THE EFFECT OF GAMMA IRRADIATION ON SELECTED GROWTH FACTORS AND RECEPTORS mRNA IN GLYCEROL CRYOPRESERVED HUMAN AMNIOTIC MEMBRANE

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by

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ACTB	actin, beta
AECs	amniotic epithelial cells
bFGF	basic fibroblast growth factor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
c-MET	proto-oncogenic c-mesenchymal-epithelial transition receptor
C_p	crossing point
C_T	threshold cycle
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphatase
E	PCR efficiency
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HAM	human amniotic membrane
HBV	hepatitis B virus
hCG	human chorionic gonadotropin

HCV	hepatitis C virus
HGF	hepatocyte growth factor
HGFR	hepatocyte growth factor receptor
HIV	human immunodeficiency virus
HLA	human leucocytes antigen
kDa	kilo Dalton
KGF	keratinocyte growth factor
KGFR	keratinocyte growth factor receptor
kGy	kilogrey
L	liter
LB	lithium boric acid buffer
MET	mesenchymal-epithelial transition proto-oncogene
MGB	minor groove binder
MIQE	minimum information for publication of quantitative real-time PCR experiments
ml	milliliter
mRNA	messenger ribonucleic acid
NFQ	non-fluorescent quencher
ng	nanogram
nm	nanometer
NTC	no template control
^{o}C	degree celsius
PBS	phosphate buffer saline
PCR	polymerase chain reaction
qPCR	quantitative PCR

R	relative expression ratio
REST-MCS	relative expression software tool-multiple condition solver
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RSD	radiation sterilization dose
RT-qPCR	quantitative reverse transcription PCR
SAL	sterility assurance level
snRNA	small nuclear RNA
TE	tissue engineering
TGF-α	transforming growth factor-α
TGF-β1	transforming growth factor-β1
TGF-β2	transforming growth factor-β2
TGF-β3	transforming growth factor-β3
tRNA	transfer RNA
V	voltage
VEGF	vascular endothelial growth factor
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar

KESAN IRADIASI GAMA KE ATAS mRNA FAKTOR PERTUMBUHAN DAN RESEPTOR TERPILIH DALAM MEMBRAN AMNION MANUSIA YANG DIKEKALKAN SECARA CRYO GLISEROL

ABSTRAK

Membran amnion manusia (HAM) telah digunakan dalam pelbagai aplikasi klinikal kerana ia mempunyai keserasian biologi yang tinggi, rendah keimunogen, anti-bakteria, anti-virus serta mengandungi faktor pertumbuhan. Faktor pertumbuhan merupakan faktor utama dalam mengawal pelbagai proses selular seperti pertumbuhan, proliferasi dan pembezaan sel. Kajian ini bertujuan untuk meneroka kesan pengekalan HAM secara cryo gliserol dan iradiasi gama ke atas mRNA faktor pertumbuhan dan reseptor terpilih yang terdapat dalam HAM. Lapan faktor pertumbuhan iaitu *EGF*, *HGF*, *KGF*, *TGF-α, TGF-β1, TGF-β2, TGF-β3* dan *bFGF* serta dua reseptor faktor pertumbuhan iaitu *HGFR* dan *KGFR* telah dinilai dalam kajian ini. Total RNA diekstrak dan ditukar kepada komplimentari DNA dengan menggunakan kit komersil. Seterusnya, ekspresi mRNA faktor pertumbuhan diukur menggunakan kuantitatif PCR dan keputusannya dianalisa secara statistik menggunakan perisian REST-MCS. Kajian ini menunjukkan kewujudan mRNA faktor pertumbuhan dan reseptor tersebut dalam HAM segar, HAM yang dikekal secara cryo gliserol dan HAM yang dikekal secara cryo gliserol serta diiradiasi. Dalam HAM yang dikekal secara cryo gliserol, keputusan menunjukkan peningkatan ekspresi mRNA *HGF* dan *bFGF* dan penurunan ekspresi mRNA 8 gen lain iaitu *EGF*, *HGFR*, *KGF*, *KGFR*, *TGF-α, TGF-β1, TGF-β2* dan *TGF-β3*. Menariknya, HAM yang dikekal secara cryo gliserol serta diiradiasi dengan 15 kGy menunjukkan

peningkatan ekspresi mRNA 7 gen iaitu *EGF, HGF, KGF, KGFR, TGF-β1, TGF-β*2 dan *TGF-β3* dan penurunan ekspresi mRNA *HGFR, TGF-α* dan *bFGF*. Walau bagaimanapun, perbezaan ekspresi mRNA berbanding kumpulan kawalan adalah tidak signifikan secara statistik. Oleh yang demikian, boleh disimpulkan bahawa pengekalan secara cryo gliserol tidak mempunyai kesan ke atas ekspresi mRNA faktor pertumbuhan dan reseptor tersebut yang terdapat dalam HAM. Iradiasi pada 15 kGy juga didapati tidak mempunyai kesan terhadap ekspresi mRNA faktor pertumbuhan dan reseptor tersebut yang terdapat dalam HAM yang dikekalkan secara cryo gliserol. Keputusan kajian ini memberi maklumat berguna kepada pengamal perubatan dan pakar bedah untuk memilih kaedah terbaik pengekalan HAM yang boleh memberi manfaat dalam rawatan pesakit.

