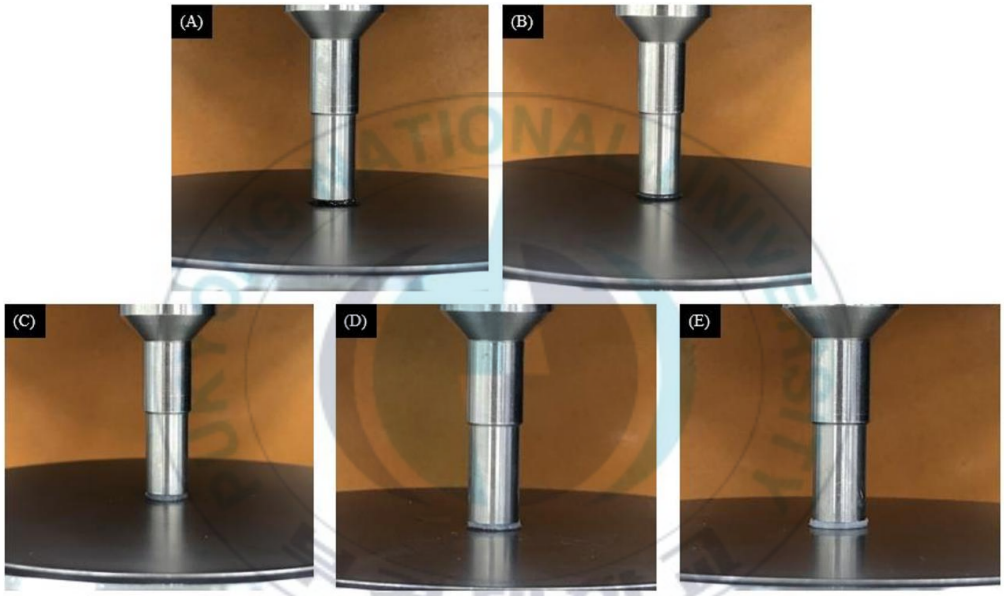

Figure 10. Measurement the storage modulus using Rheometer. (A) HA, (B) CAM-1%, (C) CAM-3%, (D) CAM-5%, (E) CAM-7%

2.4. Cytotoxicity evaluation

To observe the cytotoxicity of the prepared HA hydrogel and CAM hydrogel, passaged human fibroblasts (HFF-1, ATCC® SCRC¬1041 ™) were treated with 0.25% trypsin-EDTA solution and centrifuged to obtain 5 X 10^4. 10 ml of confocal dish (SPL, Korea) was inoculated and cultured in a 5% CO , 37 ° C incubator. After 24 hours, the cells were replaced with the culture prepared for the dissolution experiment and incubated for 6 hours.[46] After 6 hours, fluorescence staining was performed for 30 minutes with a live and dead cell viability assay kit (Invitrogen, USA) (Calcein & Ethd1 staining) to determine the ratio of viable and dead cells. And cell viability was confirmed according to the presented method. Surviving cells are green fluorescence due to calcein staining, and dead cells are red fluorescence with EthD-1 staining.

2.5. Cell proliferation

Ez-Cytox assay was performed to confirm the cytotoxicity of HA and CAM hydrogel in live and dead assay and to compare the effects on cell proliferation and survival. HA and CAM hydrogels were prepared with a height of 2 mm and a diameter of 8 mm, respectively. Human fibroblasts (HFF-1, ATCC® SCRC¬1041 ™) were dispensed on a hydrogel at a concentration of 5 X 10 to give 5% CO , 37 ° C incubator. Incubated for 1, 4, 7 days. After incubating for each period, the medium was removed and washed twice with PBS. After washing, EZ-CYTOX was added as much as 1/10 of the added medium volume, and cultured in an incubator at 5% CO and 37 ° C. for 4 hours, and the absorbance was measured at 450 nm using an ELISA Microreader.

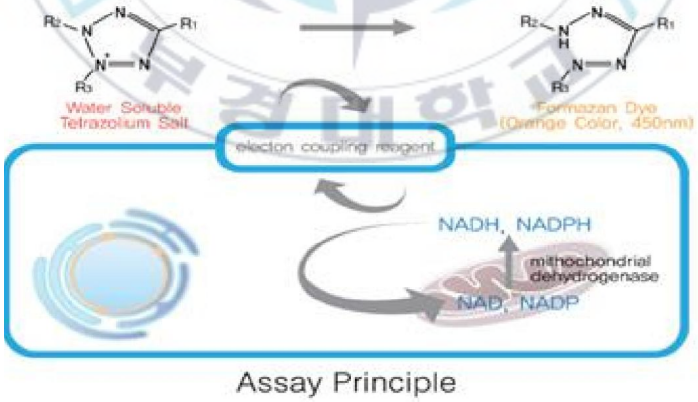

Figure 11. Principle of Ez-Cytox assay

2.6. Statistical analysis

All experiments consisted of three replicates and statistical programs (Statistical Package for Social Sciences 22.0 for Windows; SPSS, Chicago, IL) were used. Oneway analysis of variance (ANOVA) and Turkey post-hoc comparison test were performed to confirm the statistical significance between the control group and the experimental group. The statistical significance level was defined as statistical significance when the p-value was less than 0.05.

