



Histopathological evaluation of human amniotic membrane effect on early stage healing of induced defects in rabbit's oral mucosa

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Abstract

This study aims to investigate the role of using dried Human Amniotic Membrane (dHAM) on rabbit's oral mucosal wound healing in its early stages. Six male rabbits were used in this study. In each rabbit, two elliptical incisions were done on both sides of tongue dorsum; one of them was left uncovered and the other was covered by dHAM. The animals were divided into two groups with three rabbits each according to the sacrifice periods which were three days and one week. After sacrifice, histopathological examination was performed to assess re-epithelialization and inflammation. Within the first three days, the re-epithelialization was much more faster in dHAM group than that in control one, and the difference between the control and study group was statistically highly significant ($p=0.005 < 0.01$). Moreover, the rate of inflammatory process show significant difference between the two groups with advantage to HAM group ($p = 0.046 < 0.05$). However, after one week, this difference was statistically non-significant ($p = 0.317 > 0.05$). Human Amniotic Membrane could be considered as an option to enhance oral wound healing especially in its early stages.

Keywords: Human amniotic membrane, early stage healing, rabbits' oral mucosa.

Introduction

Superficial wounds are considered as a common problem managed by emergency departments and surgeons; they can reach up to 90 million annually. These wounds include lacerations, bites, burns, punctures and incisions for minor surgeries. Nearly half of those cases usually involve head, face or scalp regions ^{[1], [2]}. Not all wounds require special medical

consideration; however, local wound care is essential to ensure uncomplicated healing process. Amniotic Membrane (AM) represents a remarkable modern option to improve local healing conditions in acute or chronic wounds ^[3].

AM is the membrane enveloping the fetal side of the placenta; it is composed of epithelial cell layer,

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basement membrane rich with collagen and a spongy fibroblastic layer. It was first introduced for medical use by Davis in 1910^{[3], [4], [5]}. The interesting about microscopic structure of AM is that it does not contain any blood vessels, lymphatic vessels or nerves; besides, it does not express antigens, so it has very low antigenicity^{[3],[6],[7]}. Clinically, transplantation of AM as a surgical graft has been shown to enhance epithelialization, decrease inflammation, scarring and pain^[8]. For those reasons, AM has wide clinical applications in different surgical fields like gynecology, ophthalmology, neurology, reconstructive surgery, oral and maxillofacial surgery and in treatment of burns and ulcers^{[3], [8]}. AM can be used fresh^[3] frozen^[7], lyophilized^[5] or dried. This study aims to evaluate the histopathological effect of Dried Human Amniotic Membrane (dHAM) on healing of oral wounds regarding inflammation and re-epithelialization.

Materials and methods

The study was held at the research center of Oral and Maxillofacial Surgery department in cooperation with The Unit of Oral Pathology at Dental College / University of Mosul/ Iraq.

• Experimental Model

Six Albino male rabbits of 12-18 months age were used to conduct the study. The weight of each was about 2.0-2.5 kg. The animals were housed in an animal house prepared for that purpose.

• Surgical Procedure:

The animals were generally anesthetized with intramuscular injection of kitamin (35mg/kg) /xylazin (5 mg/kg) combination. After anesthesia was secured and checked,

the rabbit's tongue was retracted and fixed using a suture stitch at its tip. After that, two elliptical shaped longitudinal incisions, of 1cm length each, were made on tongue dorsum on both sides of the midline (Figure 1). Incisions to the left side were left uncovered; those would represent the control group. On the other hand, the incisions to the right were covered with dHAM (Amnio-care Dry Amniotic Membrane, Biocover Laboratories, India) (Figure 2) giving rise to the study group. Before application, the membrane was soaked with distilled water, gently spread over the incision and finally secured in its place using an absorbable suture (5/0 Polyglycolic acid, BMR, Scotland) (Figure 3). After that, the operated animal was given intramuscular antibiotic injection of Oxytetracyclin 50mg/kg and remained isolated under supervision till fully recovered from anesthesia. All the animals resumed their normal life activities within 3-4 hrs postoperatively.