THE EFFECT OF GAMMA IRRADIATION ON SELECTED GROWTH FACTORS AND RECEPTORS mRNA IN GLYCEROL CRYOPRESERVED HUMAN AMNIOTIC MEMBRANE

ABSTRACT

Human amniotic membrane (HAM), due to its high biocompatibility, low immunogenicity, anti-microbial, anti-viral properties as well as the presence of its growth factors, has been used in various clinical applications. These growth factors are key factors in regulating many cellular processes such as cellular growth, proliferation and cellular differentiation. The current study aimed to explore the effect of glycerol cryopreservation and gamma irradiation on the selected growth factors and receptors mRNA present in HAM. Eight growth factors, namely, *EGF, HGF, KGF, TGF-α, TGF-β1, TGF-β2, TGF-β3* and *bFGF* and two growth factor receptors, *HGFR* and *KGFR* were evaluated in this study. The total RNA was extracted and converted to complimentary DNA using commercial kits. Subsequently, the mRNA expressions of these growth factors were evaluated using quantitative PCR and the results were statistically analyzed using REST-MCS software. This study indicated the presence of these growth factors and receptors mRNA in fresh, glycerol cryopreserved and irradiated glycerol cryopreserved HAM. In glycerol cryopreserved HAM, the mRNA expression showed up-regulation of *HGF* and *bFGF* and down-regulation of the rest of 8 genes which were *EGF, HGFR, KGF, KGFR, TGF-α, TGF-β1, TGF-β2* and *TGF-β3*. Interestingly, the glycerol cryopreserved HAM radiated with 15 kGy showed up-regulation in the mRNA expression of 7 genes, namely, *EGF, HGF, KGF, KGFR, TGF-β1, TGF-β2* and

TGF-β3 and down-regulated mRNA expression of *HGFR*, *TGF-α* and *bFGF*. However, these mRNA expressions did not show a statistically significant difference compared to control groups. Thus, it can be concluded that the glycerol cryopreservation did not have an effect on the growth factors' and receptors' mRNA expression levels in HAM. Similarly, 15 kGy gamma irradiation did not have an effect on the growth factors' and receptors' mRNA expression in glycerol cryopreserved HAM. This finding provides a useful information to clinicians and surgeons to choose the best method for HAM preservation that could benefit patients in their treatment.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Human amniotic membrane (HAM) lines the inner cavity of placenta. It is five-layer thick and devoid of blood vessels or nerves. The epithelial layer of the amnion has a single layer of cuboidal to columnar cells and has microvilli on it apical surface (Riau *et al.*, 2010).

HAM has been used as a surgical biomaterial for more than 100 years since its first use by Davis in 1910. Since then, it has been widely used worldwide in a variety of clinical applications such as management of burns (Lo and Pope, 2009), surgical dressing (Ravishanker *et al.*, 2003; Mohammadi *et al.*, 2013), surgical reconstruction of the oral and maxillofacial (Kesting *et al.*, 2012; Kar *et al.*, 2014), bladder (Iijima *et al.*, 2007), vagina (Georgy and Aziz, 1996; Sarwar *et al.*, 2010), occlusion of pericardium (Muralidharan *et al.*, 1991) and in the prevention of surgical adhesions (Demirel *et al.*, 2009; Yetkin *et al.*, 2009).

The first documented application of amniotic membrane transplantation in ophthalmology fields appeared as early as 1940 when live fetal membrane was used by De Rotth (1940) to repair a symblepharon with only limited success. After that, only few reports regarding the HAM transplantation appeared in the ophthalmic literature. Five decades later, Kim and Tseng (1995) reintroduced HAM into ophthalmic practice by their success in repairing corneal defects.

Because of its usefulness, the indications for its usage in ophthalmology procedures have expanded over the time mainly in the reconstruction of ocular surface. HAM has been found to be used in promoting healing in corneal diseases such as thermal and chemical burns, persistent corneal epithelial defects, neurotrophic corneal ulcers, microbial keratitis, bullous keratopathy and band keratopathy (Ganatra, 2003; Dua *et al.*, 2004; Fernandes *et al.*, 2005; Liu *et al.*, 2010).

HAM has unique properties such as anti adhesive effect as well as the potential to bring down scarring and inflammation (Tseng *et al.*, 1999; Sippel *et al.*, 2001; Spelsberg and Reichelt, 2008), enhance wound healing and epithelialization (Tsubota *et al.*, 1999), anti-microbial properties, anti-viral characteristic (Inge *et al.*, 1991; Fernandes *et al.*, 2005) and low immunogenicity because of its non-association with rejection of graft subsequent to transplantation (Akle *et al.*, 1981; Adinolfi *et al.*, 1982; Houlihan *et al.*, 1995; Banas *et al.*, 2008).

The precise mechanism on how HAM graft aids healing still remains unclear and may be attributed to many reasons. Successful transplantation of HAM releases growth factors which enable epithelialization and reduce inflammation and scarring. Growth factors which occur naturally regulate many cell processes including cellular growth, proliferation and differentiation. Growth factor is usually a steroid hormone or a protein.

Studies conducted on HAM have shown the presence of many growth factors and receptors: epidermal growth factor (EGF), transforming growth factor (TGF-α,β1,β2, and β3), keratinocyte growth factor (KGF), KGF receptor (KGFR), hepatocyte

growth factor (HGF), HGF receptor (HGFR), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) (Koizumi *et al.*, 2000; Grzywocz *et al.*, 2014). EGF, KGF and HGF particularly might play important roles in the wound healing of ocular surface after transplantation of HAM (Koizumi *et al.*, 2000).

