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The effect of freeze-dried human amnion on New Zealand rabbit tendon healing

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ABSTRACT

Background: Hand injury often causes acute morbidity and long-term disability. In the case of hand surgery, thirty percent of cases are from tendon injuries. The biomechanical strength of the tendon plays an important role in preventing post-repair rupture, where this strength is believed to be influenced by the number of strands of thread or the suturing technique used. In addition, biological materials such as amnion can reduce the risk of tendon adhesions and increase the biomechanical strength of the tendon itself.

Method: This study used an experimental randomized post-test-only control group research design. 18 male New Zealand rabbits had their right Achilles tendon severed, then divided into 2 groups, where group A was treated as a control while group B was treated with freeze-dried human amnion after tendon repair using braided suture (silk) material. On day 21, all rabbits were terminated and subjected to the macroscopic examination of tensile strength and microscopic examination of the number of fibroblasts and collagen density with hematoxylin-eosin staining. The test that will be carried out on each variable is the Mann-Whitney Test and T-test methods.

Result: The results showed that the average tensile strength in the treatment group was significantly greater than the control group (3.02±1.05 N/mm2 vs. 0.78±0.28 N/mm2, $p<0.05$). The mean number of fibroblasts in the treatment group was significantly greater than the control group (189.67±23.34 vs. 85.44±9.17, $p<0.05$). For the variable collagen density, 77.80% of the treatment group had collagen density with a score of 3 ($p<0.05$).

Conclusion: The application of freeze-dried human amnion after tendon grafting using braided suture material could increase tensile strength, number of fibroblasts, and collagen density in tendon healing.

Keywords: *braided suture, collagen density, fibroblasts, freeze-dried human amnion, tensile strength*.
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INTRODUCTION

Hand injury often causes acute morbidity and long-term disability. In the case of hand surgery, thirty percent of cases are from tendon injuries. In January – December 2009, there were 34 cases of tendon rupture in the Outpatient Installation of RSUD Dr. Soetomo Surabaya. The biomechanical strength of the tendon plays an important role in preventing post-repair rupture, where this strength is believed to be influenced by the number of strands of thread or the suturing technique used. In addition, biological materials such as amnion can reduce the risk of tendon adhesions and increase the biomechanical strength of the tendon itself.[1] Tendon healing goes through 3 phases, namely the inflammatory phase, the proliferative phase, and the remodeling phase.[2]

The largest and strongest tendon structure in the body is the Achilles tendon. During the activity, the Achilles tendon can withstand loads up to 3500 N. Rabbits have the Achilles tendon, which is composed of dense connective tissue arranged in parallel and has a strong tensile mechanical component. The normal tensile strength of the rabbit Achilles tendon is 50 – 100 N.[3] In general, the size of the Achilles tendon in rabbits is the same as the size of some tendons of the human hand, such as the flexor digitorum longus, and in terms of vascularity, a structure similar to the tendons in the human hand was found. Tendon biomechanics is influenced by the number and type of intramolecular and intermolecular bonds of collagen. At rest, the collagen fibers and fibrils contract; when stressed, these fibers flatten, and due to the intramolecular shift of the collagen triple helix, a linear change occurs. When the tension persists, the tendon becomes elastic and will return to its original length when the tension is released. When the tension increases, the collagen fibrils elongate and will rupture if the tension is continuously added. The strength of the flexor tendon repair is believed to depend on the number of strands or the number of sutures.[4] Tensile strength is the maximum stress that a tendon can withstand when stretched or pulled before it ruptures. Tendon adhesion is the most common complication after tendon repair. Amnion is a biomaterial that can reduce the risk of tendon adhesions after tendon repair.[5] Dry amnion contains mesenchymal stem cells and growth factors which can accelerate the healing of a wound to be faster and functionally perfect. In addition, the amniotic membrane also influences the even distribution of fibroblasts and collagen fibers.[6]

This study aimed to know the effect

of freeze-dried human amnion on rabbit tendon healing after repair.

