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Research Article
Biological Activity Alterations of Human Amniotic Membrane Pre and Post Irradiation Tissue Banking

[1]Waleed Nemr, [1]A.S. Bashandy, [1]Eman Araby and [2]O. Khamiss

[1]Department of Radiation Microbiology, National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority (AEA), P.O. Box 29, Nasr City, Cairo, Egypt
[2]Department of Animal Biotechnology, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City (USC), Egypt

Abstract
Background and Objective: Innate immunity of Human Amniotic Membrane (HAM) and its highly active secretome that rich with various types of growth factors and anti-inflammatory substances proposed it as a promising material for many medical studies and applications. **Methodology:** This study evaluate the biological activity of cultivated HAM pre and post tissue banking process in which freeze-dried HAM was sterilized by 25 KGray (kGy) dose of γ radiation. The HAM's antimicrobial activity, viability, growth of isolated human amniotic epithelial cells (HAECs), hematopoietic stimulation of co-cultivated murine bone marrow cells (mammalian model), scaffold efficiency for fish brain building up (non-mammalian model) and self re-epithelialization after trypsin denuding treatment were examined as supposed biological activity features. **Results:** Native HAM revealed viability indications and was active to kill all tested microorganisms; 6 bacterial species (3 Gram-positive and 3 Gram-negative) and *Candida albicans* as a pathogenic fungus. Also, HAM activity promoted colony formation of murine hematopoietic cells, *Tilapia nilotica* brain fragment building-up and self re-epithelialization after trypsin treatment. In contrary, radiation-based tissue banking of HAM caused HAM cellular death and consequently lacked almost all of examined biological activity features. **Conclusion:** Viable HAM was featured with biological activity than fixed HAM prepared by irradiation tissue banking.

Key words: Human amniotic membrane, biological activity, irradiation, tissue banking

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Corresponding Author: Waleed Nemr, Department of Radiation Microbiology, National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority (AEA), P.O. Box 29, Nasr City, Cairo, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Innate immunity of HAM plays an important role as the first defense barrier protecting the fetus against microbial infection during pregnancy. The HAM is a thin and elastic membrane composed of monolayer of microvilli covered-epithelial cell planted on basal membrane and connected to a thin connective tissue membrane by filamentous strands. These microvilli are supposed that may form intercellular canaliculi and probably has a supportive function[1].

The HAECs were reported to have highly active secretome plays a great role in homeostasis of amniotic fluid surrounding the fetus; various of secreted substances were specifically recorded for HAECs included various antimicrobial peptides improved the innate immunity of HAM, antiviral interferon, cytokines and many of growth factors. For these reasons, HAM was used as an effective surgical biomaterial for more than 100 years[2] and was recommended in various clinical applications for curing wounds, burns, corneal stem cell deficiency and others[1-4].

There are several methods were published for HAM preparation, most of these methods aimed to preserve sterile HAM before delivering it to medical application. Lyophilization, air-drying, glycerol-preservation and cryopreservation are variuos methods for preserving HAM, each method have different impacts on histological properties and cell viability of the amniotic membrane[5]. The most commercial procedure for producing packaged HAM was depending on dehydration it before sterilization by irradiation (e.g., PURION®, MiMedx Group, Georgia).

Sterility by ionizing radiation is rapid and effective to kill all live microorganisms present in biological membranes by destroying their nucleic acids[6]. However, the effect of sterilization by γ irradiation on biological properties of HAM was not clearly stated.

The advantages of dried γ-irradiated amniotic membrane have been illustrated that may concluded in three points as: Easy to proceed, low cost and long term storage and excellent material that physically is good not permeable tissue barrier to prevent microbial invasion of treated wounds and chemically act as a substrate for epithelial cell migration during skin damage healing[7]. However, the methodology of HAM tissue banking via gamma-irradiation of dried HAM have been widely used for medical application in the field of ophthalmology and wound care (e.g., leg ulcers and burns) but by this method, HAM cells then are no longer viable[8].

There are many opposite opinions from researchers regarding the recommended methodology for HAM preparation to be suitable for clinical applications; some of them recommended the using of viable HAM and others recommended the using of fixed HAM (stored form).

In this regard, this study aimed to evaluate the importance of HAM cellular viability for some of *in vitro* applications; such as tissue engineering, re-epithelialization, growth promotion for co-cultivated cells and prevention of microbial contamination of cell cultures.