• Specimen Collection and Preparation

The animals were randomly divided into two groups, with three rabbits each, according to the intended time for sacrifice. The selected periods were 3days and one week; at which the animals' tongues were collected and divided longitudinally into two halves. This gave rise into further two subgroups; the control group that include the left tongue halves that were left uncovered and the study group containing the right tongue halves that were treated by dHAM.

Afterward, all the specimens were preserved in 10% formalin and sent for histo-pathological processing where they were embedded in paraffin wax, sectioned into 5 µm thickness and stained with hematoxylin- eosin to be examined under light microscope.

The scores to be evaluated histopathologically were the degree of inflammation and the degree of re-epithelialization respectively, according to the following scoring systems:

Inflammation Scoring:

Score 1: Predominance of acute inflammation.

Score 2: Predominance of granulation tissue.

Score 3: Predominance of chronic inflammation (fibroblasts beginning to proliferate).

Score 4: Resolution and cicatrization (reduction or disappearance of chronic inflammation [2], [9], [10], [11], [12]).

Re-epithelialization Scoring:

Score 0: Re-epithelialization at the edge of the wound.

Score 1: Re-epithelialization covering less than half of the wound.

Score 2: Re-epithelialization covering more than half of the wound.

Score 3: Re-epithelialization covering the entire wound, irregular thickness.

Score 4: Re-epithelialization covering the entire wound, normal thickness

Each specimen was separately examined by three different examiners, and the final score was obtained by having the mean of the three readings.

The statistical analysis was made using SPSS software version 16.0. Friedman non-parametric test was performed to analyze the scores; the selected α values were 0.05 and 0.01.

Results

• First period (3 days):

In the first three days, both control and amniotic membrane groups showed signs of inflammation, represented by presence of inflammatory cells, formation of new

blood vessels and beginning of fibroblast proliferation. However, The stage of this inflammatory process differ between the two groups, whilst all the samples of control group showed early stage of inflammation, predominance of polymorpho-nuclear cells, fifty percent of amniotic membrane samples presented with proliferation of fibroblast cells, which represent more advance stage of inflammation, see Figure 4. The statistical analysis of collected data using Friedman test came to confirm these findings, where the p-value was $0.046 < 0.05$ (Table 1, Figure 6). Thus, the difference between the two groups regarding the inflammatory response within the first three days was statistically significant.

Regarding re-epithelialization process, it started in both groups during the first three days. In control group, the newly formed epithelium was confined to the edge of the wound, other samples showed more progression of epithelialization process to cover less than one half of the wound surface. Amniotic membrane samples epithelialized with much more faster rate than those of control group. Here the newly formed epithelium covered more than half of the wound surface, or even fully covered the wound (Figure 4). These observations were reflected as a highly significant difference between the two groups (Friedman p-value = $0.005 < 0.01$) (Table 1, Figure 6).

• Second period (1 week):

After seven days, the inflammatory process preceded to a more late stage with high rate of fibroblast formation. For both groups, resolution of inflammation signs had begun in some samples, see Figure 5. The difference between the two groups was not significant as Friedman p-value was $0.317 (> 0.05)$ (Table 1, Figure 6).

On the other hand, most of the wounds in both groups were covered by newly formed epithelium after one week. In the control group, all defects were completely covered with full thickness epithelial layer; However, in some samples of amniotic membrane group, the epithelium did not reach its regular normal thickness (Figure 5). Again the difference regarding the re-epithelialization of wound surface was not significant, as Friedman p- value was 0.317 (> 0.05) (Table 1, Figure 6).

