Processing and Bioburden Analysis of Human Amniotic Membrane for Biological and Tissue Engineering Application- A Preliminary Report.

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ABSTRACT

Amnion membrane attained from placenta coverings have been widely used for clinical applications which includes skin covers for patients with burns and traumatic defects, cornea ulceration and, more recently gynaecological and vascular surgery. However, their use in clinical tissue engineering is still in its infancy. The purpose of the present study was to determine the quality and the safety of the amniotic membrane as an implant material for direct biological and tissue engineering applications. Amniotic membranes were obtained under sterile condition from 5 healthy women undergoing routine elective Caesarian section. The amniotic membranes were processed and dried using either freeze or air dying techniques. At the end of processing, bioburden analysis revealed that freeze drying method appeared to be inferior with counts of 347 cfu/piece as compared to air drying which was only 147 cfu/piece (p=0.042; significant at $p<0.05$). The amniotic membrane was further sterilized by gamma irradiation at 25 kGy. We found that complete eradication of microorganisms was attained following irradiation of the amniotic membranes and has not affected their physical and biological properties. The results indicates that amniotic membranes processed either by freeze drying or air drying was suitable for aminion processing because despite having significantly higher bioburden counts using the freeze drying techniques, irradiation produces good quality sterile biological material potential for clinical and tissue engineering application.

INTRODUCTION

The human amniotic membrane (amnion) is part of the placenta, being closely related to the chorion. The amniotic membrane is a tough shiny membrane consisting of five layers: the epithelium, basement membrane, compact layer, fibroblastic and a spongy layer. It is devoid of any blood vessels, lymphatics or nerves. The amniotic membrane has been used in a variety of clinical situations since the last century. The first reported case using amnion as a clinical material was for skin transplantation in 1910 at Johns Hopkins Hospital (1). Since then its use for skin grafting have been extensively documented in a number of scientific litreatures (2, 3, 4). A number of other uses have also emerged which includes: Treatment of chronic venous ulcers (5, 6), epithelial cover for patients with extensive burns (7, 8), reconstruction in head and neck surgery (9, 10), and vaginal reconstruction following radical hysterectomy (11). Wounds from large extensive burns and trauma, as well as slow or non-healing ulcers of the lower third of the leg and feet due to diabetes and chronic venous insufficiency are common clinical problems that have been treated using amnion covers as biological dressings. In the field of ophthalamology, the use of amnion have been more extensively used with an introduction of a number of clinical applications, including chronic epithelial defects cover (12, 13), chemical or thermal eye injuries biological covers (14, 15), and conjunctival surface defects repair (16).

Amniotic membrane is suitable for transplantation because of a number of reasons: Firstly, it does not express HLA-A, B, DR antigens (17, 18) and does not have immunosuppressive properties (19). Secondly unlike other tissues used in transplantations, amniotic membrane has anti adhesiveness properties which does not cause wound adhesions (20), it inhibits new vessel formation at the transplant site therefore preventing increased tissue rejection (21) and also prevents local inflammatory response (22).

However, as with the use of any biological tissue as an implant material, the main concern is infectious disease transmissions from donor to recipient.

Lyophilization has been thought to be superior as it has been shown to assist in eradicating microbes as well as to extend the shelf life of the amnion to more than 5 years. Moreover, a subsequent radiation dose for sterilization added will provide a further safety margin. In some reports, the eradication of microbes (including viruses e.g. HIV and Hep B.) using these combinations of processing have reported to be as high as100% (7,12,22) The aim of the present project was to find out the best method for processing amniotic membrane to assure the quality of the amnion was maintained while ensuring the safety of the amniotic membrane as implant material for biological dressing.

MATERIALS AND METHOD

Screening and Consent

After obtaining patients' informed consent, the amniotic membranes were obtained from 5 HIV-, syphilis- and hepatitis-seronegative women following a routine elective Caesarian section. The methods employed in our study were closely adhered to the protocol approved and provided by the Medical Ethics Committee, University Malaya.

