Basic Medical Research

Effect of lyophilized amniotic membrane, hyaluronic acid, and their combination in preventing adhesion after tendon repair in New Zealand white rabbits

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Abstrak

Latar belakang: *Untuk mengurangi adhesi peritendon pasca-reparasi tendon fleksor dapat dilakukan dengan memberikan membran amnion dan cairan asam hialuronat pada tempat reparasi tendon. Penelitian ini membandingkan efek membran amnion liofilisasi, aplikasi asam hialuronat, dan kombinasinya dalam mencegah adhesi peritendon pasca-reparasi tendon pada kelinci putih Selandia Baru.*

Metode: *64 tendon flexor digitorum fibularis dari 16 kelinci dipotong dan direparasi dengan metode modifikasi Kessler. Sampel dibagi empat kelompok; kelompok I (kontrol) dilakukan reparasi tendon saja, kelompok II pasca-reparasi dibungkus membran amnion liofilisasi, kelompok III pasca-reparasi diberikan asam hialuronat, dan kelompok IV pasca-reparasi diberikan membran amnion liofilisasi dan asam hialuronat. Pada minggu ke-6, 8 tendon dari masing-masing kelompok menjalani pemeriksaan makroskopis dan histologis dengan sistem skor Tang, untuk menilai adhesi peritendon. Sisanya menjalani evaluasi* gliding *tendon di minggu ke-10 dengan menilai rentang gerak sendi aktif dan pasif pada sendi metatarsofalangeal.*

Hasil: *Pada minggu ke-6, evaluasi makroskopis dan histologis menunjukkan kelompok II, III, dan IV secara bermakna dengan adhesi peritendon yang lebih rendah dibandingkan kelompok kontrol ($p < 0,05$). Namun, tidak terdapat perbedaan bermakna antar kelompok II, III, dan IV ($p > 0,05$). Hasil serupa ditemukan pada evaluasi* gliding *tendon, kelompok II, III, dan IV rentang gerak sendi lebih baik dibandingkan kelompok kontrol ($P < 0,05$), tetapi antara ketiga kelompok tersebut tidak ada perbedaan bermakna ($p > 0,05$).*

Kesimpulan: *Penggunaan membran amnion liofilisasi, asam hialuronat, atau kombinasi keduanya menunjukkan efek yang bermakna dalam pencegahan adhesi peritendon pasca reparasi tendon fleksor digitorum fibularis pada model kelinci.*

Abstract

Background: Several promising methods to reduce the peritendinous adhesion after repair include amniotic membrane and hyaluronic acid application on the repaired tendon site. This study aimed to compare the effect of lyophilized amniotic membrane, application of hyaluronic acid, and their combination in preventing peritendinous adhesion after tendon repair in New Zealand white rabbits.

Methods: 64 flexor digitorum fibularis tendon from 16 rabbits were cut and then repaired by modified Kessler technique. Samples were allocated into four test groups; group I as control had simple tendon repair, group II had amniotic membrane wrapping at repair site, group III had hyaluronic acid application at repair site, and group IV had combination of amniotic membrane wrapping and hyaluronic acid application. On 6th week, 8 tendon from each group underwent macroscopic and histologic evaluation of peritendon adhesion by Tang scoring system. The rest of samples were evaluated for tendon gliding at tenth week by measuring active and passive range of motion of metatarsophalangeal joint.

Results: At 6th week, macroscopic and histologic evaluation showed that groups II, III, and IV had significantly lower peritendinous adhesion compared to control group ($p < 0.05$). However, there was no significant difference found among groups II, III, and IV ($p > 0.05$). Similar results were found in tendon gliding evaluation, groups II, III, and IV had significantly better range of motion compared to control group ($p < 0.05$), but there was no significant difference found between these groups ($p > 0.05$).

Conclusion: The application of lyophilized amniotic membrane, hyaluronic acid, or their combination significantly reduce the formation of peritendinous adhesion in repaired flexor digitorum fibularis tendon in rabbit model.

