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The Effect of Mummy Substance on Matrix Protein Synthesis by Human Adipose-Derived Stem Cells and Dermal Fibroblast and Their Behavior on Plated PCL Scaffold

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Abstract

Objectives: Wounds repair has always been regarded as a challenge in health world due to its high prevalence characteristics. Therefore, there are many proposed therapeutic strategies for repairing wounds in which the use of herbal medicines is highlighted because of the low costs and complications. In traditional medicine, 'Mummy' is regarded as almost a first choice for some treatments such as bone fractures, bleeding control, poisoning treatment, headache relief and wound repair. This study seeks to provide a scientific venture for the effect of mummy on wound healing.

Materials and Methods: Human fetal foreskin fibroblast cells (HFFF-2) were purchased from Pasteur Institute (Tehran, Iran). The fibroblast cell lines and stem cells derived from adipose-derived stem cells (ASCs) were isolated by means of explant culture. Synthesis of components of extracellular matrix (ECM) such as collagen type I, type III and fibronectin 1 (FN1) were determined by real-time polymerase chain reaction (PCR) and cells attachments were examined by scaffold and cell proliferation determined by MTT assay. ASCs and HFFF-2, were investigated under mono-culture experimental conditions, then the other two-cell co-culture cells were also investigated under laboratory condition with 50-50 and 30-70 ratio respectively. They were all in an experimental group including culture medium and mummy; on the other side, in the control group there were just culture medium.

Results: The results suggested that in fibroblasts, the level of mRNA expression of fibronectin was up-regulated in the treatment group ($P<0.0001$), it was up-regulated Col type I ($P<0.0001$) and Col type III ($P<0.01$) in ASCs. In co-culture, mRNA expression of Col type I, III, and fibronectin increased ($P<0.0001$). The cells were successfully penetrated and adhered, and spread on PCL (polycaprolactone) scaffolds in all groups. Higher proliferation rate of fibroblasts was observed in Mummy-treated cells compared to the control group after 24 hours ($P<0.0001$). Increased proliferation rate in ASCs was seeded on scaffolds and treated with mummy compared to control groups for 24 hours ($P<0.01$) and 96 hours ($P<0.0001$). Proliferation rate in co-cultured ASCs and fibroblasts (proportion of 50-50 and treated with mummy) was higher after 24 hours compared to the control cells ($P<0.0001$); but in co-cultured ASCs and fibroblasts, proportion of 70-30 was significantly lower in Mummy–treated group compared to the control group ($P<0.0001$) at both lengths of time.

Conclusions: mummy may improve wound healing through synthesizing ECM attachment and proliferating cells on PCL scaffold.

Keywords: Mummy substance, Wound healing, Adipose-derived stem cells, Human fetal foreskin fibroblast, Co-culture, Matrix protein, Poly(ε-caprolactone) scaffold, SEM

Introduction

Human body's skin has two layers functionally that are epidermal and deeper dermal layer. The superficial epidermal layer provides a barrier against infection and moisture loss, and the deeper dermal layer provides support for the epidermis and it is responsible for the strength and integration of the skin (1,2). Skin damage can be caused by burns, cuts, abrasions, and ulcers to varying degrees of severity. The healing of cutaneous wounds requires complex interactions between the dermal and epidermal cells, the extracellular matrix (ECM), and the nervous and vascular components of the damaged skin and its surrounding (2,3). Wound healing represents a complex interaction of cells, cytokines, and various ECM proteins. Basically, wound repair comprises five major phases: inflammation, new tissue formation due to cell proliferation, cell migration, and neoangiogenic, as well as tissue remodeling (4,5). In a highly coordinated biological process of dermal wound healing, skin fibroblasts interact with surrounding cells such as keratinocytes, fat cells, and mast cells. Fibroblasts produce ECM, glycoproteins, adhesive molecules, and various cytokines (6). Their production of growth factors, chemokine, and ECM facilitates the angiogenic recruitment of endothelial cells and pericytes (7). During tissue repair, fibroblasts migrate into the wound for producing and remodeling of ECM (8). Integrins are believed to be crucial for tissue repair, but their tissue-specific role in this process is poorly understood. During the final step of remodeling, an increase in the rate of collagen deposition causes the tensile strength of the