Procurement

With the assistance of the operating obstetrician, following delivery of the placenta, the whole tissue is passed to a dedicated technician in a sterile dish for processing. This placenta is then brought to a dedicated wash area where processing begins. The fresh placenta was washed with running water. While doing so, the amniotic membrane was gently and carefully peeled off from the rest of the placental mass. Blood clots present on the surfaces were washed away with running water and then sterile normal saline. The amniotic membrane is then placed in a bottle containing sterile saline labeled with patient's detail. The bottles were then kept at 4°C while waiting for processing the next morning. The remaining placenta were discarded as biological waste as processed accordingly.

Processing: Washing and Drying

After being taken out from refrigeration, the amniotic membrane was then transferred into another sterile bottle containing sterile distilled water. After 10 minutes in the shaker, the amniotic membrane was then transferred into 0.05 % sodium hypochlorite and shaken slowly for another 10 minutes. Further washing of the amniotic membrane was later performed using sterile saline solution. Three subsequent washes were performed in separate sterile container in sequence, each shaken for 20 minutes at 60rpm.

The cleaned amniotic membrane was stretched over sterile tray with the chorion side towards the tray and cut into appropriate shapes under the laminar flow cabinet. Each amniotic membrane attained from each donor was cut and divided into 2 groups. Each part of the amnion is the prepared for: (group 1) air dried in laminar flow overnight and (group 2) freeze drying.

Freeze drying and air drying

In the air dried group (group 1), the stretched amnion was left overnight to be dried under the laminar flow hood. The next morning, the amnion is then trimmed to size (2cm X 2cm) and then packed in 2 vacuum sealed packages. These processes were all performed under the laminar flow hood to reduce contamination.

In the freeze drying group (group 2), the stretched amniotic membrane was place on a sterile tray or plastic bag in the freeze drier. The membrane undergoes temperature changes from 40°C to 50°C to as low as -20°C at intervals of 1-2 hours. The freeze drying process was operated for 1-2 days under vacuum condition. The water content of the dried amnion at the end of the process ranged between 5-7 %. The membrane was trimmed to (2 cm x 2 cm) sized membranes from the original dried amniotic membrane and packaged in 2 packs. All processing were done in laminar flow cabinet environment to avoid contamination.

In each package, there were 3 membranes placed inside them. In both groups the packages are stored in a dry cabinet and away from direct sunlight. While one package from each group remained non-irradiated and sent directly for bioburden analysis after 24 hours of storage, the other package was sent for irradiation.

Bioburden analysis

One package of the non-irradiated membranes from each group was opened after being kept for at least 24 hours. The small pieces of amniotic membrane was transfered into 0.01% saline polysorbate solution and shaken for 15 minutes at 2,300 rpm seperately. The samples were left to stand for 15 minutes. Certain volume of aliquot washing solution (the volume that can give less than 100 cfu per filter) was filtered using sterile membrane filter (pore size 0.45 mm, diameter 47 mm).

The membrane filter was removed with sterile forceps and was transferred onto Tryptone Soya Agar plate. The inverted plate was incubated at 37°C for 7 days. Total microbial count was performed at the end of day 7.

Sterilisation and Gamma Irradiation

One of the packed amniotic membranes from each group was sent for sterilization using gamma rays (Cobalt 60) at 25 kGy. Radiation sterilization was carried out at the Malaysia Institute for Nuclear Technology (MINT) Research facility in Bangi. Following irradiation, the packed amnions are then stored in a dry cabinet and away from direct sunlight for at least 24 hours. Similar bioburden analyses that were performed on the non-irradiated membranes were also performed on all of these samples.

Sterility test

Sterility test was carried out on the 10 samples (5 samples from each group- one irradiated, one non-irradiated). The amniotic membrane was transferred into Tryptone Soya Broth agar and incubated at 37°C. Changes on the agar were observed namely for any form of microbial colonization on a regular basis. Final observations were completed at the end to 1 month.

