http://www.cjmb.org doi 10.34172/cjmb.2025.3907

Open Access Original Article

Crescent Journal of Medical and Biological Sciences
Vol. xx, No. x, xx 2025, x–x
eISSN 2148-9696

The Effect of Mummy Extract on Matrix Protein Synthesis by Human Wharton's Jelly-Derived Stem Cells and Dermal Fibroblasts and Their Behavior on Plated PCL Scaffold

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Abstract

Objectives: Wound healing as a coordinated physiological mechanism is a critical issue in medicine. Despite numerous treatment methods, the slow-healing process and scar formation have led researchers to develop new, more effective therapeutic approaches. Here, we evaluated the matrix protein synthesis in human Wharton's jelly-derived stem cells and human fetal fibroblast cell line (HFFF-2) in the presence of Mummy extract. Furthermore, cell attachment to the polycaprolactone (PCL) scaffold was examined by electron microscopy.

Materials and Methods: The effective, non-toxic dose of Mummy was determined using an MTT assay. Subsequently, cell proliferation on the PCL scaffold was assessed using the MTT assay. The expression of collagen I, III, and fibronectin was analyzed. Finally, cell attachment and morphology on a PCL scaffold were examined using scanning electron microscopy. Furthermore, cell proliferation and attachment on the PCL scaffold were reviewed using the MTT assay and electron microscopy, respectively.

Results: The results showed that Mummy extract significantly enhanced cell proliferation and increased fibronectin expression in treated fibroblasts. Surprisingly, collagen type I and fibronectin increased significantly in the co-cultured group. Scanning electron microscopy confirmed that cells successfully penetrated and adhered to the PCL scaffold in both control and Mummy-treated groups.

Conclusions: Taken together, our findings indicate that Mummy extract can positively influence the expression of key extracellular matrix (ECM) proteins and cell-scaffold attachment, suggesting its potential as a complementary approach for wound therapy strategies, warranting further *in vivo* investigation. This study provides the first *in vitro* evidence for the synergistic effect of Mummy extract and WJSC-fibroblast co-culture on enhancing ECM production in a 3D environment.

Keywords: Collagen, Mesenchymal stem cells, Mummy, Wharton's jelly, Wound healing

Introduction

Wounds represent a disruption of the normal structure of the skin and continuously increase with aging and underlying diseases (1,2). Wound healing is a coordinated physiological mechanism observed in response to tissue injury. Today, wound healing, which is influenced by many factors, is a critical issue in medicine. Despite numerous treatment methods, the slow-healing process and scar formation have led researchers to seek new, more effective therapeutic approaches (3). According to previous studies, collagen deposition and skin tensile strength are the main processes during the remodeling phase (4). Fibroblast cells are responsible for the synthesis and maintenance of extracellular matrix (ECM) components in skin (5) and have a pivotal role in the wound healing process (6). The complexity of the healing process is further highlighted by its parallels with other fibrotic conditions. For instance, the application of anti-inflammatory and anti-fibrotic agents, such as simvastatin, has shown efficacy in reducing adhesion formation during visceral surgery, demonstrating the broader potential to modulate the fibrotic response and improve tissue repair outcomes (7). In addition, the ability of mesenchymal stem cells (MSCs) in wound healing has been well understood in recent years (8,9). These cells stimulate vascular endothelial growth factor (VEGF) levels, promote vasculogenesis (10), and modulate the inflammatory response (11). According to the Arno et al study, Wharton's jelly-derived MSCs (WJ-MSCs) supernatant stimulates re-epithelialization, neovascularization, and skin fibroblast multiplication, and improves injury healing in a murine excisional skin model (12).

Besides ASCs and WJSCs, the positive effect of Mummy (Shilajit) extract, a natural exudate from mountain rocks rich in fulvic acid, humic substances, and dibenzo-α-pyrones, has been reported. It has been used in traditional

Received 7 August 2025, **Accepted** 2 December 2025, **Available online** 16 December 2025

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medicine for centuries, and modern studies indicate its anti-inflammatory and antioxidant properties (13).

