

Research Methodology

Amniotic membrane as a scaffold in wound healing and diabetic foot ulcer: an experimental technique and recommendations

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Received: 29 June 2016

Accepted: 04 July 2016

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ABSTRACT

Background: Human amniotic membrane has been used clinically in a variety of applications for over the past 100 years and produced a significant amount of data in multiple areas of medicine. Its clinical usage ranges from wound coverage for burn victims to healing of the conjunctiva after pterygium repair. The amniotic membrane natural properties provide an easy to use, safe option for various medical applications. There is need to develop a method for storage of amniotic membrane which can retain the biological properties and as well have long shelf life too.

Methods: The experimental technique was standardized for cryopreservation of amniotic membrane. For this, amniotic membrane was obtained from mothers who had delivered through caesarean section with their consent.

Results: The standardized protocol for cryopreservation of amniotic membrane was found to be safe and preserved amniotic membrane is expected to have long shelf life.

Conclusions: The advantages associated with amniotic membrane such as easily available, inexpensive, non-immunogenic and antimicrobial, anti-inflammatory properties make it a suitable graft to be used in wound healing and diabetic foot ulcers.

Keywords: Amniotic membrane, Scaffold, Wound healing, Cryopreservation

INTRODUCTION

Regenerative medicine offers tremendous potential for skin regeneration following injury and disease. Tissue engineering is one particular aspect of regenerative medicine which aims to restore, maintain and improve the functions of lost tissues by developing or reconstructing the biological substitutes. An important component of tissue engineering is the supporting matrix (known as scaffolds) upon which the cells and growth factor can be placed to induce the restoration of defective tissues. The main function of scaffold is to support structurally and provide an environment for cell attachment, growth and differentiation. Amniotic

membrane is a non-vascular tissue comprising of epithelial cells, cell-anchoring collagen which are essential factors for wound healing. In addition to this, it is non-immunogenic and has anti-inflammatory, antimicrobial, anti-fibrosis, anti-scarring and reasonable mechanical properties to play as a scaffold.

These clinical properties of amniotic membrane allow this matrix to explore it in a wide range of applications in regenerative medicine.^{1,2} In fact, the clinical use of amniotic membrane has a long history, with the first report on its application in treatment of skin burns and wounds more than a century ago and today it has been widely used by wound care practitioners not only to treat

burns and chronic wounds, but also for treating diabetic neurovascular ulcers, venous stasis ulcers etc.³⁻⁵ The amniotic membranes are most widely used in ophthalmology to treat corneal injury, conjunctival and corneal surface lesions, and as promotor for limbal stem cell regeneration.⁶⁻⁸ It has also been tried as carrier for ex-vivo expansion and carrier for transplantation of corneal and oral epithelial cells.^{9,10} Furthermore, amniotic membranes have also shown promising results as nerve wrap, in cartilage restoration, osteoarthritis and in tendon repair.¹¹⁻¹³

So far, various methods have been explored to preserve amniotic membrane such as fresh storage (store at 4°C), cryopreservation (either in minus 86°C or liquid nitrogen), freeze drying, and dehydration, each having the objective of to retain all components of the tissue as close as to fresh tissue and have longer shelf life. Each method is having its own pros and cons. While the use of fresh amniotic membrane for transplantation in humans, a special processing and sterilization is recommended to ensure consistent quality and preservation of the amniotic membrane. The use of fresh tissue is impractical in allografts and disease transmission is another concern. While dehydration and freeze drying methods allows it to store at room temperature which makes it transportation simple, but the altered physical structure and reduction in membrane thickness are concerns associated with dehydration process. Cryopreservation and freeze drying maintain the physical structure of matrix, for evidence Reodiguez et al studied the effects of lyophilization and cryopreservation on human amniotic membrane on histological characteristics and protein content and reported that both exhibited same histological characteristics but the total protein content was reduced in lyophilized membrane.¹⁴ Thus the cryopreservation method allows storage of amniotic membrane for longer period of time, effectively preserve histological, biochemical, and functional properties of the amniotic membrane tissue and sustain the associated beneficial properties and make it potentially suitable candidate to use it in long run.

The aim of this study was to develop a method for preserving the amniotic membrane for its future usage in various wound healing process and diabetic ulcers. For this purpose, we standardized an experimental technique for cryopreservation and summarized the various clinical studies of amniotic membrane on wound healing which will guide the usage of amniotic membrane as scaffold in various medical applications.

