CHANGES IN BIOPHYSICAL PROPERTIES OF HUMAN AMNIOTIC MEMBRANES AFTER DIFFERENT PRESERVATION METHODS AND RADIATION STERILIZATION

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ABSTRACT

Preservation techniques can affect biophysical properties of human amniotic membranes. A study was conducted to determine influences of gamma radiation (25 kGy) on biophysical properties and morphological structure of processed amnions following two preservation techniques. Glycerol preservation reduced oxygen transmission rate (OTR) to 4.28 cc/m²/day significantly lower (P<0.05) than the air dried amnion (15.55 cc/m²/day). Radiation reduced slightly the OTR of the air dried (12.17 cc/m²/day) but increased the OTR of the glycerol preserved (6.06 cc/m²/day). However water vapour transmission rate (WVTR) of the glycerol preserved (0.0222 g/cm²/h) was significantly higher (P<0.05) than the WVTR of the air dried (0.0159 g/cm²/h). The WVTR did not change after irradiation except in the air dried at 24 hours. Under the scanning electron microscope (SEM), the well morphological structured cells in the glycerol preserved amnion allowed water vapour transmission through the cells however small intercellular gaps limited the oxygen transmission. On the contrary, the air drying caused condensation of microvilli and cytoplasmic strands; the flat cells reduced water vapour transmission while the large gaps allowed high oxygen transmission. Radiation at 25 kGy did not cause any undesirable changes in the biophysical properties and the gross appearance of the preserved amnions. Clinicians can choose the preserved and radiation sterilised amnions ranging from the thin air dried (10 μm) to the thick glycerol preserved (50 μm) amnions to suit their clinical applications.

1.0 Introduction

Biological dressings including human amniotic membrane have been used to cover wounds or skin defects and enhance healing by providing a structural format to the underlying tissue. Human amniotic membrane was first used as wound dressing in 1910 by JS Davis [1]. The amniotic membrane obtained from human placenta possesses most of the characteristics of an ideal skin substitute even after processed into amnion grafts.

Amnion is capable to maintain a physiologically moist microenvironment that promotes healing [1, 2]. Amnion has strong structure but yet elastic and soft, making it the most currently accepted tissue replacement to reconstruct human tissue [3]. In addition, amnion does not cause immunological reactions and it is very light since the material is very thin. Amnion permits early mobilization of patients, and patients' bodies can accept amnion quite well. It also has the ability to conform well to body contour [4].

Important physical properties of amnion to be eligible for clinical use are impermeability to microbes but permeable to
amnions were stored at room temperature. The amniotic membrane. (2008) presented data on microbiologically preserved amnions. It may not always be possible to get fresh amnions when needed. Hence processed amnions can be obtained through a variety of preserving preparations i.e. air drying, freeze drying/lyophilization, freezing and glycerol preservation [7, 8].

The use of non sterilized amniotic tissue is said to increase the risk of fungal or viral disease transmission from donors. Amnion must be sterilized after being processed for a purpose of sterility during long term storage. One of the most available methods of sterilization for amnion is gamma radiation, which is said to be the most reliable and effective sterilization technique for tissue allograft. It has been used by many tissue banks to sterilize tissues. Gamma radiation does not affect the clinical function of the amniotic membrane [9]. Dose of 25 kGy is the most common dose for sterilizing medical products [10].

Rooney et al., (2008) presented data on microbiologically failed cryopreserved skin that could be rescued by sterilizing it with 25 kGy of gamma radiation, without causing any damage to its histology, biological and physical properties. These products are useful to surgeons when no viable cryopreserved skin is available or when the surgeons do not require viable cells [11]. Earlier Singh et al., (2003) concluded that exposure of air dried amniotic membrane to 25 kGy gamma rays under different environmental conditions caused no change in the amnion properties.