METHOD

This was an experimental posttest-only control group design located in the Material Physics Laboratory Faculty of Science and Technology, Animal Laboratory of the Faculty of Veterinary Medicine, and Laboratory of Anatomical Pathology Faculty of Medicine, Universitas Airlangga Surabaya, on August – September 2022.

Freeze-dried human amnion and braided suture are independent variables, and the dependent variable is tensile strength, number of fibroblasts, and collagen density. 18 male New Zealand rabbits (*Oryctolagus cuniculus*) with 8 weeks average age and body weight around 2500 – 4000 grams had their right Achilles tendon cut sharply under Ketamin 150 mg/kg body weight, then divided into 2 groups. Group A, as a control group, had tendon repair using a braided suture (silk), while group B was treated with freeze-dried human amnion application after tendon repair using braided suture (silk) material. Tendon was repaired using 4/0 silk material and modified Kessler suture techniques on the core and continuous suture on the peripheral tendons. Freeze-dried human amnion is an amnion that is produced by the Biomaterial Center Dr. Soetomo Tissue Bank (Graft No. 14151.4, Batch No. 19.1426) with a preparation size of 10 x 10 cm, cut to 1 x 1 cm in size, which technically will be wrapped over the tendon which has been sharply cut and sutured.

The rabbit's skin closed directly with a 5/0 non-absorbable interrupted suture (nylon). The sutured wound was then treated with tulle and gauze and immobilized with a splint. Each rabbit was kept in a different cage in the same room and fed with the same amount and type. On day 21, all rabbits were terminated, and the skin was opened and evaluated directly to determine whether there was any tendon adhesion or not. Tendon tissue en–block took 1 cm distal and proximal from the repair site. Each sample was put into a tube, fixated with 10% formalin, and subjected to macroscopic and microscopic examination.

The maximum load of the tendon as the macroscopic examination was measured using *a* Universal Testing Machine (UTM), which belongs to the Material Physics Laboratory, Faculty of Science and Technology, Universitas Airlangga, Surabaya. The sample was pulled with a certain loading until it broke. The maximum pulling load (Fmax) and the width of the tendon (mm^2) were recorded, then each tensile strength (N/mm^2) was calculated manually with the formula:

$$\text{Tensile strength} = \frac{\text{Fmax}}{\text{tendon cross-section area}}$$

Where:

- F_{max}: the magnitude of the maximum pulling load when the tendon breaks in Newton
- Tendon cross section area = πr^2 (π= constant 3,14 and r = tendon width)

Microscopic examination was carried out with hematoxylin-eosin staining and examined under a light microscope Olympus model U-MDOB3 SN 6J14986, belongs to Anatomical Pathology Laboratory Faculty of Medicine, Universitas Airlangga, Surabaya, with 400x magnification to assess the density of collagen and the number of fibroblasts. Histopathological scoring parameters expressed on an ordinal scale of 0-4 were used to assess collagen density based on one field of view (0 = no collagen fibrils found, +1 = Low collagen fibril density (less than 10% in one visual field), +2 = Moderate collagen fibril density (10% - 50% in one visual field), +3 = High collagen fibril density (50% - 90% in one visual field), +4 = Very high collagen fibril density (90% - 100% in one visual field)). The number of fibroblasts was examined in 3 fields of view, and the mean number of them in each specimen will be calculated. All collected data from each variable were analyzed using the two-sample T-test and the Mann-Whitney test.

RESULTS

No rabbits died during the experiment, and one rabbit was found to be included in the exclusion criteria due to infection at the treatment site. After the third week, the animals were sacrificed. Figures 1 and 2 show the difference in results between the tendons that had been tested from day 0 and the third week. In tendons that were not covered by the amnion, there was an adhesion of the tendon to the surrounding tissue.

Tensile Strength

Maximal load and tensile strength data for the two groups of experimental animals are presented in graphical form, as shown in Figures 3 and 4. The distribution of the tensile strength of each group is shown in Table 1.

The results of the Mann-Whitney test showed that there was a difference in the value of tensile strength between the control and treatment groups. The value of tensile strength of the treatment group was greater than the control group (p=0.0000).