Keywords: *hyaluronic acid, lyophilized amniotic membrane, peritendinous adhesion, repaired tendon*

pISSN: 0853-1773 • eISSN: 2252-8083 • http://dx.doi.org/10.13181/mji.v23i2.932 • Med J Indones. 2014;23:65-73
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Injuries to the hand with tendon ruptures have a fairly high incidence. Epidemiological data in a study show the incidence of flexor tendon injury about 1:7000. It occurs more frequently in males between age 15 to 30 years.[1] Despite advanced surgical technique and postoperative rehabilitation programs, the results of tendon repair is still difficult to predict because of the high incidence of peritendon adhesion.[1] Adhesion between tendons and surrounding tissue is the most common complication after flexor tendon repair.[2]

Many techniques have been developed to prevent adhesions after tendon repair such as refinement of suturing techniques, reconstruction of tendon sheath, and controlled early mobilization.[2] However, all these effort do not provide an effective solution in preventing peritendon adhesion. Several studies reported that peritendon adhesion almost always occurs in varying degrees and it is closely related to the suturing technique, damage of tendon sheath, and immobilization after repair.[3,4]

A wide variety of substances have been used to inhibit peritendon adhesions. Prostaglandin inhibitors, such as indomethacin and ibuprofen, have been demonstrated to have a minimal positive effect.[5] Other substances such as steroid, antihistamine, and beta-aminopropionitrile have shown promising effects in some experiments but can not be routinely used clinically because it may impaired healing process.[6] In another study, the use of aprotinin and 5-fluorouracil showed varying results.[7,8]

Until now, hyaluronic acid has been becoming one of the substances that are expected to have a positive effect on the tendon healing. Hyaluronic acid can be found in the extracellular matrix of soft connective tissue and in synovial fluid. Some previous experimental studies have shown that the application of hyaluronic acid in the ruptured tendon and its sheath may enhanced the healing process and reduce peritendon adhesions.[9,10]

Several types of materials, biological or synthetic, have been used to overcome peritendon adhesion by applying it as a barrier at the location of sutured tendon.[11,12] This isolation technique does not show satisfactory results. Some techniques even interfere with the healing process of the tendon and the other may trigger severe inflammatory response.[13] Another study reported that the use of tendon sheath substitute to prevent adhesion show unsuccessful result due to scar tissue formation at both of its attachment points.[14]

Amniotic membrane has been used previously to cover wounds, burns, and ulceration at varying locations on the body.[15,16] Vascular granulation tissue appeared within a few days and capillary formation was induced by angiogenic factors from the amnion. Application of amniotic membrane has been carried out with satisfactory results on conjunctival defects reconstruction, ear reconstruction surgery, and vaginal epithelization.[17-19] Amniotic membrane has also been used to prevent adhesion in abdominal or pelvic surgery.[20,21] Another study showed that amniotic membrane induces down-regulation of transforming growth factor-beta (TGF-β), which has a role in the process of wound healing by triggering fibroblasts and macrophages recruitment and stimulates collagen production.[22] Chang et al.[23] reported that antibodies which are capable of neutralizing TGF-β may reduce peritendon adhesions after flexor tendon repair surgery.

Until now, no study has evaluated the role of lyophilized amniotic membrane and hyaluronic acid in preventing peritendon adhesion after tendon repair. This study aims to determine whether the application of amniotic membrane, hyaluronic acid, and their combination may prevent peritendon adhesion after tendon repair surgery.

METHODS

This is an experimental study with post-test only control group design. All procedures undertaken in this study have been approved by Ethical Committee Faculty of Medicine University of Indonesia on 18 February 2013 (No. 84/H2.F1/ETIK/2013). Sixteen rabbits were randomly allocated into four test groups; group I as control had simple tendon repair, group II had amniotic membrane wrapping at repair site, group III had hyaluronic acid application at repair site, and group IV had combination of amniotic membrane wrapping and hyaluronic acid application.

Anaesthesia was done by administration of ketamine 35 mg/kg body weight and xylazine 5 mg/kg body weight intramuscularly. Cefotaxime 20 mg/kg body weight was administered for preoperative prophylaxis antibiotics. After anaesthesia, shaving, asepsis, and antiseptics steps were completed, and after the application of rubber tourniquet, a 3 cm longitudinal incision was done on the volar side of the leg, tendon sheath was incised and pulley was left intact. Both of the flexor digitorum fibularis tendon was cut using a scalpel, and then repaired with modified

Kessler technique using 5-0 polypropylene monofilament suture material and continuous suture using 7-0 polypropylene monofilament suture material.