MATERIALS AND METHODS

Biological materials: Human placenta were collected from Al Demerdash Maternal Hospital, Ain Shams University. After surgery of cesarean delivery, the placenta were soaked in chilled sterilized 0.9% sodium chloride normal saline solution (NS) and immediately delivered into ice box to Amniotic Tissue Laboratory, National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA).

All microorganisms used in this study were kindly provided from radiation microbiology department, NCRRT. Bacterial samples are *Bacillus subtillus*, *Staphyloccocus aureus*, methicillin resistant *Staphyloccocus aureus* (MRSA), *Salmonella typhi*, *Pseudomonas aeruginosa*, *E. coli* and *Candida albicans* were cultivated on nutrient agar medium pH 7.2 (Oxoid, UK).

HAM preparation: Post placental sample delivery to lab, HAM was separated from the placental tissue manually in sterile metal basin undercurrent of tap water by blunt dissection. Separated HAM was transferred into new sterile metal basin contained 250 mL of NS and gently shacked into shaking water bath (Baby Scientific, UK) at 37°C for 15 min, washed three times until the HAM be cleared and free from cell debris and blood aggregates.

All cell and tissue culture media and reagents used in this study were purchased from Lonza (USA). Cleared HAM was divided into ≈ 3-5 g pieces and soaked in batches into Hanks Balanced Salt Solution (HBSS) contained 1x formulated antibiotic/antimycotic mix into sterilized glass bottle and incubated overnight at room temperature (≈ 30°C). Next day, microbial contaminated bottles were eliminated from subsequent experiments.

HAM tissue banking: Radiation-based tissue banking was carried out according to methodology recommended by Singh and Chacharkar[7], in which washed transparent HAM was layered on cotton dressing gauze as a supporting material and

freeze dried prior to gamma-irradiation treatment for sterilization at dose 25 kGy in Co^{60} cell = Cobalt 60. So, tissue banked HAM prepared by this method was termed here as dried irradiated HAM (DiHAM).

HAM tissue culturing: Fresh prepared HAM and DiHAM were cultivated in 5 mL Minimal Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS) in a sterile glass petri dish, cultures incubated at 37°C in humidified CO_2 incubator[9].

HAECs isolation: Overnight cultured HAM and DiHAM pieces were washed two times in NS and transferred separately into sterile petri dishes contained 10 mL of 0.25% trypsin-versene mixture solution for HAECs separation. First trypsinization step was incubated for 10 min at 37°C with shacking and were discarded to eliminate erythrocytes contamination and cellular debris. The second and third steps of trypsinization was performed by adding new 10 mL 0.25% trypsin-versene mixture solution and incubated for 1 h at 37°C with shacking. After each incubation step, the aspirated digest solution passed through sterile gauze held on stainless steel mesh (Schleicher and Schüll Dassel, Germany) for eliminating the undigested tissue fragments, filtrate was receipt into sterile 15 mL centrifuge tube and kept in ice shortly till accomplishment of all tubes[10].

Simultaneously, epithelial-denuded amnion was washed twice in 50 mL normal sodium chloride balanced salt solution and then transferred into new T-flask containing 5 mL growth medium contained 10% FBS for verifying denuded HAM self re-epithelialization to repair its lost epithelial layer as an indication for self healing.

Subsequently, trypsinized suspensions were centrifuged at 1200 rpm for 10 min; cell pellets were washed twice by chilled 10 mL HBSS and finally resuspended with 2 mL DMEM:F12 medium. Viable HAECs were counted by trypan blue exclusion on haemocytometer slide according to manufacturer instructions[11]. About 5×10^6 of counted HAECs were cultivated with 4 mL DMEM:F12 medium contained 10% FCS. Cultures were incubated at 37°C into humidified CO_2 incubator. Cells were maintained every 48-72 h till 80-90% confluence.

Viability assay: Cultured HAM, DiHAM and isolated HAECs were washed from medium three times with PBS, then incubated with 0.4% (w/v) trypan blue solution for 5 min and then washed three times with PBS prior to examination by inverted microscope (Leica Microsystems, Germany).

Antimicrobial activity of HAM: Antimicrobial activity of HAM and DiHAM was tested in two ways; by treating microbial culture with HAM itself layered on the surface of microbial solid culture, or by loading 50 µL of aspirated waste medium, collected after 2-3 days of HAM culture medium replacement, into wells punched into agar solid medium[12].