METHODS

Materials

Cell culture tested gentamycin (TC026), cell culture tested penicillin (TC187), cell culture tested amphotericin B (TC019), 0.22 micron, 47 mm diameter nitrocellulose membrane (SF958), phosphate buffer saline (PBS)

(TL1101) and 100 mm petriplates (PW1132), were obtained from Himedia (India). Washing tube (430791) and collection tube (352070) were from Falcon (United States of America (USA)). Cell culture tested cefoperazone sodium (219969505) was from MP biomedical (USA). Dimethyl sulfoxide (DMSO) (CB100) was from Origen (USA). 98% purified glycerol was from Renkem (India). Dulbecco's modified eagle medium (DMEM F12) with Glutamax (1X); 2.438 g/L sodium bicarbonate; sodium pyruvate (10565042) was from Gibco (USA). cryo vial (337516) from Nunc (Denmark). Aerobic (279044) and Anaerobic (279045) culture bottle from Biomerieux (France). HCV Microlisa kit (HC023096), Hepalisa Ultra kit (IR0300096), Microlisa HIV Ag and Ab test kit (IR232096) and Advantage malcard kit (IR221025) were obtained from J.Mitra.CO Pvt. Ltd (India). HTLV I/ II ELISA 4.0 kit (23080192) were from MP Biomedical (USA). Vironostika anti CMV III test kit (284124) was from Biomerieux (France). IMMUTREP RPR kit (0D061/B) was from Omega diagnostics Pvt. Ltd (Scotland). EDTA tube (367844) and SST GEL tube (367812) were from Becton Dickinson (USA).

Methods

Donor selection and guidelines

The following research was carried out at CelluGen Biotech Private Limited, Gurgaon, India and the study (CR/PRJ01/AM/R03) complied with the established GLP and GMP norms. Written informed consent from the donor (participant age range 25-37 year) was obtained for sample use in this research project and methodology prepared according to previously published studies with certain modifications.¹⁵⁻¹⁷

Infectious screening and selection criteria

Eligible amnion donors are living mothers that have delivered a live birth through caesarean section. All tissues were recovered under full informed consent of the donor. Each donor was then answered a series of questions to ensure the donor has not engaged in behaviors to place her at an increased risk for the transmission of infectious diseases and to ensure the donor has not shown signs or symptoms of illnesses. Donor procurement was prescreened to prevent the transmission of infectious diseases from donors to recipients of the material. The amniotic membrane in a fresh form was collected from the department of obstetrics and gynaecology of Mayom hospital in an elective caesarean section. Mothers carrying infections like HIV, hepatitis B, hepatitis C, syphilis, CMV and malaria before child birth were not included. Donors with a history of antenatal problems e.g. gestational diabetes or placenta previa were excluded from the study. For HIV, hepatitis B, hepatitis C, malaria, CMV, HTLV and syphilis infections, serological tests of Anti-HIV 1 and 2, HBsAg, Anti-HCV, PF/PV-ICT, CMV IgM and IgG,

RPR, HTLV 1 and 2, and RPR were carried out respectively.

Collection and transportation of amniotic membrane

After the caesarean section, the junction of the cord of the placenta was separated under sterile conditions and amniotic membrane fragment of approximately 10x10 cm² was obtained by manual dissection. The peeled amniotic membrane was separated from the underlying chorion and placed into a sterile 50 ml screw-capped reagent tube containing transport medium. The medium contains balanced PBS solution along with 50 µg/mL gentamycin, 100 units/mL penicillin, 0.125 mg/ml cefoperazone sodium and 1 mg/mL amphotericin B was used for transportation. The collection tubes were placed in collection kit box and transported to the laboratory within 48 hours between 10-15°C.

Amniotic membrane processing

In the laboratory, under the Class II Type A2 biosafety cabinet, the amnion was carefully separated from the rest of the chorion by blunt dissection and using round-ended forceps. The separated chorion was discarded and the amnion was then washed thrice with washing solution that contains balanced PBS solution along with 25 µg/mL gentamycin, 50 units/mL penicillin, 0.125 mg/ml cefoperazone sodium and 0.5 mg/mL amphotericin B to remove blood and mucus. The amniotic membrane was then incubated in DMEM F12 medium with antibiotic solution: 50 µg/mL gentamycin, 100 units/mL penicillin, 0.125 mg/ml cefoperazone sodium and 1 mg/mL amphotericin B for 1 hour at room temperature.

The amniotic membrane was then flattened uniformly without folds or tears onto 47 mm size, individually sterilized 0.22 µm nitrocellulose membrane, with the epithelium/basement membrane surface up. The membrane was allowed to adhere to the nitrocellulose membrane for 10 minutes and was then cut into 5 cmx5cm² pieces.

