

Scanning electron microscopic assessment on surface morphology of preserved human amniotic membrane after gamma sterilisation

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Abstract Human amniotic membrane that has been processed and sterilised by gamma irradiation is widely used as a biological dressing in surgical applications. The morphological structure of human amniotic membrane was studied under scanning electron microscopy (SEM) to assess effects of gamma radiation on human amniotic membrane following different preservation methods. The amniotic membrane was preserved by either air drying or submerged in glycerol before gamma irradiated at 15, 25 and 35 kGy. Fresh human amniotic membrane, neither preserved nor irradiated was used as the control. The surface morphology of glycerol preserved amnion was found comparable to the fresh amniotic membrane. The cells of the glycerol preserved was beautifully arranged, homogenous in size and tended to round up. The cell structure in the air dried preserved amnion seemed to be flattened and dehydrated. The effects of dehydration on intercellular channels and the microvilli on the cell surface were clearly seen at higher magnifications (10,000×). SEM revealed that the changes of the cell morphology of the glycerol preserved amnion were visible at 35 kGy while the

air dried already changed at 25 kGy. Glycerol preservation method is recommended for human amniotic membrane as the cell morphological structure is maintained and radiation doses lower than 25 kGy for sterilization did not affect the appearance of the preserved amnion.

Keywords Human amniotic membrane · Air dried · Glycerol preserved · Gamma irradiation · SEM · Cell morphology

Introduction

Human amniotic membrane possesses most of the characteristics of an ideal skin substitute. It acts as an effective barrier as it has good adherence to wound with no immunological reaction. The membrane is bacteriostatic and has some beneficial effects such as reducing water loss, protein and evaporative heat at the wound surface, and also preventing further bacterial contamination (Rodriguez-Ares et al. 2009). The membrane is able to maintain a physiologically moist microenvironment that promotes healing. It has been used in a variety of clinical conditions, among others, in ophthalmic treatment (Dua et al. 2004), in partial thickness burn (Branski et al. 2008), as a temporary biological dressing (Maral et al. 1999) as a cover for microskin grafts (Subrahmanyam 1995), as an epidermal substitute (Rejzek et al. 2001) and as a substrate for the ex vivo expansion of limbal epithelial cells (LECs)

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used to treat corneal epithelial stem cell deficiency in humans (Riau et al. 2010). To ensure the continuous supply of the amnion, the membrane can be processed and preserved while maintaining its properties and efficacy. Generally, the amniotic membrane can be preserved by either drying or submerged in glycerol. Radiation is used to sterilize the preserved membrane to attain high sterility assurance level for safe clinical usage.

Few studies reported the effectiveness of dried and irradiated amnions for the treatment of burns. Bujang-Safawi et al. (2010) demonstrated that the dried and irradiated amnion as a biological dressing was safe, simple to use and effective in promoting wound healing while preventing wound infection, especially in the pediatric patients.

There are several factors that may influence the surface healing effect of amnion and the influence is becoming more complex after the combination of mechanical and histological factors (Hoeynck et al. 2004). From microscopic studies they found that different sterilization and preservation procedures affected the histological and biophysical properties of amnion. The influence of preservation and sterilization on microbiological safety for medical application of human amnion was also reported by Hoeynck et al. (2008) and Singh et al. (2007). However they found that the influences on histological and biophysical properties were minimal in glycerol treated amnion as compared to air drying. Glycerol preserved the physical structure of amnion by preventing loss of fluid, protein, electrolytes, heat and energy (Rejzek et al. 2001). Rooney et al. (2008) described that when skin was submerged in high concentration of glycerol, it could be subjected to gamma irradiation (25 kGy) without any changes in histological structure. They believed that glycerol as a radioprotective agent could minimize indirect effects of radiation.

One of the challenges after processing biological dressings is to ensure the sterility of the processed tissue products. Radiation is the most suitable method for sterilizing tissue allografts. Gamma irradiation has many advantages over other sterilization processes such as heating and chemical treatment (Martinez-Pardo et al. 2007; Yusof 1994; Hilmy et al. 1987). Radiation is effective in killing various bacteria, viruses and fungi. By adopting the principles of good manufacturing practice, tissue products with low level of microbial contamination could be produced hence the products

can be sterilised at doses lower than 25 kGy (Riau et al. 2010) to achieve sterility assurance level (SAL) of 10^{-6} . In ophthalmology, the preservation of amniotic membrane structure is specifically important for treating ocular surface damage as previously described by Dua et al. (2004). Any morphological changes possibly caused by preservation method in combination with gamma irradiation would lead to the degradation of the membrane (Singh et al. 2007). Therefore use of gamma radiation at doses lower than 25 kGy for terminal sterilization is becoming a new approach in tissue banking in order to minimize the radiation effect on physical properties of tissues. Although Singh et al. (2007) reported that 25–50 kGy gamma irradiation did not evoke undesirable changes in the functional properties of the air dried amniotic membrane, but Nguyen et al. (2007a, b) in their reviewed paper indicated that gamma irradiation affected mechanical and biological properties of bone allografts and muscular skeletal soft tissues that would then affect the clinical performance.