***In vitro* application of HAM:** *In vitro* feeding of cultured cells via co-cultivation with HAM was involved in this study for proving the importance of HAM secreted growth factors and other substances in promoting cellular growth of mammalian (mice) and also non mammalian (fish) cells, this was evaluated by observation of successful cellular duplication of adherent cells to flask surface or to HAM seeded surface. All media used for cellular cultivation in this experiment are antibiotic and antimycotic-free to verify the efficiency of HAM antimicrobial activity in preventing cell culture from microbial contamination, also media not supplemented with artificial growth factors. This part of work was carried out in cell culture unit, animal biotechnology department, GBRI, USC.

Murine bone marrow cells (BMCs) co-cultivation with HAM: Four weeks an old laboratory white healthy mouse was sacrificed by cervical dislocation according to recommendations contained in the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals.

Murine femur bone was dissected from the sacrificed mice, carefully cleaned from adherent soft tissue and washed several times with sterile PBS for discarding surrounding debris and blood and then immersed into 70% ethanol for a second followed by sterile water once again. BMCs were obtained according to methodology published previously[13]. Three groups of triplicates T-25 flask seeded with 1×10^5 viable BMCs resuspended in 4 mL RPMI medium contained 10% FBS. First group labeled as a control without any further treatments. Second group co-cultivated with three grams DiHAM. Third group co-cultivated with three grams viable cultivated HAM. All flasks were incubated at 37°C in humidified CO_2 incubator and the cultures were maintained and examined for cellular growth every 48-72 h.

***Tilapia nilotica* brain building up into HAM scaffold:** Viable and irradiated preserved HAM in this experiment was tested as a scaffold for attachment and growth of fish brain aggregates, mature *Tilapia nilotica* brain was minced mechanically by pestle and mortar, washed in 15 mL sterile tube by PBS twice at 1000 rpm for 10 min and resuspended in

5 mL PBS. About 500 µL of minced brain suspension were seeded on the center of HAM in a sterile glass petri dish according to methodology mentioned previously[14] with little modifications. Briefly, seeded HAM was folded toward upper side to permit diffusion of medium nutrients for feeding fish brain only through HAM scaffold. Growth medium F15 contained 10% FCS was added to about half of scaffold height with avoiding floating of cultured tissues. Control culture was prepared as above without using HAM scaffold. Cultures were incubated at 37°C in humidified CO_2 incubator.

Culture observation and maintenance were scheduled every 48-72 h by discarding the waste medium and unfolding HAM scaffold, seeded side was turned down facing glass surface for examination by inverted microscope and refolded back as previous before adding fresh medium for further incubation periods.

RESULTS

Cellular viability and growth: Trypan blue molecules are not able to permeate the vital cell membrane of viable cells unlike impaired membrane of dead cells, so dead cells appeared blue stained whereas the live cells still negative after 5 min of incubation with 0.4% trypan blue solution.

Maintaining the HAM in culture saved the viability of HAM till about one month of cultivation, in addition, the antimicrobial activity was almost not affected till the end of incubation period (Fig. 1). On the other hand, DiHAM appeared positively stained with trypan blue due to cellular death (Fig. 1b).

The HAECs isolated from viable HAM exhibit healthy cell membrane not permeable for trypan blue (Fig. 1c), unlike HAECs isolated from DiHAM that exhibit irregular and ruptured cell membrane and permeate the trypan blue revealing cellular death (Fig. 1d). Viable HAECs appeared in spherical shape in zero time of growth and adherent to the flask surface in spindle shape within 3 days of cultivation (Fig. 1e). Fourteen days from zero time cells reach to be confluent (Fig. 1f).

Antimicrobial activity of HAM: All microorganisms cultures were examined in this study rather Gram positive, negative bacteria or *Candida albicans* are sensitive to HAM extracellular secreted antibiotics that are seemed to be constitutive expressed in non stimulated condition of cultures, these were demonstrated by inhibition zone formed around wells punched into solid microbial culture loaded with consumed medium collected from only HAM culture after

