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Therapeutic Potential and Comparison of the Effect of Mummy on Mesenchymal Stem Cells Derived from Wharton's Jelly and Adipose Cultured with Human Fibroblast

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ABSTRACT

Background: The Wound healing process, as a coordinated physiological mechanism, is a critical subject in medicine. The slow healing and scar formation associated with numerous conventional therapies have led researchers to seek new and more effective therapeutics. This study evaluated the effects of Mummy material, Wharton Jelly Stem Cells (WJSCs), and Adipocyte Stem cells (ASCs) on the fibroblast migration and proliferation.
Materials and Methods: It was demonstrated that fibroblast cells could attach to three-dimensional (3D) scaffolds in the mentioned microenvironment. ASCs and WJSCs were enriched from human adipose tissue and women undergoing cesarean section, respectively. The proliferation rate, migration, expression of fibronectin, collagen I, III, and cell adhesion on PCL scaffold in the presence of mummy material were investigated.
Results: The results emphasized the importance of Mummy material, ASCs, and WJSCs in the migration and proliferation of fibroblast cells. The presence of the aforementioned components and cells enhanced the expression of fibronectin (FN1) and collagen types I and III. Additionally, the mummy material was found to promote the proliferation of ADSCs and WJSCs seeded on the PCL scaffold. Together, these findings demonstrate a valuable *in vitro* technique for studying the healing process.
Conclusion: As a result, the potential for using Mummy material and stem cell-based therapeutics in wound healing is exciting.

Keywords: Wound healing; Adipocyte stem cells; Wharton jelly stem cells; Mummy material

INTRODUCTION

Wounds mean a disruption of the normal structure of the skin that is continuously increased with aging and underlying diseases[1, 2]. Approximately 2% of the general population of the United States suffers from non-healing wounds. Wound healing is a complex biological process involving inflammation, tissue regeneration, and remodeling. Today, the wound healing process which is affected by many factors, has been considered [3] . For example, wound healing can be impaired due to chemotherapy and radiation therapy, leading to delayed healing, fibrosis, and chronic ulcers[4]. Additionally, the immunosuppressive therapies required to prevent graft-versus-host disease (GVHD) further compromise tissue repair by impairing immune cell function and reducing the availability of growth factors essential for regeneration[5]. Moreover,

transplanted stem cells may face challenges in engraftment and differentiation within damaged tissue environments, further limiting their therapeutic potential[6]. Thus, wound healing is considered a critical subject in medicine. Fibroblasts, endothelial cells, and extracellular matrix components (especially collagen) play a critical role in wound healing[7, 8] .Several studies suggest that, fibroblast cells are one of the potential therapies for wound healing. These cells synthesize macromolecules such as elastin, collagen type I, hyaluronan, and proteoglycans that preserve the tensile strength of the cutaneous tissue[9, 10] . Adipose-derived stem cells (ASCs) promote dermal fibroblast proliferation by secreting different growth factors such as platelet-derived growth factor-BB (PDGF-BB) and basic fibroblast growth factor-2 (FGF2)[11-13]. In addition, they could differentiate into myogenic, chondrogenic, adipogenic, and osteogenic lineages in response to specific stimuli[14] .In line with this, the ability of ASCs to promote cutaneous wound healing has been previously reported[15,16] .It is found that Wharton jelly-derived mesenchymal stem cells (WJSCs) stimulate re-epithelialization, neovascularization, and skin fibroblast proliferation in a mouse excisional model of skin[17] .Besides ASCs and WJSCs, the positive effects of Mummy material on gastric mucosal damage, bone fracture, and rabbit models of cutaneous wounds have been described in many clinical situations[18-20]. Several studies have suggested that ECM supports a niche for cell growth, migration, and the regulation of intercellular communication. Therefore, 3D scaffolds can be further applied to simulate the naive ECM of the specific tissue[21] .In this study, we evaluated the effect of Mummy material, adipose, and WJSCs on the proliferation and migration of fibroblasts. Furthermore, the quantitative impact of these materials on fibroblast adhesion to 3D scaffolds and the wound healing process has been studied [22] .

MATERIALS AND METHODS

The isolation and expansion process for ASCs

Samples of human adipose tissue were taken from adult patients having elective surgery with the ethical approval committee of the University of Medical Sciences (ethic code: IR.TBZMED.REC. 5/4/9875) and informed consents written from all patients. PBS (phosphate-buffered saline) with 1% penicillin-streptomycin (Gibco) was used to wash the samples twice under sterile conditions. The samples were fragmented into tiny bits (1-2 mm thick). The fragments were broken down using 0.2% collagenase type I (Cat no: C9891, Sigma) and were then shaken at 37°C for 60 min. After digestion, the same volume of working culture medium (DMEM/Gibco, USA+10% FBS) was added to the cell suspension for neutralizing the collagenase enzyme. Then, the suspension of cell was composed by centrifugation for 5 minutes at 1500 rpm. Utilizing 0.4% trypan-blue exclusion dye, the cells were counted.

The cells were sown onto a T25 culture flask in DMEM (low glucose, Gibco) which was complemented with 1% penicillin-streptomycin and 10% FBS. A new medium was used every three days. We used expanded cells in the third passage.