As reported by Versen-Hoeynck et al., (2008), there are different morphologic characteristics when comparing fresh fixed, fresh frozen, preserved frozen and freeze-dried amnions. Both, the fresh frozen and the freeze-dried amnion were 20μm in thickness, whereas the fresh fixed was 65μm, and the preserved frozen was 463μm. Based on these observations, it was recommended the use of the thicker preserved frozen amnion for ocular surface reconstruction and pterygium surgery.

The project was aimed to identify changes in biophysical properties and morphological structure of processed amnions after subjected to two preservation techniques namely air drying and glycerol preservation; and whether radiation has further influence when the amnions were sterilized.

2.0 Materials and Methods

2.1 Procurement of human amniotic membrane

Detailed medical records and behavioural history of each potential donor were screened before signed consent was obtained. 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, no multiple sexual partner, no medical records of communicable diseases, never been undergone chemotherapy treatment and not on prolonged steroid treatment. The amniotic membrane was separated from chorion during procurement. The membrane was further separated from the adjacent components and blood residues were removed before placed in triple layer polyethylene (PE) plastic pack and aseptically handled all the time.

2.2 Preservation of human amniotic membrane

2.2.1 Fresh amnion

In the control group, the fresh amnion was washed free of blood clots and mucus using sterile distilled water. Each amnion was then cut aseptically into 15cm × 15cm, 8cm × 8cm and 3cm × 3cm as required in the oxygen transmission rate (OTR), water vapour transmission rate (WVTR) and scanning electron microscope (SEM) tests respectively. The amnions were kept refrigerated at 4°C before being subjected to the tests.

2.2.2 Air dried preserved amnion

For air drying preservation, each amnion was stretched on a sterile plastic sheet and then left for drying in biological safety cabinet for 16-24 hours to dry. The dried membrane was then cut aseptically into 15cm × 15cm, 8cm × 8cm and 3cm × 3cm as required by the OTR, WVTR and SEM tests respectively. Air dried amnion were sterilized at 25 kGy by gamma radiation emitted from cobalt-60 radioactive source at Malaysian Nuclear Agency, Bangi (Model JS 8900, dose rate of 23.04Gy/min). Samples were stored at room temperature before being subjected to OTR, WVTR and SEM tests.

2.2.3 Glycerol preserved amnion

Samples for glycerol preservation were first cut aseptically into 15cm × 15cm, 8cm × 8cm and 3cm × 3cm as required by the OTR, WVTR and SEM respectively. Each cleaned amnion was submerged in a series of glycerol concentrations starting with 40% followed by 60 - 80% for dehydration process. Samples were immersed in glycerol overnight for each of the concentrations. Finally, the membrane was then
placed in a polypropylene (PP) plastic bottle containing 80% glycerol for preservation. The samples were gamma irradiated at 25 kGy. The preserved amnions were stored at room temperature and selected randomly before being subjected to the tests.

2.3 Preparation of amnions for biophysical properties experiment

2.3.1 Oxygen transmission rate (OTR)

The experiment was carried out by using Oxygen Permeation Analyzer (Model: 8501). The oxygen transmission rate (OTR) analysis was performed according to ASTM D 3985-95: Standard Test Method for oxygen gas transmission rate through plastic film and sheet using a coulometric sensor. The 15 cm x 15 cm amnion sample was mounted as a sealed semi-barrier between two chambers at ambient atmospheric pressure. The test was completed when equilibrium (or) steady state was achieved, when sensors detect a constant amount of oxygen in the nitrogen carrier stream (cc/m²/day).

2.3.2 Water vapour transmission rate (WVTR)

The WVTR determination was performed according to ASTM E96-00: Water Vapour Transmission Property for thin film, with modifications according to Yusof and Hilmy (2007). Briefly, the WVTR measurement involved weighing a crucible containing calcium chloride (CaCl₂) which was tightly covered with the tested amnion sample. The crucibles were weighed by using analytical balance at two time intervals i.e. 2 and 24 hours of exposure. The crucible weight gain was due to the water absorption by CaCl₂. The percentage of WVTR (g/cm²/h) of each sample was calculated by the increasing weight of CaCl₂ as follows:

\[
\%\text{WVTR} = \left(\frac{D_w}{W_c \times A \times H}\right) \times 100
\]

Where:
- \(D_w\) = The increase of weight CaCl₂
- \(W_c\) = The initial weight of CaCl₂
- \(A\) = The area of amnion covering the crucible
- \(H\) = The exposure time (hour)

2.4 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to examine the surface morphology of the amnion. In this study, changes in surface structure of air dried and glycerol preserved amnions after gamma irradiation at 25 kGy were evaluated using high vacuum scanning electron microscopy (HVSEM) Model: JEOL JSM 6400.

