Med. J. Cairo Univ., Vol. 92, No. 2, Accepted 28/2/2024
DOI: 10.22608/MJCU. 429-441, June 2024
www.medicaljournalofcairouniversity.net

Induction of Hyaluronic Acid Production from Amniotic Epithelia and Irradiated *Pasteurella Multocida* Against bone Marrow Cells Aging *in Vitro*

ALAA OSAMA, M.Sc.**; WALEED A. NEMR, Ph.D.*; MEDHAT HELMY, Ph.D.** and OMAIMA KHAMISS, Ph.D.**

The Department of Radiation Microbiology, National Center for Radiation Research & Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Cairo, Egypt and Department of Animal Biotechnology**, Genetic Engineering & Biotechnology Research Institute (GEBRI-USC), University of Sadat City, Egypt*

Abstract

Background: Hyaluronic acid (HA) is a biopolymer molecule that stimulates cell growth by supporting the building of the extracellular matrix (ECM). Therefore, it has been applied in many biomedical researches. The common biological resources for producing HA are animal tissues and bacteria. Cell aging is attributed to the reduction of cell division capacity and ECM quality.

Aim of Study: The current study was aimed to evaluate the effect of irradiation on the productivity of *Pasteurella multocida* to HA and compare its biological effect with a human-sourced HA.

Material and Methods: Human amniotic epithelial cells (HAECs) were isolated from the amniotic membrane and seeded *in vitro* in to cell culture dishes with optimized different experimental conditions to produce HA. This was verified by gene expression profiling of three enzymes (HAS1, HAS2, HAS3 for HA synthesis using quantitative PCR analysis. *P. multocida* was induced to produce large quantities of HA by gamma irradiation. Then, the produced HA from HAECs (HAECs-HA) and *P.multocida* (Pm-HA) were extracted by ethanol-precipitation methodology. Then the extracted HAs were analysed and compared against a standard HA (sHA) by Fourier transform infrared (FTIR) assay. Aged murine bone marrow cells (m BMCs) were isolated from a femur of a 2-year-old rat, and cultivated in replicates into a 96-well cell culture plate. Each triplicate of m BMCs cultures was treated with one of the extracted HA (HAECs-HA or Pm-HA). Non-treated cultures were assigned as control. Cellular migration and proliferation were evaluated by microscopic examination and MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay.

Results: The optimized HAECs cultures for producing HA were determined inthe multi-layering-conditioned cells and those in the agitated culture. The principalsyn thesis enzymes that express HA in HAECs were HAS2 followed by HAS3. The Pm-HA was increased in the *P. multocida* culture after the induction by a dose of 2 KGy gamma irradiation more than other irradiation doses. FTIR assay showed chemical similarities between both synthetic HA products with sHA. The microscopic evaluation revealed the superior effect of HAECs-HA in the improvement of BMCs growth (until confluence) and migration than Pm-HA and saline (couldn't reach confluence). This was also confirmed by the results of the MTT assay.

Conclusion: This observatory study consider as the first approach for producing HA from HAECs culture in vitro that has a biological effect to improve the migration and the growth of aged mBMCs more than the bacterial HA. Therefore, these outcomes encourage further studies to evaluate the clinical feasibility of using HAECs-HA as an anti-aging ingredient.

Key Words: *Hyaluronic acid – Placenta – Bacteria – Bone marrow – Cellular growth.*

Introduction

THE HA biopolymers are the superior component of ECM which regulates the structure and the integrity of tissues. For instance, HA improves skin hydration and volume due to its hydrophilic structure. In addition, HA binds with structural proteins such as collagen and fibronectin, which are responsible for maintaining the elasticity of the skin. Therefore, it is used widely as a cosmetic ingredient for improving the firmness of the skin and maintaining skin wrinkles and dryness **[1]**. Moreover, HA regulates the response of skin to regenerate injured tissues into wounds that stimulates healing action **[2]**. Naturally, HA is found abundantly in the ECM of the connective tissues and epithelial cells. This is attributed to the action of HA in the reduction of inflammation, and lubricating body's joints. On the other hand, HA forms the bacterial capsule in some pathogenic bacteria; such as *Streptococcus zooepidemicus*, *Cryptococcus neoformans*, and *Pasteurella multo-*

Correspondence to: Dr. Omaima Khamiss,
E-Mail: Omaima.khamiss@gebri.usc.edu.eg
okhamiss@yahoo.com

cida **[3].** This HA capsule plays an important role in establishing the infection in the host body, this is by promoting adherence to the targeted tissue and preventing phagocytosis of the host's immune system. So, the production of HA capsules is considered a virulence factor in such pathogenic bacteria **[4].** *P. multocida* is the predominant bacteria present in the pneumonic lungs of livestock animals. Four subtypes of *P. Multocida* were classified according to their capsule type; A, B, C, and D. In particular, the subtype A produces a capsule of HA. Specifically, *P. multocida* is the only known bacterium owning HA synthase class II, which is featured than class I (presents in Streptococci) including; larger molecular weight, not need to liposomal transportation through the excretion to the cell membrane, has 2 modules of glycosyltransferase 2 (versus one module in class I) for adding sugars during the catabolism of polysaccharide chain and adds Uridine diphosphate-sugars to the growing HA chain from the non-reducing end. Therefore, the HAS of *P. multocida* has been inserted into many expression vectors for the synthesis of Pm-HA through genetic engineering **[3].** However, no enough information was published regarding the biological activity and the cytotoxicity of Pm-HA.

Placental stem cells are used widely in the field of biomedical application, this is for many reasons; including the obtaining operation of them is not invasive, the placenta is a biomedical waste (after birth) which makes the using of placental-derived products not opposing ethics, and they are low immunogenic transplants due to their immunomodulatory effect. Particularly, scientific records cited that HAECs, which are sourced from the amniotic membrane (the innermost layer of the placenta) could secret highly desirable substances in the field of regenerative medicine; such as growth factors, anti-inflammatory cytokines, and several ECM proteins. The anti-aging effect of HAECs has been demonstrated in previous experimental trials **[6].** However, no enough molecular and in vitro explanations are available to understand their paracrine effect.

Therefore, this study tends to investigate the productivity of HAECs for HA (as a one of anti-aging molecules) under different cell culture conditions, and compare its possibility to induce the proliferation of aged mBMCs versus a bacterial-sourced HA extracted from stimulated *P.multocida* by the irradiation stress.

Material and Methods

Microbiological experiments:

P.multocida was isolated from the lungs of pneumonic sheep and identified morphologically and biochemically according to identification standards published by MacFaddin **[7]** Molecular identification of *P.multocida* Type A was based on the specific determination of hyaC-hyaD genes by PCR testing, according to the published methodology by Furian et al. **[5].**

This study was conducted laboratory of amniotic membranes, Egyptian Atomic Emergy Authority, Cairo, Egypt From October 2020 – Dec. 2022.