Various methods have been used worldwide to preserve HAM: lyophilization or freeze drying, air drying, glycerol preservation and cryopreservation. To ensure the safety of patients after transplantation, these preservation methods were combined with sterilization procedures such as gamma irradiation to inactivate bacteria and viruses (Yusof *et al.*, 2007). Earlier researchers have described different agents and techniques for preparing HAM. These include preservation, sterilization and storage (Maral *et al.*, 1999; Adds *et al.*, 2001; Ravishanker *et al.*, 2003; Riau *et al.*, 2010). Each and every technique of preparation has different effects on the HAM's physical and biological properties and also on its cell viability (Maral *et al.*, 1999; von Versen-Hoeynck *et al.*, 2008; Riau *et al.*, 2010). This could also affect the growth factors and proteins contained in this HAM. Moreover, gamma irradiation has also lot of advantages and disadvantages over other sterilization process (Maral *et al.*, 1999; Yusof *et al.*, 2007; von Versen-Hoeynck *et al.*, 2008; Riau *et al.*, 2010). Therefore, the different methods of HAM preservation, sterilization and storage could affect the growth factors present in the HAM which could in turn affect its beneficial properties.

1.2 Problem statement

HAM has been used in ophthalmic surgery either freshly or altered by methods like freezing, lyophilization or cryopreservation in glycerol. The most common and widely used method for preserving HAM is in glycerol. Positive results that have been obtained have led to its acceptance clinically. Later, cryopreservation of HAM in mixture of glycerol and Dulbecco's Modified Eagle's Medium (DMEM) in ratio 1:1 (v/v), introduced by Lee and Tseng (1997) is widely used for ocular surface reconstruction (Riau *et al.,* 2010). The glycerol cryopreserved HAM closely resembles fresh, non-preserved HAM with regard to its biological constituents. (Rodriguez-Ares *et al.,* 2009).

Despite the anti-viral property of glycerol, it is still not accepted as a sterilizing agent. Bacteria and viruses can be found even after preservation for several months (Van Baare *et al.,* 1994; Van Baare *et al.,* 1998). To minimize the risk of infections that can be disseminated by HAM, sterilization is combined with preservation.

Radiation sterilization is a process that is intended to inactivate or kill contaminating microorganisms in the tissues. Most tissue banks in the Asia Pacific region have been using ionizing radiation at 25 kGy to sterilize human tissue including HAM for safe clinical applications. The biological effects of gamma irradiation are caused by the absorption of the radiation energy in the tissues and by the distribution of that energy in the tissue matters (Dziedzic-Goclawska *et al.,* 2005). With the efficient killing effect on microorganisms, a major concern is whether gamma radiation could cause biological damages to tissue, which could result in degradation of their functional roles (Yusof, 2000).

HAM contains several growth factors such as EGF, HGF, KGF, TGF-α, TGF-β1, TGF-β2, TGF-β3, bFGF, VEGF and PDGF and growth factor receptors such as HGFR and KGFR which play important role in wound healing. Biological damage could include the effect of this gamma irradiation on growth factor and receptors mRNA expression thus reduce the quality and efficiency of HAM.Therefore, each and every step of preparation, preservation, storage and sterilization should be carefully designed so that these steps do not adversely affect the beneficial properties of HAM.

1.3 Justification of the study

The presence of growth factors in HAM is of prime importance in wound healing. The various methods of preservation and the process of gamma irradiation are likely to alter the expression of these growth factors and receptors. Since there is a dearth of information in this arena, the present study attempted to investigate on the expression of various growth factor following preservation and gamma irradiation. This will benefit clinicians and surgeons to choose the best method of HAM preservation that could be beneficial for the patients in their treatment.

1.4 Research hypothesis

There is a significant effect of gamma irradiation on the expression of growth factors and receptors mRNA in glycerol cryopreserved HAM.

1.5 Objectives

1.5.1 General objective

The purpose of this study was to determine the effect of gamma irradiation on the expression of selected growth factors and receptors mRNA in glycerol cryopreserved HAM.

1.5.2 Specific objectives

1. To determine the effect of glycerol cryopreservation on the expression of growth factors and receptors mRNA in HAM.
2. To determine the effect of 15 kGy gamma irradiation on the expression of growth factors and receptors mRNA in glycerol cryopreserved HAM.
3. To determine the effect of 15 kGy gamma irradiation and glycerol cryopreservation on the expression of growth factors and receptors mRNA in HAM.

CHAPTER 2

LITERATURE REVIEW

2.1 Human amniotic membrane

HAM is a thin tissue which constitutes the walls of the amniotic sac. The amniotic sac contains amniotic fluid in which the fetus is suspended and this provides the protection to the fetus. HAM is also known as amnion or extra embryonic membrane. The placenta which consists of amniotic membrane is discarded after the deliveries. HAM transplantation has gained popularity because of its availability, ease of obtaining, processing and transportation, its convenience and ease of use in addition to its high and reproducible success rates. It is considered to have most of the beneficial properties as a biomaterial.

2.2 Structure of HAM

HAM develops from extra-embryonic tissues. It consists of a fetal component (the chorionic plate) and a maternal component (the decidua). The fetal component is composed of two layers; an outer layer (chorion), which contacts maternal cells and an inner layer (amniotic membrane). These membranes separates the fetus from the endometrium and forms the outer limits of the sac which encloses the fetus (Niknejad *et al.*, 2008).

Furthermore, these two layers remain separable and do not fuse histologically (Baradaran-Rafii *et al.*, 2008). HAM is a collagen rich, thin, translucent and tough membrane, lining the inner side of fetal placenta. This translucent biological

structure has no blood vessels or nerves, muscles or lymph vessels. Its source of nutrients and oxygen which are supplied by diffusion, from the chorionic fluid, amniotic fluid and fetal surface vessels (Benirschke, 2000; Toda *et al.*, 2007).

