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Original Article

Effects of different processing methods of human amniotic membrane on the quality of extracted RNA

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Abstract The aim of this study was to determine the efficiency of different human amniotic membrane (HAM) processing methods on the concentration, purity and integrity of RNA. Two different techniques (Technique 1 and Technique 2) were employed for the processing of HAM, which differed in terms of washing solution, sample storage conditions and processing time. Based on preservation of HAM, three groups were formed under each technique. In Technique 1, the groups were fresh frozen 1 (F1), glycerol preserved (GP) and gamma irradiated glycerol preserved (IGP); where else in Technique 2, the groups were fresh frozen 2 (F2), 50% glycerol/Dulbecco's modified Eagle medium (DMEM) cryopreserved HAM diluted with phosphate buffered saline (GB) and 50% glycerol/DMEM cryopreserved HAM diluted with diethylprocarbonate water (GD). Total RNA was extracted from the samples and their concentration, purity and integrity were examined. The F2 sample of which there was no pre-washing step and involved direct sample storage at -80^{o}C, shorter processing time and chilled processing conditions had yielded better quality of RNA compared to the others.

Keywords: Human amniotic membrane, processing method, RNA purity, RNA integrity.

Introduction

Human amniotic membrane (HAM) is the innermost layer of the placenta that surrounds and protects the baby during pregnancy. It is a thin and elastic membrane composed of amniotic epithelial cell monolayer aligning on a basal membrane. HAM has been shown to be an effective surgical biomaterial and was used for more than 100 years (Riau *et al*., 2010) after its first use as a skin substitute by Davis (1910). The first attempt in ophthalmology transplantation was made by De Rötth (1940) and the usage of HAM in ophthalmic practice was propelled further by Kim and Tseng (1995). Kothary (1969) reported the use of amnion in a maxillofacial surgery for mouth floor reconstruction after a glossectomy. Lawson (1985) used it for maxillofacial defect reconstruction by using pure pectoralis muscle flap lines with fresh HAM. Successful applications of HAM in various clinical fields had been reported (Ravishanker *et al*., 2003; Dua *et al*., 2004; Kesting *et al*., 2012).

The advantage of HAM lies in its unlimited availability, the ease of procurement, low processing cost and its beneficial properties. Some of the beneficial properties of HAM include bacteriostatic, antiphlogistic, protease inhibiting, re-epithelializing, wound-protecting and scar formation-reducing properties (Kim *et al*., 2001; Dua *et al*., 2004). HAM also promotes epithelialization and inhibits fibrosis. It also has anti-inflammatory and antiangiogenic properties, antimicrobial and antiviral properties, has a high hydraulic conductivity (Hao *et al*., 2000; Ganatra *et al*., 2003; Dua *et al*., 2004; Gomes *et al*., 2005; Fernandes *et al*., 2005) and also shows low or no immunogenicity (Akle *et al*., 1981; Akle *et al*., 1985).

Various methods have been used worldwide to preserve HAM; lyophilization,

air-drying, glycerol-preservation and cryo-preservation. There have been many publications in the literature describing the effects of different agents or techniques in the preparation of HAM, which includes preservation and sterilization as well as the effects of the storage (Maral *et al*., 1999; Adds *et al*., 2001; Ravishanker *et al*., 2003; Riau *et al*., 2010). Each preservation technique has been reported to have different effects on the physical, biological properties of the amniotic membrane and cell viability (von Versen-Höynck *et al*., 2004). Isolation of intact RNA is essential for many techniques used in gene expression study. Degradation of RNA could occur because of many factors such as storage, preparation, processing and contamination during handling of sample.

One of the proposed mechanisms of successful action of HAM transplantation is the release of growth factors that facilitate re-epithelialization and reduction of scarring and inflammation. Studies on HAM have revealed the presence of various growth factors, such as epidermal growth factor, transforming growth factor α, keratinocyte growth factor and hepatocyte growth factor. Preserved HAM expresses mRNAs for a number of growth factors and contains several growth factor proteins that might benefit epithelialization after HAM transplantation (Koizumi *et al*., 2000). The different methods of HAM processing prior to storage could affect the growth factors present in the HAM, which could affect its aforementioned beneficial properties. Good quality RNA containing good quality growth factors will enable good gene expression, which will help in the process of re-epithelialization and reduction of scarring and inflammation. Thus, it is of importance to obtain good quality RNA with expression of growth factor proteins. Hence, the present study aimed to assess the different methods of processing on the quality of RNA obtained.