Stimulation of HA productivity from bacteria:

Mass production of *P.multocida* cells was carried out in a 250ml conical flask containing 100 ml Brain-Heart infusion (BHI) broth under shaking at 37°C overnight. The viable bacterial count was enumerated by the total plate count of colony-forming units (CFUs). Accordingly, the bacterial suspension was divided into aliquots containing $1x10^8$ CFUs. The bacterial mass of each aliquot was collected by centrifugation at 4000 rpm for 15min. The supernatant was aspirated and the pellet was re-suspended into 1 ml fresh BHI broth. Aliquot suspensions were then transferred into T-25 flasks with filter-cap, to ensure good ventilation condition, and lyophilized in a horizontal position (onto Labcono freeze-drier shelf) until complete dryness. Then, the lyophilized aliquots were divided into 7 groups; each group involves 3 flasks. Each group was exposed to a radiation doses of 0, 2, 4, 6, 8, 10, and 12 KGy gamma rays in the Gamma-cell 220 Cobalt-60 Irradiator Facility (NCRRT). The dose rate of the irradiator was equivalent to 0.84KGy/hour. Subsequently, each aliquot was reconstituted into 5ml saline Na cl 0.09% and enumerated for CFU count. The dose-response curve (DRC) was computed by plotting the exposure dose of gamma irradiation against the LOG of survival count of bacteria on the X and Y axes, respectively, using Microsoft Excel Software. In addition, the slope (b) of the DRC regression line was calculated to estimate the D_{10}-value of the prepared *P.multocida* culture according to the standard equation: D_{10}-value = -1/b, as published elsewhere **[8].** Then, two inoculums from each aliquot containing $1x10^2$ CFUs were used for fermentation test into 5ml BHI broth (in T-25 flask) and a thin layered BHI agar plates (by pour plate method). The fermentation was carried out overnight at 37°C without shaking.

The capsular formation of *P.multocida* was examined morphologically in the BHI agar platesunder a light inverted microscope, and the productivity of HA during fermentation was examined by extracting HA from the fermented medium after discarding the bacterial cells by centrifugation at 5000 rpm for 20min. The extraction of HA from the aspirated medium of the fermented *P.multocida* culture is based on ethanol precipitation.

HAM preparation:

Human placentas were collected after caesarean delivery from Kasr Al-Ainy Maternal Hospital, Faculty of Medicine, Cairo University, Egypt. Donors were checked before the operation for hepatitis B (HBV), C (HCV), and human immunodeficiency

virus (HIV). HAM was separated from the placenta by blunt dissection, and packaged into a sterile container containing 50ml of freshly formulated Hank's Balanced Salt Solution (HBSS) with 100 ug/ml Gentamicin and 0.5ml of Antibiotic/Antimycotic 100x mix (Lonza).

Separated HAM was then transferred under aseptic conditions into a new sterile metal basin containing 250ml of 0.9% Sodium chloride balanced salt solution (NS) and gently shacked into a shaking water bath at 37ºC for 15min, washed three times till the clearance of HAM from cell debris and blood *[9]*.

Isolation and cultivation of HAECs:

The experimental design, of the in vitro trials,targeted the induction of HAECs to optimize the production of HA. These trials include changing primary culture conditions and culture substrate. The traditional cultivation method of HAECs was published previously *[9]*, the HAECs culture which was prepared by this cultivation method was considered as a control culture to compare it with the modified trials.

The First modification includes the type of culture vessel. There are two types of vessels were tested; plastic T-flask and metallic basin. The second modification includes culture agitation; static culture and shacked suspension culture. The third modification includes culture additives; such as HAM collagen extract, foetal bovine serum (FBS), or both of them. The fourth modification is based on the selective adherence time of HAECs for establishing cultures of different adherent HAECs. The last modification includes culture medium; MEM and D-MEM F12 were tested (Table 1). The produced HAs were examined to maintain and restore the growth of aged murine bone marrow.

Practically, HAM was divided into ~5g (±0.5) pieces. Each piece was transferred into a T-25 cell culture flask containing 10ml of freshly formulated HBSS, and incubated at 37ºC under gentle shaking. Passed HAM pieces were washed once again with 20ml NS, and then incubated with 10ml of freshly formulated HBSS containing 0.25% Tryps in (W/V) at 37°C for 2 hours with gentle shaking. This trypsinization step was repeated three times for about 80% dissociation of HAECs which were collected from each incubation time by centrifugation at 1000 rpm for 15min. HAECs pellet was washed twice with 10ml HBSS, then, were re-suspended with a pre-warmed cell culture medium including the assigned additives, and were plated into the culture vessel according to the experimental design (Table 1). The plating count of HAECs was 105 viable cells per T-25 flask. This was estimated by counting viable cells on a haemocytometer slide under a light microscope after staining with Trypan blue. Generally, Patches of HAECs that showed a viability of less than 85% were eliminated from the subsequent experiments.

Table (1): The experimental design of modifications that were examined on HAECs traditional culture for investigating their effect on the expression of HA.

Culture Type	Adhered (A)/ non-adhered (N)	Additives: Lcg-HAM/ FBS	Media: MEM /DMEM:F12	Vessel: T-Flask /metal basin	Cultivation methodologies
Traditional (Control)	A	FBS	DMEM:F12	T-flask	Dissociated HAECs from native HAM were plating into T-25 flask with DMEM:F12 traditionally.
T1	A	FBS	MEM	T-flask	HAECs were plated as Traditional control culture, but with MEM that instead of DMEM:F12.
T2	A	FBS	MEM	T-flask	Multi-layered cells with lower adherent 5x106 HAECs taken from primary suspension culture.
T3	N	FBS	MEM	T-flask	Multi-layered cells with non-adherent 5x106 HAECs taken from primary suspension culture.
T4	A	Both	MEM	T-flask	HAECs were prepared and plated as the traditional control with supplying HAECs-HA to the medium.
T5	N	Both	MEM	T-flask	HAECs were prepared and plated as the traditional control with supplying Pm-HA to the medium.
T6	N	Without additives	MEM	Metal basin	Floated HAECs propagated from pd- HAM into a metal basin.

The control culture was performed in a T-25 flask and fed by DMEM: F12 (Lonza) supplemented with 10% Fetal bovine serum (FBS) and 1x concentration of Antibiotic/Antimycotic 100x mix (Lonza). Modified cultures were labelled from T1-T6. All static cultures (T1-T5) were incubated at 37°C in the humidified incubator and equilibrated with air (while the control cultures were equilibrated with 5% CO_2). All cultures were maintained every 2 days by replacing the conditioned medium (CM) with a fresh medium.

In detail, the T1 culture is similar to the control culture but are vary in the type of culture medium; Hank's MEM (Sigma-Aldrich)instead of DMEM: F12 without any further modifications, Also, T2-T6 cultures were fed with Hank's MEM but with certain modifications. HAECs in T2 and T3 cultures were planted selectively based on the variability of their adherence time to the flask surface. Lower adhered cells (T2) were aspirated as floats after 30min of adhering time from the primary adhered culture. While the non-adhered cells (T3) were aspirated from the T2 culture after 1 hour as adhering time at 37°C. HAECs in T4 and T5 cultures are similar to T1 but with adding 0.1ml (100ug/ml) of previously extracted HA from HAECs or P.multocida, respectively, to the medium (the method of HA extraction is described here later).

The only dynamic culture (T6) was prepared by the cultivation of trypsinized HAM, after ~80% of HAECs removal, with serum-free Hank's MEM, in a 37°C –adjusted water bath with gentle shaking. Floated HAECs released from this partially denuded HAM (pdHAM) culture were collected after 7 days of cultivation. Generally, all cultured cells were collected after ~90% confluence and subjected to RNA extraction procedure as mentioned later.