Its beneficial effects in bone fractures, a rabbit model of cutaneous wound healing, and gastric mucosal damage have been reported in many clinical situations (14,15). Polycaprolactone (PCL) scaffolds were selected for this study due to their well-established biocompatibility, suitable mechanical properties for skin tissue engineering, and proven ability to support fibroblast and stem cell attachment and proliferation.

The healing effect of Mummy on gastric mucosal damage has been reported by several investigators (16). Several studies suggest that the cells in 2-dimensional culture systems typically undergo dedifferentiation and lose their normal phenotype. Hence, three-dimensional scaffolds can be further applied to simulate the naive ECM of the specific tissue (17). Tissue engineering and regenerative medicine have provided three-dimensional scaffolds that serve as frameworks for seeded cells to control their development (18). Considering the essential roles of fibroblasts and Wharton's jelly-derived stem cells in wound healing, this study explored whether Mummy could enhance the expression of key dermal components. These components include collagens I and III and fibronectin in a three-dimensional culture. Furthermore, we quantified the effects of the aforementioned materials' proliferation, migration, and cell adhesion on three-dimensional scaffolds.

Methods

Isolation of WJSCs

Women undergoing caesarean section (n = 3) without any complications throughout their pregnancy were enrolled in the current study. Women undergoing caesarean section (n = 3) at term (37-40 weeks' gestation) without any complications (e.g., diabetes, pre-eclampsia, infections) throughout their pregnancy were enrolled for the current study. All women provided written informed consent, which included explicit consent for the use of umbilical cord tissue for research purposes, with guaranteed donor anonymity. The samples were washed twice using phosphate-buffered saline (PBS) containing 1% penicillin-streptomycin (Gibco) under sterile conditions. After three washes with PBS, samples were placed in 70% alcohol for 30 seconds, then cut into 5 cm pieces using a sharp, sterile blade. After incising lengthwise, the vessels were removed, and WJ was gently separated from the amniotic cover, further cut into 2 × 2 mm^2 pieces, and explanted in T-25 culture flasks containing DMEM/LG supplemented with 20% FBS and 1% P/S. The culture medium was replaced twice a week. After 2 weeks, WJSCs appeared in the proximity of WJ explants. After reaching 70%-80% confluence, the cells were detached by 0.25% trypsin-EDTA solution (GIBCO) and sub-cultured into new flasks. Such cells have been characterized as MSCs in previous studies (19-21). The isolated cells (passages 3-5) were characterized by flow cytometry for positive expression of CD73, CD90, and CD105, and negative expression of CD34 and CD45. Their adipogenic and osteogenic differentiation potential was confirmed per our established protocols.

Determination of the optimum concentration of Mummy (Shilajit) extract

The Mummy extract was prepared from the local market in Kermanshah city. The effective dose of Mummy was determined using the MTT assay. Due to its water solubility, it was dissolved in DMEM completely and filtered for sterilization.

Characterization and Standardization of Mummy Extract

To ensure the reliability and reproducibility of our findings, the Mummy extract underwent comprehensive phytochemical characterization and quality control before biological testing. The raw Mummy extract was procured from a certified supplier (MumioAltai Ltd., Russia) originating from the Altai Mountains. A voucher specimen (Code: SHA-2023-01) has been deposited in the Herbarium of the Department of Pharmacognosy, Tabriz University of Medical Sciences, Iran, for future authentication and traceability (22). The crude material was purified using a standardized aqueous extraction protocol. Briefly, 100 g of raw Mummy was dissolved in 1 L of double-distilled water (1:10 w/v) under continuous stirring at 60 °C for 4 hours. The resultant solution was filtered through a 0.45 µm membrane, concentrated under reduced pressure, and subsequently lyophilized to obtain a purified, dark-brown powder. The final extraction yield was 28% w/w, which is consistent with yields reported for high-quality Iranian Mumie extracts, confirming the efficiency of our purification process (23). Quality control confirmed heavy metals (e.g., Pb < 1 ppm) and microbial load (<10^3 CFU/g) within pharmacopeial limits. Chemical standardization using HPLC-DAD and LC-MS quantified fulvic acid (480 mg/g, ~48%) and dibenzo-α-pyrones (14.2 mg/g), aligning with authenticated profiles. Cytotoxicity (MTT) assays confirmed safety at 1000 µg/mL (24).