WJSC isolation and expansion procedure

Women undergoing cesarean sections without any problems throughout their pregnancy were enrolled in the current study. Written informed permission was provided by each participant with respect to the medical ethics committee and institutional review board of the study protocol. Umbilical cords were obtained from full-term newborn babies and transferred to the cell culture laboratory in sterile tubes of PBS containing 1% penicillin-streptomycin. Following three washes with PBS, after 30 seconds in 70% alcohol, samples were removed and cut into 5 cm parts. After incising lengthwise, WJ was gently sliced into 2 x 2 mm2 pieces and explanted in T-25 culture flasks with DMEM/LG (Gibco) complemented with 1% penicillin-streptomycin and 20% FBS after the vessels were removed. The cultural media was replaced three times every week. WJSCs appeared in the proximity of WJ explants after 2 weeks. The cells were separated by 0.25% trypsin-EDTA solution (Sigma) and sub-cultured onto new flasks once they had reached 70–80% confluence.

Mummy material preparation

The fresh Mummy was obtained from Kermanshah, located in the west of Iran. The purity and quality of Mummy were determined by an expert botanist. We

used a routine MTT assay to select the effective concentration. To make a working solution, Mummy material was dissolved completely in DMEM culture medium and filtered through a 0.22-µm syringe filter.

In vitro scratch test

The effect of the Mummy material on the migration of ASCs-fibroblasts (HFFF-2 cell line, Pasteur Institute of Iran) and WJSCs-fibroblast co-cultures was evaluated using an in vitro scratch assay. For co-culture experiments, 50:50 (2.5×10^4 fibroblast: 2.5×10^4 ASCs) and 70:30 (3.5×10^4 fibrobalst: 1.5×10^4 ASCs) ratios of cells were used. The same protocol was applied for the combination of fibroblast and WJSCs. The cells were cultured in DMEM/LG complemented with 10% FBS for 24 hours for the formation of a confluent monolayer. Using a sterile pipette tip, three linear scratches were made in each well. The cells in the control groups were then either treated with 1000-2000 ug/mL doses[23] of Mummy dissolved in DMEM or received fresh serum-free DMEM. On days 0, 1, 2, 3, and 4, pictures were captured using an inverted microscope and a digital camera (Olympus, Japan) at 4X magnification. By using the computer program image J (ver. 1.49), the images were analyzed, and the distance between each scratch closure was measured.

Proliferation assay

To estimate whether Mummy material can affect proliferation of both WJSCs and ASCs in the presence of fibroblasts, the same cell ratio protocol was applied as above-mentioned. Cells were exposed to 1000 µg/ml of Mummy agent over a period of 96 h. Following the treatment protocol, cells in different groups were detached and permeabilized for 3 minutes by 0.2% Triton X-100. The supernatant was removed after washing with PBS, and cells were then treated with 5 µl of Ki-67 antibody (Ref: 12-5699-41, USA) in 100 µl of PBS for 30 minutes on ice. Cells were re-suspended in 500µl of 1xPerm Wash Buffer and examined by BD caliber (BD Bioscience, San Jose, Calif, USA).

Real-time PCR analysis

The co-culture system of fibroblast-WJSC and fibroblast-ASC was exposed to 1000 µg/ml of Mummy material at the time points of 24 and 96 h. The RNX-plus kit was used to extract the total cellular RNA (Sinaclon, Tehran, Iran). The cell suspension was centrifuged at 12000 rpm for 15 minutes after being mixed with ice and chloroform. After removing the supernatant, samples were kept at -20°C with isopropanol for an overnight period. SYBRGreen PCR Mastermix (Applied Biosystems, USA) in accordance with the manufacturer's instructions, the levels of fibronectin, collagen I, and III mRNA as well as housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were determined. For each sample, each experiment was carried out in triplicate. The Pfaffl technique was used to evaluate the data, and the CT values were standardized in relation to GAPDH expression. In the current investigation, gene primers are shown in Table 1.

Characterization of scaffold

The PCL scaffold was acquired from an Iranian stem cell technology company. The PCL sheet that was nanofibrous was cured using plasma. Prior to usage, the structural morphology of the ready scaffold was detected with a scanning electron microscope (MIRA3 FEG-SEM). The scaffold samples were made from nanofiber sheet using a 7-mm dermal punch, and then they were sputter-coated with gold. On scanning electron micrographs, we measured the fiber diameter in the electrospun scaffold. Measurements taken within representative microscope fields perpendicular to the fibers' long axes allowed researchers to determine the average diameter of the fibers[24].

Scanning electron microscopy

To observe the adhesion of cells to the nanofiber PCL scaffold, scaffolds were cut into little parts (4 mm in diameter) using a skin biopsy punch. Then, they were put in 12-well culture plates and sterilized using UV for 4 hours. Further, the samples were soaked in DMEM culture medium for 12 hours. The combination of fibroblast and stem cells at a ratio of 50:50 and 70:30 was loaded on scaffold and exposed to 1000 µg/ml of Mummy material for 24 or 96 h. In

the control group, we used DMEM free FBS. Following the treatment protocol, the cell/scaffold constructs were fixed in 5% glutaraldehyde for 1 h and dehydrated in a graded ethanol series. In the next step, they were sputtered with a thin layer of gold and analyzed by scanning electron microscopy.