Gene expression assay:

The investigation of the effect of different cultivation conditions of HAECs on the gene expression of HAS1, HAS2, and HAS3 which are responsible for hyaluronic acid synthesis in humans.

Primers design:

The primer design depends on finding pairs of primers that could amplify various spliced transcripts of each targeted gene, without non-specific or arbitrary amplification of unintended genes in the human transcriptome. This was verified by carrying out sequence alignment of selected primers against human transcriptome sequences using Primer-BLAST online utility on the NCBI website. Furthermore, to avoid unintended matching of primers with chromosomal DNA genes, the selected primers were specified to span exon-exon junction locations. Detailed primers information was listed in Table (2).

Table (2): List of selected primers for RT-PCR amplification of targeted genes transcripts.

Gene	Primer	Sequence: (5' 3')	Length	Tm*	Reference
HAS1	Forward	GTGTATCCTGCATCAGCGGT	20	60.18	All are new designed primers
	Reverse	ACCTGGAGGTGTACTTGGTAG	21	58.46	
HAS2	Forward	ACGTAACGCAATTGGTCTTGTC	22	59.78	
	Reverse	TCGTACTTGTTTAAAATCTGGACAT	25	57.03	
HAS3	Forward	ACTACATCCAGGTGTGCGA	19	58.33	
	Reverse	ACGCTGCTCAGGAAGGAAAT	20	59.67	

* The calculation of Tm (melting temperature) is according to NCBI primer BLAST online utility.

RNA extraction from HAECs:

RNA was extracted from HAECs using Qiazol reagent (Qiagen, USA) according to manufacturer instructions. Briefly, each 1ml of cell suspension in isotonic saline was added to one ml of Qiazol and shacked vigorously for 1min. Then, 0.2ml chloroform was added and mixed by inversion. The extract was centrifuged at 10000 rpm for 15min. Then, the aqueous phase was aspirated and transferred into a new tube containing 0.5ml of pre-chilled Isopropanol. RNA precipitates were collected by centrifugation at 12000 rpm for 15min under cooling and washed once again with 1ml 70% Ethanol. RNA pellet was air-dried under sterile air flow into a vertical air-flow cabinet and then re-suspended with 50ul nuclease-free water at 65o C for 15min. The optical density of dissolved RNA was determined by a Nanodrop spectrophotometer.

Real-time PCR:

cDNA synthesis from mRNA was carried out by adding 20ul suspension, containing 50ng RNA, to a premade tube of Maxime RT premix kit with oligo (dT) 15 primer (Intron Biotechnology, Korea). Then, 10ul of cDNA product to PCR mix of Hot Fire Pol Eva Green qPCR Mix Plus (Solis Biodyne, Estonia)with a pair of gene-specific primers according to manufacturer instructions, PCR was conducted into Rotorgen Q HRM platform (Qiagen, USA), according to the guide manual. The thermal profile

of PCR was adjusted at 95°C for 15min, as a pre-denaturation step, followed by 45 cycles of denaturation step (95°C for 40sec), annealing step (55°C for 15sec), and extension step (72°C for 45sec). Then, high-resolution melting analysis (HRM) was conducted as a finalization step.

Hyaluronic-acid extraction:

The protein content was eliminated by treating the fermented/conditioned medium with 4% (w/v) Trichloroacetic acid; the reaction was carried out in a 15-ml centrifuge tube and incubated vertically at 4°C for 2 hours. Then, the protein pellet was discarded after centrifugation at 10 000 rpm for 30min under cooling, and the supernatant was transferred into a new tube. The HA was precipitated from the supernatant by adding two volumes of pre-cold Ethanol 99%, the tube was inverted 10 times for mixing. Mixed tubes were incubated vertically at –20°C overnight, and the HA was collected as a pellet by the centrifugation at 10 000 rpm for 30min under cooling. The pellet was left to dry under laminar airflow and dissolved in 0.5 volume sterile distilled water. The negative control sample of HA (blank) was prepared similarly but from a fresh complete medium. The positive control sample of HA was prepared as above from sHA (Sigma-Aldrich, product number 924474). The concentration of HA was calculated by spectrophotometry at 400 nm, according to Shaheen et al. **[10]**. Then, the final concentration of each extracted HA was diluted to 100ug/ml; as a working solution for the subsequent experiments.

FTIR spectroscopy:

Dissolved HA was analysed using FTIR spectroscopy VERTIX 70 (BRUKER OPTICS GmbH). The negative control sample (which was prepared from fresh medium) was assigned as blank to eliminate background interference. All extracted HA, from each prepared culture, were compared to the extracted HA from sHA which was assigned as a reference sample.

Treatment of murine bone marrow cell culture:

The effect of previously prepared amniotic and HA extracts to induce cellular growth of murine bone marrow cells (mBMCs) was investigated in this experiment. The rat femur was dissected immediately from a recently dead young, normal rat. Tissue debris was eliminated with sterile blades, and the femur was washed three times with sterile NS, under aseptic conditions. The ends were cut by sterile scissors. mBMCs were released by flashing 3 ml NS from an end using a sterile syringe. The mBMCs were washed twice with 10ml NS and collected by centrifugation at 1000 rpm for 15min, then the cells pellet was re-suspended in 5ml complete MEM containing 15% FBS.

Viable cells were enumerated using a haemocytometer slide loaded with Trypan blue-stained cell suspension (the procedure was mentioned before). Then, 10^5 /well of viable BMCs were plated into a 96-well cell culture plate. The culture was incubated for 2 days at 37°C in a 5% CO_2- atmospheric incubator. Subsequently, the medium was replaced with 0.1ml fresh complete medium containing 10% FBS and 10ul of one HA-preparation (HAECs-HA or Pm-HA). Each preparation treated triplicates of wells during the maintenance of cultures. As well, control cultures were supplemented with 10 ul saline (instead of HA) in their media. The cultures-plate was incubated at 37°C in a 5% CO_2-atmospheric incubator and maintained every 2 days, by replacing the conditioned medium with fresh similar formulated one, for 14 days. Then, the cultures were examined by MTT assay as described below.

Proliferation assay by MTT:

This assay is based on the ability of viable cells (not dead) to produce NAD(P)H-dependent oxidoreductase which reduces the yellow-MTT solution to purple Formazan crystals. Practically, mBMCs (in the 96-well plate) were incubated with 0.1ml complete MEM containing 10% (V/V) of 5mg/ml MTT-reagent (Sigma-Aldrich) at 37°C for 4 hours. The reaction was stopped by replacing the medium with 50:50 Isopropanol: DMSO. The cell culture plate was left overnight under shaking to dissolve the formed Formazan crystals which was determined using Microtiter plate reader (Huma Reader HS, Germany) at wavelength = 570nm.

Results

Bacterial identification:

P.multocida was identified as non-hemolytic colonies in blood agar medium; short rods cells; negative for Gram stain; positive for catalase,oxidase, indole, and nitrate reduction tests; and it cannot ferment lactose in Maccon key agar medium.