Materials and methods

Ethical approval

Ethical approval for carrying out this study was accorded by the Research Ethics Committee (Human), Universiti Sains Malaysia vide reference USMKK/PPP/ JEPeM [233.4.(1.11)] dated 5th January 2011.

Technique 1

Initial preparation of HAM

HAM was procured after obtaining informed consent from the donors who delivered in Hospital Universiti Sains Malaysia. The HAM was transferred immediately to Tissue Bank of Universiti Sains Malaysia and upon arrival; the membrane was washed 5 times in sterile distilled water. It was then stored at -20°C until further use. At the time of sample processing, the HAM was removed from -20°C and thawed at room temperature for one hour. Then it was washed with sterile distilled water to remove blood clots and mucus and cut into 5 cm x 5 cm pieces. The cut HAM pieces were placed in 0.05% v/v sodium hypochlorite solution for one hour to remove bacterial contaminants on the membrane after which they were washed 3 times for 15 minutes each with sterile distilled water.

Processing of HAM

The HAM was divided into 3 groups; the preparative procedures are detailed as below:

a. Fresh frozen HAM (F1)

After following the procedure as mentioned in initial preparation of HAM, the F1 was placed in RNA later solution. The F1 was then stored at -80°C until RNA extraction was carried out. This acted as the control group.

b. Glycerol preserved HAM (GP)

After following the procedure as mentioned in initial preparation of HAM, the HAM was soaked in serial concentrations of glycerol starting with 40% followed by 60%, 80% and 90% for the dehydration process to take place. For each concentration of glycerol used, the HAM was soaked overnight. The GP was then placed in a plastic bottle containing 95% glycerol and stored at room temperature until RNA extraction was carried out.

c. Gamma irradiated glycerol preserved HAM (IGP)

After following the procedure as mentioned in initial preparation of HAM, the HAM was

soaked in serial concentrations of glycerol starting with 40% followed by 60%, 80% and 90% for the dehydration process to take place. For each concentration of glycerol used, the HAM was soaked overnight. The glycerol preserved HAM was then placed in a plastic bottle containing 95% glycerol. The glycerol preserved HAM was sent to the Malaysian Nuclear Agency for gamma irradiation from cobalt-60 radioactive source at 25 kGy. The IGP was then stored at room temperature until RNA extraction was carried out.

Technique 2

Initial preparation of HAM

In this method, the HAM was stored at -20ºC immediately after collection of HAMs from the donors. At the time of sample preparation, the HAM was removed from -20°C and thawed at 4ºC in a refrigerator for an hour. Then, it was washed with phosphate buffer saline (PBS) to remove blood clots and mucus and cut into 5 cm x 5 cm pieces. However, instead of using 0.05% sodium hydrochloride solution as in Technique 1, the cut HAM was washed 3 times for 15 minutes each with chilled PBS.

Processing of HAM

The HAM was divided into 3 groups; of which the preparative procedures are detailed as below:

a. Fresh frozen HAM (F2)

After following the procedure as mentioned in initial preparation of HAM (Technique 2), the washed F2 samples were placed in a cryo vial containing RNA later solution and stored at -80°C until RNA extraction was done. This acted as the control group.

b. Fifty per cent glycerol/DMEM cryopreserved HAM diluted with PBS (GB)

After following the procedure as mentioned in initial preparation of HAM (Technique 2), the washed HAM was placed in a cryo vial containing 50% glycerol/DMEM solution and stored at -80°C until RNA extraction was performed. The 50% glycerol was prepared by diluting it with PBS.

c. Fifty per cent glycerol/DMEM cryopreserved HAM diluted with DEPC water (GD)

After following the procedure as mentioned in initial preparation of HAM (Technique 2), the washed HAM was placed in a cryogenic vial containing 50% glycerol/DMEM solution and stored at -80°C until RNA extraction was carried out. Here, the 50% glycerol was prepared by diluting it with DEPC water.

The differences between Technique 1 and Technique 2 are presented in Table 1.