2.5 Thickness of human amniotic membrane

The thickness of the human amniotic membrane was determined according to DIN 53370 using a digimatic caliper with precision 0.01 mm measuring at three different positions of the amniotic membrane surface.

2.6 Statistical analysis

These data were statistically analysed by the Predictive Analytics Software (PASW) version 18. The data from the OTR experiment was evaluated by Independent t-test and one-way analysis of variance (ANOVA) as the data was normally distributed. Mann Whitney U-test and Kruskal-Wallis were used to analyze the WVTR of amnion at 24 hours as the distribution of the data was skewed. The cut-off point was set at P value of less than 0.05 (p<0.05).

3.0 Results

3.1 Oxygen transmission rate (OTR) evaluation

Table 1 shows the mean values of the OTR and standard deviation (SD) for all types of preservation, before and after sterilization. The mean value of the OTR for the glycerol preserved (4.28 cc/m²/day) was significantly lower (p<0.05) than the fresh amnions (16.67 cc/m²/day) and the air-dried (15.55 cc/m²/day) indicating that the glycerol preservation method markedly reduced the transmission of oxygen. After irradiation at 25 kGy, the OTR of the air dried amnions significantly reduced (p<0.05) to 12.17 cc/m²/day, but the OTR of the glycerol preserved amnions significantly increased (p<0.05) to 6.06 cc/m²/day.

<table>
<thead>
<tr>
<th>Type of preservation</th>
<th>Radiation dose (kGy)</th>
<th>n</th>
<th>Mean ± SD* (cc/m²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>0</td>
<td>22</td>
<td>16.67 ± 2.61*</td>
</tr>
<tr>
<td>Air dried</td>
<td>0</td>
<td>22</td>
<td>15.55 ± 2.92*</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>22</td>
<td>12.17 ± 2.97*</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0</td>
<td>22</td>
<td>4.28 ± 0.73*</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>22</td>
<td>6.06 ± 1.49*</td>
</tr>
</tbody>
</table>

* Means with different superscripts were significantly different at p<0.05 due to the preservation methods and/or radiation
3.2 Water vapour transmission rate (WVTR) evaluation

Table 2 shows the median and standard deviation (SD) for human amniotic membranes after different types of preservation methods before and after sterilization. In general, the WVTR median values increased with exposure time. At 2 hours the fresh amnion had significantly the lowest median of WVTR (0.0095 g/cm²/h) as compared to the air dried (0.0159 g/cm²/h) and glycerol preserved (0.0222 g/cm²/h). The WVTR median values of the fresh amnion (0.0168 g/cm²/h) at 24 hours exposure were also significantly lower than the air dried (0.0252 g/cm²/h) and the glycerol preserved (0.0296 g/cm²/h) amnions.

Radiation seemed not to have any effect on the median values of WVTR at 2 hours for both the air dried and the glycerol preserved amnion. However, at 24 hours radiation significantly reduced the value of WVTR for the air dried (0.0078 g/cm²/h) but not for the glycerol preserved amnions.

