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In vitro characterization of a novel human acellular dermal matrix (BellaCell HD) for breast reconstruction

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Abstract

In vitro characterization of a novel human acellular dermal matrix (BellaCell HD) for breast reconstruction

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Introduction: Over the last two decades, acellular dermal matrices (ADMs) have been commonly used in implant-based breast reconstruction. Although several factors affect the clinical performance of ADMs, there is a lack of systematic characterization of ADMs. The present study characterized BellaCell HD in comparison with the two commercially available ADMs, AlloDerm RTU® and DermACELL®, under in vitro settings.

Methods: Each ADM (BellaCell HD, AlloDerm RTU®, and DermACELL®) was characterized with regards to decellularization, biocompatibility, and mechanical properties. Decellularization status was evaluated with hematoxylin and eosin (H&E) staining under a light microscope. Biocompatibility including cell proliferation and cytotoxicity was evaluated

with MTT assay using NIH3T3 and L-929 mouse fibroblasts. Mechanical properties were determined through uniaxial tensile testing, stiffness testing, and suture retention strength testing with specimens.

Results: BellaCell HD showed complete decellularization. Compared to other ADMs (AlloDerm RTU® and DermACELL®), several fibroblasts grew inside BellaCell HD without cytotoxicity. Furthermore, the proliferation rate of fibroblasts on BellaCell HD was significantly higher compared to that on AlloDerm RTU® on day 1 ($p < 0.01$), 7 ($p < 0.05$), and 14 ($p < 0.01$) and DermACELL® on day 1 ($p < 0.05$). And BellaCell HD had maximum load value of 389.92 N, tensile strength of 26.48 MPa, which are higher than that of AlloDerm RTU® ($p < 0.001$). The elongation ratio of BellaCell HD was 123.4%, which is higher than that of DermACELL® ($p < 0.001$). The stiffness testing showed that BellaCell HD had the lower stiffness of 0.44 N/mm, which lower than that of DermACELL®. However, there was no significant difference in the results of suture retention strength test.

Conclusions: BellaCell HD showed complete decellularization, high biocompatibility, low cytotoxicity, high tensile strength, high elongation, low stiffness and high suture retention strength, which is comparable to the two commercially available ADMs. This demonstrated the potential for BellaCell HD to be used effectively and safely for implant/expander-based breast reconstruction.

Keywords: Biomaterial, acellular dermal matrix, breast reconstruction, in vitro technique, biocompatibility testing, mechanical phenomenon

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Introduction

Acellular dermal matrix (ADM) is a type of biological skin substitute that is obtained from cadaveric skin through processes such as decellularization and terminal sterilization. ADM consists primarily of fibrillary collagen, elastin, glycoproteins (fibronectin and laminin), proteoglycan, glycosaminoglycan, growth factors, and basement membrane[1]. Elastin and collagen contribute to tensile strength and elasticity, whereas proteoglycans and laminin contribute to the induction of angiogenesis and binding to connective tissues, respectively[2]. Furthermore, growth factors control cell behavior and cross-linked ADM degrades and releases biochemical signals at a rate similar to that of native tissue extracellular matrix (ECM). Therefore, when implanted into the human body, ADM influences host remodeling responses including cell migration, proliferation, and differentiation, and ultimately serves as an inductive scaffold for in situ formation of site-specific functional host tissue[3-5].

Currently, ADM is used in various fields such as abdominal wall surgery, cleft palate repair, nasal septal reconstruction, breast reconstruction, and the indications for its uses are evolving and increasing[4,6-9]. Because of improved detection of breast cancer, increased demand for breast reconstruction, and advances in implant manufacturing techniques, the implant/expander-based reconstruction, which has no donor site morbidity and is simple to perform, has become increasingly popular[10-11]. One of the most common techniques in implant-based breast reconstruction is the placement of the breast implant or expander beneath the pectoralis major muscle[12]. Since the fan-shaped pectoralis major muscle is unable to cover the inferolateral aspect of the tissue expander or implant, the ADM is routinely inserted as a sling between the pectoralis major muscle and the inframammary fold. The professed benefits of ADM not only include the support of inferolateral

aspect, but also greater initial fill volume, better definition of the inframammary fold, and reduced capsular contracture[12-14]. Through refinement of the surgical technique and the manufacturing process of ADM, ADM has now become an indispensable biomaterial in breast reconstruction.

