



Comparison of human acellular amniotic membranes with acellular amniotic membranes pretreated with MPLA for repair of fascia in rats

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Abstract A decellularized amniotic membrane matrix is an ideal scaffolding system. This study compared the effectiveness of amniotic membrane and amniotic membrane pretreated with monophosphoryl lipid A (MPLA) for repairing the fascia of the abdomen with tension sutures. A total of 24 rats were randomly divided into three groups of eight each. In the control group, the fascia was repaired only with non-absorbable monofilament yarn under tension. In the second and third groups, in addition to the control treatment, acellular amniotic membrane was used and MPLA pretreated amniotic membrane was used, respectively. At 3, 7 and 14 days after treatment, the fascia repair was examined macroscopically and microscopically in all groups. Macroscopic examination showed that the use of MPLA pretreated

significantly different from the other groups only in the fibrin exudate. Changes in the fascia rupture pressure showed significant differences between groups. Group three, which was sutured with MPLA pretreated acellular membrane, showed greater amounts of collagen, monocytes and neovascularization, especially at days 7 and 14. The results show that MPLA pretreated acellular amniotic membrane helped to repair abdominal fascia to some extent.

Keywords Amniotic membrane · Acellular · Dehiscence · Fascia

Introduction

Despite recent advancements in surgical and risk management techniques, wound dehiscence following abdominal laparotomy remains highly prevalent (Carbonell et al. 2005). Several studies have reported that the occurrence of this complication after major surgery is 0.4–3.5%, with a mortality of 10–45% (Çiğdem et al. 2006). Factors such as technical problems related to fascia closure, emergency surgery (Canale and Beaty 2012), vascular procedures, colon and esophageal surgery (Riou et al. 1992), intra-abdominal infection, advanced age, male gender, weight loss, wound infection, hematoma, obesity, chronic use of corticosteroids, malnutrition and other factors increase the risk of wound opening (Zinner et al. 2019).

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Surgical wound dehiscence is a complication that causes pain, psychological stress and infection, as well as significant financial costs for both the patient and the medical system. Surgical experience, type of incision, type of suture thread, surgical site infection, patient nutritional status, persistent cough, abdominal distension, anemia, obesity, diabetes, advanced age, emergency surgery and procedures such as colon surgery are all factors that can affect wound opening after abdominal surgery (Sørensen et al. 2005). Increasing the nutritional status of a patient, the use of prophylactic antibiotics, achieving hemodynamic stability and proper tissue perfusion as well as the use of mesh are all recommendations for preventing this complication (Khorghami et al. 2013).

Modification of amniotic membrane for biomedical applications

The innermost layer of the fetal membrane, the human amniotic membrane (HAM), surrounds and protects the fetus. It is a valuable substance with various applications in regenerative medicine because of its physical and biological features (Lacorzana and Lacorzana 2020). The amnion shows therapeutic promise in speeding up the healing of skin and eye wounds as well as ulcers. It has also been used for vaginal reconstruction, abdominal hernia repair, surgical adhesion prevention and pericardium closure.

The addition of further modifications to the HAM is a relatively new. Such changes are designed to increase the characteristics of HAM and to ease handling and durability (Dadkhah Tehrani et al. 2021). In some circumstances, HAM can be applied to other materials as a coating to improve their biocompatibility. The traditional treatment of hernias is to utilize polypropylene (PP) mesh, which has drawbacks such as tissue adhesion. The efficiency of HAM-coated PP mesh in preventing abdominal adhesions in comparison with the use of PP mesh alone has been evaluated in several *in vivo* models. The results have shown less inflammation and adhesion, higher epithelialization and improved wound healing with the use of HAM (Najibpour et al. 2016). Tissue engineering commonly uses decellularized tissues, which are natural or biological scaffolds because the extracellular matrix (ECM) of the tissue is preserved following decellularization. Such a natural scaffold mimics the

ECM and is frequently employed in clinical applications for tissue restoration (Hussey et al. 2018).

Monophosphoryl lipid A (MPLA) is a low-toxicity (100-fold lower) derivative of lipopolysaccharide and is commercially available as a toll-like receptor-4 ligand (Romero et al. 2011). This derivative has useful immunostimulatory properties and is used as a human vaccine adjuvant and as a prophylactic drug for septic shock (Mata-Haro et al. 2007).