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Key Messages

- Due to the challenging nature of the wounds repair process, a lot of therapeutic strategies have been proposed during years. In the majority of these strategies herbal medicines stand in a vital position because of their affordable prices and complications. "Mummy' is regarded as a priority for treatments almost a first choice for some many treatments and wound repair. This study tries to shed light on the scientific effects of mummy on wound healing.

cutaneous tissue which coincides with fibronectin removal (9, 10). In addition to secretory activity, dermal fibroblasts crosstalk with the neighboring cells and produce various cytokines, adhesive molecules, glycoproteins, and ECM. Therefore, paracrine and juxtacrine activities as well as synthetic interdependencies enable skin fibroblasts to conform to fibroblast-keratinocyte-endothelium complex during wound repair, and maintain the integrity of the skin (11).

Over the last few decades, stem cells have been in the focal point of consideration serving as a means of developing new methodology for recovery and regeneration of various tissues (12). Adipose tissue is the most well-known and practical tissue for seclusion of adipose-derived stem cells (ASCs) with insignificant patient uneasiness and death rate. These cells have characteristic intensity to differentiation into adipogenic, chondrogenic, myogenic, and osteogenic lineages because of stimuli (12).

The application of ASCs to facilitate the wound healing process and tissue regeneration has been studied in different in vitro and in vivo experiments. It has been shown that ASCs accelerates dermal fibroblast proliferation and promotes cutaneous re-epithelialization via paracrine secretion of different growth factors such as fibroblast growth factor-2 and platelet-derived growth factor (12,13). In a rat model of full thickness excisional injury, ASCs was found to enhance the formation of neo-vascularization and wound closure with ability to differentiate into endothelial and epithelial cells (14).

For about 5000 years, nature has been considered as a potential source for management of different diseases such as wounds.

In recent years, the revival of growing enthusiasm for traditional medicine has prompted examinations for better comprehension of the tools underlying unknown impacts of various conventional compounds; however, the low cost, minor negative side effects, and improved patient acceptance have drawn more attention to the role of these organic compounds. Mummy – popularly known as a substance for mummifying famous Egyptian kings, and locally called mummy in most parts of Persia – is a pitch-like secretion found in some fractures of the earth and rare caves. It is dark brown to black in color, and is produced through the oil oxidation process. It contains magnesium, sulfur, nitrogen, oxygen, and polysaccharide (15). Mummy is of two types: fat soluble and water soluble. For topical application, it is solved in boiling water and applied to the injury site such as wound or inflamed joint (16). Various effects of mummy on fracture healing, gastric ulcers, and animal model of wound healing have been recently investigated by some Persian researchers (16). Considerable impacts of mummy in quickening the wound mending have been acknowledged by local people and were specified in Old Persian books (e.g., Avicenna' The Canon of Medicine) with respect to the part of fibroblasts and ASCs during the time spent on injury recuperating.

To acquire a perfectly-designed skin looking like the ordinary one with physiological capacity, it is vital to seed cells on a three-dimensional network and give appropriate condition to cell-cell and cell-ECM interactions, as well as lessen dedifferentiation of them which occurs ordinarily in 2-D conditions (17).

In spite of the important role of fibroblasts and stem cells in wound recuperating development, it is crucial to empower the capacity of these cells using a few enhancers (17). This study aimed to assess the impact of mummy on combination of dermal ECM components in a 3-dimensional culture condition.

Materials and Methods

ASCs Isolation and Expansion Procedure

Human adipose tissues were obtained from patients undergoing laparotomic surgery. According to Ethics Committee exclusion criteria included any malignancy, administration of hormones, and chemotherapeutic agents. The samples were collected from patients undergoing liposuction surgery Written informed consents were obtained from all patients.

Preparation of ASCs

As for the sampling, adipose tissue samples were washed twice by phosphate-buffered saline (PBS) containing 1% penicillin-streptomycin (Gibco). The samples were minced into small pieces and, then, were digested with 0.2% collagenase type I (Cat no: C9891, Sigma) with gentle shaking for 60 minutes at 37°C. Digestive solution was further blocked by addition of fetal bovine serum (FBS, Gibco) and passed through a 70-µm cell strainer (Fischer Scientific). Next, cell suspension was collected by centrifugation at 1500 rpm for 5 minutes. The cells were counted through using 0.4% trypan-blue exclusion dye and seeded into the T25 culture flask in DMEM (Dulbecco's Modified Eagle Medium) low glucose (DMEM/LG, Gibco) which was supplemented with 10% FBS and 1% penicillin-streptomycin. The medium was exchanged once every three days. In the third passage, expanded cells were used. Previous studies from our lab provided evidence with the same characteristics as mesenchymal cells (17,18). Human fetal foreskin fibroblast cell line (HFFF-2) was purchased from Pasteur Institute (Tehran, Iran). After thawing, the cells were counted and plated

at density of 5 ×10^5 cell/mL in the T75 culture flasks and used for the experiment.

