ORIGINAL ARTICLE

Comparison of Efficacy of Storage of Amniotic Membrane at -20 and -80 Degrees Centigrade

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ABSTRACT

Objective: To compare the efficacy of storage of amniotic membrane at -20 and -80 degrees centigrade.
Study Design: Interventional quasi-experimental study.
Place and Duration of Study: Department of Ophthalmology, Unit 1, Dow University of Health Sciences and Civil Hospital, Karachi, from January 2009 to December 2010.
Methodology: Amniotic membrane was obtained from patients undergoing elective Caesarean section screened for HIV, Hepatitis B and C and gave informed consent. It was washed with solutions containing benzyl penicillin, streptomycin and amphotericin B and frozen at -20 degrees centigrade for upto 6 weeks or at -80 degrees centigrade for upto 6 months. Fisher's exact test was used to check significance. P-value less than 0.05 was considered as significant.
Results: Amniotic membrane was stored at -20 degrees centigrade on four occasions and was used in 25 (48.07%) patients. On 3 other occasions it was stored at -80 degrees centigrade and was successfully used in 27 (51.92%) patients. The association between different degrees of storage of graft and type of cases is insignificant (p = 0.99).
Conclusion: Compared to use of fresh amniotic membrane use of amniotic membrane stored at -20 and -80 degrees is safe and cost effective. It also excludes the chances of disease transmission. Although freezing at both temperatures is equally efficacious, freezing at -80 degrees centigrade can preserve the graft upto 6 months compared to -20 degrees centigrade which can be used only for 6 weeks.

Key Words: *Amniotic membrane. Ocular surface disorders. Chorion. Storage.*

INTRODUCTION

Amniotic membrane is increasingly being used in the management of difficult ocular surface disorders. It has been used in different fields of surgery for almost hundred years.[1] The fetal membranes consist of amnion and chorion which can either be used together or only amnion can be used. When amnion and chorion are used together, there is a higher chance of graft rejection but risk of dehiscence of the graft is less.[2] If used with the epithelial surface up, amnion gets incorporated into the tissues of the recipient. On the other hand, with the epithelial surface down, it can be used as a patch which eventually disappears and has to be replaced by corneal graft.[3] Amniotic membrane is also a good medium for propagation of limbal stem cells.[4] Since it does not express antigens of histocompatibility, the membrane is never rejected by the receiving tissues.[2]

Amniotic membrane can be used fresh. However, there is a risk of infection and transmission of hepatitis and HIV. Moreover, the membrane may not be available when needed urgently. Preserved amniotic membrane obtained from an elective Caesarean section has the advantage of pre-screening and consent of donors and can be readily available when needed.

Current methods of preservation and storage include spreading on a plastic sheet and allowing it to dry (dried membrane), passing through liquid nitrogen at -19°F (frozen membrane), keeping at -60°C under vacuum for 48 hours, then irradiated with 2.5 mega-rads (freeze dried) and gluteraldehyde fixation (stabilized amniotic membrane, SAM).[5]

Amniotic membrane can be frozen at -80°C and stored for use upto 6 months.[6] However, facility of freezing at -80°C is only available in research laboratories and is not readily available in hospitals where it may be required at a short notice. On the other hand, most hospital blood banks have refrigeration facility of upto -20°C where amniotic membrane can be stored for use upto 6 weeks and can be made available even in emergency.

The objective of this study was to assess the efficacy of preservation of amniotic membrane at -20°C and to compare it with that at -80°C.

METHODOLOGY

This study was conducted at the Department of Ophthalmology, Unit I of Dow University of Health Sciences and Civil Hospital, Karachi, between January 2009 and December 2010. It was an interventional quasi-experimental study.

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Patients undergoing elective Caesarean section were selected for the study. Informed consent was taken and they were screened for Hepatitis B and C and HIV. Under full surgical preparation, with scrubbing , donning of gowns, caps and masks and surgical gloves the placenta was collected in a sterile kidney tray. The amnion and chorion were separated and the amnion was cut off at the umbilical cord and was taken in a separate sterile container. It was then washed in a solution containing Amphotericin B in a strength of 2.5 microgram/ml, Streptomycin (50 microgram/ml) and Benzyl Penicillin (50 microgram/ml).