3. Results

3.1. Microstructures

The fabricated HA hydrogels and CAM hydrogels were freeze-dried, and cross-sections were observed by SEM after freeze drying (Fig A-E). All specimens exhibited a highly porous microstructure. However the surface and the cross-sectional thickness differed significantly between specimens. HA hydrogels (Fig 1. A) and CAM hydrogels (Fig 1. B-E) showed porous structures, but HA hydrogels had smooth surfaces and fiber bundle structures were observed on the surface of CAM hydrogels. The cross-sectional thickness of HA hydrogel is about 0.617 um, CAM 1%, 3%, 5%, and 7% cross-sectional thickness of 0.427 um, 1.52um, 4.034 um, 5.345um, respectively.

Table 1. The cross-sectional thickness of hydrogels

HA	0.617 ㎛
CAM-1%	0.427 ㎛
CAM-3%	1,520 ㎛
CAM-5%	4.034 ㎛
CAM-7%	5.345 ㎛

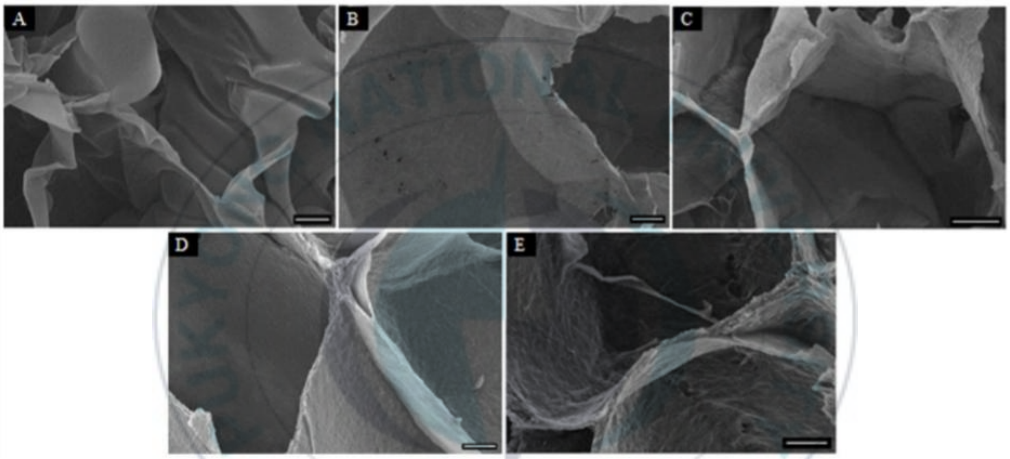

Figure 12. SEM images of the freeze-dried fracture surfaces of crosslinked (A) HA, (B) CAM-1%, (C) CAM-3%, (D) CAM-5%, (E) CAM-7% (scale bar = 20um)

3.2. Optimization of storage modulus

Rheometer was used to analyze the rheological properties of HA and CAM hydrogels (Fig. 2). The storage modulus, G′ (i.e., the amount of energy stored due to the elastic deformation, commonly referred to as the elasticity) were measured after the hydrogel reached an equilibrium swelling state in DPBS at room temperature. In all frequency ranges, CAM hydrogel showed increased storage modulus (G ') compared to HA hydrogel, and G' value increased with increasing CAM concentration. HA hydrogels are about 100Pa, CAM 1%, 3%, 5%, and 7%, respectively, about 300Pa, 1,000Pa, 4,000Pa, and 10,000Pa, respectively, the strength that CAM hydrogels can withstand shear deformation better than HA hydrogels. Indicated. Measuring range Angular frequency (rad / s) unlike the aspect of CAM hydrogel between the 10^1 and 10^2 intervals, it can be observed that the HA hydrogel graph is inconsistent, when the HA hydrogel becomes larger when the shear deformation angle is increased. It was because they could not overcome the transformation and broke. Therefore, these rheological results confirmed that the CAM hydrogel retained its shape better when the force applied from the outside than the HA hydrogel.