Since AlloDerm® was first introduced in 1994, a variety of ADM products for breast reconstruction are available in the market today[15,16]. Each product is derived from different source tissue and is subjected to different processing, sterilization, and storage conditions. Recently, a human-derived ADM, BellaCell HD, was developed by Hans Biomed Corp (Seoul, Korea). For a newly developed biomaterial to be used in living organisms, in vitro studies of their effectiveness and stability must be undertaken first. Here, we conducted an in vitro study to evaluate the decellularization status, biocompatibility, and mechanical properties of BellaCell HD.

Methods

ADMs

The study subjects included BellaCell HD (Hans Biomed Corp., Seoul, Korea) and two other human ADMs for comparison, which are AlloDerm Ready to Use (RTU)® (LifeCell Corp., Branchburg, N.J., USA) and DermACELL® (LifeNet Health, Virginia Beach, Va, USA). We conducted the following studies on these three human ADMs to evaluate the novel human ADM, BellaCell HD.

Decellularization assessment

ADM specimens were stained by hematoxylin and eosin (H&E) using the following steps: fixation, paraffin embedding, paraffin sectioning, deparaffinization, rehydration, and staining. The specimens were evaluated with a light microscope at 40×, 100×, 200×, and 400× magnifications.

Cell culture

NIH3T3 mouse fibroblasts (American Type Culture Collection [ATCC] #CRL-1658, Manassas, VA, USA) and L-929 mouse fibroblasts (ATCC #CCL-1, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA). Cells were cultured at 37 °C in 5% CO2/95% air.

For experiments, the ADM specimens were cut to 1 × 1 cm pieces, put into 24 well cell culture dishes, washed with PBS twice, and then incubated in culture media at 37 °C and 5% CO2 for 10 min before cell seeding.

Proliferation assay

For cell proliferation assay, NIH3T3 fibroblasts were seeded on the grafts at

5×10^4 cells/cm^2 and incubated for 1, 7, and 14 days. Cells on the ADMs surface were imaged by a light microscope. Cell proliferation was assessed using an MTT assay. MTT solution at 100 μl/well was added and incubated for 4 h at 37 °C. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the optical densities were measured at 570 nm by an ELISA reader.

Cytotoxicity assay

For cytotoxicity assay, different amounts of sterile physiological saline were added and eluted at 37 ± 2 °C for 72 ± 2 h. Minimum essential medium (1 ×) with Earle's salts (MEM-E, Flow Labs., Rockville, USA) was used as a negative control after cell exposure to the same environment as the test group and DMSO as a positive control, and sterile physiological saline as a solvent control. The eluate was centrifuged at 3000 rpm for 5 min, and the supernatant was used as the test group. L-929 fibroblasts were seeded at a concentration of 5×10^4 cells/well and incubated for 24 h in a 37 °C humidified incubator with 5% CO2. The medium was removed after 24 h, and the eluate from the negative control group, the positive control group, the solvent control group, and the test group was replaced with a medium mixed in the same amount with a 2-fold concentration of the MEM medium. After incubation for 48 h at 37 °C in a humidified incubator with 5% CO2, cell morphology was examined under a light microscope at 200× magnification, and cell viability was evaluated by MTT assay.

Uniaxial tensile testing

Uniaxial tensile testing was performed as previously described[17-20]. Seven specimens (n = 7) measuring 10 × 10 mm were prepared from each of the ADMs (based on the American Society for Testing and Materials [ASTM] specification #D638-03)[18-20]. The average thickness of the specimens for each

ADM was 1.5 mm for BellaCell HD, 1.3 mm for AlloDerm RTU®, and 1.9 mm for DermACELL®. Each specimen was oriented vertically in the Instron® material testing system (Instron Corporation, Norwood, MA, USA) with a 3.0 cm gauge length, and approximately 2.0 cm of the specimen was fastened in each pneumatic grip. Specimens were subjected to uniaxial tension at a rate of 30 mm/min until failure. When the ADM was broken, the elongation ratio (%) (the maximum length divided by original length) and maximum load (N) were determined, and tensile strength was calculated by dividing the maximum load by the cross-sectional area (mm^2) of the specimen to yield the value in units of megapascals (MPa) where 1 N/mm^2 = 1 MPa.