HAM's thickness varies from 0.02 mm to 0.50 mm and consists of three main histological layers (Figure 2.1): an epithelial monolayer, a thick basement membrane and an avascular mesenchymal tissue which again consists of three layers: compact, fibroblast and intermediate layers (Benirschke, 2000; Toda *et al.*, 2007; Niknejad *et al.*, 2008).

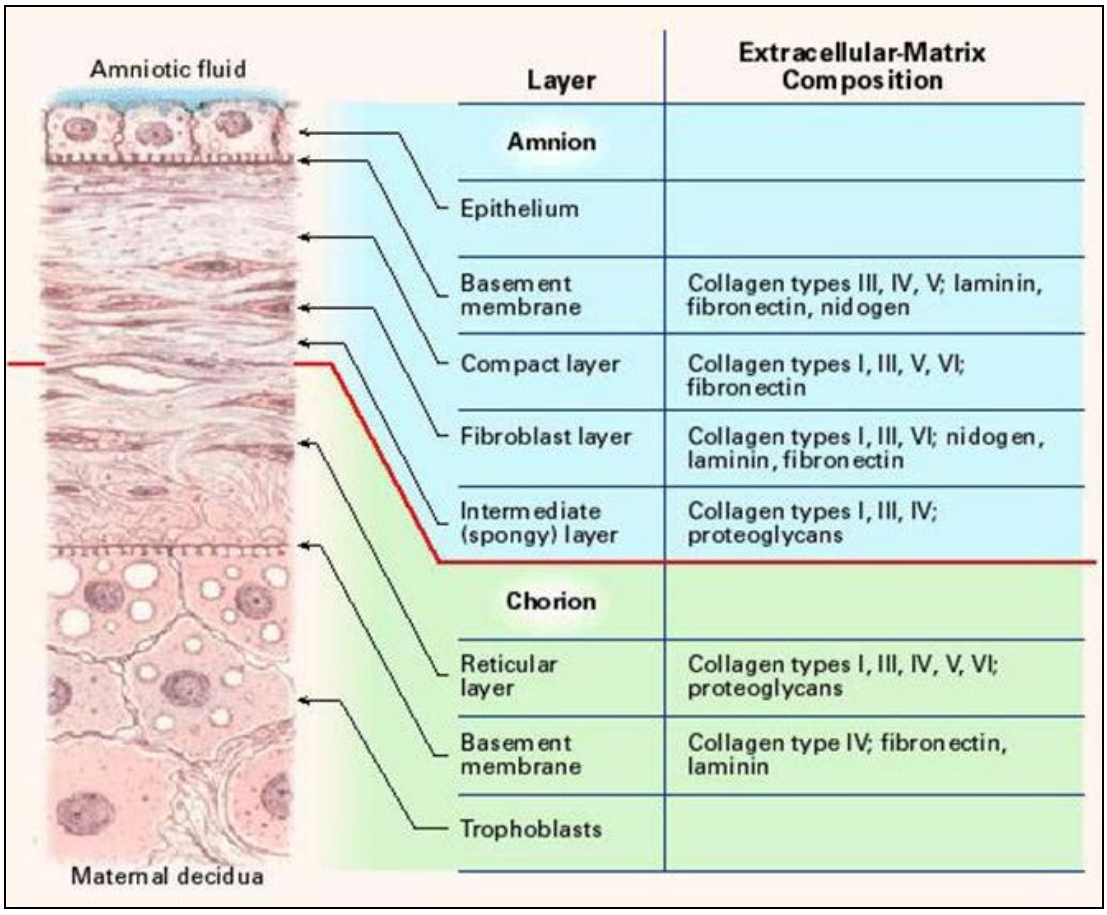

Figure 2.1 Schematic presentation of the structure of the fetal membrane at term. Adapted from Niknejad *et al.* (2008)

The innermost layer adjacent to the amniotic fluid and nearest to the fetus is termed the amniotic epithelium. It consists of a single homogeneous layer of cuboidal epithelial cells which is firmly fixed to the basement membrane. Amniotic epithelial cells (AECs) have many microvilli at the apical surface and probably have an active secretory function and intra- and trans-cellular transport function (Pollard *et al.*, 1976). These cells have many intracytoplasmic, pinocytic vesicle organelles, and a large irregular nucleus with a large homogenous nucleolus (Ab Hamid *et al.*, 2014). AECs express epidermal markers, such as glycoprotein CA125 and oxytocin receptors and are also positive for antigen CD44 and desmin (Benedetto *et al.*, 1990; Toda *et al.*, 2007). Erythropoietin and its receptors are expressed in human AECs. The production of erythropoietin in human AECs is stimulated by progesterone but is not stimulated by hypoxia or 17β-estradiol (Ogawa *et al.*, 2003).

The HAM's basement membrane is one of the thickest membranes found in all human tissues. It provides support to the fetus throughout gestation. The basement membrane has large quantities of proteoglycans that are rich in heparin sulphate and serve as a permeable barrier to amniotic macromolecules (King, 1985) and several molecules such as actin, α-actinin, ezrin, spectrin, several cytokeratins, vimentin, desmoplakin and laminin which are important in maintaining the membrane integrity (Wolf *et al.*, 1991; Akashi *et al.*, 1999). Laminin contributes to the survival of cells, its differentiation, shape and movement and is involved in the maintenance of tissue phenotypes (Takashima *et al.*, 2008).

The outer layer of HAM is composed of mesenchymal fibroblast-like cells which are rich in collagen. This collagen is secreted by mesenchymal cells. The compact layer

of stromal matrix forms the main fibrous skeleton of the HAM. The mechanical integrity of HAM is maintained by interstitial collagens (types I and III) which form parallel bundles. Interstitial collagens and the epithelial basement membrane are connected by collagen types V and VI which form filamentous connections (Niknejad *et al.*, 2008).