Cryopreservation method

The nitrocellulose membrane along with the adherent amniotic membrane was placed in 50 ml screw-capped sterile vial containing preservative medium. The preservation media consists of Dulbecco's modified eagle medium-nutrient mixture Ham's F-12(1:1) with Glutamax (1X); 2.438 g/L sodium bicarbonate; sodium pyruvate and glycerol in a ratio 1:1 along with 25 µg/mL gentamycin, 50 units/mL penicillin, 0.125 µg/ml cefoperazone sodium and 0.5 mg/mL amphotericin B. The vials were frozen at -86°C.

Stability of amniotic membrane (post preservation)

Cryopreserved membranes were allowed to thaw for 10 minutes at room temperature followed by at 40°C until thawing was complete. To reduce cell damage due to osmotic changes, the DMSO was removed by sequential washing twice with 0.9% NaCl twice and once with pre-chilled PBS along with 25µg/mL gentamycin, 50units/mL penicillin, 0.125µg/ml cefoperazone sodium and 0.5mg/mL amphotericin B at 4°C.

Preparation for treatment and administration techniques

The final washed tissue graft was placed in a respective inner pouch of sterile pack. To check for further suitability of amniotic membrane, it was examined for different testing parameters such as holes, broken seals, tears, contamination, or other physical defects, uniformity of appearance, including the absence of spots or discoloration as per the listed parameters (Table 1). To the extent possible, oxygen was removed from the inner pouch and sealed. Each inner pouch was separately packed in an outer pouch for further protection, storage and shipment. The final inspection was made of both the inner and outer pouches to ensure that the amniotic membrane contained therein matches the product specifications, such as shape, size, thickness and tissue type.

Table 1: Testing criteria for amniotic membrane after retrieval.

Testing criteria	Acceptance specification
Transparency of tissue	Should maintain the opaque-white in appearance
Sign of damage	Should be free of holes/ breakage of the membrane
Odour	Should be free from any unacceptable odour
Physical defect	Should maintain smooth and elasticity in nature
Contamination	Free from any debris present
Discoloration of Tissue	Scaffold should pass the discoloration
Sterility	Negative
Infectious screening	Sero-negative

RESULTS AND DISCUSSION

Burns and wounds are major problem worldwide and posing social and economic burden to society. Moreover, diabetic foot ulcers and chronic non-healing wound make a significant cost to the patients, so there is need of clinical approach which can reduce the burden of patients. Amniotic membrane as a wound allograft material has a number of beneficial properties inherent in their makeup. It is non-immunogenic; so easily accepted by host; and further its antimicrobial action eliminates the

risk of post-operative infection. As an effective biological dressing, it diminishes the oozing of plasma, bacterial count, and fluid, protein and heat loss in the affected area, and possesses various growth factors and bio-macromolecules important for wound healing.