Histologic Examination

Following storage for 24 hours for group 1 and irradiation for group 2, histological examination was carried. Each amnion membrane from each group was fixed in formalin for at least 24 hours. Specimens were decalcified and embedded in paraffin, and sectioned at approximately 5µm thickness. Sections were spread on slides and deparafinised in xylene and transferred in aqua dest. using decreasing concentration of ethanol. Using standard histochemical techniques, tissue sections were stained with Hematoxylin & Eosin (H&E).

RESULTS

There were 5 amnions harvested from placentas attained during caesarian section. All patients provided written consent to donating their placenta at least 24 hours prior to going for surgery. Blood screening performed in all subjects revealed no positive results to infectious diseases. The subjects were of young mothers aged less than 30 years old. Time from surgery to time of harvest for all samples was approximately 60 minutes. Time from harvest to time of processing was approximately 22 hours. All amnion membranes attained from the harvest were in one piece with no defects noted on the surfaces.

Gross examination of the amnion membrane following washing, air and freeze drying and, following irradiation did not show any distinctive changes in their appearances. In all instances, sub-maximal manual stretching by the same examiner (using pincer to pincer grip) did not appear to cause a tear to the membrane at either stages of the process.

According to the International Standard ISO 11137 (23), bioburden is defined as the population of viable microorganisms (excluding viral contamination) on a product. In the context of sterilisation, bioburden is the total count of viable microorganisms on a product determined immediately prior to the sterilisation process. In reference to table 1, the result of bioburden analysis with five dried-amniotic membranes either using freeze drying or air drying shows significant differences between the bioburden present. The freeze drying method appeared to have on average 347 cfu/piece, whereas air drying was found to only have147 cfu/piece. The result was further analysed using Wilcoxon Signed Ranks Test (non-parametric mean comparison analysis), which showed significant differences between the bioburden count using freeze and air drying (p=0.042, significant at $p<0.05$).

Table 1: Result of bioburden analysis with five dried amniotic membranes either using freeze drying or air drying.

Sample	Freeze Drying Group 2 (colonies)	Air Drying(colonies) Group 1 (colonies)
1	4	1
2	921	30
3	799	702
4	3	0
5	7	3
Average	347 cfu/piece	147 cfu/piece

Following irradiation, no infection was found from the amnion membranes of both groups. All agar plates did not show any colonization at the end of one month. It appears that irradiation has completely eradicated all microbials as evidence noted from both the bioburden analysis as well as sterility test (Figs. 1A and 2A).

Tests performed on amnion that were not irradiated proved to be colonized with microbes regardless of the preparation techniques used (Figs. 1B and 2B). It is therefore important to note the washing and drying

processes of amniotic membrane were not sufficient to eliminate microorganism contamination.

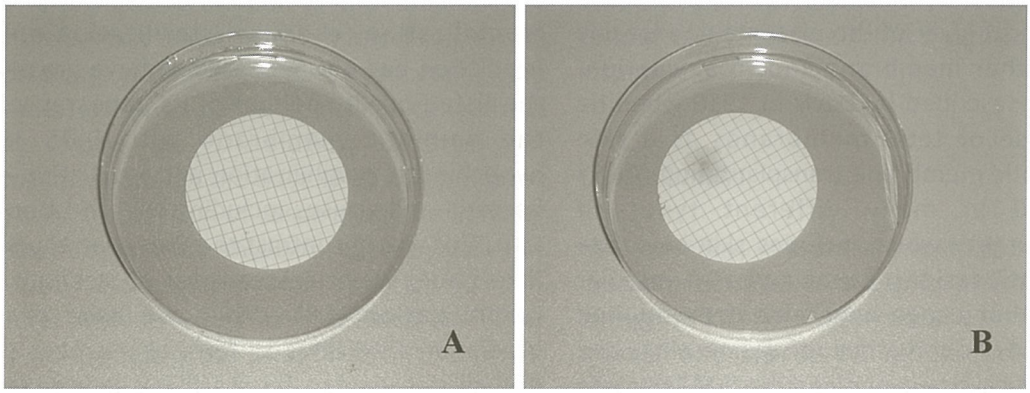

Fig1. No colonies were found on the membrane filter taken from bioburden analysis from amnion that was irradiated following lyophilization (A). Colonies were found on the membrane filter from amnion that were not irradiated but lyophilized (B).