Number of fibroblasts

Figure 5 shows the histopathological picture of fibroblast cells in the control

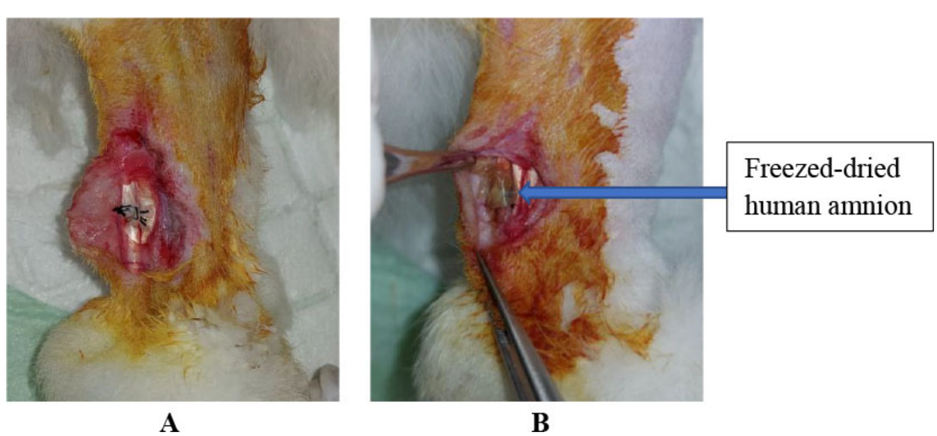


Figure 1. Right tendon Achilles New Zealand Rabbit, day 0. **A)** Group A as the control group, after tendon repair using braided suture and; **B)** Group B as the treatment group, freeze-dried human amnion was applied after tendon repair using braided suture.

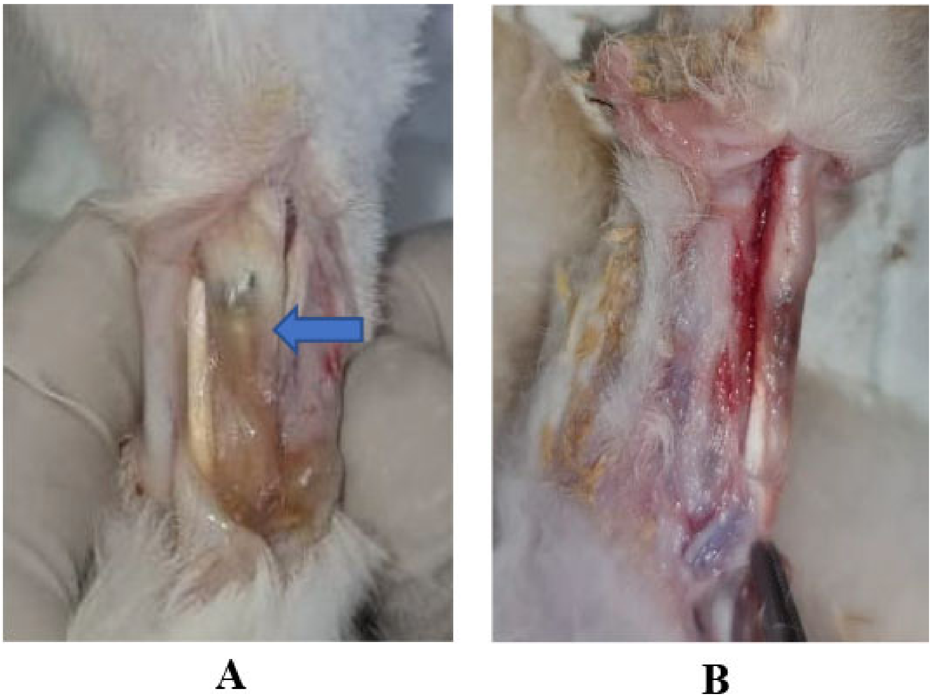


Figure 2. The appearance of the right *achilles* tendon New Zealand rabbit after 3 weeks. **A)** Control group and; **B)** treatment group, the blue arrow indicates the adhesion area.

