의학석사 학위논문

돼지 창상 모델에서 지방 유래 줄기 세포가 창상의 인장 강도 회복에 미치는 영향

2022년 2월

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이 논문을 의학석사 학위논문으로 제출함
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김진현의 석사 학위논문을 인준함
2022년 1월

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초 록

배경 및 목표

흉터를 최소화하기 위해서는 봉합사를 가능한 조기에 발사하는 것이 필요하다. 봉합사가 오래 유지될 경우 흉터 주위에 흔적이 남게 되기 때문이다. 지방유래 줄기세포로 인해 상처회복이 촉진되어 상처의 긴장력이 조기에 회복된다면, 봉합사의 조기 발사가 가능할 것이다. 이번 연구를 통해 이를 실험적으로 확인하고 향후 임상적용의 가능성을 타진해 보고자 한다.

방법

돼지 3 마리의 등 양쪽에 3cm 길이 창상을 각각 7개씩 만들었다. 이 때 자체 제작한 장력계를 활용하여 창상 봉합시 필요한 장력이 각각 0.1kgf, 0.2kgf, 0.3kgf, 0.4kgf, 0.5kgf, 0.6kgf 이 되게끔 하였다. 각각의 창상을 봉합하였고, 한 쪽에는 식염수를 주입하고, 반대쪽에는 지방 유래 줄기 세포를 주사하였다. 3주, 4주, 9주 뒤 시점에 3 마리의 돼지로부터 창상 봉합의 수직 방향으로 피부를 절제하여 장력 및 흉터 조직 크기와 더불어 사이토카인 수치를 측정하였다.

결과

지방 유래 줄기세포는 상처의 인장 강도 회복을 촉진시키며, 이를 사용하여 인장력을 많이 필요로 하는 창상의 합병증을 줄일 수 있을 것으로 생각된다. 또한 지방 유래 줄기 세포는 흉터 형성을 저해하는 사이토카인 분비에도 영향을 끼쳐 흉터 형성을 줄이고, 조기 봉합사 제거를 가능하게 할 것이다.

주요어: 지방, 줄기세포, 동물 실험, 인장 강도, 상처 회복
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목 차

그림 목차

I. Introduction

Adipose-derived stem cells (ADSC) are mesenchymal stem cells that have the ability to differentiate into other mesodermal tissues such as the bones, cartilages and muscles. (1) Previous studies have identified ADSC to also play an important role in tissue regeneration and wound repair. Though not fully clear, this is thought to be done through cell differentiation, proliferation and migration, along with increased secretion of various cytokines relating in the wound healing process. (2, 3)

Tensile strength is the force needed to break a tissue perpendicular to the direction of the force which is applied. It is the most accurate measurement in assessing the tissue ability to withstand tension. (4) The recovery of wound tensile strength is an important part of the healing process. Faster recovery of tensile strength may enable fewer sutures to be applied on wounds and also may allow early removal of suture materials. This will not only lead to reduced scar formation but also enable patients to recover to their daily physical activity in a shorter period of time. (5) In this aspect, attempts have been made to enhance tensile strength in

various wound situations. Brown et al. have investigated the effects of epidermal growth factor (EGF) and transforming growth factor-beta(TGF-β1) on tensile strengths of wounds on rodents. Also, a number of animal studies have shown low energy laser irradiation to improve wound tensile strengths. (6) Recently, a study by Lee et al. reported that activated mesenchymal cells increase wound tensile strength recovery via macrophages in aged mouse models. (7)

Though our previous study has shown faster wound healing process using adipose-derived stem cells in mouse models, we were not able to determine whether or not ADSC facilitates tensile strength recovery of the skin. (8) Considering previous studies mostly conducted on rodents which the skin properties greatly differ to that of the humans, we aimed to evaluate the effect of ADSC on tensile strength recovery using porcine skin, which closely resembles the human skin anatomically. (9) We attempted to determine the efficacy of ADSC on skin tensile strength recovery using a customized tensiometer.

II. Materials and methods

1. Customized tensiometer and wound tensile strength

A customized tensiometer (Figure 1.) was manufactured for objective measurement of wound tension and evaluation of tensile strength. A strain gauge was attached to a towel clamp. When force such as skin tension was applied, a slight bending of the towel clamp occurred and the strain gauge was able to detect this change. The tensile strength was displayed on an instrument panel connected to the tensiometer. Device calibration was needed for objective measurement. The towel clamp was fully opened, then closing it at a constant speed of 0.1mm/s, the tensile strength was measured and recorded. Calibration was done and was compared to the reference force sensor, which showed significant concordance. (Figure 1.) Using the customized tensiometer, we were also able to determine the tension of the skin strip as force was increased in both sides of the strip in opposite direction. We were also able to determine the breaking strength, which is the force applied when the skin strips ruptured. This breaking strength was considered as the tensile strength of the tissue.