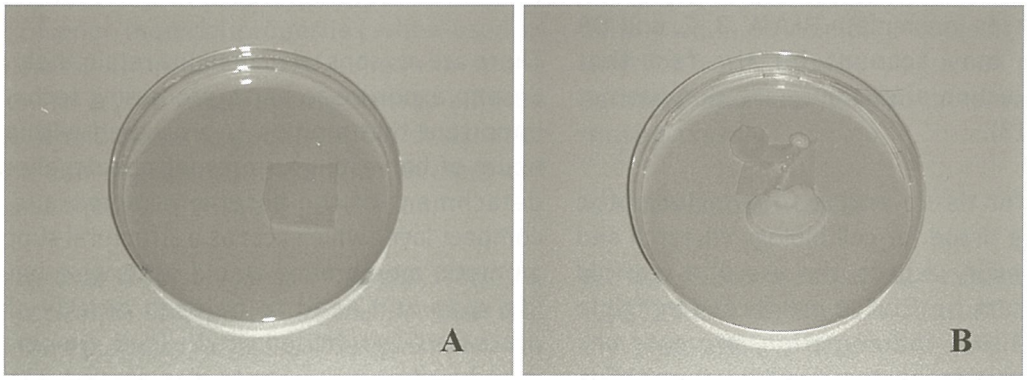

Fig2.Sterility test: No colonies were found around the amniotic membrane that were lyophilized and irradiated (A). Colonies were found around the inlaid amniotic membrane of lyophilized but not irradiated (B).

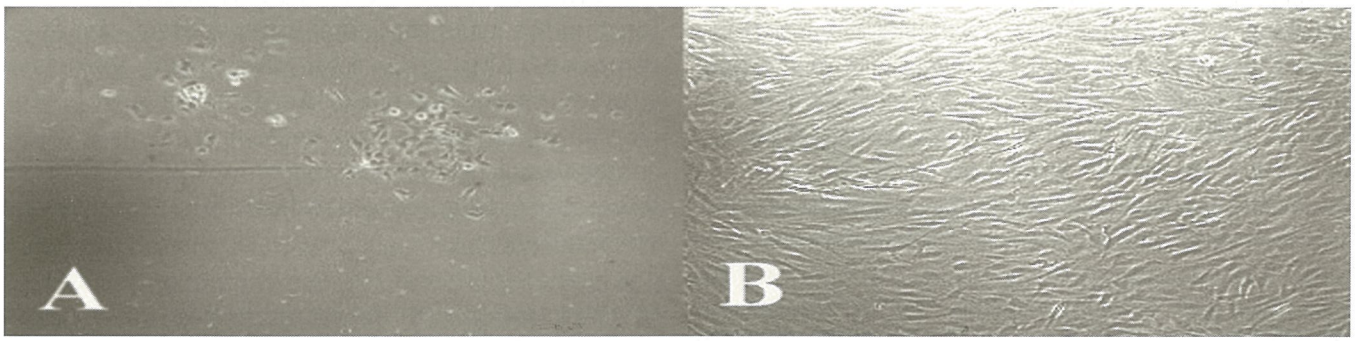

Fig3. H&E staining for amniotic membrane, 400X. (A) Washed amniotic membrane but non air dried or irradiated. (B) Washed, air dried and irradiated amniotic membrane. (C) Washed, freeze dried and irradiated amniotic membrane.