The molecular identification of this bacterium is based on the specific determination of HA-producing genes (hyaC-hyaD) by PCR testing. The loaded PCR product into the gel electrophoresis system appeared as a single band at 1044 bp (Fig. 1). This confirms that this bacterial isolate is sub-classified under *P.multocida* subtype A which can produce HA capsule.

Productivity of bacterial HA-capsule:

The selection of well encapsulated bacteria was based on the ability of the encapsulated bacteria (post-fermentation) to resist gamma irradiation stress. Therefore, fermented bacterial cultures contain viable bacterial cells after irradiation up to 10 KGy. The calculated D10-value was 0.81 KGy. This indicates that *P.multocida* is somewhat radio-resistant.

The morphology of the capsule was variable among cultures exposed to different doses of gam-

ma radiation. It was noted that the size and the intensity of the bacterial capsule increased with the increment of irradiation dose (Fig. 2). According to the quantitative analysis of HA via spectrophotometer assay, it was noted that a lower irradiation dose (2KGy) induced the productivity of HA in the fermented medium. But, higher irradiation doses caused alterations in the capsular structure, as a defence action from the bacteria against the irradiation stress (Fig. 3).

Impact of modifications on HAECs cultivation:

Each modified preparation of HAECs culture showed alterations in the traditional culture regarding the cellular morphology, cellular migration on the culture vessel's surface or on a substrate, confluency form and time, the structure of the extracellular matrix (ECM), and the productivity of HAS1-3; as shown in Figs. (4-5) and Table (3).

Table (3): Variability of HAECs cultures in response to their cultivation modifications.

Culture	Cellular morphology [a]	Confluence formation and time	Appearance of ECM [a]	HA productivity [b]
Control	Adherent HAECs appeared in a spherical shape after plating for 3 days and then turned to the spindle shape.	12 days, mono-layered horizontal confluence.	Not observed	Normalized
T1	Adherent HAECs appeared in a spherical shape after plating for 3 days and then turned to the spindle shape.	14 days, mono-layered horizontal confluence.	A meshwork of collagenous matrix was formed in agglomerations above a mono-layer of adhered cells.	n HAS1 ↑ HAS2 ↑ HAS3
T2	Adherent HAECs appeared constantly in a spherical shape from the plating time till the end of culture (14 days).	Cells grew vertically in multiple layers. But, horizontal confluence was not completely reached till the end of culture.	A large amount of white-coloured ECM was appeared surrounding multiple-layered cells.	↑ HAS1 ↑ HAS2 ↑ HAS3
T3	Small sized spherical cells were loosely adhered to the flask surface during the cultivation time.	7 days	Not observed	↓ HAS1 n HAS2 ↓ HAS3
T4	HAECs adhered to a high dense ECM and appeared constantly in a spherical shape.	No growth was observed	A high dense ECM appeared in dark colour since 2 days of cultivation	↓ HAS1 ↑ HAS2 ↑ HAS3
T5	HAECs grew into floated clusters made up of high dense ECM and appeared constantly in a spherical shape.	HAECs grew into clusters without horizontal confluence.	A high dense ECM appeared in a dark coloured- network which formed between the clustered HAECs.	↑ HAS1 ↓ HAS2 ↑ HAS3
T6	Small-sized, Non-adhered HAECs appeared constantly in a spherical shape.	HAECs appeared in a high density growth; the confluence in this case was not sensed for stopping the growth.	Separated HAECs produced dark ECMs surrounding them.	↑ HAS1 ↑ HAS2 ↑ HAS3

a The morphology of HAECs and their ECMs was visualized and described in Fig. (4).

b HAECs productivity of HA is depending on the quantitative gene expression results of real time RT-PCR compared to the normalized genes of the control cells in the traditional culture, this is referring to GAPDH housekeeping gene (data shown in Fig. 5).

↑ Indicates to up-regulated expression of the gene, compared to the control cells.

↓ Indicates to down-regulated expression of the gene, compared to the control cells.

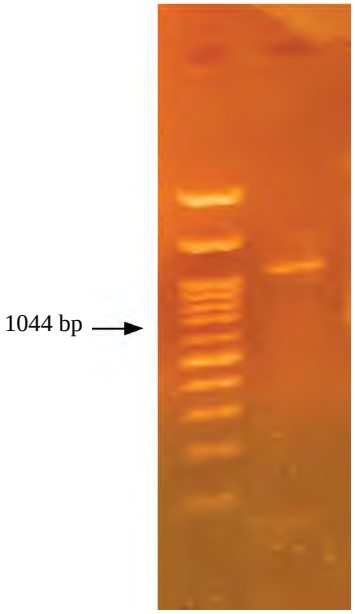
n Indicates to normal or unchanged expression of the gene, compared to the control cells.

FTIR analysis:

Spectrum characterization of HAECs-HA and Pm-HA by FTIR showed a similar profile to the sHA sample. Particularly, all samples showed similar absorption at ~3425cm^{-1} which represents OH and NH bonds, ~2980cm^{-1} indicates CH symmetrical and CH_2 asymmetrical stretching, the spectra from ~1200 to ~1655cm^{-1} are the range of amide groups, and the polysaccharide bands are ranged from ~950 to ~1200 cm^{-1} (Fig. 6).

Induction of aged mBMCs growth:

The control culture of age dmBMCs showed less metabolic activity with no ability to grow, while cultures supplied with any of both sourced HA showed better metabolic activity than the control culture. In particular, as shown in Fig. (7), many dead mBMCs appeared in the control culture, and even the remaining live cells were still spherical (as the primary time) and poorly adhered to the plate surface. On the other hand, cells in the supplied cultures with any HA maintained their viability in different morphologies depending on the type of HA; HAECs-HA induced cellular growth and migration on the plate surface in a spindle-shaped morphology, and Pm-HA preserved the viability of mBMCs in the primary morphology without inducing the cellular division or migration. Notably, no decrement in the viable count of mBMCs has been observed due to the treatment with Pm-HA or HAECs-HA during the time of experiment, this indicates lower cytotoxicity. In harmony, the optical density (OD) readings at 570nm of MTT assay indicated the greater amount of the formed Formazan crystals in treated mBMCs cultures with HAECs-HA (OD = 0.878±0.006) than those treated with Pm-HA (OD = 0.480±0.031) and the untreated cultures (OD = 0.427±0.042). Noticeably, Formazan crystals are formed only into metabolic-active cells. Thus, the increment of OD value in MTT assay indicates the large quantity of viable cells (Fig. 7).



Fug. (1): Gel electrophoresis image of positive PCR determination ofhyaC-hyaD genes (amplicon size = 1044), this indicates the ability of *P. multocida* to produce HA capsule. The 100 bp-DNA ladder showed in the left lane.