The applied tension was categorized into 2 groups. The high tension group was wound tension greater than 0.3kgf and low tension group was wound tension lesser than 0.3kgf. For each of the previously made wounds, 3 skin strips were harvested as in section *Animal model and histologic slides.* Using our customized tensiometer, the tensile strengths for each skin strips were recorded.

2. ADSC isolation and culture

Three pigs were used for ADSC harvest in this study. Under anesthesia with ketamine, shaving was done and skin preparation with 7.5% povidone iodine solution was done. A linear incision was made at the inguinal region of the pigs, and approximately 15ml of subcutaneous adipose tissue was harvested. The wound was then closed layer by layer. The harvested tissue was then extensively washed with sterile phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA). They were then minced into small fragments of $1mm^3$ size and were incubated with 0.2% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 60 minutes at 37℃. Then, after neutralization of the collagenase, the resulting cell suspension was centrifuged at 1200rpm for 10 minutes. The

supernatant was then discarded and the sediments were placed in 100mm^2 tissue culture plates containing Dulbecco's Modified Eagle's Medium (DMEM) and 50 mL/L fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). The plates were kept in a humidified atmosphere of 5% carbon dioxide at 37℃. At 90% confluence, cells were detached with 0.25% trypsin-EDTA and passaged.

3. Immunofluorescence staining

The passaged ADSCs were seeded in 6-well culture plates at 2 x 10^5 cells/well. The cells were then cultured for 3 days in medium containing DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). They were then washed with PBS and went through fixation process using 4% paraformaldehyde for 30 minutes. After fixation, the cells were incubated overnight with antibodies CD34, CD45, CD73 and CD 105 (1 : 200, Abcam, Cambridge, UK). The cells were washed with PBS 3 times and then rabbit anti-mouse immunoglobulin G (IgG; 1:200, Abcam, Cambridge, UK) were added and incubated for another 45 minutes. The fluorescence images of the samples were taken under the inverted fluorescent microscope (Leica-DMI4000B+DFC405C,

Leica Microsystems, Wetzlar, Germany) and then processed using Leica Application Suite X imaging software. (Figure 2.)

4. Animal model and histologic slides

All animal experiments were performed in accordance with the International Guiding Principles for Animal Research and guidelines provided by our institution review board. Three pigs were anesthetized using general anesthesia. First, the customized tensiometer was applied on the pig skin to determine the amount of skin excision needed to achieve certain amount of skin tension. Seven 3cm skin excisions were made on the back of the pig on both sides, so that the tension needed for wound closure were 0.1kgf, 0.2kgf, 0.3kgf, 0.4kgf, 0.5kgf and 0.6kgf respectively (Figure 3.). The wound was closed by applying 3 stitches in each wound using nylon 5-0 which was removed at week 3 across the board. Then on the right side wounds, 100 μL of ADSC with the concentration of 5 x 10^6 cells/ml was injected, while on the left side, normal saline of the same amount was injected as a control. The same procedure was carried out on the rest of the 2 pigs. Three different timelines of 3 weeks, 4 weeks and 9 weeks after skin excision were set for tensile strength evaluation on 3 experimental pigs respectively.

Skin harvest was done by harvesting the skin in vertical strips 5mm x 35mm in size, perpendicular to the direction which was skin was excised, parallel to the direction of the previously applied nylon stitches. The length of each strips were 3cm and tensile strength of each skin strip was examined using the customized tensiometer until the breakpoint. The harvested skin strips were also stained using Hematoxylin and eosin to compare the scar width according to the tension and the injected material. Scar width was measured using the Image J software (National Institutes of Health Image, Bethesda, MD, USA) and analyzed.

5. Quantitative RT-PCR analysis

To identify the changes in gene expression of the healing wound, quantitative real-time PCR analysis was performed with 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) Samples were harvested from skin harvested at week 9. Both from the ADSC injected group and the control group, 4 μg of total RNAs were harvested for each tension groups (0kgf, 0.1kgf, 0.2kgf, 0.3kgf, 0.4kgf, 0.5kgf, 0.6kgf). RNAs were treated with RNAse followed by inactivation at 65℃. Then, reverse transcription using Stratagene RT-PCR kit (Stratagene, LaJolla,

CA). The samples were assessed for GAPDH which is a housekeeping gene used for control. TGF-β1, HSP-47, MMP-2 and MMP-9 were subsequently analyzed and the quantitative values were recorded. For normalization, the values for the skin harvested from the control group without any tension (0kgf) was set as 100% and the rest of the values were calculated as percentage of this.

6. Statistic analysis

Statistical analysis of data comparing results from the control group and the ADSC injected group was performed using SPSS version 26.0 (IBM, Armonk, NY). Statistical significance was determined using the Mann-Whitney U nonparametric test, and multiple comparisons were tested with two-way ANOVA. P-value of <0.05 was considered statistically significant.