MTT assay

The proliferation rate of cells in a co-culture system loaded on scaffolds was evaluated by an MTT assay. Scaffolds were placed in 96-well plates. Cells were seeded at a density of 2×104 per cm2 and incubated at 5% CO2 at 37°C. The cells were divided into control (DMEM free) and treatment (Mummy 1000 µg/ml). The proliferation rate of cells on the scaffold was evaluated after 24 and 96 h using the MTT technique. Briefly, MTT solution (5 mg/ml in DMEM) was applied to each well (n = 6) and incubated for the next 4 h. The supernatant was removed and replaced by DMSO. The final absorbance of each sample was measured at 540 nm by using an ELISA reader plate (Bio-Tek, USA). The cell survival rate was expressed as a percentage of control.

Table 1: The details of primers

Gene	Forward, Reverse primer and size (5′-3′)
COLLAGEN type 1a1	Forward primer: 5′ GCCAAAGAAGCCTTGCCATC3′ Reverse primer: 5′TCCTGACTCTCCTCCGAACC 3′ (154 bp)
COLLAGEN type 3a1	Forward primer: 5′ GCTGGCTACTTCTCGCTCTG 3′ Reverse primer: 5′ TTGGCATGGTTCTGGCTTCC3′ (221 bp)
FIBRONECTIN 1	Forward primer: 5′ CCTCACCAACCTCACTCCAG 3′ Reverse primer: 5′GTCGCAGCAACAACTTCCAG3′ (196 bp)
GAPDH	Forward primer: 5′ CAAGATCATCAGCAATGCCTCC 3′ Reverse primer: 5′ GCCATCACGCCACAGTTTCC 3′ (189 bp)

Statistical analysis

GraphPad Prism version 6.01 software was used to evaluate the results. Values from independent, duplicate tests were presented as Means ± SD. To find the significant difference between groups at $p < 0.05$, Tukey's multiple comparisons test, Bonferroni's multiple comparisons test, and one-way and two-way analysis of variance (ANOVA) were used.

RESULT

The effect of Mummy material on the migration of ADSs and WJSCs in co-culture with fibroblasts at a period of 96 hr

To examine whether Mummy material could affect cell migration in a co-culture system, a wound scratch assay was performed (Figure 1). For this purpose, firstly, WJSCs and ASCs were cultured under 1000 or 2000 µg/ml of Mummy. Then, 50:50 (2.5×10^4 fibroblast: 2.5×10^4 ASCs or WJSCs) and 70:30 ratios (3.5×10^4 fibroblast: 1.5×10^4 ASCs or WJSCs) of cells were utilized. Data analysis showed that the treatment of ASCs with 2000 µg/ml Mummy significantly increased the migration rate compared to control groups during days 2, 3, and 4 (A). Surprisingly, the migration rate of ASCs was significantly higher in the presence of Mummy on day 4. Whereas, no significant difference was observed in migration rate in WJSCs during the early and late stages of the experiment. In addition, Mummy material stimulated the migration of fibroblast-ASC co-cultured with fibroblast at a ratio of 70:30 in both 1000 and 2000 µg/ml, as compared with the parallel co-culture system of fibroblast-WJ-MSCs for 96 hr (C). Notably, the migration rate of fibroblasts-ASCs co-cultured at a ratio of 70:30 was significantly higher than that of fibroblasts-ASCs co-cultured at the ratio of 50:50, both with the same concentration of 1000 µg/ml and in 96 hr.