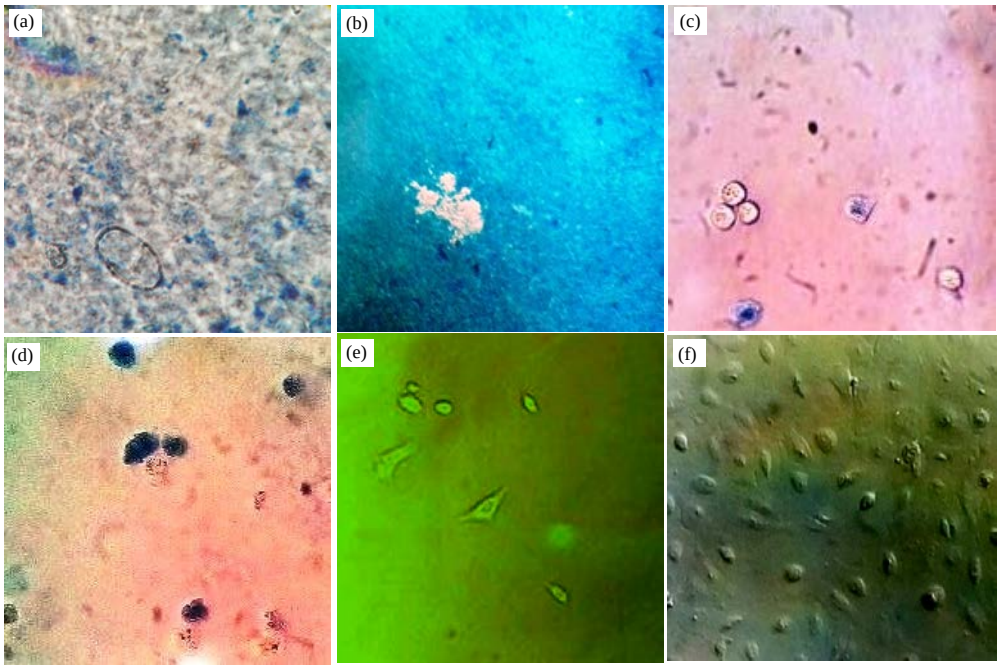

Fig. 1(a-f): Trypan blue permeability test for (a) Non-treated cultivated HAM, (b) Cultivated DiHAM, (c) HAM-isolated HAECs, (d) DiHAM-isolated HAECs, (e) Viable HAECs appeared in spindle shape adhered to T-flask surface and (f) Be confluent after 14 days of cultivation