Our H&E images revealed that amniotic membrane was denuded of cells while the basal lamina remained intact (Figs. 3A, 3B and 3C). Stromal fiber arrangements were maintained from the time of harvest to the time processing and irradiation were completed. Hemotoxilin staining also remained good at the end of our processing. We can therefore we conclude that the process of drying and irradiation did not affectively altered much of the physical or biological properties of amniotic membrane.

DISCUSSION

The amniotic membrane (or amnion) is a layer of tissue found the innermost layer of the placenta and loosely attached to another membrane known as chorion. Since it was first described by Davis in 1910 when he reported the use of fetal membranes as a skin substitute, amniotic membrane transplantation (AMT) has been used in many different types of reconstructive surgery which includes skin coverage following traumatic lesions, burns and also vascular ulcers (1,21). Other usage have also been applied namely in the field of ophthalmology, gynaecology and more recently clinical tissue engineering. AMT became important because of its ability to diminish the occurrence of adhesions and scarring, its ability to enhance wound healing and epithelialisation, and its antimicrobial potential (17). In particular, the amniotic membrane expresses incomplete HLA-A, B, C, and DR antigens, which may account for the fact that immunological rejection after transplantation has not been observed (18).

The application for tissue engineering includes the formation of living tissue for cell biology research and therapeutics. In every aspect, the use of a scaffold design system must be that it produces negligible tissue reaction, tissue inflammation, promote cell adhesion and migration, increase cellular expression and strong enough to function as tissue construct. More importantly, recent findings have also concluded that the need for biocompatibility of scaffold used in transplant must be addressed as a major factor in choosing a suitable scaffold design. We found that the use of amnion raises hope for the finding of the right biological scaffold especially in the treatment of skin defects (27). Other areas of interest would be the use as patch cover following articular cartilage repair. In a separate research which we conducted recently, amnions have shown to posses gross properties that almost mimics the periosteum used for autologous chondrocyte transplantation (ACT). However, considering that this research is in its early stage, it would be premature for us to definitely confirm its usefulness as a substitute for ACT although early results have been rather promising.

Unlike the use of other tissues in organ or tissue transplantation, the use of amnion does not initiate an immune response therefore is easily accepted by its recipient. The risk of infection is also relatively low (21). This is because amnions are relatively durable withstanding a number of processing stages allowing sterilization to be done more extensively. As seen in our study, amnion membranes which had undergone lyophilization, chemical sterilization and gamma irradiation has not appeared to have grossly changed the tissue quality neither of its appearance. Washing the amniotic membrane with 0.05 % sodium hypochlorite, lyophilization and irradiation eliminates bacteria and viruses including HIV and Hep B viruses (30). This was in contrast to other even tougher tissues (like bone) which losses most of strength following lyophilization (28). While we have proven that irradiation eradicates all form of microbes effectively, not all tissues can be sterilized using this method because tissue properties are altered by radiation exposure. It is evident that tissues like bone and tendons further looses it strength following irradiation (29).

There are reasons why amnions retain their properties despite exposure to harsh processing techniques. It is important that amnion is processed within 24 to 48 hours of harvesting to prevent cell degeneration and detachment of the basement membrane from the compact layer which acts as a structural support. Fresh amniotic membranes would otherwise have a short life span and need to be used quickly after being processed. Lyophilised or air dried membranes were introduced as measures to give amnion a longer shelf life. Under freeze dying condition, amniotic membrane can maintain its flexibility and strength whilst preserving tissue quality by almost completely removing its water content. Although air drying function the same way, total water content cannot be removed completely from the tissue therefore shortening its life span. Another advantage for lyophilized amnion is that because it has low water content and in theory assist in eliminating microbes, a lower dose of gamma irradiation is required for sterilization.