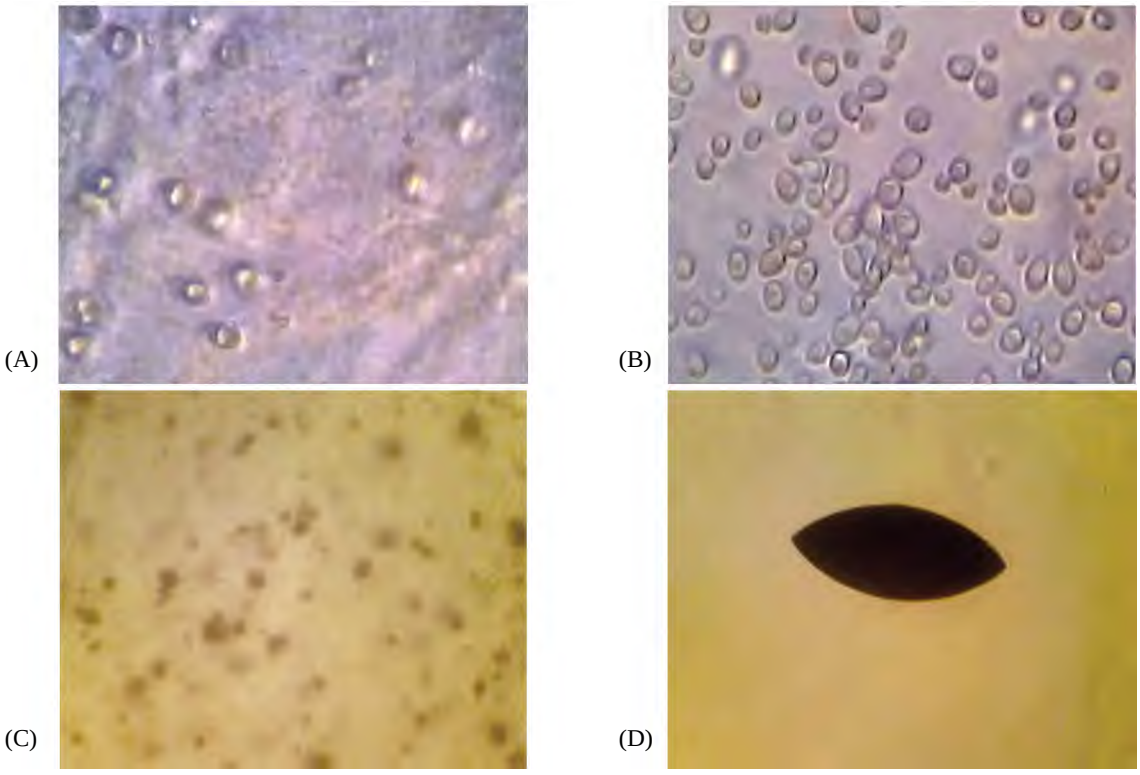


Fig. (2): Differential formation of *P. multocida* capsule in response to irradiation treatment; (A) 0 KGy, (B) 2 KGy, (C) 4 KGy, and (D) 6-10 KGy,

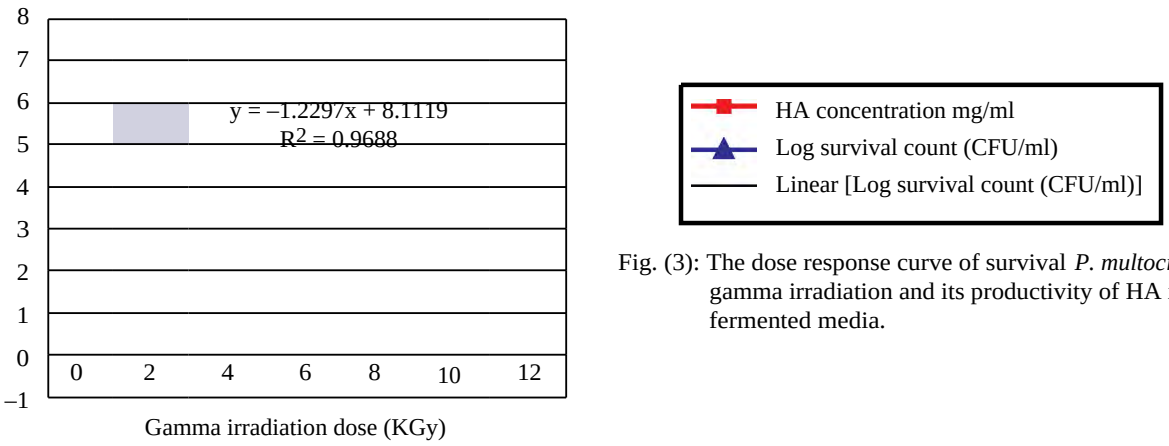
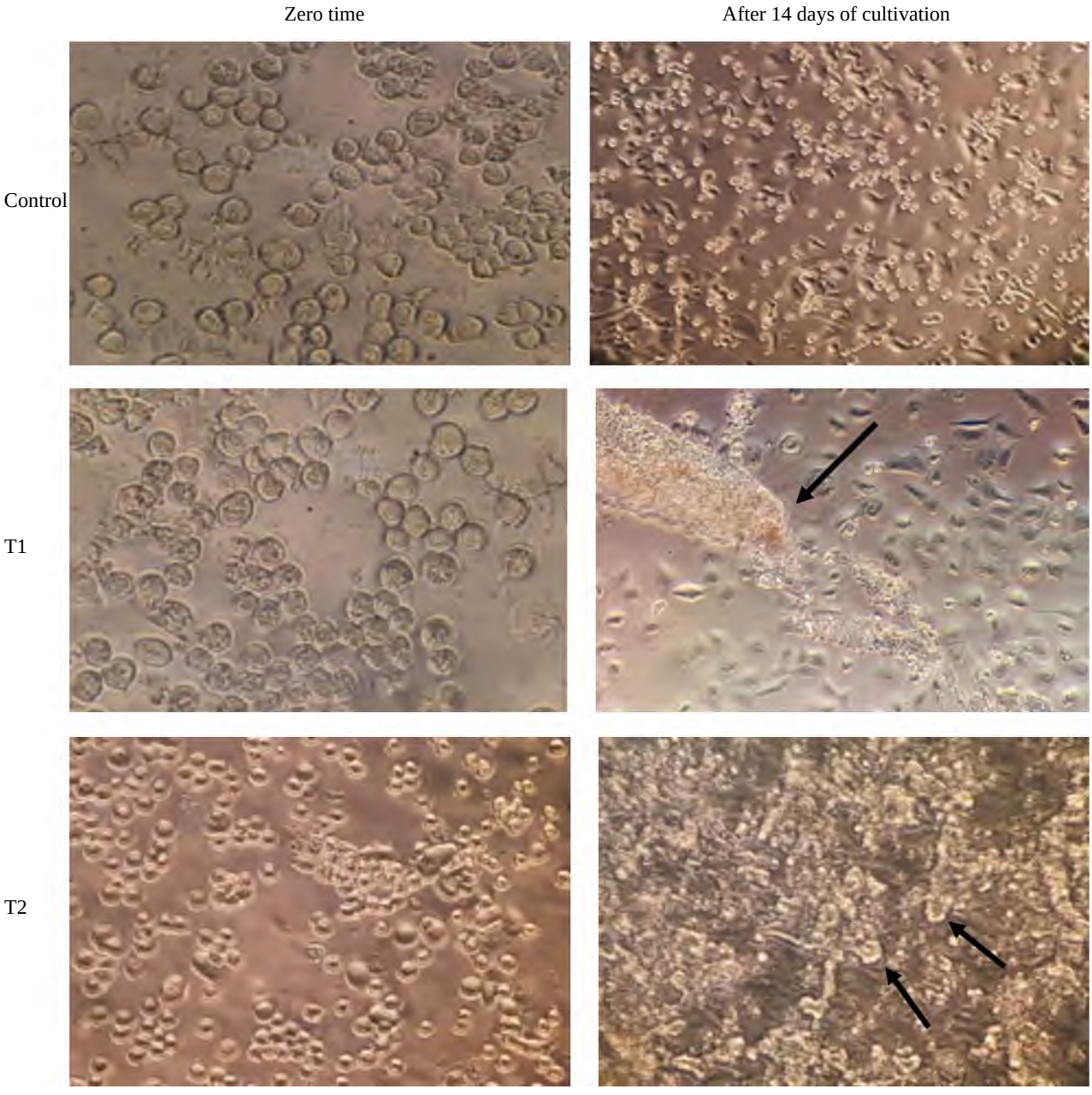


Fig. (3): The dose response curve of survival *P. multocida* to gamma irradiation and its productivity of HA in the fermented media.