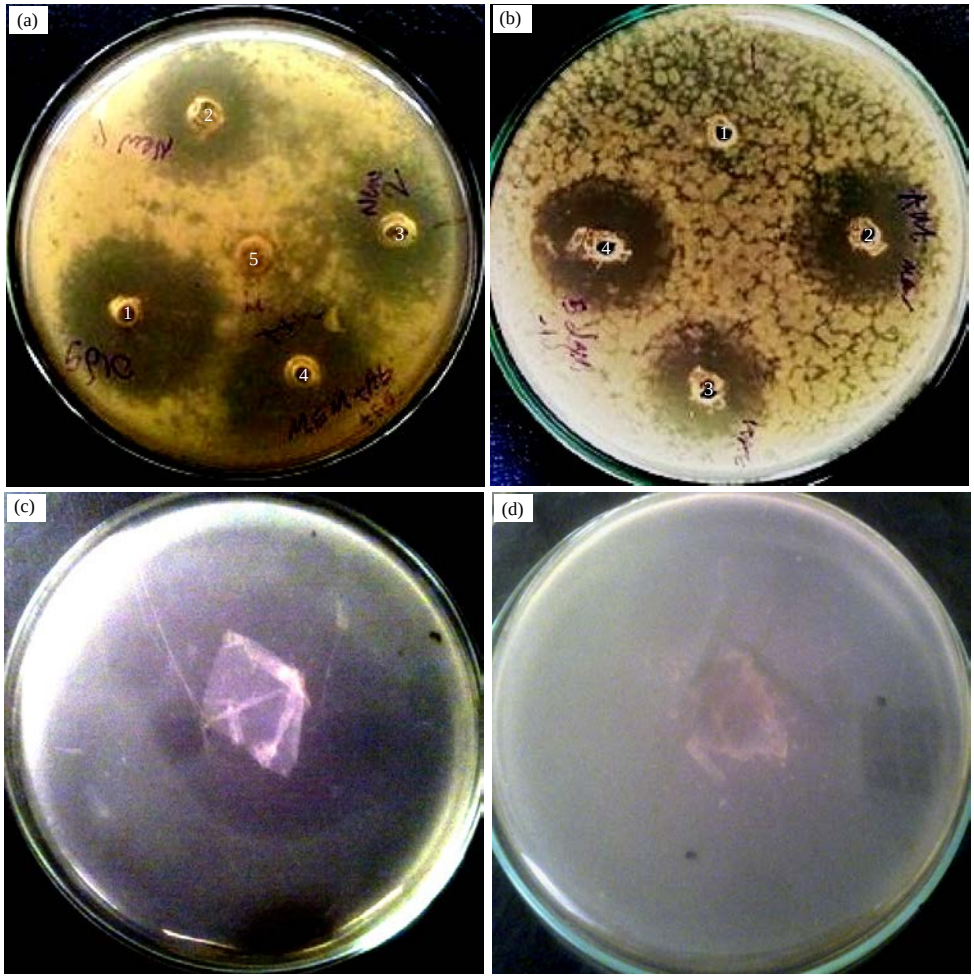

Fig. 2(a-d): Inhibition zone in microbial cultures, (a) MRSA culture; wells (1-3) loaded with collected medium from HAM culture from different batches, well (4) positive control loaded with medium contained 1x antibiotic/antimycotic mix, well (5) loaded with collected medium from DiHAM culture, (b) *Candida albicans* culture; well (1) loaded with collected medium from DiHAM culture, wells (2-4) loaded with collected medium from HAM culture derived from different batches, (c) *E. coli* culture inhibited by viable HAM layered on its solid culture surface and (d) *E. coli* culture layered with DiHAM and no inhibition zone formed

2-5 days post incubation at 37°C and not formed for DiHAM (Fig. 2a, b). Also, it was noted that HAM itself resulted extended inhibition zone in microbial solid cultures layered with HAM tissue and not formed in cultures layered with DiHAM tissue (Fig. 2c, d).

Cellular growth enhancement: Viable HAM co-cultivated with murine BMCs promoted hematopoietic stem cells colony formation during 10 days post cultivation in culture medium not supplemented with colony stimulation substances such as methylcellulose and growth factors. Neither independent culture nor co-culture with DiHAM revealed any healthy growth and subsequently cultures affected contamination by microorganisms (Fig. 3).

Also, fish brain growth also promoted via feeding through viable HAM more than DiHAM. However, cellular adhesion to HAM surface noted to be similar in zero time with both viable HAM and DiHAM. But, afterwards it was also noted that seeded cells were supported with filamentous connective canaliculi extended from only viable HAM microvilli that covered surface of epithelial side (Fig. 4), the same observation for adhered BMCs with viable HAM (Fig. 3) and not noted for DiHAM co-cultures.

HAM self healing: Denuded viable HAM resulted after three incubation times with trypsin/EDTA solution was found viable and higher metabolic active that able to reduce the red color of phenol red indicator to yellow color within only 24 h