In the control group (group I), only tendon repair was done without applying amniotic membrane and hyaluronic acid. In group II, after tendon repair, the repair site was covered by amniotic membrane which had been previously soaked in normal saline for one minute. In group III, after tendon repair, 0.3 mL of hyaluronic acid was applied at the repair site before closure of the wounds. In group IV, after tendon repair, the repair site was covered by amniotic membrane and 0.3 ml of hyaluronic acid was applied inside the membrane. Amniotic membrane used was lyophilized amniotic membrane obtained from *Badan Tenaga Nuklir Nasional* (BATAN) research tissue bank. Hyaluronic acid used was a commercial product (Suplasyn®) with a concentration of 10 mg/mL and a molecular weight of 10^6 Daltons. Surgical wound was closed with simple interrupted sutures using 4-0 braided polyglactin suture material. Immobilization was carried out on the limb in semiflexed position using casts for two weeks.

Animal subjects were kept in separate cages at temperature of 25-28°C. All animals were fed *ad libitum* with special rabbit food and have free access to drinking water. For analgesics and antibiotics, ketoprofen 0.2 mg/kg and cefotaxime 20 mg/kg were administered intramuscularly for 3 days postoperatively. After six and ten weeks, animals were sacrificed for macroscopic, histological, and tendon gliding by administering 1 mL/kg body weight 100% $MgSO_4$ intravenously. Measurement was done by assessing the macroscopic and histopathologic scores of adhesion, and also biomechanical evaluation of tendon gliding. The measurement was done blindly.

Macroscopic and histologic evaluation

On sixth week, 8 tendons from each group underwent macroscopic (Table 1) and histologic (Table 2) evaluations of peritendon adhesion by Tang scoring system.[14] Macroscopic grading was done to evaluate length, density, and tendon excursion.

From each group there were 16 tendons. Eight of them were evaluated at week-6. Finger and its tendon complex was separated and fixed with 10% formalin solution for one day. After the specimens were processed into paraffin block, each of them was cut longitudinally using a microtome with 5 µm thickness. Slides were stained for hematoxyline-eosine before microscopic evaluation. Histopathologic evaluation was done using Tang Scoring System[14] to assess how extensive adhesions formed on peritendon area (Table 2).

Tendon gliding evaluation

From each group, eight tendons underwent tendon gliding evaluation by measuring angle formed by

Table 1. Evaluation of macroscopic adhesion according to Tang scoring system*

Score	Adhesion
Length	
0	No adhesion
1	< 5 mm
2	5-10 mm
3	> 10 mm
Density and toleransion of mobility	
0	No adhesion
1	Loose, elastic, mobile
2	Moderate mobility
3	Rigid, dense, immobile
Degree of adhesion	
0	Absent
1-2	Inferior
3-4	Medium
5-6	Severe

*Data are from Tang, et al.[14]

Table 2. Evaluation of histologic adhesion according to Tang scoring system*

Score	Adhesion
Quantity	
0	No adhesion
1	Few filaments
2	Many filaments
3	Enormous filaments
Quality	
0	No adhesion
1	Regular filaments, long, and fine
2	Irregular filaments, short, and mixed
3	Dense, no filaments
Degree of adhesion	
0	None
2-1	Slight
3-4	Moderate
5-6	Severe

*Data are from Tang, et al.[14]

active and passive flexion of metatarsophalangeal joint at tenth week. Measurements done with 50 grams load indicate the level of adhesion formed on peritendon. Tendon gliding evaluation was performed using a special tool (goniometer) to measure the angle formed by the metatarsophalangeal joints after 50 g loading on the proximal (simulated active motion) and distal (simulated passive motion) from the location of repaired tendon. Then the active, passive, and difference between active and passive range of motion were determined (Figure 1).

Statistical analysis

Macroscopic and histological scores were ordinal variables thus statistical analyzes were done with Kruskal-Wallis test and post-hoc analysis by Mann Whitney test. The results of the angle of tendon gliding were numeric variable thus statistical analyzes were done by one way ANOVA test and post-hoc analysis with LSD test.