III. Results

1. Tensile strength

Average skin tensile strength was superior in skin strips that were injected with ADSC and harvested 4 weeks and 9 weeks after excision, compared to the control groups. The average tensile strength of the low tension group, harvested on week 3 was 4.47N in the control group and 3.96N in the ADSC injected group. In the high tension group, the strength was 3.85N in the control group and 3.36N in the ADSC injected group.

In wounds harvested at week 4, the ADSC injected group showed higher strength compared to the control group in both the high and low tension group. In the high tension group, the ADSC injected wound showed average of 5.60N while the control group showed3.38N. In the low tension group the tensile strength of ADSC injected group was 7.29N and that of the control group was 6.59N. The wounds harvested 9 weeks after the excision showed similar pattern with the tensile strength recovery showing statistically significant difference compared to the normal saline injected group. For the high tension group, average tensile strength was 8.15N in the ADSC injected group (P−value = 0.008), while

the strength of the control group was 4.47N. In the low tension group, tensile strength was 10.6 in the ADSC injected group and 7.73N in the control group (P-value = 0.00003).

2. PCR analysis

Four cytokines TGF-β1, HSP-47, MMP-2, MMP-9 were evaluated using the RT-PCR system. The average TGF-β1 level was higher in the control group compared to the ADSC injected group. For each specimen with wound tension ranging from 0kgf to 0.6kgf, TGF-β1 level was higher in all but in wound tension of 0.4kgf and 0.5kgf. (Figure 5.) Average level of HSP-47 was also higher in the control group. Not like that of the control group, ADSC injected group showed similar levels of HSP-47 despite the wound tension difference. (Figure 5.) However, MMP levels showed opposite results. Both the MMP-2 and MMP-9 was higher in ADSC injected group. MMPs of the control group did not show great differences according to the tension difference. (Figure 5.)

3. Scar width

The harvested skin strips for tensile strength recovery evaluation were also histologically analyzed for the effect of ADSC on scar width formation. The specimens were cut at the center of the strip and hematoxylin and eosin (H&E) staining was done for cross-sectional analysis. The measurements were done using ImageScope software (Aperio Technologies, Inc., Vista, CA). For the skin strip without any tension (0.0kgf) the average scar width was 345㎛ for the ADSC group and 247㎛ for the control group. Skin strip with 0.1kgf tension showed 237㎛ in the ADSC group and 230㎛ in the control group. For the 0.2kg tension skin strip, 233㎛ was measured in the ADSC group and 402㎛ in the control group. Skin strips with 0.3kgf tension showed 323㎛ width of scar in the ADSC group, and 321㎛ in the control group. For the 0.4kgf skin tension, 294㎛ was measured in the ADSC group, and 332㎛ in the control group. The scar width of 0.5kgf skin tension showed 204㎛ scar width in the ADSC group and 285㎛ in the control group. For the highest measured tension group (0.6kgf) the scar width was 319㎛ in the ADSC injected group and 274㎛ in the control group. In the low tension group, the average scar width was shorter in the ADSC injection group (272㎛) compared to that of the control group (293 ㎛) (P value = 0.6424). For the high tension group, the results also showed shorter scar width in the ADSC group (285㎛) compared to

that of the control group (303㎛) (P value = 0.2920). Although the results were not statistically significant, the results showed a tendency of lesser scar width formation in the ADSC injected group.

IV. Discussion

ADSCs have been recognized to play a role in repair of damaged tissues and cell regeneration. (10) The mechanism of ADSCs involved in the regenerative process remains unclear. Besides wound healing, in vitro studies have noted ADSCs to promote cartilage and neural regeneration. (11, 12) Also, a study on diabetic rats have identified that intradermally injected ADSCs enhance skin wound healing, but not through angiogenesis or collagen accumulation. (13) Intravenous injection of ADSCs to rats who have received hepatectomy have shown beneficial effects in hepatic regeneration. (14) Our previous study has demonstrated that ADSC showed improved wound healing regardless of method of ADSC administration. (8) Still, not much study has been done to identify the effect of stem cells on wound tensile strength regeneration.

Lee et al. reported that activated mesenchymal stem cells (MSCs) can increase wound tensile strength in aged mouse model via their effects on host macrophages. (7) In this study, both young and aged mice achieved increase in tensile strength when mesenchymal stem cells were injected. However, the study did not indicate whether injection of MSCs affect the amount of wound strength of spontaneous recovery to reach the same amount of

wound strength of spontaneous recovery when MSCs were injected. Also the research was conducted on rodents which bears many differences to the human skin appendage structure and the mechanism of wound recovery.