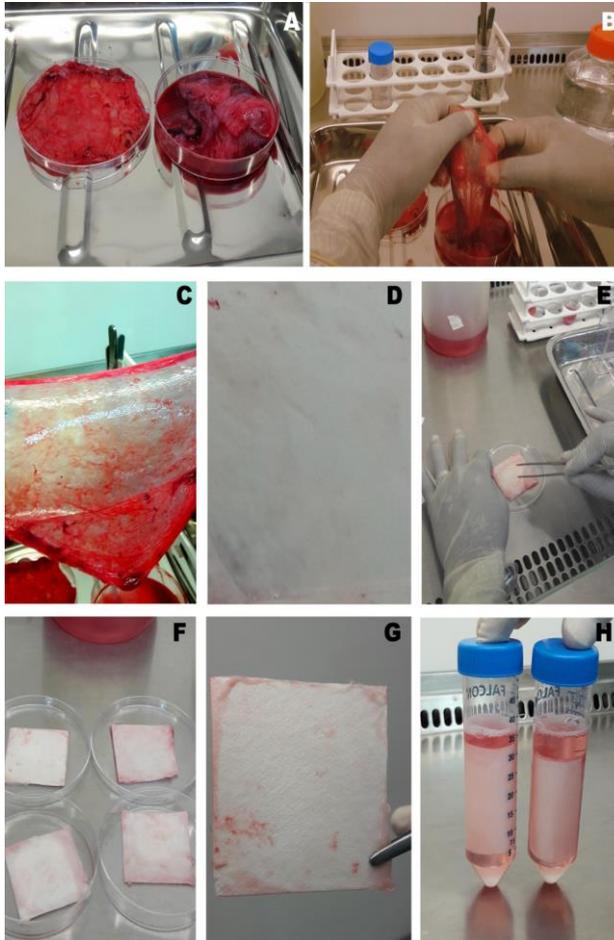


Figure 1: Illustrations of the developed methodology for cryo preservation of amniotic membrane. (A) Chorion attached to amniotic membrane and blood clots are gently peeled off. (B) With the epithelial/basement layer surface up, the amniotic membrane is standing apart. (C) Dissected amniotic membrane; before washing. (D) Processed amniotic membrane; after treated with antibiotic washing solution and washed free of blood clots. (E) Amniotic membrane is spread uniformly without folds or tears on the sterile membrane. (F) The sterile membrane of 5X5cm² is placed on the spread-out amniotic membrane. (G) The amniotic membrane is cut around the membrane and allowed to adhere to it. (H) The filter membrane along with the adherent amniotic membrane is fully placed into the preservative medium in wide-mouthed screw-capped tube.

In the present study, we collected, processed and standardized a preservation method for storing amniotic membrane. The detailed steps involved in the collection, processing, cryopreservation and preparation of amniotic membrane for wound healing are illustrated in Figure 1

and Figure 2. The protocol has been found to be extremely safe and amniotic membrane so harvested is expected to have a long shelf life.

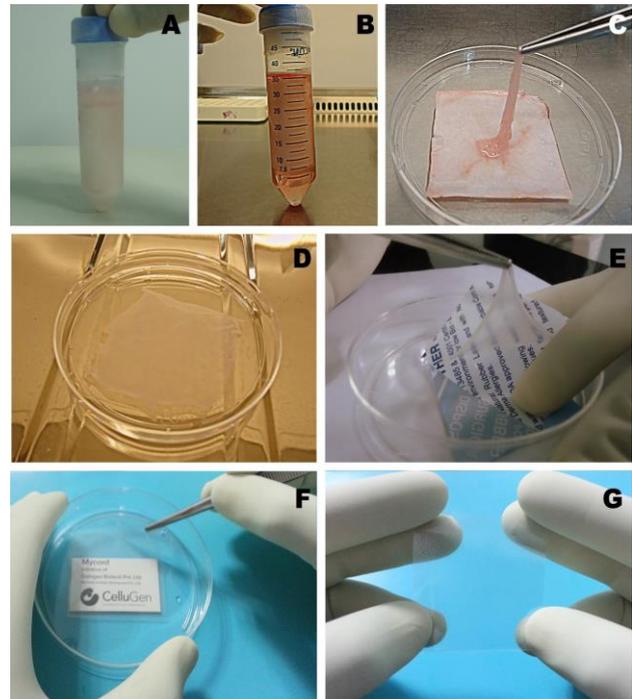


Figure 2: The steps involved in the preparation of amniotic membrane for clinical use. (A) Amniotic membrane cryo-preserved in the preservative medium at -86°C till further use. (B) Amniotic membrane is retrieved from the cryogenic environment; after thawed at 40°C. (C) Post thawed amniotic membrane is placed in petri dish and recovers from the filter membrane. (D) DMSO is removed by sequential washing and progressive dilution with PBS and along with antibiotics. (E) Investigation of retrieved amniotic membrane for transparency, smoothness, elasticity, discoloration, free of damages for releasing. (F, G) Final processed amniotic membrane for assess to clinical use.

The use of amniotic membrane is increasing day by day to promote wound healing and skin regeneration. Various clinical studies done with amniotic membrane for treatment of different kind of wounds such as surgical wounds, venous ulcers, diabetic foot ulcers, varicose ulcers, dehisced wounds, cavity wound are listed in Table 2. The amniotic membrane usage is not limited to only above mentioned wounds, but can also be used for treating pressure ulcers, palliative wounds, exuding wounds, granulating wounds, deep and acute wounds.

The cryopreservation of amniotic membrane effectively preserves histological, biochemical, and functional properties of the amniotic membrane tissue. Biochemically, cryopreservation reduced total protein and human serum albumin contents, but retained high molecular weight hyaluronic acid species including the

heavy chain-hyaluronic acid complex that is known to exert anti-inflammatory and anti-scarring effects.³¹ In the present study, we have analyzed minus 86°C cryopreservation method for the layers of amniotic

membrane, in accordance to the previous studies.¹⁵⁻¹⁷ Further cryopreservation by storing at minus 196°C using liquid nitrogen for long term shelf life has to be evaluated in future studies.