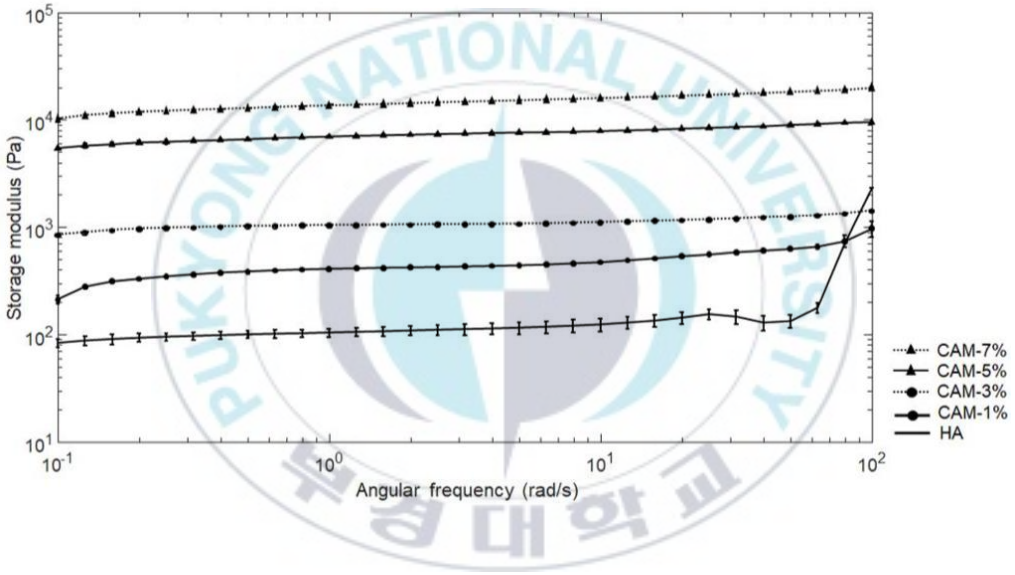

Figure 13. Storage modulus of hydrogels

3.3. Cell cytotoxicity

Human fibroblasts was immersed in a cell culture in which each of the hydrogels was immersed for 24 hours to determine whether the crosslinking agents used to prepare HA hydrogels and CAM 1%, 3%, 5%, and 7% hydrogels remained cytotoxic. After treatment for 6 hours in human fibroblast cells (HFF-1, ATCC® SCRC¬1041 ™), live and dead cell cytotoxicity assays were performed (Fig. 3). Calcein fluorescence staining for cell survival is stained green and ethidium homodimer fluorescence staining for cell damage is stained red. Therefore, the cross-linked HA hydrogels and CAM 1%, 3%, 5%, 7% hydrogels in all experimental groups confirmed that the cell viability was not more than 90%.

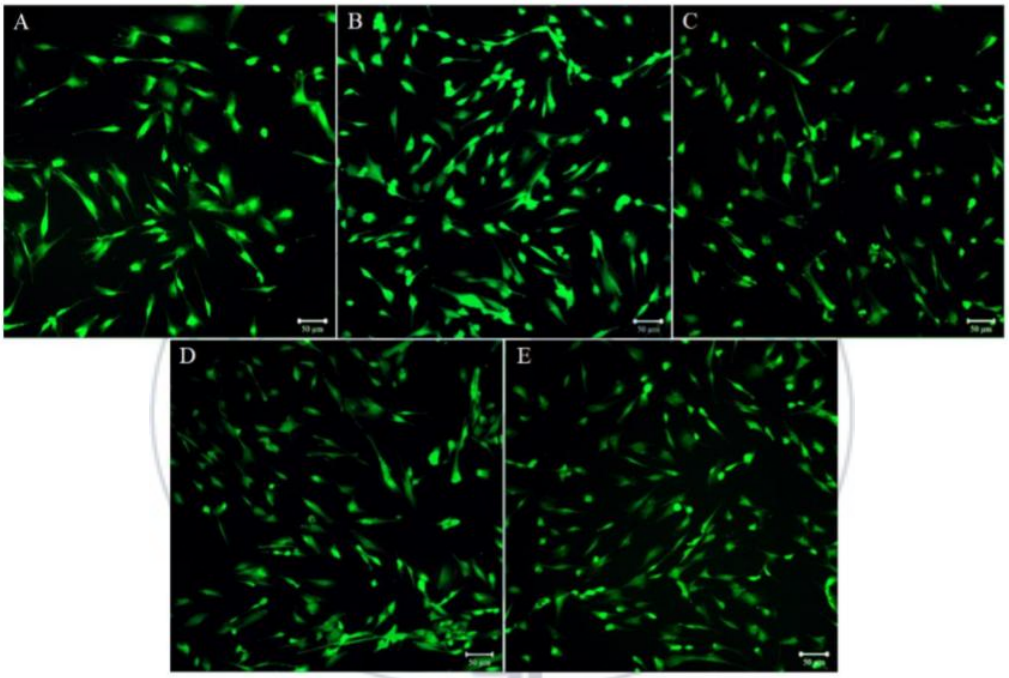

Figure 14. Fluorescent images of hFbs (A) HA, (B) CAM-1%, (C) CAM-3%, (D) CAM-5%, (E) CAM-7% (scale bar = 50 ㎛)