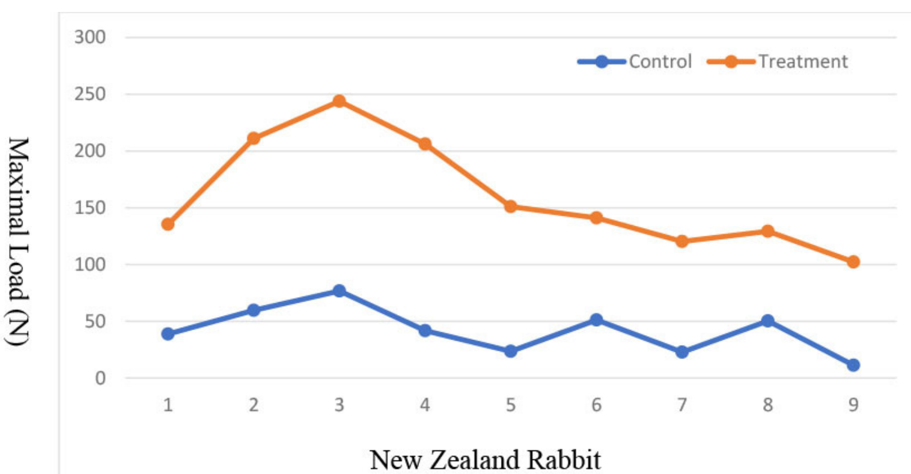


Figure 3. Maximal load (N) of tendon Achilles New Zealand Rabbits at control and treatment group.

and treatment groups. It can be seen microscopically that there are more fibroblast cells in the treatment group than in the control group. The fibroblast cells were then counted in 3 fields of view with a 400x microscope magnification. Figure 6 shows the number of fibroblasts counted in 3 fields of view under a 400x magnification microscope.

As seen in Table 2, the results of the two-sample T-test showed that the mean number of fibroblasts in the treatment group was significantly higher than the control group (p=0.000).

Collagen Density

Histopathologically, as seen in Figure 7, the density of collagen in the treatment group was denser than that of the control group. Collagen density was made in a scoring system, with the criteria mentioned in the method.

Figure 8 shows that a score of three for collagen density was found more in the treatment group, while the majority of collagen density in the control group was a score of one. The results of the Mann-Whitney test showed that there is a difference in the value of collagen density between the control and treatment groups. The value of the collagen density of the treatment group is greater than the control group (p=0.0000).

DISCUSSION

Tendon is a flexible cable that connects dynamic muscle structures with rigid bone structures, so this tissue can absorb shock and withstand tension (tensile strength). The goal of a tendon repair is the return of finger function with a complete return of joint movement. The results of statistical analysis in this study showed that there were significant results in the value of tensile strength, number of fibroblasts, and thickness of collagen in the experimental group of animals compared to the control group.

A study on the comparison of tensile strength using the 2-strand, 4-strand, and 6-strand Kessler techniques in the healing of rabbit Achilles tendon rupture was conducted by Arif *et al.* gave the result that the tendon suturing technique with a modified Kessler 4-strand had tensile strength that could withstand early active flexion. The 4-strand technique is preferred because it does not produce a large bulk in the tendon and does not impede the movement of the tendon through the pulley, and the flexion load is not large, reduces the risk of thread breaking, is technically simpler, and takes less time.[7] Caksana *et al.* in their research found differences in tensile strength values in tendons sutured with the 4-strand modified Kessler technique (62.34 ± 7.72) and tendons sutured with the continuous-cores technique (50.37 ± 3.83).[8] Referring to the two studies, the researchers used a modified Kessler 4-strand suturing technique followed by a continuous suture in the treatment after tendon rupture.