However, porcine skins shares some characteristics with human skins that they both heal partial-thickness wounds largely through re-epithelialization, and are anatomically and functionally similar. (9) Because of this similarity, pig skin has been used as an excellent animal model to investigate human wound healing. (15) In this regard, we used porcine skins to test wound tensile strength recovery. We developed a customized tensiometer using a towel clamp to measure the tensile strengths. Since towel clamps are commonly used for wound approximation during operations, the tensiometer may be used intraoperatively in the future especially when facing wounds with considerable tensions.

Our results showed that in wounds made 4 weeks and 9 weeks prior to harvest, the tensile strength recovery of ADSC injected group was enhanced compared to that of the control group, both in the high tension group and low tension group. (Figure 4.) Only the measurements at week 9 were statistically relevant. Though the tensile strength of wounds harvested at week 3 did not

show improved recovery, this may imply that ADSCs accelerate tensile strength recovery at a later phase of the healing process rather than the early phase. Also, since the ADSC injected wounds at week 9 showed improved tensile strength in both the high and low tension group, it can be said that ADSCs can improve the tensile strength regardless of the initial wound tension.

According to the PCR analysis, the group with ADSC injection showed lower levels of TGF-β1 and HSP-47 and higher levels of MMP-2 and MMP-9 compared to that of the control group, especially in wounds with high tension. (Figure 5.) TGF-β1 is a fibrogenic cytokine related to the formation of cutaneous scars. HSP-47 is known to modify and assemble collagen fibers which also relates to scar tissue formation. The increase of these 2 cytokines may imply that scar formation is actively being done. The MMPs however, are known to degrade the extracellular matrix and therefore alter the micro-environments in tissue remodeling, which has a preventative effect of scar formation. Our results imply that ADSC injection not only improves tensile strength formation but also has a positive effect on scar attenuation. This was confirmed in the histologic analysis measuring the scar width, which represents the amount of scar tissue formation. The results show greater scar width in the control group specimen compared to the ADSC injected

tissue. (Figure 6.)

There are numerous clinical situations where wounds with high tension are dealt with. Cancer wound sites such as the mastectomy wound or flap donor sites can be examples. The use of ADSCs injection on theses wound sites will be of help in wound tensile strength recovery and decrease the amount of scar tissue formation. Through this effect, it will also eventually make it possible for the early removal of sutures.

Though we used porcine skins known to be similar to that of the humans, using only one pig per measurement period can be said as a limitation of our study. Clinical studies will be needed for ADSCs to be used in the future.

V. Conclusion

ADSC injection can accelerate tensile strength recovery in wounds and therefore may decrease complication in high tension wounds. ADSCs also have an effect of modifying cytokine levels that lead to scar tissue attenuation. These positive effects of ADSCs in wound healing will also eventually make it possible for early removal of sutures.

Figure 1. Customized tensiometer and calibration

(Top row) A customized tensiometer was manufactured for measurements by attaching strain gauge to a towel clamp.

(Bottom row) Device calibration with a reference force sensor was done for objective measurements

Figure 2. Immunofluorescence staining of harvested ADSCs

The fluorescence images of the samples were taken under the inverted fluorescent microscope (Leica-DMI4000B+DFC405C, Leica Microsystems, Wetzlar, Germany) and then processed using Leica Application Suite X imaging software.