Discussion

AM has unique properties that can be helpful in treating a variety of oral surface defects. In this study, the choice of dried AM was based on many factors. First, proper sterilization of the AM is vital. Fresh human AMs are obtained at the time of elective cesarean section under sterile conditions; however, this procedure does not guarantee a completely sterile AM because of its biological origins. Second, frozen AM needs an expensive and bulky -80°C deep freezer [7]. dHAM gives many advantages for clinical use. It is kept sterile and free of contamination in its package; besides, it is easy to obtain, reasonably affordable and can be stored for long periods without deterioration.

The time to re-epithelialization is essential to be estimated in most wound healing studies, as its acceleration, even by several days, would reduce pain and scarring [2], [13], [14]. In this study, re-epithelialization was highly significant in AM group at three days period. This is consistent with many other studies [5], [8], [15], [16], [17]. This can be attributed to many factors. AM acts as a mechanical barrier covering the wound surface and protecting the newly formed epithelium which would enhance the overall re-epithelialization process.

Also, it was found that AM contains growth factors and matrix proteins promoting the migration, adhesion, and differentiation of epithelial cells [15], [18]. Besides, AM contains mesenchymal stromal cells, which display some characteristic properties of stem cells; therefore, AM has been proposed as a good candidate to be used to treat damaged or diseased tissues [19].

On the other hand, Nordback et al found that AM did not have any significant effect on wound re-epithelialization [3]. Moreover, Unger and Roberts mentioned that AM delayed healing and that it was ineffective in the management of wounds [20].

This study revealed that AM significantly reduced inflammatory process after mucosal injury. Many studies agreed with this outcome [3], [15], [18]. The membrane adapts well to the wound surface underneath which would reduce granulation tissue and edema formation and reduce inflammation [5]. Besides, this tight attachment would prevent microbial contamination. It was also found that AM suppresses neutrophils migration and infiltration which would reduce inflammation [15], [18].

In this study, there was no significant difference between control and amniotic membrane groups at one week period in regard to both re-epithelialization and inflammation. The possible explanation for this is that the effect of AM on wound healing is mainly noticeable in its early stages. Studies explained that according to a fact that AM reduces wound contracture that occurs 3-4 days after injury; contraction process usually delays healing. Besides, AM gradually degrades in few days (up to 7 days) making its action concentrated within the early phase of healing [3], [21].

Conclusion

This study demonstrated that using amniotic membrane could accelerate healing process especially in its early stage. It reduces the overall inflammatory phase of healing and remarkably enhances re-epithelialization of oral wound surfaces.