Sterility test performed on 10 irradiated amniotic membranes to determine any viable microorganism had cause contamination to processed amniotic membrane were negative despite being incubated for up to 1 month. Based on recommendation made from ISO 13409 (26), this would prove than complete eradication of bacterial and yeast were complete following gamma irradiation at doses of 25 kGy. The high bioburden noted in group 2 (lyophilized amnion) would suggest that the high contamination of

microorganisms using freeze drying technique was due to the non-sterile environment of the lyophilizer as compared to processing amnion in the laminar flow cabinet where particle counts are very low (i.e. 1/10000 ppm). Regardless, in both methods of processing, the final outcomes (as far as contamination is concerned) were the same as irradiation cleared all microorganisms. Although the dose of 25 kGy has been widely used for sterilization of tissue allografts in many tissue banks, in theory the lower dosage may assist to preserve their biomechanical properties (25). A biomechanical analysis would have deemed useful for this study. Although gross examination did show strength of the membrane were still maintained at the end of the experiment, a value would have been useful for quantitative analysis. A strong membrane is useful for clinical application. Structural breakdowns as with instances where early degradation occurs (as the result of poor material properties when used as biological dressing) are unlikely to happen in strong constructs. In tissue engineering sense, amnion membrane would serve as good scaffold designs for cell seeding and in-growth providing convenient cell transfers and therefore tissue regeneration.

Amnion retrieval from placenta removed from the womb via caesarean section is much cleaner than that harvested from placenta retrieved via vaginal delivery. This is because caesarean section avoids contamination of amnion via the vaginal canal while being removed in a clean surgical field. This finding would be in contrast to cadaveric donors whereby the risk of infection are much higher depending the length of time the donor has been deceased (30). In addition, screening of donors in women planned for routine elective Caesarian section are unnecessary as most of these results would have been available during their antenatal check up. There are further advantages of harvesting tissue from live donors as compared to cadaveric donors: First, a detailed medical history can be attained to identify high risk donors. Secondly, as a requirement for allograft transplant, a second infection screening which is usually six months from the first is required and can easily be performed in live donors but not in the cadavers.

There were limitations to the current study: Because the numbers of subjects used in this study were small, our analyses were confined to non-parametric tests allowing a wide confidence interval. If the numbers of sampling were larger, the significance would have shown an extremely strong value with narrower confidence interval. Nevertheless the values were significant and more importantly, regardless of which process have been chosen to process amnion, complete eradication of microbes is achieved following irradiation whilst maintaining its original structure.

CONCLUSION

In summary, we conclude that human amniotic membranes processed either by freeze drying or air dying, and following irradiation will provide good quality and sterile biological material for clinical application. It may also provide useful constructs in tissue engineering for biological application, as processed tissues seem to maintain its original integrity whilst providing the qualities needed as a scaffold. We would recommend that irradiation be performed in all amnion processing but the choice of whether freeze or air drying to be performed should be based upon the need to increase their shelf life in these tissues.

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REFERENCES

1. Davis J. W. Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. Johns Hopkins Medical Journal. 1910: 15; 307.

2. Subrahmanyam M. Amniotic membrane as a cover for microskin grafts. British Journal of Plastic Surgery.1995; 48: 477-478.

3. Troensegaard-Hansen E. Amniotic grafts in chronic ulceration. Lancet. 1950; 1: 859-860.

4. Sabella N.Use of fetal membranes in skin grafting. Medical records NY. 1913: 83; 478-480.

5. Ward D. J. and Bennett J. P. The healing of chronic venous ulcers with prepared human amnion. British Journal of Plastic Surgery. 1989; 42: 463-467.