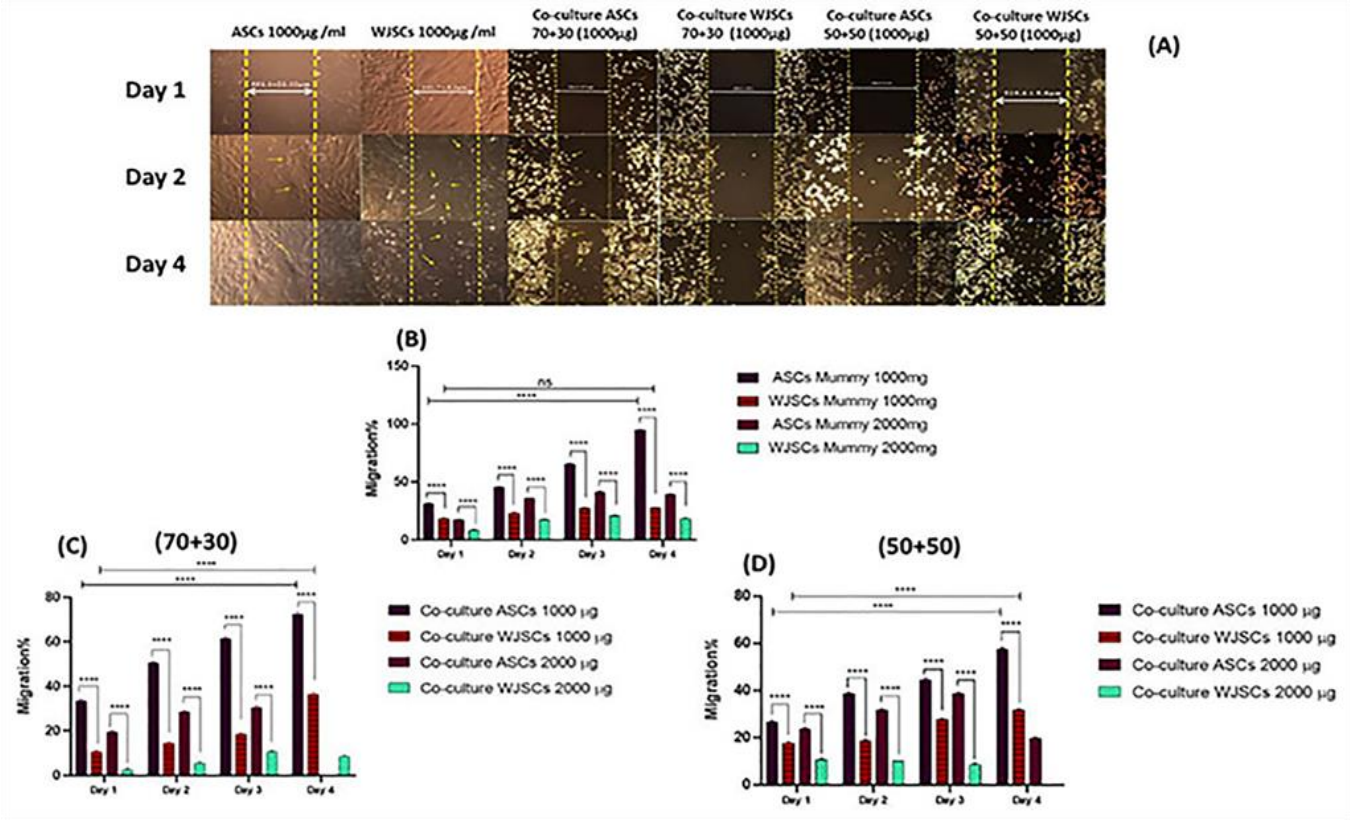

Figure 1. Mummy material increases cell migration. Sample images of the effect of Mummy material on ASCs and WJ-MSC migration capacity alone and co-cultured with fibroblast at ratios (70:30) and (50:50) on days 1, 2, and 4 (A). The wound scratch assay was performed to demonstrate the effect of Mummy material, with different concentrations (1000 and 2000 µg), on the cell migration capacity of ASCs and WJ-MSCs (B), ASCs and WJ-MSCs co-cultured with fibroblast at a ratio of 70:30 (C), and ASCs and WJ-MSCs co-cultured with fibroblast at a ratio of 50:50 (D). The values are shown as the mean ± SD of 3 independent experiments, ns: non-significant, ****P ≤ 0.001.

Mummy material stimulated cell proliferation

The effect of Mummy material on cell proliferation was evaluated by monitoring the expression of Ki-67 (Figure 2). ASCs and WJ-SCs were treated with concentrations of 1000 µg/ml Mummy material for 24 and 96 h. As shown in Figures 2A and B, the expression rate of Ki-67 was significantly increased in treated groups in both ASCs and WJ-SCs at the indicated times. Although the percentage of Ki-67 was increased in fibroblast-WJSCs co-cultured, in contrast to the fibroblast-ASCs groups in 24 h. There was not a significant distinction between the two groups in 96 h (C and D). Taken together, the findings demonstrated that Ki-67 expression was increased in a time independent manner in WJ-MSCs co-cultured with fibroblast at the ratios of 70:30 and 50:50.

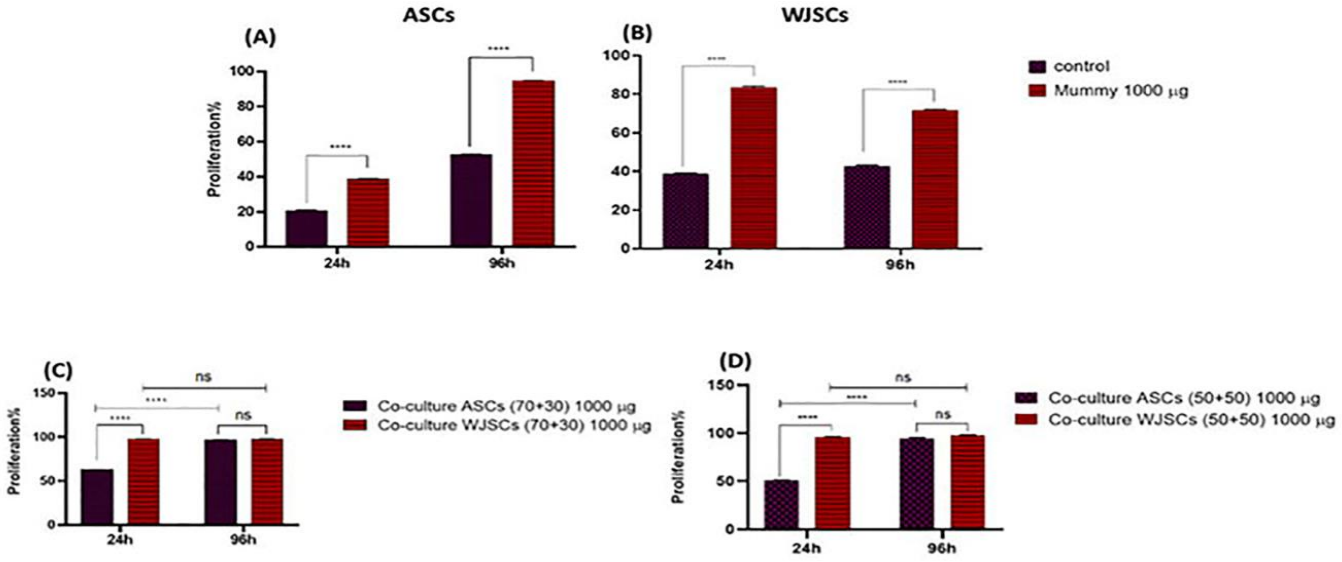

Figure 2. Mummy material augmented the cell proliferation. After the incubation of ASCs and WJ-MSCs alone (A and B) and in co-culture with fibroblast (C and D) in different ratios (70:30 and 50:50) with Mummy material, the cell proliferation rate was demonstrated using Ki-67 staining. The values are shown as the mean ± SD of 3 independent experiments, ns: non-significant, ****P ≤ 0.0001.