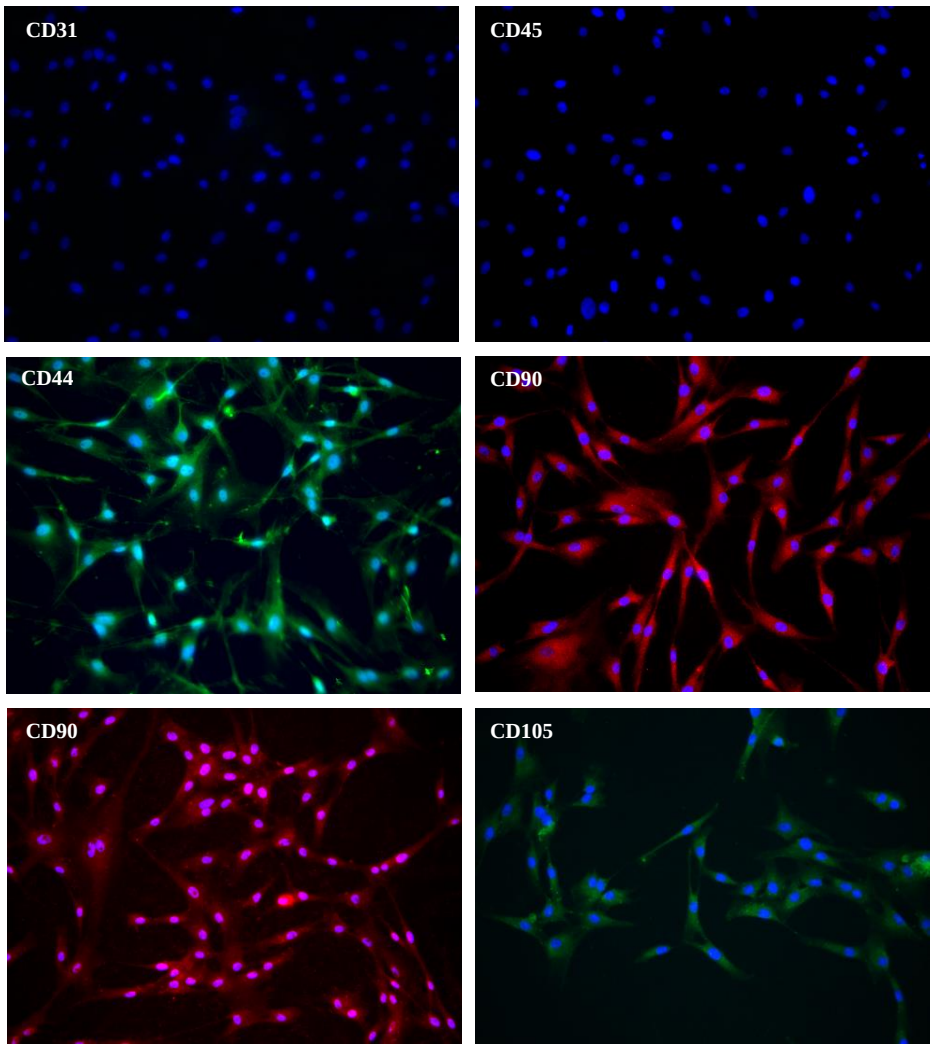


Figure 3. Porcine animal model wound and skin harvest

Seven 3cm skin excisions were made on the back of the pig on both sides (Top) Wound closure done with 3 stitches using nylon 5-0 (Middle) Skin harvest in vertical strips (Bottom left) Harvested skin strips in 5mm x 35mm size (Bottom right)

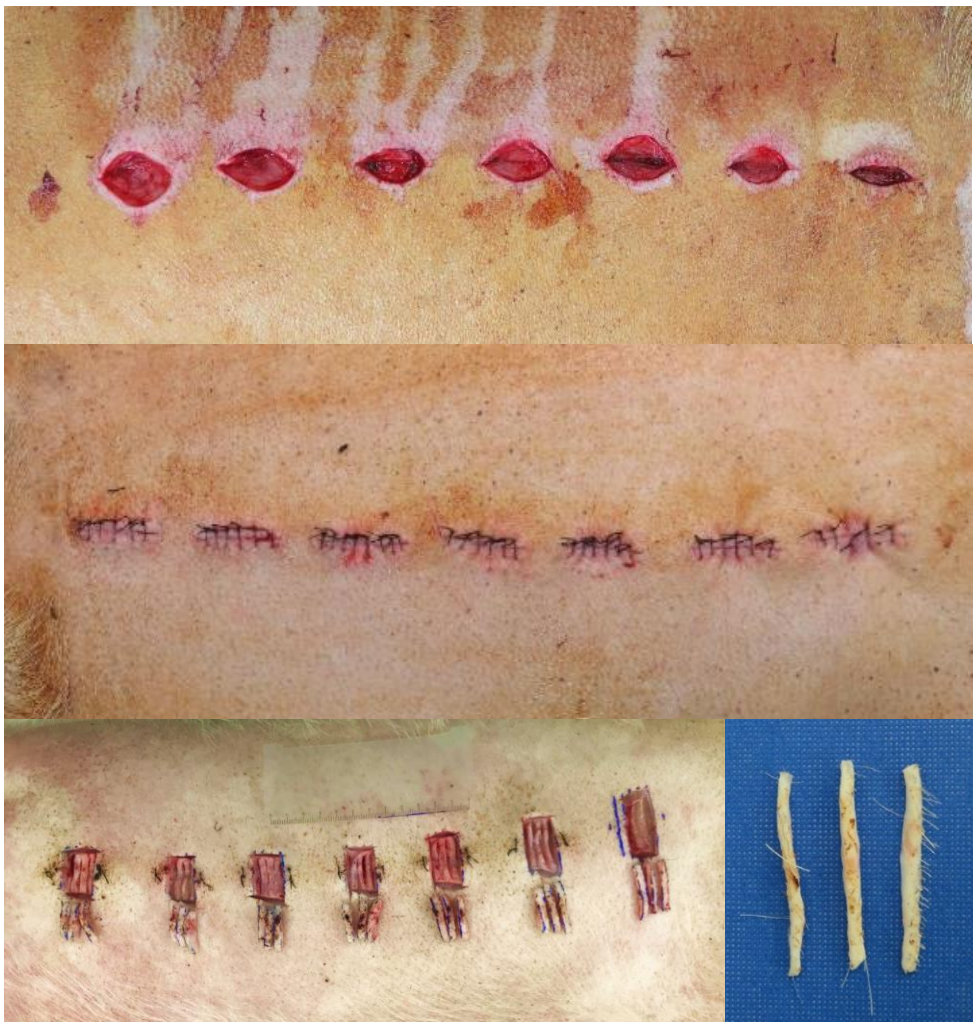


Figure 4. Tensile strength recovery according to tension and time

Tensile strength recovery of ADSC injected group was enhanced compared to that of the control group, both in the high tension group and low tension group. (Asterix indicates statistically significant result)