This work was conducted to study the influence of two preservation methods namely air drying and glycerol preservation, on the surface morphology of human amniotic membrane by using (SEM). Effects of different doses of gamma irradiation on the preserved amnion were then studied to determine the influence of sterilization in combination with preservation. Amniotic membranes used in the study were separated from chorion during procurement and amnion as the general term after the amniotic membrane was processed and preserved.

Materials and methods

Processing and preservation of human amniotic membrane

Human amniotic membranes were obtained from placenta of healthy mothers and separated from chorion during procurement. The mothers were previously screened according to the Donor Exclusion Criteria of the Tissue Bank, Universiti Sains Malaysia. Among the criteria, donors must have no history of drug or alcohol abuse, not single mothers and no multiple sexual partner, no medical records of communicable diseases, do not undergone chemotherapy treatment and not on prolonged steroid treatment. Consent for donation was

obtained from the potential mothers prior to serological test. The procured amniotic membranes were kept frozen at -20°C while waiting for the results of the serological test. Only amniotic membranes with seronegative for human immunodeficiency virus (HIV), hepatitis B and C, and syphilis were processed and preserved under clean environment and aseptically handled at all time.

The frozen human amniotic membrane was thawed at room temperature then washed free from blood clots and mucus by using sterile water. After cleaning, the samples were rinsed in 0.05 % v/v sodium hypochlorite solution to remove bacterial contamination and complete removal of hypochlorite residue. The human

amniotic membrane was further rinsed three times with sterile distilled water and then preserved by either air drying or submerged in glycerol.

For air drying preservation, the samples were stretched on sterile plastic sheets inside biological safety cabinet and then left for 16–24 h to dry at room temperature (25°C , 65–75 % humidity). The air dried amnion was packed into a double layer polyethylene (PE) bag. As for glycerol preservation, the cleaned human amniotic membrane was submerged in increasing concentrations of glycerol starting with 40 % followed by 60–80 % for dehydration process. They were immersed in the glycerol overnight at each concentration at room temperature. The membrane