Stiffness testing

Stiffness testing was performed as previously described using a Instron® material testing system[17-20]. Three specimens (n = 3) measuring 50 × 50 mm was fabricated from each of the ADMs (based on ASTM specification #F1306)[20]. The average thickness of each ADM specimen was 1.88 mm for BellaCell HD, 1.16 mm for AlloDerm RTU®, and 1.3 mm for DermACELL®. The specimen was fixed between the upper and lower jigs and the probe moved downward to compress the specimen at a rate of 25 mm/min. The stiffness was calculated by dividing the load that the specimen sustained (N) during the stiffness test by the movement (mm) of the probe.

Suture retention strength testing

Suture retention strength was measured as previously described using a Instron® material testing system[17-20]. Four specimens (n = 4) measuring 20 × 40 mm were prepared from each ADM[20]. From the bottom of the sample, a suture was passed through the mesh (1.0 cm). A pulling rate of 20 mm/min was applied as the suture tore out of the ADMs. The maximum load (N)

was recorded as mean ± SEM (standard error of the mean).

Statistical analysis

All values are reported as mean ± SEM. Statistical analyses were performed using SPSS statistical software (SPSS 26.0, Armonk, NY, USA). For all data, significant differences were determined using an unpaired t-test. For all analyses, $p < 0.05$ was defined as statistically significant.

Results

Decellularization assessment

Under a light microscope after H&E staining, BellaCell HD and DermACELL® showed complete decellularization. AlloDerm RTU® showed some cellular debris between collagen fibers (Figure 1).

Biocompatibility assessment

After incubation for 1, 7, and 14 days with NIH3T3 fibroblasts, invasive cells on the ADM's surface were photographed using a light microscope at 100× magnification (Figure 2A). On the first day, all of the specimens showed similar degree of cell adhesion. On days 7 and 14, a lot more cells invaded and over-grew inside BellaCell HD compared to that in the other matrices. In AlloDerm RTU®, cell proliferation was not even, and cell growth was mainly observed on the surface of the dent. In the MTT assay, cell proliferation in BellaCell HD was statistically significantly higher than that in AlloDerm RTU® on days 1 ($p < 0.01$), 7 ($p < 0.05$), and 14 ($p < 0.01$) (Figure 2B). It was also statistically significantly higher than that in DermACELL® on day 1 ($p < 0.05$), although there was no statistically significant difference in day 7 and 14.

In the cytotoxicity assay, a significant decrease in cell count was observed in the positive control using DMSO and an increase in cell count was observed in the negative control, as observed under a light microscope at 200x magnification (Figure 3A). In all the test groups using BellaCell HD, AlloDerm RTU®, and DermACELL®, the cells grew well with a spindle-like structure. MTT assay showed that all three products had a cell viability of over 90%, indicating no cytotoxicity (Figure 3B).

Uniaxial tensile testing

Uniaxial tensile testing showed that the maximum load at the ADM break was 389.92 N for BellaCell HD, which was significantly higher than 181.92 N for AlloDerm RTU® ($p < 0.001$) and lower than 492.11N for DermACELL® ($p < 0.01$) (Figure 4A). The tensile strength of BellaCell HD was 26.48 MPa. It was stastically significantly higher than 11.34 MPa observed for Alloderm RTU® ($p < 0.001$) and not significantly different from the 26.12 MPa observed for DermACELL® (Figure 4B). The elongation ratio at the ADM break was 123.4% for BellaCell HD, 126.38% for Alloderm RTU®, and 104.13% for DermACELL® (Figure 4C). The difference between those of BellaCell HD and DermACELL® was statistically significant ($p < 0.001$).

Stiffness testing

The stiffness testing showed that BellaCell HD had the stiffness of 0.44 N/mm, while those of AlloDerm RTU® and DermACELL® were measured 0.28 N/mm and 0.90 N/mm, respectively (Figure 5).