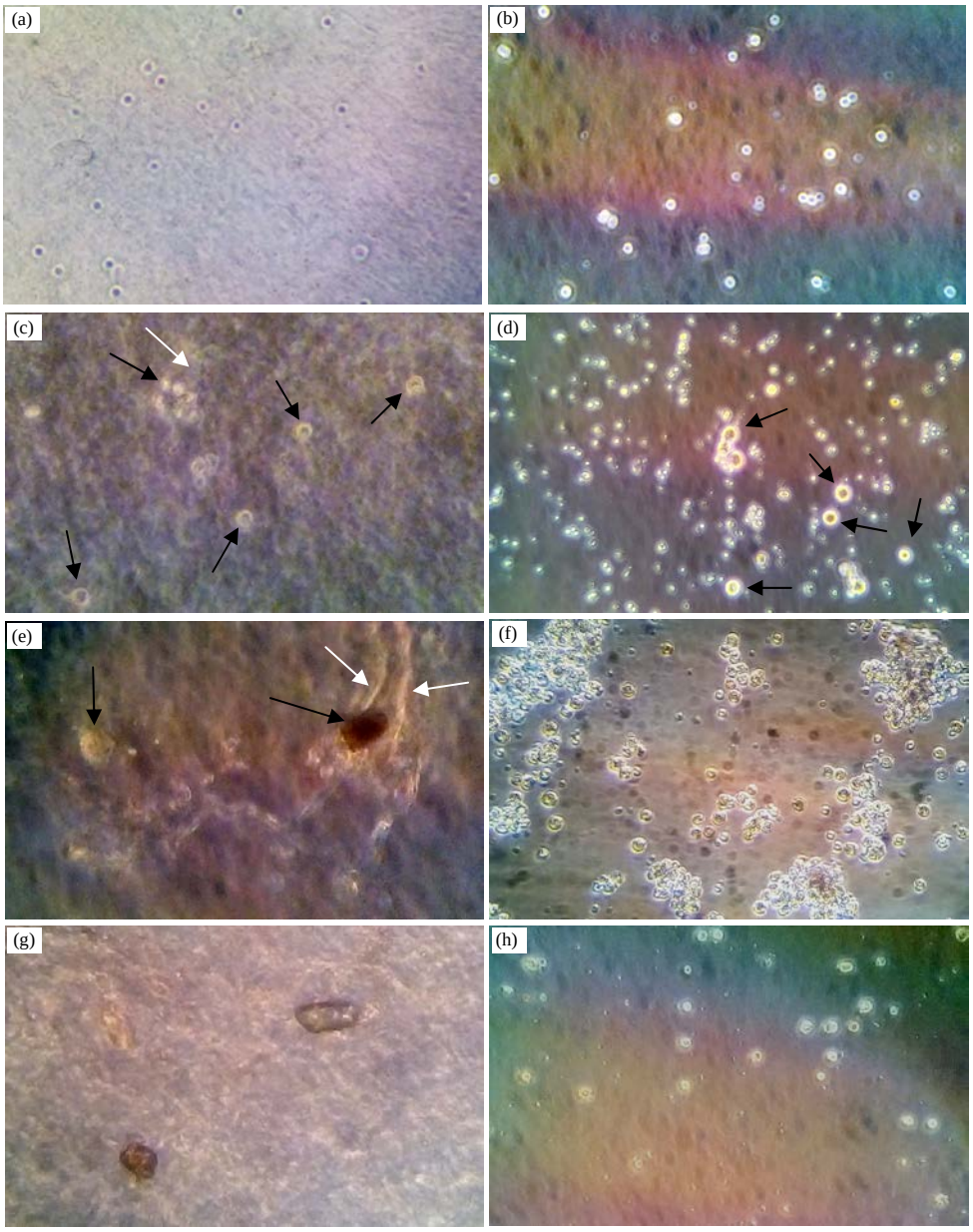

Fig. 3(a-h): Murine BMC co-cultivated with HAM, (a) Zero time of BMC adhered spontaneously to HAM villi, (b) Adhered on T-flask surface, (c and d) Multipotent progenitors-like morphology were noted in the 3rd day of co-cultivation with viable HAM (signed by black arrows), (e) Colony Forming Units (CFU) (signed by black arrows) appeared after 10 days of co-cultivation (viable HAM) and it was noted large amount of extracellular connective tubules (signed by white arrows) between adherent colonies and viable HAM villi, (f) CFU extended on T-flask surface after 10 days of co-cultivation with viable HAM and (g and h) using DiHAM in contrary

when comparing with intact viable HAM that able to reduce the red color to yellow within 2-3 days of cultivation in same conditions. Contrary, DiHAM showed no biological activity at all conditions of cultivation and appeared dead by trypan blue staining.

Morphologically, denuded HAM appeared after 4-5 days of cultivation folded on itself and compacted into smaller space by tight sealing of regenerated cells (may be epithelia) formed between surface splits and migrated over closed active denuded sites, these changes were not occurred in cultures of intact viable, or DiHAM (Fig. 5).

DISCUSSION

Importance of biological features of viable HAM as a promising material for clinical treatments and cell culture