3.4. Cell proliferation

In order to examine the proliferation and viability of cells for HA hydrogel and CAM hydrogel, EZ-CYTOX assay was performed by quantitative analysis of cells after dispensing human fibroblasts into each hydrogel. On day 1 of cell culture, there was no statistical effect between HA hydrogel and experimental group CAM hydrogel, but on day 4 of cell culture, cell growth rates decreased at CAM 1% and 3% rather than HA hydrogel and CAM 7%. Confirmed. And on the 7th day of culture, there was no statistically significant cell growth rate between HA hydrogel and CAM 7%, and cell growth rate was significantly increased compared to other groups. In general, HA favorably interacts with many cell types through specific and non-specific interactions. In particular, transmembrane binding receptors of fibroblasts, namely CD44, and receptor for HA-mediated motility play a key role in cell–cell and cell–matrix interactions by regulating a variety of downstream biochemical signals. In particular, it has recently been shown that the interaction between HA and these receptors brings the upregulation of several growth factors production such as transforming growth factor-β and epidermal growth factor enhancing the motility and proliferation of fibroblasts. Additionally, fibroblast cells have been found to prefer stiff materials to soft materials. Therefore, HA hydrogel and CAM-7% hydrogel, which have very high cell proliferation and survival rate, showed similar results, indicating that the extracellular matrix could be useful as a molding filler.

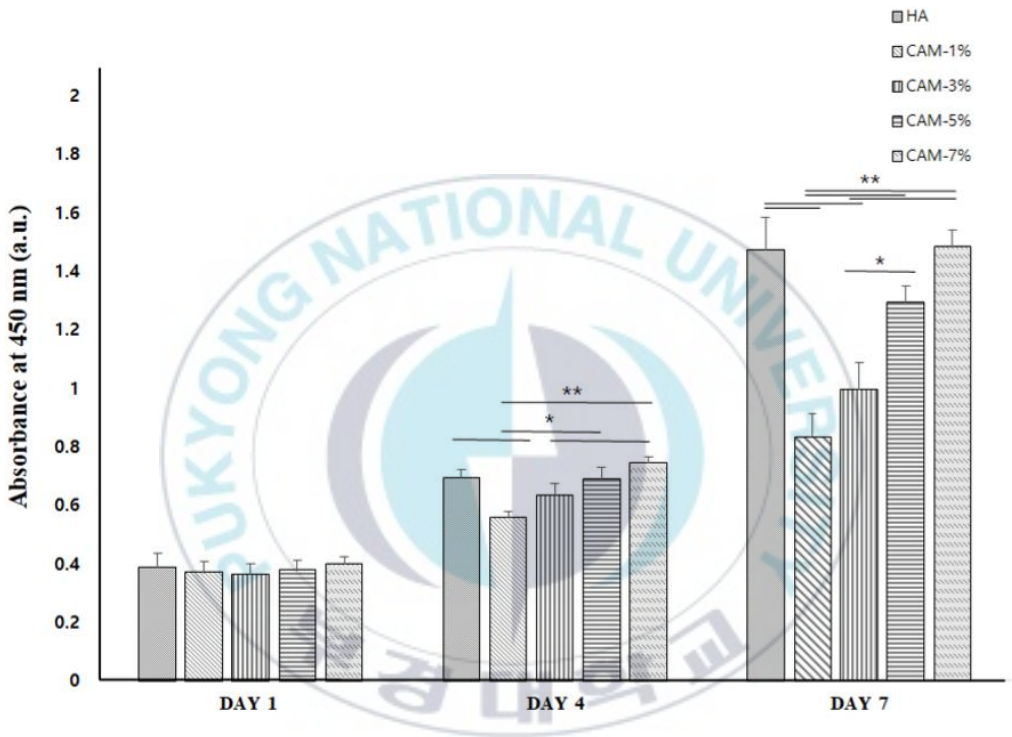

Figure 15. EZ-CYTOX assay for proliferation (*p<0.05, **p<0.005)