Suture retention strength testing

The results of suture retention strength testing showed that the maximum load for BellaCell HD was 87.06 N, which was higher than 79.65 N for AlloDerm RTU®, and 80.48 N for DermACELL® (Figure 6). However, these differences were not statistically significant.

Discussion

This study characterized the novel ADM, BellaCell HD, in an in vitro setting and compared it with two commercially available human ADMs for it to be successfully used in implant/expander-based breast reconstruction. First, BellaCell HD showed complete decellularization observed using H&E staining under a light microscope. Second, BellaCell HD showed high cell adhesion and cell proliferation with no cytotoxicity in the biocompatibility assessment. Third, BellaCell HD showed high tensile strength, high elongation, low stiffness, and high suture retention strength in the mechanical property assessment.

The manufacturing process of ADMs includes decellularization, preservation, and sterilization steps[16]. The most important step is the decellularization by physical, chemical, or biological methods, and each product is manufactured in a different way[21]. The goal of decellularization is to remove antigenic material while preserving extracellular matrix biochemistry and structure. It has been shown that the presence of residual DNA within biologic scaffold materials may be responsible for an inflammatory response[22]. A previous study has also reported that the presence of cells within a biologic scaffold is associated with increased macrophage M1 polarization, high amounts of proinflammatory cytokines, and poor remodeling outcome in a primate model[23]. Thus, this study suggests that BellaCell HD is immunologically safe to be implanted into the human body.

When an ADM is implanted during breast reconstruction, the original ADM is recolonized by fibroblasts and myofibroblasts, and also by other immune cells such as lymphocytes, macrophages, granulocytes, and mast cells[3,24]. The fibrosis and neovascularization progress to form a capsule and capillaries, followed by lymphomagenesis. As such, for the ADM to integrate with host tissues, it must be biocompatible, capable of inducing biologic responses

such as host cell adhesion and cell proliferation without cytotoxicity to the host tissue[25]. Bio-incompatibility due to lack of incorporation causes an imbalance resulting in implant mobility, which may contribute to infection, seroma, and reconstructive failure[26,27]. Thus, the high biocompatibility of BellaCell HD is likely to result in a more favorable outcome when applied in breast reconstruction.

One of the advantages of using ADM in implant/expander-based breast reconstruction is that it physically supports the implant and prevents shifting or bottoming out[12-14]. A high tensile strength ADM is a prerequisite for this. If an ADM with low tensile strength is used for breast reconstruction, it will be vulnerable to matrix rupture and this might lead to implant malposition. Moreover, after expander-based breast reconstruction, the expander is inflated for several months to obtain a sufficient amount of skin similar to that in the contra-lateral breast; thus, the ADM should have sufficient tensile strength to withstand the inflating pressure of the expander. Therefore, BellaCell HD, which has a high tensile strength, will provide sufficient physical support when used in the implant/expander-based breast reconstruction.

An interesting outcome of this study is that BellaCell HD showed not only high tensile strength but also a high elongation ratio, which means elasticity and pliability, respectively. While AlloDerm RTU® had a low stiffness value, it had a much lower tensile strength. Moreover, the tensile strength of DermACELL® was as high as that of BellaCell HD, but with much higher stiffness. Not only tensile strength but also high elasticity and pliability are important factors of ADM in implant/expander-based breast reconstruction. These help the surgeons handle the ADM easily and overcome the size discrepancy between the standardized ADM product and the space that needs to be covered for each patient. Surgeons use a variety of surgical techniques to deal with size discrepancy. Some surgeons make a

vertical or horizontal stab incision to the ADM, whereas others mesh the ADM using a skin graft mesher[28,29]. However, all of these methods have the disadvantage of increasing the area of contact between the implant and the mastectomy flap. Therefore, the high elasticity and pliability of the BellaCell HD will help to bridge the gap simply by stretching it. Recently, prepectoral implant placement with complete coverage by ADM has become popular because of the increase of direct to implant reconstruction after nipple sparing mastectomy, which has advantages of low postoperative pain and low animation deformity[30,31]. Since more complex techniques are required for total wrapping with ADM, the high pliability of the ADM will enable easy handling.