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References

- 1- Trott A.T. Wounds and Lacerations: Emergency Care and Closure. 2005; Elsevier and Mosby Inc., Philadelphia, USA. pp: 1-2; 30-31.
- 2- Albanna R.F. and Hasouni M. Kh. Epiglu Surgical Adhesive versus Silk Suture in Oral Incisions Closure; A Histopathological Comparative Study on Rabbits. Lap Lambert Publishing, Germany. 2012.
- 3- Nordback P.H, Miettinen S., Kääriäinen M., Pelto-Huikko M., Kuokkanen H. and Suuronen R. Amniotic membrane reduces wound size in early stages of the healing process. *Journal of wound care.* 2012. 2 1 (4): 190-197.
- 4- Davis, J.W. Skin transplantation with a review of 550 cases at the Johns Hopkins hospital. *Johns Hopkins Med J.* 1910; 15: 307–396.
- 5- Güler R., Uran N. and Dilek F. A Comparative Histopathological Investigation of the Effect of Lyophilized Amniotic Membrane on Wound Healing as an Allograft Material in Rats. *Journal of Islamic Academy of Sciences.* 1993. 6(3): 209-219.
- 6- Mohamad H. Anatomy and embryology of human placenta, amnion and chorion. *Sci Basis Tissue Trans.* 2001; 5: 139–148.
- 7- Nakamura T., Yoshitani M., Rigby H, Fullwood N.J., Ito W., Inatomi T., Nakamura T., Sotozono C., Shimizu Y. and S. Kinoshita. Sterilized, Freeze-Dried Amniotic Membrane: A Useful Substrate for Ocular Surface Reconstruction. *Invest Ophthalmol Vis Sci.* 2004; 45:93–99.
- 8- Kheirkhah A., Johnso D.A. and Paranjpe D.R. Temporary Sutureless Amniotic Membrane Patch for Acute Alkaline Burns. *Arch Ophthalmol.* 2008; 126(8):1059-106.
- 9- Camacho-Alonso F., López-Jornet P. and Bermejo-Fenoll A. Effects of Scalpel (with and without Tissue Adhesive) and Cryosurgery on Wound Healing in Rat Tongues. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2005; 100:E58-63.
- 10- Camacho-Alonso F. and López-Jornet P. Clinical-Pathological Study of the Healing of Wounds Provoked on the Dorso-Lingual Mucosa in 186 Albino Rats. *Otolaryngology–Head and Neck Surgery.* 2007; 136: 119-124.
- 11- López-Jornet P, Camacho-Alonso F, Molina-Miñano F and Vicente-Ortega V. Effects of Plasma Rich in Growth Factors on Wound Healing of the Tongue. Experimental Study in Rabbits. *Med Oral Patol Oral Cir Bucal.* 2009; 14(9):e425-8.
- 12- Sinha U.K and Gallagher L.A. Effects of Steel Scalpel, Ultrasonic Scalpel, CO2 Laser, and Monopolar and Bipolar Electrosurgery on Wound Healing in Guinea Pig Oral Mucosa. *Laryngoscope* 2003; 113:228 –36.
- 13- Monafó W.M. Initial management of burns. *N Engl J Med.* 1996; 335:1581–6.
- 14- Singer A.J. and McClain S.A. Development of a Porcine Excisional Wound Model. *Acad Emerg Med.* 2003; 10(10): 1029-1033.
- 15- Rinastiti M, Harijadi, Santoso ALS, Sosroseno W. Histological evaluation of rabbit gingival wound healing transplanted with human amniotic membrane. *Int J Oral Maxillofac Surg.* 2006;35:247–251.
- 16- Insausti C.L., Alcaraz A. García-Vizcaíno E.M., Mrowiec A., López-Martínez M.C, Blanquer M., Piñero A., Majado M.J., Moraleda J.M., Castellanos G. and Nicolás F.J. Amniotic membrane induces epithelialization in massive posttraumatic wounds. *Wound Repair and Regeneration.* 2010. 18 (4): 368–377.
- 17- Forbes J. and Fetterolf D.E. Dehydrated amniotic membrane allografts for the treatment of chronic wounds: a case series. *Journal of Wound Care.* 2012. 21(6): 290 – 296
- 18- Samandari m.H., Adibi S., Khoshzaban A., Aghazadeh S., Dihimi P., Torbaghan

- S.S., Keshel S.H. and Shahabi Z. Human amniotic membrane, best healing accelerator, and the choice of bone induction for vestibuloplasty technique (an animal study). *Transplant Research and Risk Management* 2011. 3: 1-8.
- 19- Díaz-Prado S, Muiños-López E, Hermida-Gómez T, Cicione C, Rendal-Vázquez ME, Fuentes-Boquete I, de Toro FJ, Blanco FJ. Human amniotic membrane as an alternative source of stem cells for regenerative medicine. *Differentiation*.
- 20- Unger M.G. and Roberts M: Lyophilized amniotic membranes on graft donor sites. *Br J Plast Surg*. 1976. 29:99-101.
- 21- Tosi, G.M., Traversi, C., Schuerfeld, K. *et al.* Amniotic membrane graft: Histopathological findings in five cases. *J Cell Physiol*. 2005; 202: 3, 852–857.



Figure 1: two elliptical incisions made on the Rabbit tongue dorsal surface.



Figure 2: Amnio-care, Dry Amniotic Membrane, Biocover Laboratories, India



Figure 3: Two elliptical incisions made on the both sides of Rabbit tongue dorsal surface. Left defect is the control; right defect is covered and sutured by dHAM.