Mummy material altered the mRNA expression of fibronectin, collagen I, and III

Collagen I, III, and fibronectin expression was demonstrated in Mummy material-, ASCs-, and WJ-MSCs-treated groups by real-time PCR in 24 and 96 h (Figure 3). Additionally, the difference in collagen I expression at 96 h was significantly higher in comparison to 24 h in ASCs (A). However, no significant change was observed in WJ-SCs treated with Mummy material at 24 and 96 h. The results of the collagen III evaluation showed that although the expression rate was significantly increased in 96 h in both ASCs and WJSCs, the collagen III expression rate was more pronounced in the ASCs group (B). Furthermore, the percentage of fibronectin increased significantly in WJ-SCs compared to ASCs treated with Mummy material in both 24 and 96 hours. In addition, the results showed that fibronectin expression rate was not time dependent in ASCs and WJ-SCs-treated groups (C). The real-time PCR analysis revealed an enhanced upregulation of collagen I, III, and FN1 in the fibroblast-ASCs system treated with Mummy material when compared to the fibroblast-WJ-SCs co-culture in a proportion of 30:70 or 50:50 (D, E, F, G, H, and I). Therefore, it was concluded that Mummy material induced of COLLAGEN I, III, and FN1 expression in mature stem cells rather than in them of neonatal counterparts.