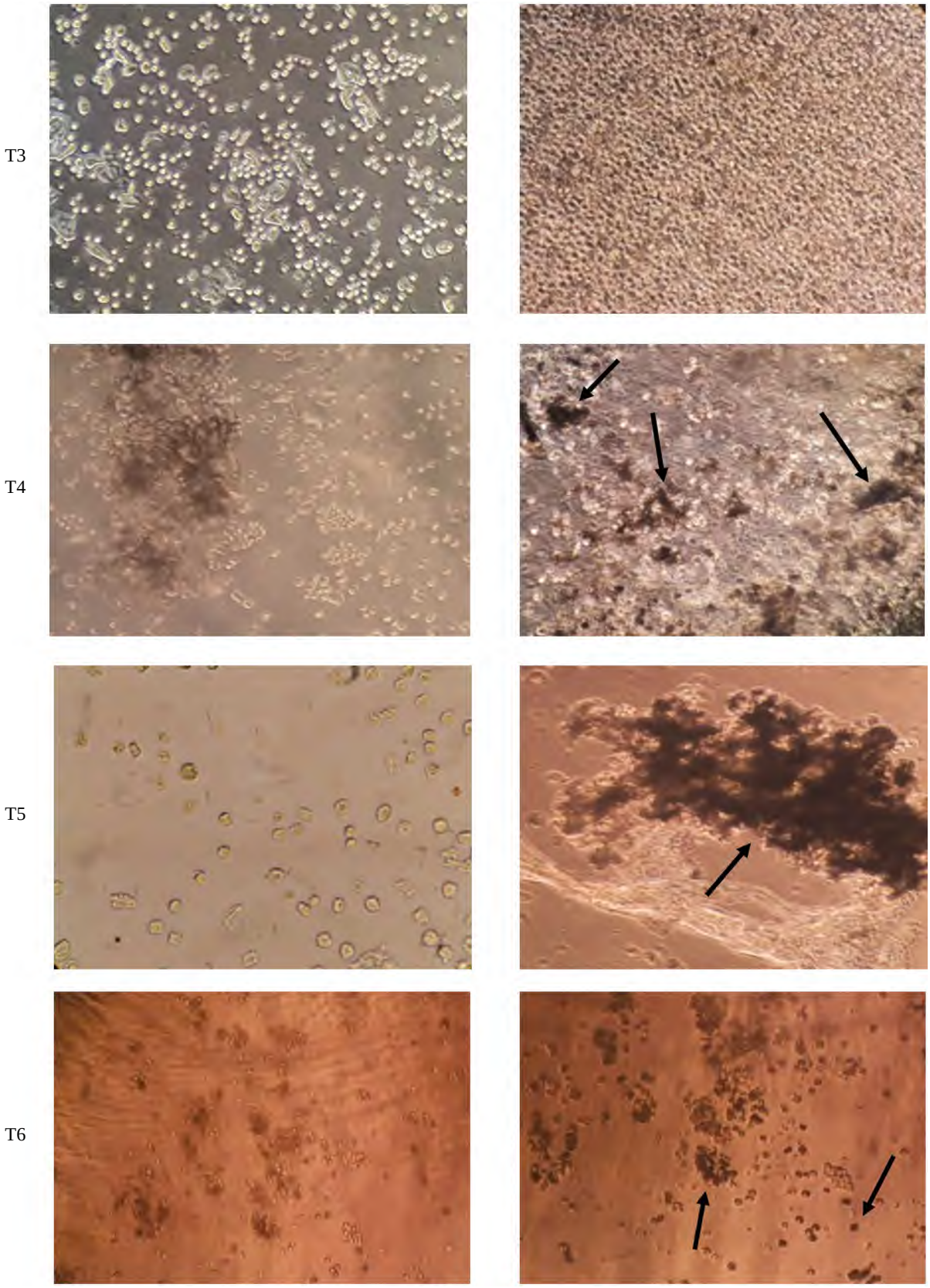


Fig. (4): Photographs of HAECs and the appearance of ECM (indicated by arrows) during 14-days of cultivation in different cultures (T1-T6) compared to the control culture (magnification = 40 x).

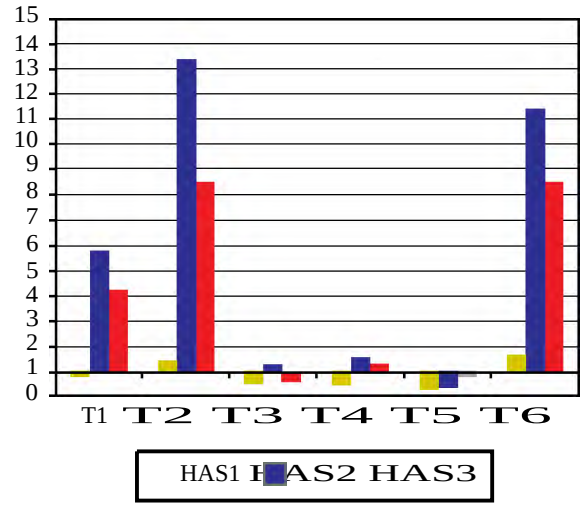


Fig. (5): Bar-chart graph of quantitative gene expression assay for three Hyaluronic acid synthase genes (HAS 1-3) in HAECs after cultivation through different conditions (T1-T6), compared to the traditional cultivation condition. Genes were normalized to the control cells in the traditional culture referring to GAPDH housekeeping gene.

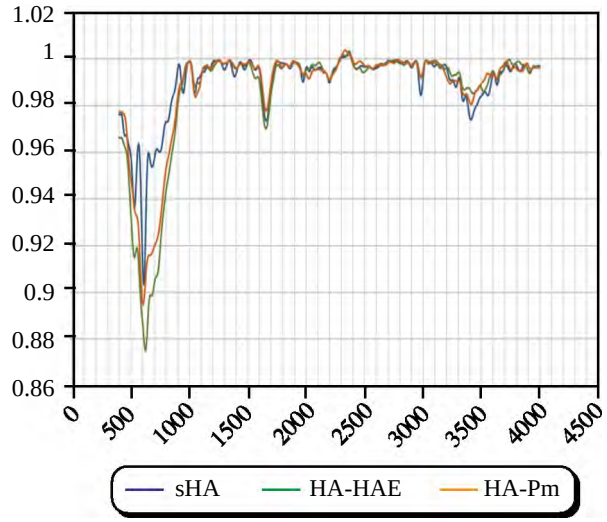


Fig. (6): FTIR analysis graphs of the extracted HA from cm-HAECs (HA-HAE), *P. Multocida* (Pm-HA), and the standard HA (sHA).