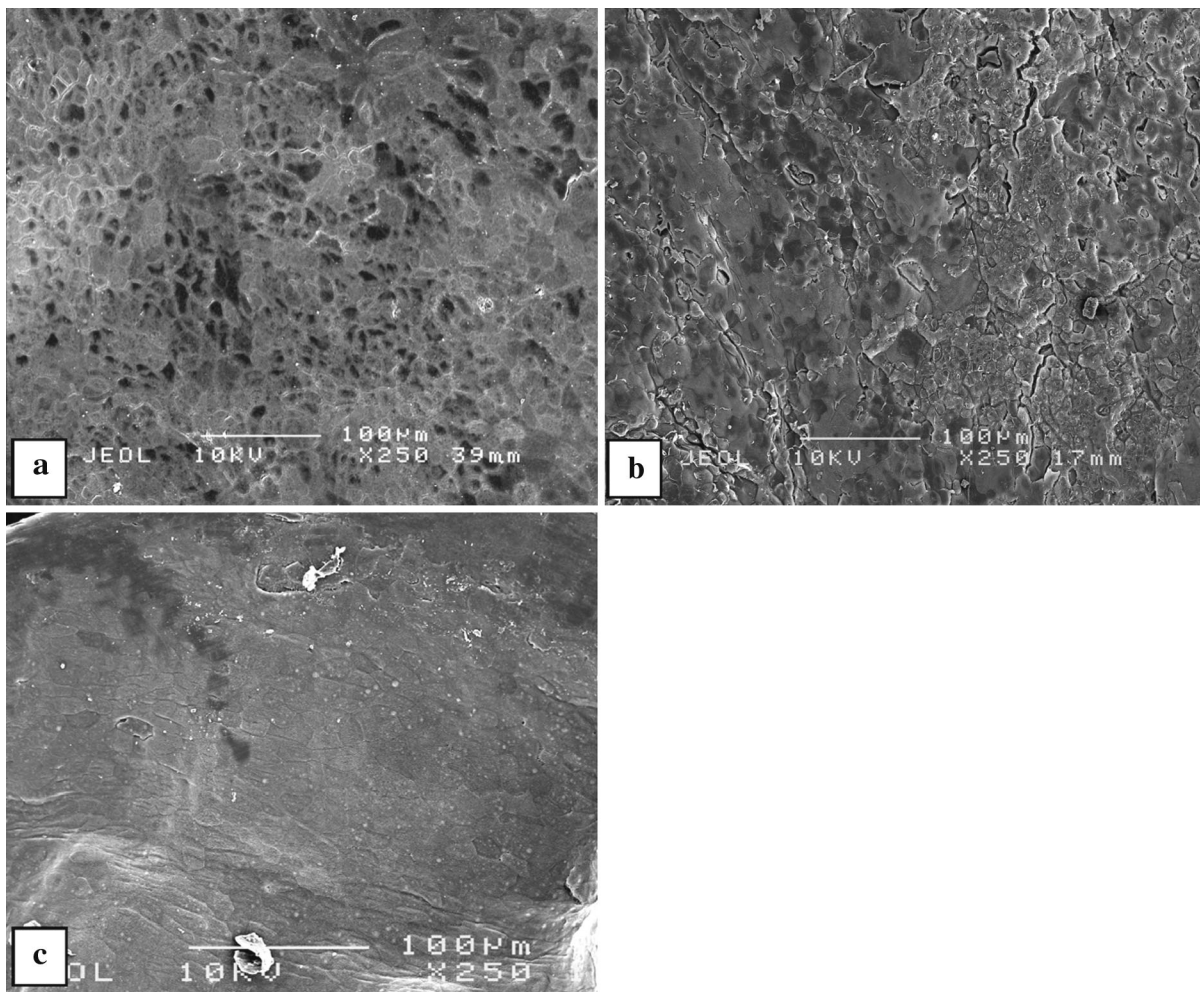


Fig. 1 Surface morphology of amnion at low magnification ($\times 250$). **a** Air dried preserved, **b** glycerol preserved, **c** fresh human amniotic membrane (control)

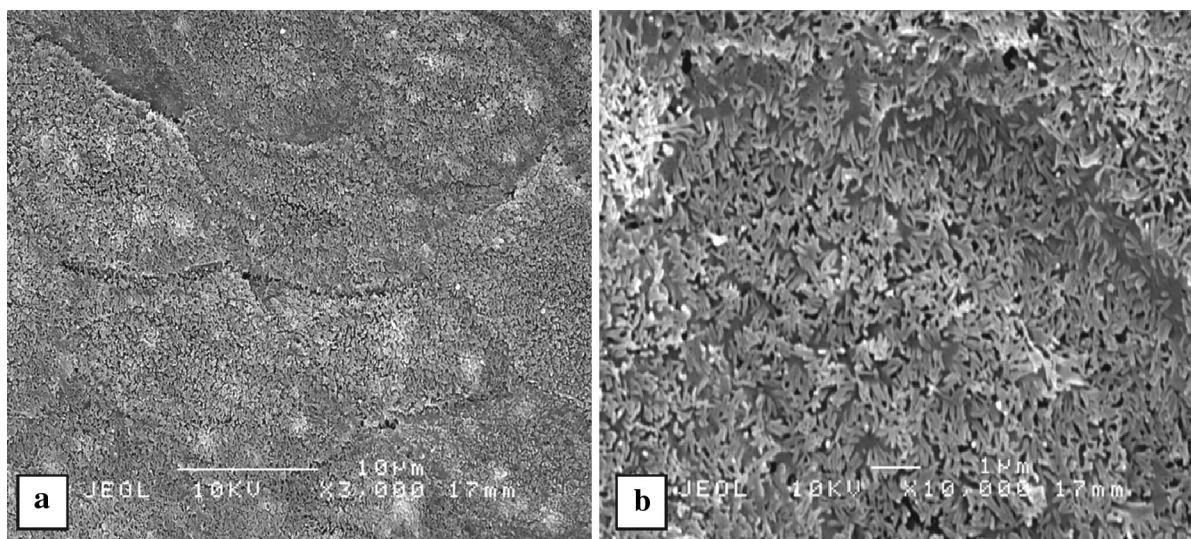


Fig. 2 Surface morphology of fresh (non-preserved and non-irradiated) human amniotic membrane. **a** Polygonal cells at magnification $\times 3,000$. **b** Microvilli extended to lateral border of cells up to intercellular channels at magnification $\times 10,000$

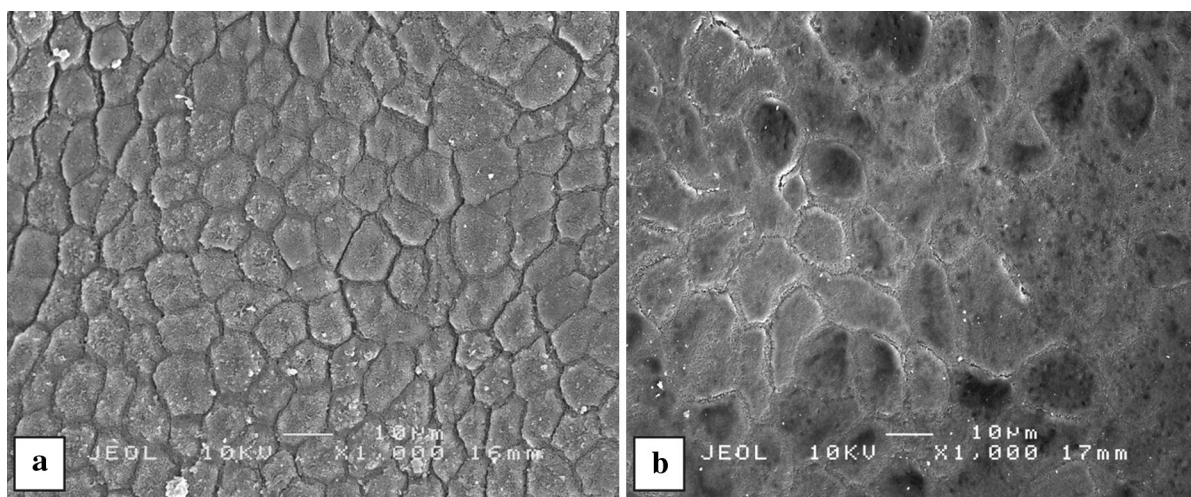


Fig. 3 Surface morphology of processed and preserved amnion (non-irradiated) at magnification $\times 1,000$. **a** Glycerol preserved, **b** air dried

was then placed in a polypropylene (PP) plastic bottle containing 87 % glycerol for preservation and packed into a double layer plastic PE bag before irradiated at varying doses of gamma irradiation and stored at room temperature.