In a study conducted by Wong, braided sutures are 194% stronger than monofilament sutures in terms of repair strength. Based on this, here, the researchers used threads with braided suture material as the basis for selecting the control group. The normal tensile load of the tendon is 50-100 N.[3] From the results of research conducted by

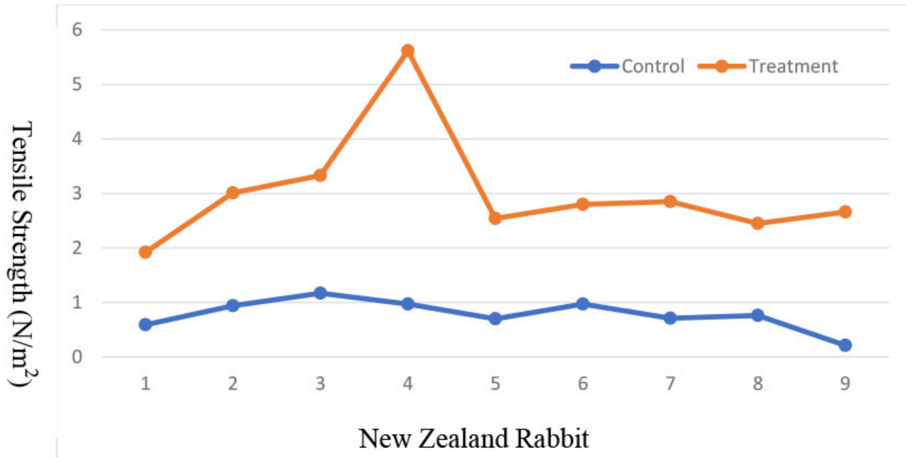


Figure 4. Tensile strength (N/mm2) of tendon Achilles New Zealand Rabbits at control and treatment group.

Table 1. **Tensile strength distribution between control group (A) and treatment group (B).**

Rabbit	n	Median (min-max)	p*
Group A	9	0,76(0,21-1,17)	<0,001
Group B	9	2,8 (1,92-5,62)	

*p-value analyzed using Mann-Whitney Test.

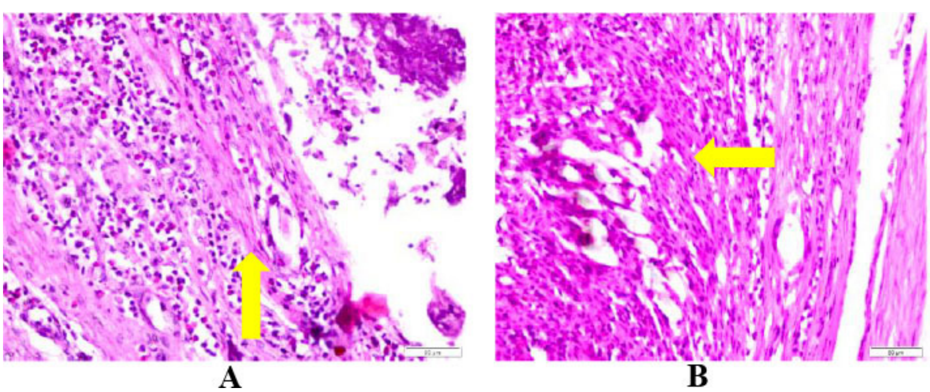


Figure 5. Microscopic fibroblast cells, viewed with an Olympus microscope model U-MDOB3 SN 6J14986 400x magnification in group A and group B.

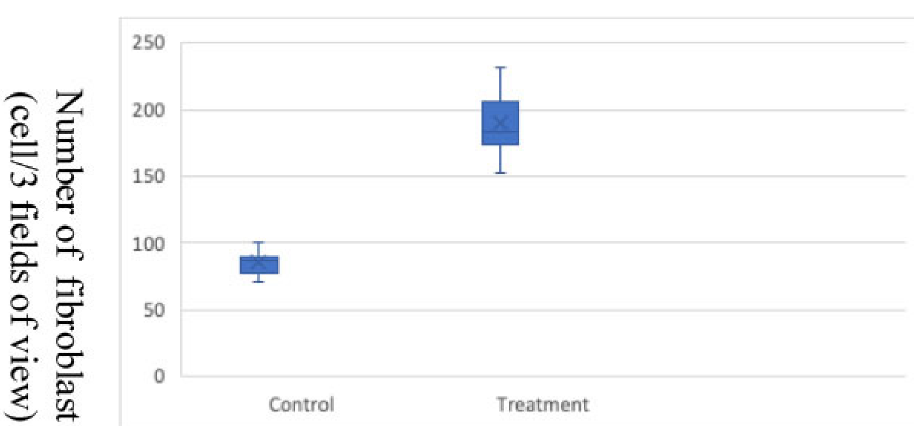


Figure 6. Number of fibroblasts (cell/3 fields of view), counted with an Olympus microscope model U-MDOB3 SN 6J14986 400x magnification.