Real-time PCR analysis

Collagen I, III, and fibronectin were selected as target genes due to their critical roles in dermal ECM synthesis during wound healing. GAPDH was validated as a stable housekeeping gene across all experimental conditions. The 24-hour time point was chosen to assess early cellular responses, while the 96-hour time point was selected to evaluate longer-term effects on ECM gene expression. WJSCs were treated with 1000 µg/mL of Mummy extract. Following 24 and 96 hours, total cellular RNA was extracted using the RNX-plus kit (Sinaclon, Iran). After adding chloroform and mixing with ice, the mixture was centrifuged at 12000 rpm for 15 minutes. Then, the supernatant was removed, and the samples

were incubated with Isopropanol at -20 ˚C overnight. cDNA was synthesized using the AccuPower® RT PreMix (Bioneer), and collagen I, III, and fibronectin mRNA, as well as the reference gene, housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were assessed using the gene-specific SYBR Green PCR Master Mix (Applied Biosystems, USA) according to the manufacturer's protocol.

- Collagen type 1a1 (forward, 5′-GCCAA AGAAG CCTTG CCATC-3′; reverse, 5′-TCCTG ACTCT CCTCC GAACC -3′)
- Collagen type 3a1 (forward, 5′-GCTGG CTACT TCTCG CTCTG-3′; reverse, 5′-TTGGC ATGGT TCTGG CTTCC-3′)
- Fibronectin 1 (forward, 5′- CCTCA CCAAC CTCAC TCCAG-3′; reverse, 5′-GTCGC AGCAA CAACT TCCAG-3′)
- Housekeeping gene GAPDH (forward, 5′-CAAGATCATCAGCAATGCCTCC-3′; reverse, 5′-GCCATCACGCCACAGTTTCC-3′).

Scaffold

Scaffold Characterization

The PCL scaffold was obtained from a stem cell technology company in Iran. The nanofibrous PCL scaffolds were fabricated by electrospinning a 12% w/v PCL solution at a flow rate of 1 mL/h with an applied voltage of 15 kV. Scaffolds were sterilized with ultraviolet (UV) light for 1 hour on each side before use. The nanofibrous PCL sheet was treated with DMEM. Before implementation, a scanning electron microscope (MIRA3 FEG-SEM) was used to assess the auxiliary morphology of the scaffold. The scaffold cases were cut from a nanofibrous sheet using a 7 mm dermal punch and coated with gold using a sputter coater. The diameter of fibers in the electrospun scaffold was measured on scanning electron micrographs. The PCL scaffolds were fabricated by electrospinning a 12% w/v PCL solution at a flow rate of 1 mL/h with 15 kV voltage. Scaffolds were sterilized with UV light for 1 hour per side. Fiber diameter and pore size were measured from SEM images using ImageJ software (n=50 measurements per group). The average diameter of fibers was resolved from estimations perpendicular to the long axis of the fibers inside the delegate microscope fields (10 estimations for every field). The extent of the pores framed between the fibers was measured using image analysis software. No fewer than five scanning electron micrographs were captured at an amplification of 2000x from arbitrary spots (25).

Scanning Electron Microscopy

To monitor cell attachment to the PCL nanofiber scaffold, scaffolds were cut into small pieces (7 mm in diameter) using a skin biopsy punch. Then, scaffolds were placed in 12-well culture plates, sterilized with UV, and incubated in DMEM for 12 hours. Fibroblasts and WJSCs alone or in a mixture of 50-50 or 70-30 were seeded at a density of 1×10^5 scaffolds and treated with FBS-free DMEM (control) or 1000 µg/mL of Mummy extract for 24 hours. Then, the cell/scaffold builds were placed in 5% glutaraldehyde (1 hour) and dried out in a graded ethanol series. Subsequently, they were sputtered with a thin gold layer and examined by SEM. Cell adhesion was quantified by counting cells in three random SEM fields (5000× magnification) per scaffold using ImageJ. Cell density was expressed as cells/mm^2.