Mummy Preparation

The fresh mummy was purchased from local market in Kermanshah. Due to the absence of any in vitro study regarding dosage of mummy in the first step, its effective concentration was determined using MTT assay technique (19). Since mummy is water soluble, it was solved in DMEM culture medium completely, and filtered through 0.22 µm syringe filter to be sterilized.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction

ASCs and fibroblasts were treated with 1000 µg/mL of mummy material (20). After 24 and 96 hours, the total cellular RNA was extracted using RNX-plus kit (Sinaclon, Iran). After adding chloroform and mixing it with ice, the ultimate mixture was centrifuged at 12 000 rpm for 15 minutes. Then, supernatant was removed and the samples were incubated with isopropanol at -20˚C overnight. cDNA was synthesized using the AccuPower® RT PreMix (Bioneer); and the levels of collagen I, III and fibronectin mRNA, the reference gene, as well as the housekeeping gene – glyceraldehyde-3-phosphate dehydrogenase (GAPDH) – were assessed using the gene-specific SYBRGreen PCR Mastermix (Applied Biosystems, USA)
The gene primers used in current experiment were as follows:
Collagen type 1a1:
(forward, 5′-GCCAAAGAAGCCTTGCCATC-3′; reverse, 5′-TCCTGACTCTCCTCCGAACC -3′)
Collagen type 3a1:
(forward, 5′-GCTGGCTACTTCTCGCTCTG-3′; reverse, 5′-TTGGCATGGTTCTGGCTTCC-3′)
Fibronectin 1:
(forward, 5′- CCTCACCAACCTCACTCCAG-3′; reverse, 5′-GTCGCAGCAACAACTTCCAG-3′)
Housekeeping gene GAPDH:
(forward, 5′-CAAGATCATCAGCAATGCCTCC-3′; reverse, 5′-GCCATCACGCCACAGTTTCC-3′).

All experiments were performed in triplicate for each sample. Data interpretation was performed by Pfaffl method and the CT values were normalized with respect to GAPDH expression.

Scaffold

Scaffold Characterization

PCL scaffold was obtained from Yakhte Company (Tehran, Iran). The nanofibrous PCL sheet was treated with DMEM. Before utilizing, filtering electron magnifying lens (MIRA3 FEG-SEM) was used for the perception of the auxiliary morphology of the scaffold. The examples of the scaffolds were taken from the nanofibrous sheet using a 7 mm dermal punch, and they were covered with gold by a sputter-coater. Diameter of fibers in the electrospun scaffold were measured by scanning electron micrographs. The average diameter of fibers across the fibers were resolved through estimations from perpendicular to the long hub of the fibers inside delegate infinitesimal fields (21). The size of the pores shaped between the fibers was measured using ImageJ scanning electron micrographs were captured at an amplification of ×2000 from arbitrary spots (21).

Scanning Electron Microscopy

Firstly, the scaffolds were cut using a 7 mm biopsy punch, and placed in 12- well culture plate. In order for sterilization to occur, ultraviolet (UV) irradiation was used, then the scaffolds were placed in culture medium for 12 hours. The cells in fourth passage were detached by Trypsin /EDTA (0.25% trypsin/ 0.2% EDTA; Sigma, USA) and seeded on scaffolds with a density of 1×10^5 fibroblast and ADCs alone. For the co-culture condition, equal amounts of cells (0.5×10^5 of each) or 70/30 proportion (0.70×10^5 fibroblasts and 0.30×10^5 ADCs) were plated and incubated in DMEM containing 10% FBS + 1% P/S for 24 hours to reach monolayer confluence. After adhesion to the culture plate, cells were received culture medium as control or mummy at concentration of 1000 µg/mL during 24 hours. To evaluate cell adhesion on the scaffold, samples were fixed using 5% glutaraldehyde (Merck, Germany) for 1 hour at room temperature and dehydrated in a graded ethanol series. After drying, they were coated by a thin layer of gold palladium and viewed using SEM.