In previous research (Rahmati et al. 2022), we have hypothesized that the MPLA could stimulate the expression of migration genes and cause recruitment of cells; thus, we pretreated decellularized foreskin (Rahmati et al. 2022) with this substance and placed it in a cell culture plate next to the cells to compare the rate of cell entry into the tissue with that of the control group which received no treatment, it was found that treatment of human umbilical cord mesenchymal stem cells with MPLA increased migration-related gene expression *in vitro* (Rahmati et al. 2022). The present study examines the *in vivo* effect of HAM pretreated with MPLA to repair the fascia in rat model.

Materials and methods

Amniotic membrane decellularization

Human placentas were obtained under sterile conditions from planned, uneventful cesarean sections. They were washed several times with distilled water. The placenta and its appendages were separated from the amniotic membrane. The HAM was placed in 0.2% EDTA solution for 30 min at 37 °C and then was treated with 0.5 M NaOH solution for 30 s. The HAM then was transferred to 5% NH₄Cl solution and vigorously shaken, after which it was washed three times with PBS and stored in a freezer at –80 °C (Khosravimelal et al. 2020).

Pretreatment of decellularized HAM with MPLA

After decellularization, the acellular tissue (2 × 2 mm) was placed on plates containing 2 ml of DMEM medium containing 15% FBS and 1% penicillin-streptomycin and was stored in an incubator overnight. For MPLA treatment, pieces of the acellular amniotic membrane were placed in the MPLA

solution (3–10 µg/ml) overnight to be used during surgery the following day.

Surgical procedure on rats

Twenty-four male Wistar rats of 7–8 weeks of age weighing 200–250 g were purchased from Pasture Institute (Iran). They were retained in a light:dark cycle of 12:12 h with free access to food and water.

In preparation for treatment, the animals were anesthetized with ketamine (25 mg/kg) and xylazine (3 mg/kg) by intraperitoneal (IP) injection. The abdominal side of each rat then was shaved and disinfected with 75% ethanol. In each animal, crushed fascia edges with an area of 4 cm² were created using clamps.

Experimental groups and treatment

The prepared animals were randomly divided into three groups of eight rats: (a) control (fascia was only sutured with 3–0 monofilament nylon thread using a simple continuous suture technique); (b) in addition to the use of monofilament thread, the wound edges were brought together and the decellularized amniotic membrane was used to suture the fascia, so that the amniotic membrane was placed on each edge and then the edges were sutured to the membrane ; (c) MPLA-pretreated decellularized amniotic membranes were used with monofilament for suturing. After recovery from anesthesia, the rats were placed in individual cages with unlimited water and food.

At 3, 7 and 14 days post-treatment, samples were taken from all three groups and the rats again underwent surgery. The sutured skin of the rats was reopened for macroscopic examination of the surgical wound to determine the existence of any infection

and wound fracture. After the macroscopic examination, the animals were sacrificed using a lethal dose of anesthesia.

The intra-abdominal pressure was measured using a sphygmomanometer as an innovative method. The test was performed with a balloon catheter constructed using a surgical glove finger attached to a 16 F Foley catheter and connected to a mercury sphygmomanometer (Fig. 1). After the membrane ruptured, samples were taken from all three groups and were used for Hematoxylin and Eosin Staining examination.

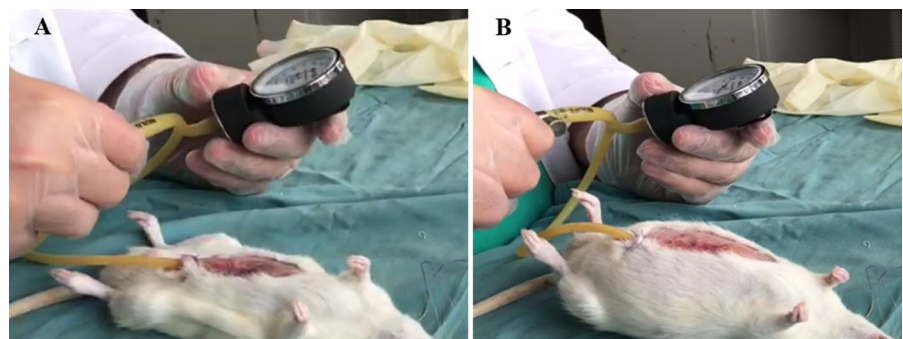
Hematoxylin and eosin staining

The biopsy specimens were embedded in paraffin after being preserved in 10% formalin solution. Then, using a rotary microtome instrument, 5–6 µm thick sections were created (microtome Leitz1512, Marshall Scientific, USA). The samples were stained with hematoxylin and eosin dye according to the standard protocols (Fischer et al. 2008). The wound biopsies of each treatment group were examined under a light microscope by a pathologist who was blind to the treatment groups for re-epithelialization, angiogenesis, and granulation tissue development.