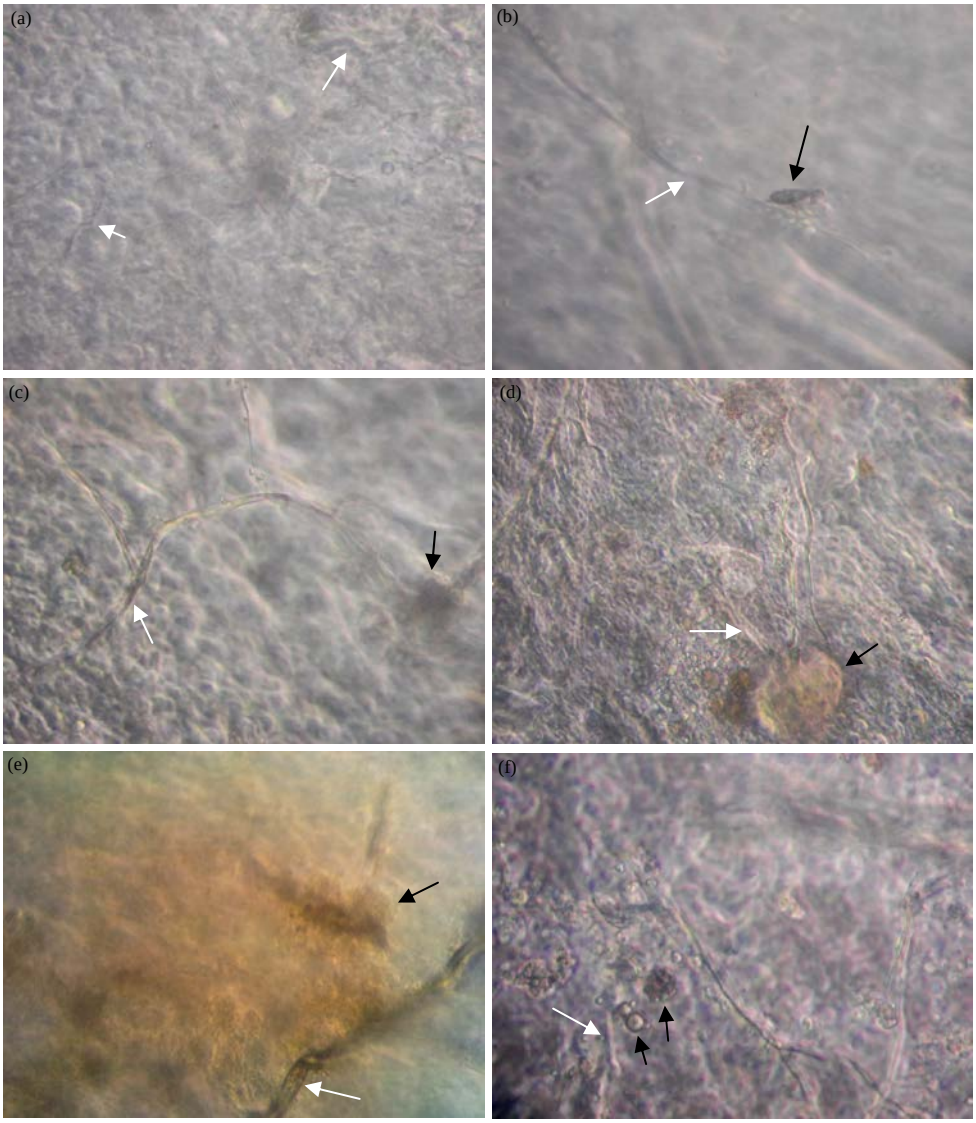

Fig. 4(a-f): Fish brain minced and seeded on epithelial side of HAM as a scaffold, (a) HAM before seeding, (b) 15 min after seeding and cultivation, (c) 5 days old culture, (d) 8 days old culture, (e) 14 days old culture and (f) 14 days old culture seeded onto DiHAM. White cursor signed the HAM extended connective tubules and the black cursor signed brain fragments

application were *in vitro* demonstrated in this study by verification the ability of viable HAM for enhancing the growth of applied cells with protecting cultures against microbial contamination, in order to investigate the presence of these features if still remained after tissue banking of HAM or not.

There are many studies recommended storage of HAM by tissue banking before medical application. The most common and economic method for HAM tissue banking was recommended by many studies[7,15], in which dehydrated HAM sterilized by γ irradiation, by this method HAM become more easy to store, fast handling, safe and have a longer shelf life. So, this study evaluated the end product of HAM tissue bank concerning the biological features those proved in viable HAM.

Microbial inhibition of HAM was proved by this study results and other related studies[16], which is due to its extracellular secretion of various types of low molecular weight peptides such as defensins which are considered as broad-spectrum antibiotics[17]. These defensins impair the cytoplasmic membrane of susceptible bacteria[18] and some types of these seemed to be constitutively expressed as a house keeping genes in HAM cells[19,20], which is also proved here by the antimicrobial activity of non stimulated HAM cultures.

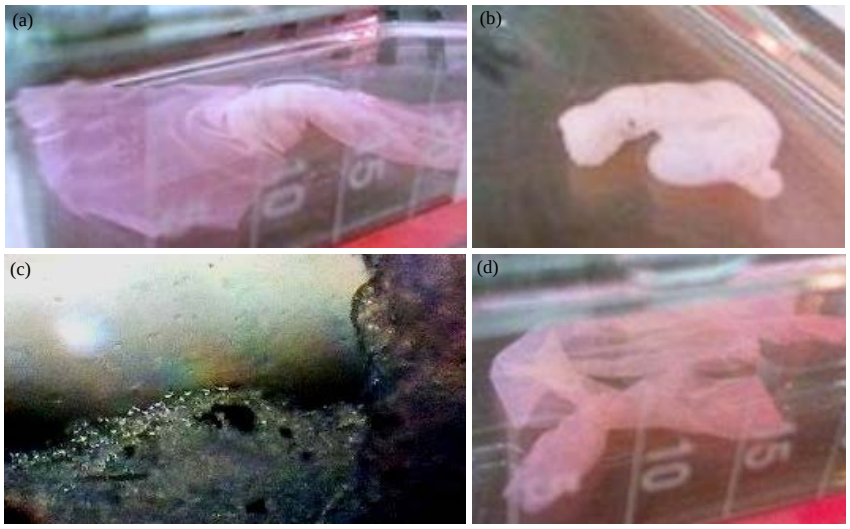

Fig. 5(a-d): Denuded HAM self healing after digestion by trypsin and cultivation into T-25 flask, (a) Zero time post treatment, (b) Viable HAM self folding for repairing, (c) viable HAM digest site healing supposed via migration of epithelial cell between closed healing sites and (d) DiHAM denuded culture