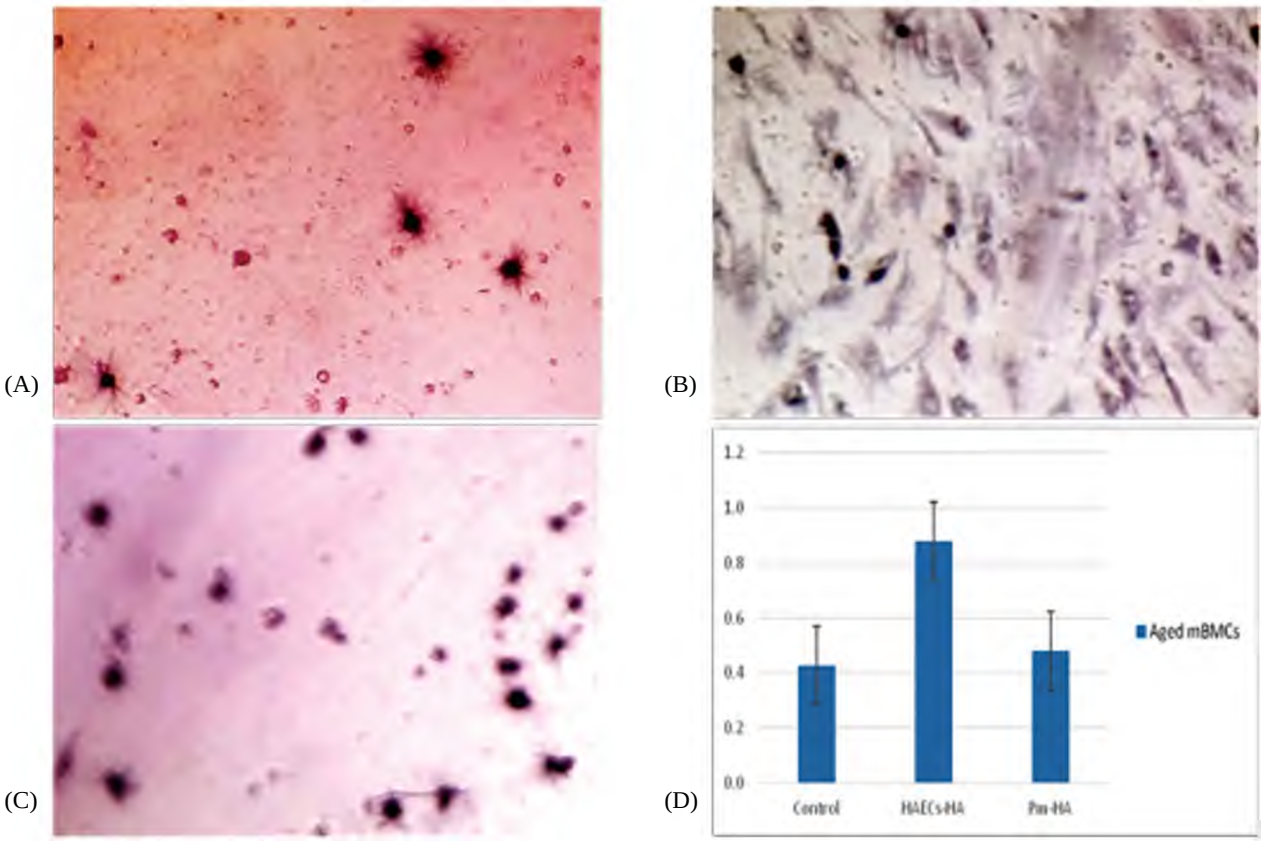


Fig. (7): Microscopic photographs (magnification= 40x) of MTT-treated mBMCs prepared from aged rat after 13 days of primary cultivation. Formed Formazan crystals indicate the viability of the cells (line arrows pointed examples), while no Formazan has been formed in dead cells (curved arrows pointed examples) (A) Control culture which was not supplied with HA in the medium resulted low viable cells without ability for division.(B) Culture supplemented with HAECs-HA; the cells appeared in a healthy spindle-shaped morphology as mBMCs proliferated and migrated on the flask surface until confluence. (C) Culture supplemented with Pm-HA;m BMC spreserved its viability without ability to divide or migration.(D) Data of MTT-assay as represented in a bar-graph chart indicating the proliferation of treated and untreated mBMCs.

Discussion

The main object of this study is to induce the production of HA from two different organisms; bacteria and humans. The ability of *P. multocida* type A to produce HA was demonstrated previously *[5]*. But, there is no scientific record proving the productivity of HAECs for HA. This study also aimed to provethe impact of the source of HA on its quality to improve stem cell growth, although after aging. Therefore, these goals will be useful in the field of geriatric plastic medical research.

The identification of *P. multocida* subtype A from infected lungs of sheep was confirmed using biochemical tests and specific detection of HA-producing genes via PCR. This is the first record to isolate this strain from Egypt. Furthermore, the morphological changes, which occurred on the outer membrane capsule complex in response to irradiation stress, have not been published before for this type of bacteria.

In general, the natural role of the HA capsule for bacteria is to mediate the adherence of the bacterial cells to the target tissue for establishing invasive infection and to prevent phagocytosis of the host's immune defense system *[4]*. Accordingly, scientists considered the production of HA capsules as a virulence factor in pathogenic bacteria *[11]*. However, bacterial-sourced HA has been used extensively for various biomedical applications. This is because bacterial-sourced biomaterials may be safer than those sourced from animals regarding the risk of animal disease transmission. However, there are many publications aimed at the extraction of animal HA; such as from rooster combs and human umbilical cords for medical application *[12]*.

In this regard, to ensure the safety of such bio-products from animals, it is preferable to use cell culture systems which are used to grow cells under fully aseptic conditions. Consequently, the chance to present pathogens during the growth of healthy cells is nothing and subsequently in their bio-products. However, recent literature lacks information about how to produce HA from animal cell culture systems.

Generally, the therapeutic uses of HA include wound treatment, dermal filling, and viscosupplementation of arthritic joints. This is because HA is biocompatible with minimal risk of immunological rejection of the body *[12]*. However, limited scientific publications compared the therapeutic effect of bacterial- versus animal-sourced HA, in regenerative medicine research. So, this study focused on *in vitro* investigation of treating aged BMCs (as a source of mesenchymal stem cells in the animal body) with one of these two types of HA, in comparison, to conclude the impact of each HA on the growth induction and on restoring the healthy ECM's structure of BMCs.

In regenerative medicine research, BMCs are considered the most common source of adult mesenchymal stem cells (MSCs) that are used for cell-based therapy of several diseases Corradetti et al., *[13]*. The self-renewal of such MSCs needs certain cell culture conditions including the control of growth factors and ECM *[14]*. The role of HA in the maintaining of ECM structure *[15]* is attributed to the interaction between HA and CD44 receptor on MSCs, this stimulates the cellular homing and migration of MSCs on the surface of the cell culture vessel *[13]*.