Statistical analysis

All data were analyzed in IBM-SPSS statistical software (version 25; SPSS; USA). The data were reported as means ± standard deviation (SD). Two-way ANOVA was used to determine the difference between the groups where $P < 0.05$ was considered significant. Analytical graphs were also created by Graphpad Prism, version 8 (Graphpad Software, California, USA).

Fig. 1 Evaluation of intra-abdominal pressure tolerance (fascia rupture pressure) using sphygmomanometer. **A** Initial pressure by inflating the sphygmomanometer. **B** Inflate the sphygmomanometer until the membrane ruptures



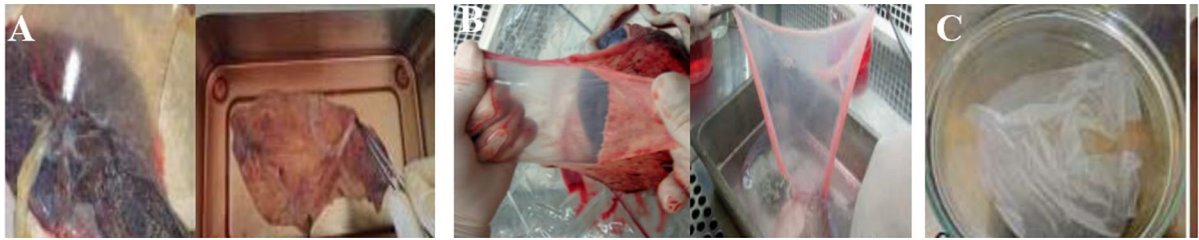


Fig. 2 Decellularization steps of HAM: **A** Amniotic membrane immediately after cesarean section; **B** separation of the fatty layer; **C** Acellular HAM after chemical treatment

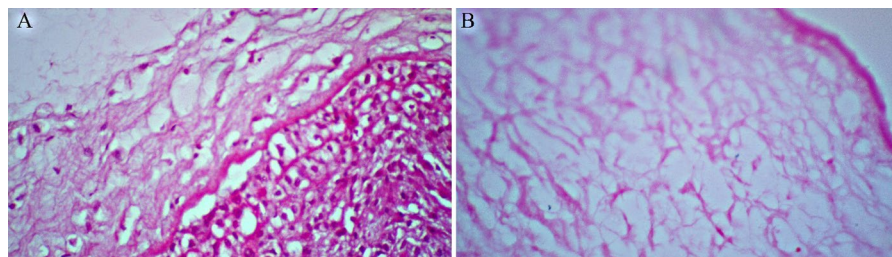


Fig. 3 Decellularization assessed using H&E staining (40× magnification). **A** Native HAM before decellularization showing a high density of cells in native tissue. **B** Decellular-

ized HAM showing complete cell removal as well as regular maintenance of matrix and collagen structure

Results

Decellularized HAM

As shown in Fig. 2, the placenta and its appendages were separated from the amniotic membrane, furthermore fatty layer was physically separated. HAM after chemical decellularization have a white and soft appearance. (Fig. 2)

H&E staining revealed that the HAM had been completely decellularized with no evidence of cells or nucleic acid (Fig. 3).

Macroscopic and microscopic results

Macroscopic observation in day 14 showed significant differences in the fibrin exudate and hernia rate all three groups. However statistical analysis of mean values these criterias showed the higher rate in control group. Furthermore tissue adhesion bonds were observed more in control group. The width of granulated tissue was significantly more detectable for MPLA-pretreated group (Fig. 4.)