MTT Assay

Cell proliferation were evaluated by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Scaffolds were placed in a 96 well culture plate, and then seeded with a density of 2×10^4 fibroblast and ADCs alone, for the co-culture condition, equal amounts of cells (1×10^4 of each) or 70/30 proportion (1.4×10^4 fibroblasts and 0.6×10^4 ADCs) were plated and incubated in DMEM containing 10% FBS + 1% P/S for 24 hours to reach monolayer confluence. In the next step, scaffold constructs were divided into two groups: control group which received only DMEM, and treated group with concentration of mummy 1000 µg/mL. After 24 hours or 96 hours, MTT solution (5 mg/mL in DMEM+FBS 10%) was added to each well (n = 96). The plates were incubated at 37°C for 4 hours, and MTT converted to Formazan crystals by mitochondrial dehydrogenases of living cells. Then supernatant was removed and DMSO 150 mL per well was added for 20 minutes in dark room for scaffold removing, 69-well plate was centrifuged, and then 100 µL of obtained solution was transferred into a new plate. The absorbance at 540 nm wavelength was measured using ELISA reader (BioTek, USA).

Statistical Analysis

The data were expressed as mean ± SD. Statistical analysis

was performed using Two-way ANOVA (for more than two groups). Statistical analyses were done using GraphPad InStat software version 2.02. Statistical difference between groups is shown by brackets, and $P < 0.05$ is considered as significant.

Results

Optimal Dosage of Mummy Material

Taking into account the previous study (20) and using MTT assay, the effective concentration of mummy on fibroblasts and ASCs was determined by our workgroup (unpublished). It was revealed that the mummy at concentration of 1000 µg/mL led to the highest proliferation rate and stimulation of fibroblasts. Similarly, the most effective dosage of mummy on ASCs was found at the level of 1000 µg/mL (20).

Expression of Extracellular Matrix Related Genes

The expression of type I and III collagens was not significantly different in fibroblast treated with mummy compared to control group, but the mRNA expression level of fibronectin was up-regulated on both 24 and 96 hours (Figure 1A, B, C). Real-time RT-PCR analysis revealed up-regulation of collagen I in ASCs treated with mummy in comparison to control for both 24 hours and 96 hours lengths of time,however, col type III up-regulated only for 96 hours, and the expression level of fibronectin was not different between experiment and control groups (Figure 1D, E, F). The expression level of collagen types I, III and fibronectin was up-regulated in fibroblasts and ASCs co-cultured in proportion of 50-50 or 70-30, and received mummy compared to control groups (Figure 1G, H, I, J, K, L). All experiment was carried out in triplicate.

Cell Attachment and Proliferation to Scaffold

Cell adhesion and proliferation to the nanofiber PCL scaffolds were tested by SEM and MTT assay techniques. The average diameter of nanofibers was approximately 0.976 ± 0.044 µm and pore size was 134.4 ± 3.83 µm. The orientations of fibers were random (Figure 2A). As can be understood from Figure 2B-2I, cells were successfully penetrated, adhered and spread on the scaffolds with a flattened and polygonal phenotype in both control and mummy-treated groups. Cell density was relatively similar