In the control group, the fresh human amniotic membrane was washed free from blood clots and mucus but not preserved and not sterilized. The amnion was kept refrigerated at 4 °C before being subjected to SEM test.

Gamma irradiation

All preserved human amniotic membranes were sent to the MINTec-Sinagama, Malaysian Nuclear Agency (Model JS 8900) for sterilisation by gamma rays emitted from cobalt-60 radioactive source with the dose rate of 23.04 Gy/min. The samples were irradiated at 15, 25, and 35 kGy. Dosimetry was performed with ceric/cerous sulphate solution and measured using potentiometric method of analysis. The amnions

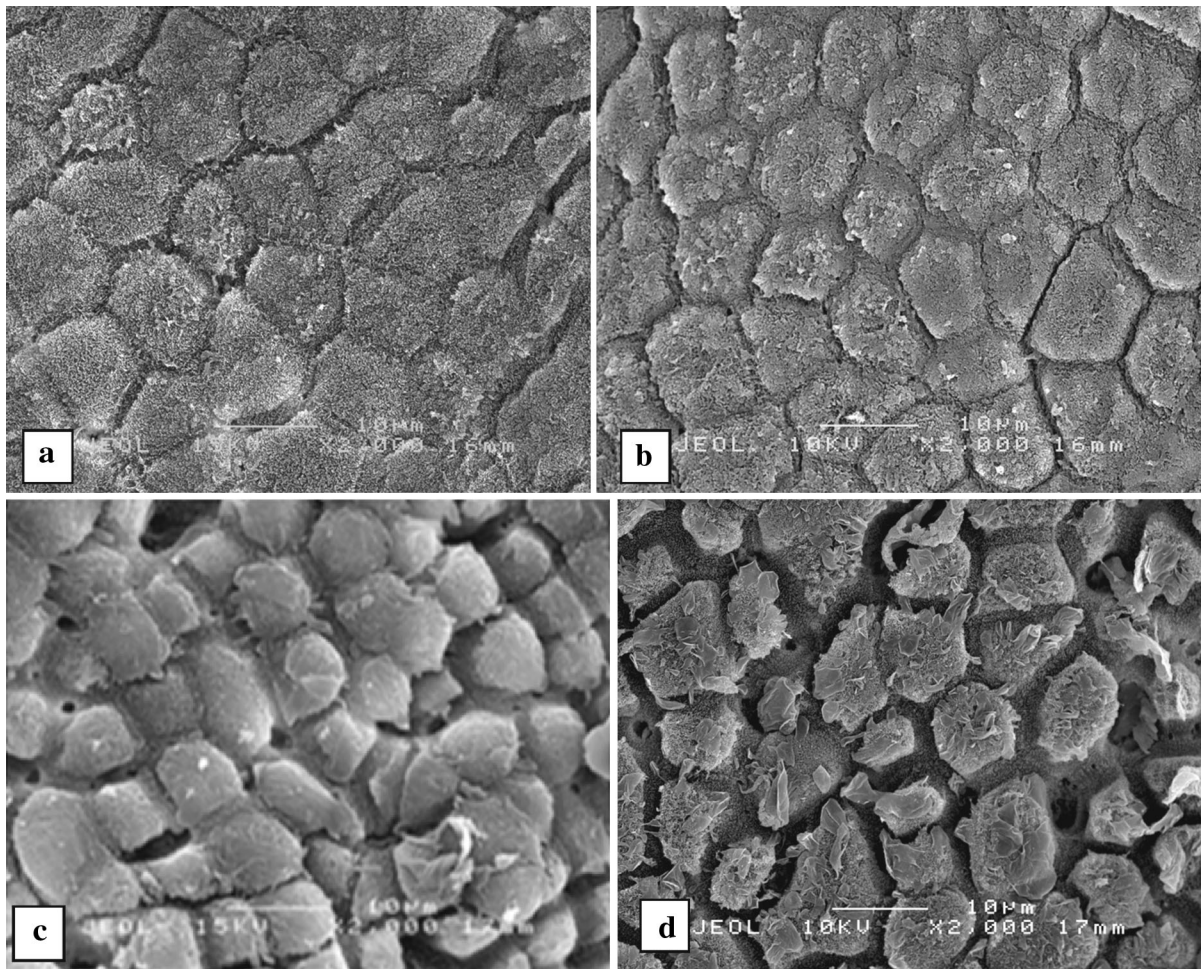


Fig. 4 Surface morphology of glycerol preserved and gamma irradiated amnion at magnification $\times 2,000$. **a** Non-irradiated, **b** 15 kGy, **c** 25 kGy, **d** 35 kGy

of the control group for each preservation method were not sterilized.