Figure 1. Evaluation of tendon gliding 10 weeks after tendon repair using goniometer

RESULTS

This study was conducted in 16 New Zealand white rabbits aged 7-10 months (mean: 8.4 months) weighing 2600-3000 g (mean: 2737.0 g)

Macroscopic evaluation

After sutures were removed, exploration was performed through previous incision site. No signs of infection found around tendon in all treatment groups. From macroscopic evaluation (Figure 2), severe adhesion found mostly in the control group, medium adhesion found mostly in amnion group, and inferior adhesion found the most on the combination group. These results are summarized in table 3.

In Kruskal-Wallis test followed by Mann-Whitney post-hoc analysis, statistically significant differences ($p \leq 0.05$) were found between the three treatment groups as compared to the control group. However, there was no significant difference ($p > 0.05$) between treatment groups; amnion, hyaluronic acid, and their combination.

Histologic evaluation

Histologic examination was performed using light microscopy with 200x magnification (Figure 3). On histologic examination, severe adhesions were

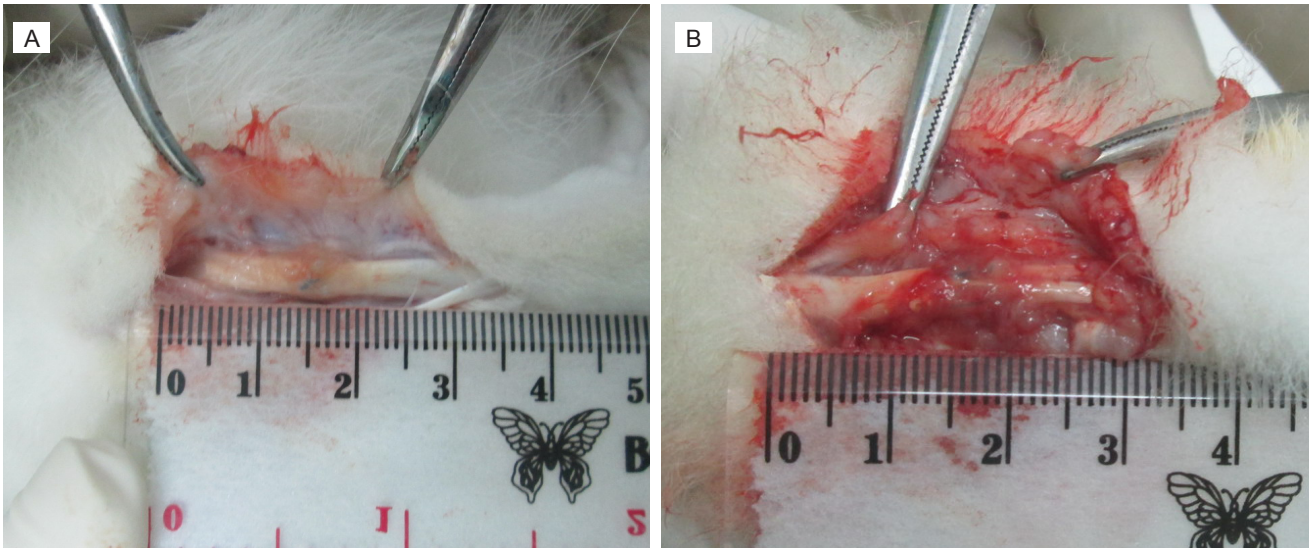

Figure 2. Macroscopic evaluation 6 weeks after tendon repair. (a) inferior adhesion, (b) severe adhesion

found mostly in control group (Figure 3A), medium adhesions were found mostly in amnion group (Figure 3B), and minor adhesions were found mostly in combination group (Figure 3C). These results are summarized in table 4.

In Kruskal-Wallis test followed by Mann-Whitney post hoc analysis, statistically significant differences ($p \leq 0.05$) were found between the three treatment groups when compared to the control group. However, there was no significant difference ($p > 0.05$) between treatment groups; amnion, hyaluronic acid, and their combination.

Evaluation of tendon gliding

The results of tendon gliding measurement were summarized in Table 5. Statistically significant differences were found between the three treatment groups as compared to the control group in active range of motion (ROM) and difference between active and passive ROM. However, no significant difference was found in passive ROM. ANOVA test followed by LSD post hoc test found statistically significant differences in active ROM between control group and the other treatment groups ($p \leq 0.05$), but no significant difference between treatment groups: amnion, hyaluronic acid, and their combination ($p > 0.05$). In the evaluation of the difference between active and passive ROM, statistically significant differences were found between control group and the other treatment groups ($p \leq 0.05$), but no significant differences were found between treatment groups: amnion, hyaluronic acid, and their combination ($p > 0.05$). The results of the statistical analysis are summarized in Table 6.