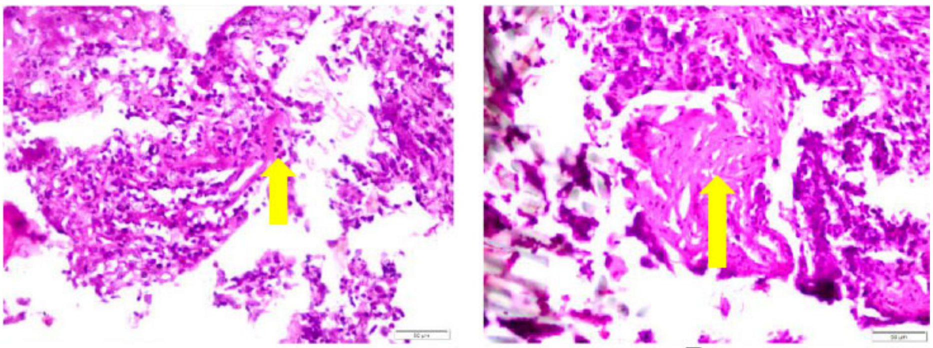
researchers, and it was found that braided suture material covered with amnion was able to withstand a maximum force of up to 169.48 N, while braided suture material without amnion was able to withstand a maximum force of 74.70 N. In research conducted by Effendi *et al.* in 2007 – 2010, with the same treatment of termination of the tendon of the rabbit but connection using monofilament suture material, a force of 51.40 N was obtained.[9] Research conducted by both researchers and Effendi *et al.* supports the research by Wong *et al.* that braided suture material can exert a greater force on tendon repair than monofilament suture. The strength of the flexor tendon repair depends on the number of threads or the number of sutures. However, the weakness of using silk thread as a braided suture material was found in this study, namely the presence of a tissue reaction in the form of infected and necrotic skin in 1 rabbit treated with A. Even though silk can provide good suture tension, accumulation of debris and bacteria can form on the surface of the material and causes inflammation around the wound.[10] In group B experimental animals, there was no tissue reaction in the form of infected skin because the amnion produces human-beta-3-defensin, which is an antimicrobial peptide that increases epithelial resistance to bacterial colonization obtained from the effect besides the use of silk suture material.[11-15]

As previously mentioned, the healing process for injured tendons goes through 3 phases, namely: the inflammatory phase, the proliferation phase, and the remodeling phase. Movement of the finger as early as possible postoperatively is possible if, after the connection of the injured tendon, the tendon has high tensile strength. High tensile strength is obtained in conditions where tendon adhesion does not occur, thus allowing good gliding of the tendon. Some other researchs stated that the amnion could reduce the risk of tendon adhesions after the connection of tendon injuries, and also, dry amnion contains mesenchymal stem cells and growth factors which can accelerate the healing of a wound to be faster and functionally perfect.[5] The amnion has components of epidermal growth factor (EGF), keratinocyte growth

Table 2. Number of fibroblasts distribution between control group (A) and treatment group (B).

Rabbit	n	Mean ± SD	p*
Group A	9	85,44 ± 9,17	<0,001
Group B	9	189,67 ± 23,34	

*p-value analyzed using two-sample T-test.



A B

Figure 7. Microscopic collagen density, viewed with an Olympus microscope model U-MDOB3 SN 6J14986 400x magnification, group A and group B, collagen B density was denser than A.