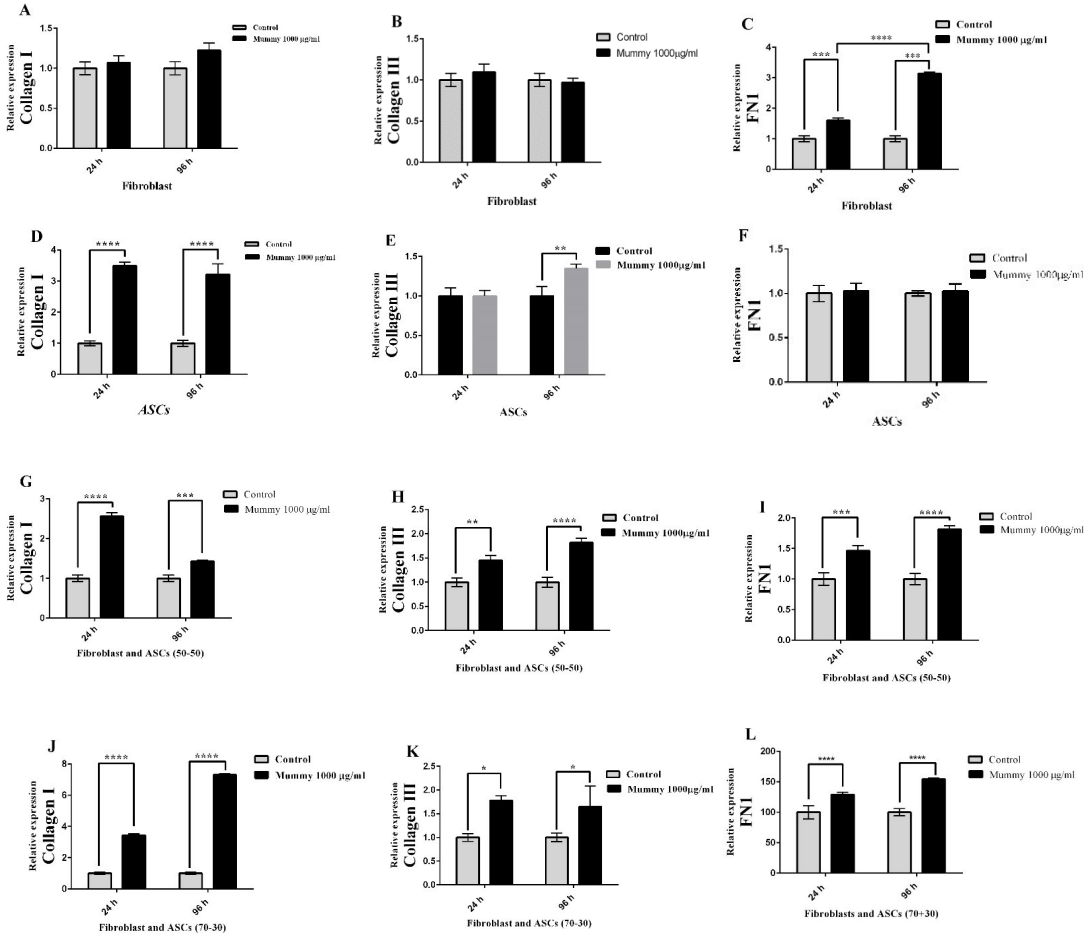

Figure 1. Comparison of Transcript Level of Collagen I, III, FN1 Between Control Group and Various Cells After Treatment With Mummy Substance for 24 h and 96 h. A, B, C- Fibroblast Cells, D, E, F ASCs, G, H, I Co-cultured Fibroblasts and ASCs With 50/50 Proportion, J, K, L Co-cultured Fibroblasts, and ASCs With 70/30 Proportion. Data are expressed as mean ± SD. $^{*}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$

in all groups, and visible lamellipodia was observed on fibers.

Cell proliferations on the PCL scaffolds were measured using MTT assay. Higher proliferation rate of fibroblasts was detected in Mummy-treated cells compared to the control group after 24 hours ($P<0.0001$). As the culture time passed, the number of cells decreased in both control and treated groups on 96 hours with no significant difference (Figure 3A). Results in Figure 3B shows an increased proliferation rate in ASCs seeded on scaffolds treated with mummy compared to control groups on 24 hours ($P<0.01$) and 96 hours ($P<0.0001$).

Statistical analysis demonstrated that proliferation rate in co-cultured ASCs and fibroblasts proportion of 50-50 treated with mummy was higher after 24 hours compared to the control cells ($P<0.0001$), the difference was not significant ($P>0.05$) on 96 hours (Figure 3C). The cell number in co-cultured fibroblast and ASCs with proportion of 70-30 was significantly lower in mummy-treated group compared to control group ($P<0.0001$) in both lengths of time (Figure 3D).

Discussion

Cutaneous wound healing is known as a complicated process, comprising multicellular overlapping and coordinated steps of inflammation, angiogenesis, formation of granulation tissue, re-epithelialization, proliferation, migration, matrix formation, and remodeling (22).