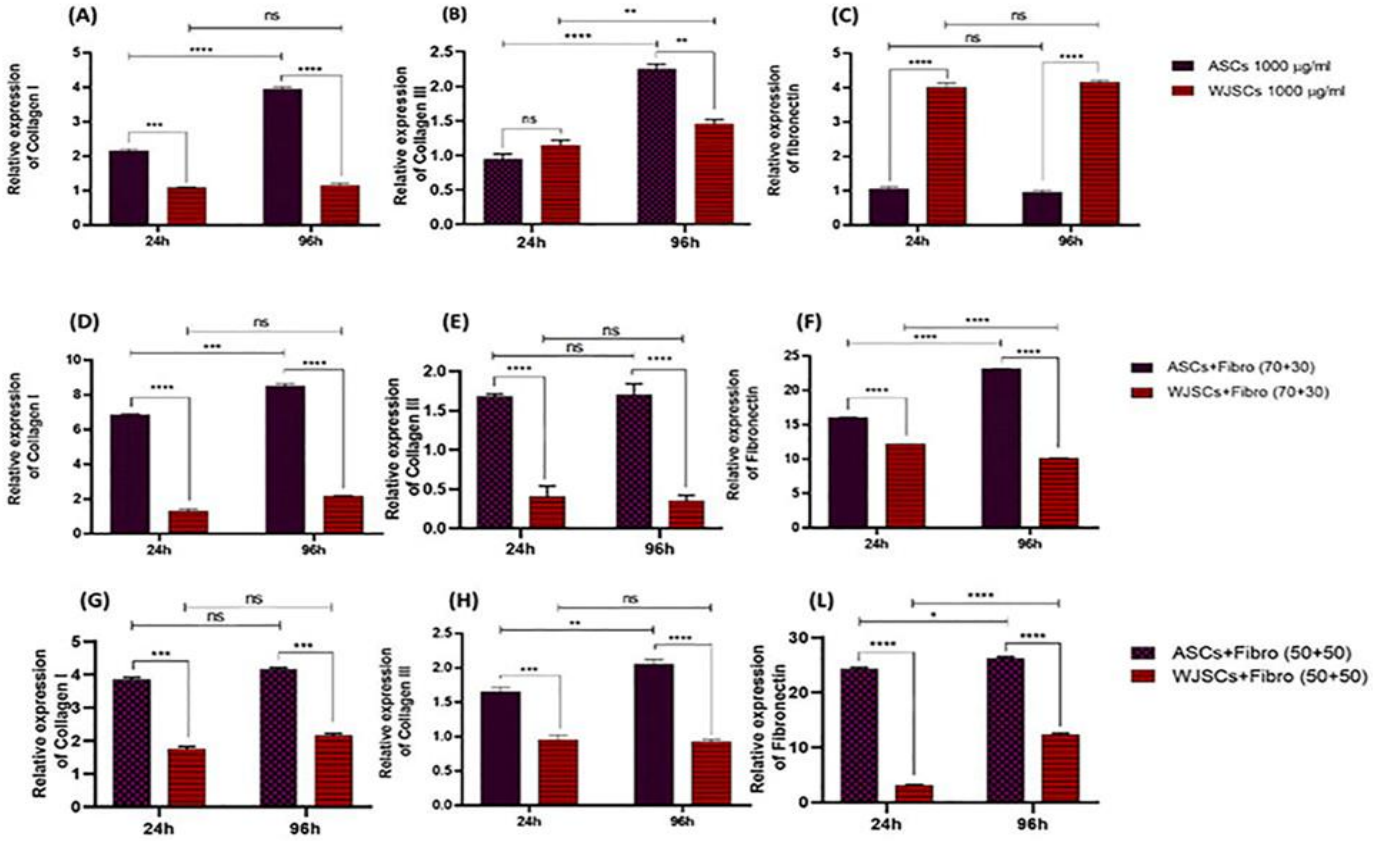

Figure 3. Mummy material altered mRNA expression levels of collagen I, collagen III, and fibronectin. The relative mRNA expression of the collagen I, collagen III, and fibronectin genes was evaluated after 24 and 96 hr. of incubation with Mummy material on ASCs and WJ-MSCs, alone (A, B, and C) or co-cultured with fibroblast in different ratios of [70:30 (D, E, and F) and 50:50 (G, H, and L)]. The values are depicted as the mean ± SD of 3 independent experiments, ns: non-significant, *p < 0.05, **p < 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

Cell adhesion to the nanofiber PCL scaffolds was evaluated using SEM technique

(Figure 4A) shows the fibers of PCL scaffold knobbed to form a strong, flexible, and porous 3D matrix. The nanofibers average diameter was roughly 0.976 ± 0.044 µm and the pore size were approximately 134.4 ± 3.83 µm. As shown in Figure 4B, the cells in the ASCs and WJ-MSCs groups interacted, attached, spread, and formed a polygonal flattened shape with lamellipodia on the scaffold, with no significant difference between the two groups. The cell density after incubation with Mummy material was relatively similar in ASCs and WJ-MSCs co-cultured with fibroblast groups in a ratio of 70:30. However, the cell density in ASCs cultured with fibroblast was more significant when compared to WJ-MSCs cultured with fibroblast in 50:50 ratios.