MTT Assay

The proliferation rate of cells (fibroblasts, WJSCs, and co-culture of fib-WJSCs (70-30, 50-50)) on the scaffold was evaluated using the MTT assay. Scaffolds were placed in a 96-well plate and sterilized using UV. Cells were seeded at a density of 2×10^4 per well and incubated at 5% CO_2 at 37 ℃. The 50-50 and 70-30 co-culture ratios were selected to simulate different cellular microenvironments. The 24-hour time point assessed initial attachment/proliferation, while the 96-hour time point evaluated long-term growth. All experiments were performed with n=6 independent replicates. The cells were divided into control (DMEM-free) and treatment (Mummy at 1000 µg/mL). Cell proliferation on the scaffold was evaluated after 24 hours or 96 hours using the MTT assay. Briefly, MTT solution (5 mg/mL in DMEM + FBS 10%) was added to each well (n=6) and incubated for 4 hours. The supernatant was removed and replaced with DMSO. After 20 minutes, cell/scaffold constructs were centrifuged at 300 rpm for 5 minutes. To remove the scaffold, 100 µL of solution was transferred into a new 96-well culture plate. The metabolized MTT was evaluated by measuring its optical density in a spectrophotometer (Bio-Tek) at 570 nm. The 50-50 and 70-30 co-culture ratios were selected to simulate different cellular microenvironments, specifically to investigate potential paracrine interactions when stem cells and fibroblasts are present in roughly equal proportions (50:50) versus a fibroblast-dominant wound environment (70:30).

Statistical Analysis

GraphPad Prism version 6.01 was used to analyze the results. Values were expressed as the Means ± SD by triplicate independent experiments. One-way and two-way analysis of variance (ANOVA) followed by Tukey's and Bonferroni's multiple comparisons tests were used to determine significant differences among groups at $P < 0.05$.

Results

Higher Expression of fibronectin in the Treated Groups

We found that the highest proliferation rate occurred when these cells were incubated with 1000 µg/mL of Mummy extract. The mRNA expression level of type I and III collagens and fibronectin was assessed by real-time

RT-PCR. Our results showed that, in fibroblasts treated with Mummy, the expression of collagens I and III was not different from that of control cells. In contrast, fibronectin expression was up-regulated by 2.5-fold at 24 hours and 3.1-fold at 96 hours (Figure 1A-C).

In WJSCs treated with Mummy extract at 1000 µg/mL, collagen I expression was also not different. However, the levels of type III collagen and fibronectin were up-regulated by 1.8-fold and 2.2-fold, respectively, at 96 hours (Figure 1D-F). The mRNA expression of type I collagen and fibronectin was up-regulated by 3.5-fold and 3.0-fold, respectively, in the co-culture of fibroblasts and WJSCs (50-50), (70-30) treated with Mummy, while collagen III type was not significantly changed (Figure 1 G-L).

Stimulated Cell Adhesion on the PCL Scaffold in the Treated Groups

Cell attachment to the PCL nanofiber scaffold was evaluated via SEM. The average diameter of nanofibers was approximately 0.976 ± 0.044 µm, and the pore size was 134.4 ± 3.83 µm. The orientation of fibers was random (Figure 2A). As shown in Figure 2B-I, cells were successfully penetrated and adhered to scaffolds, with a flattened, polygonal phenotype in both the control and Mummy-treated groups. Collectively, the results showed that cell density was relatively similar in all groups, and visible lamellipodia were observed on fibers. Quantitative analysis revealed a significant increase in cell density in the Mummy-treated groups (approximately 1250 ± 150 cells/mm^2) compared with the control groups (980 ± 120 cells/mm^2; $P < 0.05$), confirming enhanced cell adhesion.