Since this process is still a challenging issue, particularly for chain smokers, burned patients, old people, and diabetics (21-24), existence of novel procedures is necessary to upgrade wound recuperating and repair. Recently, more attention has been paid to traditional medications (15, 16). Recovering fibroblasts multiply to increase cell numbers while also delivering a few extracellular grid proteins and developmental factors to create new tissue for wounds. (25). Collagens play pivotal roles in wound healing since they function as bases for intercellular grid development, so their synthesis is essential (26). Fibroblasts deliver the new ECM which is essential for cell development, and prepare a media for oxygen and supplements dispersion for cell digestion system (27). The basic molecules of the recently shaped ECM further add to the arrangement of granulation tissue by giving a framework or course to cell movement (27).

In this study, we explored the potential impact of mummy on the length of time needed for wound healing, particularly on synthesis of the additional cell framework segments in cultured adipose inferred stem cells and fibroblasts as well as their co-culture with or without scaffold. RT-PCR showed an increased expression of FN1 in HFFF alone (Figure 1C) compared to control group, and indicated up-regulation of type I, type III COL in ASCs (Figure 1D, E). The co-culture condition with proportions of 50-50 and 70-30 revealed up-regulation of col type I, type III, and NF1 (Figure 1H-L).

Several scientists have reported the MSCs assist fibroblasts cells with secreting collagen, elastin with the concealment of MMP-1, and the blend of fibronectin (13). A review by Kim et al demonstrated that the use of ASCs altogether quickened the re-epithelialization of cutaneous injuries by developing human dermal fibroblast multiplication through direct cell–cell contact or by means of paracrine emission of an assortment of development components. The review further indicated that ASCs had impacts on HDFs by expanding collagen synthesis so that it could be utilized for the treatment of wound recuperating (21,25). Expression of qualities required in re-epithelialization, neovascularization, and fibroproliferation (TGF-β2 and hypoxia-inducible factor-1α, and plasminogen activator inhibitor-1) to improve wound healing process. Moreover, in our study the up regulation of fibroblast cells was

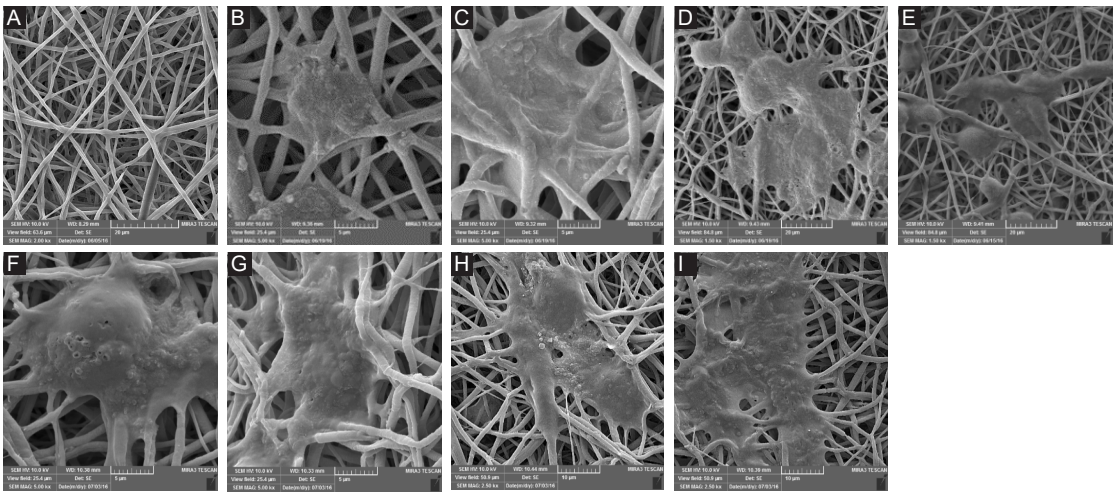

Figure 2. Scanning Electron Micrographs (SEM) Images of Poly (ε-caprolactone) Nanofibers Before and After Cell Seeding and Attachment of Cell on it in Different Groups. (A) Before cell seeding, (B, C) Fibroblast cells control and mummy, (D, E) ASCs control and mummy, (F,G) Fibroblast-ASCs co-culture in proportion of 50: 50 control and mummy (H, I) Fibroblast-ASCs co-culture in proportion of 50: 50 control and mummy in different magnifications, at ×2000 (A), ×5000 (B, C, F and G), ×1500 (D, E), ×2500 (H, I)