4. Discussion

Hyaluronic acid (HA)-based gels are now the gold standard in dermal fillers, with more cosmetic procedures in the United States using these fillers than all other fillers combined. The wide-spread acceptance of HA filler is testament to their biocompatibility (unlike protein-based fillers, they are composed of polysaccharides that exhibit no species specificity), the stability of their crosslinked HA in vivo (which promotes longevity of clinical improvement, and their good record of safety and effectiveness in other countries where they have been in use for many years.[47] Injectable dermal fillers made of various sources (e.g. human, animals, and bacterial) and by various methods (e.g. filler formulation, modifications, and cross-linking) have recently been developed for aesthetic use as well as clinical remedies.[48] Hence, the demand is continuously growing, and consumer expectations of effective fillers are also greater. In fact, recent injectable fillers are becoming more similar to human skin or tissue. However, there are still numerous concerns regarding biocompatibility, biosafety, adverse reactions, allergic reactions, inflammation, durability, physical properties, and cost. Approved dermal fillers have been shown to be relatively safe, but varying degrees of resorption make recipients require repeated percutaneous injections to maintain the expected level of collection. Therefore, new dermal filler hydrogels should be able to offer in vivo stability to ensure the longevity of the injectable implant as well as biosafety. For this reason, we considered which materials would be suitable for advanced dermal filler hydrogels. Among the material candidates, collagen, being

the major protein of the natural extracellular matrix, contains basic residues such as lysine and arginine. It also has specific cell adhesion sites such as arginine-glycine-aspartate (RGD) peptides. The RGD group actively induces cellular adhesion by binding to integrin receptors, and this interaction plays an important role in cell growth and differentiation. Eventually, it could be used to enhance the overall regulation of cell function.[49] However, collagen as a dermal filler has drawbacks, including a relatively short duration of use in vivo, the possibility of recipient hypersensitivity, and BSE concerns when using bovine-derived collagen. Thus, the use of autologous human collagen and collagen derived from various human organs or tissues has been investigated for medical applications to overcome these problems.[50] We used porcine cartilage extracellular matrix for dermal filler substance. This results indicated that the collagen from cartilage ECM is well-conserved in its active sites, which promotes cell proliferation and migration, as mentioned above. However, cartilage ECM COL also rapidly decomposes in vivo when it is used as the sole component of a dermal filler. Furthermore, if collagen is implanted alone, its viscosity increases, which acts as a barrier to cell migration.[51] Cross-linked HA is less susceptible to enzymatic degradation by elimination of carboxyls and is widely used.[52] It has been developed as a next-generation dermal filler owing to its superior properties over collagen, including no requirement of a skin test, longer duration of use, and no chemical or molecular differences between species. However, the presence of a carboxyl group (COO-) in HA can cause poor cell adhesion. Hence, we fabricated extracellular matrix of a material that possesses high biocompatibility by crosslinked porcine cartilage ECM,

because just ECM can result in short durability in vivo, as mentioned above. It can be expected that this composite material will show excellent biocompatibility. Despite the synergistic effect of ECM composites, there is no report on dermal filler applications. Thus, to offset the disadvantages of CAM as well as to make use of the advantages of these materials, we fabricated a new type of dermal filler by the addition of cartilage ECM to EDC/NHS to improve the duration of the filler.

5. Conclusion

The porcine cartilage extracellular matrix (ECM) based hydrogels compared hyaluronic acid (HA) based hydrogels to determine their biocompatibility. Porcine cartilage ECM hydrogel can be prepared from an intact scaffold, and has distinct structural, mechanical, and biologic properties. ECM hydrogel properties can be manipulated by such factors as the source tissue from which the hydrogel was prepared and the final ECM concentration, and it may occur in a non-linear fashion. In this study, we constructed injectable fillers from safe sources using simple and easy fabrication methods and evaluated their effectiveness in vitro. In our demonstrations, we make new commercial fillers. In this study, microstructure analysis and rheological analysis using crosslinked HA hydrogel and CAM hydrogel, which is an extracellular matrix, showed higher physical strength and decreased deformation from externally applied force. In addition, CAM hydrogels, especially CAM-7% hydrogels, have excellent biocompatibility and cell proliferation and survival rates as well as HA hydrogels, which are highly biocompatible through live and dead cell cytotoxicity assays and cell proliferation assays. It was confirmed that it has. In conclusion, the physical properties that extracellular matrix can be used as a filler were confirmed through this experiment, and further studies on the volume change and the transplantation safety of CAM hydrogels after transplantation through animal experiments will be conducted. The CAM-based fillers to be developed will be useful for cosmetic and skin disease improvement applications.

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