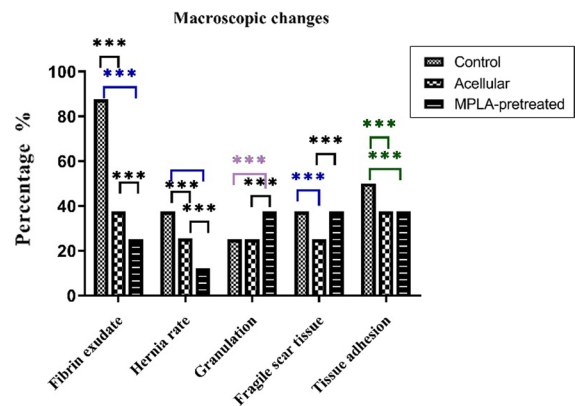


Fig. 4 Comparison of the average score of macroscopic changes in the study groups. (***) is $P < 0.0001$)

Differences between groups in terms of fascia rupture pressure were determined using the Kruskal–Wallis test. The differences in the group pretreated with MPLA compared to the acellular and control groups was significant on days 7 and 14 ($P < 0.0001$). As shown in Fig. 5, fascia rupture pressure is significantly higher on days 7 and 14 in

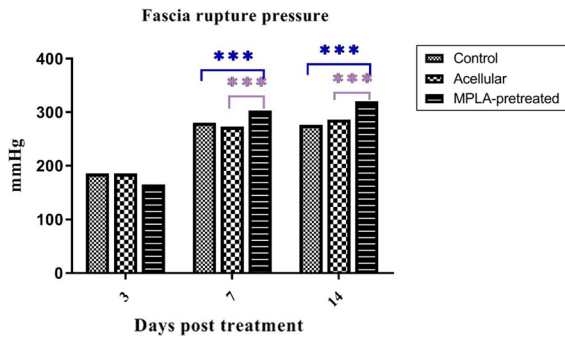


Fig. 5 Comparison of the average fascia rupture pressure in the study groups. (***) is $P < 0.0001$

MPLA- pretreated group compared to the other two groups. (Fig. 5)

Based on H&E results of samples, the formation of new endothelial tubes was considered as neovascularization (Fig. 6) in which demonstrated no particular differences between groups. However statistical analysis of mean values of endothelial tubes showed the higher neovascularization rate for MPLA- pretreated group compared to acellular group ($P < 0.0001$), and control ($P < 0.0001$) groups. The acellular group also showed more neovascularization rate compared to control group. Collagen production, which causes fibrosis, was greater in the MPLA-pretreated group than in the other two groups (not significantly).

Granulation tissue formation was almost similar for all groups (Fig. 6). Statistical analysis of granulation tissue formation also demonstrated the significant difference in formation and maturation of granulated tissue for MPLA- pretreated group compared to other groups ($P < 0.001$), (Fig. 7).

Discussion

The effects of decellularized HAM pretreated with MPLA for repair of facia in rats was compared with the effects of groups treated with decellularized HAM only and the monofilament control. The results showed that the group with decellularized HAM pretreated with MPLA had a more enhancing effect on fascia rupture pressure compared to the decellularized HAM and monofilament group.

In surgery, wound dehiscence, a rupture or reopening at the site of a previous surgical incision, can increase the rates of mortality and morbidity as well as increase the cost of treatment (van Ramshorst et al. 2010; Mangram et al. 1999). The incidence of fascia destruction after major abdominal surgery is estimated to be 1–3% and wound dehydration associated with this destruction is responsible for 15–20% of postoperative mortality. Various systemic factors have been associated with non-wound healing after surgery and mechanical factors, including wound infection,