Scanning electron microscopy (SEM)

SEM has been used to image the surface morphology of the amniotic membranes. In this study, the changes in surface morphology of human amniotic membranes at different doses of gamma irradiation were assessed using high vacuum scanning electron microscopy (HVSEM) (Model JEOL JSM 6400).

The processed amniotic membranes were aseptically cut into 1 cm \times 1 cm samples as required by the SEM test procedure. Each sample was fixed in 2 % glutaraldehyde for 12 h at 4 °C in separate vials. After

fixation, the samples were washed thoroughly with 0.1 M sodium cacodylate buffer for three changes of 10 min each. For post fixation process, the samples were post fix in 1 % osmium tetroxide for 1 h at 4 °C to prevent post mortem degradation and washed again with the buffer. Following this, the samples were dehydrated by immersing in a series of alcohol concentrations (up to 100 %).

The dehydration was continued by washing the samples with 100 % acetone. The samples were then transferred into specimen baskets containing 100 % acetone and were placed in critical point dryer of Leica EM CPD 030 for 1.5 h for dehydration and to avoid gross specimen deformation due to temperature and pressure of carbon dioxide prior to examination in the

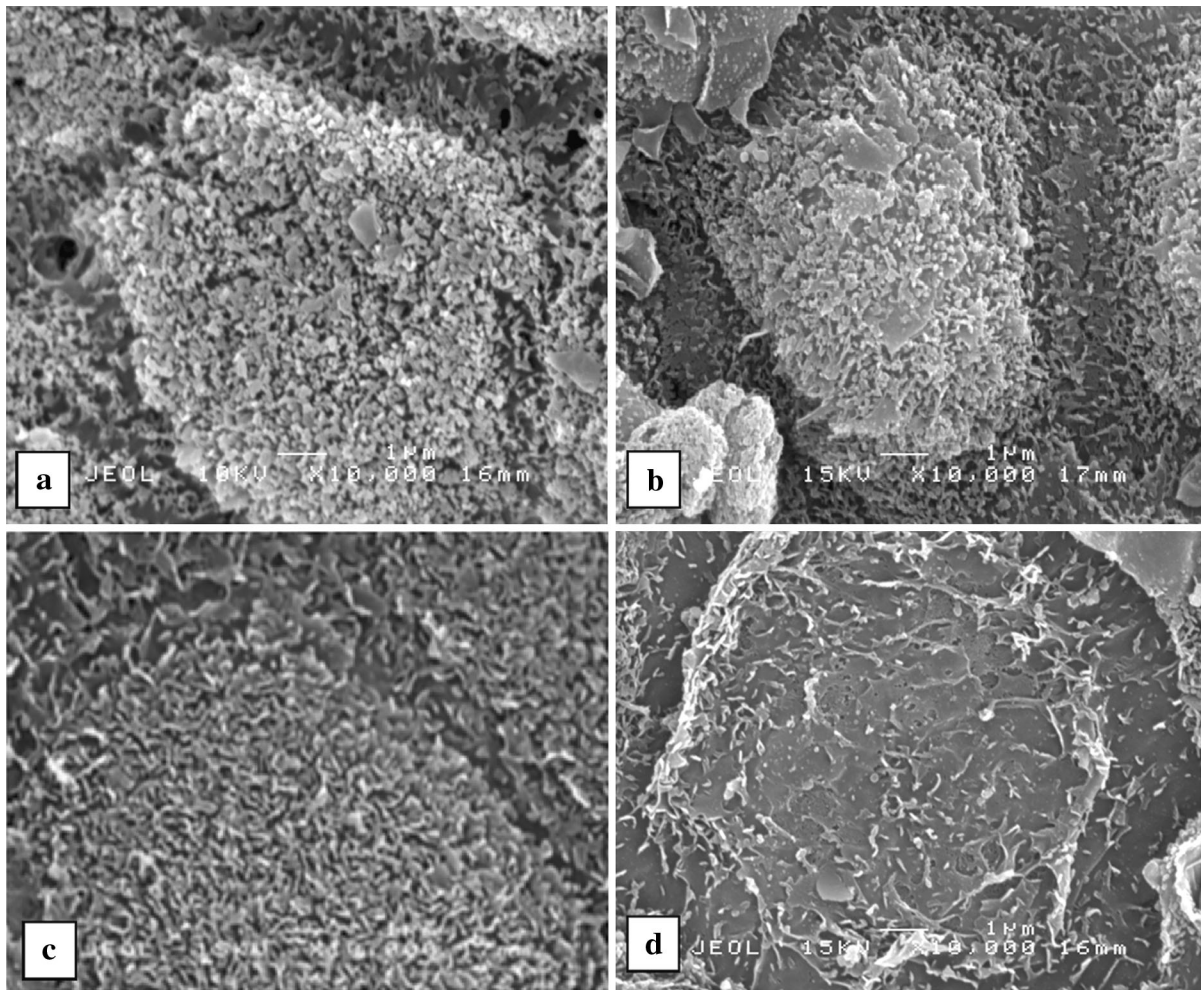


Fig. 5 Surface morphology of glycerol preserved and gamma irradiated amnion at magnification $\times 10,000$. **a** Non-irradiated, **b** 15 kGy, **c** 25 kGy, **d** 35 kGy

SEM. The samples were mounted onto aluminium studs with double-sided cellophane tape, and the studs were sputter coated by SCD 005 with 60 % gold and 40 % palladium under vacuum. The preserved irradiated human amniotic membrane was viewed under HVSEM. The morphological structures of the control and the gamma irradiated samples were then compared.