Table 2: Summary of descriptive statistic on WVTR median values for fresh, air dried and glycerol preserved amnion before and after sterilization

<table>
<thead>
<tr>
<th>Type of preservation</th>
<th>Radiation dose (kGy)</th>
<th>n</th>
<th>Median ± SD furry</th>
<th>Median ± SD furry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Median ± SD furry</td>
<td>Median ± SD furry</td>
</tr>
<tr>
<td>Fresh</td>
<td>0</td>
<td>2</td>
<td>0.0095 ± 0.0050a</td>
<td>0.0168 ± 0.0151d</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>0.0050b</td>
<td>0.0118</td>
</tr>
<tr>
<td>Air dried</td>
<td>0</td>
<td>2</td>
<td>0.0159 ± 0.0034b</td>
<td>0.0252 ± 0.0258e</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2</td>
<td>0.0150 ± 0.0024b</td>
<td>0.0078 ± 0.0037f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.0024b</td>
<td>0.0037f</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0</td>
<td>2</td>
<td>0.0222 ± 0.0072c</td>
<td>0.0296 ± 0.0283f</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2</td>
<td>0.0195 ± 0.0618c</td>
<td>0.0290 ± 0.0263f</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>0.0072c</td>
<td>0.0283f</td>
</tr>
</tbody>
</table>

*Means with different superscripts in the same column were significantly different at p<0.05 due to the preservation methods and radiation.

3.3 Surface structure evaluation

The morphological structure of all the amnion samples regardless of preservation methods was not distinctively clear at low magnification (x250) as has been discussed previously by Suzina et al., (2014). At higher magnification of x2000, the intercellular channels and the cell surface covered with microvilli were clearly observed especially in the fresh amnion (Figure 1).

However in the air dried amnion, the microvilli and cytoplasmic strands on most of the cells were thinner, the cells were flattening with large gaps. After radiation the cells closely arranged together and intercellular channels were not clear (Figure 2). The changes in the morphological structure of the air dried cells under the HVSEM appeared to be due to the condensation of microvilli and cytoplasmic strands when compared to the fresh amnion. When looking closely in each individual cell, the irradiated cells appeared slightly different however the shape was intact and almost similar to non-irradiated.
The cell structure appeared more preserved when stored in glycerol (Figure 3), as compared to air dried preservation (Figure 2). Cell structure for the glycerol preserved amnion did not appear to be affected by radiation. Interestingly, the cells of glycerol preserved seemed to be homogenous, exhibited a rounded of vascular shape and were neatly arranged. The microvilli were fairly uniform and relatively plump in almost all of the cells with and without gamma irradiation.

![Cells of glycerol preserved human amniotic membrane were homogenous, rounded of vascular shape and microvilli were relatively plump in a) non irradiated b) irradiated samples.](image)

### 3.4 Thickness of human amniotic membrane

Table 3 shows a significant variation in the thickness before and after sterilization for air dried and glycerol preserved when compared to the fresh amnion (40 μm). There were significant differences (p<0.05) in the thickness of the processed amnions before and after sterilization. Air dried amnions were found to be the thinnest (10 μm) and not affected by radiation while the glycerol preserved amnions were the thickest (70 μm) but reduced after radiation (50 μm).

<table>
<thead>
<tr>
<th>Type of preservation</th>
<th>Radiation dose (kGy)</th>
<th>n</th>
<th>Median ± SD* (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>0</td>
<td>20</td>
<td>40 ± 10.98</td>
</tr>
<tr>
<td>Air dried</td>
<td>0</td>
<td>20</td>
<td>10 ± 5.94</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>20</td>
<td>10 ± 3.08</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0</td>
<td>20</td>
<td>70 ± 17.90</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>20</td>
<td>50 ± 17.19</td>
</tr>
</tbody>
</table>

Means with different superscripts were significantly different at p<0.05 due to the preservation methods and/or radiation

### 4.0 Discussion

Results revealed that the biophysical properties of the amnions were influenced by the preservation methods both air drying and glycerol preservation, and radiation sterilization. The study on morphological surface structure of amnion evidently showed that the surface cells also changed following preservation and sterilisation. However, the changes were not in line with the changing values of OTR and WVTR.