Table 1 The differences between Technique 1 and Technique 2 employed in the processing of human amniotic membrane

Process	Technique 1	Technique 2
Pre-washed before storage	Yes	No
Washing solution	Sterile distilled water	PBS
Chemical treatment	Yes	No
Processing condition	Room temperature	10^{o}C
Storage condition	Room temperature	-80^{o}C
Processing time	~ 5 days	~ 2 hours

Total RNA extraction

Total RNA of HAMs prepared by Technique 1 and Technique 2 were extracted by a combination of TRIzol® reagent (Ambion, USA) and RNAqueous®-Micro kit (Ambion, USA). Initially, the HAM was lysed using TRIzol® reagent. TRIzol® reagent is a ready-to-use, monophasic solution of phenol and guanidine isothiocyanate suitable for isolating total RNA from cells and tissues. It is a strong chaotropic agent that disrupts cell membranes and rapidly inactivates ribonucleases. The lysate was then mixed with ethanol and applied to a RNA-binding glass fibre filter (RNAqueous®-Micro kit). Protein, DNA and other contaminants were removed in three rapid

washing steps and the bound RNA was then eluted in a concentrated form. The kit contains reagents for optional post-elution like DNase to remove trace amounts of genomic DNA that could interfere with Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assays. The DNase was removed after digestion using resin that removes DNase without heat inactivation, phenol extraction or alcohol precipitation.

Determination of RNA concentration and purity

The concentration and purity of the extracted RNA from the HAMs processed by Technique 1 and Technique 2 were quantified by measuring the absorbance at 260 nm and 280 nm in Nanodrop ND-1000 spectrophotometer (Thermo Fisher, USA). The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of purity of RNA with respect to contaminants such as protein that might also be absorbed in the UV spectrum.

Determination of RNA integrity

The integrity of total RNA purified from a combination of TRIzol® reagent (Ambion, USA) and RNAqueous®-Micro kit (Ambion, USA) was determined using 1% denaturing agarose gel electrophoresis. The RNA was pre-stained with SYBR Green dye and visualized under ultra violet light. Intact RNA will demonstrate sharp and clear 28S rRNA (4.5 kb) to 18S rRNA (1.9kb) bands. The apparent ratio of 28S rRNA to 18S rRNA band is approximately 2:1; which is a good indication that the RNA is completely intact, whereas partially degraded RNA will have a smeared appearance, lack sharp rRNA bands, or will not exhibit the 2:1 ratio of high quality RNA. Completely degraded RNA will have a very low molecular weight and hence appear as a faded smear on gel electrophoresis.

Results

Technique 1

The concentration and purity of extracted RNA based on different types of preparation as per Technique 1 is presented in Table 2. Based on Technique 1 (Table 2), it was observed that the F1 had good concentration of RNA (421.6 ng/µl) with high purity (1.94) compared to the GP (18.1 ng/µl and 1.45) and IGP (12.2 ng/µl and 0.60) HAMs. The RNA integrity was completely absent in GP and IGP compared to F1, which presented as a smear on gel electrophoresis (Figure 1).

Technique 2

The concentration and purity of RNA based on different types of preparation as per Technique 2 is presented in Table 2. Based on Technique 2, the F2 showed the highest concentration (594.9 ng/µl) and purity of RNA (1.97). The other two namely, GB and GD showed lesser concentration and purity of RNA (42.2 ng/µl and 1.87 for GB and 49.2 ng/µl and 1.90 for GD). In Technique 2 also, the RNA integrity was not good as it also represented as a smear on gel electrophoresis (Figure 1).

Table 2 Comparison of RNA concentration and purity obtained from human amniotic membranes based on Technique 1 and Technique 2

	Concentration (ng/µl)	**Purity (A_{260}/A_{280})**
Technique 1		
Fresh frozen HAM (F1)	421.6	1.94
Glycerol preserved HAM (GP)	18.1	1.45
Gamma irradiated glycerol preserved HAM (IGP)	12.2	0.60
Technique 2		
Fresh frozen HAM (F2)	594.9	1.97
50% glycerol/DMEM cryopreserved HAM diluted with PBS (GB)	42.2	1.87
50% glycerol/DMEM cryopreserved HAM diluted with DEPC water (GD)	49.2	1.90