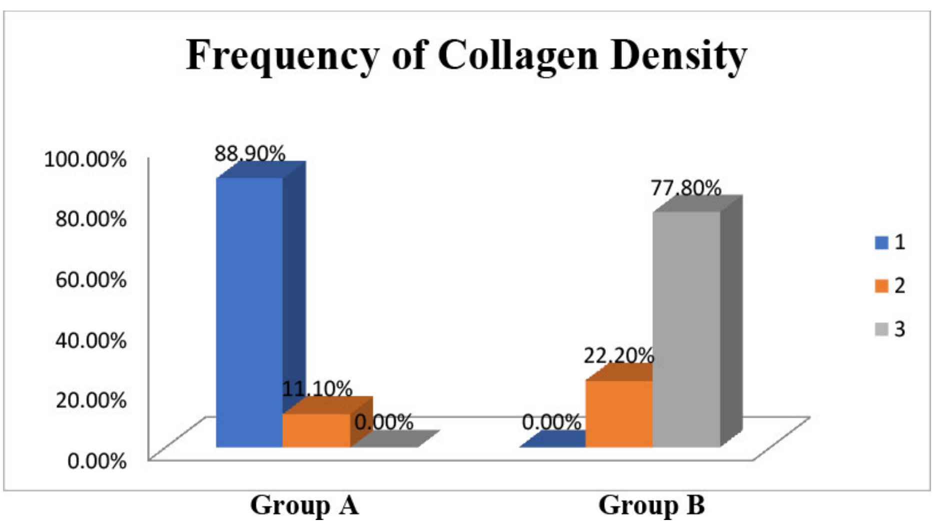


Figure 8. Frequency of collagen density in groups A and B.

factor (KGF), keratinocyte growth factor receptor (KGFR), hepatocyte growth factor (HGF), and hepatocyte growth factor receptor (HGFR), which play a role in the phases of proliferation, migration, and differentiation. of epithelial cells triggering the process of epithelialization. Besides that, the amnion also has basic components of fibroblast growth factor (bFGF) and transforming growth factor (TGF)-b1, b2, b3. The basic fibroblast growth factor (bFGF) plays a role in the formation of granulation tissue to the proliferation of fibroblasts. Transforming growth factor (TGF)-b plays a role in the synthesis and deposition of extracellular matrix (ECM) proteins and regulates the transformation of fibroblasts into myofibroblasts.[11] In addition, the amniotic membrane also influences the even distribution of fibroblasts and collagen fibers.[6]

In the research conducted, the researchers found an increase in the average tensile strength of tendon injuries that only received sutures compared to those that were covered with the amnion after suturing, namely 0.78 ± 0.28 N/mm2 to 3.02 ± 1.05 N/mm2 (p<0.05). This is possible because the amnion inhibits the process of adhesion, flattens the spread of fibroblasts and collagen fibers, and increases the production of fibroblasts through basic fibroblast growth factor (bFGF) which in turn stimulates the increased synthesis of collagen.

Histologically, the tendons that were severed and repaired by suturing showed an increase in fibroblast proliferation on the third day. The more fibroblasts that are formed, the more collagen formation will also be. In this study, a significant difference was found between the average number of fibroblasts in 3 visual fields with 400x magnification in Group A (control) obtained 85.44 ± 9.17 while Group B (treatment) 189.67 ± 23.34 (p <0, 05). The thickness of the collagen in the amnion-coated tendon group after suturing was greater than that of the non-amnion-coated group (p<0.05).

CONCLUSION

Freeze-dried human amnion has an effect on New Zealand rabbits' tendon healing by increasing tensile strength, the number of fibroblasts, and collagen density. Suggestions for further research are to conduct a larger scale study to compare the differences in tensile strength between tendon repair with monofilament and braided sutures, whether or not the amnion is covered; research differences in tendon maturation after tendon repair with braided sutures, whether or not the amnion is covered; and conduct research on the inflammatory phase and the incidence of infection after tendon repair with braided sutures, whether or not covered with amnion.

RESEARCH ETHICS

This research has been approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga, with ethic number 2.KEH.090.07.2022, and has carefully studied the proposed animal use protocol.

CONFLICT OF INTEREST

There is no conflict of interest in writing this research report.

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This research was conducted without grants, sponsors, or other sources of funding.

AUTHOR CONTRIBUTION

All authors have made the same contribution in writing the report on the results of this study, from the stage of proposal preparation, data search, and data analysis, to the interpretation of research data and presentation of the final report.