The intermediate layer, which is the outmost layer of HAM is also known as spongy layer or zona spongiosa of the stromal matrix and is adjacent to the chorionic membrane. Its abundant content of glycoproteins and proteoglycans produces a spongy appearance histologically. It also contains a non-fibrillar meshwork of mostly type III collagen (Parry and Strauss, 1998). The spongy layer consists of wavy bundles of reticulum bathed in mucin and is loosely connected to the chorionic membrane. Hence, HAM is easily separated from the chorion by blunt dissection (Niknejad *et al.*, 2008).

2.3 Functions of HAM

The basic function of HAM is to protect the developing embryo against desiccation. It also provides an ideal suspension environment for the embryo to grow without distortion by pressure from surrounding structures throughout pregnancy.

HAM plays an important role during birth. The amniotic epithelium is the main source of prostaglandins, especially prostaglandin E2 (Okazaki *et al.*, 1981), and it also expresses prostaglandin-biosynthesis enzymes such as cyclooxygenase, phospholipase and prostaglandin synthase (Bryant-Greenwood *et al.*, 1987). Prostaglandins play a pivotal function in the initiation and maintenance of uterine

contraction. The receptor of these enzymes is located on the HAM epithelium and is regulated by human chorionic gonadotropin (hCG) (Toth *et al.*, 1996).

Human amniotic epithelium is responsible for keeping and maintaining the pH of the amniotic fluid at a constant value of 7.10 throughout gestation and it is metabolically highly active (Shumway *et al.*, 1999; Toda *et al.*, 2007).

2.4 Properties of HAM

2.4.1 Anti-inflammatory and immunological characteristics

HAM can serve as a physical barrier confining the inflammatory cells to the area affected, thus reducing the inflammatory mediator in non-affected areas. A study shows that when HAM is applied as a patch, it attracts and entraps inflammatory cells infiltrating the ocular surface which explains some of its anti-inflammatory properties (Shimmura *et al.*, 2001). HAM also induces apoptosis of interferon-γ activated macrophages *in vitro* (Li *et al.*, 2006). Furthermore, the epithelial and mesenchymal cells of HAM express various anti-angiogenic and anti-inflammatory proteins (Hao *et al.*, 2000). Hyaluronic acid in HAM stroma may play an important role in the entrapment of inflammatory cells including lymphocytes when used as a patch in ocular surface disease (Higa *et al.*, 2005).

Several studies have demonstrated no immunological responses after transplantation with HAM in human volunteers who did not show any clinical signs of acute rejection (Akle *et al.*, 1981; Kamiya *et al.*, 2005). It was attributed to the reason that AECs do not express human leucocytes antigen system; (HLA) -A, -B or -DR antigens (Adinolfi *et al.*, 1982). HAM also contains some immunoregulatory factors

including HLA-G and Fas ligand and Kubo *et al.* (2001) concluded that HAM may be useful to supplement corneal collagen and need not be applied only to the ocular surface but also can be applied intracorneally.

2.4.2 Antimicrobial

Antimicrobial effect of HAM *in vitro* is due to their close adherence to the wound surface (Inge *et al.*, 1991). HAM may serve as a barrier against bacterial infiltration by adhesion to the wound surface and thus reduces the bacterial load. Another mechanism of action against infection is by preventing dead space formation and serous discharge accumulation when HAM adheres to the wound surface (Baradaran-Rafii *et al.*, 2008).

There was no effect on microbial permeability of membranes processed by air drying as was observed during storage under different temperature and humidity conditions (Singh *et al.*, 2003). Even in contaminated wounds, it was reported that HAM may decrease bacterial proliferation (Baradaran-Rafii *et al.*, 2008).

2.4.3 Promotion of epithelialization

The basement membrane of HAM serves as a safe and a suitable bed for the growth of epithelial cells. Laminin isoforms which are present in the basement membrane, facilitate adhesion and expansion of corneal epithelial cells (Baradaran-Rafii *et al.*, 2008). HAM also acts as a basement membrane that facilitates epithelial cell migration (Tseng *et al.*, 1997), reinforces adhesion of basal epithelial cells (Shimazaki *et al.*, 1998), promotes epithelial differentiation (Guo and Grinnell,

1989), prevents epithelial apoptosis (Boudreau *et al.*, 1995) and reduces excessive fibrosis (Mermet *et al.*, 2007).

HAM can provide a wet media for ocular surface re-epithelialization when it is moistened by tear. In contrast to other synthetic materials, HAM has a good permeability providing sufficient oxygenation for epithelial cells (Baradaran-Rafii *et al.*, 2008).

HAM stimulates epithelialization by the production of various growth factors (Koizumi *et al.*, 2000) and reduces fibrosis (Mermet *et al.*, 2007). The presence of proteinase inhibitors also might facilitate wound healing (Kim *et al.*, 2000).

2.5 Clinical applications of HAM

2.5.1 Ocular surface reconstruction

HAM graft transplantation was first introduced in ophthalmology in 1940 by De Rotth in the treatment of conjunctival defects and symblepharon (De Rotth, 1940) and then by Sorsby in 1947 for the treatment of acute chemical burns (Sorsby *et al.*, 1947). Thereafter, its use was abandoned or went unreported until 1995 when Kim and Tseng reintroduced its usage in ophthalmology (Kim and Tseng, 1995).