Cell Proliferation

Based on the MTT assay results, fibroblast proliferation was increased after 24 hours compared to the control group.

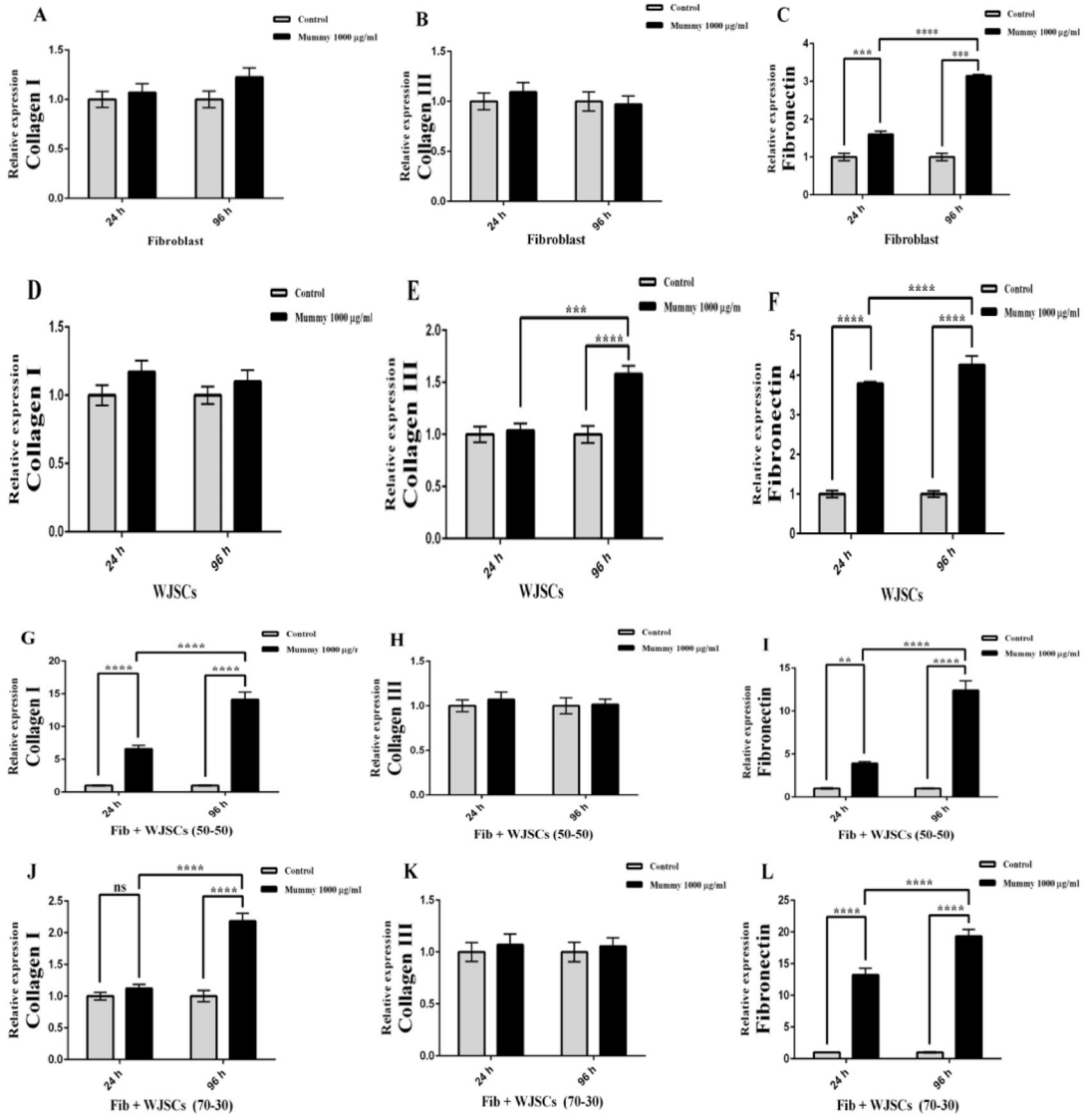

Figure 1. Comparison of Transcript Level of Collagen I, III, and FN1 Between the Control Group and Various Cells after Treatment With Mummy Extract at 24 and 96 hours. (A, B, C) Fibroblast cells. (D, E, F) WJSCs. (G, H, I) Co-cultured fibroblasts and WJSCs with a 50/50 proportion. (J, K, L) Co-cultured fibroblasts and WJSCs with a 70/30 proportion. Data are expressed as mean ± SD. ***P*<0.01, ****P*<0.001, *****P*<0.0001 and ns = *P*> 0.05.

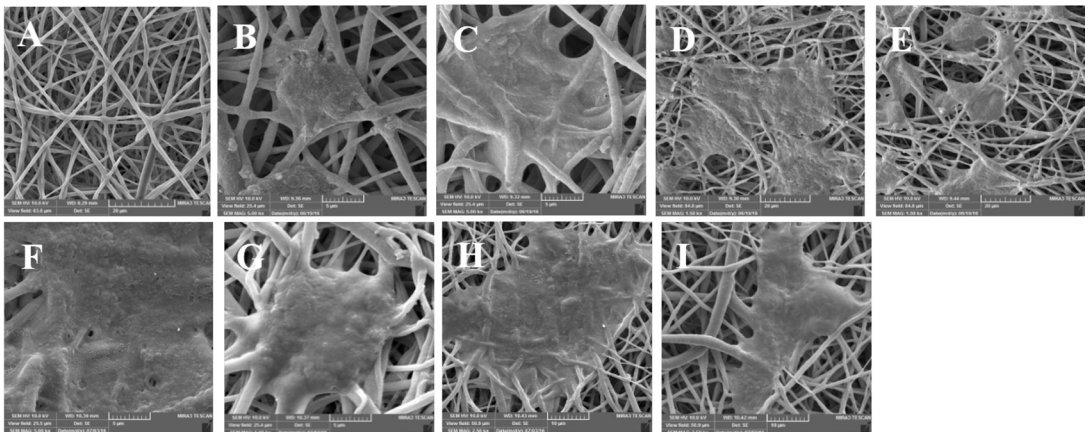

Figure 2. Scanning electron microscope (SEM) Images of Poly (ε-Caprolactone) Nanofibers Before and After Cell Seeding and Attachment of Cells on Them in Different Groups. (A) before cell seeding, (B, C) Fibroblast cells control and Mummy, (D, E) WJSCs control and Mummy, (F, G) Co-cultured Fibroblasts and WJSCs with 50/50 proportion control and Mummy, (H, I) Co-cultured Fibroblasts and WJSCs with 70/30 proportion control and Mummy, in different magnifications at 1500× (D and E), 2000× (A), 2500× (H and I) and 5000× (B, C, F and G).

Despite the increased number of cells with the culture time in both groups, the difference between the control and treated groups was not significant after 96 hours (Figure 3A). As shown in Figure 3-B, no significant difference was observed in WJSCs in both control and treated groups after 24 and 96 hours. The cell proliferation in the co-culture of fibroblasts and WJSCs on the scaffold (50-50) was significantly lower in Mummy-treated constructs compared to the control on 24 hours and 96 hours. There was no significant difference in the proliferation of co-cultured cells between the 70-30 proportion in the control and treatment groups at 24 and 96 hours (Figure 3C-D). The transient increase in fibroblast proliferation at 24 hours may reflect an initial metabolic activation by Mummy. In comparison, the return to baseline at 96 hours could indicate a shift in cellular activity from proliferation to matrix production. The reduced metabolic activity in the 50-50 co-culture group, as measured by the MTT assay, might suggest more complex cell-cell interactions leading to a differentiated, matrix-producing phenotype rather than a proliferative one.