The air drying slightly lowered the OTR but not significantly different from the fresh. The surface structure of the air dried amnion seemed rough, drier and clear gap between cells. However, the glycerol preserved with less structural changes and almost similar to the fresh had significantly the lowest OTR (p<0.05). The SEM study suggested the flat cells with clear gaps due to the dryness of the air dried amnion allowed oxygen transmission higher than in the glycerol preserved.

Looking at the WVTR results, the glycerol preserved had the highest WVTR, followed by the air dried and then the fresh at both 2 and 24 hours exposure. The well preserved cells by glycerol allowed better transmission of water vapour through the cells, but less intercellular gaps led to less oxygen to pass through. The pathway of water vapour transmission and oxygen transmission through the amnions seemed to be different.

According to Versen-Hoeynck et al., (2008), glycerol preservation leads to an insertion of hydrophilic glycerol to replace intracellular water resulting in swelling of the cells. In contrast, air drying leads to withdrawal of water during the drying process making the cell structure flatten and thinner.
through which less water vapour could transmit. As the intercellular gaps were not so affected by drying, the transmission of oxygen was comparable to fresh.

Looking at the thickness, Versen-Hoeynck et al., (2004) observed significant variation in the tissue’s thickness after different preservation procedures. Air and freeze-dried amnions were found to be the thinnest tissues varying from 20 to 30μm, while the thickest amnions preserved in glycerol varied from 45 to 50μm. Agreeable with this finding, the thickness of the fresh amnion (40 μm) reduced to 10 μm after air drying however glycerol increased the thickness to 70 μm. Radiation did not affect the thickness of the air dried but in glycerol preserved, radiation reduced the thickness to 50 μm.

Based on the observations under the SEM, the well preserved cell structure of the glycerol preserved amnion had less pores and narrow gap between the cells hence smaller pore size for oxygen to transmit. This might explain the low OTR as observed in the glycerol preserved. The cells rounded up and the microvilli were relatively plump which is in agreeable with the findings by Maral et al., (1999) and Rejzek et al., (2001). Glycerol dehydrates tissue by physically replacing most of the intracellular water but does not change the cells' ionic concentration. As a result, it is an efficient agent that preserves tissue by protecting cell integrity.

Rooney et al., (2008) reported that skin preserved in a high concentration of glycerol (85%) could be subjected to 25 kGy of gamma irradiation without adversely affecting its histological structure or biophysical properties. The structure of any particular collagen, whether in the fresh, air dried and glycerol preserved amnions would probably depend upon the internal adjustments of the cell structure and collagen during the processing of tissue including preservation and irradiation.

In clinical usage, both air dried and glycerol preserved amnions have comparable effectiveness as biological barrier in wound dressing. Bujang-Safawi et al., (2010) reported that fresh amnion has a short shelf life and not always be available when needed therefore preservation will enable amnions to be stored allowing continuous supply for clinical use.

Even though minor changes in the microscopic structure of the air dried amnion were observed after irradiated at 25 kGy, the gross appearance of the membrane was not affected. In this study, gamma irradiation at 25 kGy did not evoke undesirable changes in the morphological structure of the preserved human amniotic membrane and this finding supports the previous report by Rooney et al., (2008) that there was no change in the histological properties of amnion at 25 kGy.

5.0 Conclusion

Preservation techniques cause variation in biophysical properties (water transmission, oxygen transmission, thickness) of the processed human amniotic membranes. Preservation techniques also influence the cell morphological structure. The variations were less pronounced in the glycerol preserved as compared to the air dried amnions. Under the HVSEM, the surface morphological in glycerol preserved amnions showed minimal changes, while in the air dried condensation of microvilli and intercellular channels was observed. Gross appearance of both preserved amnions was not affected by 25 kGy. The present study suggested that glycerol treatment is the best method to preserve the surface structure as the morphology remained closely similar to the fresh amniotic membrane. Depending on the specific use of the human amniotic membrane, one may choose either thin (air dried) or thick (glycerol preserved) amnions. Thus, in relation to biophysical properties and morphological structures, the common sterilization dose of 25 kGy is acceptable to sterilize both the air dried and glycerol preserved amnions.

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