Since 1995, HAM transplantation has been successfully used in ocular surface reconstruction in patients with a variety of ocular surface diseases, including conjunctival surface reconstruction (pterygium surgery, conjunctival tumour excision, symblepharon release, repair of leaking blebs, scleral melt, fornix formation, socket reconstruction, chemical burns, cicatrizing keratoconjunctivitis), corneal surface reconstruction (persistent epithelial defects, non-healing stromal

ulcers, partial and total limbal stem cell deficiency, painful bullous keratopathy, band keratopathy) and as a substrate for the *ex-vivo* expansion of limbal and conjunctival stem cells (Burman *et al.*, 2004; Dua *et al.*, 2004; John, 2004; Gomes *et al.*, 2005; Sangwan *et al.*, 2007; Liu *et al.*, 2010).

2.5.2 Wound management

The HAM was first used by Davis in 1910 for skin transplantation (Davis, 1910) and later Stern used it for management of skin burns (Stern, 1913).

HAM has most of the qualities as an ideal skin substitute which include anti-adhesive effects, anti-inflammatory, anti-angiogenic, bacteriostatic action, wound protection, pain reduction, ability to initiate epithelialization and reduced excessive fibrosis. The membrane can act as an effective barrier as it has a good adherence to wound with no immunological reactions.

It has been used effectively in a variety of wound management such as a temporary biological dressing (Maral *et al.*, 1999; Ganatra, 2003; Salehi *et al.*, 2013), partial thickness burns (Branski *et al.*, 2008), cover for microskin grafts (Subrahmanyam, 1995), epidermal substitute (Rejzek *et al.*, 2001) and other skin applications.

2.5.3 Regenerative medicine

HAM gains importance as a potential source for scaffolding material for tissue engineering (TE) and regenerative medicine because it is easy to obtain, readily available, can be used with specific applications and has a low risk of infection or immune reaction. TE is defined as the development of biological substitutes for the

purpose of restoring, maintaining or improving tissue function and requires the application of principles and methods from both engineering and life sciences (Langer and Vacanti, 1993). The aim of TE and regenerative medicine is to promote tissue regeneration, to replace failing or malfunctioning organs by combining a scaffold/support material, adequate cells and bioactive molecules. Therefore, the design and selection of the biomaterials to be employed in scaffolding is a critical step in TE (Mano *et al.*, 2007).

The extracellular matrix components (ECM) of the basement membrane of HAM form almost a native scaffold for seeding cells in TE (Niknejad *et al.*, 2008). HAM can be used as a support matrix (scaffold) in regeneration, as it promotes re-epithelialization, reduces inflammation and suppresses fibrosis (Burman *et al.*, 2004; Yeh *et al.*, 2005; Portmann-Lanz *et al.*, 2007).

It has been reported that the ECM of HAM is an efficient medium for peripheral nerve regeneration (Mohammad *et al.*, 2000; Mligiliche *et al.*, 2002) and beneficial for neuronal differentiation (Miyamoto *et al.*, 2004; Meng *et al.*, 2007). It can serve as a carrier matrix for cartilage regeneration (Jin *et al.*, 2007). Cultivation and seeding of epithelial cells on HAM scaffold is a commonly used technique for ocular surface and skin reconstruction (Capeans *et al.*, 2003; Fatima *et al.*, 2006; Yang *et al.*, 2006). It has also been reported as a potential approach for vascular TE (Ishino *et al.*, 2004; Tsai *et al.*, 2007) and regenerative medicine (Grzywocz *et al.*, 2014).

AECs have many characteristics that make them as a great source of stem cells in TE. These cells express the surface markers associated with embryonic stem cells, e.g. SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. AECs also express pluripotent

stem cell-specific transcription factors such as Oct-4 and Nanog (Ilancheran *et al.*, 2007; Miki *et al.*, 2007a; Miki *et al.*, 2007b). This has been supported by studies using AECs in repairing damaged ocular surfaces (Tseng *et al.*, 1998; Solomon *et al.*, 2002; Uçakhan *et al.*, 2002) and to improve congenital lysosomal storage disease (Kosuga *et al.*, 2000).

2.6 Preparation and preservation of HAM

After isolation from the human placenta, the HAM can be used fresh (Mejía *et al.*, 2000) or preserved using different types of preservation method. Compared to the preserved HAM, the fresh HAM must be used immediately. Cryopreserved HAM is the fresh HAM which is submerged in glycerol and stored at sub-zero temperature (i.e. -80^{o}C). It is widely used in ocular surface reconstructive procedures because it resembles the fresh HAM (Rama *et al.*, 2001).

A study has shown that the duration of storage could affect the viability of amniotic cells. In their study, it was reported that after freezing for 2 months, at least 50% of the amniotic cells were viable and capable of proliferation but they noticed that after 18 months of cryopreservation, they were unable to demonstrate a significant amount of cell survival (Kubo *et al.*, 2001).

There are many techniques that have been used worldwide to preserve HAM such as hypothermic storage (at 4°C), freezing, air drying, lyophilization, glycerol preservation and glycerol cryopreservation. A combination of tissue preservation and sterilization by gamma irradiation has been recommended to minimize the risk of infections that may be transmitted by HAM.

It is well established that during some preservation processes such as lyophilization, air drying or glycerol preservation, viability of the epithelial cells is reduced or lost (Kim and Tseng, 1995; Tseng *et al.*, 1997; Kruse *et al.*, 2000; Singh *et al.*, 2003; Nakamura *et al.*, 2004). It was noticed that the viability of HAM cell decreased during storage and was more pronounced when stored frozen. At the same time, it was also observed that the higher viability of HAM cells was obtained by storage at above 0^{o}C with medium. Unfortunately, it was limited to a short period of storage of about 28 days (Hennerbichler *et al.*, 2007).