REFERENCES

1. Liu C, Yu K, Bai J, Tian D, Liu G. Experimental study of tendon sheath repair via decellularized amnion to prevent tendon adhesion. PLoS One. 2018;13(10):e0205811. Published 2018 Oct 16. doi:10.1371/journal.pone.0205811.
2. Buchanan EP, Longaker MT, Lorenz HP. Fetal skin wound healing. Adv Clin Chem. 2009;48:137-161. doi:10.1016/s0065-2423(09)48006-5.
3. Han S, Gemmell SJ, Helmer KG, et al. Changes in ADC caused by tensile loading of rabbit achilles tendon: evidence for water transport. J Magn Reson. 2000;144(2):217-227. doi:10.1006/jmre.2000.2075.
4. Wong YR, Loke AMK, Tay SC. The Effect of Suture Materials on the Biomechanical Performance of Different Flexor Tendon Repairs and the Concept of Construct Efficiency. J Hand Surg Asian Pac Vol. 2018;23(2):243-247. doi:10.1142/S2424835518500285.
5. Liu C, Bai J, Yu K, Liu G, Tian S, Tian D. Biological Amnion Prevents Flexor Tendon Adhesion in Zone II: A Controlled, Multicentre Clinical Trial. Biomed Res Int. 2019;2019:2354325. Published 2019 Apr 3. doi:10.1155/2019/2354325.
6. Yang JJ, Jang E, Song KS, and Lee JS. The effect of amniotic membrane transplantation on tendon-healing in a rabbit Achilles tendon model. Tissue Engineering and Regenerative Medicine. 2010;7(3). pp. 323–329.
7. Arif A, Chaidir R, Ismono D, and Hidajat N. Comparison of post repair tensile strength using a modified Kessler 2, 4, and 6 strand technique assessed in the third week of rabbit Achilles tendon rupture healing. MOI. 2010;38(1). pp8–16.
8. Caksana P, Kawiyana I, Dusak I, and Nindhia T. Comparison of tensile strength of continuous-core method and 4 strand modifies method of after healing of repaired Kessler rabbit's Achilles tendon injury. IOP Conference Series: Materials Science and Engineering. 2018;622. pp. 1–4. DOI:10.1088/1757-899X/622/1/012021.
9. Effendi T, Martiana IK, and Suroto H. The comparison of biomechanical properties between autograft flexor tendon with freeze dried flexor tendon composite and auto mesenchymal stem cell for the reconstruction of flexor tendon defect. 2012;1(1). p1-9.
10. Selvi F, Cakarer S, Can T, et al. Effects of different suture materials on tissue healing. J Istanb Univ Fac Dent. 2016;50(1):35-42. DOI:10.17096/jiufd.79438.
11. Fairbairn NG, Randolph MA, Redmond RW. The clinical applications of human amnion in plastic surgery. J Plast Reconstr Aesthet Surg. 2014;67(5):662-675. DOI:10.1016/j.bjps.2014.01.031.
12. Ozbölük S, Ozkan Y, Oztürk A, Gül N, Ozdemir RM, Yanik K. The effects of human amniotic membrane and periosteal autograft on tendon healing: experimental study in rabbits. J Hand Surg Eur Vol. 2010;35(4):262-268. DOI:10.1177/1753193409337961.
13. Dadkhah Tehrani F, Firouzeh A, Shabani I, Shabani A. A Review on Modifications of Amniotic Membrane for Biomedical Applications. Front Bioeng Biotechnol. 2021;8:606982. DOI:10.3389/fbioe.2020.606982.
14. Kang M, Choi S, Cho Lee AR. Effect of freeze dried bovine amniotic membrane extract on full thickness wound healing. Arch Pharm Res. 2013;36(4). p472-478. DOI:10.1007/s12272-013-0079-5.
15. Schmiedova I, Ozanova Z, Stastna E, Kiselakova L, Lipovy B, Forostyak S. Case Report: Freeze-Dried Human Amniotic Membrane Allograft for the Treatment of Chronic Wounds: Results of a Multicentre Observational Study. Front Bioeng Biotechnol. 2021;9:649446. DOI:10.3389/fbioe.2021.649446.