DISCUSSION

This study used New Zealand white rabbits as experimental animals with the consideration that rabbit is a mammal that has flexor tendon structure

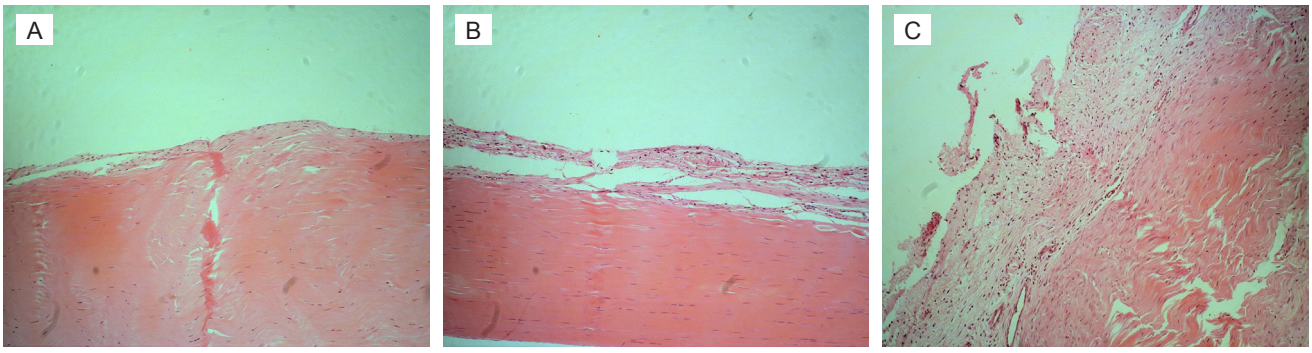

Figure 3. Histopathologic evaluation 6 weeks after tendon repair, 200x magnification. (a) inferior adhesion, (b) medium adhesion, (c) severe adhesion

Table 3. Number of tendon according to degree of macroscopic adhesion according to tang scoring system

Group	Number of tendon				
	Absent	Inferior	Medium	Severe	Total (n = 32)
Control	-	-	4	4	8
Amnion	-	1	6	1	8
Hyaluronic acid	-	2	5	1	8
Amnion and hyaluronic acid	-	3	5	-	8

Table 4. Number of tendon according to degree of macroscopic adhesion according to tang scoring system

Group	Number of tendon				
	None	Inferior	Medium	Severe	Total (n = 32)
Control	-	-	3	5	8
Amnion	-	1	6	1	8
Hyaluronic acid	-	1	5	2	8
Amnion and hyaluronic acid	-	2	6	-	8

Table 5. Result of tendon gliding evaluation

ROM	Mean ± SD*	p†
Active		0.001
Control	35.88 ± 2.47	
Amnion	43.75 ± 2.98	
Hyaluronic acid	44.13 ± 3.67	
Amnion & hyaluronic acid	45.88 ± 3.64	
Passive		0.81
Control	11.38 ± 1.92	
Amnion	10.75 ± 2.66	
Hyaluronic acid	10.75 ± 2.38	
Amnion & hyaluronic acid	10.25 ± 1.98	
Active-passive		0.002
Control	24.50 ± 3.63	
Amnion	33.00 ± 2.62	
Hyaluronic acid	34.63 ± 3.31	
Amnion & hyaluronic acid	35.00 ± 3.39	

*in degree †ANOVA test, ROM: range of movement

Table 6. Statistical analysis of active range of motion dan active-passive range of motion

Group	Active range of motion (p)*	Active-passive range of motion (p)*
Control and amnion	0.001	0.004
Control and hyaluronic acid	0.001	0.001
Control and amnion-hyaluronic acid	0.000	0.000
Amnion and hyaluronic acid	1.000	1.000
Amnion and amnion-hyaluronic acid	1.000	1.000
Hyaluronic acid and amnion- hyaluronic acid	1.000	1.000

*LSD post-hoc test

resembling the shape of flexor tendon in humans. In this study, methods used to minimize bias were by using homogeneous experimental animals, treatment techniques, and aftercare. Tendons were severed and repaired with the same suture technique and materials.