Although not statistically significant in the suture retention strength test, BellaCell HD had the highest suture retention strength of the three human ADMs tested. When the ADM is implanted as a hammock shape in implant/expander-based breast reconstruction, ADM is sutured with the elevated pectoralis major superiorly and the chest wall at the inframammary and lateral mammary fold inferolaterally[28,32]. Previous studies have shown that the suture retention strength of human ADM is usually lower than that of the maximum load[18,33-35]. Although few human studies have been performed, it is speculated that the suture site wound is more susceptible to dehiscence than the ADM itself until the ADM is fully integrated with the host tissue. Therefore, the high suture retention strength of BellaCell HD will help to prevent implant herniation through the suture site. Moreover, for BellaCell HD to expand its indications like other popular ADMs, it must have sufficient suture retention strength that can withstand high strains such as abdominal walls, which is supported by these findings[36].

Since the different results of each ADM in this study should be caused by the specific manufacturing processes, we tried to find out what difference in the processes made the difference in the test results. BellaCell HD is known

to be manufactured by a decellularization method using sodium deoxycholate which is one of the ionic surfactant. Ionic surfactant-assisted decellularization methods efficiently remove cells and genetic materials compared to the other methods such as nonionic surfactant, acid/base, and enzyme-assisted methods[37]. Among the ionic surfactants, sodium deoxycholate was reported to produce more biocompatible scaffolds than the other surfactant such as sodium dodecyl sulfate, while preserving the structural proteins necessary for the mechanical function[37,38]. This might make the positive result of BellaCell HD in this study. However, we could not get the information about the manufacturing processes of the other two ADMs, because it was proprietary and not open accessed. So it was difficult to clearly figure out the causative factors for the test result.

This study has a limitation that the biocompatibility and mechanical properties of the ADM were tested only in an in vitro setting. We used only NIH3T3 and L-929 mouse fibroblasts to examine cell proliferation and cell viability. However, not only fibroblasts but also myofibroblast, lymphocytes, macrophages, granulocytes, and mast cells are involved in the ADM integration[2,24]. Furthermore, antibodies, complement, and cytokines also play an important role in the host response to ADM. Therefore, to identify the exact mechanism by which ADM integrates into the human body and to use it to create an ideal ADM, more detailed studies will be needed in the future. Second, variable thicknesses of each ADM might affect the results of mechanical property testing. However, because an ADM comes into the market in the form of a finished product with a predetermined thickness, it was difficult to alter the thickness to the same value, as which the previous studies were performed[17,18]. Also, since an ADM will ultimately be utilized out of the package without altering the thickness prior to implantation, using the product as it is for the test can better reflect the actual clinical situation. Third, an ADM accommodates the biochemical change through a

process called ‘stretching’ after implantation, which varies from product to product[39,40]. However, it is difficult to reproduce individual multi-vector forces in an in vitro setting. In vivo studies will be needed to address this, which will help surgeons predict the need for an ADM sling overcorrection in implant/expander-based breast reconstruction.

Conclusions

This study characterized BellaCell HD and compared it to two commercially available ADMs, AlloDerm RTU® and DermACELL®, under in vitro settings. BellaCell HD showed complete decellularization, high biocompatibility, low cytotoxicity, high tensile strength, high elongation, and high suture retention strength, which demonstrate the potential for BellaCell HD to be used effectively and safely for implant/expander-based breast reconstruction. Additional in vivo studies with long term follow-up, will be required to validate the novel product before its use in humans, and our efforts to identify the ideal biomaterial for breast reconstruction will continue.

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Figure 1. Decellularization assessment. The extracellular matrix of ADMs was assessed under the light microscope at × 400 magnification after staining with H&E.

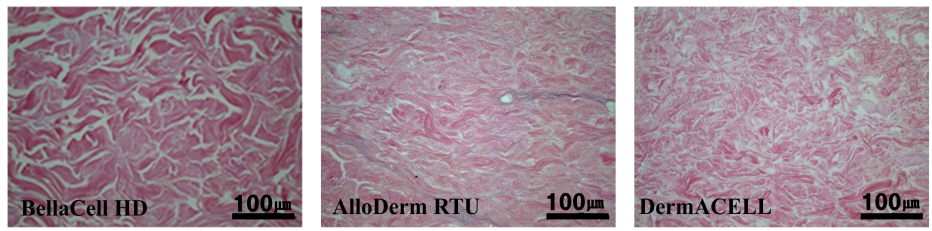


Figure 2. Biocompatibility assessment. (A) Cell adhesion on the surface of ADMs was observed under the light microscope at × 200 magnification after staining with H&E. (B) Cell proliferation was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay.