Discussion

Skin injuries are characterized by damage, swelling, the development of granulation tissue, and re-epithelialization (25). Today, wound healing, which is influenced by many factors, is a critical issue in medicine. Approximately 2% of the general population of the United States has non-healing wounds (26). Hence, researchers have focused on employing appropriate methods and novel procedures for wound repair.

In recent years, greater attention has been paid to the use of traditional prescriptions (27). To create new tissue and heal the injury, fibroblasts multiply to build more

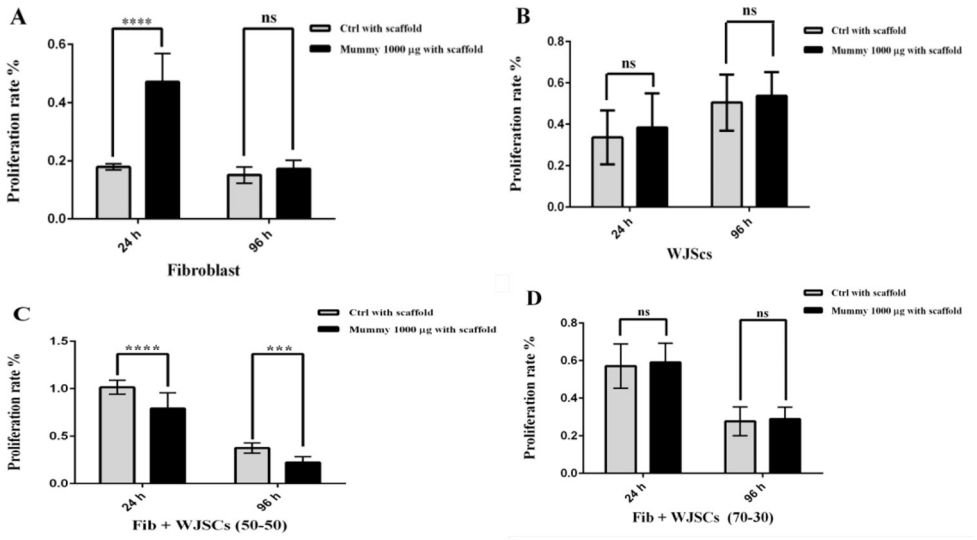

Figure 3. Representative Images of Cells: A- Fibroblast cells, (B) WJSCs, (C) Co-cultured fibroblasts and WJSCs with a 50/50 proportion, (D) Co-cultured fibroblasts and WJSCs with a 70/30 proportion loaded on a PCL scaffold. Error bars represent standard error of the mean, ****P<0.0001, ***P<0.001, and ns P> 0.05.

cells and deliver a few ECM proteins and growth factors (28). Collagens play a pivotal part in injury healing and intercellular matrix development. Fibroblasts produce new ECM, which is essential for cell development and provides a medium for oxygen and nutrient transport to the cell's digestive system (29). In this study, we investigated the potential effect of Mummy extract in wound healing. In addition, ECM synthesis in cultured Wharton's jelly stem cells and fibroblasts, and in their co-culture with or without a scaffold, was evaluated. Real-time RT-PCR results showed increased FN1 expression in HFFF compared with the control group. The co-cultured conditions at 50-50 and 70-30 proportions showed upregulated COL1 and FN1. In line with this, the researchers showed that MSCs, along with fibroblasts, play a pivotal role in collagen and elastin secretion (30). Expression of qualities required in re-epithelialization, neovascularization, and fibro proliferation (TGF-β2 and hypoxia-inducible factor-1α, and plasminogen activator inhibitor-1) was upregulated in WJ-MSC-conditioned medium (WJ-MSC-CM) treated fibroblasts, and VEGF, fibroblast development factor-2, connective tissue development factor, collagen I, and collagen III were not changed. A review by Kim et al demonstrated that adipose-derived stem cells (ASCs) stimulate re-epithelialization of cutaneous injuries by promoting the proliferation of human dermal fibroblasts. So, it can be utilized for the treatment of wound healing by collagen synthesis (16,31). The MTT assay clearly showed HFFF proliferation. Based on the study by Ghorbani et al, fibroblasts and heart cells showed higher proliferation than in two-dimensional culture scaffolds (32). According to the previous results, scaffolds supported the multiplication of adipose-derived MSCs in the presence of zinc ion (33). Therefore, it seems that proliferation of fibroblast and Wharton's Jelly-derived stem cells in the presence of Mummy enhances the Biocompatibility of the PCL scaffold. Mummy extract supported cell attachment and ECM production on the PCL scaffold, indicating a favorable cell-material interaction. Mummy extract increased the levels of several components of the ECM, suggesting that Mummy enhances wound healing in vitro.