The preparation of each preservation might differ from one institution to another but the concept is still the same, which is to maintain the integrity of the HAM for the intended safe clinical usage. Many studies have been done to evaluate the effectiveness of these preservation methods and made modification of the method itself so that it could cater to the need of patient care.

Procurement of HAM for all types of preservation is usually almost the same. HAM was collected after obtaining proper informed consent of the mother and basic screening was done. Only HAM from seronegative donors was then subjected to the respective type of preservation.

2.6.1 Glycerol preservation of HAM

In general, there are two types of glycerol preservation, which are different in the way HAM is stored. They are glycerol preservation and glycerol cryopreservation of HAM. The glycerol preserved HAM is usually stored at room temperature or 4^{o}C, whereas the glycerol cryopreserved HAM is stored at -80^{o}C.

Glycerol preservation has been the most widely used method of preserving HAM. The usage of glycerol to preserve donor skin for transplantation was introduced in the Netherlands in 1984 (Hermans, 1989). A good prognosis of its usage as a method of HAM preservation over the last decades has led to its clinical acceptance. Glycerol preserves the physical structure of amnion by preventing loss of fluid, protein, electrolytes, heat and energy (Rejzek *et al.*, 2001). Glycerol serves as the basic mainstay for tissue preservation (von Versen-Hoeynck *et al.*, 2008).

Glycerol has the strongest virucidal action at the highest concentrated form of 98 percent (Van Baare *et al.*, 1994). However, the usage of high concentrated glycerol solution could abolish the viability of HAM cells (Prabhasawat *et al.*, 2000). Although glycerol is known to have an anti-viral effect and antimicrobial action, it is not a sterilizing agent (Van Baare *et al.*, 1994; Pegg, 2006). It has been shown that even after glycerol preserved HAM was stored for several months, bacteria and viruses were still viable (Van Baare *et al.*, 1998).

2.6.2 Glycerol cryopreservation of HAM

The preservation method was originally described by Kim and Tseng (1995). Initially, it was used to treat rabbits with limbal stem cell deficiency. Later, the method was slightly modified when it was transplanted to human patients where the graft was cryopreserved in 50% glycerol and stored at -80°C (Lee and Tseng, 1997). In this method, the placenta is normally washed using balanced salt solution comprising a cocktail of antibiotics (50 mg/ml penicillin, 50 µg/ml streptomycin, 100 mg/ml of neomycin and 2.5 mg/ml of amphotericin B) prior to its storage. The HAM

is stored in glycerol which is usually mixed with DMEM in a ratio of 1:1 (vol/vol). The HAM can then be stored at -80°C for several months.

This preservation method has been proven to produce high success rates in HAM transplantation (Koizumi *et al.*, 2000; Rama *et al.*, 2001; Nakamura *et al.*, 2004; Nakamura *et al.*, 2006). Almost 95% of the existing literature on the utilization of HAM for ocular surface reconstruction has employed this particular preservation procedure. The biological constituents and structure of the glycerol cryopreserved HAM closely resemble fresh or non-preserved HAM (Rodriguez-Ares *et al.*, 2009). The data show that the general morphology of the HAM is maintained during cryopreservation but its epithelial and the fibroblastic cells lose viability (Kruse *et al.*, 2000).

2.6.3 Lyophilization and air drying of HAM

Lyophilization or freeze drying is a preservation method that removes water from a tissue by sublimation. Removal of water at the relatively high temperatures can cause the tissue to collapse and may also cause unwanted molecular cross linkage which leads to a major effect on mechanical properties (Pegg, 2006). Further, this results in the inhibition of destructive chemical reactions that could cause tissue alteration.

The freeze drying of HAM according to Nakamura *et al.* (2004) involves washing HAM with sterile phosphate buffered saline which contains antibiotics and antimycotics. AECs are removed by incubating with 0.02% ethylene diamine tetra-acetic acid. Denuded HAM was then freeze dried under vacuum conditions, vacuum-packed at room temperature and finally sterilized by gamma irradiation (25 kGy).

The resultant material was wafer-like, very lightweight and thin, easy to handle and could be sutured without tearing. The advantage is that it could be easily stored at room temperature and became relatively smooth and flexible upon hydration. Recent publications have disclosed the relevance of a combination of lyophilization technique and gamma irradiation sterilization to enhance safety usage of the graft (Nakamura *et al.*, 2004).

Air drying method is the simplest preservation method for HAM but the dehydration or air drying can cause damage to the structure of HAM. In addition, air drying was found to cause condensation of microvilli and intercellular channels and the individual cells were further damaged when irradiated at 25 kGy (Ab Hamid *et al.*, 2014). However, the functional physical properties of air dried amnion were not affected even up to 50 kGy as reported by Singh *et al.* (2007). The processing cost for air dried HAM is cheaper compared to other preservation methods. The advantage of both lyophilized and air dried HAM is that it can be stored at room temperature for a long period of time and also can be transported easily.

2.7 Sterilization of HAM

The risk of using allograft for transplantation is that it could transmit bacteria, viruses or prions from donor to recipient. Studies have shown the possible presence of pathogenic microbes in allograft tissues such as viruses (eg. HIV, hepatitis viruses (HBV, HCV) and bacteria (Campbell *et al.*, 1994; Adds *et al.*, 2001; Kainer *et al.*, 2004; Villalba *et al.*, 2007; Wang *et al.*, 2007).

The success of the donation and transplantation of tissue grafts relies on many confounding factors which start from proper selection of donor until the clinical usage by the surgeon or clinician. A proper donor screening along with aseptic surgical technique during tissue retrieval, processing and storage could reduce the potential of infection transmission (Delmonico, 2000; Rao and Mirza, 2005; McAllister *et al.*, 2007; Fishman *et al.*, 2009).