Table 2: Clinical studies of amniotic membrane for wound healing and diabetic ulcers.^{18,30}

Type of wound	Author	Journal	Study subject	Results
Burns	Eskandarlou et al (2016)	World Journal of Plastic surgery	Human	Appliance of amniotic dressing cause rapid epithelialization and wound healing and improve pain. ¹⁸
Non healing surgical wound	Riordan et al (2015)	Journal of Translational Medicine	Human	Dehydrated amniotic patch appliance have proven effective in complete healing of an otherwise non healing wound. ¹⁹
Chronic non healing wound	Mrugala et al (2015)	International Wound Journal	Human	Dehydrated amniotic membrane has proven to be safe, effective therapy for treating non healing chronic wound. ²⁰
Neuropathic foot ulcer	Shah et al. (2014)	Journal of American Podiatric Chemical Association	Human	Dehydrated amniotic membrane was found to be an effective dressing in treatment of neuropathic foot ulcers. ²¹
Infected wound	Duarte et al (2014)	Acta Cirurgica Brasleira	Rabbits	Amniotic membrane did not have effect on inflammation, epithelialization and fibroplasia, but increased angiogenesis. ²²
Diabetic foot ulcer	Zelen et al (2013)	International Wound Journal	Human	Appliance of dehydrated amniotic membrane allograft enhanced healing of diabetic foot ulcers. In 6 week of treatment, the healing rate was 92% while it was only 8% with standard healing method. ²³
Varicose ulcers	Hanumanthappa et al (2012)	International Journal of Biological and Medical Research	Human	Amniotic membrane dressing proved to be safe and cheap method for treating varicose leg ulcers. The appliance of amniotic membrane promoted epithelialization and granular tissue development. ²⁴
Venous ulcers	Alsina-Gibert M et al (2012)	Actas-Dermosifilograficas	Human	Complete wound epithelialization was achieved. Pain intensity was reduced. Amniotic membrane transplantation was found to an effective treatment for refractory chronic vascular ulcers on the lower limbs. ²⁵
Cavity wounds	Sham et al (2011)	International Journal of Dental Clinics	Human	Amniotic membrane showed its proficiency in reconstruction of buccal mucosal defect. ²⁶
Superficial Burns	Qader et al (2011)	Journal of Sulaimani Medical College	Human	Amniotic membrane dressing was found to be effective in superficial burns. ²⁷
Local wound	Francis Pesteil et al (2009)	Phlebolympology	Human	Complete wound healing was obtained in 2 patients after 19 and 26 weeks. Reduction in wound size of at least 50% was obtained in another 3 patients after 26, 31, and 32 weeks, in out of 8 patients. ²⁸
Venous leg ulcers	Mermet et al (2007)	Wound Repair Regeneration	Human	Amniotic membrane transplantation promoted epithelialization, suppressed fibrosis and proved safe to use. ²⁹
Dehiscenced wound	Kawakita et al (2007)	Journal of Medical case Reports	Human	Amniotic membrane transplantation in patient having wound dehiscence after deep lamellar keratoplasty reduced the inflammation and complete epithelialization observed in 10 days. ³⁰

CelluGen Biotech Private Limited has taken an initiative towards establishing a facility to preserve amniotic membrane for usage in future for mankind. This

cryopreserved membrane can be an ideal source of scaffold in skin tissue engineering to heal burns and wounds efficiently specially in developing countries

where treatment cost is a major issue. Further research studies using amniotic membrane as matrix for mesenchymal stem cell proliferation are needed to substantiate the usage of the same for treating various wounds, diabetic ulcers in large masses in a cost effective ways.

CONCLUSION

Management of wounds and burns using amniotic membrane has a long history, but was deserted because of the risks of bacterial and viral infections. Amniotic membrane can now be made safe for use by current methods of processing by cryopreservation, and serological testing. It is well tolerated when used for wound coverage, limits the risk of infection, and also has analgesic and anti-inflammatory properties. The present study provides additional evidence that the incorporation of cryopreserved human amniotic membrane into standard of care for patients with various wound injury could be beneficial. The experimental techniques and recommendations suggest that amniotic membrane is a viable option for the treatment of different categories of wounds.

ACKNOWLEDGEMENTS

Authors would like to express sincere gratitude to Dr. Firdosh Mahuvawala and Mr. Lalit Jaiswal, Directors for making it possible and funding the study and special thanks to Mr. Anand Bhushan, COO of Cellugen Biotech Private Limited for supporting throughout the study.

Funding: No funding sources

Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Jaianand K, SuriyaNarayanan S, Palaniyandi M, Rajangam B, Chhabra H, Pandey A. Amniotic membrane as a scaffold in wound healing and diabetic foot ulcer: an experimental technique and recommendations. *Int J Res Med Sci* 2016;4: 3654-60.