The effects may be mediated by bioactive components, such as fulvic acid, which are known to enhance mitochondrial function and modulate key signaling pathways, such as TGF-β, crucial for fibroblast activation and collagen synthesis (34). The significant up-regulation of fibronectin is particularly relevant for the initial stages of wound healing, as it facilitates cell migration, adhesion, and the assembly of a provisional ECM matrix.

In summary, we found that Mummy extract has a potent effect on the healing capacity of WJSCs exposed to dermal fibroblasts in 2D and 3D models, and that Mummy can be used for tissue engineering applications.

Limitations of the Study

This study has several limitations that should be considered. The findings are based on *in vitro* models, and their translation to *in vivo* wound healing requires further validation. We investigated a single, optimized concentration of Mummy extract; a dose-response study could provide more comprehensive insights. Furthermore, the precise molecular mechanisms behind the observed synergistic effects in co-culture remain to be fully elucidated. While our research demonstrates the potential of Mummy extract to enhance ECM production and cell adhesion, it is essential to note the complexities of its effects. For instance, we observed that Mummy extract did not uniformly increase the expression of all ECM components; collagen III remained unchanged in several treatment groups. Furthermore, the reduced metabolic activity in the 50:50 co-culture group, as measured by MTT assay, suggests that the interaction between Mummy and different cell populations can influence cellular phenotypes in non-uniform ways, potentially shifting the balance from proliferation to matrix synthesis or other functions. These nuanced findings highlight that the net effect of Mummy on wound healing is multifaceted and likely context-dependent, warranting further investigation into the underlying signaling mechanisms. Furthermore, we investigated a single, optimized concentration of Mummy extract based on initial MTT assays; a comprehensive dose-response study in future work could provide more detailed insights into its efficacy and therapeutic window. It should also be noted that our ECM data are at the mRNA level; future studies incorporating protein-level analyses, such as western blot or immunofluorescence, would be essential to confirm the translation of these genetic changes into functional protein synthesis. Additionally, while our Mummy extract underwent rigorous chemical standardization via HPLC and LC-MS, the absence of representative chromatograms within this manuscript is a limitation. Future studies would benefit from including such spectra to provide a visual benchmark for the material's composition and facilitate direct comparisons across different batches and sources.

Conclusions

Taken together, this study presented an effective in vitro method for the healing process. Hence, the prospect of utilizing Mummy extract and stem cell-based therapies in wound healing as a novel therapeutic approach is promising.

Authors' Contribution

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Resources: Jafar Soleimani Rad, Leila Roshangar.
Software: Shahnaz Sabetkam, Sepideh Hassanpour Khodaie.
Supervision: Jafar Soleimani Rad, Leila Roshangar.

Validation: Jafar Soleimani Rad, Leila Roshangar.
Visualization: Shahnaz Sabetkam, Sepideh Hassanpour Khodaie.
Writing–original draft: Shahnaz Sabetkam, Sepideh Hassanpour Khodaie.
Writing–review & editing: Shahnaz Sabetkam, Sepideh Hassanpour Khodaie, Jafar Soleimani Rad, Leila Roshangar.

Conflict of Interests
Authors have no conflict of interest.

Ethical Issues
This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (ethic code, 5/4/9874).

Financial Support
This study was supported by the Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Acknowledgments
The authors are especially grateful to the Stem Cell Research Center at Tabriz University of Medical Sciences for their support and assistance.

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