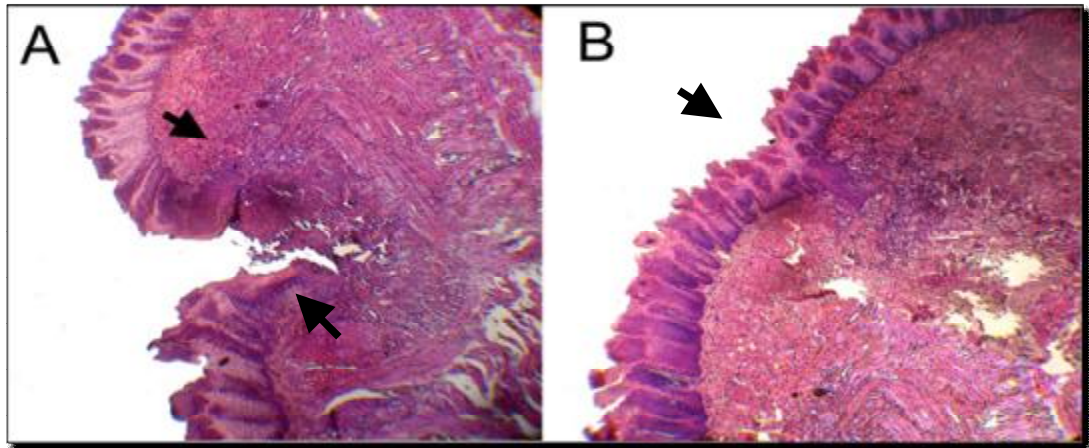


Figure 4: Three days period Hematoxylin-eiosin section (X 4/0.10). **A:** control group, newly formed epithelium (arrows) covering less than half of wound surface. **B:** Amniotic membrane group; full wound epithelialization (arrow).

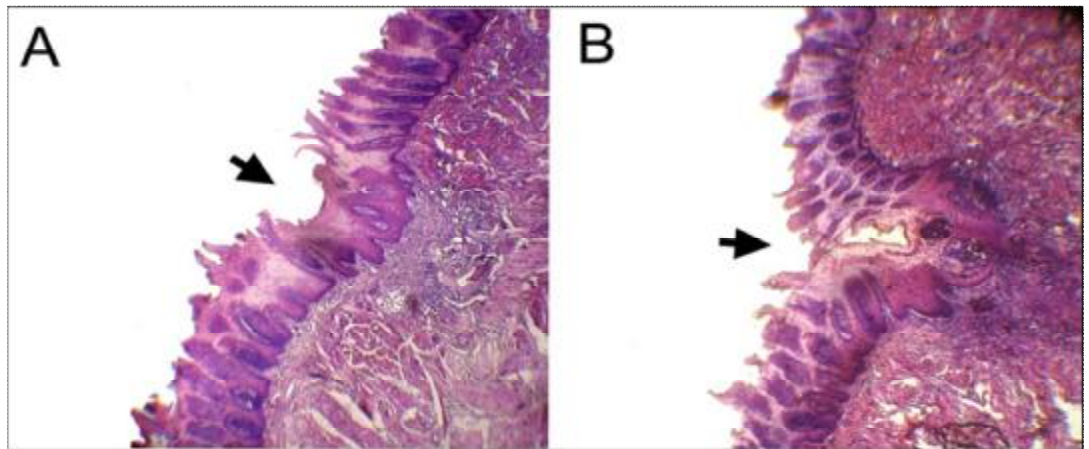


Figure 5: One week period Hematoxylin-eiosin section (X 4/0.10). **A:** control group, **B:** Amniotic membrane group; both groups showed full wound epithelialization (arrow).

Table 1 : Friedman p-values

Score measure	3 days	1week
Inflammation	0.046*	0.317
Re-epithelialization	0.005**	0.317

(*): Significant Difference < 0.05.
(**): Highly Significant Difference < 0.01.