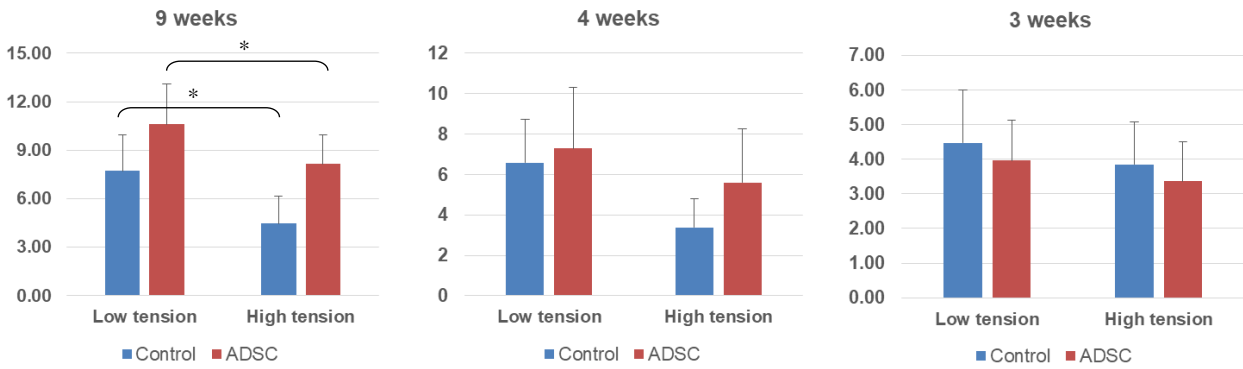


Figure 5. PCR analysis of cytokines

PCR analysis showing ADSC injected group with lower levels of TGF-β1, HSP-47 and higher levels of MMP-2, MMP-9 compared to that of the control group, especially in high tension wounds.