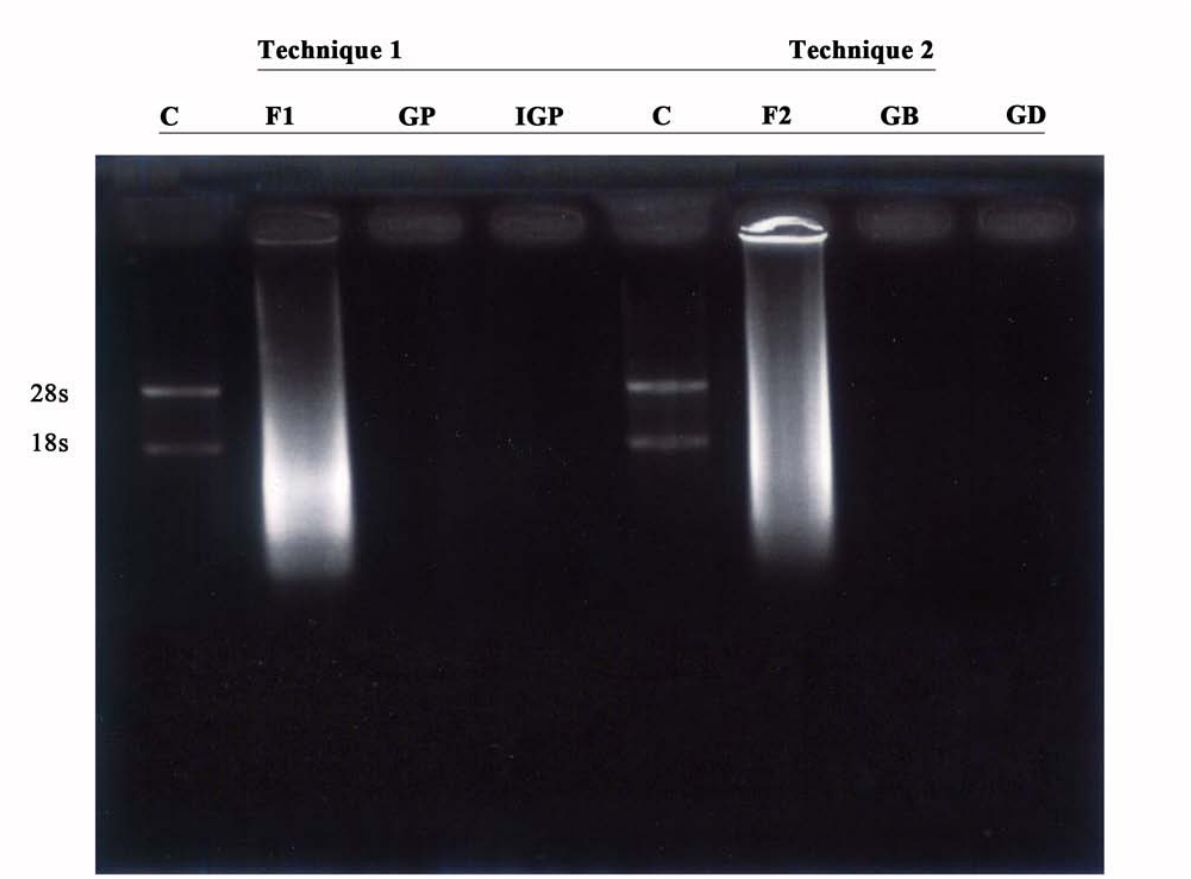

Fig. 1 Agarose gel electrophoresis of RNA obtained by different methods in the two techniques. Technique 1: F1 - Fresh frozen HAM, GP - Glycerol preserved HAM, IGP - Gamma irradiated glycerol preserved HAM. Technique 2: F2 - Fresh frozen HAM, GB - 50% glycerol/DMEM cryopreserved HAM diluted with PBS, GD - 50% glycerol/DMEM cryopreserved HAM diluted with DEPC water, C – Control RNA.