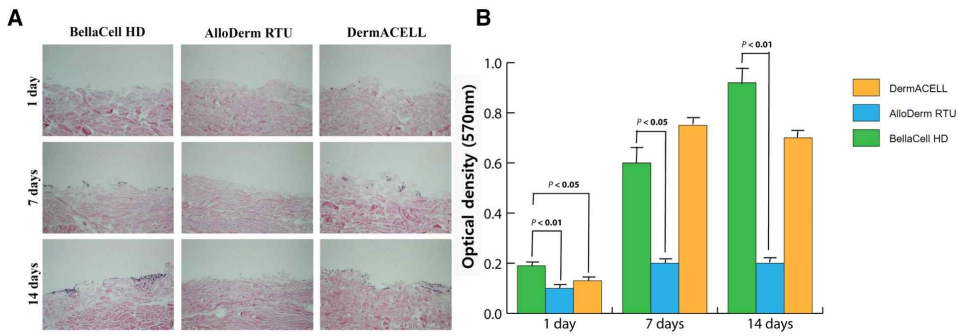


Figure 3. Cytotoxicity assay. (A) Cell morphology was observed under the light microscope at × 200 magnification. (B) Cell viability was assessed by a MTT assay.

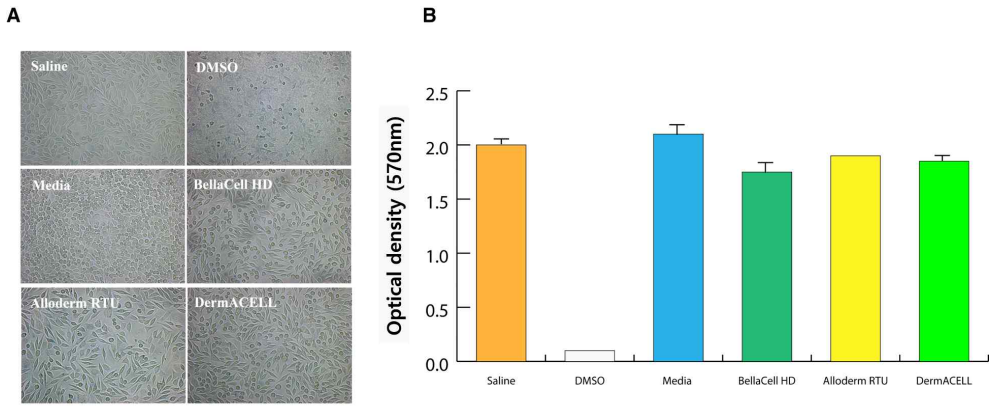


Figure 4. Uniaxial tensile testing (A) Maximal load (B) Tensile strength (C) Elongation testing.

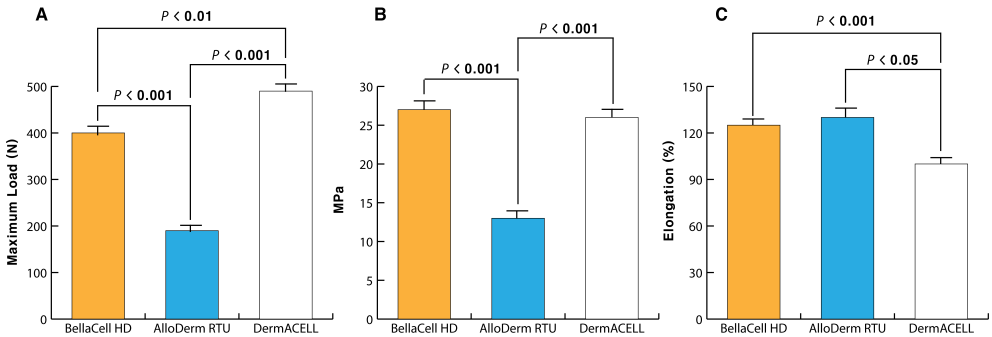


Figure 5. Stiffness testing. Stiffness testing was performed using a custom test fixture. The custom test fixture was fabricated on the basis of ASTM specification #F1306.