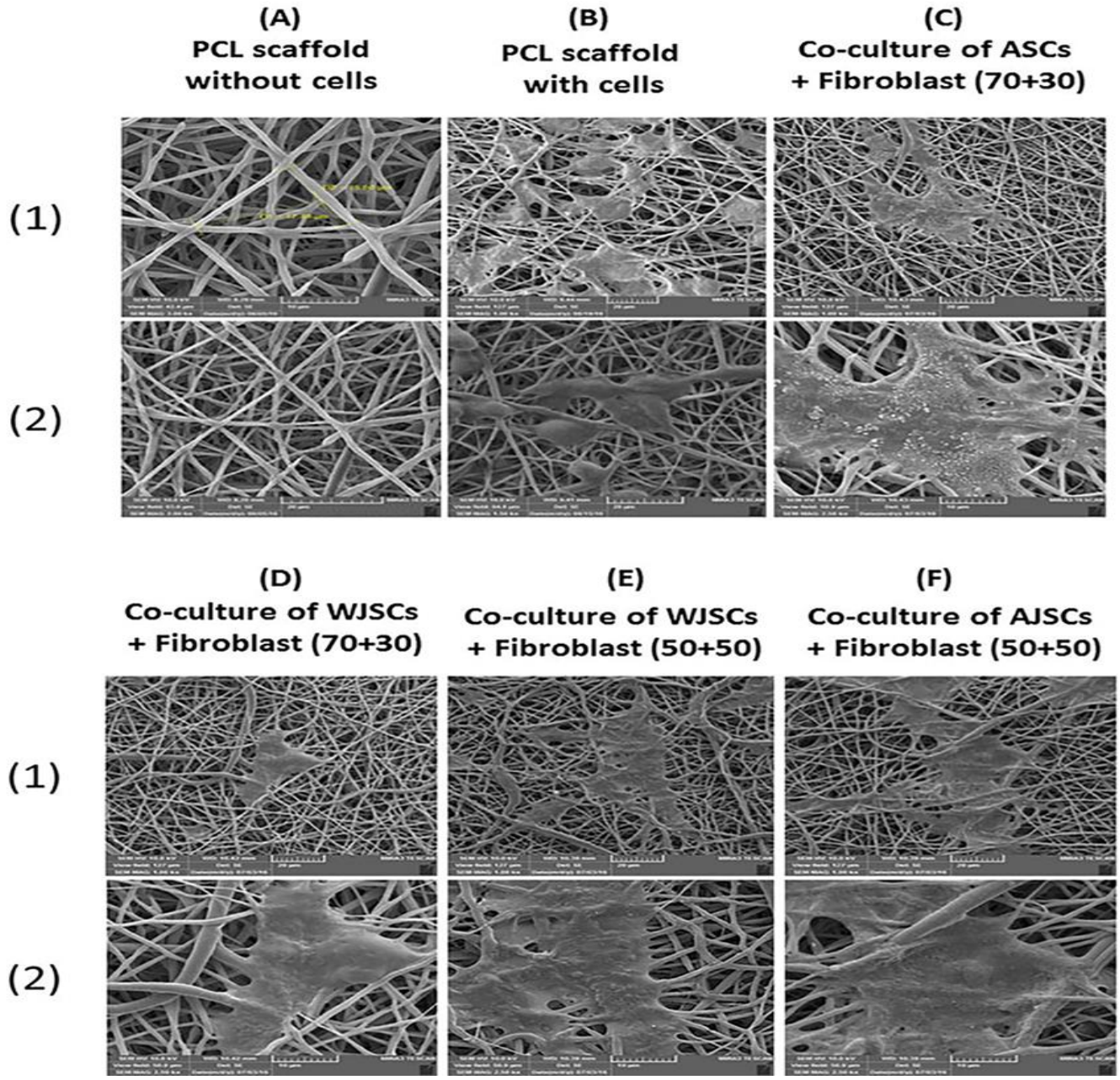

Figure 4. Sample scanning electron micrographs of PCL scaffold. SEM images of PCL scaffold without or with WJ-MSCs and ASCs (A and B). After 24 h of co-culture of ASCs and WJ-MSCs with fibroblast in different ratios (70:30 and 50:50) in the presence of Mummy material, their attachment to the nanofibers PCL scaffold was evaluated (C, D, E, and F). Images are shown in different magnifications at ×2000 (row 1) and ×5000 (row 2).

Mummy material changed the cell proliferation of ASCs and WJSCs loaded on PCL scaffolds

The cell proliferation rate was evaluated after 24 and 96 h by MTT assay on 3D scaffolds (Figure 5). The outcomes revealed no significant difference between the cell proliferation of ASCs and WJ-MSCs treated with Mummy material after 24 h. Surprisingly, the proliferation rate of ASCs treated with Mummy material increased significantly between 24 and 96 h, in contrast to the WJ-MSCs at the same time (A). As shown in Figure 5B, the proliferation rate in fibroblast-WJ-SCs co-cultured at a ratio of 70:30 and cured with Mummy material was more evident after 24 h when compared to parallel fibroblast-ASCs co-cultured (B). In contrast, cell proliferation was increased after 96 h in the co-culture system with ASCs and fibroblast when compared to WJ-MSCs and fibroblast (B). Interestingly, the combination of fibroblast-ASCs showed significantly a higher proliferation rate in a time dependent manner, but in the fibroblast-WJSC system, a lower proliferation rate was observed. A similar pattern was found at the ratio of 50:50 (C).

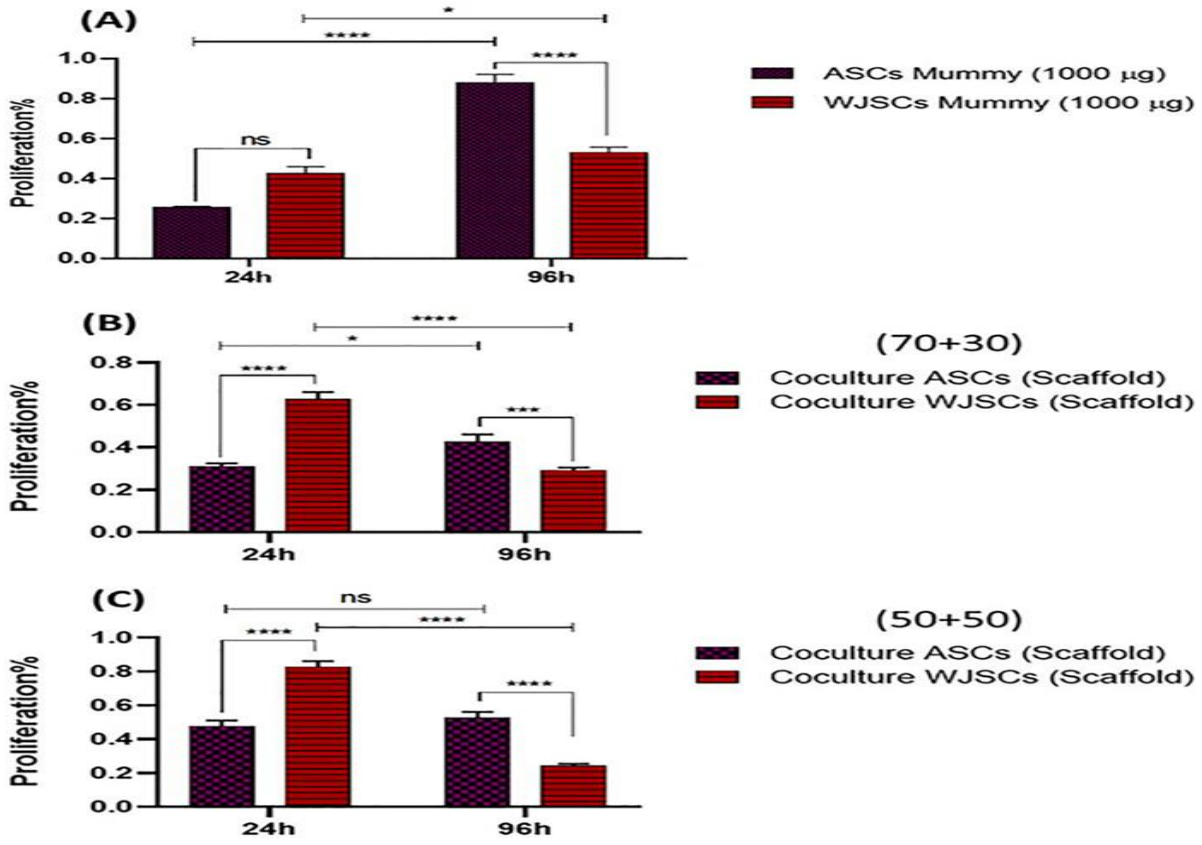

Figure 5. Mummy material changed the cell proliferation of ASCs and WJ-SCs loaded on PCL scaffolds. The effect of Mummy material on the cell proliferation of ASCs and WJSCs loaded on PCL scaffold after 24 and 96 hr. was assessed by the MTT assay (A). The effect of Mummy material on cell proliferation of fibroblast-ASCs and-WJ-MSCs systems loaded on PCL scaffold with different ratios (70:30 and 50:50) (B and C). ns: non-significant, *p < 0.05, ***P ≤ 0.001, ****P ≤ 0.0001.