These findings give a good explanation for the manner of HAECs when high levels of HA are presenting in the medium. According to the results of the current study, it was noted that when HAECs express high levels of HAS, they appeared as embedded cells into a highly dense structure of ECM (as appeared in T2 and T6 cultures). However, the middle expression of HAS (as in T1 culture) promotes the migration of HAECs in a fibroblast-like morphology. While, the addition of external HA to the medium, at the primary time, suppresses the expression of HAS gene, and mediates the growth of HAECs into clusters.

Cellular aging may be caused by the reduction of ECM quality; which may result from one or more alterations occurring in mechanical properties, viscoelasticity, and the chemical compositions of structural ECM's proteins (such as collagens, elastin and fibronectin). Therefore, the self-renewing of aged stem cells may undergo malfunctioning due to the mechanical alteration or stiffness of ECM *[16]*.

The use of HA, as an anti-aging ingredient for improving aged skin, has been widely applied in many commercial products. The in vitro studies attribute this anti-aging effect to the role of HA in decreasing the accumulation of oxidative radicals, supporting the production of structural ECM components (such as collagen and elastin), and inducing the synthesis of Glycosaminoglycan. These biological features of HA helpin restoring the bioactivities and cellular mechanisms of aged fibroblasts and keratinocytes; this was revealed when supplying the culture medium with a matrix of HA *[17]*.

In harmony, the present study proved that theory when treating the aged mBMCs with a synthesized HA. MTT assay outcomes confirmed that both types of HA (whether HAECs-HA or Pm-HA) maintained the viability of all treated cells. In comparison, HAECs-HA helped the aged cells to restore their proliferation capability and migrated on the flask surface in a fibroblast-like morphology as young cells, while Pm-HA induced the formation of a different structural ECM than young cells with a little viability improvement. On the other hand, the non-treated aged cells in the negative-control cultures appeared in a declined quality of ECM structure and cellular viability.

These results reflected that the using of a low-cost extraction method for HA (such as the ethanol precipitation method) is suitable for the production of HAECs-HA not Pm-HA, because this method not eliminates bacterial toxins from the extract which affect the metabolic activity of the treated mBMCs with Pm-HA. So, the production of bacterial HA needs further purification steps to improve its biological effect without adverse complications. These outcomes support the clinical feasibility of using HAECs-HA for medical uses.

Conclusion:

The current study optimized the production and purification of HA from bacteria and human cell culture systems, this help to avoid the biological risk when extracting HA from crude animal tissues. The induction by irradiation increased the productivity of HA in p. multocida without cytotoxicity effect. Furthermore, this study approached the first trial to produce HA from HAECs culture that improved the proliferation and the healthy morphology of aged mBMCs. Accordingly, further complementary studies are recommended to prove the safety and clinical feasibility of using HAECs-HA and Pm-HA.

Competing interests:

The authors declare that they have no competing interests.

Data availability:

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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تحفيز إنتاج حمض الهيالورونيك من الغشاء الامنيوسى وبكتيريا الباستوريلاماالتوسيدا المشعة واختباره ضد شيخوخة خلايا نخاع العظم فى مزارع الخلايا

حمض الهيالورونيك (HA) هو جزيء بوليمر حيوى يحفز نمو الخلايا من خلال دعم البناء للمحتوى الشبكى المساعد خارج الخلية (ECMextracellular matrix). ولذلك، فقد تم تطبيقه فى العديد من البحوث الطبية الحيوية. المصادرالبيولوجية المشتركة لإنتاج HA هى الأنسجة الحيوانية والبكتيريا. يُعزى شيخوخة الخلايا إلى انخفاض قدرة انقسام الخلايا وجودة ECM.

تهدف الدراسة الحالية إلى تقييم تأثير HA من مصدر بشري ومقارنة تأثيرها البيولوجى مع التشعيع على إنتاجية بكتيريا Pasteurellamultocida لـ HA على خلايا النخاع العظمي في المختبر.

تم عزل الخلايا الطلائية البشرية (HAECs) من الغشاء الامنيوسى وتم استزراعها فى المعمل في أطباق زراعة الخلايا تحت ظروف تجريبية مختلفة مُحسّنة لإنتاج HA. تم التحقق من ذلك من خلال تحديد ملامح التعبير الجينى لثلاثة إنزيمات (HAS1، HAS2، HAS3) الدالة عن تخليق HA باستخدام تحليل PCRReal time الكمى. تم حث P. multocida لإنتاج كميات كبيرة من HA بواسطة تشعيع جاما. ثم تم استخلاص HA المنتج من HAECs (HAECs-HA) وP. multocida (Pm-HA) بواسطة تقنية الترسيب بالإيثانول. ثم تم تحليل HAs المستخرجة ومقارنتها مع HA (sHA) القياسي بواسطة مقايسة فوريه لتحويل الأشعة تحت الحمراء (FTIR). تم عزل خلايا نخاع عظم الفئران المسنة (mBMCs) من عظم الفخذ لجرذ يبلغ من العمر عامين، وتم استزراعها في الأدوات المخصصة لزراعة الخلايا المكونة من ٩٦ ثقب (96 well). تمت المعاملة لخلايا العظم المستزرعة mBMCs بواحدة من HA المستخرج (HAECs-HA أو Pm-HA) في ثلاث مكررات لكل معاملة وتم اتخاذ ثلاث مكررات غير معاملة كمرجعية قياسية للمقارنة السالبة تم تقييم الهجرة الخلوية والانقسام والانتشار عن طريق الفحص المجهرى ومقايسة MTT (٣ - [٤،٥-ثنائى ميثيل ثيازول-٢-ييل] -٢،٥ بروميد ثنائي فينيل تيترازوليوم).

م تحديد طرق التنمية الـHAECs المثلى لإنتاج HA فى الخلايا المحفزة ،ولوحظ ان إنزيمات التوليف الرئيسية التي تعبر عن HA في HAECs كانت HAS2 متبوعة بـ HAS3،كذلك تمت زيادة Pm-HA في مزارع الخلايا P. multocida بعد التحفيز بجرعة قدرها 2 KGy من تشعيع جاما أكثر من جرعات التشعيع الأخرى. أظهر اختبار FTIR التشابه الكيميائي بين منتجات HA الاصطناعية مع sHA. كشف التقييم المجهرى عن التأثير المتفوق لـ HAECs–HA فى تحسين نمو مزارع خلايا النخاع العظمى mBMCs والهجرة مقارنة بـ Pm-HA والمحلول الملحى. تم تأكيد ذلك أيضًا من خلال نتائج اختبار MTT.

تعتبر هذه الدراسة الرصدية هى الطريقة الأولى لإنتاج HA من مزارع خلايا الغشاء الامنيوسى HAECs فى المختبر والتى لها تأثير بيولوجى لتحسين هجرة ونمو مزارع خلايا النخاع العظمى mBMCs المسنة أكثر من HA البكتيرى. ولذلك، فإن هذه النتائج تشجع على إجراء المزيد من الدراسات لتقييم الجدوى السريرية لاستخدام HAECs–HA كعنصر مضاد للشيخوخة.