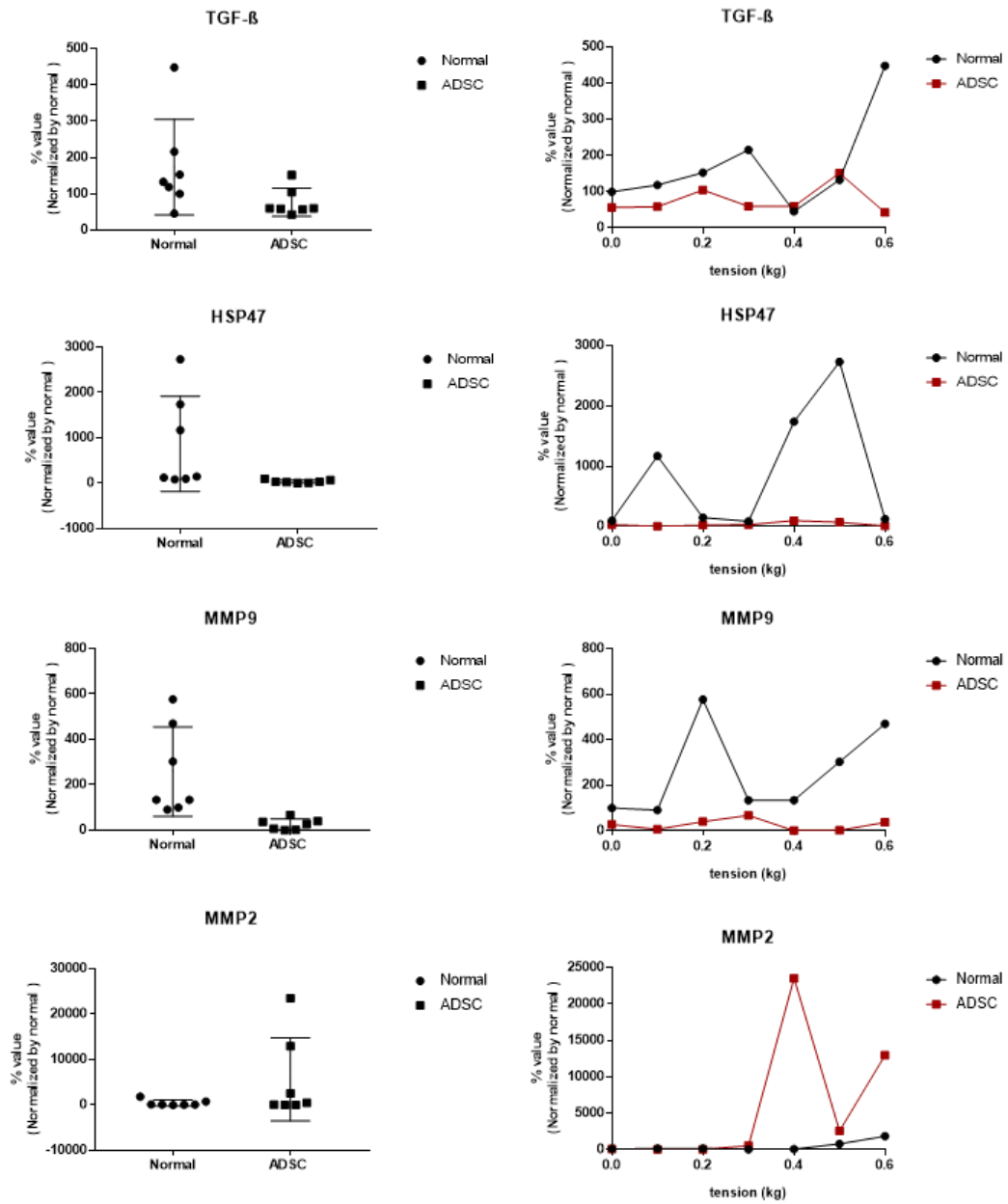
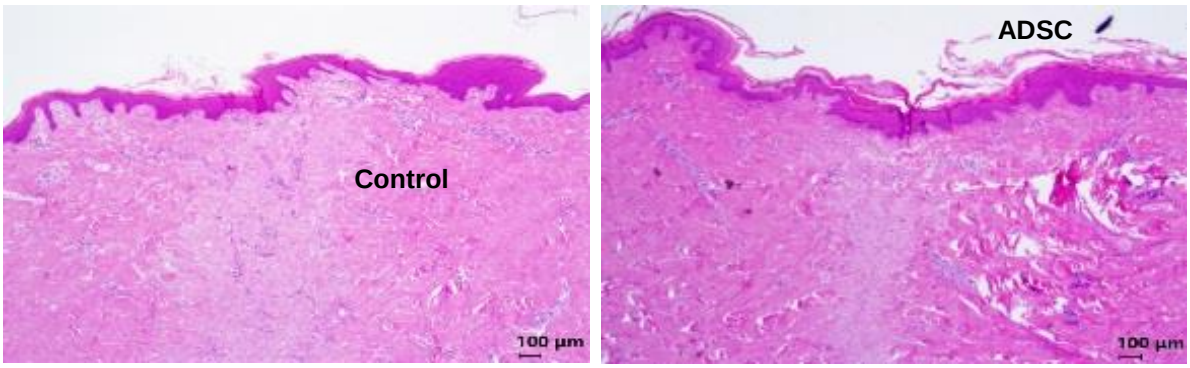
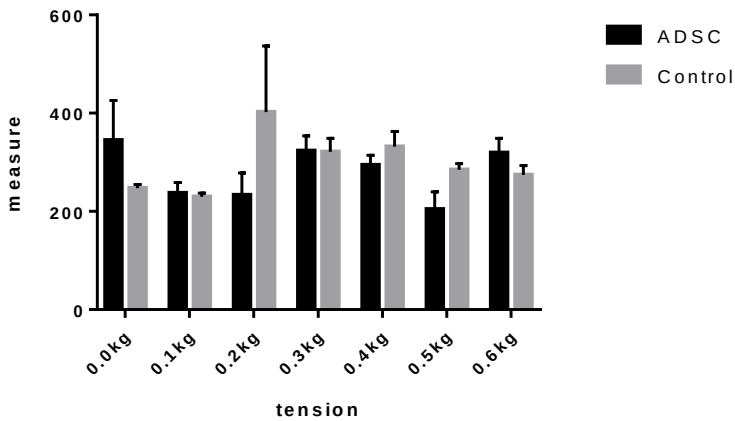


Figure 6. Histologic analysis of scar width

Representative cross-sectional histologic slide of skin strip stained with hematoxylin and eosin showing greater scar width in the control group specimen compared to the ADSC injected





참고 문헌

1. Minteer D, Marra KG, Rubin JP. Adipose-derived mesenchymal stem cells: biology and potential applications. Mesenchymal stem cells-basics and clinical application I. 2012:59-71.