Discussion

Various methods have been used to preserve HAM; fresh storage, freezing, freeze drying and glycerol preserved. It has been reported that the cell viability in HAM decreases during storage with more pronounced affect when stored frozen (Hennerbichler *et al*., 2007). During some preservation processes in use (e.g. lyophilizing, air-drying, glycerol-preserving) viability of the epithelial cells in HAM is lost (Kim and Tseng, 1995; Tseng *et al*., 1997; Kruse *at al*., 2000; Singh *et al*., 2003; Nakamura *et al*., 2004). Some other conditions (tissue culturing, refrigerating or cryopreserving with DMSO) allow retention of cell viability in HAM of about 40-90% (Burgos and Faulk, 1981; Kruse *et al*., 2000; Rama *et al*., 2001). One of proposed mechanisms of action of HAM transplantation is the release of growth factors that facilitate corneal re-epithelialization and reduction of corneal scarring and inflammation (Solomon et al., 2001).

In the present study, based on Technique 1 (Table 2), it was observed that the fresh frozen HAM (F1) had good concentration of RNA with high purity, whereas, the glycerol preserved (GP) and gamma irradiated HAM (IGP) yielded very low concentration and purity of RNA, indicating that the latter two methods were unsuccessful. Furthermore, the RNA integrity was completely absent in glycerol preserved (GP) and gamma irradiated HAM (IGP) compared to fresh frozen (F1), which presented as a smear (Figure 1). The smear of the fresh frozen sample (F1) could be due to inappropriate sample preparation and storage condition. In Technique 1, the factors involved in the processing of HAM like pre-washing step before storage, the

use of washing solution (sterile distilled water), chemical treatment with sodium hypochlorite, processing condition and storage of HAM at room temperature and time duration of processing involving 5 days could be the reasons that can be attributed to the degradation of extracted RNA, which could have eventually resulted in RNA yield with poor concentration, integrity and purity.

Based on Technique 2 (Table 2), the fresh frozen HAM (F2) showed the highest concentration and purity of RNA. The other two methods namely, 50% glycerol/DMEM cryopreserved HAM diluted with PBS (GB) and 50% glycerol/DMEM cryopreserved HAM diluted with DEPC water (GD) showed lesser concentration and purity of RNA. In Technique 2 as well, the integrity of the extracted RNA was not good as seen by the gel electrophoresis. However, the concentration and purity of RNA was better compared to Technique 1. This may be due to the slight modifications employed in the Technique 2 which encompassed no pre-wash storage, no chemical treatment, processing of HAM under chilled conditions and shorter processing time involving only 2 hours compared to 5 days in Technique 1. Moreover, in this Technique 2, all the HAM samples were stored at -80°C. Also, the preservation of samples in DMEM with PBS/DEPC could have also attributed to the good concentration and purity of RNA.

DEPC is used to inactivate RNase enzymes in water and on laboratory utensils. It is used to reduce the risk of RNA being degraded by RNases. However DEPC is suspected to be a carcinogen and should be handled with great care (AppliChem, Germany). In contrast, PBS is a buffer solution commonly used in biological research. It is used to maintain a constant pH and osmotic balance as well as to provide cells with water and essential inorganic ions. The osmolarity and ion concentrations of the solution usually match those of the human body (isotonic) (CytoSprin, USA). Moreover, the concentration and purity of RNA obtained from GB and GD in Technique 2 showed no difference and therefore, HAMs preserved in DMEM diluted with PBS is suggested for clinical use and further study. PBS has also the advantage of being non-toxic to cells compared to DEPC, which makes it safe for handling.

From Table 2, it can be noted that F1, F2, GB and GD all yielded good concentration of RNA, which can be attributed to the cryopreservation of samples employed in these methods in contrast to the samples stored at room temperature which did not yield good quality RNA. Therefore, it can be concluded that storage of HAMs at room temperature may not deem fit for studies and clinical use which requires good quality RNA. The use of 50% glycerol/DMEM (GB) cryopreserved HAM under Technique 2 was better than HAM preserved in glycerol (GP) in terms of quality of RNA and proved that the former method was better for preservation. There was no remarkable difference between the concentrations and purity of RNA between the latter two methods. From this preliminary pilot study, we can conclude that shorter processing time, chilled processing conditions and 50% glycerol/DMEM cryopreserved HAM could attribute to a better quality of RNA. However, further study in larger samples is necessary to authenticate and throw more information on these findings.

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