Amniotic membrane was spread on sofratulle on the back of a sterile kidney tray. It was then cut into 5 x 5 cm pieces. Each piece was individually wrapped in sterile gauze. Ten to 15 pieces were prepared from each amniotic membrane and stored in a sterile clear container and stored at -20°C.

When preserved at -80°C the membrane was kept in DMSO (dimethyl sulphoxide) solution of concentration 4, 8, and 10% for 5 minutes each under a laminar flow hood. It was then spread on sofratulle on the back of the kidney tray and was cut into 5 x 5 cm pieces and stored in test tubes containing 10% DMSO. Approximately thirty pieces could be prepared from each amniotic membrane and stored at -80°C.

These pieces of amniotic membrane were used as ocular grafts. The amniotic membrane grafts were applied to the eye using 10/0 nylon sutures. Those pieces left after the period of preservation (6 weeks for -20 degree grafts and 6 months for -80 degree grafts) were discarded.

In the first 2 months of the study, the amnion was stored at -20°C in PWA's blood bank at Civil Hospital, Karachi. At this temperature it can be stored for 6 weeks. In the later part of the study it was stored at -80°C at the pathology laboratory of Sindh Institute of Urology and Transplantation where it could be stored for 6 months.

Data analysis was performed by using SPSS version 10. Percentages and frequencies were calculated for categorical variable. Fisher's exact test was used to check the association of amniotic membrane at -20°C and -80°C. $P < 0.05$ was considered as significant.

RESULTS

A total of 52 patients were part of the study. Amniotic membrane obtained on four occasions was stored at -20°C and was used in 25 (48.07%) cases. In the later part of the study on 3 occasions it was stored at -80°C and was successfully used in 27 (51.92%) patients (Table I). Total number of patients was 52. There were 29 (55.76) cases of corneal ulcers, 18 (34.61%) cases of pterygium, 3 (5.76%) cases of burn and one (1.92%) each of Bowen's disease and exposed lateral rectus.

Table I: Storage of amniotic membrane.

Storage temperature	Number of times amniotic membrane was taken	Number of patients
-20°C	4 (16%)	25 (48.07%)
-80°C	3 (11.11%)	27 (51.92%)

Categorical variables are presented as frequencies and percentages.

Table II: Amniotic graft application in cases of pterygium (n=18).

Cases of pterygium	-20°C	-80°C	Total	p-value
Recurrent pterygium	7 (38.9%)	4 (22.2%)	11 (61.1%)	0.999*
Primary pterygium	4 (22.2%)	3 (16.6%)	7 (38.8%)	
Total	11	7	18	

**Insignificant p-value is calculated by Fisher's exact test.*

Out of the 18 cases of pterygium, 7 (38.8%) were primary and 11 (61.1%) recurrent. -20 degrees graft was used for 4 cases of primary and 7 cases of recurrent pterygium. Whereas -80 degrees graft was used for 3 cases of primary and 4 cases of recurrent pterygium. The association between different degrees of graft and types of cases is insignificant (p=0.999). In all cases the graft was successfully incorporated and there was no case of infection or graft rejection.

DISCUSSION

The use of amniotic membrane in ophthalmology started in 1940 and since then it has variously been used for treatment of persistent corneal defects, corneal perforation, conjunctival reconstruction following malignancy, burns, symblepharon in Stevens Johnson syndrome, limbal stem cell transplantation in severe ocular surface disease, dry eye, prevention of recurrence of pterygium, large leaking filtering blebs and preventing scar after photorefractive keratectomy.

When using autografts the problem of healing at the donor site can occur, which is not so when using amniotic membrane.[7] Amniotic membrane stimulates re-epithelialization and facilitates migration of epithelial cells over it.[8] It is not invaded by blood vessels, reduces fibrosis in healing tissue and unlike other allografts does not trigger immunological rejection. In addition it has antimicrobial effects.[9] The method of preparation affects the angiogenic factor profile of the amniotic membrane.[10]

Although the use of amniotic membrane in ocular surface disorders is well established, its application in clinical practice is not widespread, the reason for this being the lack of ready availability. Fresh amniotic graft carries the risk of transmission of infections including HIV and hepatitis etc. Preservation allows for screening of donors and preparation costs are considerably reduced. Upto 30 samples can be prepared from one placenta.