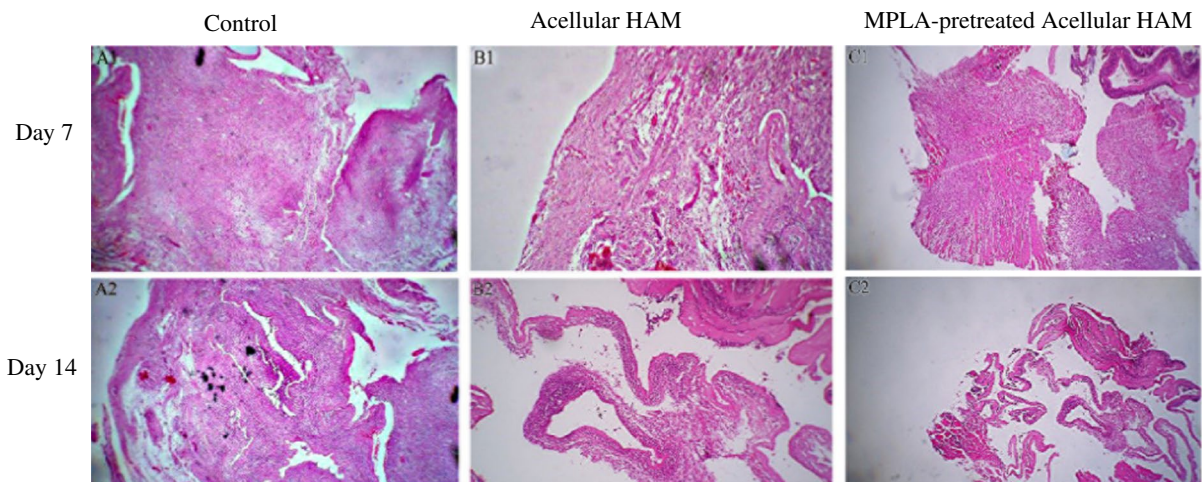


Fig. 6 The H&E staining of fascia in different groups on days 7 and 14. **A1, A2** H&E staining on days 7 and 14 in control group. **B1, B2** H&E staining on days 7 and 14 in Acel-

lular HAM group. **C1, C2** H&E staining on days 7 and 14 in MPLA-pretreated Acellular HAM group

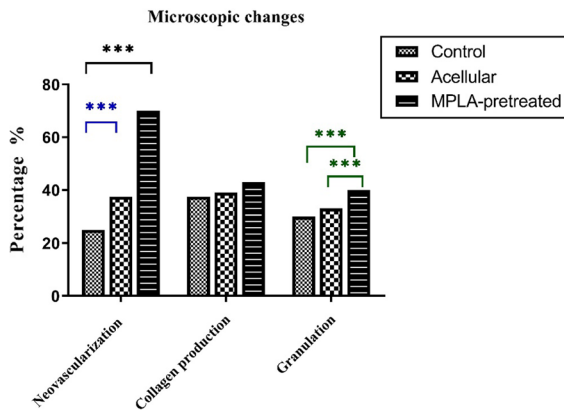


Fig. 7 Comparison of the average score of microscopic changes in the study groups. (***) is $P < 0.0001$)

abdominal distention and associated pulmonary problems, should also be prevented or during this period. Wound healing should be treated more seriously in connection with adjuvant treatments to repair the abdominal wall after surgery. In this regard, the use of biological barriers such as HAM or Seprafilm, a translucent absorbable biological membrane containing carboxymethylcellulose and hyaluronic acid, can help prevent intra-abdominal adhesion after surgery (Szabo et al. 2000).

Because the number of tissue donors is low and because of the risk of viral contamination, attempts have been made to design tissue with the help of various cells and scaffolds. The use of decellularized tissue instead of synthetic polymers is advantageous because their structure is similar the actual cells and can trigger chemical and mechanical signals which allow the cells to continue their normal physiological behavior (Khazaei et al. 2021). The results show that natural biomaterials usually require prior chemical or physical treatment to protect them from biodegradation, reduce their immunity and sterilize the tissue (Schmidt and Baier 2000). Studies have shown that such matrices contain structural components such as collagen, glycoproteins, hyaluronic acid, glucosamine glycans, reticulin and elastin that are suitable for binding them to cellular receptors (Khazaei et al. 2021).

The current study employed HAM, which offers little antigenicity and, when used as a biological coating for wounds, decreases the loss of water, electrolytes and proteins from the body, reduces the risk of infection

and prevents bacterial growth and increases the speed of wound healing. The results showed that pretreatment of HAM with MPLA increased the tolerance of fascia rupture at higher pressures and attracted more cells to the wound site, resulting in faster wound healing.

These results were in line with the results of our previous study on this substance (in vitro). The current study was designed to evaluate the previous results in vivo in a rat model. The results showed that, the pressure of fascia rupture changed significantly with the use of HAM, so in general, the use of HAM, especially MPLA pre-treated HAM, increased the rate of wound healing by increasing collagen production, angiogenesis and the number of monocytes.

Conclusion

Human amniotic membrane (HAM) pretreated with MPLA was used as a biological scaffold to promote healing of the fascia of rats. It was demonstrated that it is possible to use this as a biological dressing to improve wound healing in rats.

Authors contributions All the authors contributed in experiments and data analyzing, and writing the manuscript.

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Data availability Data will be available on request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethical approval All experiments were approved by the Ethics and Clinical Studies Research Committee of SKUMS according to Helsinki Declaration (IR.SKUMS.REC.1400.084).

Consent to participate All the authors are in agreement to participate.

Consent for publication All the authors agree for publication.

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