Antibiotic effect of HAM was verified in this study by taking inoculums from the clear zone around positive well of inhibition to be stroke on new agar medium optimal for bacterial growth. After 14 days of culture incubation it was found that no sign for microbial growth or colonies were detectable which is proved that inhibition zone formed in bacterial cultures were resulted by bacterial killing not just growth inhibition.

Antimicrobial features of HAM gave excellent protection from bacterial contamination of co-cultivated cultures, in addition to growth promotion due to growth factors donation; were proved in this study using culture media not supplied with growth factors additives, antibiotics and antimycotics. These results may advance the using of intact HAM as an alternative cell culture feeder instead of using commercial non-divided cell feeders (which are almost not featured with antimicrobial activity as HAM) for enhancing the growth of co-cultivated cells but needing more confirmation in subsequent work.

Furthermore, importance of HAM in tissue engineering application as a scaffold for mammalian tissue building up was previously demostrated[14]. In addition, results of this current study proved that HAM also promotes the growth of non mammalian cells as resulted with fish derived cells as well as mammalian cells of murine BMCs.

Growth of co-cultivated cells on HAM surface shown to be attached via filamentous connective tubules; those noted to be enlarged by adhering stimulation with co-cultivated cells, which is may act as a feeding channels to enhance the exchanging of nutrients supplying and metabolites disposal between HAM and seeded cells, this feature was found only in viable HAM, which is may be useful for tissue building up using HAM scaffold.

Yatim *et al.*[21] found that gamma irradiation of HAM at dose 25 kGy affect the quality of the extracted RNA and caused completely degradation of RNA molecules depending on the visibility of RNA on stained agarose gel electrophoresis, the irradiation sample shown absent RNA when compared with integrated RNA extracted from fresh HAM sample. It is known that intact RNA only presented in metabolic active cells. This fact proved the importance of HAM viability to utilize its own healthy secretome especially for genes encoding antimicrobial peptides, cytokines and growth factors to accelerate the healthy growth of targeted cells.

Subsequently, it was expected that cells derived from irradiated biopsy at sterility dose of γ-irradiation will be dead and unable to grow in cell culture. So, HAECs derived from DiHAM appeared in irregular shape due to cell membrane damages and not able to be established in cell culture. Same outcomes were found with keratinocytes and fibroblasts derived from γ-irradiated (dose = 25 kGy) dermal biopsy in related study[22].

Cellular adhesion of seeded cells to amniotic membrane was referred to the rich content of HAM with fibronectin[23], results of this study reflected that this feature was not affected by HAM irradiation during tissue banking process. So, it was deduced that fixed HAM may be useful as a substrate for targeted cells migration but will not provide

secretions for growth factors needed for cellular duplication. For this reason, cellular growth enhancement and HAM self re-epithelialization were succeed with viable HAM not DiHAM.

So, in relative study it was recommended to apply the fresh prepared HAM as a viable tissue for the treatment of damaged ocular surface to utilize both antiangiogenic and anti-inflammatory factors delivered from HAM epithelial cells surface when be in contact with the treated ocular surface[1].

Exploring clinical trials used HAM grafting for leg ulcer healing in 50 patients, to compare the treatment with viable HAM versus the treatment with 25 kGy gamma-irradiated/dried HAM; Faulk *et al*.[9] succeeded to cure the leg ulcer in 5 days only using viable cultivated HAM, whereas treatment with irradiated dried HAM took about 2-6 weeks for healing[24].

Consequently, this study recommend the using of viable HAM than DiHAM for utilizing the benefits of active HAM secretome in several medical applications and also as a feeder for *in vitro* promoting of animal cell culture growth.

CONCLUSION

Biological activity of viable HAM promoted the growth of cell cultures and fight microorganisms. But after irradiation tissue banking of HAM, HAM was no longer viable and subsequently was not featured with biological activity.

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