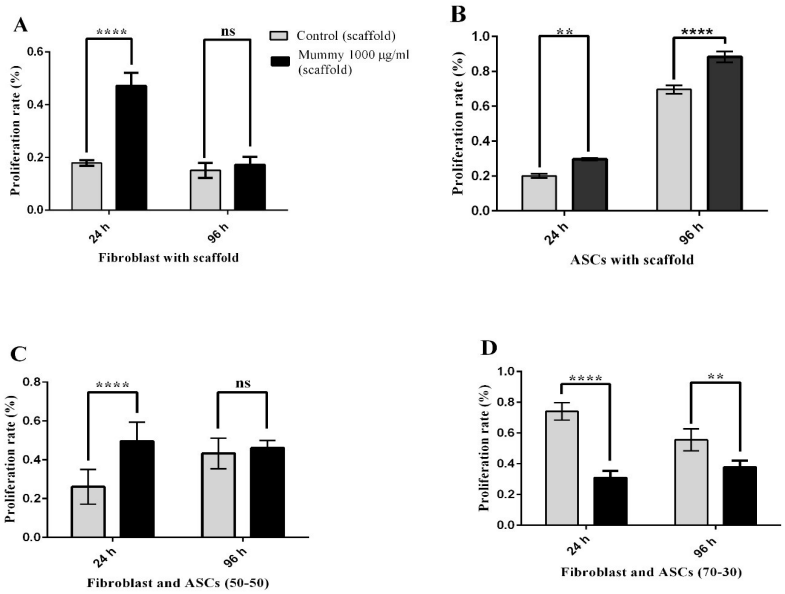

Figure 3. Representative Images of Cells A- Fibroblast Cells, B- ASCs, C- Co-cultured Fibroblasts and ASCs With 50/50 Proportion, D- Co-cultured Fibroblasts and ASCs With 70/30 Proportion Loaded on PCL Scaffold. Error bars represent standard error of the mean, $^{****}P<0.0001$, $^{**}P<0.01$

observed. Nevertheless, the quantity of collagen I and collagen III stayed constant.

The present study further aimed to produce ECM mimics and, therefore, the nanofibrous scaffolds were used. Since nanofibrous scaffolds made of nanofibers like extracellular lattice particles such as collagen, laminin, and fibronectin (0.005-0.5 nm) and proteoglycans like hyaluronic (0.45-1 nm), they have been found to build cell adherence, expansion, and separation (25). The interesting aspect of our study is that the cells (HFFF-2, ASCs, co-culture) attached to the scaffolds after 24 hours. Also, our study result from MTT assay clearly showed continuous proliferation of fibroblast in 24 hours and ASCs in both of the times, as well as indicated an increase in co-cultures 50-50. Furthermore, biocompatibility of the scaffolds was tested by MTT assay with fibroblast cells and cardiac cells, which showed higher proliferation of the cells scaffolds compared to the two-dimensional culture system (25). Scaffolds supported adhesion and proliferation of adipose derived mesenchymal stem cells in presence of Zn^{+2} ion (21).

Several reviews have already demonstrated that MSCs can cause such regenerative limit (15,26). In our review, first and foremost, we needed to demonstrate that mummy played key role in the advancement of gene-expression including injury repair, which is the foundation of granulation stage. Mummy substance expands over vast amount of segment of ECM, so it upgrades injury repair in vitro conditions. In the present study, we also exhibited that mummy substance affected the gene expression collagen type 1, 3, fibronectin in fibroblasts and ASCs, as well as co-culture of them required in wound recuperating, which was the foundation of granulation stage. In addition, we found that mummy substance had a strong impact on the acceleration of adipose-derived stem cells which presented dermal fibroblast in 2D and 3D models. Therefore, it could be used for tissue engineering applications. In addition, collaboration amongst ASCs and fibroblast cells could help develop different types of healing techniques.

Conclusions

Mummy substance could be offered as a traditional solution for healing wounds. Taking into account the results of this study, there is an underlying relationship of the mummy substance to the skin tissue designing areas by controlling ECM and expanding fibroblasts and ASCs.

Authors' Contribution

SH and JS: Concept and design. SH and SHH: data collection and interpretation of the data. SH, JS, SHH and LR: performing of the study and the writing of the draft. All authors read and approved the study.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

This study was approved by Ethics Committee on August 25, 2014 (ethics No. 5/4/9875). All phase of the current study were completed according to the guidelines of Local Medical Ethics Committee of Tabriz University of Medical Sciences and the Declaration of Helsinki (1964).

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