Results

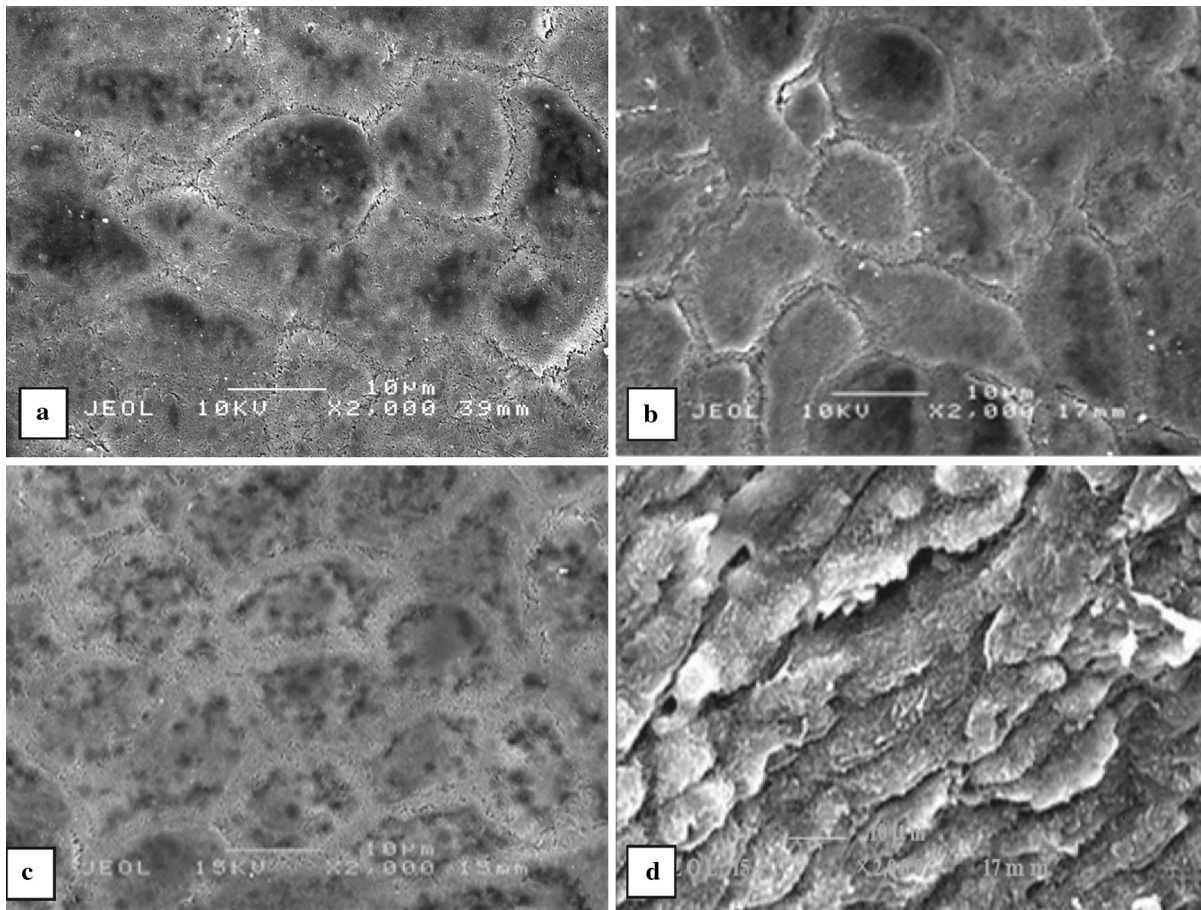
Effect of processing and preservation methods

The SEM examinations revealed some changes in epithelial surface of the cell morphology of air dried

and glycerol preserved compared to the fresh non preserved amniotic membranes. Comparatively, the cell morphology of air dried was more affected than glycerol preserved amnion.

Initially the morphological structure of all the amnions regardless of preservation methods was not distinctively clear at low magnification ($250\times$) as presented in Fig. 1a–c. At higher magnifications (more than $1,000\times$) the intercellular channels and the cell surface covered with microvilli were clearly observed especially in the fresh amniotic membrane of the control group (Fig. 2a).

The fresh membrane was composed of polygonal cells forming mosaic pattern and homogenous. At $3,000\times$ magnification, the SEM showed the cell



Figs. 6 Surface morphology of air-dried and gamma irradiated amnion at magnification $\times 2,000$. **a** Non-irradiated, **b** 15 kGy, **c** 25 kGy, **d** 35 kGy

surfaces were filled with a large number of microvilli (Fig. 2a). Looking closely at higher magnification at $10,000\times$, the microvilli obviously covered the entire surface of the cells (Fig. 2b). Moreover, the microvilli extended to the lateral border of the cells up to intercellular channels and neighbouring cells causing the cytoplasmic strands less clear.