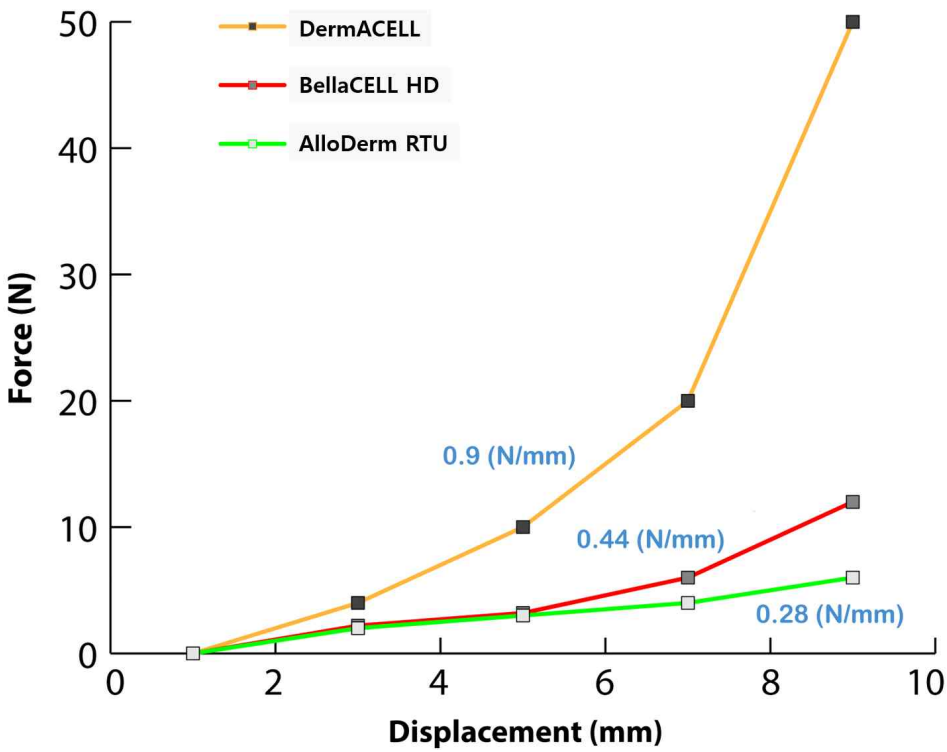
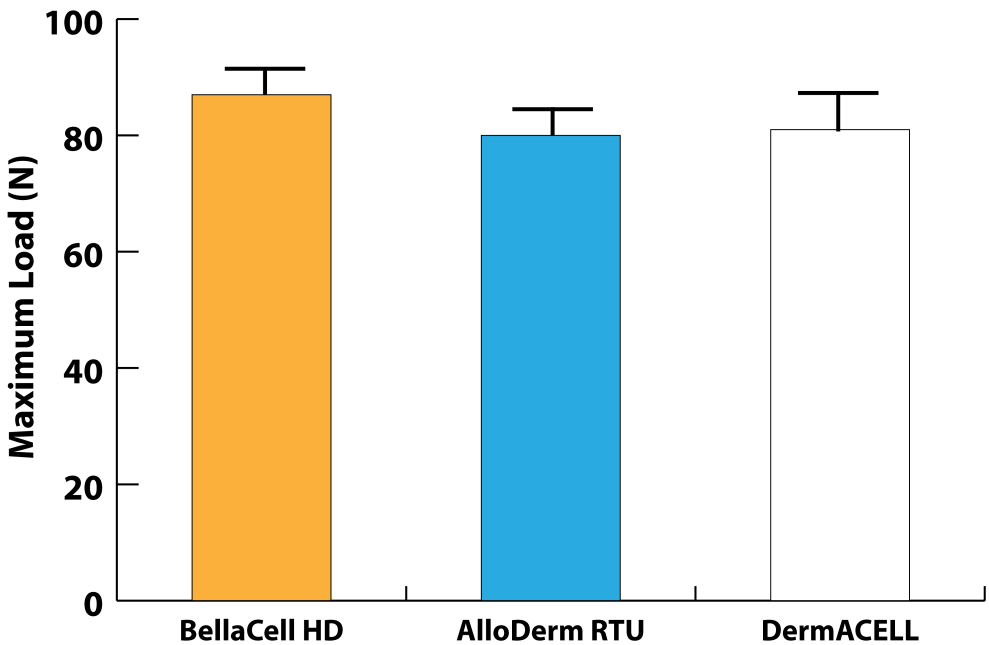