6. Ward D, J. and Bennett J. P. The long term results of the use of amnion in the treatment of leg ulcers. British journal of Plastic Surgery. 1984; 37: 191-193.
7. Kirschbaum S. M. and Hernandez H. Use of amnion in extensive burns. Proceedings in the Third International Congress of Plastic Surgery. Excerpta Medica, Amsterdam. 1963
8. Quinby W. C., Hoover H. C. et al. Clinical trials of amniotic membranes in burn wound care. Plastic Reconstructive Surgery. 1982; 70: 711-716.
9. Zohar Y. et al. Use of human amniotic membrane in otolaryngologic practice. Laryngoscope. 1987; 97: 978-980.
10. Talmi Y. P. et al. Use of human amniotic membrane as a biological dressing. European Journal of Plastic Surgery. 1990;13: 160-162.
11. Georgy M.S. and Aziz N. L. Vaginoplasty using amnion graft. Journal of Gynaecological Surgery. 1996;16: 262-264.
12. Anderson S. B. et al. Corneal ulceration after amniotic membrane transplantation. British journal of Ophthalmology. 2003;18(5): 587-591.
13. Baum J. Amniotic membrane transplantation: Why is it effective? Cornea. 2002;21(4): 339-341.
14. Meller D., Pires R. T. F. and Mack R. J. S. Amniotic membrane transplantation for acute chemical or thermal burns. Ophthalmology. 2000;107: 980-990.
15. Ucakhan O. O. et al. Non preserved human amniotic membrane transplantation in acute and chronic chemical eye injuries. Cornea. 2002;21: 169-172.
16. Tseng S. C. G. Amniotic membrane transplantation for conjunctival surface reconstruction. American journal of Ophthalmology. 1997;124:765-774.
17. Houlihan J. M. et al. The human amniotic membrane is a site of MHC class 1b: evidence for the expression of HLA-E and HLA-G. Journal of Immunology. 1995;154: 565-574.
18. Akle C. A. et al. Immunogenecity of human amniotic epithelial cells after transplantation into volunteers. Lancet. 1981;2: 1003-1005.
19. Ueta M. et al. Immunosuppressive properties of human amniotic membrane for mixed lymphocyte reaction. Clinical and Experimental Immunology. 2002;129(3): 464-470.
20. Shimazaki J. et al. Transplantation of amniotic membrane and limbal autograft for patients with recurrent pterygium associated with symblepharon. British Journal of Ophthalmology. 1998;82: 235-240.
21. Trelford J. D. and Trelford-Sauder M. The amnion in surgery, past and present. American Journal of Obstetrics and Gynaecology. 1979;134: 833-845.
22. Choi Y. S. et al. Effect of application of human amniotic membrane on rabbit corneal wound healing after excimer laser photorefractive keratectomy. Cornea. 1998;7: 389-395.
23. International Standard (ISO). Sterization of health hare products – requirement for validation and routine control – radiation sterilization. ISO 11137:1995, Geneva.
24. Farazdaghi M. et al. Preserved human amniotic membrane grafts: An evaluation of morphology, elasticity, and biocompatibility. Proceedings of the European Eye Bank Association 12th Conference, January 2000.
25. Singh R., Purohit S. and Chacharkar M.P. Effect of high doses of gamma radiation on the functional characteristics of amniotic membrane. Radiation Physics and Chemistry, in press
26. International Standard (ISO). Sterilization of health care products – radiation sterilization – substantiation of 25 kGy as sterilization dose for small or infrequent production batches. ISO 13409:1996, Geneva.
27. Eu Kyung Yang, Jung Keug Park, Jae Il Ahn et al. Tissue engineered skin substitutes using collagen scaffold with amniotic membrane component. Key Engineering Materials. 2005; 277-9: 12-18.
28. Nather A, Thambyah A, Goh JC. Biomechanical strength of deep-frozen versus lyophilized large cortical allografts. Clin Biomech (Bristol, Avon). 2004 Jun;19(5):526-33.
29. Godette GA, Kopta JA, Egle DM.Biomechanical effects of gamma irradiation on fresh frozen allografts in vivo. Orthopedics. 1996 Aug;19(8):649-53
30. Procurement of cadaveric bone allografts: How long will it remain infection free? Haris Ali, T. Kamarul, Ng LL, R. Penafort. The Journal of the Asean Orthopaedics Association. 2005; 17(1): 45-48.

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