As for the processed amnions, the cell structure was more preserved when stored in glycerol (Fig. 3a), than air dried preservation (Fig. 3b). Interestingly, the cells of the glycerol preserved amnion seemed to be homogenous, tended to round up and were beautifully arranged.

Effect of gamma irradiation on preserved amnion

Cell structure for the glycerol preserved amnion at $2,000\times$ magnification seemed not affected by

radiation doses (Fig. 4a–d), probably due to glycerol known as a radioprotectant agent. However, the cells started to have gap between intercellular channels at 25 kGy (Fig. 4c) and appeared more obvious at 35 kGy (Fig. 4d). The microvilli were fairly uniform in almost all of the cells with and without gamma irradiation. At higher magnification ($10,000\times$), the SEM revealed that the cell surface of the glycerol preserved amnion was rough and the microvilli were fairly flattened at 35 kGy (Fig. 5b). However, despite of these variations in the surface morphology, the gross appearance of the glycerol preserved amnions irradiated at 15 and 25 kGy (Fig. 5c, d) were still similar to those non-irradiated (Fig. 5a).

The cell morphology of air dried amnion seemed to be as a flat sheet of polygonal cells and the cytoplasmic strands were flattened and condensed before and after irradiation at all doses (Fig. 6a–d). In addition, at higher

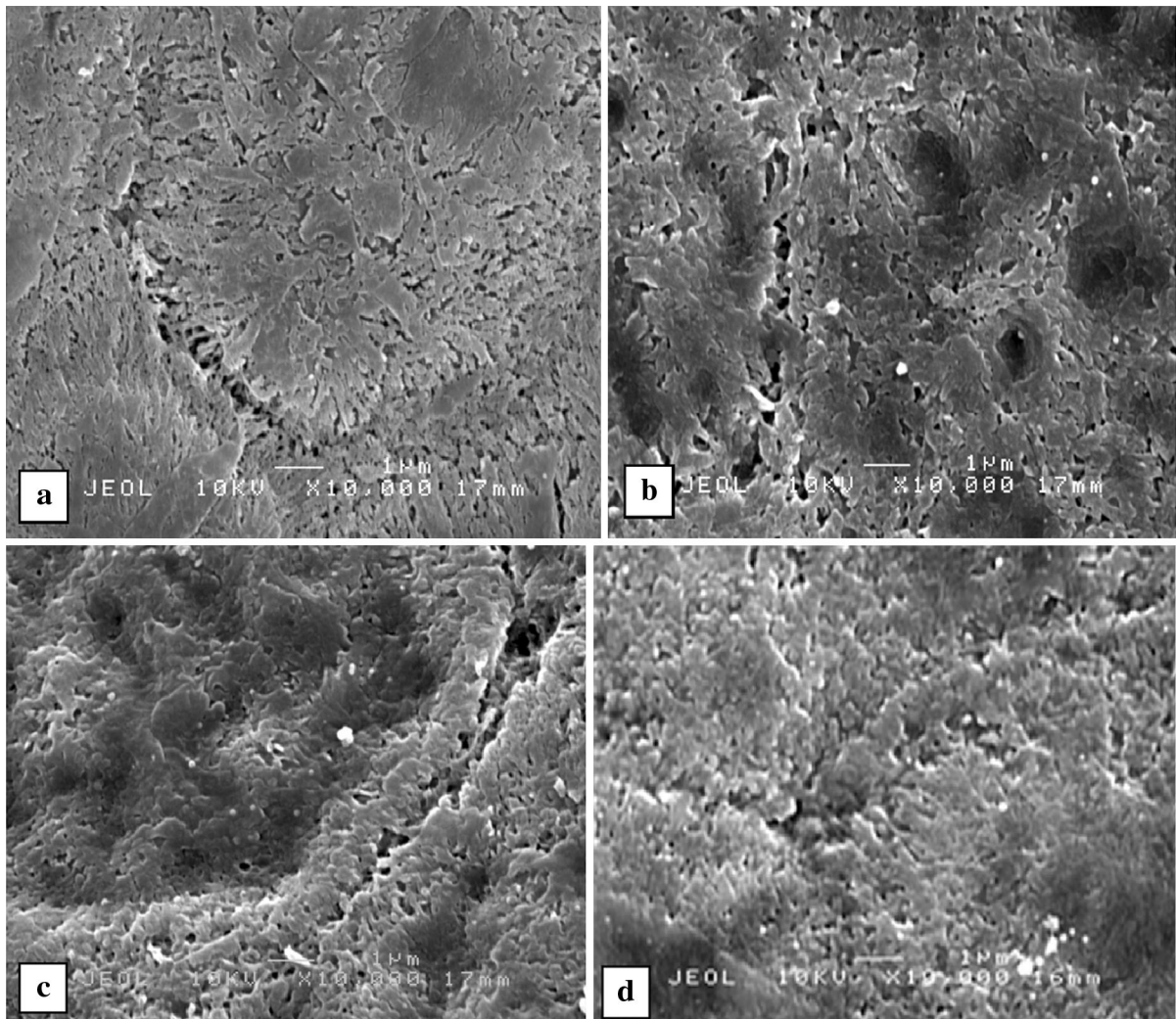


Fig. 7 Surface morphology of air dried and gamma irradiated amnion at magnification $\times 10,000$. **a** Non-irradiated, **b** 15 kGy, **c** 25 kGy, **d** 35 kGy