Effect of lyophilized amniotic membrane on peritendon adhesion

In this study, there was positive effect of lyophilized amniotic membrane on the prevention of adhesion of repaired tendon. These results were obtained through three types of evaluation; macroscopic, histological, and measurement of tendon gliding. The amniotic membrane group showed a significant difference compared to the control group in reducing peritendon adhesion. The number of filaments formed by fibrotic tissue was significantly lower than control group. This is consistent with previous studies which show that amniotic membrane may reduce peritendon adhesion by reducing fibrotic tissue formation.[21-25]

The use of amniotic membrane to cover burns, surgical wound, and ulceration have been reported since several decades ago.[15,16] Amniotic membrane also gave satisfactory result on conjunctival defect reconstruction, ear reconstruction, vaginal epithelialization, and abdominal surgery.[17-21] The use of amniotic membrane on tendon repair has been developed in recent years. Bennett, et al[22] reported that amniotic membrane may reduce adhesion by suppressing the number of fibroblasts and macrophages in wound healing process.

In another study, Chang, et al[23] reported that the application of a neutralizing antibody to TGF-β can reduce peritendon adhesion after flexor tendon repair. Amniotic do not express HLA-A, HLA-B, HLA-C and HLA-DR antigens, thus has a positive effect on the reduction of inflammatory reaction and fibrotic tissue formation and may reduce adhesion process.[26] Other studies reported that amniotic membrane may suppress the expression of inflammatory cytokines; IL-1α, IL-1β, thus reducing the inflammatory reaction and reduce the amount of fibrotic tissue in tendon healing process.[27] Rahmadian, et al[28] also reported that lyophilized amniotic membrane has a positive effect on the prevention of adhesion after Achilles tendon repair in rabbits.

Increased tendon gliding may also occur due to increased of relative tendon excursion mechanism with decreasing peritendon fibrotic tissue.[1] A study by Potenza reported different results where peritendon adhesion may occur despite barrier application as granulation tissue propagated from the edge of the tendon sheath to the repaired gap.[25] However, the study did not use amniotic membrane as a barrier but an artificial tendon sheath. Selection of lyophilized amniotic membrane in this study, in addition to its role as a mechanical barrier and its biological effects that may prevent adhesions, was also because it was less expensive and easy enough to implement.

Effect of hyaluronic acid on peritendon adhesion

This study shows that hyaluronic acid had a positive effect on the prevention of peritendon adhesion. The results of macroscopic, histological, and tendon gliding evaluation showed significant difference of hyaluronic acid group compared to control group. Hyaluronic acid is a substance naturally found in extracellular matrix of soft tissue, synovial fluid, and tendon sheath.[29] The positive effects of hyaluronic acid on the prevention of peritendon adhesion obtained in this study is in accordance with several studies; Hagberg, et al[9] reported that hyaluronic acid may reduce peritendon adhesion in rabbit flexor tendon after repair, Tuncay, et al[10] also reported similar effect on rat's calcaneal tendon. Yoneda, et al[30] explained that concentration of hyaluronic acid plays a role in determining the rate of fibroblast proliferation which result in peritendon adhesion. That study stated that in concentration of less than 1 mg/mL hyaluronic acid may increase fibroblast proliferation, whereas in concentrations of more than 1 mg/mL hyaluronic acid may inhibit fibroblast proliferation, thereby reducing the amount of adhesions.[30]

Rydell reported in a study that hyaluronic acid may reduce extrinsic tendon healing by inhibiting the migration of leukocytes into repaired tendon, thereby reducing inflammatory reaction and adhesion.[31] This is because hyaluronic acid has the ability to increase the negative charge and hydrophobicity on the surface of leucocyte membrane.[32] Extrinsic tendon healing has an important role in the formation of peritendon adhesion. Hyaluronic acid suppresses that process and on the other hand also promotes intrinsic tendon healing through increase in epitenon and endotenon cellular migration on the repaired tendon gap.[29]

The use of hyaluronic acid with concentration of 10 mg/mL and molecular weight of 10^6 Daltons in this study has a preventive effect on the formation of adhesion as described by several studies that suggest that the concentration and molecular weight of hyaluronic acid affect peritendon adhesion inhibition.[29,33] The molecular weight of hyaluronic acid that may give the inhibition effect of adhesion ranged from 10^5 to 10^6 Daltons.[33,9] Hagberg, et al[29] stated that the duration of elimination of hyaluronic acid from the tendon sheath is affected by its concentration and molecular weight. Meyers, et al[34] reported that the high concentration and molecular weight of hyaluronic acid has an inhibitory effects on the granulocytes movement and phagocytosis, thereby resulting in less inflammation and adhesion. The reverse is also apply in low concentration and molecular weight.