Figure 6. Suture retention strength testing. Four samples (2 × 4 cm) were prepared from each ADM. From the bottom of the sample, a suture was passed through the mesh (1.0 cm). A pulling rate of 20 mm/min was applied as the suture tore out of the ADMs. The maximum load (N) was recorded as mean ± SEM (standard error of the mean).



국문초록

연구 배경

지난 20년 동안 무세포 진피 기질은 보형물을 이용한 유방 재건에 널리 사용되어왔다. 무세포 진피 기질의 임상적인 효과에 영향을 미치는 요인은 다양하게 연구되었는데, 무세포 진피 기질 자체의 특성에 관한 연구는 미미한 실정이다. 이 연구는 유방 재건에 사용되는 새로운 인체 유래 무세포 진피 기질(벨라셀 수화 진피)을 현재 상용화된 두 종류의 인체 유래 무세포 진피 기질(알로덤®, 더마셀®)과 비교하여 생체 외 특성화 연구를 진행한 것이다.

연구 방법

각각의 무세포 진피 기질(벨라셀 수화 진피, 알로덤®, 더마셀®)을 대상으로 탈세포화 상태, 생체적합성, 기계적 특성 평가를 시행하였다. 탈세포화 상태는 헤마톡실린 에오신 염색법을 이용하여 광학현미경 하에 평가하였다. 생체적합성 평가를 위해 쥐의 섬유아세포에 대한 세포 독성, 섬유아세포의 세포 증식을 MTT법을 이용해 측정하였다. 그리고 기계적 특성 평가를 위해 인장 강도, 강직도, 봉합사 유지 강도를 측정하였다.

연구 결과

벨라셀 수화 진피는 광학 현미경 상에서 완전한 탈세포화 상태를 보였다. 또한, 섬유아세포를 접종하여 배양했을 때, 낮은 세포 독성과 높은 섬유아세포의 침투를 보였다. 각 무세포 진피 기질에서 섬유아세포의 증식을 측정하였을 때 벨라셀 수화 진피의 세포 증식이 1($p<0.01$), 7($p<0.05$), 14($p<0.01$)일 째 알로덤®보다 높게 측정되었고, 1($p<0.05$)일 째 더마셀®보다 높게 측정되었다. 그리고 벨라셀 수화 진피는 알로덤®보다도 높은 389.92N의 최대 인장력, 26.48MPa의 최대 인장 강도를 보였다. 연신율은 더마셀®보다 높은 123.4%로 측정되었고, 강직도는 더마셀®보다 낮은 0.44 N/mm로 측정되었다. 그러나 봉합사 유지 강도 연구에서

는 유의한 차이를 보이지 않았다.

결론

벨라셀 수화 진피는 생체 외 특성화 연구에서 완전한 탈세포화, 높은 생체적합성, 낮은 세포 독성, 높은 인장 강도, 높은 연신율, 낮은 강직도, 그리고 높은 봉합사 유지 강도를 보였는데, 이는 현재 상용화된 두 종류의 무세포 진피 기질과 비견할 만한 수치였다. 이러한 결과는 벨라셀 수화 진피 또한 보형물과 조직확장기를 이용한 유방 재건에서 효과적이고 안전하게 사용될 수 있는 인체 유래 무세포 진피 기질임을 시사한다.

주요어 : 생체재료, 무세포 진피 기질, 유방 재건, 생체 외 연구, 생체적합성 평가, 기계적 현상
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