magnification ($10,000\times$), the microvilli and cytoplasmic strands on most of the cells were thinner causing the cells closely arranged together as no intercellular channels were seen in non-irradiated and irradiated at 15 and 25 kGy (Fig. 7a–c). These changes were clearly observed in air dried membranes after gamma irradiated at 35 kGy where cells seemed to shrink as illustrated in Fig. 7d. The changes in the morphological structure of the cells of air dried amnion under the SEM appeared to be due to the condensation of cytoplasmic strands. When looking closely in each individual cell, cells irradiated at 25 kGy appeared different even though the shape was intact and still similar to non-irradiated.

Discussion

Tissue preservation and sterilization are essential steps in producing amnion grafts that are free from bacterial and fungal contamination and also safe for clinical use. Previous studies on human amnion membranes irradiated with 25 kGy sparked an interest when gamma irradiation caused changes in biophysical properties of the epithelium of amnion (Rejzek et al. 2001). Recently, attention has been drawn to detrimental effects of gamma irradiation on physical properties of soft tissues (Nguyen et al. 2007b).

In the present study, we managed to observe clearly under the HVSEM changes in cell morphological

surfaces of the human amniotic membrane after undergone preservation treatment and gamma irradiation doses ranging from 15–35 kGy. There were minimal changes where the gap between intercellular channels started to appear at 25 kGy, the dose which is generally used as terminal sterilization for tissue grafts.

As described earlier by Hoeynck et al. (2008), glycerol serves as the basic mainstay for tissue preservation as compared to air drying method. Our study is in agreeable with the findings whereby our results showed that the cell structure of the glycerol preserved samples were almost similar to the non-irradiated amnion, only started to show structural change with bigger intercellular gap at 35 kGy while cell morphology of air dried amnion already changed at 25 kGy. Glycerol as a radioprotectant can remove water as a target molecule for indirect effects of ionizing radiation thus limiting the formation of free radicals. As a result, the secondary effects of irradiation which result in inducing tissue damages cannot take place (Rooney et al. 2008). Ravishanker et al. (2003) reported that the effectiveness of the glycerol preserved amnion was comparable to the fresh amniotic membrane with regard to adherence, pain relief, structural maintenance and decreasing bacterial counts in the wound healing. Perhaps with this reason, the ophthalmologists prefer to use glycerol preserved amnion in their work.

The non-irradiated and irradiated glycerol preserved amnions showed folding of cells at epithelial surfaces than those in the air dried. This appearance was illustrated by Pollard et al. (1976) as plump microvilli. This may well be due to diffusion of hydrophilic glycerol and water into the cells of the human amniotic membrane. Thus, the diffusion caused the swelling of the cells (Hoeynck et al. 2008) and this could prevent dehydration of the wound. Our findings that gamma irradiation at 15 and 25 kGy did not evoke undesirable changes in the morphological structure of the glycerol preserved amnion support the previous report by Rooney et al. (2008) that there was no change in the histological properties at 25 kGy.

Upon dehydration or withdrawal of liquid during drying, the air dried amnion was noticeably flattened as compared to the fresh and glycerol preserved, suggesting that the dehydration caused damage to the structure of human amniotic membrane. In addition, the intercellular channels and cytoplasmic strands of the air dried were not visible after the cells collapsed during drying and the individual cells were further damaged when irradiated at 25 kGy. Gamma irradiation at 35 kGy led

to a marked change in the cell shape and the intercellular channels of the amnion cells, fortunately the influence of radiation at 35 kGy were less pronounced in the glycerol preserved. Even though changes in the microscopic structure of the air dried amnion were observed earlier at 25 kGy, the gross appearance of the membrane was not affected. In fact the functional physical properties of air dried amnion were not affected even up to 50 kGy as reported by Singh et al. (2007).

Conclusion

The influence of the tissue preservation methods on the cell morphology of human amniotic membranes were well illustrated under the HVSEM. Air drying was found to cause condensation of microvilli and intercellular channels. The results suggested that glycerol is the best method to preserve the membrane structure as the surface morphology remained closely similar to the fresh amniotic membrane. The glycerol preservation method is recommended for those clinical applications where we need to maintain the surface structure of the human amniotic membrane. However the air drying preservation technique is still acceptable for amnions as a wound dressing because the processing cost is cheaper than the glycerol preservation and easy to store. Radiation sterilization seemed to cause no gross damage to the preserved amnions, only resulted in rough surface with bigger gap in the glycerol preserved amnion at 35 kGy and minimal change in cell appearance in the air dried amnion at 25 kGy. Radiation at 15 kGy did not affect the surface morphology in air the dried and the glycerol preserved amnions. Therefore, radiation doses lower than 25 kGy are recommended to sterilize preserved amnions.

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