Another study by Hagberg, et al[29] in 1991 stated that hyaluronic acid as a permeable barrier may inhibits the formation of adhesions between the tendon and its sheath. This finding was reinforced by another study stated that hyaluronic acid form webs of macromolecules which function as a barrier to the diffusion of fibrinogen and fibronectin, thereby reducing the formation of a fibrin web, which in turn suppress adhesion formation.[35] Another controlled clinical study by Hagberg reported different results, in which the group with hyaluronic acid applications did not show different outcomes compared to the control group.[36] This was most likely because that study did not use additional modalities ie. mechanical barrier (such as amniotic membrane) to slow down the process of hyaluronic acid dispersion to the tissue around the tendon.

The ability of hyaluronic acid to suppress adhesion did not impede the healing process of the tendon.[9] Hellstrom, et al[37] found that hyaluronic acid may help tendon healing process by affecting the orientation of fibroblasts and collagen fibers thus accelerate reorganization process of fibrous layer. Bentley, et al[38] reported that there is an increase in the concentration of hyaluronic acid in the surrounding tissue after trauma. Another study stated that hyaluronic acid may trigger the growth of cells that contribute to tendon healing (tenocyte and fibroblasts). Macroscopic and histological evaluation in this study was performed at sixth week because at the time the formation of adhesion reaches its maximum condition.[24] Selection of hyaluronic acid in this study, in addition to its advantages as liquid that had a modulating effect in preventing adhesion, also because it is relatively affordable, readily available, and relatively easy to implement.

Effect of combined lyophilized amniotic membrane and hyaluronic acid to peritendon adhesion

This study found that the combination of lyophilized amniotic membrane and hyaluronic acid had positive effect on the prevention of peritendon adhesion. The results of macroscopic, histological, and tendon gliding evaluation in treated group showed significant reduction in adhesion compared to control group. This result is consistent with several

studies mentioned above which state amniotic membrane and hyaluronic acid, separately may give positive effect in prevention of peritendon adhesion. Study by Ozgenel, et al[1] stated that combination of amniotic membrane and hyaluronic acid may give better effect on the prevention of peritendon adhesion compared to their separate application. This was likely because amniotic membrane wrapping reduce dispersion of hyaluronic acid into surrounding tissue thus preventing adhesion more effectively. Those result differ from our study due to several factors, including; differences in experimental animals, different types of amniotic membrane preparation, and different types of hyaluronic acid used. Ozgenel, et al[1] used leghorn chicken as experimental animals, fresh amniotic membrane (less than 4 hours since harvesting), and hyaluronic acid with concentration of 15 mg/mL and molecular weight of 10^6 Daltons. While this study used New Zealand white rabbits, lyophilized amniotic membrane, and hyaluronic acid with concentration of 10 mg/mL and molecular weight of 10^6 Daltons.

Until now there are no clear interaction known between amniotic membrane and hyaluronic acid in the prevention of peritendon adhesion. A study by Higa, et al[39] reported a possible role of hyaluronic acid in the amniotic membrane stroma as a mediator of inflammatory cell adhesion (usually lymphocytes) which is found on the surface of the cornea. However, those interactions could not explain the biomolecular mechanism of both substances in reducing inflammatory process and peritendon adhesion.

From the results obtained in this study, it could be concluded that the application of lyophilized amniotic membrane, hyaluronic acid, or their combination significantly reduce the formation of peritendinous adhesion in repaired flexor digitorum fibularis tendon in rabbit model.

Acknowledgment

I would like to express my gratitude to my colleague at Department of Orthopaedic Ciptomangunkusumo Hospital and secretariat staff at our department.

Conflict of interest

The authors hereby affirm that there is no conflict of interest in this study.

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