In a study conducted to assess influence of storage time on sterility, histological and biological properties of cryopreserved amniotic membrane where membranes

from different donors were stored in cell culture media containing 50% glycerol for different time periods from 4 to 24 months at -80°C, none of the examined samples showed bacterial or fungal contamination.[9]

In the current study amniotic membrane stored at -20°C and at -80°C were variously used for different indications. In all cases, there was good graft incorporation and in none of the cases there was any infection or marked inflammatory response. The grafts were stored at -20°C in the Blood Bank of Civil Hospital, Karachi and were readily available. The samples preserved at -80°C required storage at a research laboratory in Sindh Institute of Urology and Transplantation. Although the graft could be safely preserved for a long period, these had to be brought to the theatre at a pre-arranged time.

In the present study, the grafts were secured by 10/0 sutures. Although there was no inflammatory reaction this method has a risk of initiating inflammatory response. In current practice, chemically defined bioadhesives which are being used to secure amniotic membrane cause no inflammation or scaring and reduce operative time.[11] Amniotic grafts pre-coated with fibrin are also being used with good results.[12] Use of bandage contact lens after amniotic membrane grafting is known to improve surgical outcome.[13] Cryopreserved amniotic membrane has also been found superior to air-dried human amniotic membrane in ophthalmologic applications.[14] Another method of preparing amniotic graft is radiation sterilization.[15]

The latest in the horizon of amniotic grafting is cells procured from amnion having stem cell like properties. They have use in ocular surface problems.[16] Also the making of a standard artificial membrane from collagen is being planned.[17] When used in non-healing corneal ulcers, there was re-epithelialisation in 80% cases.[18] Different methods of preparation and preservation of amniotic membrane can cause disintegration of RNA. It is for gene studies that it is important to have undamaged RNA.[19] Moreover, prolonged storage affects cell viability.[20]

Preservation of amniotic membrane at -80°C has the advantage of availability for 6 months. However, the facility of storage is only available in research laboratories. Moreover, it requires laminar flow hood and dimethyl sulfoxide solution and the graft pieces are stored in test tubes. These pieces are not available in emergency situations such as burns and corneal ulcers where grafts may be needed at short notice.

Most blood banks at major hospitals have the facility of refrigeration of blood products at -20°C temperature. At this temperature the graft can be stored safely for use for 6 weeks. Since the blood banks are within the hospital premises there is ready availability of graft material even in cases of emergency. The preparation for storage is also much simpler and does not require laminar flow hood and DMSO.

In the present study there was no issue regarding graft procurement. After informed consent all donor mothers agreed for the use of placenta for harvesting the graft. Likewise, once the purpose and implications of the study had been explained none of the potential recipients of the study opted out. The association between different degrees of graft and type of cases was found to be statistically insignificant.

It appears that the case for use of amniotic membrane in ocular surface disorders is well established although the preferred method of preservation remains debatable. In developed countries tissue banks for amniotic membranes are well established as are in some centres of Pakistan such as Shifa Trust Hospital in Islamabad and the Aga Khan Hospital in Karachi. However, given the size of clinical practice in ophthalmology many such tissue banks are required in the country.

CONCLUSION

Storage of amniotic membrane at -20°C is as safe and efficacious as that at -80°C, although the 6 weeks period of storage of the former method is much shorter than the 6 months availability of the latter method. Ready availability of storage at -20°C at major hospitals makes it a more attractive method of storage in local setting.