It is recommended that HAM use in ocular surface procedures should be retrieved only from placentas following elective caesarean deliveries because it is shown to be with lesser risk of pathogenic bacterial contamination compared to those procured from vaginal deliveries (Adds *et al.*, 2001). Regular training programs should be implemented by tissue banks which focus on improved donor screening and proper aseptic technique for tissue retrieval. It is also suggested that tissue banks should periodically check the rate and types of tissue contaminations to find system faults and to update processing methods (Aghayan *et al.*, 2013).

Even though aseptic technique practices in tissue bank can reduce the risk of contamination, it cannot totally eliminate the microbial load. Therefore, many tissue banks envisage that it is essential for tissue allograft to be terminally sterilized using ionizing radiation such as gamma and electron beam or other sterilization methods such as peracetic acid and trehalose (Riau *et al.*, 2010).

Radiation sterilization is a process that is intended to inactivate or kill microorganisms contaminating a product. It has been proven to be an efficient technique for inactivating bacterial and viral contaminants of allograft. Sterilization

using gamma radiation is widely applied and can provide an additional safety measure against infection (Czitrom, 1992; Yusof, 2000). It is used to complement and never to substitute for proper screening of donor and effective processing practices in tissue banking (Czitrom, 1994).

The advantages of gamma irradiation over other sterilization techniques are that it does not include any significant temperature rise that causes any physical or chemical changes and thus the grafts retain their clinical characteristics; high penetration of gamma ray enables hard and soft tissues to be sterilized in their final packaging in bulk; absence of toxic residuals and with irradiation time being the only variable, the process control is precise and simple (Phillips, 1988).

The results of radiation sterilization depends on several factors such as the amount of energy transferred, the number of contaminating micro-organisms and their resistance to ionizing radiation which is characterized by D_{10} values (Dziedzic-Goclawska *et al.,* 2005). The commonly used term 'bioburden' or initial contamination is defined as the population of viable micro-organisms or active pathogens that exists on or inside a product before sterilization. Bioburden count is one of the factors that can influence the efficiency of irradiation procedure. Clearly, the lower the bioburden, the more effective is the sterilization process.

The sterility of an irradiated graft cannot be assured in an absolute sense. A sterility assurance level (SAL) is derived mathematically and defined as the probability of non-sterility for each individual item. Graft with SAL of 10^{-6} means one graft in a million has the probability of being non-sterile. The use of gamma radiation to

sterilize tissue grafts is proven to be a safe and reliable method for achieving sterile tissue at SAL of 10^{-6} for clinical use. The recommended radiation sterilization dose (RSD) for health care products which include tissue allograft is 25 kGy. This dose was set based on the bioburden and radiation resistance of micro-organisms that are found on health care products (IAEA, 2004) and is routinely applied in many tissue banks worldwide (Hilmy *et al.*, 2000; Djefal *et al.*, 2007; Hilmy *et al.*, 2007; Nguyen *et al.*, 2007b; Yusof *et al.*, 2007).

Tissue deterioration produced by gamma radiation occurs via two mechanisms which are direct and indirect actions. The direct influence of gamma rays occurs in the dry state where polypeptide chains is disbanding or splitting, whereas, in indirect action, gamma rays cause radiolysis of water molecules and the formation of short-lived hydroxyl radicals which are highly reactive (Dziedzic-Goclawska *et al.*, 2005).

Even though International Atomic Energy Agency (IAEA) recommended the RSD of 25 kGy, some tissue banks are adopting higher doses. They found that the irradiation dose of 25 kGy is not sufficient to guarantee sterility of human tissue grafts and they also discovered that the undesired radiation induced damage can be diminished through modification of tissue preservation methods even if they irradiate the graft at 35 kGy (Dziedzic-Goclawska *et al.*, 2005).

Any morphological changes possibly caused by preservation method in combination with gamma irradiation would lead to the degradation of the membrane, which affects on the physical and biological properties of the HAM (Riau *et al.*, 2010). Selection of RSD has become a controversial issue in the bone bank community as

the industry still debates whether 25 kGy (standard dose) can be set as a gold standard, or whether a lower dose for sterilization could still minimize the risk of infection and reduce the adverse effects of radiation.

By complying with the principles of good manufacturing practice, tissue grafts with low level of microbial contamination could be produced. Hence, it can be sterilized at doses lower than 25 kGy to achieve a SAL of 10^{-6} (Riau *et al.*, 2010). The possibility of lowering the sterilization dose could be feasible for processed HAM with the initial bioburden less than 100 colonies per graft (Yusof, 1994; Baker *et al.*, 2005). Furthermore, gamma irradiation at 15 kGy and 25 kGy did not evoke undesirable changes in the morphological structure of air dried and glycerol preserved HAM (Ab Hamid *et al.*, 2014). Therefore, the use of gamma radiation at doses lower than 25 kGy for terminal sterilization has the potential to become a new approach in tissue banking.

2.8 Growth factor

Growth factor is a naturally occurring substance which is able to stimulate cellular growth, proliferation, healing, and cellular differentiation. Usually, it is a protein or a steroid hormone. Growth factors are very vital for regulating diverse cellular processes. Growth factors characteristically act as signalling molecules between cells. This present study focuses on mRNA expression of eight growth factors, namely epidermal growth factor *(EGF)*, hepatocyte growth factor *(HGF)*, keratinocyte growth factor *(KGF)*, transforming growth factor-α *(TGF-α)*, transforming growth factor-β1 *(TGF-β1)*, transforming growth factor-β2 *(TGF-β2)*, transforming growth factor-β3 *(TGF-β3)* and basic fibroblast growth factor *(bFGF)*