2. Mojallal A, Lequeux C, Shipkov C, Breton P, Foyatier J-L, Braye F, et al. Improvement of skin quality after fat grafting: clinical observation and an animal study. Plastic and reconstructive surgery. 2009;124(3):765-74.

3. Schäffler A, Büchler C. Concise review: adipose tissue-derived stromal cells—basic and clinical implications for novel cell-based therapies. Stem cells. 2007;25(4):818-27.

4. Williams D, Harrison I. The variation of mechanical properties in different areas of a healing wound. Journal of biomechanics. 1977;10(10):633-42.

5. Ireton JE, Unger JG, Rohrich RJ. The role of wound healing and its everyday application in plastic surgery: a practical perspective and systematic review. Plastic and reconstructive surgery Global open. 2013;1(1).

6. Brown GL, Curtsinger LJ, White M, Mitchell RO, Pietsch J, Nordquist R, et al. Acceleration of tensile strength of incisions treated with EGF and TGF-beta. Annals of surgery. 1988;208(6):788.

7. Lee S, Szilagyi E, Chen L, Premanand K, DiPietro LA, Ennis W, et

al. Activated mesenchymal stem cells increase wound tensile strength in aged mouse model via macrophages. journal of surgical research. 2013;181(1):20-4.

8. Kim H, Hyun MR, Kim SW. The effect of adipose-derived stem cells on wound healing: comparison of methods of application. Stem cells international. 2019;2019.

9. Sullivan TP, Eaglstein WH, Davis SC, Mertz P. The pig as a model for human wound healing. Wound repair and regeneration. 2001;9(2):66-76.

10. Mazini L, Rochette L, Admou B, Amal S, Malka G. Hopes and limits of adipose-derived stem cells (ADSCs) and mesenchymal stem cells (MSCs) in wound healing. International journal of molecular sciences. 2020;21(4):1306.

11. Han C, Zhang L, Song L, Liu Y, Zou W, Piao H, et al. Human adipose-derived mesenchymal stem cells: a better cell source for nervous system regeneration. Chinese medical journal. 2014;127(2):329-37.

12. Wu L, Cai X, Zhang S, Karperien M, Lin Y. Regeneration of articular cartilage by adipose tissue derived mesenchymal stem cells: perspectives from stem cell biology and molecular medicine. Journal of cellular physiology. 2013;228(5):938-44.

13. Maharlooei MK, Bagheri M, Solhjou Z, Jahromi BM, Akrami M, Rohani L, et al. Adipose tissue derived mesenchymal stem cell (AD-MSC) promotes skin wound healing in diabetic rats. Diabetes research and clinical practice. 2011;93(2):228-34.

14. Seki T, Yokoyama Y, Nagasaki H, Kokuryo T, Nagino M. Adipose tissue-derived mesenchymal stem cell transplantation promotes hepatic regeneration after hepatic ischemia-reperfusion and subsequent hepatectomy in rats. Journal of Surgical Research. 2012;178(1):63-70.

15. Breuing K, Andree C, Helo G, Slama J, Liu PY, Eriksson E. Growth factors in the repair of partial thickness porcine skin wounds. Plastic and reconstructive surgery. 1997;100(3):657-64.

Abstract

Adipose-derived stem cells (ADSC) accelerate recovery of wound tensile strength in a porcine model.

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Background

The recovery of wound tensile strength is an important part of the healing process. Faster recovery of tensile strength may enable fewer sutures to be applied on wounds and also may allow early removal of suture materials. Not much study has been done to identify the effect of stem cells on wound tensile strength regeneration. We attempted to determine the efficacy of ADSC on skin tensile strength recovery using a customized tensiometer.

Methods

Seven 3cm skin excisions were made on the back of the pig on both sides, so that the tension needed for wound closure were 0.1kgf,

0.2kgf, 0.3kgf, 0.4kgf, 0.5kgf and 0.6kgf respectively. Three different timelines of 3 weeks, 4 weeks and 9 weeks after skin excision were set for tensile strength evaluation. Skin harvest was done by harvesting the skin in vertical strips 5mm x 35mm in size, followed by tensile strength measurement using customized tensiometer. Quantitative PCR analysis of cytokines along with histologic evaluation of scar width was also done.

Results

The tensile strength recovery of ADSC injected group was enhanced compared to that of the control group, both in the high tension group and low tension group. According to the PCR analysis, the group with ADSC injection showed lower levels of TGF-β1, HSP-47 and higher levels of MMP-2, MMP-9 compared to that of the control group, especially in wounds with high tension. Scar width was greater in the control group compared to the ADSC injected specimen.

Conclusion

ADSCs not only accelerate tensile strength in wounds but also modifies cytokine levels that lead to scar tissue attenuation. These positive effects of ADSCs in wound healing will eventually make it possible for early removal of sutures.

Keywords: Mesenchymal Stem Cells, Tensile Strength, Wound healing, Animal research

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