DISCUSSION

Despite the current application of a wide range of ointments, wound healing and wound dressings stays on a clinical challenge, and novel therapeutic strategies for wound healing are needed. Recently, regenerative and herbal medicine, notably Mummy has emerged as an alternative to the potential healing of wounds and the restoration of the natural structure of the skin[25,26]. The combination of scaffolds and stem cells serves as a promising strategy for regenerative medicine, especially[27] for the treatment of skin wounds[28]. It has been reported that ASCs have efficiency for regeneration because they possess the ability to self-renewal, differentiate into different tissues, migrate to damaged sites, and low immunogenicity[29]. In addition, WJ-SCs share some properties that are unique to fetal-derived MSCs, such as a faster proliferation rate and a higher rate of ex vivo expansion capabilities when compared to adult types [30] .Additionally, they do not express Major Histocompatibility Complex (MHC) class II, which can trigger an immunity response in recipients [31] .Therefore, in this study, the potential of Mummy agents in wound healing, peculiarly the migration, proliferation, and ECM synthesis of ASC and WJ-SC co-cultured with fibroblasts on the surface of PCL substrate, is evaluated.

The present study revealed that Mummy material helped the migration and proliferation of the fibroblast-ACS complex when compared to the combination of WJ-SCs and fibroblasts. Consistent with us in vitro results, several studies showed that conditioned medium enhanced the migration and proliferation of dermal fibroblasts when treated with ASCs[32] .Earlier works by different groups revealed the positive effect of Mummy material on the healing of bone fractures[33] and promising advantages in the acceleration of wound healing in mice[34] .This report is supported by the literature as The results show that ASCs significantly reduced the size of the lesion and enhanced re-epithelialization from the wound edge, indicating that ASC transplantation is a more effective therapeutic treatment for skin wounds than BMSCs or ASCs transplantation[35]. According to previous studies, human WJSCs provoke fibroblast proliferation and migration to coopt wound borders in vitro [36].

It was found that collagen I, III, and FN1 in ASC-fibroblast co-culture were more expressed than those of WJSC when exposed to fibroblast. MSCs were found to help fibroblasts in the secretion of collagen and elastin, governed by the repression of MMP-1 and fibronectin[37]. Concurrently, the therapeutic effects of ASCs were confirmed on the human dermal fibroblast by increasing collagen synthesis, which makes these cells an optimal candidate for wound healing[38] . Other underlying mechanisms were confirmed in the context of ASC application. ASCs, for instance, successfully accelerated the development of neo-vascularization and wound healing in a full thickness excisional rat model damage while having the potential to differentiate into endothelial and epithelial cells and also promoted wound regeneration[39] .

The construction of 3D structural and functional cell-scaffold complexes has been rapidly developing in the field of tissue engineering research over the past decades[40] .In this study, it was further shown that nano-fibrous scaffolds affected cell behavior in terms of proliferation and adhesion. A distinct cell behavior based on the used stem cell type was also notified. The indices increased, especially when ASCs were co-cultured with fibroblasts. Compared to the 2D plastic surface, the biocompatibility of the scaffolds used augmented a higher proliferation rate in cultured cells. It was previously mentioned that PCL scaffolds supported ASC adhesion and proliferation when enriched with Zn^{+2} ions[41]. Here, Mummy material accelerates wound healing on a 3D co-culture system. The project's outputs adapt to popular culture and traditional medicine. Regardless of the application of PCL scaffold, Mummy could alone improve the migration and proliferation of stem cells in co-cultured in vitro systems. These effects were found to be more obvious in 3D culture. However, ASCs showed their capabilities to simulate the orderly composition of ECM proteins better than WJ-SCs in a co-culture in vitro model. As a result, the effects depicted by ASCs, recommend that the phase of wound healing known as proliferative could be efficiently stimulated by these cells. Although our study showed that Mummy materials have a strong impact on the wound healing process, the lack of studies on animal samples, in vivo validation, and

scalability were limitations of this project. In future research, it would be beneficial to explore not only the effects of Mummy materials in controlled lab settings but also their performance in real-life scenarios, particularly focusing on different types of wounds and varying conditions. Additionally, expanding the research to include larger animal studies could provide invaluable insights into the practical applications and effectiveness of these materials in clinical settings.

CONCLUSION

In summary, it was found that Mummy material has a potent effect on the healing capacity of different stem cells exposed to dermal fibroblast in 2D and 3D models. Additionally, the interaction between stem cell type and fibroblast cells could predestinate the process of wound healing.

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Ethics statement

This study was approved by the Ethical Committee of Tabriz University of Medical Sciences (ethic code: IR.TBZMED.REC. 5/4/9875). The study was performed in accordance with the Declaration of Helsinki and its later amendments.

CONFLICT OF INTEREST

The authors declare no competing interests.

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