REFERENCES

1. Riau AK, Beuerman RW, Lim LS, Mehta JS. Preservation, sterilization and de-epithelialization of human amniotic membrane for use in ocular surface reconstruction. *Biomaterials* 2010; **31**:216-25.
2. John T. Human amniotic membrane transplantation past, present and future. *Ophthalmo Clin N Am* 2003: **16**:43-65.
3. Thatte S. Amniotic membrane transplantation: an option for ocular surface disorders. *Oman J Ophthalmol* 2011; **5**:67-72.
4. Tseng SC, Chen SY, Shen YC, Chen WL, Hu FR. Critical appraisal of *ex vivo* expansion of human limbal epithelial stem cells. *Curr Mol Med* 2010; **10**:840-50.
5. Ganatra MA. Amniotic membrane in surgery. *JPMA* 2003; **53**: 29-32.
6. Adds PJ, Hunt CJ, Dart JK. Amniotic membrane grafts, “fresh” or “frozen”? A clinical and *in-vitro* comparison. *Br J Ophtalmol* 2001; **85**:905-7.
7. Okabe M, Kitagawa K, Yoshida T, Suzuki T, Waki H, Koike C, *et al*. Hyperdry human amniotic membrane is useful material for tissue engineering: physical, morphological properties, and safety as the new biological material. *J Biomed Mater Res A* 2014; **102**:862-70.
8. Dekaris I, Gabric N. Preparation and preservation of amniotic membrane. *Dev Ophthalmol* 2009; **43**:97-104. Epub 2009 Jun 3.
9. Thomasen H, Pauklin M, Noelle B, Geerling G, Vetter J, Steven P, *et al*. The effect of long-term storage on the biological and histological properties of cryopreserved amniotic membrane. *Curr Eye Res* 2011; **36**:247-55. Epub 2011 Jan 28.

10. Wolbank S, Hildner F, Redl H, van Griensven M, Gabriel C, Hennerbichler S. Impact of human amniotic membrane preparation on release of angiogenic factors. *J Tissue Eng Regen Med* 2009; **3**:651-4.

11. Takaoka M, Nakamura T, Sugai H, Bentley AJ, Nakajima N, Fullwood NJ, *et al.* Sutureless amniotic membrane transplantation for ocular surface reconstruction with a chemically defined bioadhesive. *Biomaterials* 2008; **29**: 2923-31. Epub 2008 Apr 15.

12. Sekiyama E, Nakamura T, Kurihara E, Cooper LJ, Fullwood NJ, Takaoka M, *et al.* Novel sutureless transplantation of bioadhesive- coated, freeze-dried amniotic membrane for ocular surface reconstruction. *IOVS* 2007; **48**:1528:34.

13. Saw WPJ, Minassian D, Dart JKG, Ramsay A, Henderson H, Poniatowski S, *et al.* The Amniotic Membrane Tissue User Group (AMTUG): Amniotic membrane transplantation for ocular disease: a review of the first 233 cases from the UK user group. *Br J Ophthalmo*l 2007; **91**: 1042-7.

14. Thomasen H, Pauklin M, Steuhl KP, Meller D. Comparison of cryopreserved and air-dried human amniotic membrane for ophthalmologic applications. *Graefes Arch Clin Exp Opthalmol* 2009; **247**:1691-700. Epub 2009 Aug 20.

15. Fetterolf DE, Snyder RJ. Scientific and clinical support for the use of dehydrated amniotic membrane in wound management. *Wounds* 2012; **24**:299-307.

16. Cirman T1, Beltram M, Schollmayer P, Roman P, Kreft ME. Amniotic membrane properties and current practice of amniotic membrane use in ophthalmology in Slovenia. *Cell Tissue Bank* 2014; **15**:177-92. Epub 2013 Dec 19.

17. Rahman I, Said GD, Maharajan SV, Dua HS. Amniotic membrane in ophthalmology: indications and limitations. *Eye (Lond)* 2009; **23**:1954-61.

18. Salman GA. Value of fresh amniotic membrane graft in management of resistant non infected corneal ulcer. *J Clinic Experiment Ophthalmol* 2010; **1**:108.

19. Yatim RM, Kannan TF, Hamid SSA, Shamsudin SH. Effects of different processing methods of human amniotic membrane on the quality of extracted RNA. *Arch Orofac Sci* 2013; **8**: 47-53.

20. Irfan QZ, Fareeha A, Wajid KA. Technique for processing and preservation of human amniotic membrane for ocular surface reconstruction. *World Acad Sci Engineer Technol* 2010; **9**:28.